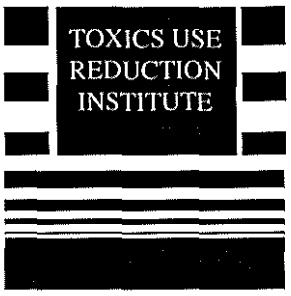



THE MASSACHUSETTS
TOXICS USE REDUCTION INSTITUTE

# TECHNOLOGY INNOVATION TO PROMOTE ALTERNATIVE CHEMICAL TECHNOLOGIES:

## DEVELOPMENT AND TESTING OF BIOSURFACTANTS IN AQUEOUS METAL CLEANING APPLICATIONS

Technical Report No. 42 **1997**

University of Massachusetts Lowell

# Technology Innovation to Promote Alternative Chemical Technologies:

# Development and Testing of Biosurfactants in Aqueous Metal Cleaning Applications

submitted by:

Toxics Use Reduction Institute
&
Biodegradable Polymer Research Center

University of Massachusetts Lowell
Lowell, MA 01854

submitted to:

Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
Washington, DC

**Final Report**

July 1997

## PRINCIPAL AUTHORS

Karen B. Thomas

Andrew L. Bray

Monica Becker

Joel Tickner

Toxics Use Reduction Institute

## CONTRIBUTORS

Dr. Richard Gross

Dr. Alexander Gorkovenko

George Allard

Jin-Wen Zhang

Biodegradable Polymer Research Center

Dr. Randall W. Swartz

Anthony Gudinas

Massachusetts Bioprocess Development Center

## EPA PROJECT OFFICER

Dr. Steven M. Hassur

Office of Pollution Prevention and Toxics

## NOTICE

The information in this document has been funded by the United States Environmental Protection Agency under the 1994 Environmental Technology Initiative (ETI) Project No. 39 - Aerospace Metal Degreasing, Project Segment: Developing Environmentally Benign Substitutes for Organic Solvents/Technology Transfer to Promote Alternative Chemical Technologies. Funding was provided through a grant (EPA Assistance ID No. X823640-01-2) to the University of Massachusetts Lowell's Toxics Use Reduction Institute and through an Interagecy Agreement with the National Science Foundation (EPA/IAG ID No. DW49936552-01-0) to the Biodegradable Polymer Research Center at the University of Massachusetts Lowell. It has been subject to peer and administrative review and has been approved by the EPA Project Officer for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

# About the Research Centers

## Toxics Use Reduction Institute

The Toxics Use Reduction Institute (TURI) is a multi-disciplinary research, education, and policy center established by the Massachusetts Toxics Use Reduction Act of 1989. The Institute sponsors and conducts research, organizes education and training programs, and provides technical support to promote reduction in the use of toxic chemicals or the generation of toxic chemical byproducts in industry and commerce.

TURI's Surface Cleaning Lab (SCL) was established to assist companies in matching specific cleaning needs with appropriate chemistry and process combinations. SCL is an industrial lab that routinely performs application-specific research on aqueous cleaning, rinsing, and drying processes with a focus on non-precision cleaning applications, such as metal finishing and metal parts fabrication. More specifically, SCL outlines cleaning options for customer situations, performs testing on actual dirty parts or standard test coupons, evaluates commercially available aqueous cleaners, and helps define cleaning specifications. The SCL has published a collection of information about currently available cleaning chemistries and equipment and is developing a database on the characteristics and compatibilities of commercially available aqueous chemistries based on vendor information and SCL test results. In order to perform its research, the SCL also has developed protocols for establishing a baseline clean standard and for contaminating, washing/rinsing/drying, and analyzing (both qualitatively and quantitatively) samples.

## Biodegradable Polymer Research Center

The National Science Foundation Center for Biodegradable Polymer Research (BPRC) at the University of Massachusetts Lowell performs exploratory and fundamental research for the development of new technologies in degradable plastic materials. The BPRC has expertise in microbial production of polymeric materials, organic transformations, plastics processing, and materials characterization. The goal of this work is to obtain suitable materials which are inherently degradable in nature as well as being environmentally benign. The BPRC fosters a broad and active cooperation with industry which ensures a fast transfer of new methods and technologies. BPRC activities are expected to be of great importance to the future economic as well as environmental health of the Commonwealth.

## Bioprocess Development Center

The Massachusetts Bioprocess Development Center (BDC) was established to help industry in the transition from research and development to the manufacture of biotechnology products. Innovations and funded research range from bioprocess development to the development of novel biological materials for advanced material applications. BDC helps the several dozen biotechnology companies in the region with training and technology needs and with the transition from research and development to manufacturing. In addition, BDC assists suppliers to the biotech industry in the evaluation and development of new products.

# EXECUTIVE SUMMARY

Cleaning and degreasing of metal parts is an essential step in the metal finishing and metal working industries. Effective cleaning assures high performance in subsequent plating or welding operations and is desirable for aesthetic reasons. Traditionally, cleaning has been accomplished by the use of chlorinated solvents in vapor degreasers or immersion systems because chlorinated solvents are highly effective at removing oils and other soils from metal parts and exhibit low flammability. However, scientific research has shown that chlorinated solvents present risks to human and environmental health, for example, chlorinated solvents cause stratospheric ozone depletion. As a result, alternative cleaning methods such as aqueous, semi-aqueous, and mechanical methods have emerged. This project was aimed at developing, testing and transferring a new technology in a new application–biosynthesized, biodegradable surfactants in aqueous surface cleaning chemistries.

Surfactants, also called wetting agents, are used in aqueous cleaners to provide certain key properties in a cleaning formulation–detergency, emulsification of soils, and wetting action. Biosurfactants are surfactants created by microorganisms. Biosurfactants offer several potential advantages over their synthetic counterparts. They tend to be more readily biodegradable, they can be produced from renewable resources, and they can be manufactured without the use or production of toxic chemicals. However, biosurfactants also have disadvantages. One disadvantage is that little research has been done on the potential adverse effects of the use of biopolymers in large quantities, particularly when they are introduced into solid or aqueous wastestreams or natural ecosystems. A second disadvantage is that biopolymers are currently two to five times more expensive to produce than synthetic resins and the production of these materials may not benefit from the economies-of-scale seen in chemosynthetic methods of production.[1]

Two biosynthesized surfactants were studied in this project; emulsan and sophorolipid. A number of studies and patents have shown emulsan to be an effective emulsifier of oily substances, initially in emulsifying oily residues in oil transport ship hulls and later in personal care products. Sophorolipid has good surface tension-lowering properties and can reportedly be produced at a cost comparable to petroleum-based surfactants.[2, 3] The research described in this report sought to take advantage of the emulsification properties of emulsan and the surface-tension lowering properties of sophorolipid in metal cleaning and degreasing applications.

---

[1]U.S. Congress, Office of Technology Assessment, *Biopolymers: Making Materials Nature's Way – Background Paper,* OTA-BP-E-102 (Washington, D.C.: U.S. Government Printing Office, September 1993).

[2]George Georgiou, Sung-Chyr Lin, and Murkul M. Sharma, "Surface-Active Compounds From Microorganisms," *Bio/Technology*, Vol. 10 (January 1992) 60-64.

[3]Michele I. Van Dyke, Hung Lee and Jack T. Trevors, "Applications of Microbial Surfactants," *Biotech. Adv.*, Vol. 9 (1991) 241-252.

The primary objectives of this project were: (1) to develop one or more biosurfactants with potential for use in surface cleaning applications, (2) to evaluate the effectiveness of the biosurfactants in laboratory simulated surface cleaning systems, (3) to evaluate the production costs of the biosurfactants, and (4) to evaluate the environmental, health and regulatory implications of biosurfactant production. In addition, if surface cleaning evaluations proved successful, a further objective was to advance the manufacture and use of the biosurfactants in commercial cleaning applications by sharing this research with companies that could manufacture and sell the biosurfactants commercially (i.e., technology transfer).

This research was supported by the U.S. Environmental Protection Agency, Office of Pollution Prevention and Toxics (OPPT) under its "Paint Stripping for Small Aircraft" Environmental Technology Initiative (Grant No. X823640-01-2 and Interagency Agreement No. DW49936552-01-0) as part of a program examining opportunities for the use of clean maintenance technologies by the aviation community. The project was a collaborative effort of the Toxic Use Reduction Institute (TURI) and two other research centers at University of Massachusetts Lowell, the Biodegradable Polymer Research Center (BPRC) and the Massachusetts Bioprocess Development Center (BDC). The BPRC team developed and scaled-up production of the several biopolymer analogues in their laboratories on campus and at the Natick Army Research, Development and Engineering Center. TURI, a multi-disciplinary pollution prevention research and education center, designed and carried-out the evaluation of the surfactants in its Surface Cleaning Laboratory and the results of TURI's evaluations influenced the direction of the biopolymer development process at the BPRC. Finally, the BDC designed an industrial scale production facility for the manufacture of emulsan and evaluated the effect of production scale on production costs.

This report provides information on the characteristics of two promising biosurfactants – emulsan and sophorolipid. The report presents background information on common soils (i.e. metal machining fluids), aqueous cleaners, and cleaning equipment. Innovative procedures developed to test the effectiveness of the biopolymers in surface cleaning applications are described. The information presented in this report is useful to an audience which includes current and future biopolymer manufacturers, biotechnology researchers, and sponsors of biopolymer research. Cost and environmental, health and safety information contained in the report may be useful to potential producers of these biosurfactants or of any biopolymer. Sponsors of research on cleaner technologies may find this project an interesting and informative example of the possibilities and challenges of collaborations between applied researchers and basic research scientists.

## SUMMARY OF CONCLUSIONS

Biosurfactant Development, Scale-up and Characterization

Research was conducted at the BPRC to further develop fermentation and purification processes in order to obtain emulsan products in yields required for evaluation of properties and surface cleaning ability. Of the several production methods tested, the continuous batch-fed process yielded the

highest purity product. However, the yields of emulsan reported in previous research were not realized in this project. It is believed that strain improvement by mutation-selection techniques (i.e., growing the polymers in the presence of another material in order to mutate the bacteria) and adjustment in the fermentation physiological operating parameters will be needed for truly high emulsan yields from triglycerides to be realized.

Emulsan analogues[4] were characterized by quantifying surface tension, interfacial tension and turbidity values. The emulsan grown on methyl myristate had the best surface tension lowering properties, was most effective at lowering the interfacial tension between hexadecane and water, and had the second highest emulsification activity of the analogues synthesized and evaluated. Emulsan grown on heptyloxyoctanoic acid/myristic acid had the highest turbidity of the analogues tested indicating higher emulsification activity. Low concentrations of sophorolipid achieved a significantly greater reduction in surface tension than that achieved by emulsan.

Design and Cost Analysis of a Commercial Emulsan Production Facility

The BDC designed a prototype industrial-scale emulsan production facility based on results from the BPRC's emulsan research and on conventional manufacturing practices employed in the biotech industry. Where environmental, health and safety (EH&S) concerns arose in the process design, e.g., the use of pentane for carbon source extraction, alternative processes were proposed. The results of this process design work were used as a basis for the EH&S analysis of emulsan production.

Cost analysis of emulsan production, based on the BDC's design, showed that the optimum plant size was approximately 100,000 L (25g/L yield) which set the selling price of emulsan at $0.08/g. Price comparisons between emulsan and potential competitor surfactants were not performed since it is not possible to compare, with any level of confidence, the gram-per-gram usage of emulsan vs. commercial surfactants in a cleaning formulation (or in any other application).

Environmental, Health, Safety and Regulatory Considerations

Biopolymers offer great promise in reducing the environmental and worker health and safety impacts of traditional synthetic chemical production and use. However, biopolymer production and use does not automatically exclude the use of hazardous materials. For example, an expedient way to purify and extract biosurfactants is with pentane–a hazardous solvent. The problem of hazardous material use needs to be addressed early in the development cycle to ensure that new hazards are not created.

While the federal framework for regulating the biotechnology industry is under modification and has not been fully challenged by a large number of submissions, government agencies are making a conscious effort to adapt and accommodate the regulatory system to these products. It is also clear

[4]Throughout this report, the term "analogue" refers to a biopolymer produced by a specific microorganism, grown on a specific feedstock, under a specific set of conditions. The chemical structure and properties of the biopolymer vary depending on all of these variables.

that biotechnology processes and products may pose new and unexplored risks that warrant regulatory oversight, investigation, and strict health and safety guidelines until these risks are better understood. There is a strong need for greater federal and state coordination, oversight, and clarification on biotechnology environmental and health and safety regulations.

Testing Biosurfactants in TURI's Surface Cleaning Lab

Biosurfactants were tested in TURI's Surface Cleaning Lab (SCL) on metals and soils commonly encountered in manufacturing. The testing was done using protocols established specifically for this project. The new protocols allowed the examination of the properties and performance of one component of an aqueous chemistry, the surfactant. The new protocols were developed by building on existing protocols and knowledge to establish more precise testing requiring quantitative analysis.

Surfactants used in surface cleaning typically reduce the surface tension to approximately 35 dynes/cm and the interfacial tension to 1-2 dynes/cm. The emulsan samples lowered the surface tension of water to 50 dynes/cm and the interfacial tension between water and hexadecane to 17 dynes/cm. Emulsan may need to be combined with another surfactant to achieve a low enough surface tension to effectively clean a surface. Test results suggested that emulsan, when combined with another surfactant, was more effective at cleaning than either surfactant alone. Although emulsan is an effective emulsion stabilizer, it must be combined with either mechanical energy or with another surfactant that further reduces the interfacial tension between water and oil in order to form an emulsion.

The sophorolipid sample exhibited promising surface tension lowering properties but it did not appear to solubilize contaminants or to cause roll-up. Additional synthesis and purification research on sophorolipid is needed to obtain samples with better surface tension lowering properties. This would enhance the sophorolipid's potential to cause roll-up.

Other Potential Uses for Emulsan and Sophorolipid

Based on what was learned about emulsan in this research, it appears to have potential use anywhere that a stable emulsion of a compatible hydrocarbon is desired. For instance, emulsan could be used in soluble emulsion oils for machining fluids, invert solvents (emulsion cleaners), or floor polishes where the polishing agent is emulsified in water.

Due to its small molecular size, sophorolipid could be used for the removal of organics from soils. A biosurfactant would be ideal in this case since it would completely biodegrade in the soil; non-biodegradable surfactants used in this application would leave a residue. In general, sophorolipid could be substituted for a synthetic nonionic surfactant in applications such as laundry and dish detergents, dyes, and personal care products.

Recommendations

Additional research and development is needed to increase the surface and interfacial tension lowering abilities of biosynthesized surfactants. If this can be achieved, biosynthesized surfactants will stand a greater chance of gaining entry into the aqueous cleaning market.

Further research is needed to improve the performance of scale-up efforts–from the laboratory to pilot-scale production–to increase yield and lower production costs. Since most of the cost to produce a biopolymer is incurred in the purification process, this process step in particular must be optimized to make biopolymer production cost effective and competitive with existing products.

Biopolymer developers should critically evaluate the proposed steps for biopolymer production so that in the quest to produce a "green product" only "green processes" are used. This research revealed the possibility that hazardous solvents could be used for purification and extraction of biopolymers in commercial-scale production and, furthermore, that there are viable, cleaner alternative processes (like supercritical $CO_2$ extraction) that will improve the life-cycle profile of these new materials. These issues should be considered and addressed at the early stages of new product development.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# ACRONYMS & ABBREVIATIONS

| | |
|---|---|
| APG | Alkyl polyglycoside (a type of surfactant) |
| ASTM | American Standards and Test Methods |
| BDC | Bioprocess Development Center |
| BPRC | Biodegradable Polymer Research Center |
| BSCC | Biotechnology Science Coordinating Committee |
| CAA | Clean Air Act |
| CDC | Centers for Disease Control and Prevention |
| CFCs | Chlorofluorocarbons |
| CMC | Critical Micelle Concentration |
| CWA | Clean Water Act |
| DCM | Dichloromethane |
| DfE | Design for the Environment |
| DI | Deionized |
| EDTA | Ethylenediamine tetraacetate |
| EH&S | Environmental Health and Safety Characteristics |
| EHS | Extremely Hazardous Substances |
| EP | Extreme pressure |
| EPA | Environmental Protection Agency |
| EPCRA | Emergency Planning and Community Right-to-Know Act |
| FDA | Food and Drug Administration |
| FIFRA | Federal Insecticide, Fungicide, and Rodenticide Act |
| GILSP | Good Industrial Large Scale Practice |
| HEPA | High Efficiency Particulate Air |
| L | liter |
| M | molarity or moles per liter |
| MCAN | Microbial Commercial Activity Notice |
| MSDS | Material Safety Data Sheet |
| NIOSH | National Institute of Occupational Safety Health |
| OECD | Organization for Economic Cooperation and Development |
| OPPT | Office of Pollution Prevention and Toxics |
| OSEE | Optically Stimulated Electron Emission |
| OSHA | Occupational Safety and Health Act |
| OSHA | Occupational Safety and Health Administration |
| OSTP | Office of Science and Technology Policy |
| OTA | Office of Technology Assessment |
| PMN | Pre-manufacture Notification |
| POTW | Publicly-owned Treatment Works |
| psi | pounds per square inch |
| RCRA | Resource Recovery and Conservation Act |
| SBO | Soy Bean Oil |
| SCF | Supercritical Fluid |

| | |
|---|---|
| SCL | Surface Cleaning Laboratory |
| SNUR | Significant New Use Reporting |
| SSO | Soap Stock Oil |
| TCA | Trichloroethane |
| TSCA | Toxic Substances Control Act |
| TURI | Toxics Use Reduction Institute |
| UMASS | University of Massachusetts |
| UNIDO | United Nations International Development Organization |
| USDA | United States Department of Agriculture |
| VOC | Volatile Organic Compounds |
| WHO | World Health Organization |

# ACKNOWLEDGMENTS

First and foremost, the authors would like to thank Dr. Richard Gross, Dr. Alexander Gorkovenko, George Allard and Jin-Wen Zhang of the Biodegradable Polymer Research Center and Dr. Randall Swartz and Anthony Gudinas of the Massachusetts Bioprocess Development Center for their collaboration on this research.

EPA Project Officer, Dr. Steven Hassur, provided thoughtful and valuable comments on the project's progress and draft reports.

The personnel in TURI's Surface Cleaning Laboratory provided guidance and advice throughout the testing.

The authors would especially like to thank John Francis of Emulsan Biotechnologies, Inc., Green Farms, CT for sharing his knowledge and providing samples for testing.

Samples provided by the following companies were greatly appreciated: The PQ Corporation, Utica, IL; Rhône-Poulenc, Surfactants & Specialties, Cranbury, NJ; Valtech Corporation, Pughtown, PA; Union Carbide Chemicals and Plastics Company Inc., Industrial Chemicals Division, Danbury, CT; Witco Corporation, Oleo/Surfactants Group, Greenwich, CT; Oakite Products, Berkeley Heights, NJ.

Finally, the authors would like to thank Maureen Hart for her editorial comments on the final document.

# CHAPTER 1 INTRODUCTION

Cleaning and degreasing of metal parts in the metal finishing and metal working industries has traditionally been accomplished by the use of chlorinated solvents in vapor degreasers or immersion systems. Effective cleaning is usually an essential step in assuring high performance in subsequent plating or welding operations or is desirable for aesthetic reasons. Chlorinated solvents tend to be highly effective at removing oils and other soils from metal parts and are highly valued for their low flammability.

As scientists began to uncover problems with chlorinated solvents – ozone depletion, adverse human and ecological health effects – aqueous, semi-aqueous, mechanical and other cleaning alternatives began to emerge. Industry observers believe that aqueous and semi-aqueous cleaners will dominate the metal surface cleaning market since large-scale cleaning operations find it easiest to make the transition to these alternatives and these systems can be chosen to remove specific types of contaminants from a range of metal surfaces.[5] Aqueous cleaning systems offer particular advantages over semi-aqueous cleaners and other alternatives since they contain virtually no volatile organic compounds (VOCs), may be biodegradable, and are excellent for removing inorganic and polar organic contaminants.[6] Furthermore, the rapid pace of development and adoption of filtration techniques to "closed loop" aqueous cleaning systems are making this alternative even more environmentally and economically attractive.[7]

Through its research program and Surface Cleaning Laboratory (SCL), the Massachusetts Toxics Use Reduction Institute (TURI) is engaged in a number of activities designed to stimulate the development and adoption of aqueous cleaning technologies for surface cleaning applications. In collaboration with other researchers at the University of Massachusetts Lowell, this project was aimed at developing, testing, and transferring a new technology in a new application--biosynthesized, biodegradable surfactants in aqueous surface cleaning chemistries.

This research was supported by the U.S. Environmental Protection Agency, Office of Pollution Prevention and Toxics (OPPT) under its "Paint Stripping for Small Aircraft" grant through the Environmental Technology Initiative (Grant No. C823640-01-2 and Interagency Agreement No. DW49936552-01-0). As part of a program examining opportunities for the use of clean maintenance

[5]Karen B. Thomas and Michael Ellenbecker, "Evaluation of Alternatives to Chlorinated Solvents for Metal Cleaning," Report to the U.S. Environmental Protection Agency, National Risk Mgmt. Research Laboratory (November 1995).

[6]U.S. Environmental Protection Agency, Office of Research and Development, *Guide to Cleaner Technologies: Alternatives to Chlorinated Solvents for Cleaning and Degreasing*, EPA/625/R-93/016 (February 1994).

[7]Toxics Use Reduction Institute, *Closed Loop Aqueous Cleaning, Technical Report No. 29* (Lowell: TURI, 1995).

technologies by the aviation community, the Paint Stripping for Small Aircraft project focuses on identifying and testing alternative coating removal technologies suitable for small aircraft. The project also addresses both existing and contemplated government regulatory activity potentially affecting the development and use of new technologies. The research presented in this report complements the Paint Stripping for Small Aircraft project by examining the production and regulatory implications of a biosynthesized surfactant with potential use in metal cleaning formulations with transferability to aircraft maintenance where the removal of oils and greases from engine parts has traditionally been accomplished by the use of chlorinated solvents. In addition, through its Alternative Chemical Synthetic Design Project, within the Design for the Environment (DfE) Program, OPPT has sought to fund academic research in new chemical technologies. This project is one such endeavor.

## Biosurfactants

Surfactants, also called wetting agents, are used in aqueous cleaners to provide certain key properties in a cleaning formulation – detergency, emulsification of soils, and wetting action. Currently, non-ionic surfactants are most commonly used for surface cleaning applications. These surfactants are chemically synthesized by attaching ethylene oxide molecules to alcohol. Ethylene oxide is produced from the petrochemical, ethylene. Alcohol may be derived from petrochemical or natural raw materials. Ethylene oxide has been shown to cause acute health hazards at high concentrations, is considered a suspect carcinogen, and is derived from non-renewable petroleum resources.[8]

Biosurfactants are another type of surfactant. Biosurfactants are a class of biopolymers, which are materials produced directly from biological processes. Biosurfactants offer several potential advantages over their synthetic counterparts: they tend to be more readily biodegradable; they can be produced from renewable resources; and they can be manufactured without the use or production of toxic chemicals. However, biosurfactants pose several disadvantages as well. Little research has been done on the potential adverse impacts of the use of biopolymers in large quantities, particularly when they are introduced into solid or aqueous wastestreams or natural ecosystems. Furthermore, biopolymers are currently two to five times more expensive to produce than synthetic resins. It is unclear whether the production of these materials will benefit from the economies-of-scale seen in chemosynthetic methods of production.[9]

Two biosynthesized surfactants were studied in this project; emulsan and sophorolipid. In a number of studies and patents, emulsan was shown to be an effective emulsifier of oily substances, initially in emulsifying oily residues in oil transport ship hulls and later in personal care products. Emulsan is currently produced commercially, albeit by one manufacturer and in very small quantities. Emulsan has found commercial use as a component of cosmetics and in oil tanker vessel cleaning. It has

---

[8]SRI International, *Chemical Economics Handbook*, 654.500G (May 1993).

[9]U.S. Congress, Office of Technology Assessment, *Biopolymers: Making Materials Nature's Way – Background Paper*, OTA-BP-E-102 (Washington, D.C.: U.S. Government Printing Office, September, 1993).

shown promise in pilot testing as an anti-adherence agent in cosmetics, toothpaste, some dermatological applications, and as a viscosity reducing agent for heavy oils and greases. Sophorolipid has good surface tension-lowering properties and can reportedly be produced at a cost comparable to petroleum-based surfactants.[10, 11] This research, aimed at metal cleaning and degreasing, sought to take advantage of the emulsification properties of emulsan and the surface-tension lowering properties of sophorolipid.

For this project, emulsan and sophorolipid were synthesized by bacteria in laboratory fermentation systems. Emulsan was produced by feeding the oil-degrading bacterium *Acinetobacter calcoaceticus* strain RAG-1 a variety of materials such as soybean oil and fatty acids (different fatty acids will modify the emulsan produced, potentially affecting its properties). The bacteria produce emulsan – a polyanionic amphipathic lipoheteropolysaccharide – extracellularly during their growth process. Sophorolipid – an amphipathic oligosaccharide with a fatty acid functional group – was produced by feeding the bacteria *Torulopsis bombicola* strain ATCC 22214 a mixture of glucose, yeast extract, and urea.

Research Objectives

The primary objectives of this project were: (1) to develop one or more biosurfactant analogues[12] with potential for use in surface cleaning applications, (2) to evaluate the production costs of the biosurfactants, (3) to evaluate the environmental, health and regulatory implications of biosurfactant production, (4) to evaluate the effectiveness of the biosurfactants in laboratory simulated surface cleaning systems, and (5) to advance the manufacture and use of the biosurfactants in commercial cleaning applications (if surface cleaning evaluations prove successful) by sharing this research with companies that could manufacture and sell the biosurfactants commercially (i.e., technology transfer).

The project was a collaborative effort of TURI (a multi-disciplinary pollution prevention research and education center) and two other research centers at University of Massachusetts Lowell–the Biodegradable Polymer Research Center (BPRC) and the Massachusetts Bioprocess Development Center (BDC). TURI designed and carried-out the evaluation of the surfactants in its Surface Cleaning Laboratory. The results of TURI's evaluations influenced the direction of the biopolymer development process at the BPRC. The BPRC team, headed up by Dr. Richard Gross, developed and scaled-up production of several biopolymer analogues in their laboratories on campus and at the

---

[10]George Georgiou, Sung-Chyr Lin and Murkul M. Sharma, "Surface-Active Compounds From Microorganisms," *Bio/Technology*, Vol. 10 (January 1992) 60-64.

[11]Michele I. Van Dyke, Hung Lee and Jack T. Trevors, "Applications of Microbial Surfactants," *Biotech. Adv.*, Vol. 9 (1991) 241-252.

[12] Throughout this report the term "analogue" refers to a biopolymer produced by a specific microorganism, grown on a specific feedstock, under a specific set of conditions. The chemical structure and properties of the biopolymer vary depending on all of these variables. The term "mutant" or "mutant strain" is used to describe a microorganism that has been genetically altered.

Natick Army Research Labs. The work at the BPRC was partially funded by OPPT. Finally, the BDC designed an industrial scale production facility for the manufacture of the biosurfactants and evaluated the effect of production scale on production costs.

The report is divided into two parts. Part I covers biosurfactant synthesis and production:

- Chapter 2 provides background on two biosurfactants, emulsan and sophorolipid.
- Chapter 3 presents the results of the research on emulsan and sophorolipid development work at the BPRC and at Natick Labs.
- Chapter 4 contains the results of emulsan production process and cost modeling.
- Chapter 5 presents the results of the environmental, health, safety, and regulatory evaluation.

Part II of the report covers biosurfactant applications:

- Chapter 6 provides background information on the need for and uses of surfactants in aqueous metal cleaning.
- Chapter 7 presents the methodology and results of the biosurfactant evaluations in the SCL at TURI.

