National Library of Canada
Canadian Theses Service
Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Services des thèses canadiennes

# CANADIAN THESES

# THÈSES CANADIENNES

## NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

## AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r 86/06)

Canada

THE UNIVERSITY OF ALBERTA

# BIOSURFACTANT PRODUCTION BY A *CORYNEBACTERIUM* ISOLATE

BY

© JANICE D. TREIT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1986

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-32426-0

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: JANICE D. TREIT

TITLE OF THESIS: BIOSURFACTANT PRODUCTION BY A *CORYNEBACTERIUM* ISOLATE

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1986

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

.....Janice Treit.....

(Signature)

13524 - 111 Street
Edmonton, Alberta
T5E 4Z8
(Permanent Address)

Date: June 19, 1986

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled BIOSURFACTANT PRODUCTION BY A *CORYNEBACTERIUM* ISOLATE submitted by JANICE D. TREIT in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

.....

(Supervisor)

.....

.....

.....

Date: May 29 1986

Dedicated to my teacher, my friend, my mother

Joyce Isabel Treit

September 27, 1924 - December 30, 1984

## ABSTRACT

Bacteria isolated from the environment were screened for production of extracellular surface active agents. It was proposed to evaluate the agents as to their usefulness in bitumen recovery. A spectrophotometric oil emulsion assay, based on a published method, was developed to detect bioemulsifier production. Surface and interfacial tension measurements were employed to detect biosurfactant production. Other detection methods were investigated, but none proved to be as suitable for rapid screening of large numbers of cultures. Four biosurfactant producing isolates were found in this survey whereas no bioemulsifier producing isolates were detected. The level of biosurfactant activity produced by these isolates was comparable to that of *Bacillus subtilis*, a recognized biosurfactant producing organism.

One of the biosurfactant producing isolates, *Corynebacterium* sp.1B-2 produced a glycolipid biosurfactant capable of reducing the surface and interfacial tension of double-distilled water to 28.5 and >0.1 dynes/cm, respectively. Production of the biosurfactant required oxygen and appeared to be hydrocarbon inducible. Maximum levels of biosurfactant were achieved during the stationary phase of growth. Optimal recovery of biosurfactant was accomplished by using pentane extraction from cell-free culture filtrates adjusted to pH 2.0. This biosurfactant was less effective at separating bitumen from oil sand than the commercial dispersant, Igepal Co 630.

ACKNOWLEDGEMENTS

[redacted] Scholarships awarded [redacted] Alberta Oil Sands Technology and Research Authority (AOSTRA) provided [redacted] support for this investigation and are gratefully acknowledged.

I thank my advisor Dr. D.W.S. Westlake for his assistance and encouragement throughout the project. I appreciate the freedom and resources that were provided to explore my own ideas.

My gratitude extends to Dr. Julia Foght and Dr. Phil Fedorak for their helpful advice and their great patience with my incessant questions.

Special thanks go to Karl Hawkinson, my friend and companion, for his shared enthusiasm throughout my studies and his demonstration of faith in my abilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# I. INTRODUCTION

Petroleum is a finite natural resource and a major component of the world's economy. Its recovery has become an important issue, especially in light of the fact that on average, only thirty to forty percent of conventional oils, which have an API gravity >20°, is recoverable from reservoir rocks by existing drilling and recovery methods (Forbes 1980, Larson *et al.* 1980, Shah 1981). Heavy oils, which have an API gravity <20°, are not recoverable by conventional technology because of their viscous nature. This leaves a large amount of oil underground which cannot be recovered economically by current methods. Large reserves of hydrocarbons, i.e. bitumen, also are contained in oil sands. For example, approximately 1 trillion barrels of bitumen are contained in the Athabasca, Cold Lake, Peace River and Wabasca oil sands in Alberta (Humphreys & Schutte 1978) and 20 to 30 billion barrels in Utah tar sands (Zajic & Akit 1983). Bitumen is a highly viscous mixture of high molecular weight hydrocarbons making up the organic solvent-soluble portion of oil sand (Wollrab & Streibl 1969). Recovery of bitumen from oil sands, especially in Alberta, has generally involved a hot water/steam, high pH (pH 8.5-9.0) process, and although the recovery is about 80% or better (Williams *et al.* 1984) there are several drawbacks to this technology. The process involves the use of very large amounts of water and energy and results in the formation of an alkaline, stable water-clay sludge containing unrecovered hydrocarbons. The disposal of these tailings presents a serious environmental problem (Gerson & Zajic 1977, Williams *et al.* 1984, Zajic & Akit 1983). For example, the storage of tailings requires large areas of land and seepage from the pits could cause contamination of surface and subsurface waters. The use of microbial biosurfactants for bitumen recovery could decrease or eliminate sludge production and reduce the energy requirements.

Since oil sand recovery operations are very large scale processes, this would have a significant impact on the economics of the oil sands operation.

The use of microbial products in a cold water process for bitumen recovery has been studied recently (Gerson & Zajic 1977, Jack 1984, Zajic & Akit 1983, Zajic & Gerson 1978), and biosurfactants produced by *Corynebacterium* species have demonstrated an ability to separate bitumen from oil sands. Spencer (1980) reported another process which uses a mixture of bacteria to release oil from sand, although the company suggests that this is accomplished enzymatically and not via biosurfactant production.

Considerable research has been directed towards using microbial methods to enhance conventional oil recovery (Jack 1984, Finnerty & Singer 1983, Moses & Springham 1982, Spencer 1980, Stosur 1981). These include: either using microorganisms *in situ*, by inoculating the reservoir with suitable microbes and nutrients (Clark *et al.* 1981, Forbes 1980, Lazar 1983a, Lazar 1983b, Moses & Springham 1982, Springham 1984); or using only microbial products, to improve the rate and extent of oil recovery (Finnerty & Singer 1984, Gerson & Zajic 1977, Gerson & Zajic 1979b, Jenneman *et al.* 1983, Singer 1985, Singer *et al.* 1983b, Zajic & Akit 1983, Zajic & Gerson 1978).

*In situ* microbially enhanced oil recovery (MEOR) depends on the growth of organisms and the production of metabolites in the reservoir to increase oil mobility (Brubela & McKay 1985, Forbes 1980, Springham 1984). This increased mobility may be achieved by several mechanisms, including: 1) repressurizing the reservoir, 2) reducing the viscosity of the oil within the reservoir, 3) increasing the porosity of the reservoir, 4) releasing trapped oil from reservoir pores, 5) increasing viscosity of the drive fluid, and 6) selective plugging of previously swept zones, where oil has been removed. Growth conditions in reservoirs are often highly selective. For example,

oxygen and nutrients, especially N and P, are limited and crude oil is usually the only available carbon source. Also, conditions of temperature, pressure and salinity can be quite severe as temperatures of >85°C are found in deep reservoirs and salinities of up to 20% exist in the connate (geological) waters of some reservoirs. Combinations of such factors has directed investigations towards anaerobic or facultative mesophilic microorganisms, as well as increased speculation about the genetic engineering of thermophilic, saline-tolerant organisms that could be used for *in situ* enhanced oil recovery .

There also has been emphasis on investigating the surface production of useful metabolites by microbes. This technology could circumvent a lack of suitable growth conditions in a reservoir. The major products of interest have been water-thickening agents, biosurfactants and bioemulsifiers. For enhancing conventional oil recovery, water-thickening polymers are generally restricted to use in the reservoir situation, whereas biosurfactants and bioemulsifiers could find application both in the conventional reservoir and in oil sands separation. Xanthan gum, a microbial product from *Xanthomonas campestris*, is currently being used as a water-thickening agent in waterflooding processes (Gabriel 1979), and has demonstrated several advantages over the conventionally used polyacrylamides (Forbes 1980). Xanthan gum is also employed to control the viscosity of drilling muds. Emulsan, a bioemulsifier which is produced by *Acinetobacter calcoaceticus* RAG-1, has been shown to be effective in oil tanker cleanup by emulsifying the oil and removing it from the metal surfaces of the fuel tanks (Gutnick & Rosenberg 1977, E. Rosenberg, personal communication). It also has shown its effectiveness at reducing the viscosity of oil in model oil transport systems (Hayes & Nestaas 1983, Jack 1984). Emulsan is now being produced commercially by Pfizer Inc. in the U.S.A. Another surface active agent, produced by a culture tentatively identified as a *Corynebacterium* sp., has been shown to reduce drastically the viscosity of heavy

oil (Finnerty & Singer 1984, Singer *et al.* 1983). These examples demonstrate the potential of water-thickening agents, biosurfactants and bioemulsifiers for the separation of bitumen from oil sands and for the recovery of heavy and conventional oils.

The objective of this study is to find microorganisms or microbial products which can stimulate the release of bitumen from oil sands. These products could also be useful in enhancing conventional oil recovery. This study will involve:

1) selection or development of a simple, rapid method to screen cultures for the production of extracellular surface active agents;

2) isolation and identification of organism(s) which produce reasonably potent extracellular surface active agent(s);

3) determination of potential usefulness of such agents with respect to bitumen release; and

4) characterization of any such agents found.

# II. LITERATURE SURVEY

## 1. MICROBES AND OIL RECOVERY

### A. *In situ* Microbially Enhanced Conventional Oil Recovery

To appreciate the role that microorganisms can play in enhanced oil recovery, it is necessary to understand the nature of the reservoir and current methods of conventional oil recovery. There is a great deal of variation with respect to the structure of the reservoirs, the composition of the surrounding geological formation and the physical and chemical conditions encountered in the reservoir. There are similarities, however, in that most reservoirs are structured with a lower layer of connate water, a middle layer of oil and an upper layer of gas. As described by Speight (1980) recovery of oil relies on natural and applied forces for the energy to drive the petroleum fluids from the formation, through the reservoir and the well, to the surface. Pressure from expanding gases in the reservoir or pressure from natural water drive are the more important forces used to drive the petroleum to the production well. Maintenance of these pressures can be aided by introducing compressed gases from the surface or from the reintroduction of natural gas which has been recovered from the well. As the initial flow of oil gradually decreases, secondary recovery methods are employed. Generally, this is accomplished by waterflooding, a process where water is pumped into the reservoir through injection wells drilled into the reservoir a short distance away from the production well(s). Produced waters, i.e. water recovered along with petroleum from the well, or water from nearby lakes or rivers is used for this purpose. Waterflooding functions by maintaining pressure in the reservoir and driving the oil to the production well(s).

Chemical additives, such as surface active agents or solvents are often added in front of the water drive to aid in the flow of oil within the reservoir (Lawson & Reisberg 1980). Also, polymers may be added to the drive water to increase its viscosity, and thereby its oil displacement ability. When the proportion of water in the produced oil becomes too great, production of the well is often halted. It is at this stage that microbially enhanced oil recovery (MEOR) is considered, for these produced wells.

The efficiency of oil recovery processes is dependent upon two factors: firstly, the properties of the oil-bearing formation, such as porosity and permeability of the reservoir and the nature of the rock surface; and secondly, the fluid properties of the oil and connate water within the reservoir (Finnerty & Singer 1983, Neumann *et al.* 1981). These factors interact to determine the flow of oil through a reservoir (Bubela & McKay 1985, Shah 1981). Porosity refers to the pore spaces or channeling throughout the formation and permeability is governed by the size of the 'entrances' and 'exits' of these pores. Thus, physical aspects such as viscosity of oil, capillary forces and interfacial tension between the various phases within the formation, control the mobility of the oil. These forces act as restraints against the drive of natural or applied pressure. Consequently, microbial activities which lead to a reduction of these restraining forces are potentially useful for enhancing oil recovery.

Springham (1984) and Janshakar (1985) have compiled lists of mechanisms by which bacteria may release oil by virtue of their growth and metabolism within the reservoir. Using the reservoir as an *in situ* fermentor, the growth of microorganisms could possibly result in the following:

1) increasing reservoir permeability by the dissolution of carbonate rock found in some reservoirs by organic acids produced by anaerobic microbial growth;

2) production of carbon dioxide, hydrogen, methane and nitrogen gases which can function to increase reservoir pressure, can dissolve in the oil thereby reducing its

viscosity, or can affect the solubility of different components in the oil, e.g. $CO_2$ precipitates asphaltenes, freeing the lighter, less viscous fractions;

3) degradation of large molecules in the oil thereby reducing viscosity (Singer *et al.* 1983);

4) production of emulsifiers and surfactants which can reduce the viscosity of oil or the interfacial tension of oil/water mixtures; and

5) production of polymers which increase the viscosity of the injected drive water; or which selectively plug regions which have been previously swept by waterflooding procedures. Organisms having these capabilities have been considered for *in situ* enhanced oil recovery.

Environmental conditions such as temperature, pH, salt concentration, nutrient supply and carbon and energy sources, vary among reservoirs and are often selective for microbial growth. For example, temperatures greater than 85°C are usually found in deep reservoirs, as temperature increases with depth. High pressures also can be encountered (Forbes 1980) and the salinity of the connate water can be as high as 20%. A computer search by Clark *et al.* (1981) shows that 27% of reservoirs in the ten top producing states in the U.S.A. have environmental conditions compatible with microbial growth. This observation is based on the reservoir having a temperature of 75°C or less, a pH range between 4.0 and 9.0, a salt concentration of 10% or less and an API gravity of the constituent crude oil of 17° or more. These criteria, however, do not take into account the possibility of using halophilic and extreme thermophilic organisms in MEOR procedures. Oxygen (air) is often absent from the reservoir due to its role in corrosion (Forbes 1980) and it's injection into reservoirs is restricted. Anaerobic or facultative, non-hydrocarbon utilizing, solvent-producing organisms, therefore, have been the microbes of choice for *in situ* oil recovery applications. Because these organisms do not

use hydrocarbons for growth they must be supplied with another cheap substrate such as molasses.

Actual field tests have shown limited success. Hitzman (1983) has compiled a detailed review of several different field trials conducted in eastern Europe and the U.S. which show widely varying results. Lazar (1983a) reported increased oil production in 2 out of 7 Romanian wells which were injected with adapted bacteria and a molasses substrate. Yarbrough and Coty (1983) found a statistically significant increase in production of oil using *Clostridium acetobutylicum* and beet molasses injected over a 6 month period in an Arkansas reservoir. McGraw-Hill's Biotechnology Newswatch reported that A.G. Swan of Science Research Centre in Abilene, Texas has seeded wells with a mixture of organisms plus molasses substrate, resulting in a 50% increase in production over 9 months. The mechanisms for these increased levels of oil production were not reported. Further reports indicate that other field studies are underway, and McInerny and co-workers at the University of Oklahoma recently patented an oil recovery enhancement process (U.S. Patent 4,522,261, 1985) which involves injection of *Bacillus licheniformis* JF-2 into the water flooded formation. Many field trials are being carried out on produced wells of oil companies, however, the results usually remain confidential. The fact that trials continue to be carried out by these companies suggests some measure of success with the microbial enhancement of oil recovery.

B. Injection of Microbial Products

Polymers are sometimes added during waterflooding to increase the viscosity of the drive fluid (Shah 1981) as this results in a more stable displacement front to push out oil (Gabriel 1979). The chemical water-thickening agents most often used are polyacrylamides, but these have limitations as they are sensitive to shear degradation,

have limited temporal stability, are salt sensitive and are highly polar so they are easily adsorbed out of a fluid system. Polyacrylamides also have been found to stimulate the growth of some aerobic soil organisms (Grula & Sewell 1983) and may be subject to microbial degradation. The most common biopolymer used is xanthan gum and this polysaccharide has several characteristics which make it advantageous for this application (Forbes 1980, Gabriel 1979). It is very efficient, having high thickening power per unit of concentration and it exhibits a marked pseudoplastic or mixotrophic behavior. This means that it is sensitive to shear and can therefore be pumped and handled quite easily, but when the shear forces are removed its thickening properties are rapidly recovered. Xanthan gum is also reasonably stable to temperatures up to 90°C, to high salt concentrations and is relatively unaffected by the ionic environment. *Xanthomonas* sp. polysaccharides also have shown their usefulness in reducing friction in the flow of viscous fluids such as drilling muds and in oil transport (Hoyt 1985).

Surface active agents, which include emulsifiers and surfactants, can be used to solubilize or displace trapped oil in the formation while emulsifiers which reduce viscosity might find application in reservoirs with heavy oils. Oil tends to get trapped in pores within the formation because the necks of these pores are too narrow to allow for free movement of the oil. Energy is required to alter the interface of the oil such that it can move through the neck of the pore (Shah 1981). A reduction of interfacial tension reduces the amount of energy required to achieve this deformation. Chemical surfactants, especially sulfonates, are currently used in the field for this purpose (Lawson & Reisberg 1980, Wasan & Mohan 1977). There are no examples of biosurfactants or bioemulsifiers being injected into the reservoirs, although this has been proposed (Finnerty & Singer 1983, Gerson & Zajic 1979b, Singer 1985).

### C. Bitumen Recovery from Oil Sands

Oil sand separation is a relatively recent area of investigation and has not been included in the term microbially enhanced oil recovery. Athabasca oil sand is a mixture of quartz sand, clays, bitumen and water (Schramm *et al.* 1985). Williams *et al.* (1984) described a high grade oil sand as having a 12% bitumen and 4% water content, whereas 8% bitumen and 8% water is considered a low grade oil sand. Bitumen is an extremely viscous mixture of hydrocarbons consisting of approximately 45% oils, 35% resins and 20% asphaltenes. The recovery method involving hot water/steam at 80 - 85°C and high pH of 8.5-9.0, causes the bitumen film on the sand particles to rupture and separate from the sand. As previously mentioned, the settling of the sludge that is formed is a very slow process and its storage is expensive as tailings storage requires large land areas. The environmental hazard is due to the alkaline nature of the tailings, which results from the large amounts of NaOH (0.2 Kg/tonne ore) used to attain the elevated pH required for the extraction process. Foght *et al.* (1985) have reported a range in pH of 8.1 to 8.4 for tailings pond samples. The successful development of cold water extraction procedures involving biosurfactants would circumvent the distinct disadvantages of current recovery practices.

## 2. SURFACTANTS AND EMULSIFIERS

### A. Mode of Action and Structure

An interface is a boundary between two immiscible phases and a surface refers to

an interface where one of the phases is a gas, usually air. Surface active agents act at interfaces and include both surfactants and emulsifiers. Hayes and Nestaas (1983) define a surfactant as a substance which reduces the mixing energy, which is the mechanical energy required for the creation of additional interfacial area between two phases, e.g. water and a hydrocarbon. A surfactant, then, reduces the energy required for the formation of an emulsion whereas an emulsifier is a substance which stabilizes an emulsion that is already formed, so that the emulsified material will not readily coalesce. Surfactants and emulsifiers have an amphipathic molecular structure, in that each molecule is composed of groups with opposing solubilites (Gutcho 1977). The presence of both hydrophilic and hydrophobic moieties causes the surfactant molecules to distort the liquid structure at the interface and increase the free energy of the system, thus promoting emulsion formation. Coalescence of the dispersed droplets of an emulsion is thermodynamically more favourable, so these dispersions are never completely stable (Zajic & Panchal 1976). An emulsifier, however, is able to stabilize an emulsion because it adsorbs around a dispersed drop as a film which will not adhere, nor thin if two drops collide. Biosurfactants and bioemulsifiers, therefore, are defined as surface active agents obtained from a biological source.

## B. Methods of Detection of Biosurfactant and Bioemulsifier Activity

The methods for detecting the production of biosurfactants and bioemulsifiers by microorganisms involve observing the physical effects of these agents' action. Reductions in surface and interfacial tension indicate the production of biosurfactants while emulsification tests determine emulsion stability and have been used to establish bioemulsifier production.

Biosurfactant production is monitored by a tensiometric assay since biosurfactants

reduce surface and interfacial tension of liquids. Almost all of the surface and interfacial tension measurements reported were made with an instrument using a du Nuoy ring. The ring is submerged in the liquid and then pulled back through the liquid to determine the tension as the ring breaks the surface. Interfacial tension is determined in a similar manner when a second phase, usually hexadecane, is layered over the first phase, after the ring is submerged. The interfacial tension is measured as the ring breaks through the first liquid into the second layer. More accurate measurements of interfacial tension of low value, i.e. < 1 dyne/cm, have been determined using the drop weight method (Singer *et al.* 1983a).

Because the tensiometric assay is a direct measure of the physical parameter of activity it can be used directly to measure biosurfactant production. As discussed previously, surfactant accumulates at the surface or interface of a liquid which, when that surface or interface area is saturated by surfactant, leads to a reduction of the surface or interfacial tension of that liquid to a minimum value. Any further increase in surfactant concentration leads to aggregation of these molecules to form micelles and this phase transition point is called the critical micelle concentration (CMC). The determination of the critical micelle concentration is achieved by measuring successive dilutions of the liquid containing the purported surfactant. The dilution at which the surface tension begins to increase is designated the critical micelle concentration and the inverse of this dilution ($CMC^{-1}$) is used as a measure of the concentration of that surfactant (Gerson & Zajic 1978). These values can be compared for a particular surfactant, but not among different surfactants. This measure of surfactant concentration is very useful when attempting to optimize microbial production.

