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RICE UNIVERSITY

Removal of Residual Hexadecane from Sand Columns
Using Neodol 25-9 and a Biosurfactant Isolated
from *Rhodococcus* species H13-A

by

Theodora May Overfelt

A THESIS SUBMITTED
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APPROVED, THESIS COMMITTEE

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ABSTRACT

Removal of Residual Hexadecane from Sand Columns
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The biosurfactant produced by a common soil isolate, *Rhodococcus*
species H13-A (*Rhodococcus erythropolis*), is effective in removing residual
hexadecane, a representative light non-aqueous phase liquid, from sand
packed columns. Increased removal occurs at capillary numbers in the range of
10^{-3} to 10^{-2}. While the results are inconclusive, the biosurfactant appears to
increase mobilization of residual hexadecane comparable to a commercial
surfactant, 1.2% (w/w) Neodol 25-9 in mineral salt solution, with a similar
interfacial tension.
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CHAPTER 1

Introduction

1.1 Problem Statement

Pollutants, such as industrial solvents, petroleum products and by-products, and fertilizers, can find their way into soil and ground water from various sources, including leachate from hazardous waste and landfill sites, leaking underground storage tanks, and run-off from agricultural and industrial areas. Contaminated ground water can cause a variety of environmental and public health concerns, and therefore, the need for aquifer restoration is very important.

One possible remediation technique is pump and treat, involving the removal of soluble contaminants from the aquifer through ground water withdrawal. This process, even at highest possible pump rates, does not remove residual contaminants, those sorbed or trapped in the pore spaces between soil particles. Estimates of 100 to 200 years for removal of residual contaminants with pump and treat technology, in some cases, make the method financially unacceptable (Mansell et al., 1999). Residual contaminants are a perpetual source of pollutants and pose health and environmental problems. Decreasing the interfacial tension between the pollutant and the water with surfactants can potentially cause release of the contaminants at lower achievable flow rates for subsequent removal or treatment.

Surfactant flushing has been used for enhanced oil recovery and is currently being investigated in lab and field applications as a technology for the removal of residual contaminants. The use of bacterially produced surfactants, or biosurfactants, has also been used in microbi ally enhanced oil recovery, and
in bench scale studies for removal of residual contaminants; however, biosurfactants generally have not been shown to be as effective at removing contaminants as commercial surfactants. This could be due to the tendency of biosurfactants to reduce interfacial tensions to a lesser extent than commercial surfactants.

1.2 Hypothesis

1. The microbial biosurfactant produced by *Rhodococcus* species H13-A will enhance removal of residual hexadecane from sand packs.

2. The effectiveness of the biosurfactant to remove residual hexadecane will be comparable to that of a commercial surfactant with approximately the same ability to lower interfacial tensions between an aqueous solution and hexadecane.

1.3 Objectives

This research investigated residual contaminant removal by a commercial surfactant, a biosurfactant, and a non-surfactant solution. The principle objectives of the research were to:

1. Extract the biosurfactant from a bacterial culture grown on hexadecane.

2. Identify a commercial surfactant and an appropriate concentration to lower the interfacial tension between the mineral salt solution and hexadecane approximately the same as with the biosurfactant.

3. Determine the amount of residual hexadecane removed from sand packed columns using non-surfactant, commercial surfactant, and
biosurfactant solutions at different flow rates.

4. Calculate the capillary numbers corresponding to interfacial tension and flow rate with the reduction of residual hexadecane saturation by non-surfactant, commercial surfactant, and biosurfactant solutions to determine the capillary number necessary for removal of residual hexadecane.

1.4 Experimental Overview

Residual saturation of $^{14}$C-hexadecane was established in four sand packed columns, and each was flushed with a mineral salt solution as a control to determine the amount of residual removal by a non-surfactant solution with a high interfacial tension against hexadecane. Following the application of the mineral salt solution, a commercial surfactant, 1.2% (w/w) Neodol 25-9 in the mineral salts solution (above the critical micelle concentration), and a biosurfactant, produced by Rhodococcus species H13-A and resuspended in the mineral salt solution, were each introduced into two of the columns.

Removal of hexadecane was monitored in the presence of the surfactants, and then compared to the control and between the two surfactants. The combined effect of lower interfacial tension and increasing flow rates on hexadecane removal was determined during the surfactant applications. Capillary numbers were calculated for each solution at each injection velocity to determine the capillary number necessary for mobilization of residual hexadecane for the experimental set up. The solubility of $^{14}$C-hexadecane in the control and surfactant solutions was experimentally elucidated to allow determination of the amount of hexadecane removed by solubilization versus mobilization.
CHAPTER 2

Literature Review

2.1 Non-Aqueous Phase Contamination of the Subsurface

There are various ways by which contaminants are introduced into soil and ground water. Improper disposal of hazardous wastes, leaking storage tanks, and agricultural runoff are only a few. Many contaminants are non-aqueous phase liquids (NAPLs) which may serve as continuing sources of contamination as they slowly solubilize into the ground water (Sitar et al., 1987; Hunt et al., 1988; Mercer and Cohen, 1990; Conrad et al., 1992). Entrapped NAPLs often are the largest volume of liquid contaminants at a site (Mercer and Cohen, 1990; Conrad et al., 1992), and pump and treat technology does not effectively remove these materials from the subsurface. Potential estimates of 100 to 200 years for contaminant removal with pump and treat technology in some cases, make the method financially unfeasible (Mansell et al., 1993). Therefore, the removal of NAPLs during cleanup of a site is difficult but imperative.

NAPLs tend to move as a separate phase due to their low solubility in water. Determination of their location and movement through the subsurface depends on whether they are lighter (LNAPL) or denser (DNAPL) than water. Several factors affecting migration through the soil include the volume of contaminant released, the duration of the release, the area infiltrated, the properties of the NAPL and subsurface media, and the ground water flow conditions (Mercer and Cohen, 1990; Conrad et al., 1992). If enough NAPL is released to cause local saturation, gravity will cause it to migrate downward through the unsaturated zone regardless of the density of the NAPL (Sitar et al.,
1987; Mercer and Cohen, 1990). Areas with the highest permeabilities are the most favorable paths for NAPL infiltration. As the contaminant percolates through the soil some lateral spreading may occur, and a portion may remain trapped in the pore spaces as ganglia, which are noncontinuous blobs occupying one to several adjoining pore spaces (Payatakes, 1982), or as large lenses of contamination (Sitar et al., 1987). The saturated zone will eventually be encountered by some of the NAPL if sufficient volume is released. Once there, DNAPLs and LNAPLs differ in behavior.

LNAPLs may become concentrated at the top of the water table by spreading laterally along layers of lower permeability at the capillary fringe (Schwille, 1967) and may even cause lowering of the water table (Mercer and Cohen, 1990). Fluctuations in ground water levels caused by seasonal rainfall, discharge, and pumping can lead to increased contamination of the saturated zone by allowing the LNAPL to fall and rise with the water table. After drawdown of the water level and accompanying LNAPL, water table recoveries can leave some of the LNAPL entrapped in pore spaces within the saturated zone (Schwille, 1967; McKee et al., 1972; Thornton, 1980; Conrad et al., 1992). These contaminants can, therefore, potentially be found in the soil above the water table, floating on top of it, and entrained in the pore spaces within the saturated zone.

DNAPLs, once they overcome the entry pressure of the water table, will continue to move down through the saturated zone via gravity and pressure forces (Mercer and Cohen, 1990). DNAPLs favor migration through zones of higher permeability and fractures in less permeable layers until coming to rest upon a barrier of sufficiently low permeability (Sitar et al., 1987; Mercer and Cohen, 1990; Conrad et al., 1992).
Capillary, viscosity, and gravity or buoyancy forces control NAPL behavior in ground water (Conrad et al., 1992). Fluid potential and physical properties, such as interfacial tension, density, and viscosity initially control NAPL movement (Hunt et al., 1988). Pore network geometry, fluid-fluid properties (such as interfacial tension, fluid viscosity ratio, density ratio, and phase behavior), fluid-rock interfacial properties, pressure gradients, and gravity/buoyancy forces determine the amount of NAPL retained and the microscopic mechanism of trapping (Morrow and Songkran, 1981; Mercer and Cohen, 1990).

The term residual saturation refers to the amount of organic liquid remaining in the pore spaces after migration and is often represented as the percent void pore space occupied by NAPL. It is the saturation at which under normal ground water flow conditions the NAPL is discontinuous and trapped in place via capillary forces. These forces are represented by the capillary number \( (N_C) \), the ratio of viscous to capillary forces:

\[
N_C = \frac{\nu \mu}{\gamma}
\]  

where \( \nu \) is the interstitial velocity of the ground water (cm/s) (the Darcy velocity divided by porosity), \( \mu \) is the viscosity of water (dyne s/cm\(^2\)), and \( \gamma \) is the interfacial tension between water and the NAPL (dyne/cm). Removal of residual saturation increases at capillary numbers around \( 10^{-3} \) with essentially 100% recovery at a capillary number of \( 10^{-2} \), shown to correspond to an interfacial tension of \( 10^{-2} \) to \( 10^{-4} \) dyne/cm under reservoir conditions (Finnerty and Singer, 1983).
Buoyancy or gravitational forces can influence low interfacial systems and are represented by the bond number (NB), the ratio of gravity to capillary forces:

\[ NB = \frac{\Delta \rho \ g \ R^2}{\gamma} \]  \hspace{1cm} (2)

where \( \Delta \rho \) is the density difference between the water and NAPL (g/cm), \( g \) is the acceleration due to gravity (cm/s\(^2\)), and \( R \) is the radius of a medium particle (cm). In systems at normal water-oil interfacial tensions, the capillary forces which cause entrapment and prevent mobilization of residual NAPL are stronger than the buoyancy forces (Morrow and Songkran, 1981). Increasing the Darcy velocity and hydraulic conductivity in these systems will cause the release of a small fraction of the residual saturation. In order to move the residual through the pore spaces, the gradient must be sufficiently high (Wilson et al., 1984).

Morrow and Songkran (1981), using an aliphatic oil (Soltrol-130) as the wetting phase and air as the nonwetting phase, found that at capillary numbers less than or equal to around 10\(^{-6}\) and bond numbers less than 0.00667, capillary forces dominate and the residual saturation is constant. At capillary numbers of 10\(^{-3}\) and greater and bond numbers above 0.35, no trapping occurs and residual saturation depends on the combined effect of viscous and gravitational forces. They found that it is about five times easier to prevent entrapment of NAPL than to mobilize the NAPL once trapped.

Oil-air-water systems using oil as the nonwetting phase generally requires higher capillary numbers to prevent entrapment of nonwetting residuals than air-water systems with air as the nonwetting phase (Morrow et
al., 1988). However, prevention of trapping is easier to achieve than mobilization of the nonwetting phase in both oil-air and oil-water-air systems.

2.2 Surfactants

Improved removal of NAPLs from soil and ground water may be accomplished through the application of surfactants. Surfactants are surface active agents which contain a nonpolar hydrophobic tail and a polar hydrophilic head. There are several types of surfactants, based on the nature of the hydrophilic surface active group. Surfactants are considered anionic if the polar ionic group has a negative charge, cationic if it has a positive charge, nonionic if it has no charge, and zwitterionic if it has both positive and negative charges. The hydrophobic tail of the surfactant molecule is usually a long-chain hydrocarbon.