Finally, Chapter 8 presents conclusions and recommendations for future research.

# PART I BIOSURFACTANTS: SYNTHESIS AND PRODUCTION

## CHAPTER 2 BIOSURFACTANTS

### 2.0 INTRODUCTION

In recent years, significant research has been devoted to the development of biopolymers–materials that are biologically synthesized from renewable resources and are readily biodegradable. According to a 1993 study by the Office of Technology Assessment, biopolymer applications are beginning to emerge in packaging, food production and medicine.[13] Biosurfactants, a class of biopolymers, are surface active agents that have the ability to emulsify and/or lower the surface tension of certain liquids. There are six major types of biosurfactants: hydroxylated and cross-linked fatty acids, lipoproteins-lipopeptides, phospholipids, complete cell surfaces, glycolipids, and polysaccharide-lipid complexes.

Two biosurfactants, emulsan (a polysaccharide-lipid complex) and sophorolipid (glycolipid), were the focus of this research aimed at finding new, environmentally benign agents for metal cleaning and degreasing. Since the early 1970's emulsan has been the subject of significant research at the University of Tel Aviv[14] and more recently at the University of Massachusetts Lowell's Biodegradable Polymer Research Center. Emulsan is produced commercially at this time. In a number of studies and successful patent applications emulsan was shown to be an effective emulsifier of oily substances, initially in emulsifying residues in oil transport ship hulls and later in personal care products. Sophorolipid is of interest because it appears to have good surface tension-lowering properties and can be produced at a cost comparable to petroleum based surfactants.[15,16] This research sought to take advantage of the emulsification properties of emulsan and the surface-tension lowering properties of sophorolipid in metal cleaning and degreasing applications.

---

[13]U.S. Congress, Office of Technology Assessment, *Biopolymers.*

[14]Naim Kosaric, Neil C. C. Gray and W. L. Cairns, "Introduction: Biotechnology and the Surfactant Industry," in Naim Kosaric, Neil C. C. Gray and W. L. Cairns, eds. *Biosurfactants and Biotechnologies* (New York: Marcel Dekker, Inc., 1987).

[15]Van Dyke, "Applications of Microbial Surfactants," 241-252.

[16]Georgiou, "Surface-Active Compounds From Microorganisms," 60-64.

This chapter begins with a summary of surfactant market issues and follows with a description of the chemical structure of emulsan and sophorolipid as well as the history of the discovery and commercialization of emulsan.

## 2.1 THE SURFACTANT MARKET

In recent years, there has been an increased demand for surfactants derived from renewable resources. For the alcohol-based (ethylene as principal feedstock) products, in particular, there has been a rising competition between synthetic (petroleum-based) and natural (vegetable-based) products. Although the market is still dominated by the petroleum-based products, alkyl polyglycoside surfactants (APGs) are riding the green wave, propelled by the interest in products made from renewable resources, such as glucose and fatty alcohols from coconut and palm kernel oils.[17]

APGs are being used in the household cleaner and personal care markets and are supposed to be especially mild to the skin and eyes. They are often used as co-surfactants with other oleochemicals. From a major survey of the U.S. surfactant market conducted in 1993, it was reported that there appears to be some feeling in the industry that advertising a product as natural can provide a lift, but that the APG penetration in the market has been slow.[18] Proctor and Gamble offers a nonionic natural surfactant, alkylaminoglucoside, which is derived from their own proprietary technology, and is researching several additional sugar-based surfactants. In addition, Witco is a big player in the oleo-based surfactant market.

As surfactant manufacturers vie for market share, they also wrestle with many environmental, health and safety issues potentially associated with their products. Questions about the environmental impacts of surfactants have centered around the biodegradability of the compounds. Surfactants based on ethylene or propylene oxide do not pass certain biodegradability tests. Although manufacturers of vegetable-surfactants claim complete biodegradability,[19] scientifically based definitions and standards relating to degradability have yet to be legally established in the U.S. Some studies on degradability have been performed by EPA but the U.S. Congress has yet to mandate technical standards in this area.[20]

Although there are no widely-accepted standards for biodegradability in the US, in Europe a test method designed by the Organization for Economic Cooperation and Development is the most widely used test of biodegradability. The test method, which employs three tiers of increasingly complex

---

[17]David Richards, "Going Natural - Detergents '93," *Chemical Marketing Reporter* (January 25, 1993): SR20-24.

[18]Chemical Week, "Specialty Surfactants: A Restructured Industry Turns to Product Development" (September 28, 1994): 25-28.

[19]Alice Naude, "Family Resemblance - Detergents '93," *Chemical Marketing Reporter* (January 25, 1993): SR16.

[20]U.S. Congress, Office of Technology Assessment, *Biopolymers.*

testing, has many problems including lack of standardization, confusion over definitions, and the need for specialized testing as the current tier testing favors certain types of chemicals.[21] Regardless of these problems, manufacturers in Europe are using biodegradability as a benchmark for environmental friendliness. However, cost is still the most important criterion for the success of a particular surfactant. One producer notes that "higher biodegradability won't get you any premiums, it has to be cost effective."[22]

While the demand for "green" or natural surfactants in the personal care market is expected to increase, the metal cleaning market has quite a different history. Typically, metal cleaning has been done with chlorinated solvents. Aqueous cleaners, whether they contained petroleum-based components or not, have been introduced to the degreasing arena as "green" alternatives to their chlorinated solvent counterparts. This makes the market much more difficult to penetrate for biosurfactants. Nonetheless, there is debate over the biodegradability of aqueous metal cleaners as well as their VOC content. This debate may effectively set the stage for the emergence of biopolymers in the aqueous degreasing market.

## 2.2 EMULSAN

Emulsan is of interest in cleaning, degreasing and maintenance applications because of its ability to form stable hydrocarbon-in-water emulsions. Emulsan is a polyanionic lipoheteropolysaccharide bioemulsifier produced by the bacterium *Acinetobacter calcoaceticus* strain RAG-1. The polymer consists of a polysaccharide backbone made up of three amino sugars: N-acetyl-D-galactosamine, N-acetyl-aminouronic acid, and an unidentified amino sugar.[23] The polymer has an amphipathic structure, i.e., the structural group, or backbone, is hydrophilic while the fatty acids linked to the backbone are hydrophobic. The surface active properties of emulsan stem from the presence of both polar and nonpolar groups.[24]

---

[21]Camela Zarcone, "Degradation Testing - Detergents '94", *Chemical Marketing Reporter* (January 24, 1994): SR8.

[22]Melissa Shon, "Green and Mild-Mannered - Detergents '94", *Chemical Marketing Reporter* (January 24, 1994): SR22-23.

[23]Z. Zosim, E. Rosenberg and D. L. Gutnick, "Changes in Hydrocarbon Specificity of the Polymeric Bioemulsifier Emulsifier: Effects of Alkanols," *Colloid and Polymer Science*, Vol. 264, No.3: 218-223.

[24]Y. Shabtai and D. L. Gutnick, "Enhanced Emulsan Production in Mutants of *Acinetobacter calcoaceticus* RAG-1 Selected for Resistance to Cetyltrimethylammonium Bromide," *Applied and Environmental Microbiology*, Vol. 52, No. 1: 146-151.

The discovery of emulsan[25] is a unique story. Two scientists, David Gutnick and Eugene Rosenberg from the University of Tel Aviv were walking down a beach when they noticed a "bull's eye" in the middle of a pool of tar. They concluded that something must be "eating" the tar in the middle area. They isolated the bacterial organism, *Acinetobacter calcoaceticus* strain RAG-1, and later discovered that the organism produced a bioemulsifier which they named "emulsan". The patents and intellectual property rights relating to these discoveries were acquired by Leslie Misrock, a patent lawyer at Pennie and Edmond (a large patent law firm in New York). Misrock provided funding for the research of Gutnick and Rosenberg.

Leslie Misrock devised a commercialization plan for Emulsan™ Brand Biopolymer. This plan involved the formation of a corporation, Petroferm, Inc., to facilitate the commercialization. Later Petroferm diverted their energies from biotechnologies to developing CFC replacements for the printed circuit board industry. At that time Leslie Misrock reacquired the rights to Emulsan™ Brand Biopolymer and formed a new corporation, Emulsan Biotechnologies, Inc., to continue the development and commercialization of Emulsan™ Brand Biopolymer and other biotechnologies. According to Emulsan Biotechnologies, Inc., the company owns the right to manufacture, use and sell Emulsan™ Brand Biopolymer and has not licensed the property right to any other entity.[26] A brief overview of the patent history of emulsan is given in Table 1.

According to Emulsan Biotechnologies, Inc., Emulsan™ Brand Biopolymer has commercial utility in any application where the formation of stable emulsions is important. Current end use applications range from cosmetics to oil tanker vessel cleaning. Fabricated Emulsan™ Brand Biopolymer-based products are sold for cleaning and degreasing applications (though we were not able to get a sample for our own evaluation).[27] Reportedly this dispersant product, when used to clean a greasy lawnmower for example, will emulsify the grease and prevent an oil slick from forming when the lawnmower is rinsed.[28]

According to David Gutnick, emulsan has shown promise in pilot testing as an anti-adherence agent in cosmetics, toothpaste, some dermatological applications, and as a viscosity reducing agent for heavy oils and greases.[29]

---

[25]Note: In this report the word emulsan refers to any polyanionic lipoheteropolysaccharide bioemulsifier produced by *A. calcoaceticus* RAG-1, while Emulsan™ Brand Biopolymer refers to the biopolymer produced by Emulsan Biotechnologies, Inc.

[26]Written communication with John A. Francis, Emulsan Biotechnologies, Inc., August 30, 1995.

[27]Ibid. Production data are considered company confidential and were not made available to the researchers.

[28]Ibid.

[29]Email communication with David Gutnick, September 5, 1995.

## 2.3 SOPHOROLIPID

Sophorolipid is a nonionic biosurfactant produced by the yeast *Torulopsis bombicola* strain ATCC 22214. It is one of the few biosurfactants identified that are produced from yeasts. Several glycolipids have been characterized from the crude fermenter extract. These glycolipids contain the dimeric sugar sophorose and a long-chain carboxylic acid with a hydroxyl function on the penultimate or terminal carbon. In most of the structures the acid is attached to the carbohydrate by the hydroxyl group, leaving a free carboxyl function.[30]

Sophorolipid is of significant interest because of its characteristics. It is a small molecular weight biosurfactant that is amphipathic. Being amphipilic means that is has a structure which contains a site or sites that interact strongly with hydrophilic substances (such as water) and also contains a site or sites that interact strongly with hydrophobic substances (such as oils). It has good surface tension-lowering abilities which is a desirable quality for metal cleaning. As mentioned earlier, previous research has shown that sophorolipid can be produced at a cost significantly lower than any other biosurfactant. This is critical because cost is one of the largest hurdles to be overcome for biosurfactants to become a viable economic replacement for their petroleum-based counterparts.

[30]David G. Cooper and D. A. Paddock, "Production of a Biosurfactant from *Torulopsis bombicola*," *Applied and Environmental Microbiology* (January 1984): 173-176.

Table 1. Patent History of Emulsan Brand Biopolymer

| Patent Number | Date Filed | Date Issued | Brief Description of the Patent (All information regarding the patent history of emulsan taken from copies of patents listed above, provided by the U.S. Dept of Commerce, Patent and Trademark Office.) |
|---|---|---|---|
| 3941692 | 1/10/75 | 3/2/76 | Addition of microorganism (*Acinetobacter calcoaceticus RAG-1*), nutrients, and aeration to unpumpable residue in the tanks of oil transport ship, converting it to byproducts that do not contain oil contamination; byproducts to be recovered or discharged |
| 4230801 | 2/22/79 | 10/28/80 | Describes the fermentation process used to produce the biopolymer, α-emulsan; also describes different assays used in the characterization of emulsan |
| 4234689 | 2/22/79 | 11/18/80 | Discusses using α-emulsan in oil-contaminated vessel cleaning, and enhanced oil recovery by chemical flooding, and oil spill management |
| 4276094 | 12/21/79 | 6/30/81 | Discusses in further detail cleaning crude oil-contaminated vessels with α-emulsan in water, forming an oil-in-water emulsion that may be removed and used for fuel value or refining |
| 4311829 | 5/14/80 | 1/19/82 | Describes the process used to produce apo-β-emulsan |
| 4311830 | 5/14/80; | 1/19/82 | Describes the process used to produce apo-α-emulsan |
| 4311831 | 5/14/80 | 1/19/82 | Describes the process used to produce apo-ψ-emulsan |
| 4311832 | 5/14/80 | 1/19/82 | Describes the process used to produce proemulsan |
| 4380504 | 5/14/80 | 4/19/83 | Describes the process used to produce ψ-emulsan |
| 4395353 | 5/14/80 | 7/26/83 | Describes the chemical structure of the biopolymer |
| 4395354 | 7/17/80 | 7/26/83 | Further characterizes the different emulsan biopolymers; describes ability of polymer to flocculate aluminosilicate ion-exchangers such as bentonite and kaolin |
| 4704360 | 11/30/83 | 11/3/87 | Describes a process to produce and use an enzyme, emulsanase, that degrades bioemulsifiers, particularly emulsan; describes the demulsification of emulsan-induced hydrocarbon-in-water emulsions |
| 4818817 | 5/15/87 | 4/4/89 | Similar text to previous patent with additional claims |
| 4870010 | 4/15/86 | 9/26/89 | Describes the use of bioemulsifiers, specifically emulsan, in personal care products; describes the use of emulsan in soaps, shampoos, shampoos to treat eczema/psoriasis on the scalp, creams and soaps to treat acne, and moisturizing creams also states emulsan acts as an anti-adhering agent on surfaces |
| 4883757 | 11/24/87 | 11/28/89 | Describes process used to produce nondialyzable bioemulsifiers produced by *Acinetobacter calcoaceticus* strains; describes the production of emulsan at different grades of purity |
| 4943390 | 9/28/88 | 7/24/90 | Describes the use of bioemulsifiers, particularly emulsan, to facilitate the transport and combustion of highly viscous hydrocarbon fuels by forming hydrocarbon-in-water emulsions |
| 4999195 | 7/5/89 | 3/12/91 | Similar text to patent # 04870010, with different claims |

**Key**

α-emulsan: emulsan produced by *Acinetobacter calcoaceticus* strain RAG-1 grown in ethanol medium; also known as neoemulsan

β-emulsan: emulsan produced by *Acinetobacter calcoaceticus* strain RAG-1 grown in hexadecane medium; also known as protoemulsan

ψ-emulsan: produced by base hydrolysis of emulsan under mild conditions; also known as pseudoemulsan

apoemulsan: deproteinized emulsan produced by hot phenol extraction

proemulsan: produced by base hydrolysis of emulsan under strong conditions; deproteinized; no emulsification capacity

apo-α-emulsan, apo-β-emulsan, apo-ψ-emulsan: deproteinized α-emulsan, β-emulsan, ψ-emulsan respectively

# CHAPTER 3 BIOSURFACTANT DEVELOPMENT AND PRODUCTION SCALE-UP

## 3.0 INTRODUCTION

For this project, research was conducted at the BPRC to further develop proven fermentation and purification processes in order to obtain emulsan products in yields required for evaluation of properties and surface cleaning ability. Scale-up of the lab-scale work was performed at the U.S. Army Natick Research, Development and Engineering Center in Natick, MA. Characterization of the polymer by surface tension and turbidity testing added valuable information to the research. As the testing of emulsan proceeded to TURI's Surface Cleaning Laboratory, the research team decided that it would be beneficial to test a smaller molecular weight material with better surface tension-lowering ability, thus, work began to synthesize a sophorolipid sample to include in the cleaning tests. Due to sophorolipid being synthesized late in the project, it was not characterized as extensively as emulsan. This chapter describes the fermentation, purification and characterization work performed at the BPRC and in Natick.

## 3.1 EMULSAN

Laboratory Synthesis

*Two liter fermentation*

Gram negative bacterium *Acinetobacter calcoaceticus* strain RAG-1 can use a variety of substrates as sole carbon sources during its growth, including middle chain length alkanes, crude oil, alcohols, fatty acids, and triglycerides. In previous emulsan synthesis work at the BPRC, myristic acid was shown to be a preferred carbon source for RAG-1 growth and emulsan production. The emulsan product exhibited high emulsification activity for relatively shorter chain substrates. The emulsification activity was determined by turbidity measurements on samples of emulsan, hexadecane, water, and a buffer. The first samples of emulsan provided by the BPRC to TURI for testing in the SCL were from a 2 liter (L) fermentation, using methyl myristate as the carbon source. The sample from this fermentation is referred to as sample #1 in Chapter 7.

*Thirty liter fermentation*

Scale-up to a 30 L fermentation was performed at U.S. Army Natick Research, Development and Engineering Center in Natick, MA. An 80% methyl oleate/ 20% oleic acid carbon source was chosen for the 30 L run based on previous lab-scale studies indicating that the emulsan produced by this feed

source also showed improved emulsification activity when compared to emulsan formed on other feed sources. The 30 L fermentations were conducted for 86 hours. Two feed strategies were used for two 30 L fermentations. (Both feed strategies are considered 'fed-batch' as food is added as the fermentation is taking place as opposed to 'batch' where all the carbon source is added at the beginning of the fermentation.) One feed strategy was to feed the fermentor in steps at 12 hour intervals (coordinated feed). No carbon source was fed during the final 12 hours so that all of the carbon source would be consumed. This feed strategy produced an emulsan yield prior to purification of 14 g/L but the product was of low purity (i.e. contained cellular debris). The second feed strategy, continuous feed, appeared to produce a higher purity product though the purity of the unpurified product was not determined.

*Purification of the scale-up product*

From the first scale-up purification, it was learned that cells not removed by a continuous centrifugation step were the main product impurity. The purification strategy in the second scale-up run was modified to include a microfiltration step to remove virtually all of the cells from the broth; this provided a higher purity product. The two purification strategies are shown in Table 2. The coordinated feed schedule produced 3 g/L purified emulsan. The continuous feed schedule produced an emulsan yield of approximately 1 g/L of purified product. The sample from this fermentation is referred to as sample #2 in Chapter 7.

Table 2. Purification Steps Used in Scale-up Synthesis of Emulsan

| Coordinated Feed | Continuous Feed |
|---|---|
| Continuous centrifugation<br>Ammonium sulfate precipitation (supernatant)<br>Liquid/liquid separation of precipitate<br>Diafiltration with 30 K Dalton filter, 2 volumes<br>Concentration with 30 K Dalton filter<br>Lyophilization<br>Soxhlet extraction of carbon source from emulsan | Continuous centrifugation<br>Microfiltration, 0.3 micron<br>Diafiltration with 30 K Dalton filter, 5 volumes<br>Concentration with 30 K Dalton filter<br>Lyophilization<br>Soxhlet extraction of carbon source from emulsan |

*Additional two liter fermentation*

In an effort to improve yields in the 30 L fermentation, two additional 2 L fermentations were performed by the BPRC. The two runs were performed to reproduce results reported by Shabtai[31] who reported that, when using soybean oil as the carbon source, yields of 20 g/L were obtained. Soybean oil and methyl oleate/oleic acid were used as carbon sources for the BPRC fermentations. Using Shabtai's methods, the soybean oil experiment yielded 19 g/L of lyophilized sample. However, after performing a Soxhlet extraction (to remove the excess carbon source), an emulsan yield of 3 g/L

[31]Y. Shabtai, and D. Wang, "Production of Emulsan in a Fermentation Process Using Soybean Oil (SBO) in a Carbon-Nitrogen Coordinated Feed," *Biotechnology and Bioengineering*, Vol. 35 (1990): 753 -765.

was obtained. This was the same yield obtained by the BPRC in previous experiments using methyl oleate/oleic acid as the carbon source. This result was not entirely unexpected since both carbon sources consist of 18 carbon chain lengths.

Shabtai's methods were also used in the second 2 L fermentation, but methyl oleate/oleic acid was used as the carbon source. This experiment used a carbon-nitrogen coordinated feed, a higher initial ammonium sulfate concentration, and a reduced buffer concentration relative to previous BPRC runs. The emulsan yield after extraction was only 1 g/L. This confirmed the results of the second 30 L experiment (yield of approximately 1 g/L of purified product). However, the results by Shabtai were not confirmed for oil-based carbon sources.

*Continuing research on emulsan synthesis*

It appears that strain improvement by mutation-selection techniques (i.e., growing the polymers in the presence of another material in order to mutate the bacteria) and adjustment in the fermentation physiological operating parameters will be needed so that truly high emulsan yields from triglycerides can be realized. The work on emulsan presented here is part of a larger program at the BPRC to develop emulsan analogs with modulated fatty acids side chain composition and to study substrate specificity of new emulsions. Current work by the BPRC includes attempting to assign property changes in emulsan to specific side chain structural changes.

Characterization of Emulsan

TURI and the BPRC performed three types of tests in order to help characterize purified emulsan analogues: surface tension, interfacial tension and turbidity. Surface tension is a measure of the tension (in dynes per centimeter or dyne/cm) required to lift a platinum-iridium ring from the surface of a liquid (i.e. a surfactant in aqueous solution). This is of significant interest in metal cleaning because the surface tension of water (72-73 dyne/cm) must be lowered sufficiently in order for effective cleaning to occur. Petroleum- and oleo-based surfactants used in aqueous metal cleaning reduce the surface tension to less than 35 dyne/cm. Interfacial tension is a measure of the tension (in dynes per centimeter) required to lift a platinum-iridium ring from the interface between two liquids (in this research the two liquids are a surfactant in aqueous solution and an oil). Interfacial tension is a significant factor in metal cleaning because if a surfactant is successful at significantly lowering the interfacial tension between a contaminant and its carrier fluid then the contaminant may be removed from the substrate more easily.[32]

In this research turbidity refers to a relative measure of the amount of a given hydrocarbon dispersed in an aqueous phase. Turbidity is commonly used in the characterization of biosurfactants to measure

[32]Erik Kissa, "Kinetics of Soiling and Detergency," In Gale W. Culer and Erik Kissa, eds., *Detergency: Theory and Technology,* New York: Marcel Dekker, 1987.

their ability to solubilize and/or emulsify hydrocarbon contaminants into the aqueous phase.[33,34] It is measured in Klett units, which indicates the amount of light that is scattered by the sample. A higher value indicates greater emulsification and/or solubilization. It must be noted that interfacial tension and turbidity are dependant on the type of oil chosen for testing. Different oils will give different values for these two tests due to hydrocarbon specificity (e.g., one emulsan analogue may be more effective than another at emulsifying a particular oil).

*Surface and interfacial tension testing*

The surface tension and interfacial tension properties of biopolymers are of significant interest for aqueous cleaning because they provide direct and indirect measures of cleaning ability. Surface tension and interfacial tension measurements were obtained using a Fisher Scientific Tensiometer, Surface Tensiomat-21, and the ASTM Standard Test Method for Surface and Interfacial Tension of Solutions of Surface-Active Agents, test number D 1331-89. The surface tension of nine emulsan analogues was measured at two concentrations (see Table 3). Of the analogues synthesized and evaluated, the emulsan grown on methyl myristate had the best surface tension lowering properties (~50 dyne/cm at a concentration of 83 mg/L). [Note: A concentration of 83 mg/L was chosen as the concentration above which the surface tension of emulsan was not significantly reduced with further addition of emulsan. Likewise 484 mg/L was chosen as the concentration above which turbidity was not significantly increased with further addition of emulsan.]

Table 3. Surface Tension of Nine Emulsan Analogues at Two Concentrations

| Feedstock carbon source | Surface tension (dyne/cm) with 83 mg/L of emulsan | Surface tension (dyne/cm) with 484 mg/L of emulsan |
|---|---|---|
| Methyl myristate (sample #1) | 49.4 | 46.4 |
| 2-Hydroxyl lauric acid: myristic acid (0.75g:0.25g) | 53 | 44.2 |
| 2-Hydroxyl lauric acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 53.5 | 49.5 |
| 3-Hydroxyl lauric acid: myristic acid (0.5g:0.5g) | 57.5 | 52.9 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g) | 58 | 54.5 |
| 2-Hydroxyl palmitic acid: myristic acid (0.75g:0.25g) | 60 | 54.4 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 55.6 | 52.5 |
| 2-Hydroxyl myristic acid | 56.5 | 49.5 |
| Heptyloxyoctanoic acid: myristic acid (0.5g:0.5g) | 60 | 53.5 |
| 2-Hydroxyl stearic acid | 59.3 | 55 |

[33]Abdul S. Abdul, Thomas L. Gibson and Devi N. Rai, "Selection of Surfactants for the Removal of Petroleum Products from Shallow Sandy Aquifers," *Ground Water*, Vol. 28, No. 6, (November-December 1990): 920-926.

[34]Rosenberg, E.,A. Zuckerberg, C. Rubinovitz, and Gutnick, D. L., "Emulsifier of *Arthrobacter* RAG-1: Isolation and Emulsifying Properties," *Applied and Environmental Microbiology*, Vol. 37, No. 3 (March 1979): 402-408.

As illustrated in Table 3, there was a variance of about 10 dynes/cm in the surface tensions of the nine emulsan analogues. These differences can be attributed to a change in chemical structure and composition that occurs when the polymer is produced from different feedstocks. Note that the surface tension of water was not greatly reduced by any of the emulsan analogues. This agrees with earlier reports that emulsan does not significantly lower the surface tension.[35] All surface tensions were well above 35 dynes/cm. Because surface tension is used as an indicator of a surfactants ability to perform certain mechanisms essential to surface cleaning,[36] surfactants with surface tensions above 35 dynes/cm, such as emulsan, may need to be combined with another surfactant to obtain a low enough surface tension.

Determined by measuring surface tensions at different surfactant concentrations, the critical micelle concentration (CMC) is the concentration at which the further addition of surfactant no longer significantly reduces the surface tension. At this concentration the surfactant is at its solubility limit in an aqueous phase and the surfactant molecules begin to form aggregates known as micelles.[37] These micelles are not in free-molecular form and do not orient at the air-water interface, hence they do not have an added effect on lowering the surface tension.

The CMC is also of interest when determining the amount of surfactant required to achieve solubilization of oily contaminants from the surface of parts. Solubilization is minimal at concentrations of surfactant below the CMC, but rises abruptly once the CMC has been reached.[38] Therefore, the CMC indicates the minimum amount of surfactant required in an aqueous cleaner to achieve effective solubilization of oily contaminants.

The CMC of five emulsan analogues were determined by measuring and plotting the surface tensions at various concentrations and determining the point at which the slope of the curve leveled out (see Figure 1). For example, the CMC of emulsan grown on methyl myristate (sample #1) was 83 mg/L at a surface tension of 50 dynes/cm.

The effect of nine different emulsan analogues on the interfacial tension between hexadecane and water was determined at two concentrations of emulsan (see Table 4). Again, there was a variance in the interfacial tension values (about five dynes/cm) of different analogues as a result of their unique chemical properties.