In their early investigations, Reisfield *et al.* (1972) developed a method to determine the extent of oil dispersion caused by *A. calcoaceticus* RAG-1. This involved turbidity measurements in a Klett-Summerson colorimeter fitted with a green filter.

Spectrophotometric methods also have been used by other groups (Cirigliano and Carman 1984, Illarionova *et al.* 1984, Roy *et al.* 1979).

A qualitative test of emulsification used by several investigators is the test tube emulsification method (Cooper and Zajic 1978) where the percent of the total volume occupied by the emulsion was used as a measure of emulsion-stabilizing ability. Other investigators used this method, with different volumes of liquid culture and kerosene, and demonstrated the production of bioemulsifiers by two *Corynebacterium* species (Akit *et al.* 1981) and *Torulopsis petrophilum* (Cooper & Paddock 1983). Cairns *et al.* (1982) determined the half life of emulsions using this assay method and were able to apply this technique for establishing the production of de-emulsification agents by *Nocardia amarae* .

A more sensitive method of assessing emulsification is to determine the mean droplet diameter of the emulsion which is directly related to the stability of the emulsion. A smaller droplet diameter average indicates a more stable emulsion (Zajic *et al.* 1977a). Panchal and Zajic (1978) used the Coulter Counter to measure drop sizes in emulsions, and Zajic *et al.* (1977b) measured droplet diameter using a light microscope.

The methods previously described generally require several days for the growth of the organism, the use of large volumes of material and filtration or centrifugation to obtain cell-free culture broth prior to assay. The development of rapid screening procedures, therefore, is desirable for the detection of surface active agent production. Mulligan *et al.* (1984) devised a blood agar plate screening method which is based on the observation that surfactin, the biosurfactant produced by *Bacillus subtilis* , can rupture erythrocytes. For surfactin, the degree of clearing is proportional to the concentration of surfactant (Mulligan *et al.* 1984). Singer (1985) has shown further evidence of the correspondence between surfactant concentration and cleared zone diameter with several other biosurfactants on blood agar plates. However, for this assay, the microbes in

question must be able to produce biosurfactants without the presence of a hydrocarbon substrate, and it is necessary to assure that this clearing is not due to protease activity. The possibility of incorporating a hydrocarbon substrate, e.g. hexadecane, into blood agar plates was not examined. Singer and coworkers (Finnerty & Singer 1984, Singer 1985, Singer *et al.* 1983b) also have shown that crude oil - agar plates can be used to demonstrate surfactant production which is seen as zones of clearing on these plates. This assay correlates with bioemulsifier production as 82% of the positive isolates detected demonstrated bioemulsifier activity in emulsification testing (Singer *et al.* 1983b). The incorporation and uniform dispersion of crude oil into an agar medium is not a trivial procedure.

## 3. SURFACE-ACTIVE AGENT PRODUCING ORGANISMS

### A. Microbial Sources of Biosurfactants and Bioemulsifiers

There are many surface active agents produced by microorganisms and these have been discussed in several reviews (Cooper *et al.* 1979b, Cooper & Zajic 1980, Gerson & Zajic 1979b, Ratledge 1980, Zajic & Panchal 1976, Zajic & Steffens 1984). The data in Table 1 summarizes those discussed here. The main chemical classes of these compounds are glycolipids, polysaccharide-lipid complexes, lipopeptides, phospholipids, fatty acids and neutral lipids (Cooper Zajic & Gracey 1979, Zajic & Steffens 1984). Surface active agents can be cell-associated or extracellular and the most commonly isolated extracellular surface active compounds are glycolipids.

Zajic *et al.* (1977a) found that *Corynebacterium hydrocarboclastus* produces compounds having both surfactant and emulsifying properties. The possession of two

TABLE 1 - MICROBIAL PRODUCERS OF BIOSURFACTANTS AND BIOEMULSIFIERS

| ORGANISM | SURFACE ACTIVE AGENT | REFERENCE |
|---|---|---|
| BIOSURFACTANT PRODUCTION | | |
| *Arthrobacter paraffineus* KY4303 | glycolipid | Suzuki *et al.* 1969 |
| *Arthrobacter paraffineus* NRRL B-3453 | glycolipid | Akit *et al.* 1981 |
| *Bacillus subtilis* | lipopeptide | Arima *et al.* 1968 |
| *Corynebacterium fascians* | corynomycolic acids | Gerson & Zajic 1979a |
| *Corynebacterium fascians* ICPB | neutral lipids | Akit *et al.* 1981 |
| *Corynebacterium hydrocarboclastus* | glycoprotein | Zajic *et al.* 1977b |
| *Corynebacterium lepus* | lipopeptide | Cooper Zajic & Gracey 1979 |
| *Corynebacterium xerosis* | corynomycolic acids | Gerson & Zajic 1979a |
| *Pseudomonas aeruginosa* | glycolipid | Guerra-Santos *et al.* 1984 |
| *Pseudonomas aeruginosa* P-20 | peptidoglycolipid | Koronelli *et al.* 1983 |
| *Pseudomonas asphaltenicus* | lipopolysaccharide | Gerson & Zajic 1978 |
| *Rhodococcus erythropolis* | glycolipid | Rapp *et al.* 1979 |

TABLE 1 - Continued

| ORGANISM | SURFACE ACTIVE AGENT | REFERENCE |
|---|---|---|
| *Torulopsis bombicola* | glycolipid | Cooper & Paddock 1984 |
| *Torulopsis petrophilum* | glycolipid | Cooper & Paddock 1983 |
| BIOEMULSIFIER PRODUCTION | | |
| *Acinetobacter calcoaceticus* BD 413 | lipid-polysaccharide complex | Kaplan & Rosenberg 1982 |
| *Acinetobacter calcoaceticus* RAG-1 | lipid-polysaccharide complex | Rosenberg Zuckerberg Rubinovitz & Gutnick 1979 |
| *Corynebacterium hydrocarboclastus* | lipoprotein | Zajic *et al.* 1977b |
| *Corynebacterium insidiosum* ICPB C113A | neutral lipids | Akit *et al.* 1981 |
| *Corynebacterium lepus* | corynomycolic acid | Gerson & Zajic 1979a |
| *Corynebacterium* sp PPS-II | glycolipid | Panchal & Zajic 1978 |
| *Candida lipolytica* | protein-lipid | Roy *et al.* 1979 |
| *Candida lipolytica* | lipopolysaccharide | Cirigliano & Carman 1984 |
| *Candida petrophilum* | lipopeptide | Iguchi *et al.* 1969 |
| *Torulopsis petrophilum* | protein compound | Cooper & Paddock 1983 |

TABLE 1 - Continued

| ORGANISM | SURFACE ACTIVE AGENT | REFERENCE |
|---|---|---|
| BIOSURFACTANT / BIOEMULSIFIER PRODUCTION | | |
| *Bacillus licheniformis* JF-2 | lipopeptide | Pfiffner *et al.* 1985 |
| Isolate H13A (*Corynebacterium*) | glycolipid | Finnerty & Singer 1984 |
| *Pseudomonas* PG 1 | peptidoglycolipid | Reddy *et al.* 1983 |
| *Pseudomonas* sp MUB | glycolipid | Wagner *et al.* 1983 |

critical micelle concentrations suggests the presence of two surfactants, and preliminary chemical analysis of the compounds indicated that they were probably a glycoprotein and a lipoprotein. Panchal and Zajic (1978) reported that emulsifying agents which also demonstrate surfactant properties, are produced by *Corynebacterium* sp. PPS-II. The agents may be glycolipid in nature, although this requires confirmation. Gerson and Zajic (1979a) found that *Corynebacterium lepus* produces potent biosurfactants which also have emulsifying capability (Gerson & Zajic 1978). Analysis of these predominantly lipid products by Cooper *et al.* (1979b) showed a mixture of corynomycolic acids produced early in the fermentation, followed by production of a lipopeptide containing corynomycolic acids and small amounts of phospholipids and neutral lipids. Gerson and Zajic (1979a) also found corynomycolic acids to be the major components of the biosurfactants produced by *Corynebacterium fascians, C. hydrocarboclastus* and *Corynebacterium xerosis*. Both surfactant and emulsifying properties have been demonstrated by neutral lipids extracted from producing cultures of *C. fascians* ICPB and *Corynebacterium insidiosum* ICPB C113A (Akit *et al.* 1981). Singer *et al.* (1983 a) isolated an organism, tentatively identified as a *Corynebacterium* sp., which produced a glycolipid having both surfactant and emulsifying properties (Finnerty & Singer 1984, Singer *et al.* 1983a, Singer *et al.* 1983b).

Arima and coworkers (1968) initially isolated and characterized a lipopeptide biosurfactant produced by *B. subtilis*.. This compound, called surfactin or subtilysin, demonstrated exceptional surface activity as a reduction of the surface tension of water from 72 to 27 dynes/cm was accomplished with a concentration of 0.005% surfactin. Other biosurfactants seldom reduce the surface tension of water below 30 dynes/cm (Cooper *et al.* 1981). An equally potent biosurfactant resembling surfactin, was produced by *B. licheniformis* (Jenneman *et al.* 1983, Pfiffner *et al.* 1985). This compound also demonstrates emulsifying activity and preliminary studies indicated some

ability to mobilize oil in a sand pack column.

Gerson and Zajic (1978) reported the production of a biosurfactant by *Pseudomonas asphaltenicus* which reduces the surface and interfacial tension of the fermentation broth as effectively, but not as efficiently, as *C. lepus* . The biosurfactant is primarily carbohydrate and is completely devoid of protein. Several investigators have demonstrated production of a rhamnolipid by strains of *Pseudomonas aeruginosa*. Guerra-Santos *et al.* (1984) observed that a rhamnolipid produced by *P. aeruginosa* reduces the surface and interfacial tension of the medium to 29 and 0.25 dynes/cm, respectively. Koronelli *et al.* (1983) found that *P. aeruginosa* P-20 produces a peptidoglycolipid biosurfactant. Another group (Reddy *et al.* 1983) found that *Pseudomonas* PG 1 produces a peptidoglycolipid which demonstrates hydrocarbon emulsifying and solubilizing properties. Wagner *et al.* (1983) also have observed emulsifying capability by a glycolipid produced by *Pseudomonas* sp. MUB.

Several investigators have examined cell-associated biosurfactant production by *Rhodococcus erythropolis* (Kretschmer *et al.* 1982, MacDonald *et al.* 1981, Wagner *et al.* 1983). Many compounds with biosurfactant properties can be recovered, including long chain alcohols, phosphatidylethanolamines and trehalolipids. Rapp *et al.* (1979) found that the trehalolipids were the major compounds responsible for the surface activity. Trehalolipids and long chain alcohols with emulsifying activity, are also produced by *Arthrobacter paraffineus* KY4303 (Suzuki *et al.* 1969) and *A. paraffineus* NRRL B-3453 (Akit *et al.* 1981).

A great number of investigations have been concerned with the very potent emulsifier produced by *A. calcoaceticus* RAG-1. Initially identified as *Arthrobacter* RAG-1 (Belsky *et al.* 1979, Zuckerberg *et al.* 1979), this isolate produces an extracellular lipid-polysaccharide complex called emulsan (Rosenberg Zuckerberg Rubinovitz & Gutnick 1979, Zosim *et al.* 1982). Although it has no surfactant

properties, it is capable of binding very tightly to hydrocarbon droplets, thereby strongly stabilizing emulsions. Emulsan also demonstrates specificity to the hydrocarbon substrates involved in the emulsion (Rosenberg Perry Gibson & Gutnick 1979), in that an aliphatic/aromatic hydrocarbon mixture produces a more stable emulsion than either a strictly aliphatic or aromatic substrate. Kaplan and Rosenberg (1982) have isolated a bioemulsifier from *A. calcoaceticus* BD413, which has a slightly different chemical composition than emulsan although the precise composition of either bioemulsifier has not been determined. The lipid-polysaccharide complex from *A. calcoaceticus* BD413, has similar emulsifying capabilities, but different emulsion hydrocarbon substrate specificity, than emulsan. After testing several *A. calcoaceticus* strains, Sar and Rosenberg (1983) suggested that production of extracellular emulsifying agents is a general characteristic of this species.

Neufeld *et al.* (1983), however, isolated an organism identified as *A. calcoaceticus* 2CA2, which did not produce an extracellular bioemulsifier, but rather had only cell-associated surface activity. This organism also reduced surface and interfacial tension and produced an extracellular lipopeptide with good de-emulsifying properties (Neufeld & Zajic 1984). A strain of *N. amarae* also has shown de-emulsifying capability as well as surfactant activity (Akit *et al.* 1981), with both of these properties being cell-associated (Cairns *et al.* 1982, Gray *et al.* 1984).

Bacteria are not the only microorganisms which produce biosurfactants and bioemulsifiers. The yeast, *Candida petrophilum*, produced an emulsifying factor composed of protein and lipid (Iguchi *et al.* 1969). Ten strains of *Candida lipolytica* demonstrated emulsification properties (Illarionova *et al.* 1984) and Roy *et al.* (1979) characterized the active compound as being either a peptidolipid or a lipopeptide. Cirigliano and Carman (1984), however, recovered a carbohydrate emulsifier from *C. lipolytica*.. Another yeast, *Torulopsis bombicola*, produces a sophorolipid which

demonstrates surfactant activity (Cooper and Paddock 1984, Inoue and Ito 1982). Cooper and Paddock (1983) isolated a potent protein-containing bioemulsifier as well as a glycolipid surfactant from *T. petrophilum* .

B. Distribution of Producing Organisms

Although no surveys have been undertaken to determine the ecological distribution of organisms which produce biosurfactants and bioemulsifiers, one could assume that they are rather ubiquitous in the environment since they have been isolated from a variety of sources. Hydrocarbon contaminated sources, for example oil polluted waters and soils, have yielded many surface active agent producing organisms suggesting a positive relationship between the presence of hydrocarbons and biosurfactant production. For example, *A. calcoaceticus* RAG-1, the producer of emulsan, is an isolate from a mixed culture which was obtained from a beach sample in Tel Aviv, Israel (E. Rosenberg 1985, personal communication). Panchal & Zajic (1978) isolated a bisurfactant producing *Corynebacterium* sp.PPSII from sewage sludge which had been enriched with hydrocarbons. Akit *et al.* (1981) have isolated biosurfactant producing coryneforms from populations of phytopathogens and soil organisms and Finnerty & Singer (1984) isolated a biosurfactant producing *Corynebacterium*, isolate H13-A, from soil. Asphaltic gums, crude petroleum and paving asphalt samples have yielded *Corynebacterium* isolates which produce biosurfactants (Gerson & Zajic 1979a) and a biosurfactant producing strain of *B. licheniformis* was isolated from an oil-field injection water sample (Jenneman *et al.* 1983).

It has long been noted that degradation of hydrocarbons is often accompanied by the production of biosurfactants and bioemulsifiers (Floodgate 1978, Rambeloarisoa *et al.* 1984, Zajic & Panchal 1976). Gutnick and Rosenberg (1977) proposed that surface

active agents function to suspend the hydrocarbon droplets formed, thus making the substrate more accessible to the microorganisms. There also has been the suggestion that extracellular lipid compounds may function in alkane uptake (Finnerty 1977). Floodgate (1978) and Ito and Inoue (1982) are among several investigators who noted that the presence of the biosurfactants and bioemulsifiers markedly stimulated the growth of the organisms on hydrocarbon substrates. These observations suggest that bioemulsifier and biosurfactant production may be related to the presence of hydrocarbon.

It does not follow, however, that a hydrooarbon substrate is always required for the production of surface-active agents. For example, *A. calcoaceticus* RAG-1 produces large quantities of emulsan with ethanol rather than hexadecane as the carbon source (Rosenberg Zuckerberg Rubinovitz & Gutnick 1979). The production of surfactin by *B. subtilis* is actually inhibited by the presence of hydrocarbons although hydrocarbons have no effect on growth of the organism.

## C. Isolation and Growth of Biosurfactant and Bioemulsifier Producers

Because of the common association of the growth on hydrocarbons with the production of surface active agents, enrichment conditions usually involve a hydrocarbon substrate, for example crude oil, kerosene or hexadecane. Even when screening for biosurfactant producers from a sample free of hydrocarbons, e.g. with the phytopathogens investigated by Akit *et al.* (1981), hexadecane was used as a carbon source to stimulate biosurfactant production. Hydrocarbon substrates may function as inducers of biosurfactant production and not just enrich for producing organisms. Therefore, they should be included in the medium when screening for surface active agent producers.

Four percent hexadecane or 4% kerosene has been employed for growth and optimum biosurfactant production of various coryneform bacteria (Akit *et al.* 1981), *C. lepus* (Cooper *et al.* 1979b) and *R. erythropolis* (MacDonald *et al.* 1981). One yeast, *T. bombicola* was grown on 2% alkane for the production of a sophorolipid biosurfactant (Inoue & Ito 1982). The reduction in the amount of alkane, from 4% to 2%, was employed to reduce its toxic effect on the organism. *C. hydrocarboclastus* demonstrated good production of surface active agents when grown on 2% hydrocarbons or 1.5% carbohydrates, although production was higher on $C_{13}$- and $C_{14}$-alkanes than on carbohydrates (Zajic *et al.* 1977a). Both *B. subtilis* (Cooper *et al.* 1981) and *B. licheniformis* JF-2 (Javaheri *et al.* 1985) produce biosurfactant when grown on 4% glucose. Guerra-Santos *et al.* (1984) found that the best production of biosurfactant by *P. aeruginosa* was achieved using either a hydrocarbon or glycerol rather than glucose. Neufeld *et al.* (1983) observed that *A. calcoaceticus* 2C-2 grew better on 1% hexadecane than on 1% kerosene. However, Rosenberg, Perry, Gibson and Gutnick (1979) observed that *A. calcoaceticus* RAG-1 produced greater emulsifying activity when grown on 0.1% ethanol than when grown on 0.2% hexadecane. This may be due, however, to a better recovery of the emulsan from the non-hydrocarbon culture medium rather that differences in production levels.

## 4. RECOVERY AND CHARACTERIZATION OF BIOSURFACTANTS AND BIOEMULSIFIERS

### A. Methods of Recovery of Surface Active Agents

In most cases, the biosurfactants and bioemulsifiers which have been identified are

extracellular products. Therefore, it is cell-free culture liquids that are subjected to various procedures for recovery of these agents. Cells are usually removed by centrifugation and filtration procedures.

The methods used for the recovery of biosurfactants and bioemulsifiers from cell-free broths have generally involved either precipitation or solvent extraction. Surface active agents have been most commonly recovered by acid precipitation, although emulsan has been recovered by ammonium sulphate precipitation (Kaplan & Rosenberg 1982). Acid precipitation has been used to recover biosurfactant from *Bacillus* species (Cooper *et al.* 1981, Pfiffner *et al.* 1985) and from *Corynebacterium* species (Zajic *et al.* 1977b). Organic solvents have been used extensively to extract bioemulsifiers and biosurfactants from cell-free broths. Biosurfactants and bioemulsifiers have been recovered by extraction using ethanol (Hilton & Kapur 1982, Iguchi *et al.* 1969), alkanes (Cooper *et al.*, Cooper Zajic & Gracey 1979, Rosenberg Zuckerberg Rubinovitz & Gutnick 1979), ethyl acetate (Ito & Inoue 1982) and various solvent mixtures (Cirigliano & Carman 1984, Kretschmer *et al.* 1982, Rapp *et al.* 1979, Singer *et al.* 1983a, Suzuki *et al.* 1969, Wagner*et al.* 1983). Acetone precipitation has also been used for bioemulsifier recovery ( Floodgate 1978, Reddy *et al.* 1983).

B. Methods of Characterization of Surface Active Agent Components

Since the major classes of components making up biosurfactants and bioemulsifiers are lipid, carbohydrate and protein, standard analytical procedures have been used for the detection and identification of these components (Cooper *et al.* 1979b, Finnerty & Singer 1984, Javaheri *et al.* 1985, MacDonald *et al.* 1981, Rambeloarisoa *et al.* 1984, Reddy *et al.* 1983).

# 5. APPLICATIONS OF BIOSURFACTANTS AND BIOEMULSIFIERS

## A. Oil Sand Separation

The environmental problems associated with the disposal of tailings from the hot water/alkaline processes used to recover bitumen from oil sands have been a major incentive factor for research into a cold water process using biosurfactants. Measuring the release of bitumen from oil sand by microbial products under laboratory conditions has taken into account two main mechanisms of separation. The first is the release of bitumen from the oil sand through flotation and solubilization of bitumen and the second is the separation of sand and clay from the oil sand leaving a residue with high bitumen concentrations (Zajic & Gerson 1978). It has been observed that tar balls result with the complete removal of the sand and clay from oil sand. Zajic and Gerson (1978) found that *Corynebacterium* IBD released 64% of the sand from oil sand samples and *Corynebacterium* OSGB1 released 10.1% of the total bitumen, as well as increasing the bitumen concentration of the residue. The whole broth of *Corynebacterium* OSGB1 was more effective than the crude recovered biosurfactant at separating bitumen from oil sand (Gerson & Zajic 1977). Zajic and Akit (1983) found that a culture of *C. fascians* was almost as effective at oil sand separation as their most effective commercial surfactant, Nalco 7710.