Biosurfactants, usually either neutral or negatively charged, are classified based on their nonpolar hydrophobic tail lipid types: neutral lipid, phospholipid, glycolipid, or lipopeptide (Cooper, 1986). Glycolipid-containing biosurfactants, usually consisting of diglycosyl diglycerides, trehalose, rhamnose, sophorose, or lipoteichoic acids, are the most common extracellular biosurfactants (Zajic and Seffens, 1984). Biosurfactants may be preferred over commerical surfactants for use in residual contaminant clean up because of their biodegradability, potential lower toxicity, potential for low cost production, allowance for genetically engineered specific and more effective properties, and their non-petroleum derivation as petroleum costs increase (Singer, 1985; Van Dyke et al., 1991). In addition to residual contaminant release, biosurfactants are also believed to play an integral role in the uptake of
hydrocarbon substrates by bacteria during biodegradation (Zajic and Seffens, 1984).

Surfactants remove liquid contaminants from the subsurface through solubilization and mobilization. Solubilization is the partitioning of the organic contaminant into the interior of a micelle. Micelles are aggregates of surfactant molecules of various types and occur at and above the critical micelle concentration (CMC). Micelles orient in aqueous solutions so as to minimize contact of the hydrophobic tails while increasing the contact of the hydrophilic heads with the aqueous phase. They vary in structure from spherical, cylindrical, and lamellar to vesicles. The shape of the micelles is important in determining properties of the surfactant solution, such as viscosity and capability to solubilize water-insoluble material (Rosen, 1989). The CMC is a function of the surfactant structure, temperature of the solution, concentration of added electrolytes, and concentration of solubilizes (Harwell, 1992). Interfacial tension, surface tension, osmotic pressure, detergency, and electrical conductivity can be used to experimentally determine the CMC, noted by a sudden change in the slope of a plot between the physical characteristic and concentration of the surfactant (Rosen, 1989). Generally, as the hydrophobic character of the surfactant increases, the CMC of the aqueous medium decreases (Rosen, 1989).

The location or combination of locations in a micelle where solubilization occurs is dependent primarily on the contaminant's water solubility (Harwell, 1992). Solubilization of the contaminant may occur in the nonpolar hydrophobic core of the micelle, on the polar surface, or in the palisade layer, a transition region between the core and the surface of the micelle (Harwell, 1992). With surfactant concentrations at or above the CMC, substantially larger
amounts of contaminants can be solubilized than in water alone (Miller and Neogi, 1985).

Mobilization removes the contaminant from the site of entrapment by releasing it into the aqueous phase and allowing it to migrate through the formation. At normal interfacial tensions between NAPLs and ground water, 30 to 50 dyne/cm (Wilson et al., 1990), large pressure drops between injection and extraction wells are needed to remove residual contaminants from subsurface pore spaces. Lowering the interfacial tension decreases the amount of pressure differential needed (West and Harwell, 1992) and recovery occurs at lower flow rates. Work by Patel and Greaves (1987) indicates that lowering the interfacial tension is necessary for initial mobilization. A relatively large increase in residual recovery occurs with only a small increase in viscosity, which can be caused by accelerating the flow rate, or with a small decrease in capillary forces, which can be obtained by lowering the interfacial tension. Mobilization can be riskier than solubilization for remediation because of free phase movement of the contaminant, but is more likely to result in more timely remediation (West and Harwell, 1992).

Selection of surfactant for use in remediation is dependent on three processes: the capability of micelles to solubilize the contaminants, the capability of the surfactant to produce ultra-low interfacial tensions of $10^{-3}$ or less, and the capability of the surfactant to maintain its activity in the subsurface (Harwell, 1992). In mobilization ultra-low interfacial tensions and minimized loss of surfactant must be considered. In solubilization only minimized surfactant loss is considered (Mansell et al., 1993). Surfactant sorption onto soil surfaces can influence surfactant performance and the amount required for remediation. Nonionic surfactants can sorb to soil and thereby cause the dose of surfactant to achieve solubilization to be greater than in aqueous systems.
without soil (Edwards et al., 1992). The use of surfactant adsorption curves along with other surfactant parameters, such as surface tension minimization, critical micelle concentration, and solubility efficiency, is the most accurate estimate of enhancing aquifer restoration by surfactants (Vigon and Rubin, 1989).

The use of commercial surfactants to aid in the removal of residual NAPLs has been demonstrated in the lab and to a lesser extent in the field. Nash and Traver (1986) used a two surfactant solution of ethoxyxlated fatty acid and ethoxyxlated alkyl phenol to remove 88% of Murban crude oil from a sand column in 10 pore volumes. In a pilot treatment operation, the expected reduction of contaminants to 50% of the original level was not obtained. A Texas Research Institute (1985) study compared three different surfactant application techniques to remove gasoline from a sand packed tank. Single percolation application, multiple percolation application, and multiple injection application removed 6, 76, and 83% respectively, of the gasoline using 2% Richonate YLA with 2% Hyonic PE-90.

A study comparing the removal of residual automatic transmission fluid from sand by ten commercial surfactants, including nonionics (ethoxyxlated alcohols and ethoxyxlated nonylphenols) and anionics (sulfates and sulfonates), found removal of the residual ranging from 23% by water to more than 80% by an ethoxyxlated alcohol (Abdul et al., 1990). A further study using an alcohol ethoxylate surfactant to wash residual levels of automatic transmission from sandy soil found 0.5, 1.0, and 2.0 percent solutions of surfactant removed 55, 60, and 72.8 percent of the residual. Water alone only removed 25.5% (Ang, 1991).

Solubilization can be used without mobilization for removal of DNAPLs, such as tetrachloroethylene, from horizontal sand packed columns using a
surfactant solution designed with a lowered interfacial tension of 5 dyne/cm or less (Fountain et al., 1991). West (1992) found the solubilizing efficiencies of the nonionic surfactants Triton X-100, Brij-30, Igepal CA-720, and Tergitol NP-10 as basically the same for enhanced solubilization of tetrachloroethylene, trichloroethylene, and 1,2-dichloroethylene. In another study, solubilization of dodecane by a nonionic surfactant, polyoxyethylene sorbitan monooleate, removed five orders of magnitude of oil from sand packed columns (Pennell et al., 1993).

Bacterially produced surfactants have been used in microbially enhanced oil recovery (MEOR) for decades. The indigenous bacteria are stimulated to produce biosurfactants in situ or biosurfactants are produced above ground and injected into the formation (Van Dyke et al., 1991). The two mechanisms by which MEOR primarily work are selective plugging of the formation and changing physical and chemical properties of reservoir fluids (Knapp et al., 1991). Plugging of high permeability zones can occur by accumulation of either non-viable or viable cells. Increases in residual removal can occur due to the production of gases such as carbon monoxide as microbial by-products, and from the production of biosurfactants which can lower the interfacial tension at the oil-water interface (Knapp et al., 1991). In an unconsolidated thin reservoir flow cell for microscopic observation, in situ growth of injected bacteria, Bacillus licheniformis, Pseudomonas aeruginosa, Clostridium acetobutylicum, decreased residual oil saturation by 9 to 24% (Kianipey and Donaldson, 1986). From MEOR work extends the use of biosurfactants for clean up of the contaminated subsurface.

Pilot plant production of a rhamnolipid biosurfactant by Pseudomonas aeruginosa followed by anion-exchange chromatography, yielding 60% of pure activated product, demonstrated the capacity for substantial production without
large scale extraction (Reiling et al., 1986). Pilot plant production of bacterium Pet 1006, grown on 2% (v/v) hydrocarbon, yielded a culture broth that, after sterilization, had a surface tension of 33.5 dyne/cm in synthetic deposit water, and recovered 91% crude oil from cleanup of an oil storage tank at a worth of $110,000 (Banat et al., 1991). Biosurfactants can also reduce the viscosity of crude oil. Bacterial *Rhodococcus* isolate H-13 reduces the viscosity up to 98% (Singer, 1983).

Removal of bitumen from Utah tar sands using whole culture broth containing biosurfactants from *Acinetobacter calcoaceticus*, *Corynebacterium fasicans*, *Corynebacterium lepus*, and *Arthrobacter* SFC has been shown to be just as effective as synthetic surfactants (Zajic and Akit, 1983). Biosurfactants produced from bacterial growth on gasoline and a mixture of glucose with vegetable oil acted similarly to commercial surfactants by increasing the solubility of contaminants (Falatko and Novak, 1992). Arthrofactin, a biosurfactant isolated from *Arthrobacter* species MIS 38 has been reported as a "better oil remover" than the biosurfactant surfactin and commercial surfactants Triton X-100 and sodium dodecyl sulfate (SDS), based on the measured cleared area of an oil-agar plate by each surfactant, even with increasing surfactant amounts up to 17 nM (Morikawa et al., 1993).

Comparison of eight extracellular biosurfactants from various bacterial strains showed *Pseudomonas aeruginosa* UG2 and *Acinetobacter calcoaceticus*-Rag-1 to partition hydrophobic compounds, such as hexachlorobiphenyl, into the aqueous phase of soil slurries at 48.0 and 41.9% respectively. Results suggest that the *Pseudomonas aeruginosa* UG2 biosurfactant has potential for remediation of soil containing hydrophobic contaminants (Van Dyke et al., 1993).
The rhamnolipid biosurfactant from *Pseudomonas aeruginosa* removed 23-59% of the hydrocarbon from unsaturated soil columns which were not at residual saturation. Removal was a function of increasing surfactant concentration and was augmented by the addition of sodium pyrophosphate (Scheibenbogen et al., 1994). Whole culture broth of ST-5, *Rhodococcus*, grown on 2% (v/v) n-paraffin, removed 86% of residual crude oil from a sand packed column at 33-35% oil saturation, and was more effective than commercial surfactants 0.1% SDS, 1% Spolene, and 1% petroleum sulfonate with 0, 63, and 58% removal, respectively (Abu-Ruwaida et al., 1991). Whole culture broth of thermophilic *Bacillus* strain AB-2, grown on 2% (v/v) oleic acid with an interfacial tension between the two liquids of less than 1.5 dyne/cm, removed 95% of the light crude oil from a sand packed column (Banat, 1993).

The use of biosurfactants for the removal of residual oil is an area with many possibilities in research and field application. Since not all biosurfactants have been found to be useful because of their tendency to plug pores and be removed from solution, the effectiveness of each biosurfactant must be determined before use in the field. Work in this area can be very useful for the clean up of contaminated aquifers with residual NAPLs.
CHAPTER 3

Materials

3.1 Media and Chemicals

Liquid cultures of *Rhodococcus* species H13-A culture were grown in 0.8% nutrient broth with 0.5% yeast extract (NBYE, Difco Laboratories, Detroit, MI). Agarose plates and stock slants were prepared by adding 1.6% Bacto agar (Difco). Basal salts minimal medium E (BSE) (Finnerty and Singer, 1984), the chemically defined medium used for the column control flush and for growth of cultures when amended with hexadecane, consisted of: 10 g K$_2$HPO$_4$, 5 g NaH$_2$PO$_4$, 2 g (NH$_4$)$_2$SO$_4$, 200 mg MgSO$_4$·7H$_2$O, 1 mg CaCl$_2$·2H$_2$O, 1 mg FeSO$_4$·7H$_2$O at pH 7.0, amended with 0.05 mM thiamine (Singer et al., 1990) per liter water. The water used for media preparations and final rinses of glassware was deionized and passed through a Milli-Q$^{50}$ ultra-pure water system (Millipore, Bedford, MA) at 18 MΩ. Phosphate buffered saline (PBS), consisting of 1.24 g Na$_2$HPO$_4$, 0.18 g NaH$_2$PO$_4$·H$_2$O, and 8.5 g NaCl at pH 7.0, was used for all dilution series. All chemicals used were reagent grade, except NaH$_2$PO$_4$ which was enzyme grade.