---

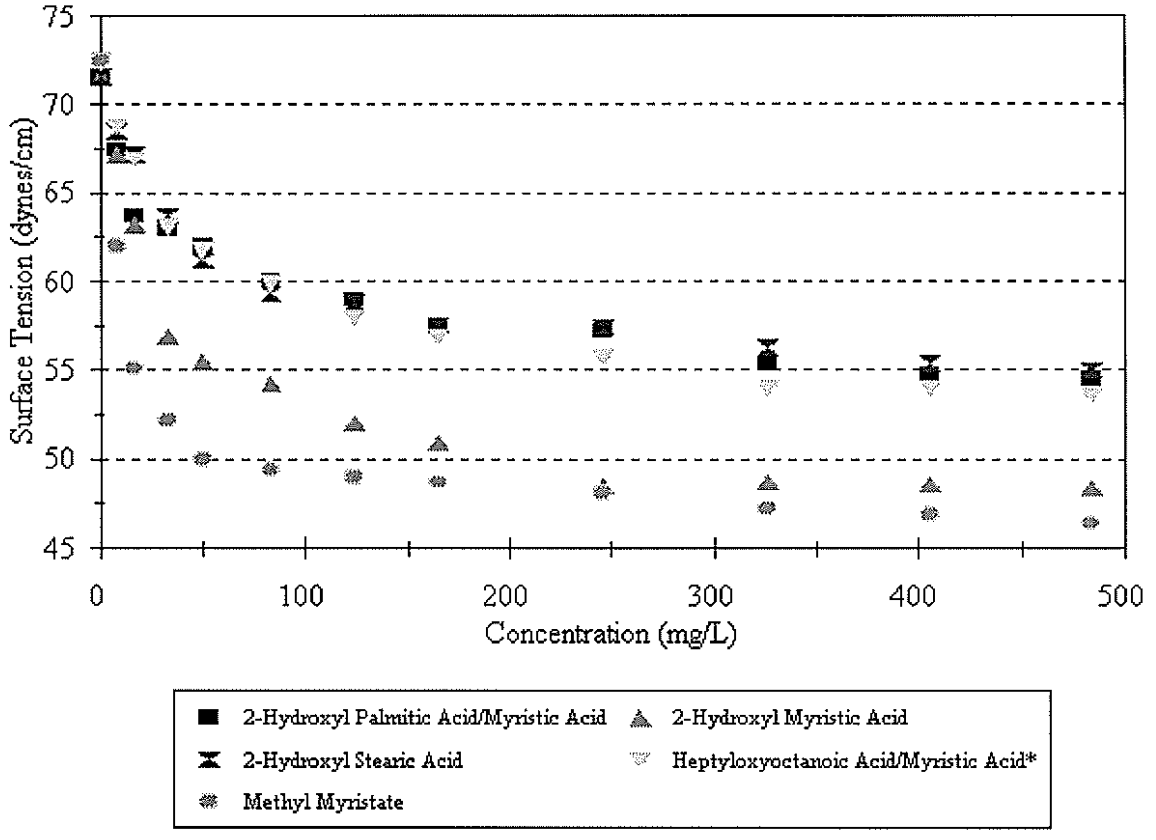
[35]Michael E. Hayes, Eirik Nestaas and Kevin R. Hrebenar, "Microbial Surfactants," *CHEMTECH*, April, 1986.

[36]Kissa, "Kinetics of Soiling and Detergency," 196.

[37]Georgiou, "Surface-Active Compounds From Microorganisms," 60-64.

[38]Robert Laughlin, "Solubilization of Solutions of Surfactants: Micellar Catalysis," *The Aqueous Phase Behavior of Surfactants*, London and San Diego: Academic Press, 1994.

Figure 1. Surface Tension of Five Emulsan Analogues at Various Concentrations




* weight ratios: Hydroxyl Palmitic Acid/Myristic Acid (0.75 g:0.25g);
Heptyloxyoctanoic Acid/Myristic Acid (0.5 g:0.5 g)

Of the nine emulsan analogues evaluated, emulsan grown on methyl myristate (sample #1) was most effective at lowering the interfacial tension between hexadecane and water (~17 dyne/cm at a concentration of 83 mg/L) at the concentrations evaluated. Typically surfactants reduce the interfacial tension to 1-2 dynes/cm. This reduction is essential to performing the basic mechanisms responsible for the removal oil from substrates.[39]

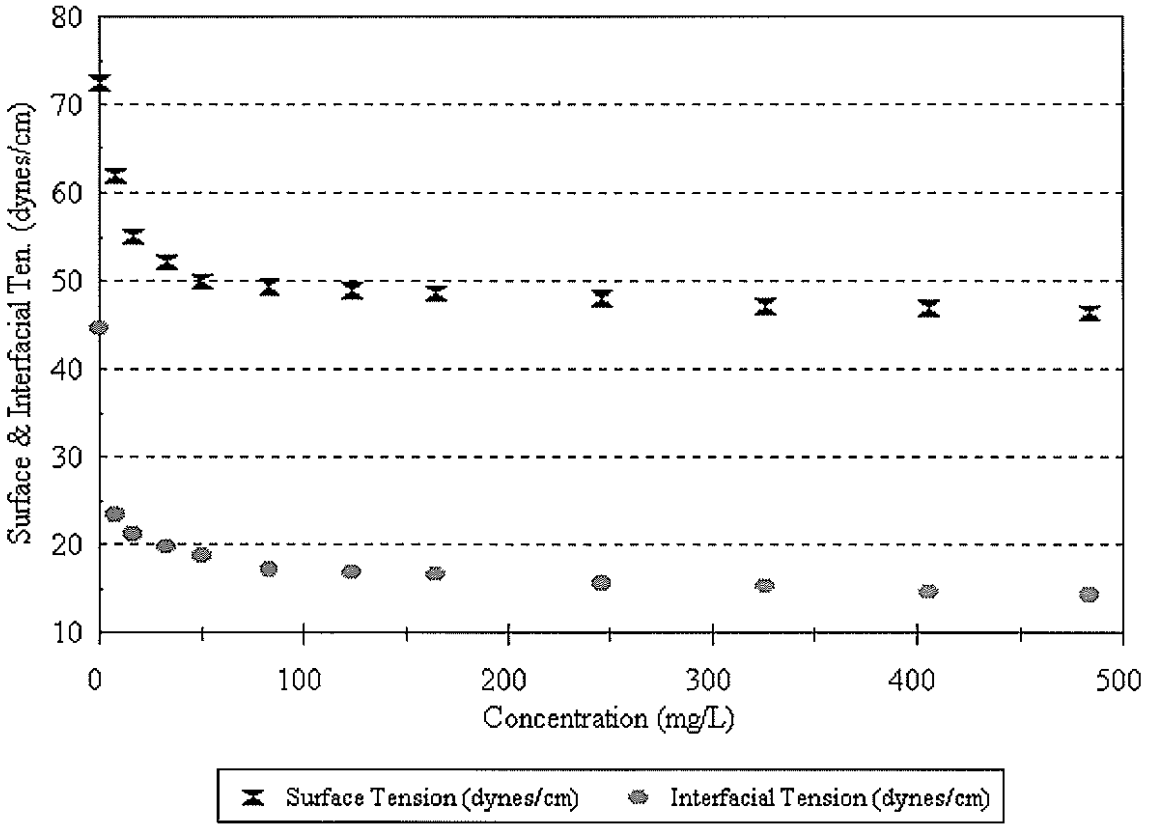
The interfacial tension is a function of the surface tension of the two solutions that make up the interface. Because the presence of a surfactant lowers the surface tension of its carrier fluid (water), it also lowers the interfacial tension at the same rate. This is illustrated in Figure 2, which shows the surface tension and interfacial tension of emulsan from methyl myristate at various concentrations.

[39]Guy Broze, "Mechanisms of Soil Removal," In Robert K. Large, *Detergents and Cleaners: A Handbook for Formulators*, (Cincinnati: Hanser/Gardner Publications, Inc., 1994).

Table 4. Interfacial Tension of Nine Emulsan Analogues at Two Concentrations

| Feedstock carbon source | Interfacial tension (dyne/cm) with 83 mg/L of emulsan | Interfacial tension (dyne/cm) with 484 mg/L of emulsan |
|---|---|---|
| Methyl myristate (sample #1) | 17.2 | 14.3 |
| 2-Hydroxyl lauric acid: myristic acid (0.75g:0.25g) | 15 | 13.5 |
| 2-Hydroxyl lauric acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 16.9 | 15.3 |
| 3-Hydroxyl lauric acid: myristic acid (0.5g:0.5g) | 19.9 | 18.1 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g) | 19.2 | 18.1 |
| 2-Hydroxyl palmitic acid: myristic acid (0.75g:0.25g) | 18.4 | 17.5 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 17.9 | 17 |
| 2-Hydroxyl myristic acid | 19.5 | 15.4 |
| 2-Hydroxyl stearic acid | 19.5 | 18.1 |

Figure 2. Surface and Interfacial Tension for Emulsan from Methyl Myristate at Various Concentrations



Although surface tension and interfacial tension are directly related, a given decrease in surface tension does not indicate the exact decrease in interfacial tension resulting from the presence of emulsan. This is attributed to the hydrocarbon specificity of the polymer. A certain emulsan analogue grown on a given substrate will have a specific affinity for a given hydrocarbon-water interface. This is also why there is no direct correlation between the surface and interfacial tensions and the turbidity. An analogue grown on a specific substrate may have a higher capacity to emulsify hexadecane than an analogue grown on a different substrate due to hydrocarbon specificity.

*Turbidity*

Turbidity measurements were determined to indicate the emulsification activity of emulsan analogues. In a 250 mL buffled flask, 0.2 mL of hexadecane were added to 15 mL of Tris buffer (0.02 M[40] Tris, pH 7.2, supplemented with 0.01 M $MgSO_4$) [41] containing 83 or 484 µg/mL of emulsan. After gyrator shaking in a Labline incubator shaker (at 250 rpm) for one hour at 30°C the contents of the flask was transferred to a Klett tube. The turbidity was measured after the sample stood undisturbed for ten minutes. Turbidity tests were run on nine different emulsan analogues at two concentrations (see Table 5). Measurements varied for the nine different analogues as a result of the unique chemical properties of each analogue.

Table 5. Turbidity Test Results for Nine Emulsan Analogues

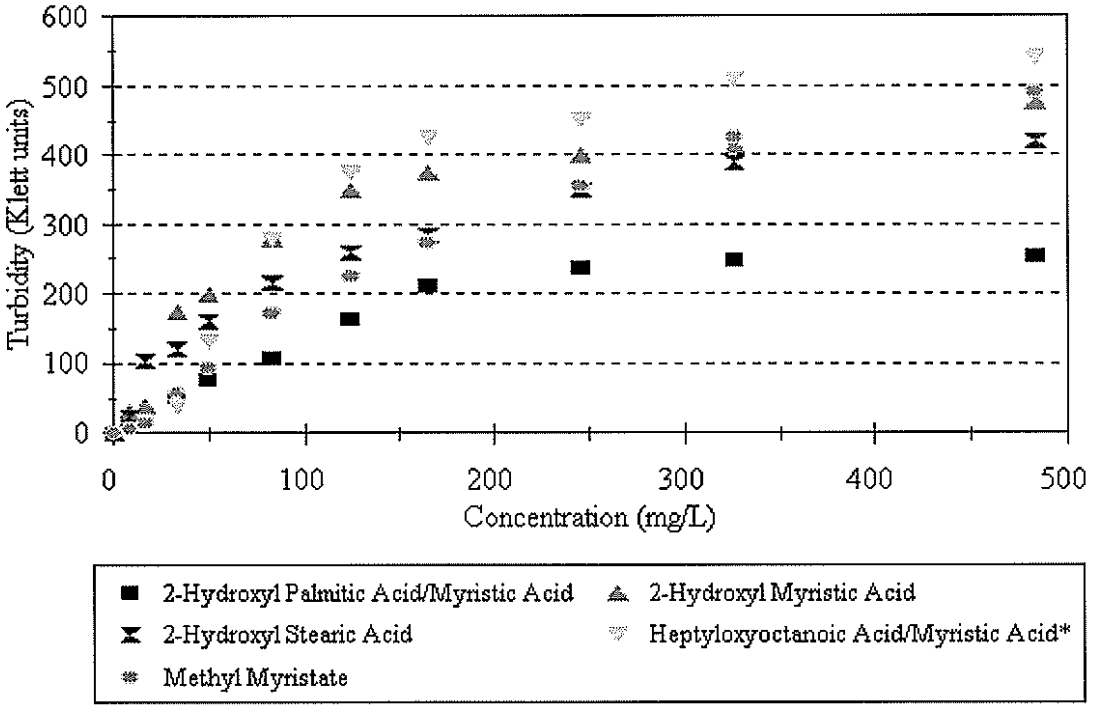
| Feedstock carbon source | Turbidity Measurement (Klett units) with 83 mg/L of emulsan | Turbidity Measurement (Klett units) with 484 mg/L of emulsan |
|---|---|---|
| Methyl myristate (sample #1) | 72 | 490 |
| 2-Hydroxyl lauric acid: myristic acid (0.75g:0.25g) | 72 | 405 |
| 2-Hydroxyl lauric acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 180 | 345 |
| 3-Hydroxyl lauric acid: myristic acid (0.5g:0.5g) | 27 | 270 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g) | 206 | 415 |
| 2-Hydroxyl palmitic acid: myristic acid (0.75g:0.25g) | 106 | 254 |
| 2-Hydroxyl palmitic acid: myristic acid (0.5g:0.5g), 0.02 mM iodoacetamide | 200 | 315 |
| Heptyloxyoctanoic acid: myristic acid (0.5g:0.5g) | 286 | 550 |
| 2-Hydroxyl myristic acid | 286 | 475 |
| 2-Hydroxyl stearic acid | 216 | 420 |

Turbidity as a function of emulsan concentration for five emulsan analogues was measured and plotted (see Figure 3).

[40]Throughout this report "M" represents molarity, or moles per liter.

[41]$MgSO_4$ is the chemical symbol for magnesium sulfate.

Figure 3. Turbidity of Five Emulsan Analogues at Various Concentrations



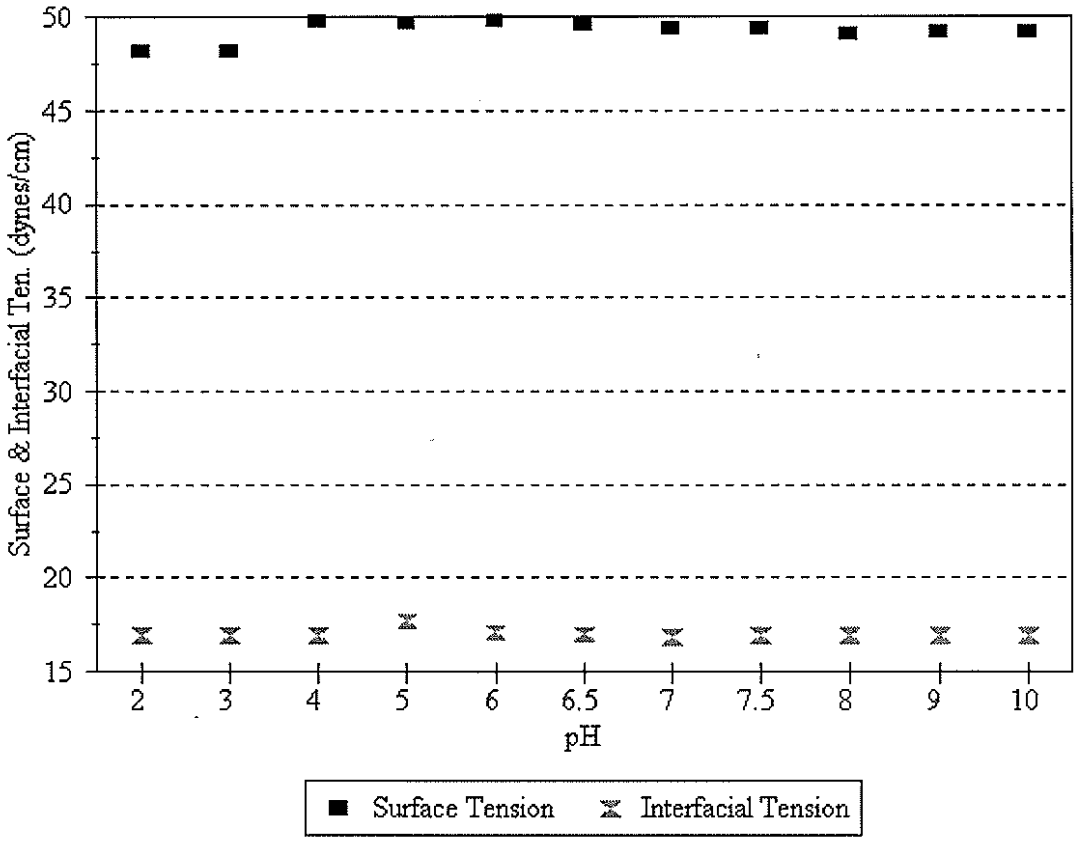

* weight ratios: Hydroxyl Palmitic Acid/Myristic Acid (0.75 g:0.25g);
Heptyloxyoctanoic Acid/Myristic Acid (0.5 g:0.5 g)

The emulsan grown on heptyloxyoctanoic acid/myristic acid had the highest turbidity of the analogues tested (550 Klett Units at a concentration of 484 mg/L) indicating higher emulsification activity than the other analogues synthesized and evaluated. This emulsan analogue also had the second highest turbidity (490 Klett Units at a concentration of 484 mg/L) of the analogues synthesized and evaluated.

*Effect of pH*

In aqueous cleaning processes the pH of cleaning baths varies greatly. The effect of pH on the characteristics of emulsan was therefore evaluated. The surface tension and interfacial tension of emulsan grown on methyl myristate were measured while varying the pH from 2 to 10 (see Figure 4).

Figure 4. Surface & Interfacial Tension of Emulsan From Methyl Myristate at Various pH

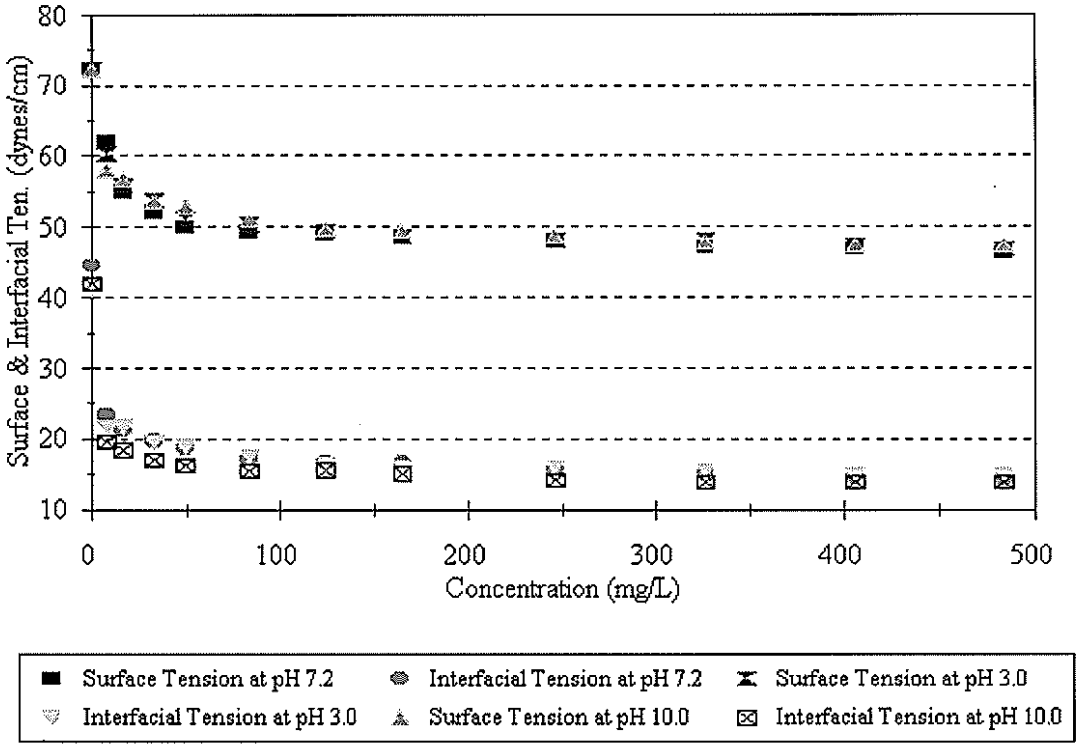



The surface tension and interfacial tension of this analogue were also measured at pH 3, 7.2, and 10 for varying concentrations of emulsan (see Figure 5). The pH had no effect on the surface and interfacial tension properties of emulsan. For turbidity some variability was noted, but there was no peak optima. However, higher turbidity was observed at pH values greater than 5. The fact that pH had little effect on the surface active properties of emulsan is evidence the polymer is stable over the range of pH tested.

*Continuing Research* on *Emulsan Characterization*

It is not possible at this time to clearly define the relationship between an emulsan analogue grown on a given substrate and the changes in its chemical structure and properties because of the number of variables introduced when the carbon source is changed which include a change in the side chain structure of the molecule, a change in its molecular weight, and/or a change in its chemical composition. In addition to continued research regarding the synthesis of modified emulsan analogues, the BPRC will attempt to assign property changes in these analogues to specific side change structural changes.

Figure 5. Surface & Interfacial Tension of Emulsan at Various Concentrations at Three pH




## 3.2 SOPHOROLIPID

Laboratory Synthesis

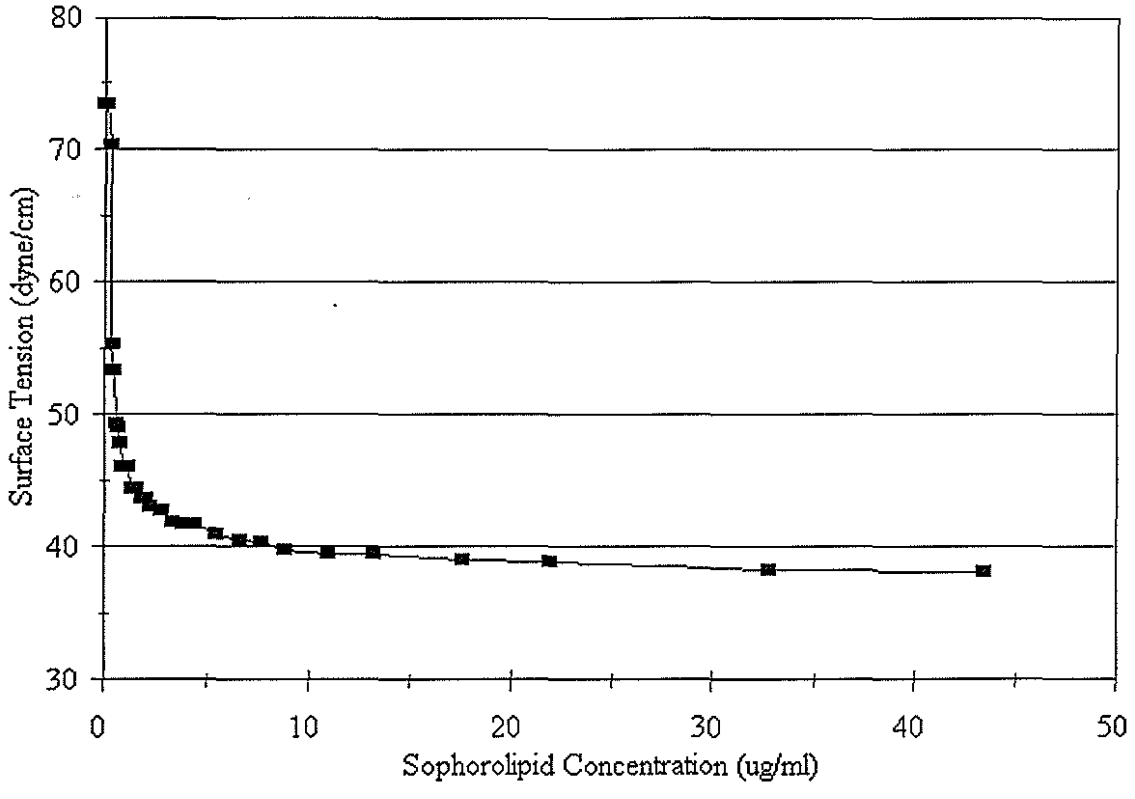
Based on the conditions determined in previous laboratory experiments at the BPRC, a 15 L (10 L working volume) fermentation of sophorolipid was undertaken at the BPRC. The basic medium used was 100 g/L glucose, 10 g/L yeast extract and 1 g/L urea. The conditions of the bioreactor were those of Asmer[42]. The 15 L fermentation resulted in a production yield of approximately 20 g/L of unpurified product. The product was isolated from the broth by centrifuging and extracting with ethyl acetate. The crude product yield from the biomass alone was 15 g/L broth. The isolation of sophorolipid from lipids and other residual materials was achieved by gravimetric and flash chromatography. A sample of sophorolipid was transferred to TURI for analysis in the Surface Cleaning Lab.

[42]H. Asmer, S. Lavy, F. Wagner and V. Wray, "Microbial Production, Structure, Elucidation and Bioconversion of Sophorose Lipids", *JAOCS*, Vol. 65 No. 9 (September, 1988).

Characterization

Surface tension testing was done on the sophorolipid at various concentrations to determine the critical micelle concentration (see Figure 6).

Figure 6. Surface Tension of Sophorolipid at Various Concentrations




The surface tension dropped significantly with the addition of small amounts of sophorolipid (at a concentration of 3.33 mg/L the surface tension was 44.6 dyne/cm ). This is significantly lower than the surface tension of emulsan at the same concentration (approximately 67 dynes/cm). As shown in Figure 6, the surface tension curve for sophorolipid levels out at about 37 dyne/cm at a concentration of 35 mg/L. This reduction in surface tension is significantly greater than that achieved by emulsan. However, as discussed earlier, surfactants used in aqueous metal cleaning reduce the surface tension to about 30 dynes/cm, which is necessary to perform various mechanisms responsible for the removal of oil from substrates.

# CHAPTER 4 DESIGN AND COST ANALYSIS OF A COMMERCIAL EMULSAN PRODUCTION FACILITY

## 4.0 INTRODUCTION

Two objectives of this research were to examine the environmental, health and safety (EH&S) characteristics and production costs of biosurfactant manufacturing. By developing a design for a biosurfactant production process, one can evaluate potential EH&S problems and estimate production costs.

The Bioprocess Development Center (BDC) designed a prototype industrial-scale emulsan production facility based on results from the BPRC's emulsan research and on conventional manufacturing practices employed in the biotech industry. Where EH&S concerns arise in the process design, e.g., the use of pentane for carbon source extraction, alternative processes are proposed. The results of this process design work were used as a basis for the EH&S analysis of emulsan production in Chapter 5.

Cost analysis of emulsan production, based on the BDC's design, was accomplished with the help of a chemical engineering process design software system BioPro Designer® from Intelligen Inc. The software was used to examine the effects of economies of scale on emulsan selling price. Three production scales were examined: 10,000, 100,000 and 1,000,000 liters. This chapter describes the results of the BDC's work.