## B. Viscosity Reduction of Heavy Oil

The reduction of the viscosity of heavy crude oil can be effective in the *in situ* displacement of oil. The formation of stable heavy oil-in-water emulsions can lead to

substantial viscosity reductions of these fluids where the viscosity of this mixture then resembles that of water rather than crude oil (Jack 1984). Hayes and Nestaas (1983) have found that emulsan-stabilized oil-in-water emulsions have an optimum composition of 70% oil and 30% water. A model system of pipeline transport demonstrated the stability of emulsan and the emulsion to the stress of shear degradation found in this mode of fluid transport. This resistance to shear forces has also been demonstrated for *Xanthomonas* polysaccharides (Hoyt 1985) which reduce the turbulent friction in fluid flow thereby reducing drag.

Finnerty and Singer (1984) found that isolate H13-A, substantially reduced relative oil viscosity when grown on heavy crude oils. Viscosity reductions of 93-98% were observed with growth on Mongas crude oil and reductions of up to 99% were observed with growth on Cerro negro crude oil supplemented with hexadecane. The production of a glycolipid-protein polymer and the subsequent formation of a stable oil-in-water emulsion accounts for this phenomenon. The extracellular glycolipid, at a concentration of 295 mg/L, resulted in a 50% reduction of the viscosity of Monagas crude oil (Singer *et al.* 1983b).

# III. MATERIALS AND METHODS

## 1. MICROORGANISMS

### A. Sources of Organisms

Identified organisms capable of producing surface active agents were used as controls for testing the methods used in this study and are listed in Table 2. Cultures known to produce biosurfactants or bioemulsifiers were obtained from the American Type Culture Collection (ATCC): *B. subtilis* ATCC 21332, a surfactin producer and *A. calcoaceticus* ATCC 31012, an emulsan producing RAG-1 isolate. *A. calcoaceticus* BD 413, a bioemulsifier producer (Kaplan & Rosenberg 1982), was provided by Dr. D. Gutnick, Tel Aviv University, Israel. *C. petrophilum* ATCC 21404 and *Corynebacterium* sp. ATCC 15529 were included in this survey because they were described as utilizers of petroleum fractions and n-alkanes. *R. erythropolis* ATCC 25544, formerly known as *Nocardia erythropolis*, was included as a biosurfactant producer.

Other "new" isolates were investigated for their ability to produce biosurfactants and bioemulsifiers. Enrichments of bacteria which have been maintained in the laboratory for several years by regular transferring demonstrated immediate dispersion of oil when it was added to the cultures. These enrichments and pure isolates obtained from these cultures were screened for surface active agent production. The enrichment cultures studied included the Shell Lake culture which was obtained from a sample site at a sea plane base at Inuvik, Northwest Territories (Cook & Westlake 1973) and was very active in oil degradation. This culture and its isolates are listed in Table 3. Other

TABLE 2 - IDENTIFIED ORGANISMS -- GROWTH CONDITIONS AND METHODS OF ASSAY

| CULTURE | MEDIA * | GROWTH TEMPERATURE °C | SUBSTRATE † | ASSAY METHODS ** BAP / Tension / O.D. | | |
|---|---|---|---|---|---|---|
| *A. calcoaceticus* ATCC 31012 | 2a | 27° | K/n-$C_{16}$ | + | - | + |
| | 1 | 27° | K/n-$C_{16}$ | + | - | + |
| *A. calcoaceticus* BD 413 | 5 | 27° | glucose | + | + | + |
| *B. subtilis* ATCC 21332 | 4 | 27° | glucose | + | + | + |
| *C. petrophilum* ATCC 21204 | 2a | 25°/27° | K/n-$C_{16}$ | + | + | + |
| *Corynebacterium* sp. ATCC 15529 | 2a | 17°/27° | n-$C_{14}$ | + | + | + |
| *R. erythropolis* ATCC 25544 | 2b | 25° | yeast extract | - | - | + |
| | 2a | 25° | K/n-$C_{16}$ | - | - | + |

* Media composition are found in the Appendix

† K = kerosene; n-$C_{16}$ = hexadecane; n-$C_{14}$ = tetradecane

** BAP = blood agar plates; Tension = surface and interfacial tension; O.D. = optical density, Spectrophotometric oil emulsion assay; - = assay not done

TABLE 3 - FRESHWATER CULTURES -- GROWTH CONDITIONS AND METHODS OF ASSAY

| CULTURE | GROWTH TEMPERATURE °C | SUBSTRATE * | ASSAY METHODS † BAP / Tension / O.D. | | |
|---|---|---|---|---|---|
| Shell Lake (mixed) | 25° | oil/n-$C_{16}$ | - | + | + |
| Isolate #1A | 25° | oil | + | + | - |
| Isolate #2A | 25° | oil | + | - | - |
| Isolate #3A | 25° | oil | + | - | - |
| Isolate #4A | 25° | oil | + | - | - |
| Isolate #5A | 25° | oil | + | - | - |
| Isolate #6A | 25° | oil | + | - | - |
| Isolate #7A | 25° | oil | + | - | - |
| Isolate #8A | 25° | oil | + | - | + |
| Isolate #9A | 25° | oil | + | - | - |
| Isolate #10A | 25° | oil | + | - | + |
| Isolate #11A | 25° | oil | + | - | - |
| Isolate #12A | 25° | oil | + | - | - |
| Isolate #13A | 17°/25°/27° | oil/ n-$C_{16}$/n-$C_{14}$ | + | - | + |
| PsSLNW8a | 27° | K | - | - | + |
| PsSLNW8aC | 27° | K | - | - | + |

* All cultures were grown in B+N+NuBr (0.05%) with: oil = Prudhoe Bay crude oil;n-$C_{16}$= hexadecane; n-$C_{14}$ = tetradecane; K = kerosene

† BAP = blood agar plate; Tension = surface and interfacial tension; O.D. = optical density, Spectrophotometric oil emulsion assay; - = assay not done

enrichment cultures were obtained from sample sites in northern Puget Sound and the Strait of Juan de Fuca, Washington State (Westlake & Cook 1980) and were designated 1B and 4B (beach) and 4S (sediment). Pure cultures solated from these sources were designated as 1B-1, 1B-2, etc. Many known biosurfactant producers, e.g. species of *Corynebacterium*, *Arthrobacter* and *Bacillus*, are Gram positive organisms. Therefore, Gram positive isolates from the Washington State sites (Westlake & Cook 1980) were tested for surface active agent production and are designated as "NOAA isolates". These organisms and the other marine cultures surveyed are listed in Table 4.

Hydrocarbon enriched sources provided other cultures for this investigation. Samples of water from oil storage tanks were obtained from Interprovincial Pipelines in Edmonton, Alberta, and enrichment cultures from these samples were designated IPL TS-1 through IPL TS-5 (Table 5). Oil degrading isolates, designated as PsL7aF, PsL7aC, PsL6a and PsL1a (Table 6) were obtained from soil samples at Lodgepole, Alberta, which were contaminated by an oil spill in 1984. An enrichment culture also was established from an Athabasca oil sand sample using 20 g oil sand/200 mL medium. This enrichment and the pure cultures isolated from this source are listed in Table 6.

Fertile soil and sewage are rich sources for a great variety of microorganisms and enrichment cultures were established from samples of garden soil using 10 g soil/200 mL medium, and a sample of sewage using 10 mL sewage/200 mL medium from the Goldbar Wastewater Treatment Plant, Edmonton, Alberta. These cultures (Table 6) were included in the screening processes and as potential sources of pure cultures. A few contaminants (Table 6) were included which were isolated from solvent rinse containers and dilute, non-sterile dispersant solutions.

TABLE 4 - MARINE CULTURES ** -- GROWTH CONDITIONS AND METHODS OF ASSAY

| CULTURE | MEDIA * | GROWTH TEMPERATURE ℃ | SUBSTRATE † | ASSAY METHODS ◊ BAP / Tension / O. D. | | |
|---|---|---|---|---|---|---|
| MIXED CULTURES | | | | | | |
| 1B | a | 25° | oil | - | + | + |
| 4B | a | 25° | oil | - | + | + |
| 14S | a | 25° | oil | - | + | + |
| ISOLATES (from Culture 1B or Culture 4B) | | | | | | |
| 1B-1 | b | 27° | K | + | [redacted] | + |
| 1B-2 | b/c | 17°/27° | oil/K/n-$C_{16}$ /n-$C_{14}$ | + | + | + |
| 1B-3 | b | 27° | oil/K | + | - | + |
| 1B-5 | b | 27° | oil/K | + | - | + |
| 1B-4 | b | 27° | n-$C_{16}$ | + | - | + |
| 1B-4A | b | 27° | n-$C_{16}$ | + | - | + |
| 4B-6 | o | 17° | n-$C_{14}$ | + | + | - |
| "NOAA ISOLATES" | | | | | | |
| 6WIST10-2 | b | 27° | oil | - | - | + |
| 6WIST10-6 | b | 27° | oil | - | - | + |
| 8WIST10-13 | b | 27° | oil | - | - | + |
| 27ST7-5 | b | 27° | oil | - | - | + |
| 27ST7-6 | b | 27° | oil/K | - | + | + |
| 27ST7-16 | b | 27° | oil | - | - | + |

TABLE 4 - Continued

| CULTURE | MEDIA * | GROWTH TEMPERATURE °C | SUBSTRATE † | ASSAY METHODS ◊ BAP / Tension / O. D. | | |
|---|---|---|---|---|---|---|
| 23ST7-1 | b | 27° | oil | + | - | + |
| 23ST7-6 | b | 27° | oil | + | - | + |
| 24ST7-3 | b | 27° | oil | - | - | + |
| 24ST7-14 | b | 27° | oil | + | - | + |
| 24ST7-16 | b | 27° | oil | + | - | + |
| 24ST7-18 | b | 27° | oil | + | - | + |
| C#6T10-8 | b | 27° | K/n-$C_{16}$ | - | - | + |
| 24SILT11-1 | b | 27° | K/n-$C_{16}$ | - | - | + |
| 24SILT11-3 | b | 27° | K/n-$C_{16}$ | - | - | + |
| 24SILT11-12 | b | 27° | K/n-$C_{16}$ | - | - | + |
| 24SILT11-19 | b | 27° | K/n-$C_{16}$ | - | - | + |
| 27SILT11-8 | b | 27° | K | + | - | + |
| 27ST11-9 | b | 27° | K | + | - | + |
| 34ST11-9 | b | 27° | K | + | - | + |
| 24SILT11-17 | b | 27° | K | + | - | + |
| 24BT11-6 | b | 27° | K | + | - | + |
| 20S20°E2-3 | b | 27° | K | + | - | + |
| 5W8°E3-5 | b | 27° | K | + | - | + |
| 5B8°E3-8 | b | 27° | K | + | - | + |
| 20W8°E3-3 | b | 27° | K | - | - | + |
| 20W20°E2-2 | b | 27° | K | - | - | + |

TABLE 4 - Continued

| CULTURE | MEDIA * | GROWTH TEMPERATURE °C | [redacted]TRATE † | ASSAY METHODS ◊ BAP / Tension / O. D. | | |
|---|---|---|---|---|---|---|
| 20W20°E2-9 | b | 27° | K | - | - | + |
| 20S20°E2-2 | b | 27° | K | - | - | + |
| 20W20°E2-1 | b | 27° | K | + | - | + |
| 20S8°E3-5 | b | 27° | K | + | - | + |
| 20W8°E3-4 | b | 27° | K | + | - | + |
| 20S8°E3-6 | b | 27° | K | + | - | + |
| 20S20°E2-6 | b | 27° | K | - | - | + |
| 20W8°E3-12 | b/c | 17°/27° | K/n-$C_{14}$ | + | + | + |
| 20W8°E3-3 | b | 27° | n-$C_{14}$ | + | + | - |
| 20S8°E3-5 | b | 27° | n-$C_{14}$ | + | + | - |
| 8BT10-15 | b/c | 17°/27° | n-$C_{14}$ | + | + | - |
| 6BT10-6 | b/c | 17°/27° | n-$C_{14}$ | + | + | - |
| 24BT11-6 | b | 27° | n-$C_{14}$ | + | + | - |
| 34ST11(B)9 | b | 27° | n-$C_{14}$ | + | + | - |

** Cultures were from Puget Sound and Strait of Juan de Fuca, Washington State, U.S.A.

* Media is ASW with: a = N,P; b = N,P+NuBr (0.05%); c = NuBr (0.8%)

† oil = Prudhoe Bay crude oil; K = kerosene; n-$C_{16}$ = hexadecane; n-$C_{14}$ = tetradecane

◊ BAP = blood agar plate; Tension = surface and interfacial tension; O.D. = optical density, Spectrophotometric oil emulsion assay; - = assay not done

TABLE 5 - CULTURES FROM PRODUCED WATERS -- GROWTH CONDITIONS AND METHOD OF ASSAY

| CULTURE | MEDIA / TEMPERATURE | SUBSTRATE * | O.D. † |
|---|---|---|---|
| MIXED CULTURES | | | |
| IPL TS-1 | - all cultures were grown in ASW+N,P+NuBr (0.05%) at 27°C | n-$C_{16}$ | + |
| IPL TS-2 | | n-$C_{16}$ | + |
| IPL TS-3 | | K/n-$C_{16}$ | + |
| IPL TS-4 | | K/n-$C_{16}$ | + |
| IPL TS-5 | | n-$C_{16}$ | + |
| ISOLATE | | | |
| IPL TS1-4 | ASW+N,P+NuBr (0.05%) at 27°C | K/n-$C_{16}$ | + |

* K = kerosene; n-$C_{16}$ = hexadecane

† O.D. = optical density, Spectrophotometric oil emulsion assay

TABLE 6 - BITUMEN, SOIL AND SEWAGE SAMPLE CULTURES AND CONTAMINANTS -- GROWTH CONDITIONS AND METHODS OF ASSAY

| CULTURE | GROWTH TEMPERATURE °C | SUBSTRATE * | ASSAY METHODS † BAP / Tension / O.D. | | |
|---|---|---|---|---|---|
| ATHABASCA OIL SAND CULTURE AND ISOLATES | | | | | |
| Bitumen (mixed) | 17°/25°/27° | K/ n-$C_{16}$/n-$C_{14}$ | - | - | + |
| Isolate B-#2B | 27° | K/ n-$C_{16}$ | + | - | + |
| Isolate B-#6B | 27° | K/ n-$C_{16}$ | + | - | + |
| Isolate B-#7B | 27° | K | + | - | + |
| Isolate B-#9B | 27° | K/ n-$C_{16}$ | + | - | + |
| Isolate B-#1B | 27° | K/ n-$C_{16}$ | + | - | + |
| Isolate B-#3B | 17°/27° | K/ n-$C_{14}$ | + | + | + |
| Isolate B-#4B | 17°/27° | K/ n-$C_{14}$ | + | + | + |
| Isolate B-#5B | 17°/27° | K/ n-$C_{14}$ | + | + | + |
| Isolate B-#8B | 17°/27° | K/ n-$C_{14}$ | + | + | + |
| SOIL SAMPLES | | | | | |
| Soil #1 (mixed) | 27° | K | - | - | + |
| Soil #2 (mixed) | 27° | oil | - | - | † |
| OIL-CONTAMINATED SOIL SAMPLES | | | | | |
| PsL7aF | 27° | K | + | - | + |
| PsL7aC | 27° | K | + | - | + |
| PsL6a | 27° | K | + | + | + |
| PsL1a | 27° | K | + | + | + |

TABLE 6 - Continued

| CULTURE | GROWTH TEMPERATURE °C | SUBSTRATE * | ASSAY METHODS † BAP / Tension / O.D. | | |
|---|---|---|---|---|---|
| SEWAGE SAMPLE | | | | | |
| Sewage (mixed) | 27° | K | - | + | + |
| CONTAMINANT ISOLATES | | | | | |
| W.D.#1 | 17°/27° | n-$C_{14}$ | + | + | - |
| W.D.#2 | 17°/27° | n-$C_{14}$ | + | + | - |
| Disp-1 | 27° | K | - | - | + |
| Co9600-1 | 27° | n-$C_{14}$ | - | - | + |
| Co7664-1 | 27° | n-$C_{14}$ | - | - | + |

* All cultures were grown in B+N+NuBr (0.05%) with: oil = Norman Wells crude oil; K = kerosene; n-$C_{16}$ = hexadecane; n-$C_{14}$ = tetradecane

† BAP = blood agar plates; Tension = surface and interfacial tension; O.D. = optical density, Spectrophotometric oil emulsion assay; - = assay not done

B. Growth Conditions

The various media, hydrocarbon substrates, growth temperatures and assay methods used for each culture are listed in Tables 2-6. Cultures, unless otherwise specified, were grown in in 500 mL Erlenmeyer flasks containing 200 mL of liquid medium with a 1% hydrocarbon substrate. The composition of the media used are listed in the Appendix. The cultures were incubated on gyrotary shakers at 250-300 rpm. Initially, inocula for the cultures were provided directly from enrichment cultures using 10 mL as inoculum, or from colonies on solid media. The resulting liquid cultures were successively transferred, using 10 mL of 72-96 hr culture as inoculum, at least twice in a medium before assessing surface active agent production.

Pure isolates were obtained from the various enrichment cultures by serial dilution and plating on solid media, incubating at 27°C for 24-72 hr and picking single colonies. Each colony selected in this manner was replated to ensure purity. Marine cultures and others grown in artificial sea water (ASW) medium, were plated on basal marine agar (BMA) while cultures grown in non-marine types of media were plated on plate count medium solidified with gellan gum (PC+K9A40). Gellan gum was used instead of agar to solidify this medium because of the clarity of the gelled medium and its lower cost.

## 2. CHEMICALS

The hydrocarbon substrates employed for growth and assay methods included Prudhoe Bay oil (Atlantic Richfield Co., Harvey Technical Center, Harvey, Illinois), Cold Lake oil (Esso Resources Canada Ltd.), kerosene (Gulf Canada), hexadecane (both Eastman Kodak Co., 98%; and Fisher Scientific, 99.8%), tetradecane (Chemonics Scientific Ltd., 99%) and 2-methyl naphthalene (Aldrich Cemical Co. Inc.).

Commercial dispersants Drew Lt (Drew Chemical Ltd., Ajax, Ontario) and Igepal Co 630 (Van Waters & Rogers, Ltd., Edmonton, Alberta) were used as reference compounds. A sample of the biosurfactant produced by *A. calcoaceticus* BD413 ("emulsan") was provided by Dr. D. Gutnick, Tel Aviv University, Israel.

Pentane, methylene chloride, ethyl acetate and acetone (Fisher Scientific) were used as extraction solvents for the recovery of biosurfactant. Toluene (Fisher Scientific) was used in the bitumen recovery assay.

Thin layer chromatography procedures involved the use of plastic sheets coated with silica gel $F_{254}$ (Merck) and Rhodamine-6-G dye (Sigma). Reference compounds included myristic acid, monomyristin, dimyristin and trimyrisitn (Sigma). Hexane, isopropyl ether and acetic acid (Fisher Scientific) were used for developing the chromatograms.

## 3. ASSAY METHODOLOGY

### A. Biosurfactants

Biosurfactants can cause the clearing of both blood agar plates (Mulligan *et al.* 1984) and oil plates (Singer 1985), and these two rapid (24-48 hr) screening methods were used to screen for biosurfactant production. Various isolates were grown aerobically on sheep's blood agar plates and both the growth of the organisms and the clearing of the plates were visually monitored. It was observed that several plates showed clearing after storage at 4°C, even though they had not shown any clearing during growth at 27°C. Therefore, after four days incubation at 27°C, those plates which did not demonstrate any clearing were further incubated for up to an additional 4 days at 4°C. Because clearing could be due to the action of proteases, organisms which

demonstrated clearing on blood agar plates were further tested for protease production using a gelatin medium. Various isolates were grown on oil plates at 27°C and their growth and clearing of oil was visually monitored.

Biosurfactant and bioemulsifier assays were carried out on cell-free culture filtrates. Culture broths were centrifuged for 25 min at 3,840 *g*, or for 20 min at 5,300 *g*, to partially pellet the cells. The supernatant was then filtered using a membrane filter (0.45 μm pore, Millipore apparatus). A prefilter (Millipore) and glass wool (Corning Glass Works) were also used to aid the filtration process. The use of this procedure means that any cell-associated biosurfactant production would not be detected in this study.