Glassware for the *Rhodococcus* species H13-A growth curves was cleaned for at least two hours in concentrated sulfuric acid and Nochromix (Godax Laboratories Inc., Pawling, NY), an inorganic oxidizer. Acid washed glassware was rinsed twelve times with deionized water followed by three rinses with Milli-Q water. All other glassware, unless otherwise noted, was cleaned in a 2% solution of Micro (International Products Corp., Burlington, NJ) overnight at room temperature or for 2 hours at 66°C, and was rinsed four to ten times with deionized water followed by three to five rinses with Milli-Q water.
Column apparatus was cleaned in 2% RBS 35 Detergent Concentrate (Pierce, Rockford, IL) at 50°C, rinsed five times with deionized water, and followed by three rinses in Milli-Q water. Column apparatus with radiolabelled hexadecane residue was cleaned using two parts 2% RBS 35 Detergent Concentrate and one part ethanol. The 50 ml beakers used for interfacial tension readings with the duNoüy ring tensiometer were either acid washed or cleaned in 2% Micro solution at 66°C.

3.2 Microorganism and Biosurfactant

3.2.1 *Rhodococcus* species H13-A

The bacterium that produced the biosurfactant used to remove hexadecane in the column studies was received from W. R. Finnerty (Finnerty Enterprises, Inc., Athens, GA). It was originally isolated from oil enriched soil via several passages on hexadecane enrichment medium (Finnerty and Singer, 1984) and identified as a *Rhodococcus* species (Singer and Finnerty, 1990). Speciation of the bacterium was performed using Biolog MicroStation System, Release 3.01 (Biolog Inc., Hayward, CA).

*Rhodococcus* species H13-A cells were gram-positive nocardioforms, which exhibit rod to coccal appearance during growth on complex medium. The culture had a strictly oxidative metabolism, and growth on chemically defined medium required thiamine (Singer and Finnerty, 1990). Liquid growth cultures of the organism in NBYE and 2% (v/v) hexadecane in BSE formed sponge-like orange-beige aggregates at the surface, while cultures grown on NBYE plates formed beige, slick, mucoid colonies.
Finnerty and Singer (1984) reported alkanes (C8 to C20) and paraffin-containing crude oils supported growth, but significant levels of biosurfactant production occurred only when grown on n-alkanes and fatty alcohol (Singer and Finnerty, 1990). Duston (1995) reported limited biosurfactant production by this isolate, indicated by reductions in surface tension, when grown on yeast extract.

### 3.2.2 Biosurfactant

The extracellular biosurfactant isolated from *Rhodococcus* species H13-A was a mixture of glycolipids consisting of trehalose and an intricate array of fatty and mycolic acids (Singer et al., 1990). The extracellular glycolipids, extracted from spent culture medium, were a mixture of trehalose derivatives (Table 3.1) with an ionization constant at 5.5, indicative of an anionic surfactant. The extracellular biosurfactant produced from growth on hexadecane exhibited a critical micelle concentration (CMC) of 1.5 mg/ml with an interfacial tension against hexadecane of 0.25 dyne/cm (Singer et al., 1990). Measurements against a range of n-alkanes showed the minimum interfacial tension of the extracellular biosurfactant to be against decane (2.0 x 10⁻² dyne/cm) and, with the presence of 0.5% pentanol as a co-surfactant, against undecane (6.0 x 10⁻⁵ dyne/cm). Growth on decane did not produce as large a quantity of biosurfactant as growth on hexadecane (Finnerty and Singer, 1984).

Conditions in the culture medium were linked to extracellular biosurfactant production (Singer and Finnerty, 1990). Nitrogen limitations were connected to synthesis and release, while increased concentrations of hexadecane and depletion of ammonium ions were coupled with increased biosurfactant concentrations.
Table 3.1. Composition of extracellular glycolipid biosurfactant (Singer et al., 1990).

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Relative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2'-Diacyltrehalose</td>
<td>7.0</td>
</tr>
<tr>
<td>6,6'-Diacyltrehalose</td>
<td>2.3</td>
</tr>
<tr>
<td>4,4'-Diacyltrehalose</td>
<td>3.9</td>
</tr>
<tr>
<td>2,3,2',3'-Tetraacyltrehalose</td>
<td>17.0</td>
</tr>
<tr>
<td>2,4,2',4'-Tetraacyltrehalose</td>
<td>10.0</td>
</tr>
<tr>
<td>2,6,2',6'-Tetraacyltrehalose</td>
<td>5.7</td>
</tr>
<tr>
<td>2,3,4,2',3',4'-Hexaacyltrehalose</td>
<td>10.0</td>
</tr>
<tr>
<td>3,4,6,3',4',6'-Hexaacyltrehalose</td>
<td>3.8</td>
</tr>
<tr>
<td>2,4,6,2',4',6'-Hexaacyltrehalose</td>
<td>2.2</td>
</tr>
<tr>
<td>2,3,6,2',3',6'-Hexaacyltrehalose</td>
<td>8.9</td>
</tr>
<tr>
<td>2,3,4,6,2',3',4',6'-Octaacyltrehalose</td>
<td>29.2</td>
</tr>
</tbody>
</table>

3.3 Commercial Surfactant

Several commercially produced surfactants, concentrations, and mixtures were considered for use in the column study (Table 3.2). Neodol 25-9, (Shell Chemical Co., Houston, TX) 1.2% (w/w) in BSE, was selected because its interfacial tension of 0.278 dyne/cm against hexadecane, as experimentally determined by a University of Texas Model 300 spinning drop tensiometer (University of Texas, Austin, TX) at 30°C, was closest of those tested to the reported value of 0.25 dyne/cm for the Rhodococcus species H13-A produced biosurfactant (Singer et al., 1990). All Neodol 25-9 solutions used in this study were 1.2% (w/w) in BSE, which was below the CMC. Neodol 25-9, a non-ionic alcohol ethoxylate, contained C_{12} to C_{15} linear alcohols with an average of 9 ethoxylates per alcohol, and a CMC of 0.0018 mg/ml. It biodegrades rapidly (Shell, 1993) and would, therefore, be a good choice for actual use in ground water remediation.
Table 3.2. Surfactant concentration (% w/w in BSE), mixture, and interfacial tension measurements against hexadecane.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Interface Tension (dyne/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Tergitol 15-S-5</td>
<td>0.12</td>
</tr>
<tr>
<td>0.1% Tergitol 15-S-7</td>
<td>0.18</td>
</tr>
<tr>
<td>0.1% Neodol 25-7</td>
<td>0.15</td>
</tr>
<tr>
<td>1.0% Neodol 25-9</td>
<td>0.30</td>
</tr>
<tr>
<td>1.1% Neodol 25-9</td>
<td>0.29</td>
</tr>
<tr>
<td>1.2% Neodol 25-9</td>
<td>0.28</td>
</tr>
<tr>
<td>Percent Mixtures of Neodol 25-7/Neodol 25-9</td>
<td></td>
</tr>
<tr>
<td>1.15/1.00</td>
<td>0.21</td>
</tr>
<tr>
<td>1.25/1.00</td>
<td>0.20</td>
</tr>
<tr>
<td>1.50/1.00</td>
<td>0.18</td>
</tr>
</tbody>
</table>

3.4 Column Medium and Apparatus

3.4.1 Medium and Characteristics

The porous medium used to pack the columns and entrap hexadecane was a 40/60 mesh Ottawa sand from the Jordan Formation, Ottawa, MN (Unimin Corp., New Canaan, CT). The reported typical mean percent retained on individual sieves was: 0.3 for 40 mesh, 23.7 for 45 mesh, 64.5 for 50 mesh, 10.8 for 60 mesh, and 0.7 for 70 mesh (Unimin, 1991). The average mean diameter, as determined by the Rice University Automated Sediment Analyzer, RUASA, (Anderson and Kurtz, 1979) was 0.352 ± 0.005 mm. The sand was sifted to insure complete mixing of particles before analysis.

The bulk density, particle density, porosity, and hydraulic conductivity were determined using sand that was dried in an oven at 105°C for 24 hours and cooled in a desiccator. The bulk density (ρb), the ratio of the oven dried mass to the volume of the medium, was determined using the equation:
\[ \rho_b = \frac{M_s}{V_b} \]  

(3)

where \( M_s \) was the mass of the oven dried sand, and \( V_b \) was the bulk volume. A graduated cylinder containing 30 ml water was the receptacle for 100.00 g sand, added in three increments with ten taps on the bench top between additions. A mean bulk density of 1.65 \( \pm \) 0.01 g/cm\(^3\) was obtained.

The particle density (\( \rho_p \)), the ratio of total sand mass to total volume (excluding pore spaces), was calculated using:

\[ \rho_p = \frac{\rho_w (W_s - W_a)}{(W_s - W_a) - (W_{sw} - W_w)} \]  

(4)

where \( \rho_w \) was the density of water; \( W_s \) the weight of sand and flask; \( W_a \) the weight of the flask (air only); \( W_{sw} \) the weight of the flask, sand, and water; and \( W_w \) the weight of the flask and water. A sample of 50.00 g of oven dried sand was added to a 100 ml volumetric flask filled with boiled/deaerated water, and the weight recorded. The volumetric flask weight was also recorded empty and with 100 ml water. The particle density was determined to be 2.63 \( \pm \) 0.01 g/cm\(^3\).

The porosity (\( f \)), the ratio of the volume of the pore spaces to the total void volume, was calculated using:

\[ f = 1 - \left( \frac{\rho_b}{\rho_p} \right) = \left( \frac{\rho_p - \rho_b}{\rho_p} \right) \]  

(5)
where \( \rho_b \) was the bulk density and \( \rho_p \) the particle density. Porosity can also be expressed as a percent, and a value of 37.26% was calculated for the 40/60 mesh sand.

The hydraulic conductivity (\( K \)), was calculated using the Carman-Kozney equation (Montgomery, 1985):

\[
K = \frac{\rho_w g f^3 d_c^2}{\mu(1-f)^2 180}
\]

(6)

where \( \rho_w \) was the density of water (g/cm\(^3\)), \( g \) the acceleration due to gravity (cm/s\(^2\)), \( f \) the porosity, \( d_c \) the mean particle diameter of the sand (cm), and \( \mu \) the viscosity of water (g/cm s). The hydraulic conductivity was determined to be 9.99\(\times10^{-4} \) cm/s.