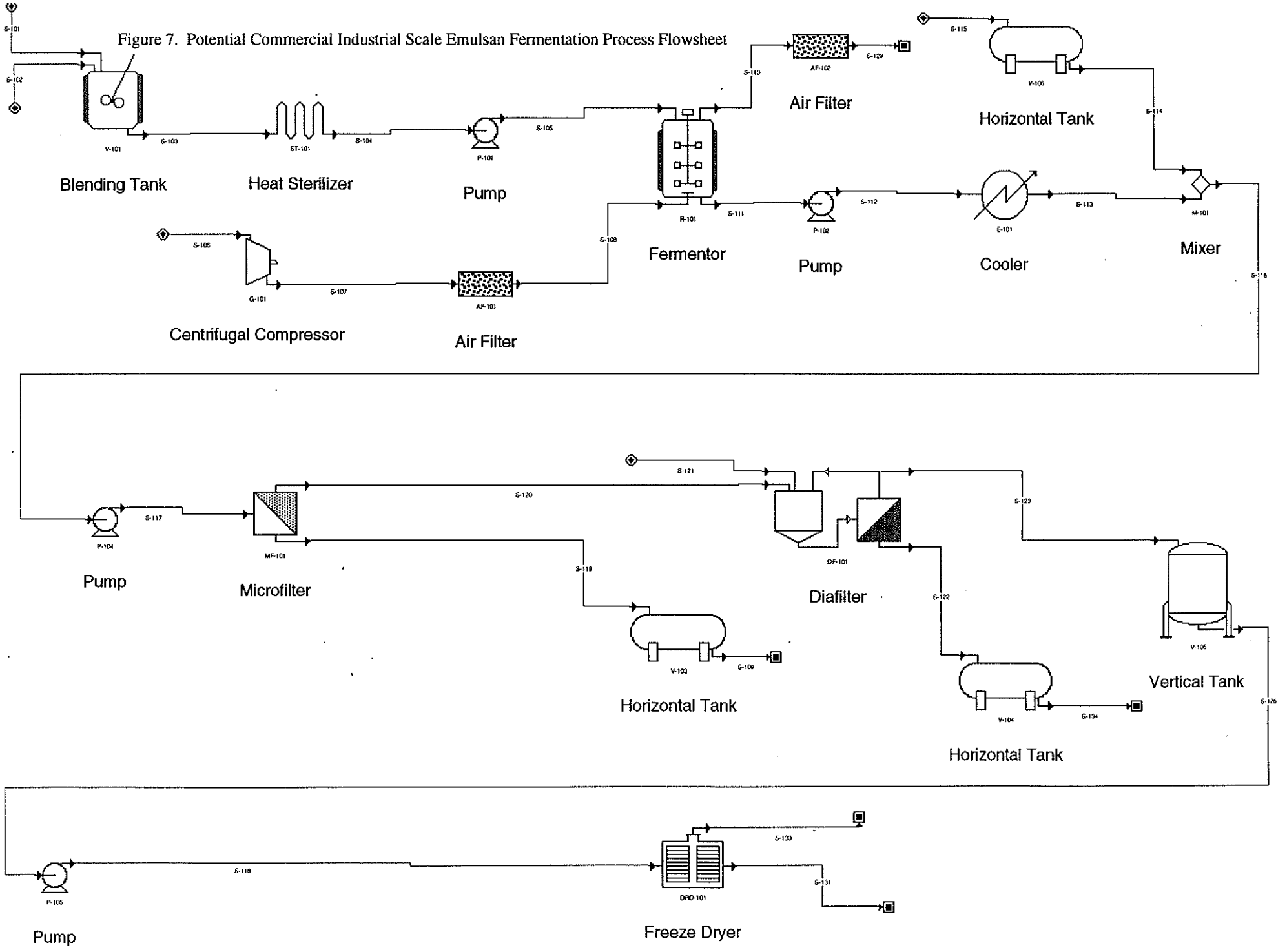
## 4.1 PROCESS DESIGN

In the process design, three fermentation volumes scales (10,000 liters, 100,000 liters and 1,000,000 liters) and three emulsan concentrations (5, 25, and 50 g/L) were considered. These emulsan concentrations were based on a crude yield of 5 g/L, obtained from a simple batch fermentation, 25 g/L obtained by a fed-batch fermentation, 50 g/L based on the yields obtained for other biopolymers, and the potential to obtain this yield for emulsan by developing a high producing strain through genetic mutations.[43, 44] Figure 7 shows the process flowsheet for a potential commercial industrial scale fermentation process for emulsan.

[43]Shabtai, "Production of Emulsan in a Fermentation Process Using Soybean Oil."

[44]Georgiou, "Surface-Active Compounds From Microorganisms," 60-64.

Figure 7. Potential Commercial Industrial Scale Emulsan Fermentation Process Flowsheet



Raw materials and river or municipal water enter a blending tank, are mixed and sent through a continuous flow sterilizer to a fermentor. The fermentor is inoculated with a seed culture to a final v/v concentration of 5% and begins in a batch mode. The fermentor is switched to a fed-batch mode after five hours when the carbon source is intermittently fed to the fermentor to maintain a concentration of 40 g/L, and run for an additional 55 hours. The addition of the carbon source is stopped near the end of the fermentation to allow the cells to deplete the carbon source level to a minimum. The whole fermentation broth is then cooled through a chiller before being pumped through a 0.33 $\mu$m microfilter to remove the cell mass. The supernatant containing the emulsan, residual salts, and residual carbon source is then pumped to a 30kd ultrafilter where it is diafiltered to remove the salts and concentrated to 15 g/L emulsan. The aqueous phase is then lyophilized to recover the emulsan. Unless the application needs FDA approval, the residual carbon source does not need removal, as it is only 0.05-0.1% of the mass of emulsan.

Process scale-up: BPRC Lab to 30 L scale at Natick Army Research Labs

The scale-up of emulsan production from 2 L quantities at the BPRC lab to 30 L quantities at the Natick Army Research Labs forms the basis for scale-up to the commercial process. The processes are essentially the same except for scale differences. The major difference is that purification steps in the lab scale are done batchwise, while the purification steps in the commercial scale are semi-continuous. Another unit operation that is distinctly different is the method for the removal of the residual carbon source if such removal is necessary. In the 30 L scale, residual carbon source is removed from the lyophilized powder via reflux in a soxhlet apparatus. This would not be practical on a large-scale commercial process, nor necessary. A commercial process would require a piece of equipment capable of operating in a semi-continuous mode such as a differential extractor to remove the residual carbon source. A differential extractor would require the use of a solvent such as pentane or ether.

Carbon Source and Emulsification Properties

The carbon source used in the scale-up work was an 80/20 mix of methyl oleate and oleic acid. This mix was selected because emulsan produced using this carbon source had improved emulsification properties compared to emulsan formed on other feed sources. This is based on earlier BPRC lab-scale results of turbidity measurements on samples of emulsan, hexadecane, water, and a buffer. For a commercial process, methyl myristate is considered optimal because it has the best combination of emulsan yield and emulsification properties. Another alternative that has been used for lab scale preparations is soap stock oil (SSO) and soybean oil (SBO)[45] and is available as a cheap commercial commodity. Depending on the desired performance properties of the emulsan, different carbon sources could be used. Using a more expensive carbon source such as methyl oleate/oleic acid or methyl myristate as opposed to SSO or SBO would not have a significant impact on the cost of emulsan per gram.

[45]Shabtai, "Production of Emulsan in a Fermentation Process Using Soybean Oil."

## 4.2 PROCESS OPTIMIZATION

Carbon Source Extraction Options

Two options for the removal of the residual carbon source were considered before the lyophilization step. The first is extraction in a counter-current column using an organic solvent such as pentane. First, the aqueous retentate stream leaving the ultrafiltration unit containing emulsan, residual carbon source and water is pumped to a holding tank where it is acidified with hydrochloric acid to pH 2.0. Emulsan is stable at this low pH for the period of time required. At this pH, emulsan does not emulsify the carbon source and the extraction is facilitated. This stream enters the top of a differential extractor as the heavy phase and pentane enters the bottom as the light phase. The flow rate of the pentane depends on the solubility of the carbon source in pentane and the corresponding solvent-to-feed ratio required to extract the carbon source with a given size extractor. The heavy stream exiting the extractor contains only emulsan and water and is pumped to a holding tank where the pH is adjusted to neutral. The emulsan purification is finished by lyophilizing the water/emulsan mixture.

The second option for removing the residual carbon source is using a supercritical fluid extraction (SCF) process. This is a "green" process, eliminating the introduction of any organic solvents and the accumulation of organic waste. This process is similar to the previous solvent extraction, except the pentane is replaced by supercritical carbon dioxide and there is no pH adjustment. In the supercritical state, carbon dioxide is a good solvent for lipids and fatty acids. Again, the flow rate of the supercritical $CO_2$ depends on the solubility of the carbon source. The extraction is carried out in a continuous process where the supercritical $CO_2$ is recycled and the carbon source is separated out and can be reused. The SCF method incurs a larger capital cost compared to the pentane-based system but $CO_2$ is a cheaper solvent and is a viable, clean process.

Fermentation Options

As discussed in Chapter 3, a number of different carbon sources can be used for the fermentation. Ethanol was first used, later SBO and commercially available SSO were used because of their higher emulsan yield than ethanol.[46, 47] At BPRC under the direction of Dr. Richard Gross, Dr. Alexander Gorkovenko and doctoral candidate Jin-Wen Zhang have evaluated the emulsification properties of emulsan produced utilizing a number of fatty acid sources in the 2 L fermentor. The two most promising candidates are methyl myristate and a methyl oleate/oleic acid mixture. Of the two, the methyl oleate/oleic acid mix seems to have somewhat superior emulsification performance and methyl myristate has a higher emulsan yield. Both have a higher yield than ethanol. (Note that these results were not replicated in the 30 L fermentations). Despite the fact that the choice of carbon source can

[46]S. Goldman, C. Shabtai, C. Rubinovitz, E. Rosenburg and D. L. Gutnick, "Emulsan in *Acinetobacter calcoaceticus* RAG-1: Distribution of Cell-Free and Cell-Associated Cross Reacting Material," *Applied and Environmental Microbiology*, Vol. 44, No. 1 (July 1982.)

[47]Shabtai, "Production of Emulsan in a Fermentation Process."

result in an increase of a few cents per gram of emulsan (which can be commercially significant), it was decided that at this stage of product development emulsification properties should be a primary consideration in the choice of carbon source.

The two fermentation strategies studied were batch and fed-batch. The batch fermentation yield was limited by the initial concentration of the carbon source. The fed-batch mode employed the addition of carbon source and nitrogen source for the duration of the fermentation, producing three times the emulsan concentration as the batch culture. On this basis, the fed-batch mode was chosen for the design of the commercial process.

## 4.3 PRODUCTION COSTS

Three scales of commercial production of emulsan were evaluated to determine the effect of scale on the price of emulsan, a 10,000, a 100,000, a 1,000,000 L plant with each of three different emulsan concentrations (5, 25, and 50 g/L). The plant design and economic analysis was performed using the design software BioPro Designer® from Intelligen Inc., and was verified by hand calculations. The calculations were based on a new, free standing facility and the selling price was based on an ROI of 30%. Table 6 shows the selling price of emulsan for the three yields and production scales. It should be noted that these results were obtained for the production of emulsan at a new facility. It is common in the biotechnology industry for small, emerging firms to contract with another company who supplies fermentation (and possibly purification) services.

As the table and graph indicate, the scale-up has a more significant effect on the selling price of the emulsan from the 10,000 L to the 100,000 L scale than from the 100,000 L to the 1,000,000 L scale. Between the 10,000 and 100,000 L scale, there was a decrease of 56% at a yield of 5 g/L, 47% at a yield of 25 g/L and 36% at a yield of 50 g/L. The decrease in price from the 100,000 liter scale to the 1,000,000 liter scale was 13% at a yield of 5 g/L, 11% at a yield of 25 g/L and the price remained the same at a yield of 50 g/L.

At 10,000 L and below, the largest single component cost is labor. The labor costs do not increase proportionately with plant size because labor costs are activity-based not volume-based. As plant size increases, equipment cost quickly becomes the largest cost component. However, equipment that produces ten times as much does not cost ten times as much. This is known as the economy of scale. Once components reach the maximum size available or constructable, to produce more volume, more components of the maximum size are added together. Here, doubling the capacity doubles the equipment cost resulting in no economy of scale. This is essentially what is occurring in going from 100,000 L to 1,000,000 L.

The purification equipment is the largest fraction of the equipment cost. Below the solubility limit of emulsan, about 15 g/L, an increase in emulsan yield (per liter) lowers the per gram processing cost. This is because the same size equipment can process more product. Above the solubility limit, however, increased yields for the same fermentation equipment requires larger purification equipment

to remain below the emulsan solubility limit. This explains the relationship between yield and per gram costs.

Based on the results shown in Table 6, the optimum plant size is about 100,000 L at a yield of 25 g/L. There are diminishing returns beyond this point.

Table 6. Selling Price of Emulsan for Different Production Scales and Emulsan Yields

| Scale (liters) | $/g<br>5 g/L yield | $/g<br>25g/L yield | $/g<br>50g/L yield |
|---|---|---|---|
| 10,000 | 0.68 | 0.17 | 0.11 |
| 100,000 | 0.30 | 0.09 | 0.07 |
| 1,000,000 | 0.26 | 0.08 | 0.07 |

Results obtained from BioPro®

## 4.4 WASTE GENERATION

The wastes generated from the fermentation process are the cell mass, salts, and organic solvent in the case of the organic solvent extraction step. From an environmental, health and safety standpoint, the extraction process of choice would be the SCF process as opposed to the organic solvent process. This would eliminate the need of organic solvent disposal, which is costly. The cell mass waste and salts can be discharged to a municipal wastewater treatment facility. The cell mass must be killed first by sterilization or with bleach and can then be discharged. The salts must be below a specified concentration (usually between 50,000 to 100,000 ppm at neutral pH) prior to discharge. In the purification step, the volume of water required to diafilter is so large that this concentration is easily achieved. The cost of wastewater disposal is estimated at $\$0.15\text{-}0.30/m^3$ and is incorporated into the total utility cost.

# CHAPTER 5 ENVIRONMENTAL, HEALTH, SAFETY AND REGULATORY CONSIDERATIONS FOR COMMERCIAL BIOSURFACTANT PRODUCTION

## 5.0 INTRODUCTION

In the process of developing and commercializing a biosynthesized product, companies must consider many different issues. Two important issues are compliance with applicable environmental regulations and potential environmental and worker effects of the process and final product. Failure to examine both these issues during the development stage of a biosynthesized chemical could be costly from both the business and environmental standpoints.

The first section of this chapter outlines the environmental and worker health and safety regulations applicable to the development and production of biosynthesized chemicals. The section begins by describing the general regulations involving biosynthetics and concludes with a discussion of how those regulations relate to the production and use of emulsan. The second section examines the potential effects of the development process and product on the environment and worker health and safety, describes ways to reduce those effects, and addresses the specific issues involved with emulsan production and use.

## 5.1 REGULATION OF BIOPOLYMERS

The regulations that potentially affect the development and production of biosurfactants such as emulsan and sophorolipid include:

- 1986 Coordinated Framework for Regulation of Biotechnology (Coordinated Framework)–describes the functions of the federal agencies involved in regulating biotechnology;
- Toxic Substances Control Act (TSCA)–regulates the biosynthesized chemical and microbes used in its production;
- Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)–regulates the use and safety of pesticide products;
- Occupational Safety and Health Act (OSHA)–regulates hazards in the workplace;
- Resource Recovery and Conservation Act (RCRA)–regulates both production and disposal/treatment processes;
- Emergency Planning and Community Right-to-Know Act (EPCRA)–provides for emergency planning, community right-to-know reporting and toxic chemical release reporting;
- Clean Air Act (CAA)–regulates air emissions;
- Clean Water Act (CWA)–regulates effluents to the Nation's waters; and

How much the regulations affect the development and production of biosurfactants depends upon whether the bacteria involved are intrageneric or intergeneric. Intrageneric bacteria consist of one bacterial strain while intergeneric bacteria contain genetic information from dissimilar source organisms. The bacteria used in producing emulsan and sophorolipid are intrageneric and are not subject to as many environmental regulations as intergeneric microorganisms. However, because development and production of alternative biosurfactant technologies may involve intergeneric as well as intrageneric microorganisms, this section describes the regulations relating to both intrageneric and intergeneric microorganisms.

The 1986 Coordinated Framework for Regulation of Biotechnology

The main piece of U.S. legislation regulating the use of biotechnology is the 1986 Coordinated Framework for Regulation of Biotechnology (Coordinated Framework).[48] The Coordinated Framework was the first attempt of the federal government to coordinate the policy of agencies involved in the review of biotechnology research and products. According to the policy, existing statutes are sufficient to adequately regulate the development and production of biotechnology products and provide a network of agency jurisdiction.[49] The Coordinated Framework describes the functions of the federal agencies involved in regulating biotechnology.

The agencies involved include the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), the Occupational Safety and Health Administration (OSHA), and the U.S. Department of Agriculture (USDA). EPA has jurisdiction over environmental emissions, waste, and other reporting. Whether or not other agencies also have jurisdiction depends on how the product is used. For example, the EPA has jurisdiction over industrial uses. If the product is used in cosmetics, it will be subject to FDA regulations.

Interagency coordination of biotechnology is achieved through a Domestic Policy Council Working Group on Biotechnology and the Biotechnology Science Coordinating Committee (BSCC). The Domestic Policy Council is responsible for the coordinated framework for the regulation of biotechnology and agency jurisdiction. The BSCC is responsible for the coordination and consistency

[48]The White House Office of Science and Technology issued its final policy statement on federal oversight for biotechnology-derived products in 1992. The policy concludes that "products should not be subjected to heightened scrutiny merely because they were created by new processes which utilize biotechnology, but rather should be scrutinized on the basis of the risks posed to human health and safety or the environment." This policy statement follows the basic themes present in the 1986 Coordinated Framework. (S. Pierce and J. Ortego, "Recent developments in biotechnology regulation," *Toxicology and Environmental Chemistry*, Vol. 40 (1993).)

[49]Office of Science and Technology Policy (OSTP). "Coordinated framework for regulation of Biotechnology; announcement of policy and notice for public comment," 51 *Federal Register*, No. 123 (June 26, 1996): 23302-23350.

of scientific policy and scientific reviews. Among the most important roles of the BSCC is the development of definitions for a common scientific approach to the Coordinated Framework.[50]

EPA requirements under the Coordinated Framework

EPA operates its biotechnology programs under the provisions of the "Statement of Policy: Microbial Products Subject to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substance Control Act" which forms part of the 1986 Coordinated Framework. EPA regulation under FIFRA applies to pesticide products produced through biotechnology methods. Microorganisms used for applications such as waste degradation (i.e., bioremediation), chemical production, conversion of biomass to energy, and other environmental and industrial uses are subject to TSCA. Microorganisms used as foods, drugs, cosmetics, or medical devices (or used as intermediaries in the production of these) are excluded from review under TSCA. Chemicals produced by microorganisms are subject to the same requirements and procedures as chemicals produced by other means.

According to the 1986 EPA Statement of Policy, TSCA provides sufficient authority for the agency to meet its goals and responsibilities in regulating the products of biotechnology.[51] Microorganisms are considered "chemical substances" under TSCA Section 3 and are subject to TSCA authority. TSCA regulates commercialization of industrial chemical substances, including testing and reporting requirements. TSCA biotechnology regulations are focused on intergeneric and pathogenic[52] microorganisms. Naturally occurring microorganisms as well as engineered organisms from similar source organisms (intrageneric organisms) are exempt from the majority of TSCA requirements. Emulsan and sophorolipid are produced by intrageneric organisms and therefore are exempt from the majority of TSCA requirements.[53]

Biosynthesized chemicals are subject to the same TSCA requirements as any other chemical substance. As defined in the 1986 Policy, the main TSCA requirements regarding the use of microorganisms in biotechnology are as follows:

---

[50]The two BSCC definitions relevant to the regulation of biotechnology and biotechnology products are those of "pathogen" and "intergeneric (new) organism." These are described in more detail in Appendix A.

[51]Office of Science and Technology Policy (OSTP), "Coordinated Framework."

[52]A *pathogen* is a virus or microorganism that has the ability to cause disease in other living organisms. (See Appendix A for a more complete definition.)

[53]In 1994, the EPA issued a proposal on "Microbial Products of Biotechnology" to consolidate requirements and procedures applicable to intergeneric organisms. The 1994 proposed rule will begin to codify EPA policy, but may not be finalized for several years.

1. Any chemical substance not already on the TSCA Inventory of Chemical Substances is considered new under TSCA section 5(a)[54] and is subject to Pre-manufacture Notification Requirements (PMN) under TSCA Section 5. Section 5(d)(1)(A) of TSCA specifies the information that should be included in a PMN submission. This information includes information identifying the microorganism and data on the risks to human health and the environment, given the organism's intended use. Using the information provided in the PMNs, EPA evaluates the risks and benefits of the new chemical substance and determines whether or not the manufacture and use of that substance may present an unreasonable risk to human health or the environment, necessitating further regulation.

   Persons intending to manufacture a new chemical substance must submit a PMN at least 90 days before manufacture and processing. EPA has 90 days to review the submission, request additional information, and issue any controls on the manufacture of the microorganism. Upon receiving a notification of intent to manufacture from the manufacturer (provided that EPA has completed its review), EPA will place the microorganism on the TSCA Inventory of Chemical Substances. Once on the Inventory, future users of the same microorganism are not subject to TSCA Section 5 PMN requirements.

2. Microorganisms other than intergeneric combinations that are subject to TSCA and are pathogenic will, if released into the environment, be subject to Significant New Use Reporting (SNUR) requirements under TSCA 5(a)(2). Persons subject to a SNUR must comply with most of the same notice requirements and regulatory procedures as submiters of PMNs. They must submit a significant new use notification 90 days prior to new uses involving environmental applications. EPA has yet to develop significant new use rules for microorganisms and is requesting that companies file information on a voluntary basis.

3. Under section 8(e) of TSCA, EPA must be notified by manufacturers, processors, or distributors of microorganisms immediately if they become aware of any new information that suggests the microorganisms present a substantial risk of injury to human health or the environment.[55]

The TSCA exemption for testing of new chemical substances accorded to other research and development activities will not be applicable to research and development of intergeneric organisms

---

[54]Microorganisms found in nature are implicitly included on the TSCA Inventory.

[55]Under section 8(a) of TSCA EPA had planned to require that manufacturers of microorganisms, if not otherwise subject to review, submit general environmental and worker health and safety data before environmental release. The EPA would have used this information to monitor environmental uses of the microorganisms and to determine if additional requirements are necessary in the future. EPA would also have used this information to monitor the progress of its biotechnology program and gain a better understanding of how microorganisms are being used. However, EPA has indicated that this rule will not be promulgated. (Giamporcaro, D., Section Chief, TSCA Biotechnology Program, U.S. EPA, Personal communications, August 29 and October 17, 1995.)

released into the environment. Thus, companies developing new microorganisms for commercial purposes will be required to notify EPA prior to environmental releases. In the meantime, EPA asks for voluntary compliance with the PMN requirements by researchers using and releasing to the environment new microorganisms in research and development activities. Intergeneric microorganisms used solely in contained systems (e.g., for research and development) and never intentionally released to the environment are exempted from PMN requirements.

Proposed Rule on Microbial Products of Biotechnology

In 1994, EPA issued a proposed rule to finalize and codify its policy relating to microorganisms. Due to the differences between microorganisms and other chemical substances, EPA is proposing to establish a new part 725 to the Code of Federal Regulations to consolidate all requirements and procedures applicable to new microorganisms.[56] In the rule, EPA plans to maintain oversight of intergeneric microorganisms as "new chemical substances," subject to TSCA Section 5. Non-pathogenic intrageneric microorganisms are not covered under the proposed rule. Reporting requirements for new microorganisms would be similar to those outlined in the previous section.

Companies intending to manufacture or process intergeneric microorganisms for commercial purposes must submit a Microbial Commercial Activity Notice (MCAN) to EPA at least 90 days before manufacture or processing.[57] Pathogenic microorganisms subject to significant new use rules would also be required to file a MCAN. Fees for filing the MCAN are: $100 for small businesses (annual sales less than $40 million) and $2,500 for all others. No other reporting fees are proposed.

In the proposed rule, EPA proposes several exemptions from reporting under Section 5 of TSCA in the case of test marketing or no significant risk. EPA proposes tiered reporting exemptions for specific recipient microorganisms, under certain conditions, as well as exemptions in the case of well-characterized genetic material or contained production processes. The proposed rule also covers issues relating to confidential business information claims.

Other Regulations Applicable to the Manufacture of Biosynthesized Chemicals

*Occupational Safety and Health Act (OSHA).* In the 1986 Coordinated Framework, OSHA determined that its general duty clause, together with several specific standards, provides an adequate and enforceable basis for the protection of the safety and health of employees in the field of biotechnology. OSHA concluded that there is no need for new regulations and that the laws that regulate chemical exposures will usually ensure that biohazards will be controlled.[58]

---

[56]U.S. Environmental Protection Agency, "Microbial products of biotechnology; Proposed regulation under the Toxic Substances Control Act; proposed rule," 59 *Federal Register*, No. 169 (September 1, 1994): 45526-45585.

[57]The MCAN is the same as a Premanufacture Notification.

[58]Office of Science and Technology Policy (OSTP), "Coordinated Framework."

The general duty clause (Section 5(a)(1) of OSHA requires that "each employer furnish to each of his employees employment and a place of employment which are free from recognized hazards that are causing or are likely to cause death or serious physical harm." The OSHA general duty clause, as well as other components of the Act, provides adequate protection for workers in biotechnology research, development, and production.[59] OSHA maintains authority to develop new standards if new biotechnology processes cause hazardous working conditions that result in a significant risk of death or harm to workers. For example, OSHA issued a standard for blood-born pathogens, mainly directed toward biomedical workers. Also, the OSHA standard on Laboratory Safety Procedures establishes, on a federal level, many of the safeguards recommended by the National Institutes of Health in its Laboratory Practices Guidelines.[60] Thus, there are no additional OSHA requirements for companies and institutions developing or producing biosynthesized chemicals.

*Resource Recovery and Conservation Act.* RCRA provides a cradle-to-grave approach to the regulation of hazardous wastes. The Act established a system for identifying and listing hazardous wastes, standards for generators and transporters of hazardous wastes, and a permit system to enforce these standards. RCRA Subtitle C requires EPA to develop criteria for determining what is a hazardous waste, as well as to establish record keeping requirements and a manifest system to be used to track shipments of hazardous waste from point of generation.

If the manufacture of a biopolymer results in the generation of hazardous waste, the generator is required under RCRA (40 CFR 262.10) to: (1) maintain record keeping to accurately identify quantities of such hazardous waste generated and constituents of the waste; (2) properly label containers used for storage, transport or disposal of waste; (3) use appropriate containers for hazardous waste; (4) furnish information on composition of wastes to transport, treatment, or disposal facilities; (5) use a manifest system to ensure that all hazardous waste generated is designated for treatment, storage, or disposal and arrives at properly permitted facilities; (6) submit reports to EPA or the state agency on quantities of waste generated, disposition, and efforts undertaken to reduce the volume of wastes. RCRA contains streamlined reporting requirements for small quantity generators, producing less than 1,000 kg during a calendar month.

*Emergency Planning and Community Right-to-Know Act.* Under EPCRA, companies using specific listed chemicals over threshold amounts must report: (1) information on chemical inventories (section 311 and 312) for extremely hazardous substances (EHS); and (2) information on environmental releases and treatment and disposal quantities (section 313). Companies must also provide a Materials Data Safety Sheet to local emergency authorities, as well as prepare emergency plans and comply with release notification requirements. These requirements would potentially apply to some of the reagents used in the production of emulsan.

---

[59]Office of Science and Technology Policy (OSTP), "Coordinated Framework."

[60]Frantz, et al, "Toxics Use in Biotechnology: Capstone III Project," Medford, Mass.: Tufts University, School of Engineering, 1992.

*Clean Air Act.* Section 112 of the CAA established the National Emission Standards for Hazardous Air Pollutants, designed to protect humans against pollutants which can cause serious illness or death. Biotechnology firms would likely be considered "area sources" under this section and subject to negotiable technology-based standards. Sections 182 and 183 of the Act establish control technologies for stationary sources of VOCs. The CAA requirements would apply specifically to the reagents used in the production of emulsan and other biosynthesized chemicals.

*Clean Water Act.* The CWA regulates waste discharges to waterways, both to public treatment works and directly to receiving waters. In the case of direct discharges to waterways, the Act establishes technology-based effluent limitations for classes of toxic pollutants by different industrial manufacturers. Effluent limitations and water-quality based discharge standards designed to protect a receiving water body are incorporated into a facility's discharge permit, administered under the National Pollutant Discharge Elimination System. The CWA requirements would apply specifically to the reagents used in the production of emulsan and other biosynthesized chemicals.

Regulatory Analysis of Production of Emulsan

Emulsan is produced through a biosynthesis process involving the non-pathogenic, naturally occurring, gram negative bacterium *Acinetobacter calcoaceticus* strain RAG-1. *A. calcoaceticus* is an intrageneric microorganism and, as such, is not subject to Premanufacture Notification requirements under TSCA section 5. The bacterium can be considered a "chemical substance" under TSCA section 3 and, as such, the manufacturer is required to comply with TSCA section 8(e) reporting in the case of new substantial hazard information.