The tensiometric assay was used as the standard method to determine the presence of extracellular biosurfactants. The surface and interfacial tensions of 25 mL of the liquid samples were determined at 25°C using an automatic tensiometer (Fisher Model 215 Autotensiomat Surface Tension Analyzer). The units of surface and interfacial tension used, are dynes/cm, which are equivalent to milliNewtons/m (mN/m). The interfacial tension was measured against 15 mL of hexadecane. All tensiometric measurements were recorded on a Hewlett-Packard 7100 B strip chart recorder. A commercial dispersant, Igepal Co 630, was used as a reference. The determination of the critical micelle concentration (CMC) was accomplished by tensiometric monitoring of successively diluted aliquots of the sample, plotting the surface and interfacial tension values on a graph, and the determining the dilution at which the surface and interfacial tension values begin to rise. This value is considered to be the CMC. The inverse of this value ($CMC^{-1}$) is the relative concentration of biosurfactant that is produced by the culture.

B. Bioemulsifiers

The production of bioemulsifiers was detected by determining the stability of the emulsions formed. The spectrophotometric oil emulsification assay method that was developed was a modification of the method of Rosenberg, Zuckerberg, Rubinovitz and Gutnick (1979). The procedure involved creating an emulsion and, after a period of time, measuring the optical density of the emulsion. In developing the method, several parameters were investigated including the mechanical procedure for creating the emulsion, the period of time allowed for phase separation, the emulsion substrate used and the selection of a suitable wavelength for monitoring emulsions. The stability of emulsions prepared by circular mixing for 15 and 60 sec using a vortex mixer and horizontal shaking for 15, 30 and 60 sec using a reciprocal shaker were compared over the period from 2 min to 60 min. The commercial dispersant, Drew Lt, was used to establish these parameters using various concentrations of three hydrocarbon emulsion substrates, Prudhoe Bay oil, kerosene and hexadecane/2-methyl-naphthalene (H/2mN). As the standard assay, 0.1 mL of hexadecane/2-methyl-naphthalene (1:1, v/v) emulsion substrate was added to 4.9 mL of liquid sample in a screw-capped test tube. The tubes were shaken horizontally for 30 sec, at 280 oscillations/min, on an Eberbach reciprocal shaker and allowed to stand vertically to settle for 10 min. A 2 mL sample was then removed from the lower third of the tube using a Pasteur pipette and the optical density determined at 523 nm (Jones, 1975) using a Pye Unicam SP8-500 spectrophotometer and a 1 cm light path. Cell-free filtrates were used for this assay and each filtrate was assayed in triplicate using unaltered filtrate as a blank. Sterile medium with emulsion substrate was used as a control. The commercial dispersant, Igepal Co 630, and a sample of "emulsan" (provided by Dr. D. Gutnick, Tel Aviv University) were used as reference emulsifying compounds. Increases in optical density over control values

indicated the presence of emulsifier.

The test tube emulsification method (Gerson and Zajic 1978) also was employed using 60 sec rather than 2 minutes of mixing by vortex agitation to create an emulsion. This method was tested using *A. calcoaceticus* BD 413 filtrates, the Igepal Co 630 standard dispersant and "emulsan".

## 4. IDENTIFICATION OF ISOLATE 1B-2 AND CHARACTERIZATION OF ITS BIOSURFACTANT

### A. Identification of Isolate

As culture filtrates of isolate 1B-2 produced significant tensiometric reduction indicating production of biosurfactant, the organism was identified and the biosurfactant produced was recovered and subjected to preliminary analysis. Light microscopic techniques were used to determine cell morphology, Gram stain reaction, motility and spore formation. Several metabolic characteristics, e.g. oxygen requirement, catalase and oxidase, were also determined to aid in identification of this isolate (Smibert and Krieg 1981). The percent guanine plus cytosine content of the deoxyribonucleic acid of isolate 1B-2 was determined using the procedures of Johnson (1981).

### B. Recovery of Biosurfactant

To obtain biosurfactant for characterization, cell-free filtrates of isolate 1B-2 cultures were subjected to different precipitation and solvent extraction methods. Ammonium sulphate precipitation (Kaplan & Rosenberg 1982), acid precipitation at pH2 and solvent extractions using methylene chloride, pentane or ethyl acetate were compared

as to efficiency of recovery of biosurfactant activity.

Pentane extraction (Cooper & Zajic 1978) was the most efficient and this method was carried out at various pH levels to determine the optimum conditions for recovery of biosurfactant. The standard method for biosurfactant recovery involved pentane extraction at pH 2. Cell-free culture filtrates were extracted twice with 35 mL pentane/200 mL filtrate. The solvent was removed by rotary evaporation at 35-40°C and the resulting crude sediment resuspended in double-distilled water. The sediment also was extracted with acetone to yield an acetone-soluble and an acetone-insoluble fraction. These partially purified preparations were assayed tensiometrically to determine biosurfactant activity. The critical micelle concentration of the crude extract was determined by successive dilution with double-distilled water. Comparisons of filtrate and pentane-extract critical micelle concentrations enabled the percent recovery of the surfactant to be calculated. Samples of the crude pentane extract and the acetone-soluble fraction were weighed, dissolved in methylene chloride and aliquots of these solutions were placed in the tensiometric apparatus. The solvent was evaporated and the residue dissolved in double-distilled water and the surface and interfacial tension was determined. This allowed the specific activity of the biosurfactant to be determined in terms of the critical micelle concentration.

C. Characterization of Biosurfactant

Chemical characterization of the partially purified biosurfactant involved determining the percent composition of carbohydrate, protein and lipid in the lyophilized pentane extract and in acetone-soluble portion of the extract. Carbohydrate was determined by Dubois' phenol-sulphuric acid assay (Dubois *et al.* 1956) using fructose as the standard, and protein was assayed by ultraviolet absorption (Kalb & Bernlohr

1977) using samples dissolved in methylene chloride. Lipid composition was determined gravimetrically as described for purified total lipids (Hanson & Phillips 1981).

Thin layer chromatography was employed to analyze the lipid composition of this biosurfactant. Silica gel 60 $F_{254}$ sheets were spotted with biosurfactant and known lipid compounds dissolved in methylene chloride. Both acetone-soluble and acetone-insoluble portions of the biosurfactant were used along with the reference lipids myristic acid, monomyristin, dimyristin and trimyristin. Chromatograms were developed in hexane/isopropyl ether/acetic acid (15:10:1) solvent as outlined (Cooper *et al.* 1979b). Visualization was achieved by spraying the chromatograms with 0.1% Rhodamine-6-G, in $CH_2Cl_2$. Lipids appeared as yellow spots under ultra-violet light.

## 5. APPLICATION OF BIOSURFACTANT - BITUMEN RECOVERY FROM OIL SAND

The effectiveness of the biosurfactant in the recovery of bitumen was determined by measuring its release from oil sand into an aqueous phase. Athabasca oil sand 1.5 g samples were placed into 50 mL Erlenmeyer flasks and autoclaved at 121°C for 15 min. Twenty mL of double-distilled water, double-distilled water with biosurfactant, or double-distilled water with 2% (v/v) Igepal Co 630 were added to the flasks, representing a control, a biosurfactant test and a reference commercial dispersant effect, respectively. The flasks were agitated on a rotary shaker at 200 rpm for 24 hr. The contents of each flask were then centrifuged at 12,100 *g* for 15 min, the supernatant collected and the bitumen extracted with 10 mL of toluene. The toluene was removed by

rotary evaporation at 50-55°C, and the recovered bitumen redissolved in 2 mL of methylene chloride. The relative amount of bitumen recovered from oil sand under these various conditions was determined by measuring the absorbance at 410 nm using a 1 cm light path.

# IV. RESULTS AND DISCUSSION

## 1. METHODS

### A. Development of Spectrophotometric Oil Emulsion Assay

The technique for the preparation of the emulsions and the emulsion substrate for the spectrophotometric oil emulsion assay were established using commercial dispersants as emulsifiers. The degree of emulsification and stability varied among dispersants. Initially Drew Lt was used to establish the conditions of the assay. Another commercial dispersant, Igepal Co 630, which is cited as a reference dispersant in the "Guidelines on the Use and Acceptability of Oil Spill Dispersants" (Environment Canada, Report EPS 1-EE-73-1, 1973) was used later in the development of the assay method. Drew Lt and Igepal Co 630 were similar in their emulsifying activity. The data in Figure 1 illustrate the degree of emulsification and stability difference observed between emulsions prepared using circular mixing for 15 sec and reciprocal shaking for 30 sec. The mixing times specified created the most stable emulsions for each mixing method. These data reveal a much greater instability and variation among samples by circular mixing than by reciprocal shaking. The data also show that emulsions prepared by reciprocal shaking are stable for 10-30 minutes. The effect of varying the reciprocal shaking time on emulsion stability also was investigated. A 30 sec shaking time produced emulsions with the least variation among triplicate samples and were the most stable emulsions for 10 to 30 min (data not shown). Consequently, a reciprocal shaking time of 30 sec and a standing time of 10 min were selected as conditions for this assay method.

Undefined hydrocarbon mixtures, i.e. Prudhoe Bay oil or kerosene, initially were

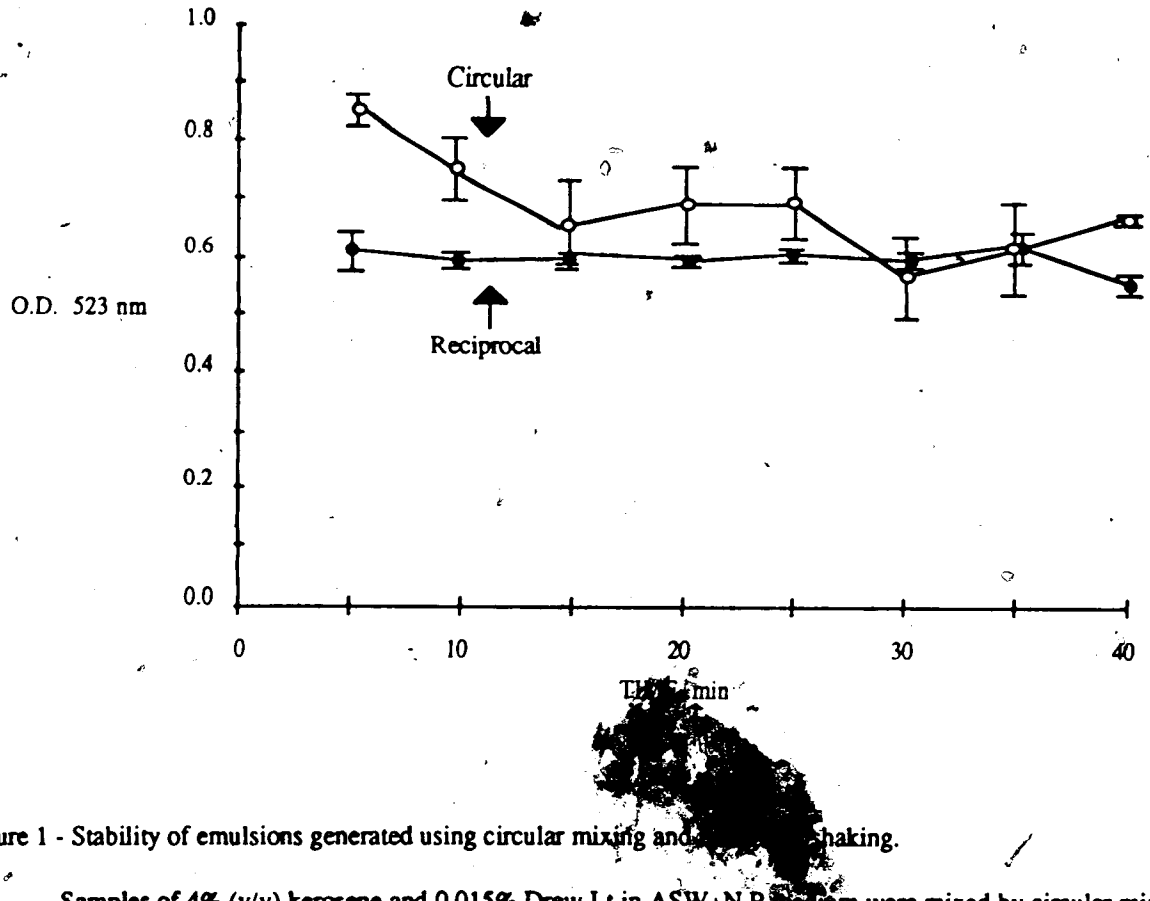


Figure 1 - Stability of emulsions generated using circular mixing and [illegible] shaking.

Samples of 4% (v/v) kerosene and 0.015% Drew Lt in ASW+N,P medium were mixed by circular mixing for 15 sec or reciprocal shaking for 30 sec. Emulsification is represented by O.D. measurements Each point is an average of triplicate samples; error bars represent 1 standard deviation.

investigated as emulsion substrates. While optical density readings were higher when using crude oil than when using kerosene as emulsion substrate, crude oil was not emulsified as easily as kerosene (data not shown). It also interfered with the pipetting of the sample from the test tube to the cuvettes for the emulsification determinations. Rosenberg, Perry, Gibson and Gutnick (1979) report that a mixture of aliphatic and aromatic hydrocarbons, e.g. hexadecane/2-methyl naphthalene (1:1, v/v), provides a more suitable emulsion substrate for *Acinetobacter* RAG i than either strictly aliphatic or aromatic hydrocarbons. The data in Figure 2 shows the increased affinity of the dispersant Igepal Co 630 for this hexadecane/2-methyl-naphthalene mixture over kerosene. Therefore, the mixture was used as the emulsion substrate. As increasing the concentration of emulsion substrate, i.e. from 2% - 6% (v/v) H/2mN, did not make any significant difference to the O.D. measurements, a volume 0.1 mL, i.e. 2%, of emulsion substrate was used in all assays.

A wavelength of 523 nm, used by Jones (1975) for detecting micelles, was chosen to determine optical density. This wavelength is within the range of a green filter, which was used by Rosenberg's group for their assay of emulsification (Reisfield *et al.* 1972).

B. Validation of Assay Methods

The spectrophotometric oil emulsion assay method was used to screen cultures for bioemulsifier production and both *A. calcoaceticus* ATCC 31012 and *A. calcoaceticus* BD 413, known bioemulsifier producers, demonstrated significant activity (Table 7). More extensive investigations of the *Acinetobacter* strains indicated that for *A. calcoaceticus* ATCC 31012 (Figure 3), bioemulsifier production essentially parallels growth with maximum levels being reached at the end of exponential growth phase. The

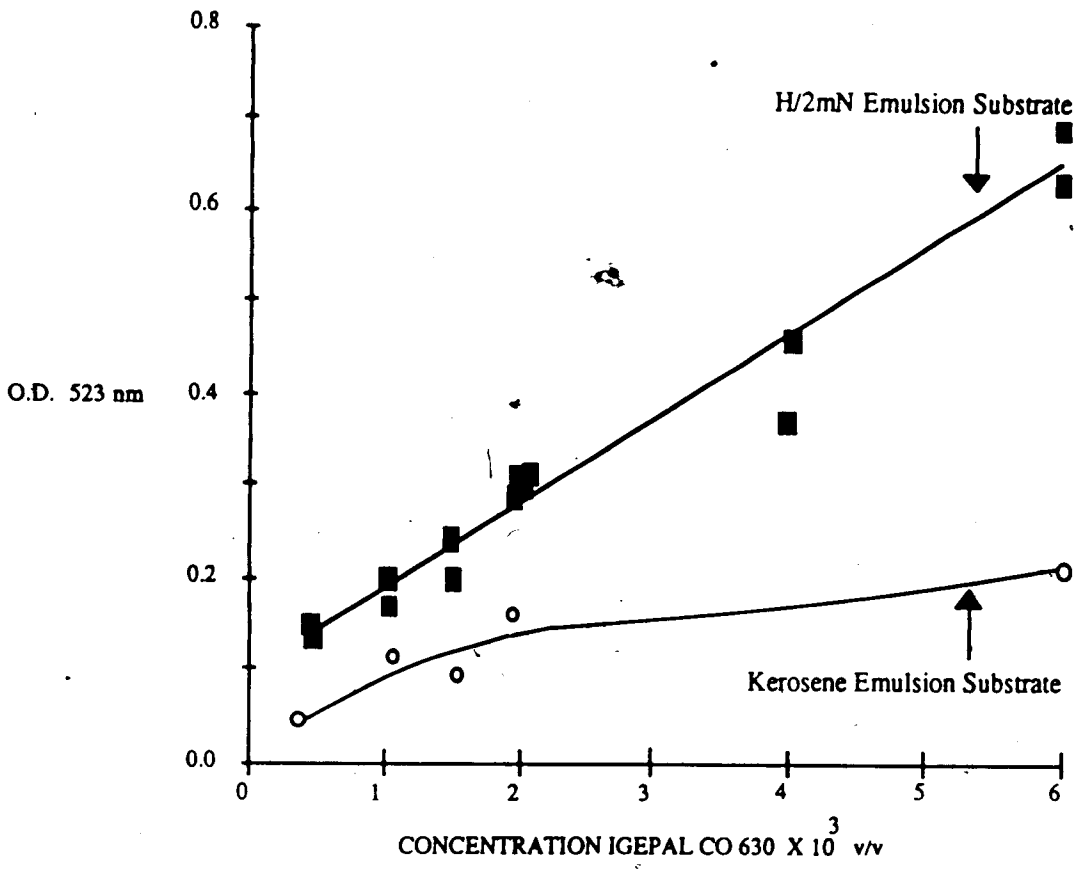


Figure 2 - Comparison of the emulsification of kerosene and hexadecane/2-methyl naphthalene.

Samples of varying concentrations of Igepal Co 630 in ASW-NuBr medium contained 2% (v/v) of either hexadecane/2-methyl naphthalene (H/2mN) or kerosene (K) as emulsion substrates. Standard conditions of 30 sec reciprocal shaking and 10 min standing were used in the preparation of these emulsions. Emulsification is represented by O.D.

TABLE 7 - IDENTIFIED ORGANISMS -- PRODUCTION OF SURFACE ACTIVE COMPOUNDS

| CULTURE | BAP at 27°C | TENSION ** Surface dynes/cm | TENSION ** Interfacial dynes/cm | O.D. ◊ ± (90% confidence limit) (Emulsion Substrate) † |
|---|---|---|---|---|
| *A. calcoaceticus* ATCC 31012 | + | no reduction | | 0.14 ± 0.03 (H/2mN) |
| *A. calcoaceticus* BD 413 | - | no reduction | | 0.35 ± 0.27 (H/2mN) |
| *B. subtilis* ATCC 21332 | + | (73.0) * 25.5 | (15.0) 0.8 | no activity (H/2mN) |
| *Corynebacterium* sp. ATCC 15529 | + (4°C) | (63.5) 42.0 | (25.5) 20.0 | no activity (H/2mN) |
| *C. petrophilum* ATCC 21204 | - | (63.5) 50.5 | (25.5) 20.0 | no activity (K/2mN, H/2mN) |

** Surface and interfacial tension to detect biosurfactant activity

◊ Spectrophotometric oil emulsion assay to detect bioemulsifier activity

* Tensiometric control (medium) values for comparison with value(s) listed below

† H/2mN = hexadecane/2-methyl naphthalene (1:1); K/2mN = kerosene/2-methyl naphthalene (1:1)

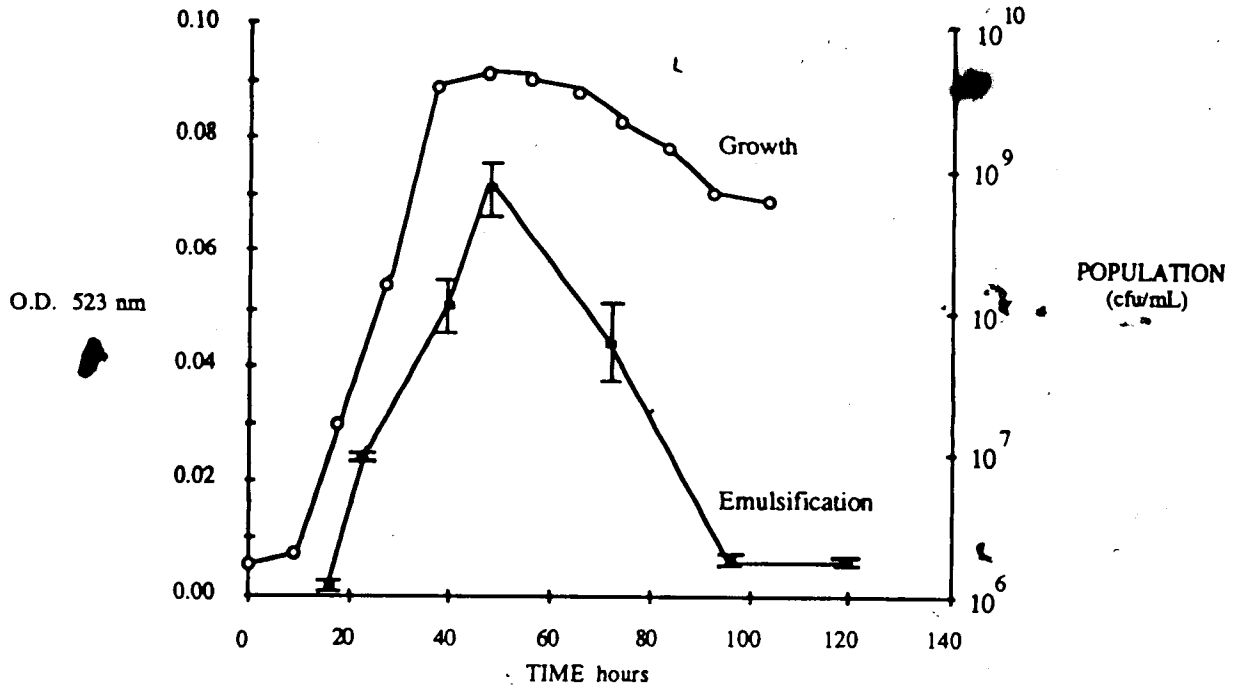


Figure 3 - *Acinetobacter calcoaceticus* ATCC 31012 growth and bioemulsifier production.