The values obtained for the 40/60 mesh Ottawa sand corresponded well with values reported for Sevilleta soil, a well sorted, medium grained, aeolian sand with a similar mean particle diameter (0.352 mm for Ottawa sand and about 0.3 mm for Sevilleta sand), as cited by Wilson et al. (1990). The Ottawa and Sevilleta sand had, respectively, a bulk density of 1.65 \(\times10^{-2} \) and 1.656 \(\times10^{-2} \) g/cm\(^3\), a particle density of 2.63 and 2.65 g/cm\(^3\), and a porosity of 37.26% and 37.5%.

The sand used in Column 1 was rinsed three times in deionized Milli Q water. Since syringe pump problems, possibly due to plugging, were encountered in Column 1, Columns 2, 3, and 4 used base/acid washed sand. Following the protocol of Veerapaneni (1991), about 300 cm\(^3\) of sand were placed in a 1 L beaker, washed and stirred seven times with deionized water, and then sonicated (Cole-Parmer 8890, Niles, IL) for 10 minutes in 0.01 M NaOH. After twelve rinses in deionized water, the sand was sonicated 10
minutes in 1 M HNO₃ followed by another twelve deionized water rinses. The sand was then stirred and autoclaved for 30 minutes on three consecutive days.

3.4.2 Column Design

The four chromatography columns (Spectrum, Houston, TX) used in this study were made of borosilicate glass, with 2.68 cm inner diameter and 15 cm in length (Figure 3.1). All fittings were Teflon. A 0.22 μm opening 25 mm diameter Magna nylon filter (MSI, Westboro, MA) was epoxied (Hardman Inc., Belleville, NJ) into the bottom endfitting after the Teflon had been chemically etched (Tetra Etch, R.S. Hughes Co. Inc., Houston, TX). The filter was covered by a 316 stainless steel screen (Anderson Wire Works, Inc., Houston, TX) with 0.177 mm openings to prevent clogging with sand. An adjustable height plunger was placed on top of the sand pack and was also covered with a stainless steel screen secured in place with Teflon tape.

The columns were operated with an upwards flow. The influent was pumped through one end of a three-way valve at the bottom of the column using a Harvard Apparatus Syringe Infusion Pump 22 (Harvard Apparatus Inc., South Natick, MA). The influent and effluent tubing used in Column 1 consisted of 1/8 inch outer diameter Teflon tubing. Columns 2, 3 and 4 had effluent tubing of 1/16 inch outer diameter in order to prevent collection of hexadecane in the curve of the tubing which led to a Retriever II fraction collector (Isco Inc., Lincoln, NB) where samples were gathered in pre-weighed test tubes for analysis. Any hexadecane that may have been trapped in Column 1 effluent tubing was collected in the final sample when the tubing was disconnected from the fraction collector.
Figure 3.1 Apparatus used in column experiments.
CHAPTER 4

Methods

4.1 Experimental Design

A residual saturation of $^{14}$C-hexadecane was formed in each of four sand packed columns after tritiated water breakthrough and flush out were monitored to determine the pore volume. Each column was then flushed with BSE, as a control, followed by either Neodol 25-9 (1.2% w/w in BSE) or biosurfactant in BSE from *Rhodococcus* species H13-A.

The flow rate was increased during each column run. The rise in flow rates during the control runs accounted for hydraulic removal of residual hexadecane (Wilson et al., 1990). During both control and surfactant runs, the capillary number and reduction in residual hexadecane saturation were calculated for each flow rate. As the capillary number approached $10^{-3}$, mobilization of the hexadecane was expected to start. A capillary number of $10^{-2}$ was predicted to exhibit free flow of residual hexadecane.

Columns 1 and 2 were run with BSE and Neodol 25-9 at flow rates increasing from 1.29 ml/min to 12.9 ml/min for both solutions while Columns 3 and 4, used for control and biosurfactant runs, flow rates were increased from 1.00 to 10.0 ml/min. Neodol 25-9 flow rates were increased until the column back pressure caused the syringe pump to stop or to 26.6 ml/min, the highest flow rate permissible with the Harvard Apparatus Syringe Infusion Pump 22 (Harvard Apparatus Inc., South Natick, MA), and the biosurfactant flow rates were increased to 17.5 ml/min. Timed fractions were collected in pre-weighed test tubes in a Retriever II fraction collector (Isco Inc., Lincoln, NB). The samples were covered with pre-weighed caps, weighed, vortexed, and dumped into
tared scintillation vials containing 10 ml of Ready Safe scintillation cocktail (Beckman, Houston, TX). A Beckman LS 3801 Scintillation Counter was used for 5 minute counts of each sample. The percent of residual hexadecane removed, capillary number, and reduced residual saturation were calculated.

4.2 Measurements of Interfacial Tension

Interfacial tension reductions were indicative of biosurfactant production. At the minimum interfacial tension, the concentration of the surfactant, whether biological or synthetic, was considered to be at or above the critical micelle concentration. Measurements of the interfacial tension were used to follow changes in biosurfactant production during bacterial growth, determine when biosurfactant extraction from growth cultures was performed, and determine the concentration of biosurfactant and commercial surfactant to use in column studies. Growth curves and early extraction readings were made from whole culture broth while later extraction readings were made from culture broth which had been centrifuged at 10,000 x g (Sorvall RC2-B, Dupont, Irving, TX or Eppendorf Centrifuge Model 5415, Fisher Scientific, Houston, TX) for 10 minutes at 4°C.

4.2.1 duNoüy Ring Tensiometer

There were several methods by which surface and interfacial tensions were measured. The ring method using a duNoüy interfacial tensiometer model 70545 (CSC Scientific, Fairfax VA) at 22°C was one method. Three samples of 8 to 10 ml were placed in a Micro or acid washed 50 ml beaker and allowed to sit for 30 minutes. A platinum-iridium ring was lowered halfway into the
aqueous solution after which an equal volume of hexadecane was carefully layered on top. The ring was then placed at the interface in order to begin the measurement. The ring was slowly pulled upward while the beaker of solution was lowered so as to keep the lever arm index of the apparatus at zero. The tension at which the ring broke into the upper phase was read from the scale and recorded as the apparent interfacial tension in dyne/cm.

The apparent interfacial tension can vary from the true value by as much as 30% (Zuidema and Waters, 1941), but usually varies by less than 5% (CSC duNoüy Tensiometer Manual). This is accounted for and adjusted with a correction factor dependent on the size of the ring wire, the circumference of the ring, the maximum pull on the ring and the density difference of the two liquids (Zuidema and Waters, 1941). Zajic et al. (1983) noted this method as accurate above 1.0 dyne/cm while CSC Scientific (personal communication) reported it accurate above 5.0 dyne/cm.

4.2.2 Spinning Drop Tensiometer

A University of Texas Model 300 spinning drop tensiometer (University of Texas, Austin, TX) at 30°C was used for interfacial tensions approximately equal to or less than 1.0 dyne/cm. The sample cell was filled with the more dense fluid (surfactant solution), one drop of the less dense fluid (hexadecane) was placed in the center, and the cell spun. Once the drop had reached the proportion of length at least four times the width, it was assumed to be cylindrical and measurements began. By assuming the shape was an elongated cylinder, the interfacial tension could be calculated without knowing the exact drop shape (Zajic et al., 1983). The height (width) of the drop, measured with the eyepiece micrometer, and the rotational velocity were
recorded over time, and the interfacial tension was calculated from the equation:

$$\gamma = \left(\frac{\pi^2}{8}\right) \left(\frac{d}{\eta}\right)^3 \left(\frac{\Delta \rho}{p^2}\right) \left(\frac{10^6}{\text{millisec}}\right)$$  (7)

where $\gamma$ was the interfacial tension (dyne/cm), $d$ was the diameter of the drop (cm), $\eta$ was the refractive index of the denser solution, $\Delta \rho$ was the density difference between the solutions (g/cm$^3$), and $p$ was the mean rotational velocity (millisec) (Miller and Neogi, 1985). The interfacial tension was then plotted against time.

When the interfacial tension between the purified diluted biosurfactant solution and hexadecane was measured using the spinning drop, problems were encountered with the biosurfactant suspension. A consistent reading could not be obtained. The measured interfacial tension was decreased initially but then increased over time. If surfactant aggregates were formed, they could have spun towards the outside of the sampling tube, away from the interface. The removal of biosurfactant from the interface may have resulted in the later increased interfacial tensions.

4.3 Speciation and Growth of Rhodococcus species H13-A

4.3.1 Biolog MicroStation System

The bacterial isolate, Rhodococcus species H13-A, was speciated or classified using Biolog MicroStation System, Release 3.01 (Biolog Inc., Hayward CA). An inoculum of Rhodococcus species H13-A was streaked onto
Biolog Ultimate Growth Medium and incubated overnight at 30°C. At 24 hours, a single colony was transferred from the plate with a sterile cotton swab into 20 ml of 8.5 g NaCl per L Milli-Q water. The suspension was vortexed and more inoculum added until an absorbance of 37 to 42 was reached on the Biolog Turbidimeter (590 nm). Each of the 96 wells in the GP MicroPlate was loaded with 150 μl of the bacterial suspension and incubated at 30°C. The wells in the GP MicroPlate contained tetrazolium violet, a redox dye, and various carbon sources. Utilization of carbon sources resulted in a purple (positive) color change while unutilized solutions of carbon sources remained clear (negative). The plate was visually read at 4 and 24 hours, and the results compared with the MicroLog 2 software.

4.3.2 Growth of *Rhodococcus* species H13-A

The paper disk impregnated with *Rhodococcus* species H13-A received from W.R. Finnerty (Finnerty Enterprises, Inc., Athens, GA) was placed in 100 ml NBYE on an Eberbach reciprocating shaker (Eberbach Corp., Ann Arbor, MI) at 1/2 inch stroke, 100 cycles/min at 28°C. After 4 days, 100 μl were transferred into 100 ml of 2% (v/v) hexadecane in BSE. The culture was maintained on NBYE agarose slants at 10°C. Two stock slants were transferred every month or two to prevent loss of the bacterial strain. One was used for inoculating culture broth as needed while the second one was used for stock transfer only.

Several growth curves were performed where the inoculum and set up varied. The viable cell count (colony forming units/ml) and interfacial tension (dyne/cm) were monitored in all cases. The viable cell counts were performed via dilution series and plating on NBYE agarose plates. Three 9 ml samples were collected for interfacial tension measurements using a duNoüy ring
tensiometer. When the spinning drop tensiometer was used, an additional 9 ml were drawn off and centrifuged for 20 minutes at 12,000g at 4°C on a Sorvall RC2-B centrifuge (Dupont, Irving, TX).

Growth curve 6/20 was performed in a 1 L Erlenmeyer flask containing 200 ml of 2% (v/v) hexadecane in BSE. A loopful of inoculum was taken from the working stock slant and placed in 10 ml PBS, vortexed, and 0.1 ml was inoculated into the 1 L flask. After the first 24 hours, aliquots were taken every 6 to 9 hours.

Growth curve 7/23 was performed in a 2 L Erlenmeyer flask containing 700 ml of 2% (v/v) hexadecane in BSE. Four consecutive 0.1 ml transfers of a growing culture were made every 12 hours before inoculation of the medium in the 2 L flask with 0.1 ml of the culture. W.R. Finnerty (personal communication) reported 12 hour transfers to increase the number of log phase cells and biomass. After the first 24 hours of the growth curve, samples were taken every 4, 6, or 12 hours.