The final chemical product, emulsan, is subject to TSCA PMN reporting requirements. Emulsan has already been produced commercially and is likely to be already on the TSCA Inventory.[61] As such, there are no additional filing requirements for subsequent emulsan manufacturers. The only exception might be a significant chemical change in emulsan or significant differences in the microorganism used.

Since the production of emulsan produces biological wastes, disposal of those wastes would be subject to RCRA regulations. Similarly, the producer of emulsan would be subject to the strict and several liability provisions under the CERCLA in the event the final disposal site of the manufacturer's (or development institution's) wastes is listed on the National Priority List or state priority list and the wastes contain listed chemicals. In the event the company uses an EHS chemical or one included under Section 313 (e.g., the solvents used for extraction), the manufacturer of emulsan will be required to comply with EPCRA requirements. In the case of larger production volumes of emulsan, the manufacturer may be subject to permit requirements under both the Clean Air Act and Clean Water Act.

---

[61]The manufacturer of Emulsan™ Brand Biopolymer, Emulsan Biotechnologies, Inc. indicated that it has already submitted a PMN under TSCA section 5. However, emulsan was not found when checking the 1995 CD-Rom version of the TSCA Inventory.

Side chain manipulations to the microorganism used to produce emulsan may require a new product PMN. EPA determines the need for a new PMN based on changes to the function of the product. If there are no changes to the function of the product, a PMN will not likely be required. EPA has no policy for these determinations and makes them on a case-by-case basis using previous determinations.[62]

## 5.2 ENVIRONMENTAL AND HEALTH ASPECTS OF BIOSYNTHESIZED CHEMICALS

This section addresses potential worker and environmental hazards caused by the production of biosynthesized chemicals. It begins with a discussion of the potential exposure routes for biosynthesized chemicals. Next, the general effects of biological agents on worker and environmental health are outlined followed by a discussion of ways to reduce those effects. Finally, the potential environmental, public and occupational health effects of emulsan and *A. calcoaceticus* are discussed.

Potential Routes of Exposure

When compared to chemical and physical agents, hazardous agents of biological origin (infectious microorganisms, biological allergens and toxins) are less well known and defined.[63] In testimony on OSHA's policy statement on biotechnology contained in the Coordinated Framework, the Director of the National Institute of Occupational Safety and Health (NIOSH) stated, "we believe that this industry presents an uncommon work environment where the potential hazards are not recognized."[64] NIOSH indicated the need for research into potential worker exposures in the biotechnology industry, which OTA has estimated will employ 80,000 to 200,000 individuals in the U.S. by the year 2000.[65] There is a lack of documented studies on the health risks of working with microorganisms.[66] Similarly, other studies have indicated that "little research has been done concerning the potential environmental impacts of biopolymers that are used in large quantities. It is possible that the widespread use of some biopolymers may have unanticipated effects on ecosystems or waste

---

[62]D. Giamporcaro, Personal communications.

[63]Dutkiewicz, et. al., "Occupational Biohazards: A Review," *American Journal of Industrial Medicine*, Vol. 14 (1988): 605-623.

[64]J.D. Millar, NIOSH comments on OSHA guidelines on biotechnology, Docket number H-042, Cincinnati: National Institute for Occupational Safety and Health, 1985.

[65]L. Elliott, et. al., "Perspectives on Opportunities Toward a Hazard-Free Bioprocessing Environment," In W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations* (Philadelphia: American Society for Testing and Materials, 1990) 20-26.

[66]K. Martinez, et al., "General Considerations for Work Practices and Personal Protective Equipment in Biotechnology Industries," In W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations* (Philadelphia: American Society for Testing and Materials, 1990): 83-90.

streams."[67] Nonetheless, a WHO working group concluded that "at present, biotechnology appears to possess no risks that are fundamentally different from those faced by workers in other processing industries."[68]

The methodologies used to identify health and safety risks in the field of biotechnology are in their early stages. Difficulties exist in defining the biologic agents that cause occupational disease among workers in the biotechnology industry. Exposures are not always due to the primary dissemination of a microorganism but may be due to accessory processes.[69] For example, natural mutations can occur during production processes, converting a previously harmless bacteria into a pathogenic or toxic one.[70] Also, environmental conditions may activate a bacteria or potentiate its actions. Finally, there has been little research on the exposure levels which cause pathogenic or immunologic effects in workers. For this reason, it is presently difficult to define exposure thresholds for biotechnology workers.

A NIOSH funded study conducted by Landrigan, et. al. identified three potential hazards of biotechnology: microbial hazards, product hazards and reagent hazards.[71,72] Hazards will vary based on the microorganism, product, and chemicals used. Thus, it is imperative to consider the hazards of each biotechnology production process on a case-by-case basis.

Potential exposures to microorganisms occur during seed growth (transfer of stock cultures and analysis of broth samples), fermentation (culture broth sampling, exhaust gas), product recovery (releasing the filter cake from filters), and disposal of biowastes. Exposure mainly occurs through inhalation, due to the aerosol-generating potential of industrial processes. Studies have shown that while some microorganisms may live short periods of time in aerosols, they can live in moist dust and similar environments for weeks. Other microorganisms are more resistant and can be carried for large distances without drying. Therefore, once a microorganism is released in aerosol form within a building, in theory it can spread as a viable organism to all locations in the building and to the

---

[67]U.S. Congress, Office of Technology Assessment, *Biopolymers: Making Materials Nature's Way.*

[68]United Nations Industrial Development Organization, *An International Approach to Biotechnology Safety.* (Vienna: UNIDO, 1990).

[69]Dutkiewicz, "Occupational Biohazards: A Review."

[70]Andrup, L, "Biosafety Considerations in Industries with Production Methods Based on the Use of Recombinant Deoxyribonucleic Acid," *Scandinavian Journal of Work Environment Health*, Vol. 16 (1990): 85-95.

[71]Landrigan, P., et. al.,"Medical surveillance of biotechnology workers: Report of the CDC/NIOSH Working Group on Medical Surveillance for Industrial Applications of Biotechnology", *Recombinant DNA Technical Bulletin*, Vol. 5, No. 3 (publication no 82-99), U.S. Department of Health and Human Services, National Institutes of Health, 1982.

[72]Elliott, "Perspectives on Opportunities Toward a Hazard-Free Bioprocessing Environment."

exterior.[73] However, experts generally agree that given strict physical containment of microorganisms in the biotechnology industry and conditions necessary for the microorganism to cause illness in a worker, the risks of adverse pathogenic impacts are generally low.[74,75]

The second set of hazards faced by workers in the biotechnology industry are product hazards. These hazards occur due to worker exposure to biological and chemical products of biotechnology. Biotechnology product hazards (toxicity, reactivity) in many cases do not differ significantly from those encountered in synthetic chemical production. The hazards of product exposure extend throughout the full manufacturing process from initial production to final processing. However, because of the physiologically reactive nature of many biological products, especially in the pharmaceutical industry, exposures to even minute quantities may cause medically significant effects.[76] These exposures may result in immunologic sensitization or the formation of endotoxins.[77]

The third set of hazards are those relating to the chemical reagents used in the extraction, separation and purification of microbial products, as well as equipment sterilization. Biotechnology processes generally use solvents (phenol, toluene), buffers (sodium hydroxide, magnesium chloride), and disinfectants (halogens). Exposures to these reagents may occur during transfer from one container to another, from noncontained or unventilated product extraction processes, or though spills or breakage.[78] Larger scale operations increase the probability of all three types of chemical exposures, especially as a result of spills.

A final potential environmental and worker hazard relates to the disposal of biowastes. Large-scale fermentation operations will produce large quantities of biowastes which can include reagents and microorganisms.[79] Biowastes can pose a hazard to workers and the environment through handling, spills and final disposal.

Review of Potential Worker and Environmental Impacts of Biological Agents

Occupational biohazards may act as infectious, allergenic, toxic, or carcinogenic agents in humans. They generally cause strong specific or nonspecific stimulation in immunological response in exposed

---

[73]Andrup,"Biosafety Considerations."

[74]Landrigan, "Medical Surveillance of Biotechnology Workers."

[75]Andrup, "Biosafety considerations."

[76]Landrigan, "Medical Surveillance of Biotechnology Workers."

[77]An endotoxin is a toxin produced by a microorganism and released upon the destruction of the cell in which it is produced.

[78]Elliott, "Perspectives on Opportunities Toward a Hazard-Free Bioprocessing Environment."

[79]UNIDO, *An International Approach to Biotechnology Safety.*

persons, which normally results in "allergic" reactions.[80] Some of the potential health effects resulting from exposure to biologic agents of biotechnology include:

1. *Immunologic sensitization to microorganisms, their components, or metabolic products.* Immunologic sensitization can lead to immune diseases such as hypersensitivity pneumonitis (extrinsic allergic alveolitis) and bronchial asthma. In addition, dermatitis and allergic rhinitis might also be expected to occur.[81]

2. *Infectious and pathogenic reactions.* Pathogenic bacteria that are inhaled into the lungs can produce symptoms ranging from fever and pneumonia. Pathogenic bacteria can produce systemic disease and involve multiple organs, eventually causing death in the exposed individual.[82] Infectious and pathogenic health hazards will likely be minimal or nonexistent in the production of biosynthesized polymers.

3. *Toxic reactions.* Many bacteria can produce endotoxins from their outer cell walls during degradation. Endotoxins, characteristically present in gram-negative bacteria, can produce both allergic and toxic reactions in exposed individuals, affecting the immune system, complement and coagulation systems, and produce pulmonary symptoms.[83,84] Endotoxins mainly produce short-term, noninfectious fevers, with general malaise, shortness of breath, headache, and occasionally pulmonary edema. Though the pathology of endotoxin effects is not well understood, chronic exposure may lead to decreased lung function.[85,86]

---

[80]Dutkiewicz, "Occupational biohazards."

[81]UNIDO, *An International Approach to Biotechnology Safety.*

[82]J. Merchant, ed, *Occupational Respiratory Diseases* (Cincinnati: National Institute for Occupational Safety and Health, pub. 86-102, 1988).

[83]Andrup, "Biosafety Considerations."

[84]S. A. Olenchock, "Health effects of Biological Agents: The Role of Endotoxin," *Applied Occupational and Environmental Hygiene*, Vol. 9, No. 1 (1994): 62-64.

[85]Merchant, *Occupational Respiratory Diseases.*

[86]Olenchock, "Health Effects of Biological Agents: The Role of Endotoxin."

General Guidance for Safety in the Production of Biosynthesized Polymers

National and international organizations, such as the United Nations (World Health Organization), the Organization for Economic Cooperation and Development (OECD), and the National Institutes of Health and Centers for Disease Control (NIH, CDC) in the U.S. have established guidelines for protection of workers and the environment from the potential hazards of biotechnology processes. The standard for biotechnology safety in the United States and abroad is the CDC/NIH guide Biosafety in Microbiological and Biomedical Laboratories. This manual and the Laboratory Biosafety Manual produced by the World Health Organization, present comprehensive information on aspects of laboratory biosafety, including: (1) classification of organisms according to risk category; (2) laboratory and facility practices, (3) design of facilities, (4) protective equipment; (5) medical surveillance, (6) training, and (7) emergency procedures.[87]

With regard to facility design and practices, OECD has developed "Good Industrial Large Scale Practice (GILSP)" guidelines for environmental and occupational safety in the biotechnology industry.[88] These guidelines include the following concepts:[89]

1. Keep workplace and environmental exposure to any physical, chemical, or biological agent to a level appropriate to the characteristics of the organism, product or process.

2. Exercise engineering control measures at source and supplement with appropriate personal protective equipment.

3. Test for the presence of viable process organisms outside the process equipment. Maintain adequate physical containment procedures.

4. Ensure adequate training and experience for personnel.

5. Establish biological safety committees among management, workers and regulatory authorities to focus on safety and hazard communication.

6. Establish a code of practice for workplace and environmental safety.

Additional guidelines for safety include: identification and mitigation of biohazards; adequate air ventilation systems equipment (using high efficiency particulate air or HEPA filters); separation of fermentation area from other production areas; maintaining emergency plans; systematic preventive

---

[87]UNIDO, *An International Approach to Biotechnology Safety.*

[88]These guidelines apply to non-pathogenic microorganisms. Also, OECD has also developed guidelines for small scale processes which are very similar to those for large scale processes.

[89]Organization for Economic Co-Operation and Development, *Safety Considerations for Biotechnology* (Paris: OECD, 1992).

maintenance of the fermentation process including seals, valves, sterilization processes, and safeguards; and adequate effluent and waste treatment.[90,91]

Medical surveillance for workers involved in bioprocessing should consist of a periodic evaluation process and include the following steps:

- preemployment examination and collection of baseline serum samples (to identify any conditions which might impair the immune system as well as other chronic illnesses);[92]
- periodic follow-up examination;
- follow-up examination of all illnesses that cause absence from work of more than 48 hours;
- epidemiologic or other longer term follow-up studies.[93]

According to Landrigan, et. al., it is unlikely that a medical surveillance program will routinely detect illnesses caused by microorganism, products or reagents, due to the difficulties of predicting both the nature and time of onset of illness. However, the fact that various occupational illnesses have been attributed to exposure to microorganisms and products of production processes involving microorganisms warrants the need for medical surveillance.[94]

Worker and Environmental Hazards Associated with the Production of Emulsan

*Bacterium Acinetobacter calcoaceticus* According to its manufacturer, *A. calcoaceticus* is a non-pathogenic bacterium. Through an in-depth literature search, Dutkiewicz, et. al. described the hazards associated with 83 biological agents. *A. calcoaceticus* was identified as a hazard among animal product processors and foundry workers. According to the study, *A. calcoaceticus* causes allergic reactions and produces respirable endotoxin. Exposure occurs mainly through organic dusts or droplet aerosols. Chronic exposure to metallic dust has been recognized as a factor predisposing foundry workers to pulmonary infection with *A. calcoaceticus*.[95] The health effects discussed in the literature address *A. calcoaceticus*. There is no literature available on the health effects specific to the strain RAG-1.

---

[90]Elliott, "Perspectives on Opportunities Toward a Hazard-free Bioprocessing Environment."

[91]S. P. Vranch, "Containment and regulations for safe biotechnology", In W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations* (Philadelphia: American Society for Testing and Materials, 1990): 39-57.

[92]Environmental sampling for microorganisms and chemical agents can be a less costly mechanism for monitoring environmental conditions, sanitation, and control systems.

[93]Landrigan, "Medical Surveillance of Biotechnology Workers."

[94]Elliott, "Perspectives on Opportunities Toward a Hazard-Free Bioprocessing Environment."

[95]Dutkiewicz, "Occupational Biohazards."

*Emulsan™ Brand Biopolymer* According to the Material Safety Data Sheet (MSDS) prepared by its manufacturer, Emulsan™ Brand Biopolymer poses no physical or health hazards. Emulsan™ Brand Biopolymer has an $LD_{50}$ of >5grams/Kg. of body weight in rates.[96] The following possible health effects are noted in the MSDS: slight irritation in the case of eye contact; slight membrane irritation in the case of inhalation contact; and slight erythema and slight edema in the case of skin contact. The MSDS notes no chronic effects associated with the biopolymer. The most important hazard identified for Emulsan™ Brand Biopolymer, when sold in powdered form, is ignition hazard in the case of contact with static electricity.[97]

*Reagents* The handling of reagents may pose some of the greatest hazards in the production of emulsan. Possible chemical exposures can occur during trace metal solution preparation, medium preparation for seed culture, inoculation, extraction, and sterilization. Hazards associated with the chemicals used in these preparations are well known and relevant safety precautions should be taken to reduce exposures to the extent possible. Some of the potential reagent hazards in the preparation of emulsan include:[98]

*Extraction chemicals*: Ether, used in laboratory extraction, is a powerful narcotic which can cause death in high doses. Effects of ether are usually acute and seldom chronic. Continued exposure to small quantities of ether causes loss of appetite and fatigue. According to the literature, pentane, used in larger scale extraction, is a narcotic in high concentrations. It can cause blisters on skin contact and may be a lung irritant when heated.

*Culture and precipitation reagents*: Hydrochloric acid (HCl) is toxic by ingestion and a corrosive irritant to eyes, lungs and skin. It has been shown to be a mutagen and teratogen in experimental studies. Copper sulfate pentahydrate ($CuSO_4$ 5H2O) is poisonous by ingestion and a mutagen in experimental studies. It causes systematic effects including jaundice and hemolysis. Cobalt chloride ($CoCl_2$) is poisonous upon skin contact. It is an experimental teratogen and reproductive hazard, as well as a potential carcinogen. Magnesium chloride ($MnCl_2$) causes experimental reproductive effects.

## 5.3 CONSIDERATIONS IN DEVELOPMENT OF BIOSYNTHESIZED CHEMICALS

A potential barrier to the development of some alternative chemical technologies and biotechnology, in the United States, is uncertain, inconsistent, and sometimes conflicting regulations and licensing

---

[96] $LD_{50}$ is the dose of the chemical at which 50% of laboratory animals (in this case rats) die in clinical experiments.

[97] Emulsan Biotechnologies, *Material Safety Data Sheet for Emulsan™ Brand Polysaccharide Biopolymer*, Greens Farms, CT, 1995.

[98] R. Lewis, *Sax's Dangerous Properties of Industrial Materials* (New York: Van Nostrand Reinhold. 1992).

processes at the national level.[99] Regulations on the state level are even more inconsistent and undeveloped. As environmental, health and safety issues (compliance, liability, etc.) are often fundamental economic concerns in the development of a product, a confusing regulatory structure can possibly hinder development of alternative chemical technologies.

As biotechnology regulations are not yet finalized, EPA encourages companies or institutions developing biosynthesized products to contact them early in the proposal development phase to determine: (1) agency jurisdiction regarding the product and process; and (2) laws and requirements applicable to the product or process. Early contact with EPA allows the agency to identify special data requests and preliminary concerns, and can ease project planning as well as the submission and review process. Thus, it is advantageous for companies or institutions that are developing biosynthesized chemicals to consider regulatory compliance and relevant testing issues from the early project planning stages. Early consideration of and consultation on the regulatory process could eliminate costly or time consuming barriers that might surface in the development and production of a biosynthesized material. To date, EPA has reviewed more than 25 biotechnology Premanufacture Notifications and is beginning to observe new and different uses of the microorganisms.

In encouraging the development of alternative chemical technologies, EPA has not developed any "benign" design certification system. In general, EPA does not recommend specific substances. EPA believes that "Benign by Design Chemistry should be the option of first choice which is built into the earliest stages of planning to manufacture a chemical product in order to ensure full consideration of the most fundamental pollution prevention methods available."[100]

---

[99]Pierce, "Recent Developments in Biotechnology Regulation," 167-178.

[100]P. Anastas, "Benign by Design Chemistry," In P. Anastas and C. Farris, ed. *Benign By Design: Alternative Synthetic Design for Pollution Prevention* (Washington, D.C.: American Chemical Society, 1994).

# PART II BIOSURFACTANT APPLICATIONS

## CHAPTER 6 AQUEOUS METAL CLEANING

### 6.0 INTRODUCTION

Degreasing, the surface cleaning of metal parts, is a common step in many manufacturing processes and maintenance procedures. The proper cleaning of parts is essential because the presence of contaminants, also known as soils, can affect the quality of the finished product or the completion of a maintenance project. The most common contaminants or soils are metal machining fluids.

There are a number of variables involved in the cleaning of metal: the part being cleaned may be made of any type of metal; soils vary depending on the situation; and there are numerous cleaners available to remove these soils. For this project, the biosurfactants were tested in TURI's Surface Cleaning Lab (SCL) on metals and soils commonly encountered in manufacturing. What was learned in examining the use of biosurfactants in general metal cleaning can be applied to many manufacturing processes and maintenance procedures including aerospace applications. This chapter presents background information on common soils ( i.e. metal machining fluids), aqueous cleaners, and cleaning equipment.

### 6.1 MACHINING FLUIDS

For over a hundred years, fluids have been used in the machining of metals as lubricants, chip removers, rust inhibitors, and coolants. These machining fluids have evolved over the years to become more compatible with the metal being machined and the machining process used. The machining fluid chemistries include substances such as silicates, chlorine, sulfur, and phosphorus. This evolution has resulted in a plethora of different fluids being used. There are three main types of machining fluids: cutting oils, water-soluble fluids, and paste or solid lubricants. All these fluids become contaminants or soils on the surface of the metal part and must be removed prior to further processing of the metal.

Cutting Oils

Cutting oils are mineral oils that are used alone or compounded with polar and/or chemically active additives. The mineral oils used are typically paraffinic (straight-chain hydrocarbon base), or naphthenic (ring-structured hydrocarbon base). Naphthenic oils are more widely used because they have a much higher solubility for many of the additives commonly used in metalworking fluids. There are two classes of cutting oils: inactive and active. Inactive cutting oils are mineral oils compounded

with chemically inactive additives. Active cutting oils are mineral oils or fatty mineral oil blends that contain sulfur, chlorine or phosphorus in an active form. Tables 7 and 8 summarize the different types of active and inactive cutting oils.

Table 7. Inactive Cutting Fluids

| Types of inactive cutting oil | Characteristics and Uses |
|---|---|
| Straight mineral oils | Used in light duty operations that require low levels of cooling and lubrication; if kept clean reusable indefinitely; lower in cost than compounded oils |
| Fatty oils | Most common types are lard and rapeseed oil; high anti-friction properties but poor anti-weld characteristics; have a tendency to emit unpleasant odors |
| Compounded cutting oils | Made by blending mineral oils with polar additives and/or chemically active additives |
| Fatty-mineral oils | Straight mineral oils blended with up to 40% fatty oil |
| Inactive extreme-pressure (EP) additives | Additives such as chlorine, sulfur or phosphorus added to mineral or compounded oils for machining applications where forces are high |

Table 8. Active Cutting Fluids

| Types of active cutting fluids | Characteristics and Uses |
|---|---|
| Fluids containing sulfur additives | Contain sulfur additives that form metallic sulfide films which act as solid films up to 1300°F; may stain aluminum, copper, brass, bronze, and magnesium |
| Fluids containing chlorine additives | Ferrous chloride film forms when chlorine reacts with ferrous work pieces or HSS (high strength steel) tools; low shear strength reduces friction at temperatures up to 750°F |
| Fluids containing phosphorus additives | phosphorus additives used as friction and wear reducers; will not stain ferrous or nonferrous work pieces |

Water-Soluble (Water-Miscible) Fluids

Water-soluble fluids are primarily used for high speed machining where heating of the work piece and the tool is a concern. They are effective at preventing thermal distortion of the tool and work piece. For high-speed machining when metal chips are formed, the oil concentrate is usually blended 1 part with 20 to 30 parts water. For many grinding operations where a lighter fluid with better cooling properties is desired, the ratio of oil to water may be from 1:40 to 1:50. The three types of water-soluble fluids are: emulsifiable oils, synthetic (chemical) fluids, and semisynthetic (semichemical) fluids. Synthetic fluids consist of inorganics and/or other material dissolved in water. They contain

no mineral oil. Semisynthetic fluids are a combination of synthetics and emulsifiable oils. Tables 9 and 10 describe the types of emulsifiable oils and synthetic fluids, respectively.

Table 9. Emulsifiable Oils

| Type of emulsifiable oil | Characteristics |
|---|---|
| Emulsifiable mineral oil | Suspension of mineral oil made by blending the oil with an emulsifying agent; emulsifiers break the oil into minute particles and keep the particles dispersed in water for a long period of time; bactericides are used to control the growth of bacteria, algae, and fungi; bactericides are commonly nonphenolic compounds specifically approved by the EPA; phenolics may be used when disposal is not a concern |
| Extreme-pressure emulsifiable oils | Sometimes referred to as heavy-duty soluble oils; contain sulfur, chlorine, or phosphorus; may also contain some fatty oils to increase lubricity |

Table 10. Synthetic Fluids

| Type of synthetic fluid | Characteristics |
|---|---|
| True solution fluids | Chemical solutions primarily contain rust inhibitors, sequestering agents, amines, phosphates, borates, glycols or ethylene oxide condensates; have a tendency to leave a residue of hard or crystalline deposits that are formed by water evaporating. |
| Surface-active chemical fluids | Fine colloidal solutions of organic or inorganic materials dissolved in water; wetting agents are usually added to provide moderate lubricity; have low surface tensions; usually contain rust inhibitors; when they dry onto a work piece they usually leave a powdered residue |
| EP surface-active chemical fluids | Similar to surface-active fluids but contain extreme pressure (EP) additives such as chlorine, sulfur, and phosphate to give the fluid extreme pressure lubrication qualities |

Paste and solid lubricants

Grinding wheels are sometimes impregnated with solids possessing lubricating qualities. In special cases grinding wheels are treated with sulfur to produce a cooling action in wet grinding. Often grease sticks are externally supplied to grinding wheels. Solid waxes in stick form are used as lubricants on grinding wheels, sanding disks or belts, and band or circular saw blades. Other solids commonly used as heavy-duty lubricants are: molybdenum disulfide, graphite, mica, talc, glass, pastes, and soaps.[101,102]

[101]Society of Manufacturing Engineers, *Tool and Manufacturing Engineers Handbook, Fourth Edition, Vol. 3, Materials, Finishing and Coatings*, 1985.

[102]American Society for Metals, *Metals Handbook, Desk Edition* (Metals Park, Ohio: ASM, 1985).

## 6.2 AQUEOUS CLEANER COMPONENTS

After a part has been machined, the machining fluid and other soils must be removed prior to further processing. This is crucial because further processing, such as electroplating, will not be successful unless the surface of the work piece is completely clean. Until recently, many companies used vapor degreasing with chlorinated solvents to clean metal parts because it was effective and, until now, inexpensive. However, the manufacturing of two common chlorinated solvents, 1,1,1 - TCA and CFC - 113, has been banned under the Montreal Protocol because of concerns about their long term environmental effects. As a result, taxes and disposal costs of these chlorinated solvents have become increasingly prohibitive.

Most observers think that water-based cleaners will be favored over the many possible replacements for chlorinated solvents. Large-scale metal cleaning operations (e.g., manufacturers of automobiles, aircraft, construction equipment, etc.) find it easiest to make the switch to these systems because aqueous-based cleaners can be tailored to remove a specific type of contaminant from a given metal surface. For diversified operations (e.g. job shops) that encounter a number of contaminants and substrates, the switch is more difficult. These companies may resign to continued use of chlorinated solvents because of their greater versatility. Another hurdle for firms interested in switching from vapor degreasing to aqueous cleaning is they may not currently have a wastewater discharge permit, which may be required for disposal of aqueous cleaning solutions.

Aqueous cleaner chemistries are very complex and cleaning formulations vary greatly. Aqueous cleaners use water as the primary solvent. There are a number of substances available that may carry out similar functions in a cleaning formulation. Surfactants are combined with builders and additives such as pH buffers, rust inhibitors, chelating agents, and saponifiers. These agents provide multiple degrees of freedom in formulating, blending, and concentrating. They also provide useful synergistic effects. A brief description of each component and its function follows.

Builders

Builders are the alkaline salts in alkaline cleaners. An aqueous cleaning formulation usually includes two or more builders. A number of functions are carried out by builders in a cleaning formulation. These functions depend on the type of builder used. There are three main chemical groups used as builders: phosphates, silicates, and carbonates.

Phosphates serve a number of purposes in an aqueous cleaner formulation. Phosphates soften water, eliminating the flocculent precipitate caused by calcium, magnesium and iron. They also act as a soil dispersant, a buffer, and a source of alkalinity. Common phosphates used as builders are trisodium phosphate, disodium phosphate, tetrasodium pyrophosphate, and sodium tripolyphosphate (known as tripoly).

Silicates also serve multiple purposes in an aqueous cleaning formulation. They provide alkalinity, prevent redeposition of soil by keeping it suspended, provide some detergency, and act as inhibitors protecting metals such as zinc and aluminum from attack from other alkaline salts.