*A. calcoaceticus* was grown at 27°C in Acinetobacter medium with 2% hexadecane. Emulsification (O.D.) indicates bioemulsifier production. Population (cfu/ml) and emulsification data were obtained from duplicate batch cultures.

maximum average bioemulsifier production observed for *A. calcoaceticus* ATCC 31012 (Figure 3) gave an O.D. reading of 0.07. Strain *A. calcoaceticus* BD 413 followed a similar growth pattern to *A. calcoaceticus* ATCC 31012, reaching stationary phase in 48 hr and the level of bioemulsifier was maximal during the stationary phase of growth. These results are consistent with those reported for *A. calcoaceticus* BD 413 (Kaplan & Rosenberg 1982), for *A. calcoaceticus* RAG1 (Rosenberg Zuckerberg Rubinovitz & Gutnick 1979) and for other *A. calcoaceticus* strains (Reisfield *et al.* 1972, Rubinovitz *et al.* 1982).

The tensiometric assay measures surfactant activity of compounds. The commercial dispersant, Igepal Co 630, reduced the surface and interfacial tension of double-distilled water from 73 and 44 dynes/cm to 32 and 3 dynes/cm, respectively (Figure 4). Culture filtrates of *B.subtilis* ATCC 21332 reduced the surface and interfacial tension of a minimal medium containing glucose from 73 dynes/cm and 15 dynes/cm to <26 dynes/cm and <1 dyne/cm, respectively (Table 7). These results are similar to those obtained by Arima *et al.* (1968), who reported that surfactin, the biosurfactant produced by *B. subtilis* reduced the surface tension of water to 27 dynes/cm.

A simple, direct, rapid screening method for the detection of surface active agent production involves monitoring growth and hemolysis on blood agar. Two of the identified organisms, *B. subtilis* ATCC 21332 and *A. calcoaceticus* ATCC 31012 (Table 7), demonstrated clearing of blood agar plates within 24 hr at 27°C, whereas the *Corynebacterium* species, ATCC 15529 and ATCC 21204, did not. Neither of these BAP-positive organisms demonstrated protease activity. The data in Table 7 also show that *B. subtilis* produced biosurfactant and that *A. calcoaceticus* ATCC 31012 produced a bioemulsifier. These results are consistent with those of Mulligan *et al.* (1984), who found that *B. subtilis* caused clearing of blood agar, while three other

biosurfactant producing *Corynebacterium* sp. did not. Mulligan's group proposed that biosurfactant producers that are BAP-negative require hydrocarbon substrate for biosurfactant production. Singer (1985) also showed that only biosurfactant producing cultures cleared blood agar.

The oil plate assay method (Singer *et al.* 1983b) also allows for rapid presumptive screening of organisms which disperse or solubilize crude oil. In this study, however, all of the identified organisms assayed, i.e. *A. calcoaceticus* ATCC 31012, *A. calcoaceticus* BD413, *B. subtilis* ATCC 21332 and *C. petrophilum* ATCC 21204, showed good growth but no clearing of oil plates. This suggests that any surface active agents produced, were unable to solubilize the crude oil (Singer 1985). Only Prudhoe Bay oil was used as substrate for this plate assay and because of the difficulty in preparing oil plates this method was not further investigated in this study.

The test tube emulsification method developed by Gerson and Zajic (1978) for detecting bioemulsifiers gave very inconsistent results in this study. Using the emulsifiers: "emulsan", Igepal Co 630 and *A. calcoaceticus* BD413 culture filtrates, emulsion ratios of duplicate and triplicate samples for each, showed relative standard deviations of >20%. Because of this, and the long incubation time required before results were obtained this method was considered inappropriate as a screening procedure.

## 2. SCREENING OF "NEW" CULTURES

### A. Biosurfactant Production

Isolate PsL1a was the only "new" isolate, of 59 cultures tested, that showed

clearing of blood agar plates at 27°C (Table 8). This isolate, however, did not show any other evidence, e.g. tensiometric or spectrophotometric, of surface active agent production. This isolate was found to be protease positive and this would account for its hemolytic behavior. Isolate 1B-2 and *Corynebacterium* sp. ATCC 15529 did not produce clearing of blood agar plates yet demonstrated surfactant production by the tensiometric procedures. This indicates that they cannot produce surface active agents without the presence of a hydrocarbon substrate (Mulligan *et al.* 1984). This hypothesis was verified for isolate 1B-2 when it was grown without a hydrocarbon substrate (see section on Production of Biosurfactant by *Corynebacterium* sp. 1B-2). The clearing of blood agar plates occurred with several isolates following a further incubation of the plates at 4°C. Although some of these organisms, e.g. isolates: 1B-2, 20W8°E3-12 and 8BT10-15, were shown to be biosurfactant producers, others, e.g. isolates: B-#3B, B-#4B and 20W8°E3-3, are non-producers (Tables 7 & 8). Therefore, cold temperature clearing cannot be correlated with biosurfactant production.

Four of the 30 cultures tested, 1B-2, 20W8°E3-12, 8BT10-15 and 6BT10-6, showed significant tensiometric reduction (Table 8). Surface and interfacial tensions were reduced to approximately 30 dynes/cm and <1 dyne/cm, respectively, for each of these isolates. This indicates that these cultures are significant producers of biosurfactants. All of these cultures are Gram-positive organisms from marine environments. The magnitude of surface and interfacial tension reduction observed for these organisms compares favorably with that achieved by *B. subtilis* which has been cited as a producer of the most effective biosurfactant reported in the literature (Zajic & Steffens 1984). Critical micelle concentrations were determined for isolates 1B-2 and 20W8°E3-12 as they showed the greatest tensiometric reduction of all the isolates tested. Average $CMC^{-1}$ values of 2.5 and 1.7 were calculated for cell-free culture filtrates of isolate 1B-2 and isolate 20W8°E3-12, respectively. Isolate 1B-2 showed more

TABLE 8 - "NEW" CULTURES -- PRODUCTION OF SURFACE ACTIVE COMPOUNDS

| CULTURE | BAP | TENSION ** Surface dynes/cm | Interfacial | O.D. ◊ ± (90% confidence limit) (Emulsion Substrate) † |
|---|---|---|---|---|
| FRESHWATER CULTURES | | | | |
| | | (65.5) * | (32.0) | |
| Shell Lake (mixed) | -- Δ | 52.5 | 22.5 | no activity (H/2mN) |
| Isolate #13A | + (4°C) | no reduction | | 0.028 ±0.007 (K/2mN) |
| PsL6a | ± (4°C) | -- | | no activity (K/2mN, H/2mN) |
| PsL1a | + (27°C) | no reduction | | no activity (K/2mN, H/2mN) |
| MARINE CULTURES | | | | |
| | | (63.5) | (25.0) | |
| 1B-2 | + (4°C) | 28.5 | <0.1 | no activity (K/2mN, H/2mN) |
| 20W8°E3-12 | ± (4°C) | 29.5 | <0.1 | no activity (K/2mN) |
| 8BT10-15 | + (4°C) | 28.5 | <0.1 | -- |
| 6BT10-6 | ± (4°C) | 30.5 | 0.5 | -- |
| 1B (mixed) | -- | no reduction | | 0.29 (PB) |
| 4B (mixed) | -- | no reduction | | 0.31 (PB) |
| 14S (mixed) | -- | no reduction | | 0.38 (PB) |
| 4B-6 | ± (4°C) | no reduction | | -- |
| 20W8°E3-3 | + (4°C) | no reduction | | -- |
| 20S8°E3-5 | + (4°C) | no reduction | | -- |
| 24BT11-6 | + (4°C) | no reduction | | -- |
| 34T11(B)9 | ± (4°C) | no reduction | | |

TABLE 8 - Continued

| CULTURE | BAP | TENSIOMETRIC Surface dynes/cm | TENSIOMETRIC Interfacial dynes/cm | O.D. ± (90% confidence limit) (Emulsion Substrate) † |
|---|---|---|---|---|
| BITUMEN, SEWAGE AND CONTAMINANT CULTURES | | | | |
| | | (65.0) * | (26.5) | |
| Isolate B-#1B | ± (4°C) | 49.0 | 21.0 | no activity (K/2mN) |
| Isolate B-#3B | ± (4°C) | no reduction | | no activity (K/2mN) |
| Isolate B-#4B | + (4°C) | no reduction | | no activity (K/2mN) |
| Isolate B-#5B | + (4°C) | 47.0 | 16.[illegible] | no activity (K/2mN) |
| Isolate B-#8B | ± (4°C) | 49.0 | 22.5 | no activity (K/2mN) |
| | | (60.5) | (26.0) | |
| Sewage culture (mixed) | -- | 46.0 | 24.0 | no activity (K/2mN, H/2mN) |
| | | (66.0) | (26.0) | |
| Isolate W.D.#1 | + (4°C) | 49.0 | 19.0 | -- |
| Isolate W.D.#2 | + (4°C) | 47.0 | 18.0 | -- |

** Surface and interfacial tension to detect biosurfactant activity

◊ Spectrophotometric oil emulsion assay to detect bioemulsifier activity

* Tensiometric control (medium) values for comparison with value(s) listed below

† H/2mN = hexadecane/2-methyl naphthalene (1:1); K/2mN = kerosene/2-methyl naphthalene (1:1); PB = Prudhoe Bay oil

Δ -- = not assayed

consistent production of biosurfactant and was chosen for further characterization.

B. Bioemulsifier Production

None of the 84 cultures screened, except for the *Acinetobacter* species, showed any significant bioemulsifier production using the spectrophotometric oil emulsion assay (Table 8). These results contrast sharply with those obtained by Singer *et al.* (1983b), where approximately 82% of the 77 bacterial isolates tested for production of surface active compounds formed stable emulsions with hexadecane. Also, several *Corynebacterium* species (Akit *et al.* 1981, Gerson & Zajic 1978, Panchal & Zajic 1978, Zajic *et al.* 1977a) and several *Acinetobacter* (Kaplan & Rosenberg 1982, Rosenberg *et al.* 1982, Sar & Rosenberg 1983) species have shown bioemulsifier production including *A. calcoaceticus* RAG-1, the producer of emulsan.

## 3. ISOLATE 1B-2: CHARACTERIZATION OF THE ORGANISM AND ITS BIOSURFACTANT

### A. Identification of the Isolate

Isolate 1B-2 produced pink, circular pulvinate colonies with entire edges and a mucoid texture when grown on Basal Marine Agar (BMA). The cells were Gram-positive, pleomorphic rods with many showing club- or V-shaped morphologies which is a distinctive characteristic of a coryneform bacterium. Other general and metabolic characteristics of this isolate are listed in Table 9 and these support the classification of the isolate as a *Corynebacterium* species belonging to the

TABLE 9 - IDENTIFICATION OF ISOLATE 1B-2

| CHARACTERISTIC | ISOLATE 1B-2 | CORYNEBACTERIUM * | ARTHROBACTER † |
|---|---|---|---|
| Morphology | Gm+ rods, pleomorphic | Gm+ rods pleomorphic | Gm+, rod to coccoid cycle |
| Motility | - | - | ± |
| Spore-formation | - | - | - |
| Oxygen requirements | facultative | facultative | obligate aerobe |
| Catalase | + | + | + |
| Gelatinase | - | - | + (slow) |
| Urease | + | + (group II) | -- |
| Acid (from glucose/lactose) | - | - | - |
| G+C content of DNA | 61% | 50-70% | 59-66% (globiformis) |

* reference: Barksdale, 1981; ± = varies with species; -- = information not obtained

† reference: Keddie & Jones, 1981

urease-positive Group II. This group includes organisms such as *C. fascians* (see Table 1), whereas Group I includes animal pathogens such as *Corynebacterium diptheriae* (Barksdale, 1981).

B. Production of Biosurfactant by *Corynebacterium* sp.1B-2

This isolate produces an extracellular biosurfactant as following the growth of the organism the surface tension of the medium was reduced from 63.5 to 28.5 dynes/cm and the interfacial tension from 25 to <0.5 dynes/cm. A known biosurfactant producer, *B. subtilis* ATCC 21332, demonstrated (Table 7) a reduction of surface and interfacial tension from 73 to about 26 dynes/cm and from 15 to about 1 dynes/cm, respectively. Therefore, *Corynebacterium* sp. 1B-2 is at least as good a biosurfactant producer as *B. subtilis* ATCC 21332. Similarly, Jenneman, *et al.*, (1983) observed that *B. licheniformis* JF-2 reduced the surface and interfacial tensions to 27 dynes/cm and <0.5 dyne/cm, respectively. Comparison of these tensiometric properties with those of the commercial dispersant Igepal Co 630 (Figure 4) establishes the relatively high potency of the biosurfactant produced by *Corynebacterium* sp.1B-2 and other bacterial species.

The data in Figure 5 demonstrates how the critical micelle concentration was determined for a culture filtrate of *Corynebacterium* sp.1B-2. Different $CMC^{-1}$ values of 2.5 and 3.3 at 72 hr (Figure 6) indicate that the amount of surfactant produced varies among separate batch cultures of the isolate. Since the growth conditions were kept constant these variations probably result from the variations in the inocula used. Each innoculum consisted of 10 mL taken directly from the enrichment cultures of *Corynebacterium* sp. 1B-2 at 72-96 hr of growth. The results of this experiment (Figure 6) show that maximum biosurfactant production occurs after approximately 72 hr of growth, which is during stationary phase. Therefore, 72 hr was selected as the time for

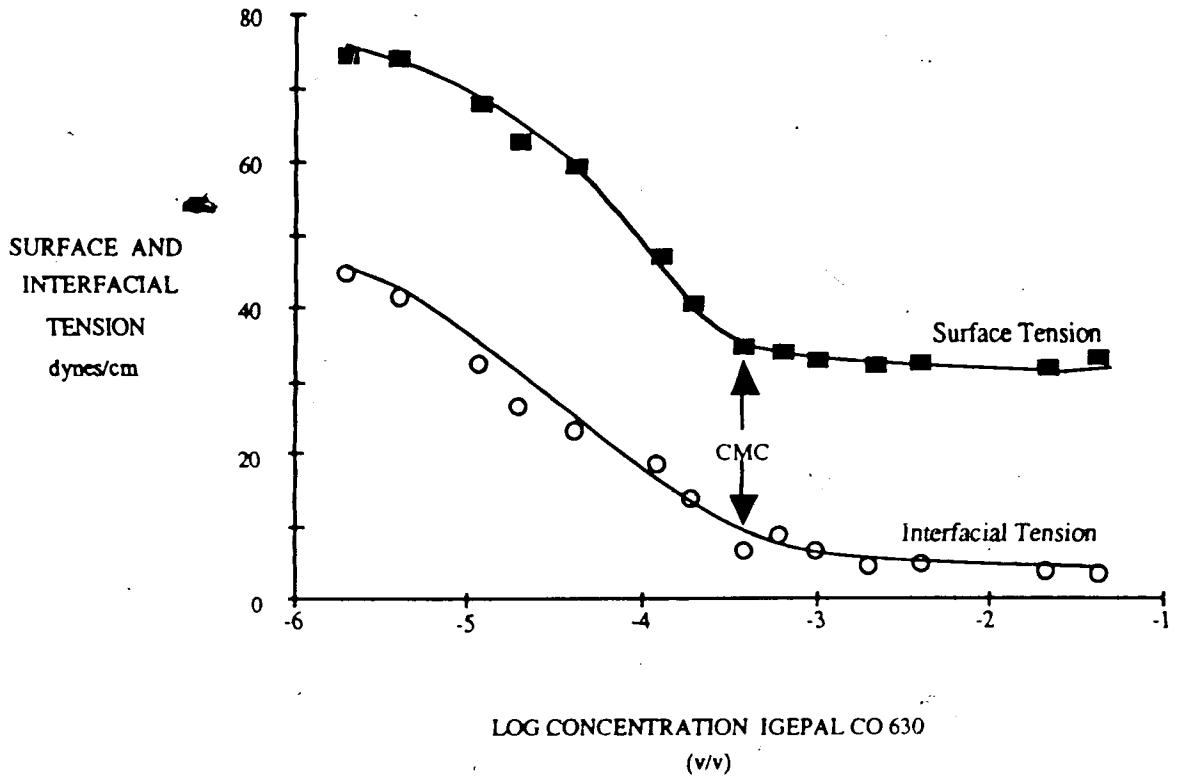


Figure 4 - Standard tensiometric curves for Igepal Co 630 dispersant.

The dispersant was dissolved in double-distilled water to achieve the specified concentrations. Surface and interfacial tensions were measured at 25°C using an autotensiomat; interfacial tension vs hexadecane. CMC is at 0.0006 (v/v) Igepal Co 630.

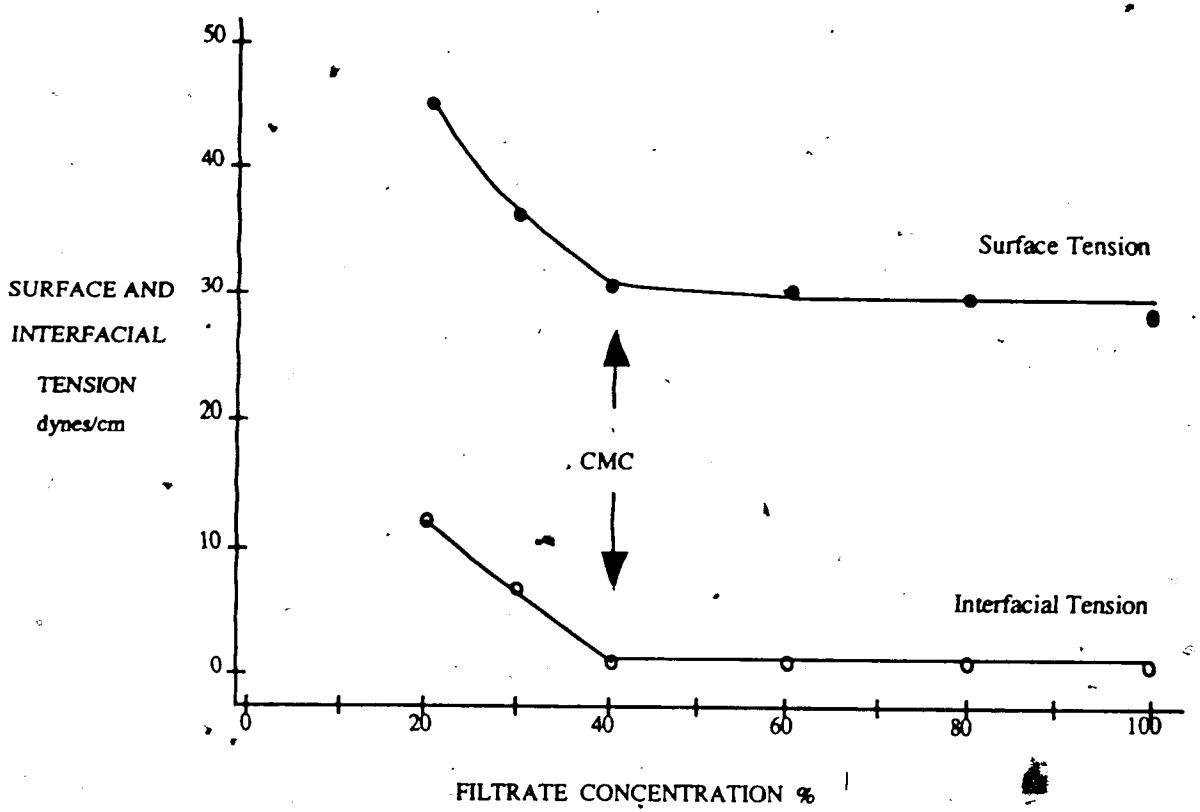


Figure 5 - Determination of critical micelle concentration of *Corynebacterium* sp. 1B-2.

*Corynebacterium* sp.1B-2 was grown at 27°C in ASW-NuBr medium with 1% tetradecane. A 72 hr cell-free culture filtrate was assayed tensiometrically as outlined in Figure 4 to determine the critical micelle concentration (CMC).