Both growth curve cultures were compared to uninoculated controls which were sampled identically to the Rhodococcus species H13-A cultures. The growth curve cultures were all grown at 28 to 30°C on an Eberbach reciprocating shaker at 100 cycles per minute, 1/2 inch stroke.

4.4 Biosurfactant Extraction and Quantification

4.4.1 Culture Conditions

A foam stoppered 250 ml Erlenmeyer flask containing 50 ml NBYE was inoculated with Rhodococcus species H13-A from a NBYE stock slant. The culture flask was placed on a rotary shaker (New Brunswick, Fisher Scientific,
Houston, TX) at 150 to 250 rpm, 28°C. After 2 to 6 days, 20 ml were transferred into 29 ml BSE and 1 ml hexadecane (50 ml total). After 2 days, 25 ml were transferred into 24 ml BSE and 1 ml hexadecane. The 25 ml transfers were continued every 8 to 12 hours for 2 to 3 days before inoculation for biosurfactant extraction. Transfers were initially performed every 12 hours but were changed to every 8 hours to further increase the concentration of cells. The serially transferred cultures then provided 100 ml inoculum into each 2 L Erlenmeyer flask containing 390 ml BSE and 10 ml hexadecane.

Several methods of culturing the *Rhodococcus* species H13-A in batch, to maximize biosurfactant production for subsequent extraction, were tested. A 13 L carboy was filled with 12 L BSE and 2% hexadecane. Oxygen was provided by air sparging using an aquarium pump at 28°C, and the culture was stirred. This large scale growth culture provided very low biosurfactant yields, most likely due to low oxygen levels in the system and not allowing the culture a long enough growth time. One liter flasks of 500 ml medium and inoculum were also tried. The flasks were maintained at room temperature and were equipped with one aquarium pump per two flasks and stir bars on stir plates to provide mixing of the oxygen. Interfacial tension reductions occurred much slower, and after 21 days were still not as low as the 2 L flask set up. Therefore, the 2 L flasks containing 500 ml medium were used to produce the biosurfactant in batch.

4.4.2 Biosurfactant Chloroform Extraction

The extraction of the biosurfactant from the growth culture and subsequent chloroform phase was modified from the protocol from Singer et al. (1990). Once the interfacial tension of the growth culture against hexadecane
decreased to 0.01 dyne/cm against decane or 0.3 dyne/cm against hexadecane, the culture was extracted. The pH of the growth medium was lowered to 5.5 using hydrochloric acid, and the bacterial suspension centrifuged at 10,750 rpm for 30 minutes at 4°C. The supernatant fluid was removed into Erlenmeyer flasks, and equal volumes of chloroform were added. The mixture was placed on a rotary shaker overnight at 250 rpm. The more dense chloroform phase was separated from the cell-free growth culture using a separatory funnel. Once most of the chloroform had been drawn off, the remainder of the chloroform/cell-free growth culture was centrifuged at 5,000 rpm for 5 min at 4°C to further distinguish the phases at the interface to aid in separation. A second extraction of the supernatant with half volume chloroform was performed, and the chloroform phases from both extractions were combined.

The chloroform phase, containing the lipids from the culture medium including the glycolipid biosurfactant, was then dried with anhydrous Na₂SO₄ and filtered through a Whatman No. 1 paper filter (Whatman International Ltd., Maidstone, England). A flash-evaporator (Buchler Instruments, Fort Lee, NJ) reduced the volume to near dryness, and the rest was evaporated in a fume hood. The residue was resuspended in 5 ml chloroform and introduced into a silicic acid chromatography column.

4.4.3 Silicic Acid Chromatography

Silicic acid chromatography columns were used to separate the glycolipid biosurfactant from the neutral lipids and phospholipids in the chloroform residue (Makula and Finnerty, 1970). The glass chromatography columns (Dave Lanman, Chemistry Glass Shop, Rice University) were 0.22 cm
inner diameter by 30 cm in length with a 20 mesh fritted disk above the stopcock to prevent the silicic acid from leaving the column. The Unisil 100 - 200 mesh silicic acid (Clarkson Chemical, S. Williamsport, PA) was activated by heating at least 8 hours in a 100°C oven. Silicic acid, 40 g, was weighed out, and chloroform was added to form a slurry. The slurry was poured incrementally into the column, and the column was tapped between additions to remove any trapped air and to tightly pack the medium. The silicic acid was then washed with 200 ml of chloroform, and the 5 ml chloroform suspended residue was loaded onto the column. The neutral lipids were removed with a flush of approximately 10 pore volumes of chloroform (800 ml) while 800 ml of acetone were used to remove the glycolipid fraction containing the biosurfactant followed by 800 ml of methanol to flush out the phospholipids, and thereby cleaning the silicic acid for reuse.

The acetone was removed from the glycolipid by rotovaporization. The glycolipid biosurfactant residue was resuspended in chloroform and was transferred to a sterile, pre-weighed screw cap test tube. The chloroform was allowed to evaporate. Once the biosurfactant dry weight was recorded, the residue was resuspended in 5 ml BSE using vigorous vortexing and heating of the test tube in a water bath.

4.4.4 Biosurfactant Concentration

The biosurfactant BSE solutions, both those extracted in the lab and those provided by Dr. William F. Finnerty (Finnerty Enterprises, Inc., Athens, GA), were all combined in a 50 ml test tube. The majority of the biosurfactant was then transferred to a bottle with a final volume of 84 ml. Some pellets of biosurfactant that would not resuspend were left in the 50 ml test tube with 5 to
10 ml of BSE. A 4 L solution of $1.93 \times 10^{-3}$ dilution of the stock was used for the biosurfactant column experiments and solubility test. The interfacial tension of the biosurfactant against hexadecane was taken to be 0.25 dyne/cm, as reported by Singer et al. (1990) because a consistent reading could not be obtained from the spinning drop tensiometer.

4.5 Column Packing and Pore Volume Determination

4.5.1 Column Packing

Four sand packed columns were used for control flushes followed by the experimental surfactant flushes. The column apparatus was autoclaved prior to packing. The integrity of the nylon filter in the bottom endfitting was tested by drawing water through the filter under a 120 mm Hg vacuum. After water was seen in the tubing leading to the vacuum pump, the endfitting was lifted from the water and the tubing observed for signs of air bubbles, indicative of a breach in filter integrity. If no air bubbles were present, the filter was considered sound and was used (Wilson et al., 1990). Each column was packed with 50.000±0.003 grams of sand. The column was filled with approximately 20 ml of sterile water. The sand was added incrementally with 10 taps of the column on the bench top in the sterile hood between additions. The plunger was then inserted and adjusted to sit on top of the sand pack. The column was perfused with 60 ml of sterile water to flush the column at 0.645 ml/min for columns 1 and 2 and 1.00 ml/min for Columns 3 and 4, and the dead volume determined.
4.5.2 Tritiated Water Breakthrough

The pore volume of each column was determined by using a tritiated water breakthrough curve. The columns were perfused with $^{3}$H$_{2}$O (5μC/L) (Sigma Chemical, St. Louis, MO) after the initial water flush at the same flow rate: 15 ml for Columns 1 and 2; 23 ml for Columns 3 and 4. Samples were collected over 75 ml of effluent. Aliquots of 500 μl were taken from select samples, suspended in 10 ml Ready Safe scintillation cocktail (Beckman, Houston, TX), and counted for 10 minutes on a Beckman LS 3801 Scintillation Counter. The concentration of tritiated water in the effluent was normalized to the initial concentration (C₀), and the cumulative volume (ml) versus the normalized effluent concentration (C/C₀) was graphed. The pore volume was determined at the breakthrough of the tritiated water (defined as 0.5 C/C₀).

4.6 Entrapment of Residual Hexadecane

A residual of 0.15 μC/ml $^{14}$C-hexadecane (Sigma Chemical, St. Louis, MO) was trapped in the sand packed column following the technique of Wilson et al. (1990). The stem of a Swagelok TFE sealed quick-connect valve (Houston Center Valve & Fitting Co., Houston, TX) was connected to the top of the column and the valve body to a side arm port on the lower end of a 50 ml screw top glass bottle (Dave Lanman, Rice University Chemistry Glass Shop, Houston, TX) containing $^{14}$C-hexadecane via Tygon F-4040A tubing (Fisher Scientific, Pittsburgh, PA). Using gravity, the water in the column was displaced by hexadecane. The Magna nylon filter in the endfitting retained the hexadecane. The column was weighed after the initial water flush (M₁) and during the organic flush (M₂). The hexadecane was allowed to flood the
column for at least 24 hours and was stopped once the mass of the column \( M_2 \) remained constant. The column was then flooded with sterile water at a rate of 0.645 ml/min for columns 1 and 2, and at 0.500 ml/min for columns 3 and 4, until the non-residual hexadecane had been removed and the column mass remained constant \( M_3 \). At this point, the column was considered to contain only residual amounts of \(^{14}\text{C}\)-hexadecane.

4.6.1 Determination of Residual Hexadecane: Mass Difference

The volume of residual hexadecane \( V_{or} \) in the columns was calculated using the equation:

\[
V_{or} = \frac{M_1 - M_3}{\Delta \rho}
\]  

(8)

where \( M_1 \) was the mass of the column full of water before the hexadecane flood, \( M_3 \) was the mass of the column at residual hexadecane, and \( \Delta \rho \) was the density difference between water and hexadecane. The percent residual saturation \( S_{or} \), the volume of discontinuous entrapped hexadecane per void volume, was calculated from the difference of the column masses using equation:

\[
S_{or} = \frac{M_1 - M_3}{\Delta \rho \ V_p} = \frac{V_{or}}{V_p}
\]  

(9)

where \( V_{or} \) was the volume of residual hexadecane, and \( V_p \) was the pore volume of the column.
The calculated volume and saturation of residual hexadecane, using equations (6) and (7), were limited by the capability of the Mettler P2000 balance (Mettler, Inc., Princeton, NJ) to record only to 0.1 gram and the potential operator error in reading the balance. The column apparatus was too heavy to use a more sensitive balance. The accuracy of the column mass and precision of readings could have had a considerable effect on the determined amount of residual hexadecane (potentially 0.2 g error per reading). A discrepancy of only 0.1 g would change the volume of residual hexadecane by 0.45 ml (Table 4.1). Therefore, another method for volume and saturation of residual hexadecane, by destructive sampling of the sand pack, was investigated.