Carbonates are an inexpensive source of alkalinity and act as buffers in solution. Carbonates can also be an adsorbing media for the liquid components of the cleaner when the cleaner is in a powdered form.

Two other substances that are used as builders in aqueous cleaning formulations are hydroxides and borates. Hydroxides are a relatively inexpensive source of alkalinity. Borates act as buffers and provide some detergency and metal protection.

Additives

Additives are organic or inorganic compounds that are used in aqueous cleaner formulations to provide a number of functions. Additives provide additional cleaning or surface modification. They also soften water and complex or tie up metal ions. There are three main groups of additives: chelating agents, inhibitors, and sequestering agents.

Chelating agents solubilize metal salts by forming chemical complexes. Some widely used chelating agents are sodium glucona, sodium citrate, tetrasodium ethylenediamine tetraacetate (EDTA), trisodium nitrilotriacetate (NTA), and triethanolamine.[103] Chelating agents are sometimes used as builders in place of phosphates to eliminate the possible problem of eutrophication of water bodies that is attributed to phosphates.

Inhibitors minimize the negative effects alkaline cleaners may have on certain metal substrates. They prevent rusting of the part being cleaned and of the cleaning equipment. Inhibitors are found in high pH cleaners to prevent the cleaner from attacking non-ferrous metals. Low pH cleaners do not usually contain inhibitors. Inhibitors may be used in the wash stage of single stage cleaning operation. They may be used in the rinse phase of a multiple stage cleaning process. Inhibitors may deposit a film on a part as soon as it is cleaned. This may interfere with future processing of the part. The use of inhibitors may increase the difficulty of rinsing the cleaner from the part.[104] Some commonly used inhibitors are aldehydes, amines, benzoates, borates, carboxylates, molybdates, nitrites, thiols, triazoles, and urea.[105]

Sequestering agents combine with heavy metal ions, calcium and magnesium ions, in hard waters. They form molecules in which ions are held and can no longer react. Sequestering agents also

---

[103]JoAnn Quitmeyer, *Aqueous Cleaning Handbook* (Lexington, Mass.: W.R. Grace&Co.-Conn., January 1996).

[104]U.S. Environmental Protection Agency, *Solvent Alternative Guide, Version 2.1*, 1995

[105]JoAnn Quitmeyer, *The Aqueous Cleaning Handbook.*

prevent salts from recontaminating parts. Types of sequestering agents commonly used are orthophosphates, orthosilicates, and phosphates.

Surfactants

Surfactants are organic compounds in aqueous cleaning formulations that provide solubilization, emulsification and wetting. Surfactants have unique chemical characteristics. The surfactant molecule has two ends that have different charges. The hydrophilic end of the molecule is polar and is attracted to the solvent, water. When a surfactant is placed in water the hydrophilic end of the molecule bonds with the water molecules and lowers the water's surface tension. By lowering the surface tension of water the surfactant enables the water to wet small areas previously inaccessible to the water.

There are a number of mechanisms at work when a surfactant in an aqueous solution comes in contact with a contaminated substrate. The contaminant, a machining oil in this research project, adheres to the substrate as a result of the interfacial tensions between the oil and the substrate. There are also interfacial tensions between oil molecules, a result of the force of attraction between like molecules (van der Waals force). The forces between oil molecules at the interface between the oil and the substrate is less then the forces between the outer oil molecules and those on the inside of the oil droplet. As a result, the molecules at the surface of the oil are pulled inward. When a surfactant in an aqueous cleaner comes in contact with the oil, the hydrophobic end of the surfactant molecule orients itself in the oil droplet causing it to swell. The force caused by the swelling of the oil surface acts against the interfacial tension forces that pull inward on the oil. This results in a reduction in the net interfacial tension. By lowering the interfacial tension, the surfactant allows the oil to be broken into smaller droplets more easily.[106]

There are four main types of surfactants: nonionic, anionic, cationic and amphoteric. The two main types used in aqueous cleaning formulations are nonionics and anionics. Cationic surfactants are typically only used in germicides and fabric softeners. Amphoteric surfactants are usually only used for specialty purposes such as hydrotroping less soluble cleaner components.

Nonionic surfactants are the most widely used in metal cleaning because they are low-foaming. Nonionic surfactants do not have a charged group and they are effective at solubilizing nonpolar soils. They are effective over a wide range of pH resistant but their properties are temperature dependent. Two groups of chemicals that are commonly used as nonionics surfactants are nonylphenol ethoxylates and primary alcohol ethoxylates.

Anionic surfactants are the most widely used in the surfactant industry due to their low production costs. However, due to their foaming characteristics they are not as commonly used in metal cleaning as nonionics. Anionic surfactants do work effectively in immersion applications where foaming is not

---

[106]Laurier L. Schramm, *Emulsions: Fundamentals and Applications in the Petroleum Industry* (Washington D.C.: American Chemical Society, 1992).

a factor. They have a negatively charged end and can solubilize polar soils. Anionic surfactants are not temperature dependent. Typical anionics are sulfates, sulfonates, and phosphate esters.

Cationic surfactants have a positively charged end. They are not commonly used in aqueous cleaner formulations although cationic surfactants may act as an effective emulsifier. Cationic surfactants will often deposit on the surface of the metal being cleaned. This effectively defeats the purpose of attempting to clean with them.

Amphoteric surfactants are pH dependent. The surfactant becomes anionic in an alkaline medium and cationic in an acidic medium. There is generally no advantage to this but amphoterics are sometimes used in specialty applications.

*Biosurfactants*

The general term biosurfactant is used to describe all biologically synthesized surface active agents. Based on function, however, there are two distinct types of surface active agents: biosurfactants and bioemulsifiers. Surfactants facilitate the emulsification of oil in water by lowering the surface tension of water or by lowering the interfacial tension between water and oil.[107] This is true of low molecular weight biosurfactants such as sophorolipid. Emulsifiers stabilize an emulsion by orienting at the oil/water interface. Emulsan, a large molecular weight bioemulsifier, neither significantly reduces the surface tension of water nor does it greatly reduce the interfacial tension between water and oil. However, emulsan is an effective emulsion stabilizer.[108]

The reaction of emulsan with a contaminant does not follow the mechanisms discussed above for petroleum based surfactants. The molecule is amphipathic (i.e., the molecules have both a hydrophobic and hydrophilic end) in nature, but it is not soluble in any substance. Therefore, when emulsan in solution contacts a water/hydrocarbon interface it orients itself between the two phases without entering either. Because emulsan does not orient itself inside the oil droplet it does not cause the interfacial tension to be greatly reduced. This is why only a slight reduction in surface and interfacial tension can be realized from the use of emulsan. The presence of emulsan at the water/hydrocarbon interface is the reason for any reduction in interfacial tension. Emulsan molecules connect with each other to form a layer around the hydrocarbon approximately 20 A thick. These bonds are strong and are the reason emulsan is an excellent emulsion stabilizer.[109]

---

[107]Kosaric, *Biosurfactants and Biotechnology*.

[108]David L. Gutnick and Yossef Shabtai, "Exopolysaccharide Bioemulsifiers," in *Biosurfactants and Biotechnology* (New York: Marcel Dekker, Inc., 1987).

[109]Jitendra D. Desia, "Microbial Surfactants: Evaluation, Types, Production and Future Applications," *Journal of Scientific and Industrial Research,* Vol. 46 (1988): 443-444.

## 6.3 CLEANING EQUIPMENT

Process equipment used with aqueous-based cleaners can be divided into three categories: immersion, spray, and ultrasonic. In addition, filtration techniques are sometimes used to achieve 'closed loop' cleaning. Each of these types of equipment are described briefly below.

Immersion

The immersion method cleans the parts by immersing them in a solution and using some form of agitation to add the energy needed to displace and float away contaminants. Soil is removed from the metal surface by the aqueous cleaner and convection currents in the solution. The currents are created by heating coils or by mechanical action. Table 11 highlights the advantages and disadvantages of immersion cleaning of metal parts.

Table 11. Advantages and Disadvantages of Immersion Cleaning

| Advantages | Disadvantages |
|---|---|
| Usable with parts on trays | Requires rinse water for some applications |
| Will flush out chips | Difficult to automate |
| Simple to operate | Requires proper part orientation and positional changes while in solution |
| Cleans complex parts and configurations | Can use existing vapor degreasing equipment with some simple engineering changes. |
| | May require separate dryer. |

Spray Washing

The spray washing method cleans parts with a cleaning solution sprayed at medium-to-high pressure. Spray pressure can vary from as low as 2 psi to more than 400 psi. Higher spray pressures deliver more mechanical action to help remove soils from metal surfaces. Spray cleaning solutions are prepared with low foaming detergents. As cleaners, they may be less effective chemically than those used in immersion cleaners but they are still effective because of the increased mechanical action. Although spray cleaning is effective on most parts, certain part configurations, such as the interior of an automobile tail pipe, have soiled areas that are inaccessible to the spray stream. In these instances, immersion cleaners are more effective. Table 12 highlights the advantages and disadvantages of spray washing of metal parts.

A high pressure water spray is an effective final rinse step. Pressures may range from 100 psi in less critical applications up to 2,000 psi in critical applications. Optimization of nozzle design parameters such as spray pattern, drop size and formation, pressure/velocity, and volume is very important and

may have a major impact on effectiveness. The final spray can be much cleaner than in an immersion bath, since the final spray touching the part can be pure water.

Table 12. Advantages and Disadvantages of Spray Washing

| Advantages | Disadvantages |
|---|---|
| High level of cleanliness | May require rinse water to eliminate film residues. |
| Relatively inexpensive | Not effective in cleaning complex parts with blind areas. |
| Will flush out chips | May require a separate dryer.[110] |
| Simple to operate | |
| Can process a high volume of parts at one time. | |
| Some units are portable. | |

Ultrasonics

In this cleaning method ultrasonic waves are generated in the cleaning bath which cause tiny bubbles to form and collapse at the surface of the part. This is known as cavitation. Process design requires caution to insure that cavitation erosion of the part's surfaces is not a problem. Certain part geometries are also ultrasonic sensitive. The number of parts and their orientation is very important for good cleaning. In some instances final rinsing with DI water or alcohol, such as isopropanol, is used to remove residues and prevent water spots.

Table 13. Advantages and Disadvantages of Ultrasonics

| Advantages | Disadvantages |
|---|---|
| Highest level of cleaning; cleans complex parts/configurations | Higher costs |
| Can be automated | Long lead time |
| | Low maintenance applications |
| | Can not handle heavy oils [111,112] |

[110]U.S. Environmental Protection Agency, Office of Air and Radiation, *Alternatives for CFC-113 and Methyl Chloroform in Metal Cleaning* (June 1991).

[111]U.S. EPA, "Alternatives for CFC-113 and Methyl Chloroform in Metal Cleaning."

[112]Thomas, "Evaluation of Alternatives to Chlorinated Solvents for Metal Cleaning."

Closed Loop Aqueous Cleaning

Regardless of which of the above cleaning methods are used, when aqueous cleaning is performed it is frequently done as a closed loop process. Closed loop aqueous cleaning involves the removal of contaminants from the cleaner bath. This extends the useful life of the cleaner and reduces the quantity of waste disposed. A variety of methods exist for removing contaminants from aqueous solutions. Methods selected for a particular application are often chosen on the basis of contaminant size. Particulates may be removed using settling tanks, chip baskets, media filtration, or canister filters. Tramp oils are removed using skimmers and coalescers. The majority of contaminants can be removed using membrane filtration techniques (micro or ultrafiltration).[113]

There are numerous reasons for closed loop aqueous cleaning. Many firms currently switching to aqueous cleaning do not have a water discharge permit. When aqueous cleaners are spent and discharged to a POTW they can cause problems with the normal operation of the wastewater treatment system. Closed loop processes are also of interest because of the relatively high cost of aqueous cleaners.

[113]Toxics Use Reduction Institute, *Closed Loop Aqueous Cleaning*.

# CHAPTER 7 METAL CLEANING WITH BIOSURFACTANTS

## 7.0 INTRODUCTION

Matching a specific cleaning need with an appropriate chemistry and process combination is a challenge because of the variety of substrates and contaminants to be cleaned and the numerous aqueous chemistries and processes with which to clean. TURI established the Surface Cleaning Lab (SCL) to assist companies with this challenge. Because the SCL focuses on non-precision cleaning in industrial applications, its main focus is the functionality of cleaners, not precise testing and analysis of specific cleaners. For this project however, some more basic research was required to examine the properties and performance of one component of an aqueous chemistry, the surfactant. The specific goals of the work performed in TURI's SCL were threefold:

- To assess the ability of the biosurfactants to wet a metal surface, cause oil contaminants to roll up and then lift from a metal surface, and to determine if (or at what concentration) solubilization of the oil contaminants would occur.

- To evaluate the ability of the biosurfactants to act as a cosurfactant (i.e., to enhance the performance of another surfactant) was evaluated.

- To determine if emulsan's emulsification ability alone was enough to clean a metal surface.

A combination of tests and analytical procedures were used to explore these three areas.

To accomplish this research, new protocols were developed, which built on existing protocols and knowledge to establish more precise testing requiring quantitative analysis. As these protocols were developed, it became obvious that additional characterization of the surfactants was necessary. The characterization work was conducted jointly by TURI and the Biodegradable Polymer Research Center; the results were discussed in Chapter 3.

This chapter includes a discussion of the theory behind each of the three goals, a description of the lab testing and analytical procedures employed, results of each series of tests, a general discussion of the tests in relation to the goals, and conclusions and recommendations from the testing in the SCL.

## 7.1 THEORY

Surfactants are crucial ingredients in cleaning formulations because their unique chemical nature causes the lowering of interfacial tensions and the formation of surfactant micelles. When these characteristics are added to a system consisting of an oil-contaminated substrate, the reactions caused

by the surfactant result in numerous physio-chemical changes in the system. These changes have been classified according to the different mechanisms that perform the removal of oil from a substrate. The mechanisms of wetting, roll-up, solubilization, co-surfactant, and emulsification are described below along with how each was explored in the SCL for emulsan and sophorolipid.

Wetting

In surface cleaning, the term wetting refers to the ability of a cleaning solution to come in complete contact with the surface to be cleaned. If a part contains areas that are not readily accessible to the solution, the addition of a surfactant to the solution may reduce the surface tension enough that the solution penetrates the areas. Wetting also refers to the movement of contaminant oil away from the point of contact of a cleaning solution. Because the interfacial tension between the oil and the cleaning solution is lower than the tension between oil molecules (van der Waals forces), the oil is pulled to the region of higher tension.[114] An example of this is a kitchen sink full of water with an oily slick on the surface. When a drop of dish detergent comes in contact with the center of the slick, the oil is pulled from the point of contact.

The ability of emulsan and sophorolipid to provide spreading of oil away from the point of contact was tested using coupon screening tests. The tests involved contaminating metal test coupons with various oils and then applying a drop of surfactant solution to the center of the contaminated area. The reaction of each was observed and recorded.

Roll-up

Roll-up is one of the driving forces causing oils to separate from a surface (see Figure 8). It has been referred to as the most important mechanism by which oils are removed from substrates.[115] Roll-up results from changes in interfacial tensions between oil, water, and substrate. The equilibrium condition of an oil on a substrate is illustrated by Young's equation,

$$\gamma_{sw} = \gamma_{os} + \gamma_{ow} \cos\theta$$

where $\theta$ represents the contact angle of the oil with the substrate and $\gamma_{sw}$, $\gamma_{os}$, and $\gamma_{ow}$ represent the interfacial tensions between the substrate-water, oil-substrate, and oil-water interfaces, respectively (see Figure 9).[116]

---

[114]Anthony M. Swartz and James W. Perry, *Surface Active Agents: Their Chemistry and Technology* (New York: Interscience Publishing, Inc.,1949).

[115]Kissa, "Kinetics of Soiling and Detergency."

[116]Kissa, "Kinetics of Soiling and Detergency," 257.

Figure 8. Roll-Up[117]

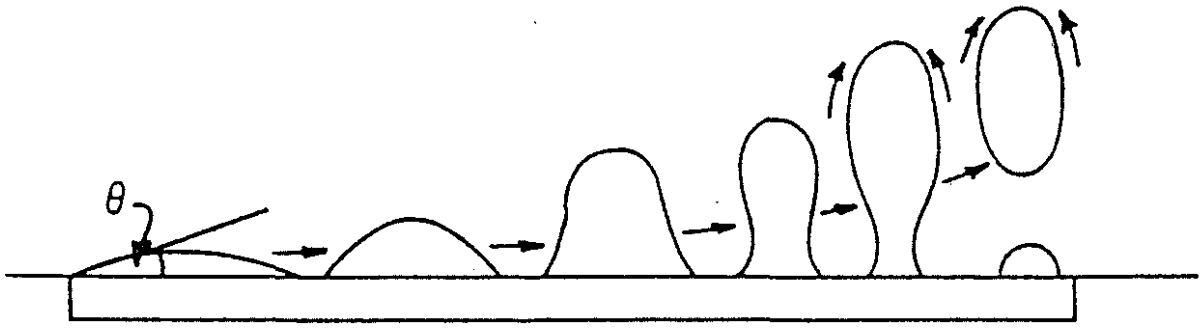
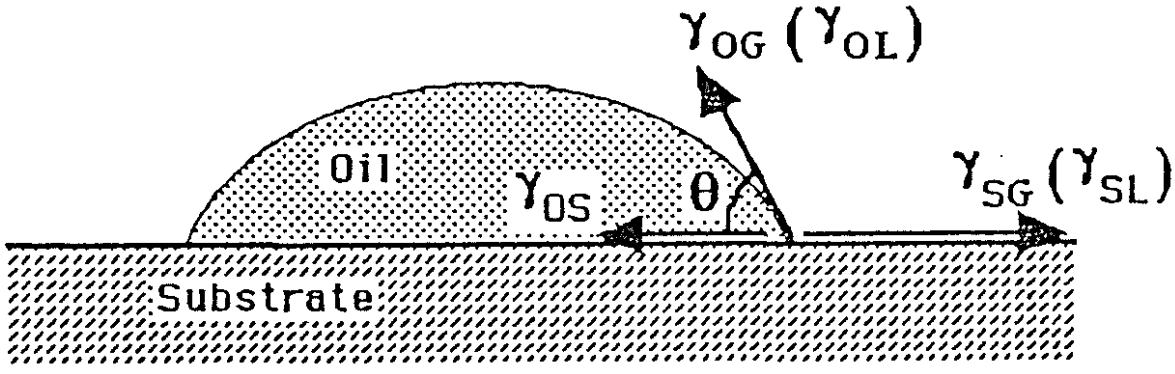



Figure 9. Drop of Oil on a Solid Substrate[118]

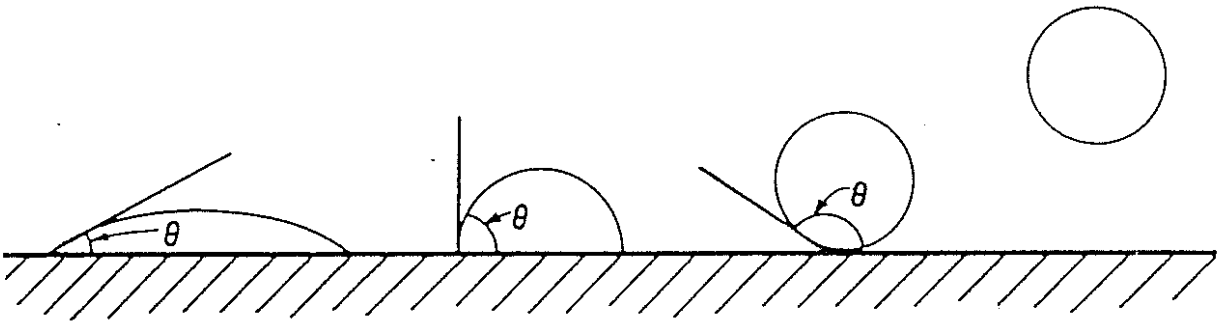



[117]Kissa, "Kinetics of Soiling and Detergency," 258.

[118]Broze, "Mechanisms of Soil Removal," 258.

When an appropriate surfactant is added to water, it orients at the oil-water and substrate-water interfaces, causing the tensions at these interfaces to be greatly reduced. Because the oil-substrate interfacial tension is not affected, the system is driven to a new equilibrium condition that results in an increase in the contact angle. When the reduction in tension at the oil-water and substrate-water interfaces is great enough that their sum is equal to the oil-substrate interfacial tension, the contact angle becomes 180° and complete oil removal is achieved (see Figure 10).[119]

Figure 10. Roll-Up Mechanism[120]



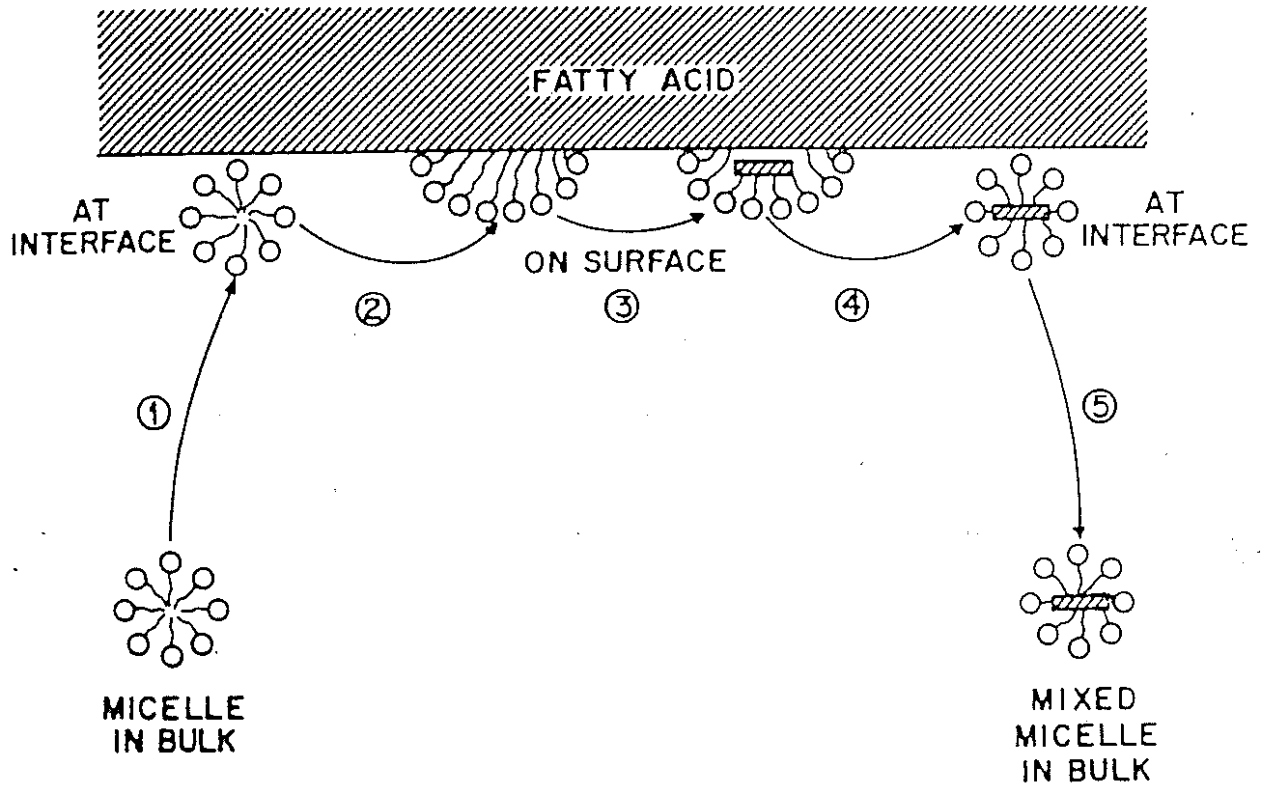

When the reduction in interfacial tensions is not great enough to result in a contact angle of 180°, other forces, such as mechanical energy, must complete the removal. Also, in practice, roll-up does not occur in the ideal sequence as shown in Figure 10 because the buoyancy of the oil and hydrodynamic forces of the cleaning bath can cause necking and drawing of the oil droplet. In these cases removal may involve several droplet roll-ups.

Two tests, immersion and immersion with mechanical agitation, were used to evaluate the ability of emulsan and sophorolipid to cause roll-up. In these tests, metal coupons contaminated with oil were lowered into unagitated or agitated baths. The reaction of the oil during the immersion time was observed and the amount of oil removed was measured.

[119]Broze, "Mechanisms of Soil Removal."

[120]Kissa, "Kinetics of Soiling and Detergency," p. 257.

Figure 11. Solubilization of an Oil by a Micelle[121]




Solubilization

Solubilization is a term used to describe the process of dissolving an otherwise insoluble substance, such as a hydrocarbon oil, by incorporating it into the micelles of a surfactant solution. As discussed in Chapter 3, surfactants in water form aggregates known as micelles in which the hydrophilic segment of the surfactant is oriented toward the water. For nonpolar contaminants, the solubilized substance is located in the interior of the micelle. For polar contaminants, the solubilized substance is located between the surfactant molecules forming the micelle.[122] The solubilization mechanism is best described in the following five steps (see Figure 11):

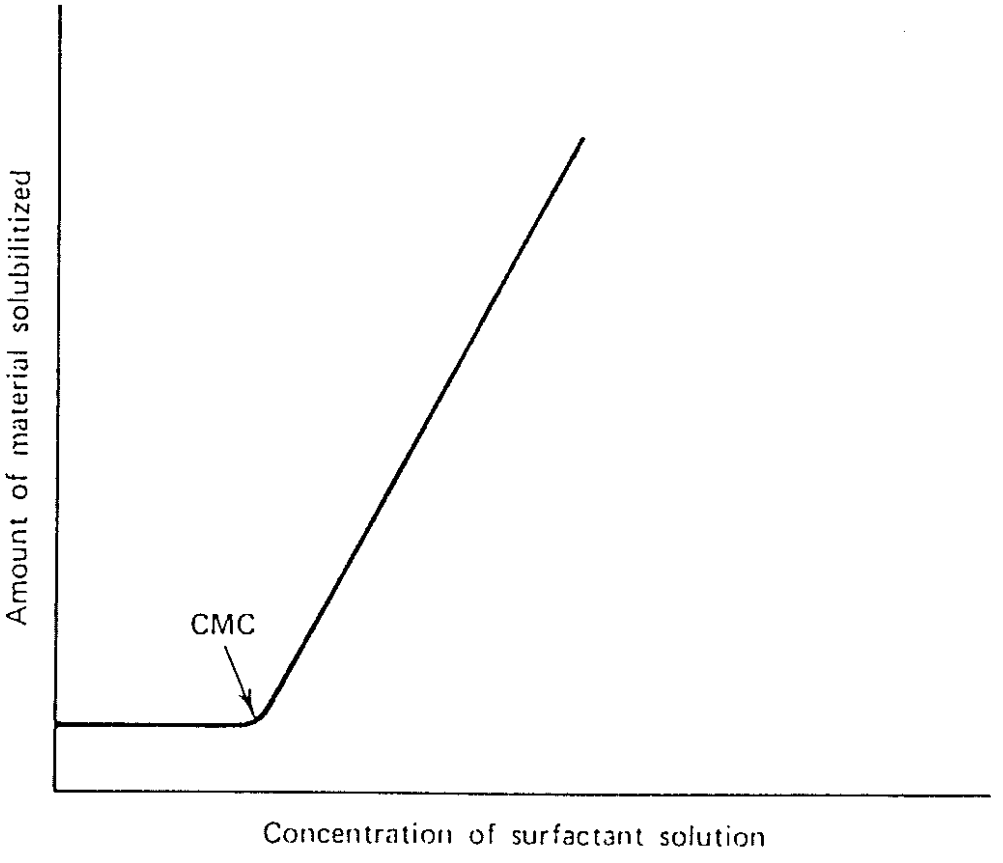
1. The micelle diffuses to the surface of the contaminant.
2. The micelle is adsorbed at the contaminant/water interface.
3. Contaminant molecules mix with the adsorbed surfactant molecules.
4. The surfactant micelle is desorbed from the contaminant surface.
5. The micelle containing solubilized contaminant diffuses into the bulk of the water.