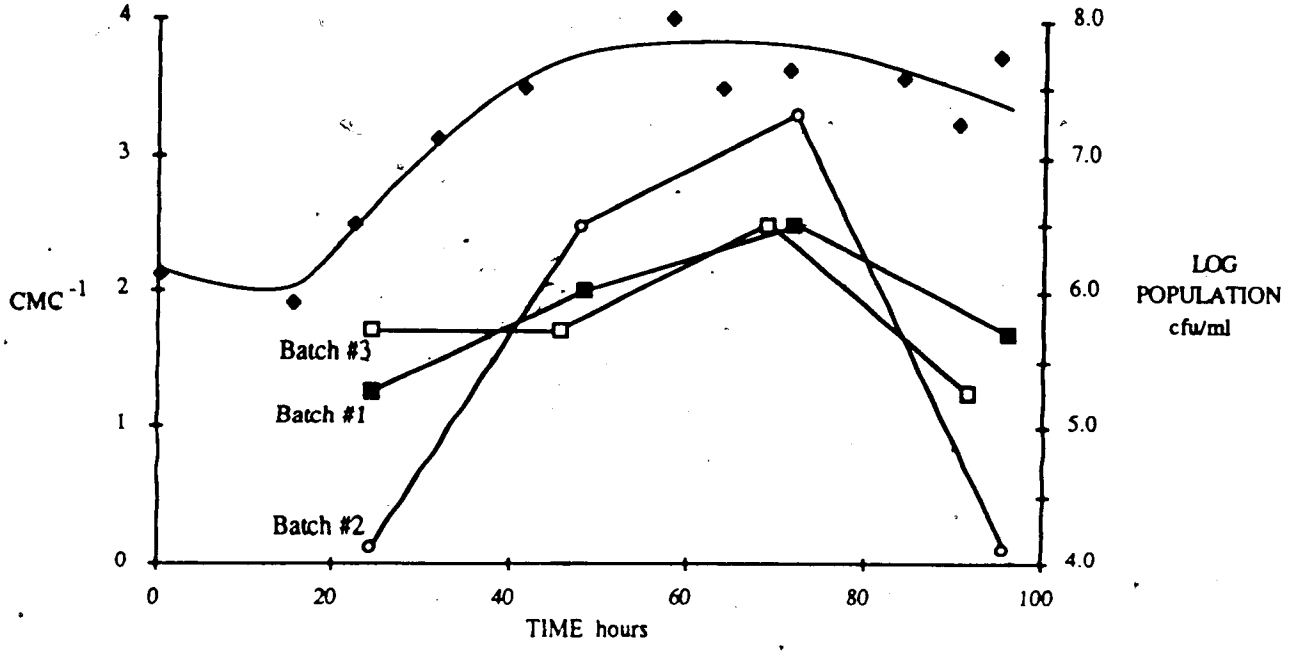


Figure 6 - Determination of maximum biosurfactant production by *Corynebacterium* sp. 1B-2.

Batch cultures were inoculated with 5% of 72-96 hr enrichment cultures and incubated as described in Methods and Materials. $CMC^{-1}$ values (■ o □)were calculated from CMC determinations established for each sampling. The typical growth curve population data (◆ ) was obtained from duplicate batch cultures.

harvesting cultures of *Corynebacterium* sp. 1B-2, to recover biosufactant for further characterization. Biosurfactant production by the isolate H-13A, tentatively identified as *Corynebacterium* (Finnerty & Singer 1984), occurs during stationary phase. *C. lipolytica* strains (Illarionova *et al.* 1984) also have their maximum bioemulsifier production in the period following exponential growth. In contrast, *B. licheniformis* JF-2 (Javaheri *et al.* 1985) apparently produces its surface active agent during exponential growth.

Growth of *Corynebacterium* sp.1B-2 without hydrocarbon substrate did not adversely affect the growth of the organisms, however, no biosurfactant was produced under these conditions. These results suggest that production of the biosurfactant is induced by hydrocarbon. This behavior is similar to that found for *R. erythropolis* (Rapp *et al.* 1979) where biosurfactant production also was found to be hydrocarbon inducible by n-alkanes and as may be the case with *P. aeruginosa* where the greatest biosurfactant production is achieved using hydrocarbon substrate (Guerra-Santos *et al.* 1984). Biosurfactant production by *B. subtilis* ATCC 21332 (Table 2) and *B. licheniformis* JF-2 (Jenneman *et al.*,1983) does not require hydrocarbon for induction as production occurs without hydrocarbon in the medium. Similarly, bioemulsifier production by *Acinetobacter* species does not appear to be hydrocarbon inducible as neither *A. calcoaceticus* BD 413 (Table 2) nor *A. calcoaceticus* 2CA2 (Neufeld *et al.* 1983) require hydrocarbons to produce bioemulsifier. *A. calcoaceticus* ATCC 31012, showed the best bioemulsifier production when grown on ethanol rather than hydrocarbons (Rosenberg, Zuckerberg, Rubinovitz and Gutnick 1979). The production of a biosurfactant by *A. calcoaceticus* 2CA2 (Neufeld *et al.* 1983) does appear to be hydrocarbon inducible as only cultures grown with hydrocarbon substrate demonstrate tensiometric reductions. Recently, Duvnjak and Kosaric (1985) found that *C. lepus* produces a considerable amount of biosurfactant when grown on either hexadecane or

glucose as sole carbon source. However, the biosurfactant is cell bound with glucose as substrate but extracellular with hexadecane as substrate. Addition of hexadecane or tetradecane to glucose grown cells leads to the release of the biosurfactant into the medium within 2-4 hr. Similar observations were made with *A. paraffineus*, and the amount of biosurfactant produced per biomass is approximately two fold higher with a hydrocarbon as growth substrate (Duvnjak & Kosaric 1985). These results suggest that or certain bacteria hydrocarbon may induce the release rather than inducing production of extracellular biosurfactant. This hypothesis was not tested with *Corynebacterium* sp.1B-2.

*Corynebacterium* sp.1B-2 was grown under low oxygen conditions, i.e. 300 mL of medium in 500 mL flasks without agitation, but no biosurfactant was produced. Although growth was not monitored, visual examination of culture turbidity suggested that sufficient growth occurred, so that biosurfactant production requires more aeration than was obtained under these conditions. According to Barksdale (1981), Group II *Corynebacterium* exhibit a predominantly oxidative metabolism, and these results suggest that biosurfactant production also requires oxygen.

C. Recovery of Biosurfactant from *Corynebacterium* sp.1B-2

Acidification of the culture filtrates to pH 2, a method used for recovery of biosurfactant from *B. subtilis* (Cooper *et al.* 1981) and *B. licheniformis* JF-2 (Pfiffner *et al.* 1985), did not result in the precipitation of the *Corynebacterium* sp. 1B-2 biosurfactant. Ammonium sulphate precipitation, as used to recover emulsan from *A.* [redacted] RAG-1 (Zuckerberg *et al.*,1979), also was ineffective in recovering biosurfactant from *Corynebacterium* sp.1B-2 filtrates.

The use of ethyl acetate to extract biosurfactant from culture filtrates at pH 6.4 was

unsuccessful since difficulties were encountered in separating the ethyl acetate phase from the aqueous solution even after allowing several days for separation to occur. Both methylene chloride and pentane effectively extracted biosurfactant from the filtrates at pH 6.4 but phase separation occurred faster with pentane. Pentane was used to extract *Corynebacterium* sp.1B-2 filtrate at pH 6.4. The tensiometric measurements of a medium control, the original culture filtrate, the resuspended extract and the extracted culture filtrate are shown in Figure 7. The reduced surface and interfacial tensions of the resuspended extract and the loss of activity in the extracted filtrate suggested good recovery of biosurfactant using this method. However, a comparison (Figure 8) of critical micelle concentrations of a culture filtrate and its resuspended pentane extract, i.e 2.2% and 40% respectively, showed that the relative amount of biosurfactant recovered, based on tensiometric activity, was very small. Taking into account that the pentane extract wass 7.1 times as concentrated as the original filtrate, the actual recovery of activity in the extract was 0.8%. This suggests that there may be some form of inhibition or loss of biosurfactant activity through the pentane extraction recovery process at pH 6.4.

In an attempt to improve biosurfactant recovery, aliquots of a *Corynebacterium* sp.1B-2 culture filtrate were adjusted from their normal pH 6.4 prior to extraction. Adjustment to pH 10 resulted in the formation of copious precipitate in the filtrate which interfered with both, tensiometric measurements and extraction procedures. However, extractions at pH values of less than 6.4 led to increasingly better recovery of biosurfactant. For exam[illegible] amount of biosurfactant activity expressed as $CMC^{-1}$ was three-fold h[illegible]n at pH 4. Subsequent extractions of culture filtrates at pH 2 have [illegible]erage, greater than 100% recovery of biosurfactant activity (Table 10). The extracted filtrate listed in Table 10 which yielded only 71% recovery was the result of the incomplete extraction of the surfactant from the culture

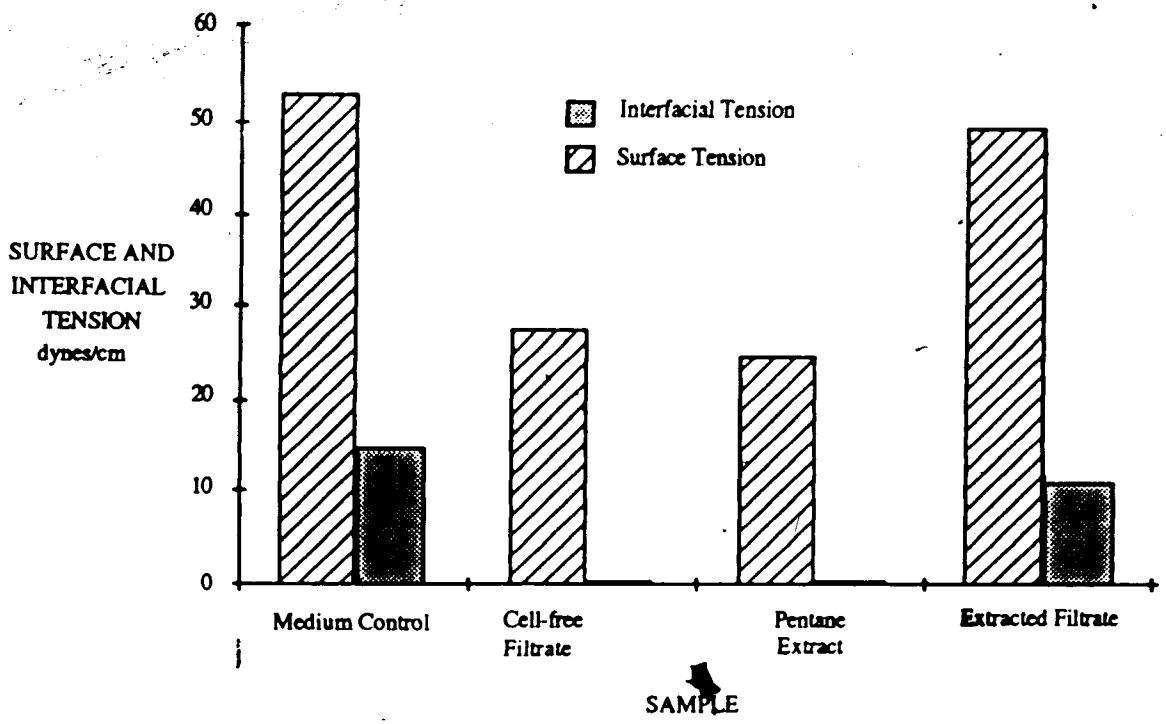


Figure 7 - Apparent recovery of biosurfactant activity using pentane extraction at pH 6.4.

Pentane extraction of biosurfactant was carried out on a 72 hr *Corynebacterium* sp.1B-2 cell-free filtrate adjusted to pH 6.4. Tensiometric assays as outlined in Figure 4 were carried out on a medium control, the original filtrate, the pentane extract and the extracted filtrate. The pentane extract was resuspended in double distilled water at the original filtrate concentration.

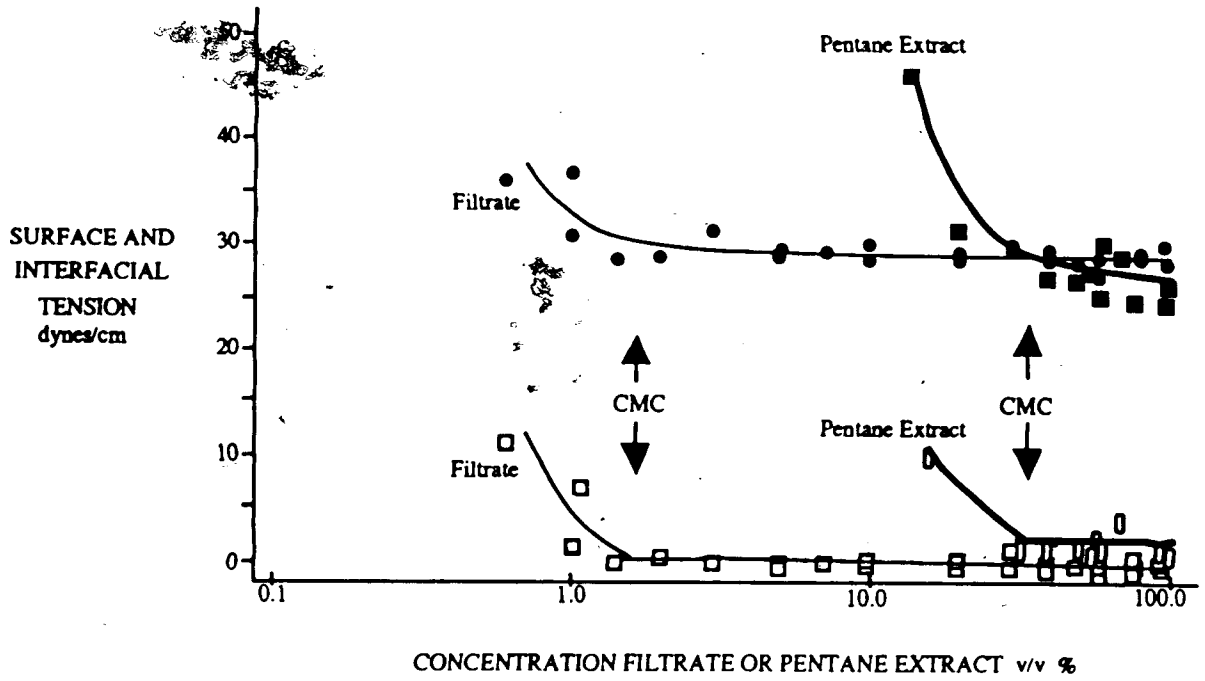


Figure 8 - Determination of biosurfactant concentration in filtrate and pentane extract by CMC determination.

The extraction of biosurfactant and assay conditions are described in Methods and Materials. The tensiometric assay data of the original filtrate (● surface, □ interfacial) and the pentane extracted biosurfactant (■ surface, ▯ interfacial) are plotted to determine the CMC for each. Dilutions of both filtrate and biosurfactant were made using double-distilled water.

TABLE 10 - BIOSURFACTANT RECOVERY FROM *Corynebacterium* sp. 1B-2

| FILTRATE CMC [1] | EXTRACT CMC [1] * | % RECOVERY |
|---|---|---|
| 2 | 8 | 400 |
| 1.7 | 2 | 120 |
| 2 | 1.4 | 71 † |
| 2 | 5 | 250 |
| 1.1 | 5 | 455 |

* Pentane extractions of filtrates at pH 2.0

† This low value of recovery is due to incomplete pentane extraction of biosurfactant, as residual tensiometric activity was found in the extracted filtrate.

filtrate. All of the other *Corynebacterium* sp.1B-2 extractions reported in Table 10 showed no residual biosurfactant activity in their extracted filtrates. Recoveries of >100% could be a result of the loss of inhibitors or activation of the biosurfactant due to the pentane extraction at pH 2.0. Tensiometric assays were not performed on adjusted culture filtrates to determine as to whether activation was due to low pH.

D. Characterization of the Biosurfactant

The lyophilization of the pentane extract yielded a semi-solid yellowish mixture which was designated as the crude biosurfactant. When left for several days at room temperature, this mixture separated into a viscous golden-colored oil and a whitish particulate material. The visual appearance of the pentane extract was the same, regardless of the pH of the filtrate at which the extraction was carried out. Acetone extraction (Cooper Zajic & Gracey 1979) of the crude biosurfactant resulted in solubilization of the oil which and it left an acetone-insoluble white particulate residue. The particulate portion was only slightly soluble in water and tensiometric measurement showed no significant surfactant activity. The oil was recovered by evaporation of the acetone and showed substantial tensiometric reduction activity. The oil, then, was considered to be the concentrated biosurfactant. The critical micelle concentration was achieved with 40 µg/mL of the acetone-soluble biosurfactant (Figure 9).

Chemical characterization of the biosurfactant, as previously described, revealed it as being primarily lipid. The results in Table 11 show that the biosurfactant was made up of over 75% lipid, about 6% carbohydrate and less than 3% protein. It is probable that the protein content, because of its low value, represents contaminating protein rather than an actual protein component of the biosurfactant. The biosurfactant is considered to be a glycolipid.

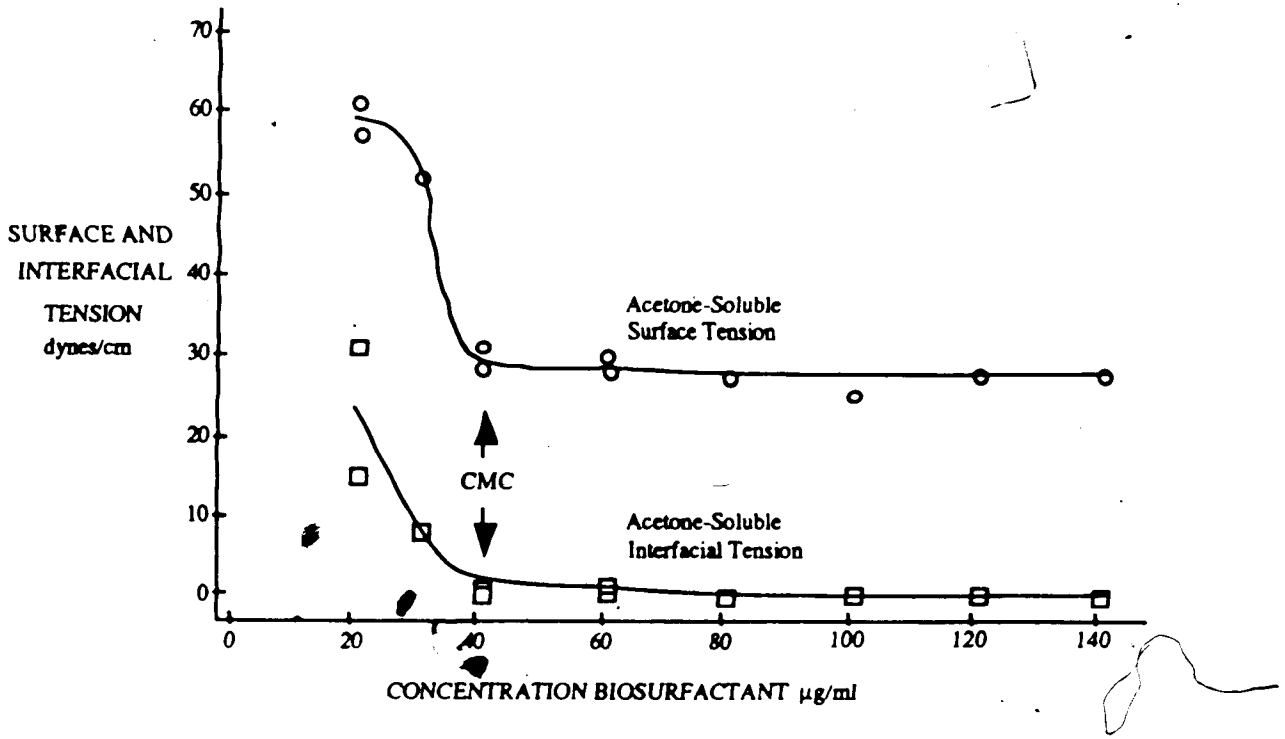


Figure 9 - Determination of CMC values of acetone-soluble extracts of concentrated biosurfactant.

The acetone-soluble fraction of biosurfactant was obtained as described in Methods and Materials. This fraction was resuspended in double-distilled water to the concentrations specified and assayed tensimetrically as outlined in Figure 4.