<table>
<thead>
<tr>
<th>mass difference (g)</th>
<th>volume hexadecane (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.79</td>
</tr>
<tr>
<td>0.5</td>
<td>2.23</td>
</tr>
<tr>
<td>0.6</td>
<td>2.68</td>
</tr>
<tr>
<td>0.7</td>
<td>3.13</td>
</tr>
<tr>
<td>0.8</td>
<td>3.57</td>
</tr>
<tr>
<td>0.9</td>
<td>4.02</td>
</tr>
<tr>
<td>1.0</td>
<td>4.46</td>
</tr>
<tr>
<td>1.1</td>
<td>4.91</td>
</tr>
</tbody>
</table>

4.6.2 Determination of Residual Hexadecane: Destructive Sampling

Columns 2 and 4 were destructively sampled after the studies were complete to determine the amount of hexadecane remaining in each column and to back calculate the initial amount. The hexadecane remaining in the sand in columns 2 and 4 after the runs was determined using a biological
material oxidizer, OX-600 (R.J. Harvey Instrument Corp., Hillsdale, NJ). The technique was not available for Columns 1 and 3. All 50 g of sand from each column was tested, using samples of approximately 2 g of sand per reading. The biological material oxidizer combusted each sample with oxygen at 900°C, passed the products through a series of catalysts at 680°C, bubbled the resultant carbon dioxide through 15 ml of Carbon Trapping Solution, and the sample was purged with nitrogen to remove any free oxygen which might inhibit counting efficiency. The Carbon Trapping Solution was then placed in scintillation vials and counted for 10 minutes on a Beckman Scintillation Counter. The efficiency of the $^{14}$C-hexadecane biooxidation from Column 2 sand containing $^{14}$C-hexadecane and Neodol 25-9 was 69.9±2.0%, as determined from oxidation of a known volume of $^{14}$C-hexadecane in sand. Column 4 sand containing $^{14}$C-hexadecane and biosurfactant had a recovery efficiency of 97.6%. These efficiencies were used as correction factors in the calculation of the amount of hexadecane remaining in Columns 2 and 4.

After all of the sand in the column was sampled, the column apparatus was rinsed with acetone to remove any remaining hexadecane and the rinse and Teflon tape which held the top screen in place were counted. A wipe test was performed to insure the $^{14}$C-hexadecane was removed from the apparatus. The total amount of hexadecane initially in the column was determined by adding all the hexadecane found after completion of the experiments adjusted to reflect efficiency with the amount of hexadecane flushed out during the experiments. The values were compared with the initial amount of hexadecane determined from the column mass difference (8).
4.7 Solubilization of Hexadecane

The amount of hexadecane solubilized by BSE, Neodol 25-9, and the biosurfactant extracted from Rhodococcus species H13-A were experimentally determined. Duplicate scintillation vials with septum-lined, mininert-valve caps were filled with 1.5 ml $^{14}$C-hexadecane (0.15 µC/ml) and 10 ml of flush solution and mixed on a Burrell wrist-action shaker (Burrell, Inc., Pittsburg, PA). Control vials containing no $^{14}$C-hexadecane were also run. The vials were inverted and spun at 2000 rpm for 30 minutes on a bench top centrifuge (ICE, Fisher Scientific Co., Houston, TX) to remove the hexadecane from the cap and break emulsions. Duplicate aliquots of 150 µl were drawn off the aqueous phase of each sample with a 250 µl gas tight syringe through the septum of the inverted vial at 16 hours, 28 hours, and after sitting one week. Each aliquot was added to 1 ml Ready Safe Scintillation Cocktail, and counted for 20 minutes on a Beckman Scintillation Counter.

4.8 Removal of Residual Hexadecane

4.8.1 Velocity, Flow Rate, and Capillary Number Calculations

The flow rates used in the column studies were calculated from the velocity in the capillary number equation,

$$N_c = \frac{v \mu}{\gamma}$$

(10)

where $\mu$ was viscosity (dyne·s/cm²), $v$ was interstitial velocity (cm/s), and $\gamma$ was the interfacial tension (dyne/cm) between the flush solution and hexadecane.
Free mobilization occurs at capillary numbers around $10^{-2}$ (Finnerty and Singer, 1983; Elsik, 1987).

The viscosity was determined experimentally using a Fluids Rheometer RFR7800 (Rheometrics, Inc.). The viscosity (dyne·s/cm²) was found as the slope of rate ($g$) vs. stress ($\mu \cdot g$) ($\mu$ in poise). The viscosity of BSE, 0.01250 dyne·s/cm², was used for the biosurfactant.

The interfacial tension was determined using a duNoüy ring or a spinning drop tensiometer. Solving for the velocity necessary for freely flowing mobilization ($Nc = 10^{-2}$),

$$v = \frac{Nc \cdot \gamma}{\mu},$$  \hspace{1cm} (11)

the flow rate (ml/min), $Q$, was then calculated from,

$$Q = A \cdot v \cdot n$$ \hspace{1cm} (12)

where $A$ was the area of the column (5.64 cm²), and $n$ was the porosity (0.3726).

The actual capillary numbers for each flow rate in each column were calculated from equation (10) using the values listed in Table 4.2 for viscosity and interfacial tension. The velocity was calculated for each flow rate from the mean flow rate. The first and last fractions were discounted due to variations of flow from starting and stopping the syringe pump and fraction collector.
Table 4.2 Characteristics of flush solutions, including calculated velocities and flow rates necessary for a capillary number of $10^{-2}$

<table>
<thead>
<tr>
<th></th>
<th>BSE</th>
<th>Neodol 25-9</th>
<th>Biosurfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>viscosity (dyne*s/cm²)</td>
<td>0.01250</td>
<td>0.01286</td>
<td>0.01250</td>
</tr>
<tr>
<td>interfacial tension (dyne/cm)*</td>
<td>48.6</td>
<td>0.278</td>
<td>0.25</td>
</tr>
<tr>
<td>interstitial velocity (cm/s)</td>
<td>38.88</td>
<td>0.216</td>
<td>0.200</td>
</tr>
<tr>
<td>flow rate (ml/min)</td>
<td>4902.3</td>
<td>27.26</td>
<td>25.22</td>
</tr>
</tbody>
</table>

*against hexadecane

4.8.2 Control Flushes

Each column was flushed with BSE as a control. The calculated velocity needed to obtain a capillary number of $10^{-2}$ with an interfacial tension of 48.6 dyne/cm against hexadecane and a viscosity of 0.01250 dyne*s/cm² was 38.88 cm/s, or a flow rate of 4902.3 ml/min, which was infeasible in laboratory or field conditions. Therefore, the flow rates used in the control BSE flushes were the same as the experimental flow rates calculated and used for the Neodol 25-9 or biosurfactant flushes.

4.8.3 Columns 1 and 2: Neodol 25-9 Flushes

Columns 1 and 2 were flushed with Neodol 25-9 (1.2% w/w in BSE) after the control run. The flow rates used in columns 1 and 2 control BSE and Neodol flushes were: 1.29, 6.45, 9.03, 11.6, and 12.9 ml/min. Only the first three flow rates were obtainable during the Neodol flush in Column 1 due to either pressure build up or a damaged syringe pump. Column 2 Neodol flush was continued with flow rates of 19.3, 21.9, and 26.6 ml/min.
4.8.4 Columns 3 and 4: Biosurfactant Flushes

After the BSE flush, columns 3 and 4 were flushed with biosurfactant extracted from *Rhodococcus* species H13-A. The actual flow rates used were calculated from an estimated interfacial tension of 1.0 dyne/cm while the experimental value was trying to be determined. The initial flow rates used in columns 3 and 4 control BSE and biosurfactant flushes were: 1.00, 2.50, 5.00, 7.50, and 10.0 ml/min. Subsequent flow rates of 12.5, 15.0, and 17.5 ml/min were also used during the biosurfactant flushes.
CHAPTER 5
Results

5.1 *Rhodococcus* species H13-A

5.1.1 Speciation

The *Rhodococcus* species H13-A culture was further classified using Biolog MicroStation, Release 3.01. It was identified as *Rhodococcus erythropolis* (72.8% similarity).

5.1.2 Growth on Hexadecane

Growth (colony forming units/ml) of *Rhodococcus* species H13-A on hexadecane and subsequent reduction of interfacial tension (dyne/cm) were monitored over time. Culture set up and growth conditions were described previously.

In growth curve 6/20, the interfacial tension of the growth medium decreased from 48.3 to 1.8 dyne/cm against hexadecane (Figure 5.1). Biosurfactant production, as indicated by interfacial tension reductions, apparently occurred during the exponential growth phase. An interfacial tension reduction of 49.7 to 1.3 dyne/cm, also during exponential growth, was seen in growth curve 7/23 (Figure 5.2). The lag phase and time for interfacial tension reductions to occur was longer for growth curve 7/23 than in growth curve 6/20 due to less inoculum.
From these growth curves the doubling time was determined to be $1.73 \pm 0.15$ hours (1 hour and 43 minutes). The growth rate constant ($k$) was $0.58 \pm 0.05$ hours$^{-1}$, using the equation

$$k = \frac{\log N - \log N_0}{0.301 t}$$  \hspace{1cm} (13)$$

where $N$ was cell number at time $t$, $N_0$ was cell number at time 0, 0.301 was conversion from base e to base 10, and $t$ was time (hours).

Figure 5.1 Growth and interfacial tension reduction over time of *Rhodococcus* species H13-A in growth curve 6/20
Figure 5.2 Growth and interfacial tension reduction over time of *Rhodococcus* species H13-A in growth curve 7/23

### 5.2 Column Studies

The pore volume of each column was determined from the breakthrough of tritiated water, graphed as cumulative volume (ml) versus normalized effluent concentration (C/C₀) (Figure 5.3). The total pore volumes of Columns 1, 2, 3, and 4 were 12.81, 13.06, 12.38, 13.07 ml, respectively, with a mean value of 12.83 ± 0.32 ml. Subtraction of dead volume yielded a pore volume of 8.69 ml for Column 1, 10.55 ml for Column 2, 10.48 ml for Column 3, and 11.18 ml for Column 4. The mean value was 10.22 ± 1.07 ml. Different tubing was used for Column 1 and accounts for its larger dead volume.
Figure 5.3  Breakthrough of tritiated water from all four columns, dead volume included

5.2.1 Solubility of Hexadecane

The solubility of hexadecane in BSE, Neodol 25-9, and biosurfactant solutions was determined at 16 hours, 28 hours, and after sitting on the bench top for a week. The solubility of hexadecane at 16 hours in Neodol 25-9 was 352 ppb and 1.25 ppb in the biosurfactant. At 28 hours, the hexadecane solubility was 1.16 ppb in BSE, 580 ppb in Neodol 25-9, and 0.92 ppb in the biosurfactant. The solubility in biosurfactant was comparable to the BSE value (mean 1.26 ± 0.13 ppb). After sitting for a week, the solubility of hexadecane in the solutions was basically the same: 608 ppb in Neodol 25-9, and 1.88 ppb in the biosurfactant. Therefore, the solubility of hexadecane in Neodol 25-9 was 594±20 ppb, and 1.35±0.49 ppb for the biosurfactant.
5.2.2 Residual Saturation of Hexadecane