[121]Kissa, "Kinetics of Soiling and Detergency," p.263.

[122]Ibid.

For solubilization to be effective, the surfactant must be in concentrations that exceed its critical micelle concentration (see Figure 12).

Figure 12. Solubilization as a Function of Surfactant Concentration and CMC[123]




At concentrations below the CMC very little solubilization takes place due to the absence of micelles in sufficient quantity to effect oil removal.[124] Contrary to roll-up, solubilization is not a function of how well the surfactant lowers the surface and interfacial tensions. It is more specifically a function of the micelle formation characteristics of the surfactant and how these micelles interact with the oil to be solubilized.

Immersion testing was used to determine if emulsan and sophorolipid were able to solubilize oil regardless of whether they were able to lower the interfacial tension enough to cause roll-up.

---

[123] Laughlin, Robert, "Solubilization by Solutions of Surfactants: Micellar Catalysis," *The Aqueous Phase Behavior of Surfactants* (London and San Diego: Academic Press, 1994) 263.

[124] Kissa, "Kinetics of Soiling and Detergency," 265.

Co-Surfactant

In aqueous metal cleaning formulations often more than one surfactant is used. Because different surfactants have different characteristics, these combinations are often tailored to provide numerous functions in the cleaner. One surfactant may be more effective at solubilizing a contaminant while another may be more effective at emulsifying the contaminant. When two or more surfactants are present in significant concentrations in a solution, the surfactants form aggregates known as mixed micelles. As discussed above, when surfactant solutions contact a contaminant oil on a metal part, it is the surfactant micelles that solubilize the oil.

In the development of an aqueous cleaning formulation containing emulsan and/or sophorolipid it may be desirable to combine these biosurfactants with a surfactant which has better surface tension reducing properties. These combinations would also result in the formation of mixed micelles that would have different properties than the single surfactant micelles.

The ability of mixed micelles to solubilize oils more effectively than the single surfactant micelles was tested using immersion, immersion with mechanical agitation, and coupon screening tests. Solutions of emulsan and sophorolipid mixed with other surfactants were tested.

Emulsification

Surfactant molecules are partially soluble in both the oil phase and the water phase due to their amphipathic nature (i.e., the molecules have both a hydrophobic and hydrophilic end). The nonpolar end of the molecule enters the oil phase leaving the polar end oriented in the water phase. This is referred to as chemical emulsification. Surfactant molecules at the oil-water interface impart a charge on the surface of the droplets. The droplets have like charges and therefore repel each other, preventing coalescing. When oil is chemically emulsified, it is prevented from redepositing on the substrate once it has been removed. Mechanical emulsification is the breaking of oil into tiny droplets using some mechanical action. When surfactants are present, they reduce the interfacial tension allowing less mechanical energy to be used to emulsify the oil. In the absence of a surfactant, when the mechanical energy is removed from the system, the oil coalesces.

Small molecule surfactants are thought to be effective at forming emulsions because the small size of the molecules allow the surfactant to quickly move to the interface. However, these emulsions are typically stable for only a short period of time. This is attributed to the small size of the surfactant molecules and the fact that the molecules do not bind to each other. Because the molecules do not bind to each other they are easily displaced on the surface of the droplet. When two droplets approach each other, the repelling forces between droplets cause the surfactant molecules to be displaced from the impending point of contact.

Emulsan, a large molecule surfactant, is unique from other surfactants in the way it emulsifies oils. Emulsan molecules bind to each other at the oil-water interface to form a 20 Å thick layer around the oil droplet. The hydrophilic group is thought to be adsorbed by the water phase. The hydrophilic

portion of the polymer imparts an ionic charge on the droplets causing them to have like charges, therefore repelling each other. Because emulsan molecules bind to each other and the molecules are large, they are not displaced from the impending point of contact of two oil droplets. Hence the polymer forms a stable emulsion.[125]

As discussed previously for roll-up, when the interfacial tensions are not sufficiently reduced, additional mechanisms must aid in removal of oil from the substrate if cleaning is to be achieved. It is theorized that emulsification may be one of these mechanisms. Any increase in the contact angle results in a larger interfacial area for the surfactant to react with. The presence of the surfactant at the interface imparts a like charge on oil surfaces, causing these surfaces to repel each other, aiding in the removal of oil from the substrate.

The emulsification mechanism was evaluated in the coupon screening test, the immersion test and the immersion with agitation test.

## 7.2 ESTABLISHED PROTOCOLS AND MODIFICATIONS

Testing

There are a number of variables involved with selecting a cleaning formulation for a specific application. Many contaminants and substrates are encountered in metal cleaning and there a number of aqueous cleaners available to remove contaminants from substrates. Due to this variability, screening tests have been developed to evaluate aqueous cleaners with specific contaminants and substrates.

A quick screening test is capable of testing aqueous cleaners for a specific application. The procedure involves contaminating representative metal coupons with a sample of the specific oil or oils that are encountered in the field application. The oils are allowed to age for one hour, at which time a droplet of each cleaner being screened is applied to the coupons. The droplet is allowed to stand for one minute. The effectiveness of each cleaner at removing the contaminant is then noted.

For this research the quick screening test was modified to allow surfactant solutions to be tested on different contaminants. The amount of spreading of each droplet on the oil-contaminated substrate was observed and the ability of the surfactant to remove oil from the point of contact was noted. Also, the contact angle of the surfactant solution was qualitatively measured.

[125]Kosaric, "Introduction: Biotechnology and the Surfactant Industry."

Figure 13. SCL Evaluation Protocol

Establish Baseline "Clean"

1. *Substrate Identification*: metal test coupons (2" x 2") or company supplied parts
2. *Standardized Cleaning Procedure*: must be performed prior to contamination step in order to establish a baseline clean
   example: 15 minute ultrasonics wash @ 140°F, 10 % solution Daraclean 232 cleaner, 5 minute immersion rinse @ 140°F in tap water, 5 minute immersion rinse @ 140°F in DI water, 10 minute dry @ ambient temperature Laminaire Flow Station, 30 minute dry @ 158°F oven, cool to ambient conditions
3. *Gravimetric Analysis of "Cleaned" Substrates*: determine weight of cleaned coupons before contamination (sensitivity of the balance is 0.1 mg)
4. *Supplemental Analytical Characterization*: establish baseline readings for the "clean" substrate (FT-IR/Grazing Angle Reflectance, OSEE).

Contaminate

1. *Designate Contaminant*: cutting oils, lubricants, greases, coolants, particulates, chips and fines, oxidation products, fingerprints, waxes (aqueous-based, synthetic, petroleum-based, natural products)
2. *Select Substrate Material for Contamination*: ferrous, non-ferrous, plated alloys
3. *Contaminant Application*: loading is dependent on the method used. Range: 0 mg/sq cm to customer specified level
4. *Application Method*: spray on soil/solvent solution; roll/wipe-on using brush; dip/soak in soil/solvent solution; other: "as received" from customer
5. *Drying Process*: dry/age in air for 24 hrs. or dry/process at elevated temperature
6. *Gravimetric Analysis of Contaminated Coupons*: determine contaminated loading (mg/sq cm)
7. *Supplemental Characterization*: OSEE, FT-IR to establish readings for the "dirty" substrates

Clean, Rinse, and Dry

1. *Select Cleaning Chemistry*: from technical literature, product bulletin, MSDS, manufacturer, distributor
2. *Select Appropriate Number of Test Materials*: for statistical validation and design of experimental trials based on the number of experimental variables chosen
3. *Select Process Cleaning Equipment*: ultrasonics, soak immersion, agitated immersion, pressure spray wash
4. *Selected Variables to Investigate*: wash time (1 to 15 minutes), wash temperature (70-190°F), concentration of cleaning solution (start with manufacturer's recommendations)

The standard protocol was modified as necessary for the evaluation of biosurfactants and bioemulsifiers in metal cleaning applications.

The immersion and immersion with mechanical agitation tests mentioned previously are variations on tests used by cleaning science to identify the most effective cleaner for a specific application. (See Figure 13). The tests were modified to eliminate some variables that are not important in routine testing. Because the tests for this research are sensitive to any addition of mechanical energy to the immersion baths, the coupons were lowered into and raised out of the baths at a slow, controlled rate. Also, because wetting, roll-up, solubilization, and emulsification are all a function of the amount of

surfactant relative to the amount of oil present, equal amounts of oil were applied to equal areas of the coupon.

In order to evaluate the effectiveness of the biosurfactants with a variety of contaminants commonly encountered in surface cleaning, seven different oils were used in the tests. Six oils were used in the coupon screening tests and a seventh, C.I. Hayes Quench Oil, was used in the immersion and immersion with mechanical agitation tests. This oil was selected because emulsan (using the turbidity tests described in Chapter 3) was effective at emulsifying the oil.

The commercially available surfactants Witconol SN-90 and Rhodasurf DA-630 were selected because these ethoxylated alcohol surfactants are representative of surfactants used in aqueous cleaning formulations. The two aqueous cleaning formulations Valtron SP2275 and Inproclean 2500 were selected as representative of the wide variety of aqueous cleaning formulations on the market.

Analysis

It is difficult to attribute cleaning to any one of the mechanisms discussed previously because these mechanisms work simultaneously. There are tests available to quantitatively measure the mechanisms separately for a given oil and surfactant such as the Draves Wetting Test. However, due to the numerous oils encountered in surface cleaning, it was more beneficial for this exploratory research to evaluate these biosurfactants in lab-simulated cleaning systems. The reaction of surfactant solutions with contaminated coupons was observed and recorded.

Gravimetric analysis was used to measure the total effectiveness of these mechanisms at removing oil from the substrate. Gravimetric analysis involves measuring and recording the mass of metal coupons before and after contaminating them, cleaning the coupons using whatever method and/or solution being tested, and then measuring and recording the mass of the coupon again to determine how much contaminant was removed.

Although less quantitative than gravimetric analysis, fluorescing analysis was also used to evaluate the effectiveness of biosurfactants in the cleaning tests. Fluorescing analysis uses the fluorescent characteristics of oils to determine the extent of contamination. Coupons were contaminated with a fluorescent oil then placed under fluorescent lamp in a dark enclosure. A cleaning process was used and the coupons were reexamined under the lamp. The amount of oil before and after cleaning was noted.

## 7.3 COUPON SCREENING TEST

The coupon screening test was used to compare the cleaning ability of emulsan and sophorolipid to an aqueous cleaner and a petroleum-based surfactant and to determine if emulsan or sophorolipid would allow adequate wetting of the substrate. The amount of spreading and the contact angle of

emulsan and sophorolipid solutions with six oils commonly used in the metal working industry were evaluated.

Procedure

Two-inch square, hot-dipped galvanized steel coupons were contaminated with the six oils and allowed to age for one hour. Table 14 lists the manufacturer, product name, and characteristics of each oil. The coupons were contaminated using swabs to ensure even spreading.

Table 14. Oils Used in Coupon Screening Tests

| Manufacturer | Product Name | Description |
|---|---|---|
| Cook's Industrial Lubricants | Cut 20 | Heavy duty cutting oil containing specialized combined fats, sulfur and chlorine |
| East Falls Corp. | Hydraulic Oil 8-68 | Mineral oil, solvent-dewaxed heavy paraffinic petroleum distillates |
| Exxon | Kutwell 40 | Emulsifiable cutting fluid; Mineral oil, hydrotreated light and heavy naphthenic petroleum distillates |
| W. A. Wood Corp. | C-EBLIS Cutting Oil | 100 % water soluble; Mineral oil, hydrotreated light and heavy naphthenic petroleum distillates |
| Steco Corporation | Tap Magic Protap Cutting Fluid | Biodegradable; aliphatic organic acid, aliphatic organic ester, organic polyol |
| East Falls Corp. | Hydraulic Oil 8-32 | Mineral oil, solvent-dewaxed heavy paraffinic petroleum distillates |

Cleaning solutions were prepared using mixtures of emulsan (sample #1 described in Chapter 3), sophorolipid, an ethoxylated alcohol surfactant (Witconol SN-90 produced by Witco), a formulated aqueous cleaner (Valtron SP2275 produced by Valtech Corporation, a neutral pH cleaner) and deionized (DI) water. The concentration of emulsan was taken from work by Rosenberg, Zuckerberg, Rubinovitz, and Gutnick, at Tel Aviv University where 75 microgram per milliliter was the maximum concentration used to test emulsan's emulsification activity.[126] The following solutions were evaluated:

- deionized water
- Valtron SP2275 (5 % by volume in DI water)
- Witconol SN-90 - 2% by volume in DI water (2 ml of surfactant was added to 98 ml of DI water)
- emulsan sample #1 (75 mg/L) in deionized water (15 mg of emulsan in 200 ml of DI water)

[126]A. Zuckerberg, A. Diver, Z. Peeri, D.L. Gutnick, and E. Rosenberg, "Emulsifier of Arthrobacter RAG-1: Chemical and Physical Properties," *Applied and Environmental Microbiology,* Vol. 37, No. 3 (1979).

- emulsan sample #1 (75 mg/L) and Witconol SN-90 in DI water (2 ml of Witconol SN-90 added to 98 ml of the emulsan solution)
- emulsan sample #1 (75 mg/L) and Valtron (75 mg/L) in DI water

Because little work has been done with sophorolipid to date, it was not evident what concentration to use in cleaning evaluations. Therefore six solutions of varying concentrations of sophorolipid (0.05,0.06, 0.07, 0.08, 0.09, and 0.10 %) were prepared and tested using the coupon screening test procedure described previously. This was done to explore which concentration would be appropriate to work with in future evaluations and to evaluate spreading and contact angle characteristics of the biosurfactant.

After the oil stood undisturbed on the coupons for one hour, a drop of each solution was placed on each coupon. After one minute the amount of spreading of the solutions on the coupons and the contact angle between the solutions and the coupons were noted. The coupons were then tilted on their sides and the droplets were allowed to roll off. The amount of oil remaining on the coupon was observed and recorded.

Discussion of Results

The specific results of each test are found in Appendix B. The solutions containing the formulated aqueous cleaner or the ethoxylated alcohol surfactant spread into thin layers, repelling and removing the oil as they spread. This corresponds to a low contact angle. The solutions containing emulsan or sophorolipid alone did not cause spreading. A convex droplet of each solution remained at the point of contact (i.e., the contact angle of the biosurfactant solutions with the coupon remained high). This test indicated that emulsan and sophorolipid were not successful at wetting the surface.

When the emulsan solution was placed on a coupon contaminated with Kutwell 40, the oil appeared to be emulsified. Pure deionized water reacted identically with this oil. This may be attributed to the fact that the manufacturer lists Kutwell 40 as an emulsifiable oil. The solution containing emulsan and Witconol SN-90 reacted identically to the solution containing only Witconol SN-90 on all coupons except on Cut 20. On this coupon the solution containing only the surfactant had one drop of oil left in the center of the droplet while the solution containing emulsan and Witconol SN-90 was observed to contain a number of small droplets that were grouped together but did not coalesce. This was attributed to the presence of emulsan at the oil droplet-water interface causing the droplets to repel each other, hence preventing coalescing.

Wetting is only one mechanism that provides the removal of contaminants from substrates. The abilities of emulsan and sophorolipid to perform other mechanisms essential to surface cleaning were evaluated in the immersion test.

## 7.4 IMMERSION TESTING

This test was used to evaluate the ability of emulsan and sophorolipid to roll-up, solubilize, and/or emulsify a contaminant oil from the surface of a substrate as well as to explore the ability of these biosurfactants to act as co-surfactants with an ethoxylated alcohol surfactant. This was done by making observations throughout the immersion time regarding the condition of the contaminant oil as well as performing gravimetric and fluorescing analyses.

Procedure

Two-inch square, hot-dipped galvanized steel coupons were contaminated with C.I. Hayes Quench Oil. This is a mineral oil that is used to quench metal parts after they have been machined. Three drops of oil were applied using an eye dropper to deliver approximately the same volume of oil to each coupon. The tip of the dropper was used to spread the oil over a one-inch square in the center of the coupon. The oil was then allowed to age for one hour.

The coupons, suspended by coupon hangers, were immersed in beakers for fifteen minutes. Prior to removing the coupons from the baths, the oil that had floated to the surface was removed using an eye dropper to siphon the oil from the surface. The coupons were removed slowly so that minimal turbulence was created in the bath, therefore reducing the influence of hydrodynamic forces on a solution's ability to remove contaminants from substrates. After the coupons were removed from the immersion baths they were placed in an oven at 140°F for fifteen minutes. The coupons were allowed to stand for twelve hours so they could return to ambient temperature.

A number of solutions were evaluated using this test including: emulsan (sample #2), Emulsan™, sophorolipid, two ethoxylated alcohol surfactants, an aqueous cleaner, and combinations of the ethoxylated alcohol surfactants and biosurfactants. A range of concentrations was selected to explore the effects of concentration on the cleaning mechanisms. The high concentrations for the single surfactant solutions (875 mg/L emulsan, 70 mg/L and 50 mg/L of sophorolipid, 1% DA-630, and 2% SN-90) were used to explore the ability to solubilize oils at concentrations well above the CMC. At these high concentrations there were significant amounts of micelles present to potentially perform solubilization. Also, because at these concentrations the greatest possible reduction in interfacial tension had been achieved, the maximum ability of each surfactant to perform roll-up could be evaluated.

Concentrations of two and four times the CMC (332 mg/L & 166 mg/L of emulsan; 133.33 mg/L and 66.67 mg/L of Emulsan™, 13.33 mg/L and 6.67 mg/L of sophorolipid; and 0.37% and 0.185 % DA-630) were evaluated to evaluate the cleaning mechanisms as a function of concentration. The lowest concentrations of DA-630 (0.06%, 0.055%, and 0.04%) were selected to illustrate the large decrease in solubilization and roll-up at concentrations below the CMC.

The solutions containing an ethoxylated alcohol surfactant and a biosurfactant were used to explore the ability of the biosurfactants to act as co-surfactants. The ability of the mixed micelles in these

Table 15. Results of Gravimetric Analysis for the Immersion Test

| Cleaning solution | Mass of contaminant applied (g) | Mass of contam. removed (g) | Percent of contaminant removed (%) |
|---|---|---|---|
| emulsan (875 mg/L)* | 95.50 | 51.60 | 54.03 |
| emulsan (332 mg/L ) | 52.60 | 23.60 | 44.87 |
| emulsan (166 mg/L ) | 58.00 | 27.80 | 47.93 |
| Emulsan™ (133.33 mg/L) | 56.80 | 22.00 | 38.73 |
| Emulsan™ (66.67 mg/L) | 61.30 | 27.80 | 45.35 |
| sophorolipid (70 mg/L) | 58.20 | 26.50 | 45.53 |
| sophorolipid (50 mg/L) | 65.30 | 35.80 | 54.82 |
| sophorolipid (13.33 mg/L) | 62.00 | 26.30 | 42.42 |
| sophorolipid (6.67 mg/L) | 55.00 | 24.10 | 38.91 |
| DA-630* (1%) | 103.80 | 101.40 | 97.69 |
| DA-630 (0.37%) | 63.40 | 60.90 | 96.06 |
| DA-630 (0.185%) | 56.20 | 54.20 | 96.44 |
| DA-630 (0.06%) | 54.5 | 39.80 | 73.03 |
| DA-630† (0.05%) | 55.10 | 19.13 | 34.84 |
| DA-630 (0.04%) | 61.00 | 16.00 | 26.23 |
| SN-90* (2%) | 81.00 | 78.60 | 97.04 |
| SN-90 (0.031%) | 55.00 | 34.10 | 62.00 |
| Inproclean 2500† (10%) | 55.07 | 49.43 | 89.76 |
| emulsan (875 mg/L) and SN-90* (2%) | 85.20 | 82.30 | 96.6 |
| emulsan (332 mg/L) and DA-630 (0.37%) | 59.30 | 57.40 | 96.8 |
| emulsan (166 mg/L) and DA-630 (0.185% ) | 56.50 | 54.70 | 96.81 |
| emulsan (83 mg/L) and DA-630† (0.05% ) | 56.60 | 43.83 | 77.28 |
| Emulsan™ (133.33 mg/L) and DA-630 (0.37%) | 53.20 | 50.30 | 94.55 |
| Emulsan™ (66.67 mg/L) & DA-630 ( 0.185%) | 61.70 | 59.40 | 96.27 |
| sophorolipid (13.33 mg/L) and DA-630 (0.37%) | 43.30 | 41.80 | 96.54 |
| sophorolipid (6.67 mg/L) & DA-630 ( 0.185% ) | 54.90 | 51.90 | 94.54 |
| sophorolipid (70 mg/L)and DA-630 (0.06% ) | 62.50 | 45.60 | 72.96 |
| sophorolipid (50 mg/L)and DA-630† (0.05% ) | 56.73 | 40.23 | 70.94 |
| sophorolipid (50 mg/L) and DA-630 (0.04% ) | 62.50 | 43.00 | 68.80 |
| Emulsification formulation alone* | 88.50 | 61.30 | 69.27 |
| 1% DA-630 in the emulsification formulation* | 85.10 | 83.70 | 98.35 |
| emulsan (875 mg/L) in the emul. formulation* | 89.50 | 61.10 | 68.27 |
| DA-630 (1%) and SN-90* (2%) | 97.00 | 93.20 | 96.08 |
| Pure deionized water† | 58.25 | 30.28 | 52.24 |

† These values are the average of three values, which are given in Table 16 with the standard deviation of each set.
* Five drops of oil were used on these coupons.

solutions to solubilize oil was evaluated at various concentrations. An emulsification formulation from work by Zosim, Rosenberg, Gutnick, Zuckerberg, and Rubinovitz[127,128] was included because in this work it was shown to enhance emulsan's emulsification capacity. The formulation consisted of 40 mM of magnesium sulfate, 10 mM of sodium chloride, and 0.25 mM of 1-decanol. A formulated aqueous cleaner (Inproclean 2500) was included to compare the cleaning ability of the solutions to an aqueous cleaner. Pure deionized water was included to indicate the cleaning ability of each solution relative to water.

Gravimetric analysis was performed on the coupons to obtain a quantitative measure of the total oil removed by all mechanisms combined. The initial mass of each coupon was measured and recorded. After contaminating the coupons, the mass of each was remeasured and recorded. This was repeated a third time after the immersion procedure was completed and the coupons were dried and cooled. Also, fluorescing analysis was performed to determine the location and intensity of oil remaining on the coupon after the immersion time. This provided additional qualitative information to aid in determining the effectiveness of the biosurfactants at performing the cleaning mechanisms. Additional visual observations were made on selected immersion baths and coupons. The condition of the contaminant throughout the immersion time and the condition of the cleaning bath after the immersion time was noted.

Results

Results of the gravimetric analysis are given in Table 15.

To insure that the results of the gravimetric analysis were reproducible, selected solutions were tested three times. The solutions were prepared separately for each run and the immersion procedure above was followed. The results of the gravimetric analysis are given in Table 16. The standard deviation for the percent contaminant removed of each set is given in the table.

Recorded observations of certain solutions while the coupons were lowered into the baths and while the coupons were immersed are included in Appendix B.

---

[127]E. Rosenberg, C. Zuckerberg, C. Rubinovitz, and D. L. Gutnick, "Emulsifier of *Arthrobacter* RAG-1: Isolation and Emulsifying Properties," *Applied Environmental Microbiology*, Vol. 37, No. 3 (1979): 402-408.

[128]Z. Zosim, E. Rosenberg, and D. L. Gutnick, "Changes in hydrocarbon emulsification specificity of the polymeric bioemulsifier emulsan: effects of alkanols," *Colloid and Polymer Science*, Vol. 264, No. 3 (1986) 218-223.

Table 16. Reproducibility Evaluation of Immersion Bath Test

| Cleaning solution | Mass of contaminant applied (g) | Mass of contaminant removed (g) | Percent of contaminant removed (%) |
|---|---|---|---|
| Pure deionized water | 64.80 | 29.90 | 46.14 |
| Pure deionized water | 55.00 | 32.10 | 58.36 |
| Pure deionized water | 55.90 | 30.20 | 54.03 |
| Pure deionized water | 57.30 | 28.90 | 50.44 |
| Pure deionized water | 56.10 | 31.50 | 56.15 |
| | | Standard deviation: | 4.83 |
| 50 mg/L of sophorolipid and 0.05% DA-630 | 57.30 | 41.90 | 73.12 |
| 50 mg/L of sophorolipid and 0.05% DA-630 | 55.10 | 39.80 | 72.23 |
| 50 mg/L of sophorolipid and 0.05% DA-630 | 57.80 | 39.00 | 67.47 |
| | | Standard deviation: | 3.04 |
| 83 mg/L of emulsan and 0.05% DA-630 | 56.10 | 36.00 | 64.17 |
| 83 mg/L of emulsan and 0.05% DA-630 | 58.10 | 52.90 | 91.05 |
| 83 mg/L of emulsan and 0.05% DA-630 | 55.60 | 42.60 | 76.62 |
| | | Standard deviation: | 13.45 |
| 0.05% DA-630 | 56.20 | 15.50 | 27.58 |
| 0.05% DA-630 | 53.20 | 21.80 | 40.98 |
| 0.05% DA-630 | 55.90 | 20.10 | 35.96 |
| | | Standard deviation: | 6.77 |
| 10% Inproclean 2500 | 55.30 | 52.60 | 95.12 |
| 10 % Inproclean 2500 | 52.60 | 45.80 | 87.07 |
| 10% Inproclean 2500 | 57.30 | 49.90 | 87.09 |
| | | Standard deviation: | 4.64 |

## Fluorescing Analysis

After the coupons were cleaned, dried, and weighed they were placed under a fluorescent lamp. The oil remaining on each coupon was observed and recorded. The results are listed in Table 17.

Table 17. Results of Fluorescing Analysis

| Cleaning solution | Contamination observed after cleaning |
|---|---|
| 875 mg/L of emulsan | contaminated throughout the surface with the edges clean |
| 1% DA-630 | a few tiny drops and a small, light smear |
| 2% SN-90 | a few tiny drops and a smear similar to coupon cleaned in 1% DA-630 |
| Emulsification formulation alone | contamination remained in the area were it was applied; contamination ran toward bottom of the coupon |
| 875 mg/L of emulsan in the emulsification formulation | large, solid contaminated area in the center; splotchy, large drops elsewhere with some tiny drops |
| 875 mg/L of emulsan and 2% SN-90 | about ten small drops at the top of the coupon; clean everywhere else; slightly larger drops than first few coupons |
| 1% DA-630 and 2% SN-90 | a few tiny drops and a small, light smear |
| Pure deionized water | contaminated throughout the surface except around the edges |

Discussion of Results

Emulsan, sophorolipid, and Emulsan™ alone did not exhibit the ability to solubilize or roll-up the contaminant under the conditions of this test. This was confirmed by the gravimetric analysis (see Table 15) because each solution provided a percent contaminant removal in the range of that provided by deionized water.

The solutions containing 1%, 0.37%, and 0.185% DA-630 provided greater than 96% contaminant removal (see Table15). From the observations made on the 1% solution (Appendix B), roll-up was an apparent mechanism in the removal of oil. Solubilization is difficult to observe in the presence of roll-up because it is not visible. There was no difference between the percent contaminant removed by twice the CMC and that removed by more than ten times the CMC (see Table 15). The reaction of 2% SN-90 was similar to these three DA-630 solutions.

The solutions that were below the critical micelle concentrations of the ethoxylated alcohol surfactants resulted in lower contaminant removal (see Table 15). Except for the solution containing 0.06 % DA-630, these solutions provided less contaminant removal than pure deionized water. This may be attributed to some lowering of the interfacial tension significant enough to prevent some viscous shearing of the oil from the surface of the coupon but not significant enough to provide roll-up. Solubilization was not a consideration at these concentrations due to the absence of micelles required to adsorb the oil molecules.