TABLE 11 - COMPOSITION OF *Corynebacterium* sp. 1B-2 BIOSURFACTANT

| COMPOSITION | ACETONE-SOLUBLE FRACTION | CRUDE BIOSURFACTANT |
|---|---|---|
| Carbohydrate | 6 % | 5.7 % |
| Lipid | 77 % | 76 % |
| Protein | 2.7 % | 3 % |

Thin layer chromatography of this glycolipid present in both the crude and concentrated biosurfactant showed two distinct lipid spots having Rf values of 0.35 and 0.66. It is not known which lipids have biosurfactant activity. The lack of two distinct CMC values in the tensiometric assays (Figures 5 & 9) supports the hypothesis of their being only one biosurfactant. The lipid compounds must be separated and assayed to clarify this situation. No lipid was detectable in the acetone-insoluble portion of the crude biosurfactant. Data in Table 12 shows the Rf values obtained for the biosurfactant and each of the known compounds tested as well as the Rf values reported for neutral lipids isolated from *C. lepus* by Cooper *et al.* (1979b). This group also used silica gel plates and the same solvent system so these data can be used for a general comparison. Based on this comparison, the types of lipid in *Corynebacterium* sp.1B-2 biosurfactant may be corynomycolic acid and fatty acid of triglyceride. Corynomycolic acid is more likely as it is a relatively common constituent of *Corynebacterium* biosurfactants (Table 1).

## 4. BIOSURFACTANT APPLICATION - BITUMEN RECOVERY FROM OIL SAND

The data in Figure 10 shows the relative amounts of bitumen extracted into the aqueous phase by the reference commercial dispersant (Igepal Co 630) and the concentrated biosurfactant as compared with that obtained with a double-distilled water control. This represents the amount of both surface and emulsified bitumen separated from sand and clay, although the emulsified bitumen probably represents an insignificant portion of that total. The biosurfactant from *Corynebacterium* sp.1B-2 was 2x as effective as double-distilled water but was less effective than the commercial dispersant. The spectrophotometric value for extracted bitumen by the Igepal Co 630 dispersant

TABLE 12 - THIN LAYER CHROMATOGRAPHY -- Rf VALUES OF BIOSURFACTANTS AND KNOWN LIPIDS

| SAMPLE | Rf VALUE (standard deviation) |
|---|---|
| UNKNOWN | |
| *Corynebacterium* sp. 1B-2 | 0.35 (0.07) |
| | 0.66 (0.11) |
| KNOWN LIPIDS | |
| Monomyristin | 0.05 (0.0) |
| Dimyristin | 0.25 (0.04) |
| | 0.32 (0.04) |
| Trimyristin | 0.72 (0.14) |
| Myristic Acid | 0.63 (0.13) |
| LIPIDS (data from Cooper *et al.* 1979b) | |
| Corynomycolic Acids | 0.3 - 0.4 |
| Monoglyceride | 0.03 |
| 1,2-Diglyceride | 0.2 |
| 1,3-Diglyceride | 0.25 |
| Triglyceride | 0.7 |
| Fatty Acid | 0.6 |

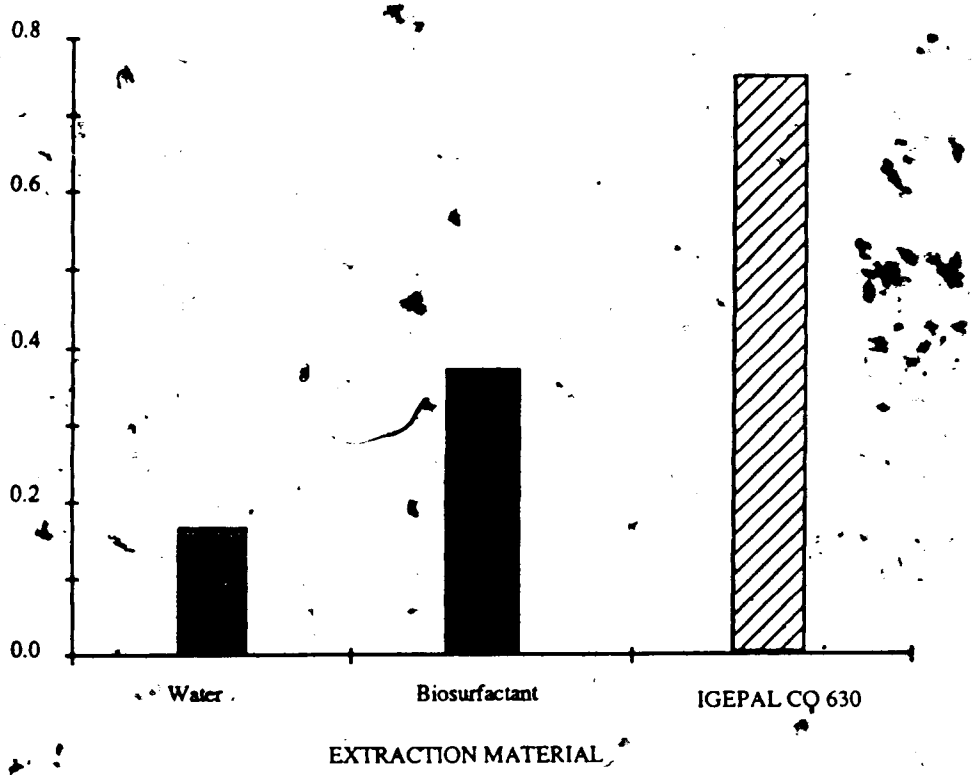


Figure 10 - Comparison of the capability of water, biosurfactant and Igepal Co 630 to extract bitumen from oil sand.

The extraction of bitumen from oil sand is described in Methods and Materials. The amount of bitumen extracted by each extraction material is measured by absorbance at 410 nm. These results are the average of duplicate samples which showed <1% variation. The concentration of biosurfactant (1µg/ml) and Igepal Co 630 (0.02, v/v) are >CMC for each.

represents approximately 1% of the total bitumen in the oil sand sample as determined by total bitumen extraction using toluene. Zajic and Gerson (1978) reported that their isolates ranged in effectiveness at separating bitumen from oil sand as from 1-10% of the total bitumen was extracted to the surface of the aqueous phase. For example, using whole culture broths, one *Corynebacterium* sp. effectively separated 6% while another *Corynebacterium* OSGB1, released 10.1% of the total bitumen to the aqueous surface. Using gravimetric values calculated from spectrophotometric data, Zajic and Akit (1983) report an improvement factor over the control of 17.4 for *C. fascians* and 14.5 for the commercial dispersant 7710 (Nalco Chem. Co.). The dispersant was more effective than *C. fascians* broths at removing sand and clay particles and thus increasing the bitumen concentration in this fraction.

Zajic and Akit (1983) stated that the ability of surfactants to extract bitumen from oil sand was neither a reflection of their ability to lower surface and interfacial tension, nor a function of concentration of surfactant. This hypothesis they based on their results which showed that dispersant 7719 (Nalco Chem. Co.) has more effective tensiometric properties, i.e. greater reduction of surface and interfacial tension, than dispersant 7710 but it is less effective at extracting bitumen from oil sands. A similar phenomenon was observed in this study. *Corynebacterium* sp.1B-2 biosurfactant was more effective at reducing surface and interfacial tension, i.e. to 28.5 dynes/cm and <0.1 dyne/cm, respectively, than Igepal Co 630, i.e. to 32 dynes/cm and 2.5 dynes/cm, respectively yet the dispersant extracted more bitumen. In all cases that Zajic & Akit (1983) tested the amounts of surfactant or biosurfactant used were greater than their critical micelle concentrations as $CMC^{-1}$ values ranged from 11 to >1000. The concentration of 2% (v/v) Igepal Co 630 used in this study represented a $CMC^{-1}$ of 33, whereas the concentrations of *Corynebacterium* sp.1B-2 biosurfactant represented $CMC^{-1}$ of 25 & 75, respectively. Higher concentrations of biosurfactant did not increase its bitumen

separation capability (data not shown). Concentrations of surfactant or biosurfactant at or below the critical micelle concentrations were not tested to determine whether critical micelle concentration is related to bitumen extraction capability as it is to tensiometric reduction. The same response of different concentrations of >CMC of *Corynebacterium* sp.1B-2 biosurfactant indicate that this relationship may exist. If so, then the statement about concentrations of surfactant not reflecting bitumen extraction ability may not be relevant, as this was based on data using concentrations greater than critical micelle concentrations. In any case for a successful biosurfactant, the minimum concentration required to effect bitumen separation should be established.

The surface active properties of a biosurfactant can function to wet the interface between the bitumen and the sand and clay particles, as well as solubilize the bitumen, and thereby separate bitumen from oil sand. The initial measurement of bitumen released to the aqueous phase gives an indication of an ability to facilitate bitumen separation from oil sand. Because the extraction of bitumen from oil sands also involves the component of removing the sand and clay particles from the oil sand, with the consequent increase in bitumen concentration of this fraction, this assessment should be included in the evaluation of the effectiveness of a biosurfactant in separating bitumen from oil sand.

# V. SUMMARY AND CONCLUSIONS

The recommended approach of research and development of industrial surfactants is directed at the process, not the surfactant, such that a surfactant is designed or chosen to fit into a process (Layman 1985). A similar sort of approach has been used in this study. Physical properties such as reduction of surface and interfacial tensions and emulsification are involved in the release of bitumen from oil sands and in the mobilization of oil for enhanced petroleum recovery. Several microbial biosurfactants and bioemulsifiers have demonstrated such physical properties, thereby indicating their possible value in such recovery applications. For this study, several oil-degrading cultures were available that visibly demonstrated oil dispersion activity and these, as well as cultures from freshwater, marine, soil, sewage, "oil-contaminated" and bitumen-associated environments were screened for the production of extracellular surface active metabolites. It was proposed that microbially produced bioemulsifiers and biosurfactants might be found that would be effective for the separation of bitumen from oil sands.

For these investigations a spectrophotometric assay was developed to detect the presence of extracellular bioemulsifiers. The validity of this method was established using commercial dispersants, Igepal Co 630 and Drew Lt and confirmed with bioemulsifier producing *A. calcoaceticus* strains. For detecting the production of extracellular biosurfactants by microbial cultures, a tensiometric assay has proven to be the most reliable method. The relative concentration of a biosurfactant produced can also be determined using this method, and the values of surface and interfacial tension indicate the potency of the biosurfactant. The reciprocal of CMC values, i.e. $CMC^{-1}$, provide a basis for comparison of different batches of a culture but not for different

cultures. Unfortunately, the tensiometric assay is neither rapid nor convenient for screening large numbers of samples. A blood agar plate assay method is limited to the detection of organisms which can produce surface active agents without hydrocarbon growth substrate and, therefore, is restricted in its usefulness for this type of survey. It may be worthwhile to investigate the possibility of incorporating a hydrocarbon, e.g. tetradecane or an aromatic hydrocarbon, into the blood agar medium, to detect hydrocarbon inducible biosurfactant producers. False positives due to lysis of blood by protease require an additional test for the presence of proteolytic enzyme. It appears from this study that the spectrophotometric oil emulsion assay and the tensiometric assay are currently the most useful methods for the detection of bioemulsifiers and biosurfactants, respectively. The lack of a rapid, general procedure for detecting surface active agents still remains a serious problem.

The use of the spectrophotometric oil emulsion assay to screen a number of cultures, failed to detect any producers of extracellular bioemulsifier. No provision was made in this study to detect any cell-bound bioemulsifier. Of the four biosurfactant producing marine isolates found, isolate 1B-2 was selected for further investigation. Based on the taxonomic tests performed, isolate 1B-2 has been identified as a group II *Corynebacterium* but identification of the isolate was not definitive since the characterization of cell wall lipids was not done. This isolate produced a biosurfactant which was capable of lowering the surface and interfacial tension of water to 28 and <0.5 dynes/cm, respectively, with 0.004% biosurfactant, which is comparable to the biosurfactant, surfactin, produced by *B. subtilis*. The production of biosurfactant by the isolate appeared to be inducible by hydrocarbon as extracellular biosurfactant was not produced in the absence of hydrocarbon substrate. Recovery of biosurfactant was accomplished by pentane extraction of culture filtrates followed by acetone extraction of the pentane extract. The recovery of tensiometric activity of the biosurfactant was

enhanced by pentane extraction at pH 2. Preliminary characterization of the biosurfactant indicated that it is a glycolipid and thin layer chromatography results suggest that the lipid composition may include corynomycolic acid, which is common to *Corynebacterium* species' biosurfactants.

The biosurfactant produced by *Corynebacterium* sp. 1B-2 did not demonstrate significant ability to release bitumen into the aqueous phase. Its ability to remove sand and clay to yield fractions of high bitumen concentration, i.e. tar balls, was not assessed. This bitumen concentrating phenomenon should be included as part of an assessment of bitumen separation capability of biosurfactants. The effectiveness of the biosurfactants from the other marine isolates, i.e. isolates 20W8°E3-12, 8BT10-15 and 6BT10-6, for bitumen separation was not investigated. Samples from oil-contaminated marine environments also may provide a source of biosurfactant producers which could be examined for bitumen separation capabilities.

The importance of finding a bitumen recovery process with lower energy requirements and less negative environmental impact than the conventional hot water and high pH process, indicates that investigation should continue into the application of biosurfactants for this purpose. Biosurfactants have properties which are potentially desirable for a bitumen recovery process. They are able to lower surface and interfacial tension which should promote separation of bitumen from the oil sand, and they do not form stable emulsions which would simplify the recovery of the bitumen from the aqueous phase. The development of more rapid screening methods to detect biosurfactant producers also would aid these investigations.

## VI. REFERENCES


Akit, J., D.G. Cooper, K.I. Manninen and J.E. Zajic. 1981. Investigation of potential biosurfactant production among phytopathogenic Corynebacteria and related soil microbes. Curr. Microbiol. 6: 145-150.

Alstinat, M.H. 1981. Surfactants for enhanced oil recovery processes in high salinity systems - product selection and evaluation. In Enhanced oil recovery. Ed. by F.J. Fayers. Amsterdam, Elsevier Scientific Pub. Co. 43-62.

Arima, K., A. Kakinuma and G. Tamura. 1968. Surfactin, a crystalline polypeptide surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31: 488-494.

Barksdale, L. 1981. The genus *Corynebacterium*. In The prokaryotes. Ed. by M.P. Starr, *et al.* Berlin, Springer-Verlag. 1827-1837.

Bayer, E.A., E. Rosenberg and D. Gutnick. 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. J. Gen. Microbiol. 127: [illegible]

Belsky, I., D.L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthr* [illegible] G-1: determination of emulsifier bound fatty acids. FEBS Lett. 101: 1[illegible]

Bubela, B., and B.A. McKay. 1985. Assessment of oil reservoirs for microbiological enhanced oil recovery. In Microbes and oil recovery, Vol.I. Ed. by J.E. Zajic and E.C. Donaldson. El Paso, Bioresources Pub. 99-107.

Cairns, W.L., D.G. Cooper, J.E. Zajic, J.M. Wood and N. Kosaric. 1982. Characterization of *Nocardia amarae* as a potent biological coalescing agent of water-oil emulsions. Appl. Environ. Microbiol. 43: 362-366.

Cirigliano, M.C., and G.M. Carman. 1984. Isolation of a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. 48: 747-750.

Clark, J.B., D.M. Munnecke and G.E. Jenneman. 1981. *In situ* enhancement of oil production. Dev. Ind. Microbiol. 22: 695-701.

Cook, F.D., and D.W.S. Westlake. 1973. Biodegradability of northern crude oil. Environmental-social Committee. Northern Pipelines, Task Force on Northern Oil Development, Report#73-20.

Cooper, D.G., C.R. MacDonald, S.I.B. Duff and N. Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42: 408-412.

Cooper, D.G., and D.A. Paddock. 1984. Production of a biosurfactant from *Torulopsis bombicola* . Appl. Environ. Microbiol. 47: 173-176.

Cooper, D.G., and D.A. Paddock. 1983. *Torulopsis petrophilum* and surface activity. Appl. Environ. Microbiol. 46: 1426-1429.

Cooper, D.G., and J.E. Zajic. 1980. Surface active compounds from microorganisms. Adv. Appl. Microbiol. 26: 229-253.

Cooper, D.G., J.E. Zajic and D.F. Gerson. 1979(a). Interfaces and the aquatic environment. In Proceedings, microbial degradation of pollutants in marine environments. Ed. by A.W. Bourguin[illegible]d P.H. Pritchard. Springfield, NTIS. 227-240.

Cooper, D.G., J.E. Zajic and D.F. Gerson. 1979(b). Production of surface active lipids by *Corynebacterium lepus*. Appl. Environ. Microbiol. 37: 4-10.

Cooper, D.G., J.E. Zajic and D.E.F. Gracey. 1979. Analysis of corynomycolic acids and other fatty acids produced by *Corynebacterium lepus* grown on kerosene. J. Bacteriol. 137: 795-801.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350.

[illegible]njak, Z., and N. Kosaric. 1985. Production and release of surfactant by *Corynebacterium lepus* in hydrocarbon and glucose media. Biotechnol. Lett. 7: 793-796.

Finnerty, W.R. 1977. The biochemistry of microbial alkane oxidation: new insights and perspectives. Trends Biochem. Sci. 2: .73-75.

Finnerty, W.R., and M.E. Singer. 1984. A microbial surfactant -- physiology, biochemistry and applications. Dev. Ind. Microbiol. 25: 31-40.

Finnerty, W.R., [illegible]M.E. Singer. 1983. Microbial enhancement of oil recovery. Bio/technol. 1: 47-54.

Floodgate, G.D. 1978. The formation of oil emulsifying agents in hydrocarbonclastic bacteria. In Microbial ecology. Ed. by M.W. Loutit and J.A.R. Miles. Berlin, Springer-Verlag. 82-85.

Foght, J.M., P.M.Fedorak and D.W.S. Westlake. 1985. Microbial content and metabolic activities in the Syncrude tailings pond. AOSTRA J. Research. 1: 3, 139-146.

Forbes, A.D. 1980. Microorganisms in oil recovery. In Hydrocarbons in biotechnology. Ed. by D.E. Harrison, *et al*. London, Heydon & Son Ltd. 169-180.

Forrester, P.I., D. Pearson and J. Roth. 1983. Microbial degradation of Athabasca bitumen. In Microbial enhanced oil recovery. Ed. by J.E. Zajic, *et al*. Tulsa, PennWell Pub. Co. 156-161.

Gabriel, A. 1979. Economic value of biopolymers and their use in enhanced oil recovery. In Microbial polysaccharides and polysaccharases. Ed. By R.C.W. Berkeley, *et al*. London, Academic Press. 191-204.

Gerson, D.F. 1983. Interfacial free energies of cells and polymers in aqueous media. In Physicochemical aspects of polymer surfaces. Ed. by K.L. Mittal. New York, Plenum Pub. Corp. 229-240.

Gerson, D.F., and D. Scheer. 1980. Cell surface energy, contact angles and phase partition III. Adhesion of bacterial cells to hydrophobic surfaces. Biochim. Biophys. Acta. 602: 506-510.

Gerson, D.F., and J.E. Zajic. 1979(a). Comparison of surfactant production from kerosene by four species of *Corynebacterium*. Antoine Leeuwenhoek. J. Microbiol. 45: 81-94.

Gerson, D.F., and J.E. Zajic. 1979(b). Microbial biosurfactants. Proc. Biochem. 14: 20-29.

Gerson, D.F., and J.E. Zajic. 1978. Surfactant production from hydrocarbons by *Corynebactrerium lepus*, sp. nov. and *Pseudomonas asphaltenicus*, sp. nov. Dev. Ind. Microbiol. 577-599.

Gerson, D.F., and J.E. Zajic. 1977. Bitumen extraction from tar sands with microbial surfactants. In The oil sands of Canada-Venezuela, 1977. Ed. by D.A. Redford and A.G. Winestock. CIMM. 705-710

Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg and D.L. Gutnick. 1982. Emulsan in *Acinetobacter calcoaceticus* RAG-1: distribution of cell-free and cell-associated cross-reacting material. Appl Environ. Microbiol. 44: 165-170.

Gray, N.C.C., A.L. Ste[illegible], W.L. Cairns and N. Kosaric. 1984. Bacteria-induced de-emulsification of oil-in-water petroleum emulsions. Biotechnol. Lett. 6: 419-424.

Gruia, M.M., and G.W. Sewell. 1983. Microbial interactions with polyacrylamide polymers. In Proceedings of 1982 International Conference on MEOR. Ed. by E.C. Donaldson and J.B. Clark. Bartlesville, U.S. Dept. Energy. 129-134.

Guerra-Santos, L., O. Käppeli and A. Fiechter. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. 48: 301-305.

Gutcho, S. 1977. Surfactants and sequestrants, recent advances. New Jersey, Noyes Data Corp. 105-117.

Gutnick, D.L., and E. Rosenberg. 1977. Oil tankers and pollution: a microbiological approach. Ann. Rev. Microbiol. 31: 379-396.

Haferburg, D., O. Asperger, U. Lohs and H.P. Kleber. 1983. Regulation of alkane utilization by *Acinetobacter calcoaceticus*. Acta. Biotechnol. 3: 371-374.

Hanson, R.S., and J.A. Phillips. 1981. Chemical composition. In Manual of methods for general bacteriology. Ed. by P.Gerhardt, *et al.* Washington, ASM. 339.