The volume of residual hexadecane saturation, as calculated from mass differences (8), were 4.02 ml, 2.68 ml, 2.23 ml, and 4.02 ml for Column 1, 2, 3, and 4, respectively, with a mean of 3.24 ± 0.92 ml (Table 5.1). The amount of hexadecane initially present in Columns 2 and 4 as determined from destructive sampling was 2.246 ml and 2.550 ml, respectively, with a mean of 2.398 ± 0.215 ml. Column 2 showed similar amounts of hexadecane present from both the mass difference and destructive sampling methods (mean of 2.46 ± 0.31 ml), and since the second method was more reliable, based on the potential for error due to the first method, the destructive sample determined hexadecane volume was used. Column 4 mass difference determined volume of 4.02 ml did not agree closely with the more reliable 2.550 ml from destructive sampling of the column; therefore, the volume was taken as 2.550 ml. Column 3 agreed closely with the residual volume of Column 2 by either method and with Column 4 by the destructive sampling method. Because the mass difference determined volume of hexadecane was reasonable and the destructively sampling technique was unavailable, the volume was taken as 2.23 ml from the mass difference for Column 3. Column 1 had a mass difference value higher than expected most likely due to errors in measurement of the column mass because of the limitation of the balance. Since the formation of residual saturation was the same as in Column 2, the destructive sample determined volume of hexadecane in Column 2 of 2.246 ml was used. This value brought the amount of residual hexadecane saturation closer to the other column values and to literature values.
Table 5.1 Initial volume of residual hexadecane in columns based on different methods of determining final hexadecane volume

<table>
<thead>
<tr>
<th>Column number</th>
<th>mass difference</th>
<th>destructive sampling</th>
<th>used/corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.02 ml</td>
<td></td>
<td>2.246 ml</td>
</tr>
<tr>
<td>2</td>
<td>2.68 ml</td>
<td></td>
<td>2.246 ml</td>
</tr>
<tr>
<td>3</td>
<td>2.23 ml</td>
<td>2.246 ml</td>
<td>2.23 ml</td>
</tr>
<tr>
<td>4</td>
<td>4.02 ml</td>
<td>2.550 ml</td>
<td>2.550 ml</td>
</tr>
</tbody>
</table>

The residual saturation of hexadecane in Columns 1, 2, 3 and 4 based on the mass difference calculation was 0.462, 0.256, 0.212, and 0.359, respectively, with a mean of 0.322 ± 0.112 (Table 5.2). The saturation based on the destructive sampling corrections were 0.258, 0.214, 0.211, and 0.228, respectively, with a mean of 0.228 ± 0.021. The corrected values correspond well to the reported residual saturation of decane in Sevilleta sand, which has similar characteristics to the Ottawa sand (see section 3.4.1) (Wilson et al., 1990).

Table 5.2 Residual saturation of hexadecane in columns based on mass difference and corrected values of $V_{or}$

<table>
<thead>
<tr>
<th>Column number</th>
<th>mass difference $V_{or}$</th>
<th>corrected $V_{or}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.462</td>
<td>0.258</td>
</tr>
<tr>
<td>2</td>
<td>0.256</td>
<td>0.214</td>
</tr>
<tr>
<td>3</td>
<td>0.211</td>
<td>0.211</td>
</tr>
<tr>
<td>4</td>
<td>0.360</td>
<td>0.228</td>
</tr>
</tbody>
</table>

The log of the capillary number was plotted against the reduced residual saturation ($S_{or}/S^{*}or$), where $S_{or}$ was the residual saturation removed and $S^{*}or$ was the initial residual saturation. The remaining residual saturation at the end of each control run was assigned as $S^{*}or$ for the following surfactant run.
5.2.3 Removal of Residual Hexadecane by BSE Control

After the residual saturation had been established, each column was run as a control using BSE to determine the amount of residual hexadecane removed due to increasing the velocity and from flooding with a high interfacial tension solution (Table 5.3). The residual hexadecane removed during the control application was due to mobilization because hexadecane was barely soluble in BSE (1.16 ppb). The results from each column were graphed as cumulative volume (ml) versus normalized effluent concentration (C/Co). The individual value of C/Co for each fraction collected and the cumulative C/Co were graphed to allow comparison. Removal of hexadecane in individual fractions and their effect on overall or cumulative percent removal could be compared.

<table>
<thead>
<tr>
<th>Column</th>
<th>flow rate (ml/min)</th>
<th>cumulative C/Co* removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.29</td>
<td>0.00718</td>
</tr>
<tr>
<td></td>
<td>6.45</td>
<td>0.0164</td>
</tr>
<tr>
<td></td>
<td>9.03</td>
<td>0.0245</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>0.0383</td>
</tr>
<tr>
<td></td>
<td>12.9</td>
<td>0.0392</td>
</tr>
<tr>
<td>2</td>
<td>1.29</td>
<td>0.00156</td>
</tr>
<tr>
<td></td>
<td>6.45</td>
<td>0.00434</td>
</tr>
<tr>
<td></td>
<td>9.03</td>
<td>0.0122</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>0.0161</td>
</tr>
<tr>
<td></td>
<td>12.9</td>
<td>0.0194</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>0.0102</td>
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<tr>
<td></td>
<td>2.50</td>
<td>0.0123</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
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</tr>
<tr>
<td></td>
<td>7.50</td>
<td>0.0277</td>
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<td></td>
<td>10.0</td>
<td>0.0302</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>0.0579</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>7.50</td>
<td>0.140</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.158</td>
</tr>
</tbody>
</table>

*C= dpm $^{14}$C-hexadecane in fraction of effluent collected

Co= total dpm $^{14}$C-hexadecane in column
Column 1 data exhibited increased removal of 3.29% of the residual hexadecane, Column 2 released 1.94%, Column 3 released 3.02%, and Column 4 released 15.8%. The residual in Column 1 was removed mostly in 6 different fractions over the first 4 flow rates (Figure 5.4). A small steady release occurred in Column 2 during the first flow rate (1.29 ml/min) and most of the second (6.45 ml/min). The rate of release increased at the end of the second flow rate and through the third (9.03 ml/min), leveled some the first third of flow rate 4 (11.6 ml/min), and increased through the rest of the run (Figure 5.5). The BSE control flush of Column 3 removed 3.02% of the residual hexadecane. Flow rates of 1.00 ml/min and 5.00 ml/min caused the release of the majority of the removed hexadecane (Figure 5.6). The BSE control flush of Column 4 released 15.8% of the residual $^{14}$C-hexadecane. A flow rate of 1.00 ml/min displaced 25% of the residual removed. Flow rates of 5.00 ml/min through 10.0 ml/min released hexadecane at a fairly steady rate (Figure 5.7).
Figure 5.4 Individual fraction and cumulative removal of residual hexadecane in control Column 1

Figure 5.5 Individual fraction and cumulative removal of residual hexadecane in control Column 2
Figure 5.6 Individual fraction and cumulative removal of residual hexadecane in control Column 3

Figure 5.7 Individual fraction and cumulative removal of residual hexadecane in control Column 4
5.2.4 Removal of Residual Hexadecane by Neodol 25-9

Columns 1 and 2 were flushed with the Neodol 25-9 solution after the controls were run (Table 5.4). Neodol 25-9 removed 32.8% of the residual hexadecane in Column 1 (Figure 5.8). The first flow rate, 1.29 ml/min, removed 7.64% of the hexadecane, with the majority of it coming off in 3 consecutive fractions. A small but steady increase in removal was apparent during the second flow rate of 6.45 ml/min. The third flow rate (9.03 ml/min) released 68.7% of the cumulative hexadecane removal. With hexadecane having a solubility of 594 ppb in the Neodol 25-9 solution, only $1.15 \times 10^{-4}$ ml of the 2.158 ml residual hexadecane could have been removed due to solubilization; therefore, the vast majority of the hexadecane was displaced by mobilization.

The Neodol flush of Column 2 released a total of 77.9% of the residual hexadecane (Figure 5.9). A slight increase in removal rate was noticed at the beginning of each flow rate with the greatest increase during flow rate 6 (19.3 ml/min). Only $3.28 \times 10^{-4}$ ml of the 2.202 ml residual hexadecane could have solubilized; therefore, mobilization was the major mode of removal in both columns. If the concentration of the Neodol 25-9 solution used had been at or above the CMC, the solubility of hexadecane would have been higher and solubilization might have accounted for more the residual hexadecane removal.
Table 5.4 Summary of results from Neodol 25-9 columns

<table>
<thead>
<tr>
<th>Column</th>
<th>flow rate (ml/min)</th>
<th>cumulative C/Co removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.29</td>
<td>0.0764</td>
</tr>
<tr>
<td></td>
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<td>9.03</td>
<td>0.328</td>
</tr>
<tr>
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<td>1.29</td>
<td>0.0335</td>
</tr>
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<td></td>
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<td>0.0778</td>
</tr>
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<td></td>
<td>9.03</td>
<td>0.104</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>26.6</td>
<td>0.799</td>
</tr>
</tbody>
</table>

Figure 5.8 Individual fraction and cumulative removal of residual hexadecane by Neodol in 25-9 Column 1
Figure 5.9  Individual fraction and cumulative removal of hexadecane by Necdol 25-9 in Column 2

5.2.5 Removal of Residual Hexadecane by Biosurfactant

Columns 3 and 4 were flushed with biosurfactant solution after the controls were run (Table 5.5). As in the BSE applied columns with a similar very low hexadecane solubility (1.16 ppb in BSE and 1.35 ppb in biosurfactant), the residual hexadecane removed by biosurfactant application was due to mobilization. The biosurfactant flush of Column 3 released 30.6% of the residual hexadecane (Figure 5.10). No large releases of residual were noted during the biosurfactant flush until the end of flow rate 3 (5.00 ml/min) and the beginning of flow rate 4 (7.50 ml/min). A fairly steady increase in residual removal rate was seen throughout the remainder of the experimental run with
an increase in rate at the beginning of flow rate 6 (12.5 ml/min) and 7 (15.0 ml/min).

The application of biosurfactant to Column 4 removed 45.8% of the residual hexadecane (Figure 5.11). The biosurfactant flush exhibited an increased rate of release during the second half of flow rate 2 (2.50 ml/min), a marked increase in rate at the beginning of flow rate 4 (7.50 ml/min) similar to what was observed in Column 3, and a steady increase over the next four flow rates.

Table 5.5 Summary of results from biosurfactant columns

<table>
<thead>
<tr>
<th>Column</th>
<th>flow rate (ml/min)</th>
<th>cumulative C/Co removed</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>5.00</td>
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<td>7.50</td>
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Figure 5.10  Individual fraction and cumulative removal of residual hexadecane by biosurfactant in Column 3
Figure 5.11  Individual fraction and cumulative removal of residual hexadecane by biosurfactant in Column 4

5.2.6  Capillary Numbers and Reduced Residual Saturation

The capillary number was calculated for each column and graphed as the log capillary number versus the reduced residual saturation ($S_{OR}/S^*_{OR}$) (Table 5.6, Figure 5.12). The residual saturation, 1.00 at the beginning of the column, was reduced as residual hexadecane was removed. As the residual hexadecane saturation decreased slightly, a corresponding increase in capillary number was observed. The control column capillary numbers exhibited an increase of one order of magnitude during the run: the capillary number of Column 1 was increased from $2.30 \times 10^{-6}$ to $2.48 \times 10^{-5}$; of Column 2
from $2.53 \times 10^{-6}$ to $2.47 \times 10^{-5}$; of Column 3 from $1.94 \times 10^{-6}$ to $1.92 \times 10^{-5}$; and of Column 4 was increased from $2.05 \times 10^{-6}$ to $1.92 \times 10^{-5}$.

The capillary number for the Neodol flush in Column 1 increased from $4.63 \times 10^{-4}$ to $2.96 \times 10^{-3}$ over only three flow rates. Only 13 of the 26 fractions for flow rate 3 in the Neodol flush were run at the correct flow rate due to problems encountered with plugging during the column run. Therefore, only those 13 fractions were used for capillary number calculation. Column 2 capillary number increased from $4.61 \times 10^{-4}$ to $9.05 \times 10^{-3}$ during the eight flow rates of the Neodol application.

Biosurfactant Column 3 capillary number increased from $3.77 \times 10^{-4}$ to $6.03 \times 10^{-3}$ over the eight flow rates while the capillary number for column 4 was increased from $3.75 \times 10^{-4}$ to $6.65 \times 10^{-3}$.
<table>
<thead>
<tr>
<th>Column</th>
<th>(ml/min) flow rate</th>
<th>Capillary number</th>
<th>Reduced residual saturation</th>
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<td>Control</td>
<td>Surfactant</td>
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<td>6.45</td>
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<td>2.96 \times 10^{-3}</td>
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</tr>
</tbody>
</table>
Figure 5.12 Capillary number and reduced residual saturation for all columns
CHAPTER 6
Discussion

6.1 Effect of BSE on Removal of Residual Hexadecane, Capillary Number, and Reduced Residual Saturation

For three of the four columns, application of a solution with a high interfacial tension against hexadecane resulted in less than 5% (2.65 ± 0.89 %) removal of the residual hexadecane (Figure 6.1). Column 4 removed 15.8% of the residual. This could have been due to the column not being at residual saturation at the beginning of the BSE flush.

Residual hexadecane was removed to a slight extent by the control solution BSE due to the increasing flow rates. At capillary numbers less than 10\(^{-3}\), trapping of hexadecane occurred, as was expected. The increasing velocities of the BSE solution were not able to decrease the capillary forces or increase the viscous forces enough to release the hexadecane. The capillary numbers calculated for these columns with the application of BSE increased an order of magnitude, 2.21 (± 0.26) \times 10^{-6} to 2.20 (± 0.32) \times 10^{-5}, for all of the columns with the increasing flow rates (Table 5.6). However, the final values were at least two orders of magnitude less than capillary numbers expected for movement of hexadecane. Little reduction in the residual saturation was observed with these increases in capillary number (Figure 5.12). As expected from the capillary number, the effectiveness of applying a solution without a surfactant to remove residual hexadecane, even at increasing flow rates, was limited.
Figure 6.1 Cumulative removal of residual hexadecane in control columns

6.2 Removal of Residual Hexadecane By Neodol 25-9

The two columns to which the commercial surfactant, Neodol 25-9, was applied could only be compared for the first three flow rates, up to 9.03 ml/min. Column 1 experienced problems of stopped flow during the third flow rate (9.03 ml/min) due to either column plugging or syringe pump problems. This flow rate removed 18.6% of the residual hexadecane compared to only 2.22% removed at the same rate in Column 2 (Figure 6.2). The mobilization of a large ganglion of hexadecane in Column 1 at the beginning of the third flow rate (9.03 ml/min), as seen in the individual fraction removal (Figure 5.8), could have caused a build up in pressure to 40-45 psi, causing the syringe pump to quit and giving the large removal.
The cumulative residual hexadecane removal was not comparable either between Columns 1 and 2. In Column 1, a total of 32.8% of the residual hexadecane was removed up through the third flow rate, while only 10.4% was removed in Column 2 up through the third flow rate. As the flow rates were continually increased for Column 2, however, the removal of entrapped hexadecane continued to increase. At the final flow rate of 26.6 ml/min, a cumulative 79.9% of the residual hexadecane had been removed. The removal of the residual hexadecane by application of the commercial surfactant at increasing flow rates was apparently more effective than application of a non-surfactant solution, as expected. However, duplication of the results at high flow rates was not achieved.

Figure 6.2. Cumulative removal of residual hexadecane by Neodol 25-9 in Columns 1 and 2
6.3 Removal of Residual Hexadecane by Biosurfactant

Application of a solution containing biosurfactant produced by a common soil isolate, *Rhodococcus* species H13-A (*Rhodococcus erythropolis*), also increased the removal of residual hexadecane as compared to the control, BSE. The percentages of residual hexadecane removed from Columns 3 and 4 by BSE with increasing flow rates were 3.02% and 15.8%, respectively (Figure 6.1). Flushing the columns with the biosurfactant solution with increasing flow rates up to 17.5 ml/min removed 30.6% and 42.7% from Columns 3 and 4, respectively (Figure 6.3). The presence of the biosurfactant apparently enhanced the removal of hexadecane.

![Graph showing cumulative C/Co vs. pore volume for Columns 3 and 4](image)

Figure 6.3 Cumulative removal of residual hexadecane by biosurfactant in Columns 3 & 4
6.4 Comparison of Removal of Residual Hexadecane by Neodol 25-9 and Biosurfactant

Both the Neodol 25-9 and the biosurfactant solutions, having similar low interfacial tensions with hexadecane, were apparently more effective in removal of residual hexadecane from packed sand columns than a solution having a higher interfacial tension with hexadecane, BSE. Likewise, the effectiveness of residual hexadecane removal appeared to increase for both applications as the flow rate was increased.

At flow rates of 12.9 ml/min for Neodol 25-9 and 12.5 ml/min for the biosurfactant, removal of residual hexadecane was comparable: 24.6% by Neodol 25-9 and 20.0% and 27.5% by the biosurfactant (24.0±3.8% biosurfactant mean) (Table 5.4 and 5.5, Figure 6.2 and 6.3). At the highest similar flow rates, 19.3 ml/min for Neodol 25-9 and 17.5 ml/min for biosurfactant, 63.2% of the residual hexadecane was removed by Neodol 25-9 while 30.6% and 42.7% were removed by the biosurfactant (36.7±8.6% biosurfactant mean). At the lower compared flow rate (12.9 ml/min and 12.5 ml/min), the commercial surfactant and biosurfactant removed similar amounts of residual hexadecane while at the higher compared flow rates (19.3 ml/min and 17.5 ml/min), the commercial surfactant appeared more effective than the biosurfactant; however, the commercial surfactant solution was injected at a slightly higher flow rate than the biosurfactant solution which should have resulted in increased hexadecane removal. An additional removal of hexadecane might have been observed due to greater solubilization of hexadecane in Neodol 25-9. Also, only one Neodol column was run at the higher flow rates; therefore, the results of Neodol 25-9 Column 2 were not verified.
6.5 Comparison of Surfactant Capillary Numbers and Reduced Residual Hexadecane Saturation

Corresponding with the increasing removals of residual hexadecane in the Neodol 25-9 columns with increasing flow rates, increases in capillary number were also calculated. For both Columns 1 and 2, an order of magnitude increase in capillary numbers occurred with increasing flow rates (velocities) as determined by the control columns. However, since the interfacial tension between the Neodol solution and the hexadecane was so much lower, the capillary numbers were increased to a range of 10^{-2} to 10^{-3} where mobilization of hexadecane was expected (Figure 5.12, and Table 5.6).

The calculated theoretical flow rate needed to achieve a capillary number of 10^{-2} for Neodol 25-9 was 27.26 ml/min, or a velocity of 0.216 cm/s. This flow rate was barely out of range for the syringe pump (maximum setting of 26.6 ml/min). The actual capillary number obtainable for Column 2 at 26.6 ml/min was 9.05x10^{-3}. At a value of 10^{-2}, mobilized hexadecane was expected to flow freely, which was approximated in Column 2 where a cumulative removal of 80% of the residual hexadecane (20% reduced residual saturation) was seen.

As with the Neodol 25-9 solution, the interfacial tension of the biosurfactant solution was low enough to increase the capillary number to a range of 10^{-3} to 10^{-2}. During the fourth flow rate (7.5 ml/min) both Columns 3 and 4 exhibited an increase in residual removal and a corresponding decrease in residual saturation as the capillary number reached 2.8x10^{-3} (Figure 5.12 and 6.3). Reduction in residual saturation was expected at this range of capillary numbers. The final capillary numbers for biosurfactant Columns 3 and
4 at 17.5 ml/min were 6.03x10^{-3} and 6.65x10^{-3} (6.34(±0.44)x10^{-3}), and a reduction in residual saturation of 69.5% and 57.3% (63.4±8.6%), respectively.

Comparisons of the Neodol 25-9 and the biosurfactant capillary numbers and reduced residual saturation were made at the highest similar flow rates used. At a flow rate of 19.3 ml/min, Neodol 25-9 application in Column 2 resulted in a capillary number of 6.76x10^{-3} and reduction of residual hexadecane saturation from 100 to 36.8%. At a flow rate of 17.5 ml/min, a mean capillary number of 6.34(± 0.44)x10^{-3} and reduced residual saturation mean of 63.4±8.6% were observed in biosurfactant Columns 3 and 4. Similar capillary numbers were obtained, but the reduction of residual hexadecane saturation by Neodol 25-9 was 32.6% greater than by biosurfactant application at the highest comparable flow rates.

The degree that capillary numbers increase with a corresponding reduction in residual saturation varies in the literature dependent on the system set up, initial wetting conditions, and flush out solution (Stegemeier, 1977). The Neodol 25-9 and biosurfactant log capillary number versus reduced residual saturation curves (Figure 5.12) correspond with the results for bead-packed column work (Figure 6.4) (Wilson et al., 1990).
Figure 6.4 Capillary number and reduced residual saturation for sandstones and glass beads (from Wilson et al., 1990)
6.6 Conclusions

An extracellular glycolipid biosurfactant was extracted from a culture of *Rhodococcus* species H13-A, subsequently identified as *Rhodococcus erythropolis* using Biolog MicroStation System, grown on hexadecane. A suspension of this biosurfactant in BSE had an interfacial tension of 0.25 dyne/cm against hexadecane. The commercial surfactant chosen for use in this study was 1.2% (w/w) Neodol 25-9 in BSE with a comparable interfacial tension against hexadecane of 0.278 dyne/cm.

The biosurfactant produced by *Rhodococcus* species H13-A was effective in removing residual hexadecane from sand packed columns. Mobilization of most of the residual hexadecane occurred at capillary numbers of $10^{-3}$ to $10^{-2}$. Since the commercial surfactant and biosurfactant columns were not run at the same flow rates and a replicate of the Neodol 25-9 column was not attained, the results comparing the two were inconclusive. However both the Neodol 25-9 in one column and the biosurfactant in two columns were able to remove residual hexadecane and the residual saturation ($S_{or}$) versus capillary number ($N_c$) graphs were similar to behavior reported in the literature for other commercial surfactants. Mobilization of the hexadecane apparently occurred at similar flow rates and capillary numbers for the two surfactants.

Future work should include investigation of the emulsification capabilities of the biosurfactant and Neodol 25-9 to further elucidate the mechanisms of removal of residual hexadecane. Running the columns at identical, linearly increasing flow rates as well as use of a commercial surfactant more comparable to the biosurfactant in regard to solubilization and emulsification capacity would allow for clearer comparison of column results. Use of a column set up with a smaller mass allowing the use of a more sensitive balance would
allow better comparison between mass difference and destructively sampled values for the volume of residual oil in a column. Removal of residual decane by the *Rhodococcus* species H13-A biosurfactant should be explored due to the lower interfacial tension between the two, as well as addition of a co-surfactant to further decrease interfacial tensions.
CHAPTER 7

Literature Cited