Hayes, M., and E. Nestaas. 1983. Microbial surface active compounds: applications and market potential in viscous hydrocarbon emulsions. Synfuel's 3rd worldwide symposium. McGraw Hill Pub. Co. (AOSTRA)

Hilton, C.L., and B.L. Kapur. 1982. Analysis and testing of amphoteric surfactants. In Amphoteric surfactants. Ed. by B.R. Bluestein and C.L. Hilton. New York, Marcel Dekker, Inc. 291-309.

Hinds, G.E. 1976. Petroleum-based raw materials for anionic surfactants. In Anionic surfactants. Ed. by W.M. Linfield. New York, Marcel Dekker, Inc. 11-86.

Hitzman, D.O. 1983. Petroleum microbiology and the history of its role in enhanced oil recovery. In Proceedings of 1982 International Conference on MEOR. Ed. by E.C. Donaldson and J.B. Clark. Bartlesville, U.S. Dept. Energy. 162-218.

Horowitz, A., D. Gutnick and E. Rosenberg. 1975. Sequential growth of bacteria on [redacted]ude oil. Appl. Microbiol. 30: 10-19.

Hoyt, J.W. 1985. Drag reduction in polysaccharide solutions. Trends in Biotechnol. 3: 17-21.

Hug, H., and A. Fiechter. 1973. Assimilation of hydrocarbons by *Candida tropicalis*. Arch. Mikrobiol. 88: 77-86.

Humphreys, R.D., and R. Schutte. 1978. The Alberta oil sands: energy for the future. In Oil sand and oil shale chemistry. Ed. by O.P. Strausz and E.M. Lown. New York, Verlag Chemie International, Inc. 1-9.

Iguchi, T., I. Takeda and H. Ohsawa. 1969. Emulsifying factor of hydrocarbon produced by a hydrocarbon-assimilating yeast. Agric. Biol. Chem. 33: 1657-1658.

Ilarionova, V.I., N.V. Shishkanova, D. Haferburg and T.V. Finogenova. 1984. Emulsifying activity of yeast grown on normal alkane. Microbiology (trans. from Russ.). 53: 423-426.

Inoue, S. and S. Ito. 1982. Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. Biotechnol. Lett. 4: 3-8.

Ito, S., and S. Inoue. 1982. Sophorolipids from *Torulopsis bombicola* : possible relation to alkane uptake. Appl. Environ. Microbiol. 43: 1278-1283.

Jack, T.R. 1985. Microbially enhanced oil recovery. In Comprehensive biotechnology (Vol. 4). Ed. by M. Moo-Young. Oxford, Pergammon Press Ltd. 295-304.

Jack, T.R. 1984. Microbial aspects of heavy oil . Exploration for heavy crude oil and bitumen. AAPG Research Conference. (AOSTRA).

Janshekar, H. 1985. Microbial enhanced oil recovery processed. In Microbes and oil recovery. Ed. by J.E. Zajic and E.C. Donaldson. El Paso, Bioresource Pub. 1: 54-84.

Javaheri, M., G.E. Jenneman, M.J. McInerney and R.M. Knapp. 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ. Microbiol. 50: 698-700.

Jenneman, G.E., M.J. McInerney, R.M. Knapp, J.B. Clark, J.M. Feero, D.E. Revus and D.E. Menzie. 1983. A halotolerant, biosurfactant-producing *Bacillus* species potentially useful for enhanced oil recovery. Dev. Ind. Microbiol. 24: 485-492.

Johnson, J.L. 1981. Genetic characterization. In Manual of methods for general bacteriology. Ed. by P. Gerhardt, *et al.* Washington, ASM. 450-458.

Kalb, F.F.,Jr. and R.W. Bernlohr. 1977. A new spectrophotometric assay for protein in cell extracts. Anal. Biochem. 82: 2, 362-371.

Kaplan, N., and E. Rosenberg. 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. Appl. Environ. Microbiol. 44: 1335-1341.

Keddie, R.M., and D. Jones. 1981. Saprophytic, aerobic Coryneform bacteria. In The prokaryotes. Ed. by M.P. Starr, *et al.* Berlin, Springer-Verlag. 1838-1878.

Koronelli, T.V., T.I. Komarova and Y.V. Denisov. 1983. The chemical composition of *Pseudomonas aeruginosa* peptidoglycolipid and its role in the process of hydrocarbon assimilation. Mikrobiologiya. 52: 767-769.

Kosaric, N. N.C.C.-Gray and W.L. Cairns. 1983. Microbial emulsifiers and de-emulsifiers. In Biotechnology, vol.3. Ed. by H.-J. Rehm and G. Reed. Weinheim, Verlag-Chemie. 575-592.

Kretschmer, A., H. Bock and F. Wagner. 1982. Chemical and physical characterization of interfacial- active lipids from *Rhodococcus erythropolis* grown on n-alkanes. Appl. Environ. Microbiol. 44: 864-870.

Larson, R.G., H.T. Davis and L.E. Scriven. 1980. Elementary mechanisms of oil recovery by chemical methods. In First Joint SPE/DOE Symposium on EOR, Proceedings: Tulsa, SPE. 305-321.

Lawson, J.B. and J. Reisberg. 1980. Alternate slugs of gas and dilute surfactant for mobility control during chemical flooding. In First Joint SPE/DOE Symposium on EOR, Proceedings: Tulsa, SPE. 388-

Layman, P.L. 1985. Industrial surfactants [illegible] strong growth. Chem. Eng. News. 63: 3, 23-48.

Lazar, I. 1983(a). Microbial enhancement of oil recovery in Romania. In Proceedings of 1982 International Conference on MEOR. Ed. by E.C. Donaldson and J.B. Clark. Bartlesville, U.S. Dept. Energy. 140-148.

Lazar, I. 1983(b). Some characteristics of the bacterial innoculum used for oil release from reservoirs. In Microbial enhanced oil recovery. Ed. by J.E. Zajic, *et al*. Tulsa, PennWell Pub. Co. 73-82.

Mac Donald, C.R., D.G. C[illegible] and J.E. Zajic. 1981. Surface-active lipids from *Nocardia erythro[illegible]* grown on hydrocarbons. Appl. Environ. Microbiol. 44: 117-123.

Mattei, G., and J-C. Bertrand. 1985. Production of biosurfactants by a mixed bacteria population grown in continuous culture on crude oil. Biotechnol. Lett. 7: 217-222.

Morgan, J.C., R.S. Schechter and W.H. Wade. 1977. Recent advances in the study of low interfacial tensions. In Improved oil recovery by surfactant and polymer flooding. Ed. by D.O. Shah and R.S. Schechter. New York, Academic Press, Inc. 101-118.

Moses, V., and D.G. Springham. 1982. Bacteria and the enhancement of oil recovery. London, Applied Science Pub.

Mulligan, C.N., D.G. Cooper and R.J. Neufeld. 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. 62: 311-314.

Neufeld, R.J., and J.E. Zajic. 1984. The surface activity of *Acinetobacter calcoaceticus* sp. ACA2. Biotechnol. Bioeng. 26: 1108-1113.

Neufeld, R.J., J.E. Zajic and D.F. Gerson. 1983. Growth characteristics and cell partitioning of *Acinetobacter* on hydrocarbon substrates. J. Ferment. Technol. 61: 315-321.

Neumann, H., B. Paczyńska-Lahme and D. Severin. 1981. Composition and properties of petroleum. In Geology of petroleum, vol. 5. Ed. by H. Beckmann. New York, Halsted Press.

Panohal, C.J., and J.E. Zajic. 1978. Isolation of emulsifying agents from a species of *Corynebatterium*. Dev. Ind. Microbiol. 19: 569-576.

Pasquarelli, C.H., and D.T. Wassan. 1981. The effect of film-forming materials on the dynamic interfacial properties in crude oil - aqueous systems. In Surface phenomena in enhanced oil recovery. Ed. by D.O. Shah. New York, Plenum Press. 237-248.

Paul, J.H., and W.H. Jeffrey. 1985. The effect of surfactants on the attachment of estuarine and marine bacteria to surfaces. Can. J. Microbiol. 31: 224-228.

Pfiffner, S.M., G.E. Jenneman, G.B. Walker Jr., M. Javaheri, M.J. McInerney and R.M. Knapp. 1985. Isolation and characterization of halotolerant, thermophilic, anaerobic, biopolymer-producing and biosurfactant-producing bacteria. In Microbes and oil recovery. Ed. by J.E. Zajic and E.C. Donaldson. El Paso, Bioresource Pub. 285-294.

Ramberloarisoa, E., J.F. Rontani, G. Giusti, Z. Duvnjak and J.C Bertrand. 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea surface foams. Mar. Biol.(Berl.) 83: 69-81.

Rapp, P., H. Bock, V. Wray and F. Wagner. 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkanes. J. Gen. Microbiol. 115: 491-503.

Ratledge, C. 1980. Microbial lipids derived from hydrocarbons. In Hydrocarbons in technology. Ed. by D.E.F. Harrison, *et al.* Cambridge, Heyden & Son Ltd. 133-153.

Reddy, P.G., H.D. Singh, M.G. Pathak, S.D. Shagat and J.N. Baruah. 1983. Isolation and functional characterization of hydrocarbon emulsifying and solubilizing factors produced by a *Pseudomonas* species. Biotechnol. Bioeng. 25: 387-401.

Reisfield, A., E. Rosenberg and D. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. 24: 363-368.

Rosen, M.J. 1978. Surfactants and interfacial phenomena. New York, John Wiley & Sons.

Rosenberg, E., A. Perry, D.T. Gibson and D.L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. Appl. Envron. Microbiol. 37: 409-413.

Rosenberg, M., and E. Rosenberg. 1985. Bacterial adherence at the hydrocarbon-water interface. Oil Petro. Pollut. 2: 155-162.

Rosenberg, E., A. Zuckerberg, C. Rubinovitz and D.L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Envron. Microbiol. 37: 402-408.

Roy, P.K., H.D. Singh, S.D. Bhagat and J.N. Baruah. 1979. Characterization of hydrocarbon emulsification and solubilization occuring during the growth of *Endomycopsis lipolytica* on hydrocarbons. Biotechnol. Bioeng. 21: 955-974.

Rubinovitz, C., D.L. Gutnick and E. Rosenberg. 1982. Emulsion production by *Acinetobacter calcoaceticus* in the presence of chloramphenicol. J. Bacteriol. 152: 126-132.

Sar, N., and E. Rosenberg. 1983. Emulsifier production by *Acinetobacter calcoaceticus* strains. Curr. Microbiol. 9: 309-314.

Schramm, L.L., R.G. Smith and J.A. Stone. 1985. The influence of natural surfactant concentration on the hot water process for recovering bitumen from the Athabasca oil sands. AOSTRA J. Research 1:1, 5-13.

Shah, D.O. 1981. Introduction. In Surface phenomena in enhanced oil recovery. Ed. by D.O. Shah. New York, Plenum Press. 1-11.

Shoham, Y., and E. Rosenberg. 1983(a). Bacterial degradation of emulsan. Appl. Environ. Microbiol. 46: 573-579.

Shoham, Y., and E. Rosenberg. 1983(b). Enzymatic depolymerization of emulsan. J. Bacteriol. 156: 161-167.

Singer, M.E. 1985. Microbial surfactants. In Microbes and oil recovery. Ed. by J.E. Zajic and E.C. Donaldson. El Paso, Bioresource Pub. 1: 19-38.

Singer, M.E., W.R. Finnerty, P. Bolden and A.D. King. 1983(a). Characterization of a biosurfactant effective in heavy oil viscosity reduction. Amer. Chem. Soc. - Div. of Petrol. Chem. Preprints. 28: 785-788.

Singer, M.E., W.R. Finnerty, P. Bolden and A.D. King. 1983(b). Microbial processes in the recovery of heavy petroleum. In Proc. of 1982 int. conf. on MEOR. Ed. by E.C. Donaldson and J.B. Clark. Bartlesville, U.S. Dept. Energy. 94-101.

Smibert, R.M., and N.R. Krieg. 1981. General characterization. In Manual of methods for general bacteriology. Ed. by P. Gerhardt, *et al.* Washington, ASM. 409-443.

Speight, J.G. 1980. The chemistry and technology of petroleum. New York, Marcel Dekker, Inc. 1-34.

Spencer, B. 1980. New chemical method developed fro removing oil from oil sands. Oilweek. 31: 56-60.

Springham, D. 1984. Microbiological methods for the enhancement of oil recovery. Biotechnol. Gen. Eng. Rev. 1: 187-221.

Stosur, J.J.G. 1981. Enhanced oil recovery research and development in the U.S. and in the U.S. department of energy. In Enhanced oil recovery. Ed. by F.J. Fayers. Amsterdam, Elsevier Scientific Pub. Co. 587-594.

Sutherland, I.W. 1986. Industrially useful microbial polysaccharides. Microbiol. Sci. 3:1, 5-9.

Suzuki, T., T. Katsunobu, I. Matsubara and S. Kinoshita. 1969. Trehalose lipid and a-branched-b-hydroxy fatty acid formed in bacteria grown on n-alkanes. Agric. Biol. Chem. 33: 1619-1627.

Una, G.V., and M.J.N. Garcia. 1983. Biodegradation of non-ionic dispersants in seawater. Eur. J. Appl. Biotechnol. 18: 315-319.

Wagner, F., U. Behrendt, A. Kretschmer, S. Lang and C. Syldatk. 1983. Production and chemical characterization of surfactants from *Rhodococcus erythropolis* and *Pseudomonas* sp. MUB on hydrocarbons. In Microbial enhanced oil recovery. Ed. by J.E. Zajic, *et al.* Tulsa, PennWell Pub. Co. 55-60.

Wasan, D.T., and V. Mohan. 1977. Interfacial rheological properties of fluid interfaces containing surfactants. In Improved oil recovery by surfactant and polymer flooding. Ed. by D.O. Shah and R.S. Schechter. New York, Academic Press, Inc. 161-203.

Waters, J., and C.G. Taylor. 1977. The colorimetric estimation of anionic surfactants. In Anionic surfactants - chemical analysis. Ed. by J. Cross. New York, Marcel Dekker, Inc. 193-220.

Westlake, D.W.S. 1984. Heavy crude oils and oil shales: tertiary recovery of petroleum from oil-bearing formations. In Petroleum microbiology. Ed. by R.M. Atlas. New York, Macmillan Pub. Co. 537-552.

Westlake, D.W.S., and F.D. Cook. 1980. Petroleum degradation potential of northern Puget Sound and Strait of Juan de Fuca. Springfield, National Technical Information Service. EPA-600/7-80-133.

Williams, K., M. Kandar, B. Patel, A. Hart and A. McMullen. 1984. Oil sands bitumen extraction process evaluation - vol. 2 - preliminary technical evaluation, final public report. A.E.R.C.B. Pub. # 860-24/3/1, Calgary. 200.

Wollrab, V., and M. Streibl. 1969. Earth waxes, peat, montan wax and other organic brown coal constituents. In Organic geochemistry. Ed. by G. Eglinton and M.T.J. Murphy. Heidelberg, Springer-Verlag Berlin. 576-598.

Yarbrough, H.F., and V.F. Coty. 1983. Microbially enhanced oil recovery from the upper cretaceous Nacatoch fromation, Union County, Arkansas. In Proceedings of 1982 International Conference on MEOR. Ed. by E.C. Donaldson and J.B. Clark. Bartlesville, U.S. Dept. Energy. 149-153.

Zajic, J.E., and J. Akit. 1983. Biosurfactants in bitument separation from tar sands. In Microbial enhanced oil recovery. Ed. by J.E. Zajic, *et al.* Tulsa, PennWell Pub. Co. 50-54.

Zajic, J.E., and D.F. Gerson. 1978. Microbial extraction of bitumen from Athabasca oil sand. In Oil sand and oil shale chemistry. Ed. by O.P. Strausz and E.M. Lown. New York, Verlag Chemie International, Inc. 145-161.

Zajic, J.E., H. Guignard and D.F. Gerson. 1977(a). Emulsifying and surface active agents from *Corynebacterium hydrocarboclastus* . Biotechnol. Bioeng. 19: 1285-1301.

Zajic, J.E., H. Guignard and D.F. Gerson. 1977(b). Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. 19: 1303-1320.

Zajic, J.E., ~~and E. Knettig~~. 1971. Flocculants from paraffinic hydrocarbons. Dev. Ind. Microbiol. 12: 87-98.

Zajic, J.E., and A.Y. Mahomedy. 1984. Biosurfactants: intermediates in the biosynthesis of amphipathic molecules in microbes. In Petroleum microbiology. Ed. by R.M. Atlas. New York, Macmillan Pub. Co. 221-297.

Zajic, J.E., and C.J. Panchal. 1976. Bio-emulsifiers. CRC Crit. Rev. Microbiol. 5: 39-66.

Zajic, J.E., and W. Steffens. 1984. Biosurfactants. CRC Crit. Rev. Biotechnol. 1: 87-107.

Zosim, Z., D. Gutnick and E. Rosenberg. 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. Biotechnol. Bioeng. 24: 281-192.

Zuckerberg, A. A. Diver, Z. Peeri, D.L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Envron. Microbiol. 37: 414-420.

## VIII APPENDIX - MEDIA COMPOSITION

### A. LIQUID MEDIA

1. Acinetobacter medium (Rosenberg, *et al*, 1979b)

1.25 g/L urea
1.25 g/L $MgSO_4 \cdot 7H_2O$
0.02 g/L $FeSO_4 \cdot 7H_2O$
0.01 g/L $CaCl_2$
0.25 g/L $K_2HPO_4$
24.2 g/L Tris(hydroxymethyl)aminomethane (Sigma)
pH 7.4

2. ASW

23.4 g/L NaCl
0.75 g/L KCl
7.0 g/L $MgSO_4 \cdot 7H_2O$
pH 7.3
a) - supplemented with N,P solution (0.42 g/L $K_2HPO_4$; 0.18 g/L $KH_2PO_4$; 0.60 g/L $NH_4NO_3$; final concentration) -- ASW+N,P
b) - supplemented with N,P solution and 0.5 g/L Nutrient Broth (NuBr) (Difco) -- ASW+N,P+NuBr
c) - supplemented with 8.0 g/L NuBr (Difco) -- ASW-NuBr

3. B+N

0.5 g/L $K_2HPO_4$
1.0 g/L $NH_4Cl$
2.0 g/L $Na_2SO_4$
0.2 g/L $MgSO_4 \cdot 7H_2O$
2.0 g/L $KNO_3$
trace $FeSO_4 \cdot 7H_2O$
a) - supplemented with 1.0 g/L NuBr -- B+N+NuBr
b) - supplemented with 1.0 g/L Yeast Extract (Difco) -- B+N+YE

4. 4% Glucose + Minimal Medium (Cooper, *et al*, 1981)

4.0 g/L $NH_4NO_3$
4.0 g/L $KH_2PO_4$
5.7 g/L $Na_2HPO_4$

| | | |
|---|---|---|
| trace | | $Na_2$·EDTA |
| 40 | g/L | Glucose |

5. HPMS (Kaplan & Rosenberg, 1982)

| | | |
|---|---|---|
| 22.2 | g/L | $K_2HPO_4 \cdot 3H_2O$ |
| 7.26 | g/L | $KH_2PO_4$ |
| 4.0 | g/L | $(NH_4)_2SO_4$ |
| 0.2 | g/L | $MgSO_4 \cdot 7H_2O$ |
| 0.1 | g/L | L·Tryptophan |
| 5.0 | g/L | Glucose |
| pH 7.0 | | |

B. SOLID MEDIA

1. BASAL MARINE AGAR

| | | |
|---|---|---|
| 23.4 | g/L | NaCl |
| 0.75 | g/L | KCl |
| 7.0 | g/L | $MgSO_4 \cdot 7H_2O$ |
| 1.0 | g/L | Proteose Peptone #3 (Difco) |
| 1.0 | g/L | Yeast Extract |
| pH 7.5 | | |
| 20 | g/L | Agar (Difco) |

2. BLOOD AGAR PLATE

| | | |
|---|---|---|
| 33 | g/L | Tryptose Blood Agar Base (Difco) |
| 50 | ml/L | Defibrinated Sheep's Blood (Vista Labs, Edmonton) |

3. GELATIN TUBES

| | | |
|---|---|---|
| 8.0 | g/L | Nutrient Broth (Difco) |
| 50 | g/L | gelatin (Difco) |

4. PC + K9A40

| | | |
|---|---|---|
| 5.0 | g/L | Tryptone (Difco) |
| 2.5 | g/L | Yeast Extract (Difco) |
| 1.0 | g/L | Dextrose |
| 0.9 | g/L | $MgCl \cdot 6H_2O$ |
| 8.0 | g/L | K9A40 Gellan Gum (Kelco) |

B+N medium

| | | |
|---|---|---|
| 0.5 | g/L | Yeast Extract |
| 0.9 | g/L | $MgCl \cdot 6H_2O$ |
| 8.0 | g/L | K9A40 Gellan Gum |

- add 1 drop from a 1mL pipette of Prudhoe Bay oil to the surface of molten media in each plate