The solutions containing an ethoxylated alcohol and a biosurfactant were used to evaluate emulsan and sophorolipid as co-surfactants. For the solutions containing ethoxylated alcohols well above their CMC value, no increase in the percent contaminant removal was noted (see 15). Because the surfactants alone provided a high percent removal, they masked any added effect provided by the

biosurfactants. However, the solutions that included lower concentrations of DA-630 (70 mg/L of sophorolipid and 0.06% DA-630, 50 mg/L of sophorolipid and 0.05% DA-630, etc.) showed an increase in percent contaminant removal over the solutions containing only DA-630 at these concentrations (see Table 15). Oil was observed to be rolled-up from the surface of the coupons and float to the top of the baths (see Appendix B). This did not occur with the single surfactant solutions at these concentrations.

The solution combining the emulsification formulation and emulsan was more effective at cleaning than the solution containing emulsan in water (see Table 15). However, the bath containing only the emulsification formulation provided similar results to the bath containing emulsan in the emulsification formulation. Subsequent surface tension testing of the emulsification formulation revealed a value of 45 dynes/cm which by itself would affect the cleaning mechanisms.

As shown in Table 16, the standard deviations (4.83, 3.04, 13.45, 6.77, and 4.64%) confirm that the immersion test was able to provide reproducible results. Because the variance was small, the percent contaminant removals were able to show differences between cleaning solutions. For example, the three solutions containing 83 mg/L of emulsan and 0.05% DA-630 provided an average percent contaminant removal of 77.28 with a standard deviation of 13.45, while the three solutions containing 0.05% DA-630 alone provided an average percent contaminant removal of 34.84 with a standard deviation of 6.77. This proves that the test was able to indicate differences in cleaning abilities.

## 7.5 IMMERSION WITH MECHANICAL AGITATION

This test was used to further explore the effectiveness of emulsan and sophorolipid at performing roll-up, solubilization and emulsification. The addition of mechanical energy was employed to potentially aid in the emulsification and roll-up processes.

Procedure

Six galvanized, stainless steel coupons were precleaned, dried and weighed. The coupons were contaminated with equivalent volumes of C. I. Hayes Quench Oil. The oil was applied to the coupons using an eye dropper as described in the immersion test. The oil was allowed to age on the coupons for one hour. The mass of each contaminated coupon was measured and recorded. The coupons were lowered into a beaker containing 500 mL of solution. The six solutions were: 0.4 g/L of emulsan (sample #2) in deionized water; 0.4 g/L of emulsan (sample #2) in the emulsification formulation, described in the immersion test; the emulsification formulation alone; pure deionized water; 0.04% Rhodasurf DA-630 in deionized water; 10% Inproclean 2000 in deionized water.

Mechanical energy was added to the cleaning solutions using a stir bar. The stir rate of the six solutions was kept constant. The coupons were lowered into the solutions while the baths were being agitated and remained submerged for fifteen minutes. The reaction of the contaminant with each bath was observed and recorded. The coupons were removed, dried in an oven for fifteen minutes at

140°F and allowed to cool to ambient temperature. The mass of each coupon was then remeasured and recorded. The coupons were then placed under a fluorescing lamp. The relative amount of oil remaining on the coupon was observed and recorded.

Table 18. Results of Gravimetric Analysis for Immersion with Mechanical Agitation Test

| Cleaning solution | Mass of contaminant applied (g) | Mass of contaminant removed (g) | Percent of contaminant removed (%) |
|---|---|---|---|
| 400 mg/L of emulsan | 0.0924 | 0.0808 | 87.45 |
| 400 mg/L of emulsan in the emulsification formulation | 0.1000 | 0.0885 | 88.50 |
| Emulsification formulation alone | 0.0918 | 0.0852 | 92.81 |
| Pure deionized water | 0.0913 | 0.0797 | 87.29 |
| 0.04% DA-630 | 0.1003 | 0.0962 | 95.91 |
| 10% Inproclean 2000 in water | 0.1020 | 0.1012 | 99.22 |

Results

The results of gravimetric analysis are given in Table 18. The results of fluorescing analysis are given in Appendix B.

Discussion of Results

It may be concluded from the observations made during the immersion procedure and the fluorescing analysis that the orientation of the contaminant remaining on the coupon during and following the cleaning process was a function of the flow conditions of the cleaning bath. The stir bar was rotating in a clockwise direction causing a vortex in the bath in the same direction. As a result, the bulk contaminant on the side of the coupon contacted by the vortex was sheared off the coupon by hydrodynamic forces. The bulk of the contaminant remaining on the coupons after the immersion process was located on the sides of the coupon that were shielded from the flow of the bath (see Appendix B). This may be attributed to the buoyant forces of the oil.

For the six samples evaluated, there was only a 11.77% difference in percent contaminant removed (See Table 18). Because this is less than the highest standard deviation obtained in the reproducibility test (13.45%, See Table 16), this test was not successful at showing differences between the solutions.

## 7.6 SURFACE TENSION

In addition to the surface tension testing of emulsan and sophorolipid discussed in Chapter 3, tests were performed on the ethoxylated alcohol surfactants to determine CMC. Also, surface tension testing was done on some of the surfactant solutions used in the coupon screening and immersion tests to further define the characteristics.

Procedure

The solutions were allowed to stand overnight. The solutions containing emulsan were refrigerated during this time period. All of the solutions were stirred and allowed to stand for five minutes before measuring surface tension.

The surface tension of each solution was measured using a Fisher Scientific Tensiometer, Surface Tensiomat-21. The procedure used to determine the surface tension is described in the ASTM Standard Test Method for Surface and Interfacial Tension of Solutions of Surface-Active Agents, test number D 1331-89. Five readings of each solution were made for accuracy. The average of these values was recorded as the surface tension of that solution.

The solutions containing the ethoxylated alcohol surfactants had surface tensions at or below 30 dynes/cm, while the surface tension of solutions containing only emulsan or sophorolipid had surface tensions above 43 dynes/cm.

The ASTM method states that the glassware and the platinum ring of the tensiometer should be precleaned using a chromic-sulfuric acid mixture. Chromic-sulfuric acid is considered a toxic chemical under the Massachusetts Toxics Use Reduction Act. A main function of the TURI and the SCL is to promote the use of alternatives to solvent cleaning. In accordance with this a nontoxic precleaning procedure for the equipment used in the experiment was designed and implemented. The equipment was cleaned using an aqueous cleaning solution. It was then rinsed with deionized water and dried under air knives. The accuracy of the equipment cleaned with the nontoxic alternative was monitored by measuring the surface tension of deionized water (72-73 dynes/cm) after every cleaning procedure.

Table 19. Surface Tension of Various Solutions

| Cleaning Solution | Surface Tension (dyne/cm) |
|---|---|
| 0.031% Witconol SN-90 | 30.3 |
| 0.37% Rhodasurf DA-630 | 27.1 |
| 0.185% Rhodasurf DA-630 | 27.2 |
| 332 mg/L of emulsan | 58.0 |
| 166 mg/L of emulsan | 65.6 |
| 13.33 mg/L of sophorolipid | 43.1 |
| 6.67 mg/L of sophorolipid | 47.0 |
| 133.33 mg/L of Emulsan™ | 46.6 |
| 66.67 mg/L of Emulsan ™ | 49.2 |
| 10 % Valtron SP2275 | 34.7 |
| 2 % Witconol SN-90 and 75 mg/L of emulsan | 30.8 |
| 332 mg/L of emulsan and 0.37% DA-630 | 27.2 |
| 166 mg/L of emulsan and 0.185% DA-630 | 27.2 |
| 13.33 mg/L of sophorolipid and 0.37% DA-630 | 27.1 |
| 6.67 mg/L of sophorolipid and 0.185% DA-630 | 27.2 |
| 133.33 mg/L of Emulsan™ and 0.37% DA-630 | 27.1 |
| 66.67 mg/L of Emulsan™ and 0.185% DA-630 | 27.2 |
| Pure deionized water | 73.0 |

Results

The surface tension of solutions used in the coupon screening and immersion tests are given in Table 19. Solutions containing Wiconol SN-90, Rhodasurf DA-630 and Valtron SP2275 were effective at significantly lowering the surface tension (below 35 dynes/cm). While sophorolipid, Emulsan™, and emulsan lowered the surface tension (43.1 to 58 dynes/cm), alone they did not achieve the 35 dynes/cm that is typical of petroleum- and oleo-based surfactants used in aqueous metal cleaning.

## 7.7 GENERAL DISCUSSION

The goals of the lab work in the TURI's SCL were threefold:

- assess the ability of the biosurfactants to solubilize or roll-up contaminants from metal surfaces,
- evaluate the ability of the biosurfactants to act as co-surfactants or enhance the performance of another surfactant,

- determine if emulsan's emulsification ability alone was enough to clean a metal surface.

When alone in solution, neither the two samples of emulsan nor the one sample of sophorolipid evaluated exhibited the ability to solubilize or roll-up contaminants under the conditions of the test. However, when combined with an ethoxylated alcohol surfactant, at concentrations well below its CMC, increased oil removal was observed both qualitatively (visual observations and fluorescing analysis) and quantitatively (gravimetric analysis). This was attributed to the solubilization of oil by the mixed micelles formed by these combined solutions. Also, roll-up was aided in these solutions by the surface tension lowering properties of the surfactant and biosurfactant combination. The emulsification ability of emulsan alone was not enough to remove oil from the substrate under the conditions tested.

## 7.8 CHAPTER SUMMARY

A summary of the SCL work is given in Table 20. In this work, two samples of emulsan and one sample of sophorolipid were evaluated. The emulsan samples exhibited the ability to lower the surface tension of water to 50 dynes/cm and the interfacial tension between water and hexadecane to 17 dynes/cm. Because greater reductions in interfacial tensions are required to cause roll-up, it would be beneficial to combine emulsan with a surfactant with better interfacial tension lowering properties when including emulsan in a cleaner formulation. Because emulsan is an emulsion stabilizer and does not greatly reduce the interfacial tension, mechanical energy is required to initially form an emulsion so the use of a cosurfactant should also be considered when using emulsan as an emulsifying agent. The interfacial tension lowering properties of another surfactant would require less mechanical energy to be added to form the emulsion.

The sophorolipid sample exhibited promising surface tension lowering properties, but it did not exhibit the ability to solubilize contaminants or to cause roll-up. Additional synthesis and purification research on sophorolipid should be done to obtain samples with better surface tension lowering properties. This would enhance sophorolipid's ability to cause roll-up.

The use of emulsan and sophorolipid as cosurfactants proved successful at providing increased oil removal under the conditions of the immersion test. Additional work in this area is suggested to explore this phenomenon over a range of surfactant concentrations.

Table 20. Summary of Experiments

| Experiment Title | Objective | Procedure | Results |
|---|---|---|---|
| Coupon screening test: emulsan, sophorolipid, an aqueous cleaner, and a surfactant | To observe emulsan and sophorolipid's cleaning abilities<br><br>To compare their cleaning abilities to those of an aqueous cleaner and a surfactant | Contaminate coupons with oils<br><br>Apply solutions to coupons with a dropper and observe reactions | Aqueous cleaner broke up the oil<br><br>Emulsan and sophorolipid solutions did not spread on the surface of the coupon; did not break up oil<br><br>Visual activity of the emulsan/surfactant combination identical to the surfactant solution |
| Immersion testing | To test emulsan and sophorolipid's abilities to remove oil from the substrate in an immersion bath<br><br>To compare activities of emulsan, sophorolipid , a surfactant, emulsan in an emulsification formulation, and combinations of each | Oil-contaminated coupons were immersed in the baths for one hour<br><br>Gravimetric and fluorescing analyses were performed | Increased cleaning was realized in the baths containing emulsan and sophorolipid as cosurfactants<br><br>The cleaning ability of the emulsification formulation was not improved by the addition of emulsan |
| Immersion with mechanical agitation testing | To evaluate the impact of agitation on emulsan and sophorolipid's cleaning abilities | Oil-contaminated coupons were lowered into agitated cleaning baths for fifteen minutes<br><br>Gravimetric and fluorescing analyses were performed | Test results were masked by the shearing forces of the mechanical agitation |
| Surface tension testing | To measure the surface tension of the solutions used in previous experiments | The surface tension of each solution was measured using a tensiometer, results in dynes/cm | Approximate values - Deionized water: 73 , Emulsan: 50 Sophorolipid: 37 Aqueous cleaner solution: 35 Surfactant solutions: 30 |

# CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

## 8.0 CONCLUSIONS

The potential for using biopolymers as replacements for nonrenewable resource-based materials has yet to be realized. While biosurfactants offer several potential advantages over their synthetic counterparts a significant challenge lies ahead in developing, producing, and applying biosurfactants in ways that are truly competitive with existing products. The research described here has made progress toward this objective.

This chapter begins with technical conclusions from the research. These conclusions directly address the primary objectives articulated in the introduction of this report. Next, more general conclusions, reflecting on the overall project are presented. The chapter concludes with recommendations for future research.

## 8.1 TECHNICAL CONCLUSIONS

Biosurfactant Development

Emulsan analogues were characterized by quantifying surface tension, interfacial tension and turbidity values. There were differences between emulsan analogues for all of the characterization test values. These differences can be attributed to changes in chemical structure and composition that occur when the polymer is produced from different feedstocks.

For the nine emulsan analogues tested, there was a spread of approximately 10 dynes/cm in surface tension values and a spread of approximately 5 dynes/cm in interfacial tension values. The emulsan grown on methyl myristate had the best surface tension lowering properties (~50 dyne/cm at a concentration of 83 mg/L). Emulsan grown on methyl myristate was most effective at lowering the interfacial tension between hexadecane and water (~17 dyne/cm at a concentration of 83 mg/L) at the concentrations evaluated.

Emulsan grown on heptyloxyoctanoic acid/myristic acid had the highest turbidity of the analogues tested (550 Klett Units at a concentration of 500 mg/L) indicating higher emulsification activity than the other analogues synthesized and evaluated. Emulsan grown on methyl myristate had the second highest emulsification activity (490 Klett Units at a concentration of 500 mg/L) of the analogues synthesized and evaluated.

There is no direct correlation between surface tension and interfacial tension values; likewise there is no correlation between surface tension and turbidity values for a given emulsan analogue. An

emulsan analogue grown on a given substrate will have a specific affinity for a given hydrocarbon-water interface. As a result, interfacial tension and turbidity measurements depend on the oil used in measuring these parameters. Since surface tension is tested in the absence of oil (i.e., it is measured using water and emulsan alone) each emulsan analogue will have only one associated surface tension value.

The pH of the testing solution had little effect on the surface active properties of emulsan. This is evidence that the polymer is stable over the range of pH tested, i.e., pH 3 to 10.

The yields of emulsan reported by Shabtai were not realized in this research. It is believed that strain improvement by mutation-selection techniques (i.e., growing the polymers in the presence of another material in order to mutate the bacteria) and adjustment in the fermentation physiological operating parameters will be needed so that truly high emulsan yields from triglycerides can be realized. The continuous batch-fed process yielded the highest purity product.

Three feedstocks were used for the synthesis of sophorolipid: 100 g/L glucose, 10 g/L yeast extract, and 1 g/L urea. The 15 L fermentation resulted in a production yield of approximately 20 g/L of unpurified product. The crude product yield from biomass alone was 15 g/L broth. In surface tension testing, the surface tension dropped significantly with the addition of small amounts of sophorolipid (at a concentration of 3.33 mg/L the surface tension is 44.6 dyne/cm ). The surface tension curve for sophorolipid leveled out at approximately 37 dyne/cm at a concentration of 35 mg/L. This reduction in surface tension is significantly greater than that achieved by emulsan.

Production Costs

Using the design software system BioPro Designer® from Intelligen Inc., the selling price of emulsan (based on an ROI of 30%) was evaluated at production scales of 10,000, 100,000, and 1,000,000 L and yields of 5, 25 and 50 g/L. The optimum plant size was approximately 100,000 L (25g/L yield) which set the selling price of emulsan at $0.08/g. Meaningful price comparisons between emulsan and potential competitor surfactants are not possible at this time since it is not possible to compare, with any level of confidence, the gram-per-gram usage of emulsan vs. market surfactants in a cleaning formulation (or in any other application).

Environmental, Health and Regulatory Issues

Biosynthesized polymers offer great promise in reducing both the environmental and worker health and safety impacts caused by the production and use of traditional synthetic chemicals. However, hazardous materials can be used in the production of biopolymers. For example, this research demonstrated that an expedient way to purify and extract biosurfactants is with pentane--a hazardous solvent. Cleaner manufacturing alternatives need to be sought out early and precautions must be taken to ensure that new hazards are not created.

While the federal framework for regulating the biotechnology industry is under modification and has not been fully challenged by a large number of submissions, government agencies are making a conscious effort to adapt and accommodate the regulatory system to these products. It is also clear that biotechnology processes and products pose new and unexplored risks that warrant regulatory oversight, investigation, and strict health and safety guidelines until these risks are better understood. There is a strong need for greater federal and state coordination, oversight, and clarification on biotechnology environmental and health and safety regulations.

Biosurfactants in Surface Cleaning Applications

The use of biosynthesized surfactants in aqueous metal degreasing formulations presents a market challenge. Aqueous cleaners, whether they contain petroleum-based components or not, are considered a green alternative to their chlorinated solvent counterparts. Furthermore, many non-biosynthesized surfactants used in aqueous cleaners today are called "biodegradable." However, there is an ongoing debate over the true biodegradability of aqueous cleaners as well as concern over their VOC content. This debate may effectively set the stage for the emergence of biopolymers as a truly biodegradable component for the aqueous cleaner market.

The emulsan samples exhibited the ability to lower the surface tension of water to 50 dynes/cm and the interfacial tension between water and hexadecane to 17 dynes/cm. Surfactants used in surface cleaning typically reduce the surface tension to approximately 35 dynes/cm and the interfacial tension to 1-2 dynes/cm. Emulsan may need to be combined with another surfactant to achieve a low enough surface tension to effectively clean a surface. Test results suggested that emulsan, when combined with another surfactant, was more effective at cleaning than either surfactant alone. For use as an emulsion stabilizer, emulsan must be combined with either mechanical energy or with another surfactant that further reduces the interfacial tension between water and oil.

The sophorolipid sample exhibited promising surface tension lowering properties, but it did not exhibit the ability to solubilize contaminants or to cause roll-up. Additional synthesis and purification research on sophorolipid is needed to obtain samples with better surface tension lowering properties. This would enhance the roll-up ability of sophorolipid.

Technology Transfer

While potentially a rich source of information and collaboration in product development, our efforts to involve potential biopolymer producers did not yield results. However, a working relationship was established between TURI and Emulsan Biotechnologies, Inc. This relationship helped to guide some of the testing in the Surface Cleaning Lab.

Other Potential Uses for the Emulsan and Sophorolipid

Emulsan™ has been successful in aiding viscous fluid transport and in making No. 2 fuel burnable as No. 6 fuel. Some of the emulsan patents demonstrate emulsan use in personal skin care products

such as acne soaps and skin cream. The polymer is said to possess anti-adherent properties which prevent bacteria from adhering to the skin. Emulsan was used to clean an oil transport ship hull by emulsifying the residual oil and allowing it to be pumped. In the same manner, emulsan could be successful at emulsifying oil to make it available for biodegradation, perhaps for oil spills at sea. According to Emulsan Biotechnologies, Inc., emulsan could be used in this way to "clean" oil from a piece of equipment, such as a lawnmower, and would prevent the occurrence of an oil slick.

Basically, emulsan has potential anywhere that a stable emulsion of a compatible hydrocarbon is desired. For instance, emulsan could be used in soluble emulsion oils for machining fluids, invert solvents (emulsion cleaners), or floor polishes where the polishing agent is emulsified in water.

Due to its small molecular size, sophorolipid could be used for the removal of organics from soils. A biosurfactant would be ideal in this case since it would completely biodegrade in the soil; non-biodegradable surfactants used in this application would leave a residue. In general, sophorolipid could be substituted for synthetic nonionic surfactants in applications such as laundry and dish detergents, dyes, and personal care products.

## 8.2 CONCLUSIONS RELATED TO THE OVERALL RESEARCH COLLABORATION

A Tale of New Product Development

Ideally, the development of a new biopolymer would begin with laboratory research where material synthesis, characterization, and scale-up would yield a product perfectly matched to a clearly-defined commercial application. The biopolymer would then be transferred to a manufacturer and end-product formulator for development and production of a commercial product. Unfortunately, a linear path to new product development is infrequently achieved, and this research effort was a case-in-point.

The project began with an expectation that the biosurfactant emulsan could be formulated into an effective aqueous cleaner. Research work at TURI began with some initial screening tests to evaluate the effectiveness of emulsan alone as a surface active agent. When the results of this work showed no surface activity, it quickly became evident to the TURI research team that not enough was known about whether and how the biopolymer would function in an aqueous cleaning chemistry. Does emulsan affect the surface tension of a liquid? Would emulsan increase the emulsifiability of a formulation and would this property be an advantage in a cleaning chemistry? Much work had to be done to identify the unique qualities of emulsan, i.e., to characterize its surface tension and emulsification properties, and then to formulate the biopolymer with other components that would enhance or complement its action. This need to "step back", to establish a firmer foundation of knowledge about the biopolymers, required significant research effort and inhibited us from achieving all we had expected to achieved in the time allowed.

At the same time, the BPRC's difficulty in obtaining reasonable emulsan yields in their scale-up efforts called into question the viability of the commercial-scale production of the polymer at this early stage of development. Based on the characterization and scale-up results, BPRC researchers chose to alter their course somewhat to evaluate a small molecule biosurfactant--sophorolipid--that promised better surface tension lowering ability and lower production cost. This new direction was chosen as a result of the findings of the characterization work and the surface cleaning experiments. Given more time, this report might have included more results regarding sophorolipid scale up, characterization, cleaning tests, economic and environmental health and safety analysis. The BPRC and the BDC are continuing to pursue work in this area.

Collaboration Across the University

This project succeeded in bringing together three research centers at the university, engaged in different stages of new product development - synthesis, commercial production and application. The collaboration of these three groups challenged and enriched the project. Through basic research, the BPRC provided the biopolymers, knowledge and capabilities in biopolymer characterization. By defining the requirements of a surface cleaning chemistry, TURI added direction to the characterization work and provided feedback to the BPRC on the strengths and weaknesses of the biopolymers with regard to commercial applications. The BDC's production cost estimates influenced the work of the BPRC in improving yield and evaluating a lower cost polymer. In addition, the BDC's production system design efforts led all three centers to explore less hazardous synthesis, purification and production process options.

## 8.3 RECOMMENDATIONS

Based on our research, it appears that more research and development is needed to increase the surface and interfacial tension lowering abilities of these biosurfactants. If this can be achieved, biosurfactants will stand a greater chance of gaining entry into the aqueous cleaning market. Specifically, for cleaning applications, additional research should be conducted to develop a sophorolipid analogue with a surface tension below 35 dynes/cm.

More research is needed to improve the performance of scale-up efforts--from the laboratory to pilot-scale production--to increase yield and lower production costs. Since most of the cost to produce a biopolymer is incurred in the purification process, this process in particular must be optimized to make biopolymer production cost effective and competitive with existing products.

Biopolymer developers should critically evaluate the proposed steps for biopolymer production so that in the quest to produce a "green product" only "green processes" are used. This research demonstrated that hazardous solvents could be used for purification and extraction of biopolymers in commercial-scale production, and furthermore, that there are viable, cleaner alternative processes (like supercritical $CO_2$ extraction) that can improve the life-cycle profile of these new materials. These issues should be considered and addressed at the earliest stages of new product development.

## BIBLIOGRAPHY


Abdul, Abdul.S., Thomas L. Gibson and Devi N. Rai, "Selection of Surfactants for the Removal of Petroleum Products from Shallow Sandy Aquifers," *Ground Water,* Vol.28, No.6 (November-December, 1990): 920-926.

Ainsworth, Susan J., "Soaps and Detergents," *C&EN* (January, 1995).

American Society for Metals, *Metals Handbook*, Desk Edition, Metals Park, Ohio: ASM, 1985.

American Society for Testing and Materials (ASTM), "Standard Test Method for Interfacial Tension of Oil Against Water by the Ring Method," D971, 1991.

American Society for Testing and Materials (ASTM), "Standard Test Methods for Surface and Interfacial Tension of Solutions of Surface-Active Agents," D1331, 1989.

Anastas, P., and C. Farris, *Benign By Design: Alternative Synthetic Design for Pollution Prevention.* Washinton, D.C.: American Chemical Society, 1994.

Andrup, L., et. al., "Biosafety Considerations in Industries with Production Methods Based on the Use of Recombinant Deoxyribonucleic Acid," *Scandinavian Journal of Work Environment Health,* Vol.16 (1990).

Asmer, H., S. Lavy, F. Wagner and V. Wray, "Microbial Production, Structure, Elucidation and Bioconversion of Sophorose Lipids," *JAOCS*, Vol. 65, No.9 (September, 1988).

Baird, J.K., P.A. Sandford and I.W. Cottrell, "Industrial Applications of Some New Microbial Polysaccharides," *Bio/Technology* (November, 1983).

Bodzek, M., and K. Konieczny, "The Use of Ultrafiltration Membranes Made of Various Polymers in the Treatment of Oil-Emulsion Wastewaters," *Waste Management*, Vol.12 (1992).

Borbely, M., Y. Nagasaki, J. Borbely, K. Fan, A. Bhogle and M. Sevoian, "Biosynthesis and Chemical Modification of poly (γglutamic acid)," *Polymer Bulletin,* Vol.32 (1994).

Brooks, K., and G. Meyer, "Emulsion Know-How Lightens Heavy Oil," *Chemical Week* (February 4, 1987).

Broze, Guy, "Mechanisms of Soil Removal," In Robert K. Large, ed., *Detergents and Cleaners: A Handbook for Formulators*, Cincinnati: Hanser/Gardner Publications, Inc., 1994.

Cairns, William L., David G. Cooper, James E. Zajic, Joan M. Wood and N. Kosaric, "Characterization of *Nocardia amarae* as a Potent Biological Coalescing Agent of Water-Oil Emulsions," *Applied and Environmental Microbiology*, Vol.43, No.2 (February, 1982).

Chemical Marketing Reporter, "Surfactant Makers Push Exports to U.S.," *Chemical Marketing Reporter* (September, 1994).

Chemical Week, "Specialty Surfactants: A Restructured Industry Turns to Product Development" (September 28, 1994): 25-28.

Chen, Gordon C., "Application of a Surfactant as a Kraft Pulping Additive," *Tappi Journal*, Vol.77, No.2 (February, 1994).

Cirigliano, Michael C., and George M. Carman, "Isolation of Bioemulsifier from *Candida liolytica*," *Applied and Environmental Microbiology*, Vol.48, No.4 (October, 1984).

Cohen, L.E., and J.A. Hook, "Optimization of Alkaline Soak Cleaners for Ferrous Metal Surfaces," *Plating and Surface Finishing* (March 1985).

Cooper, David G., and Beena G. Goldenberg, "Surface-Active Agents from two *Bacillus* Species," *Applied and Environmental Microbiology*, Vol.53, No.2 (1987).

Cooper, David G., and D.A. Paddock, "Production of a Biosurfactant from *Torulopsis bombicola*," *Applied and Environmental Microbiology* (January, 1984): 173-176.

Cooper, David G., James E. Zajic and Donald F. Gerson, "Production of Surface-Active Lipids by *Corynebacterium lepus*," *Applied and Environmental Microbiology*, Vol.37, No.1 (January 1979).

Davila, A.M., R. Marchal, N. Monin and J.P. Vandecasteele, "Identification and Determination of Individual Sophorolipids in Fermentation Products by Gradient Elution High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection," *Chromatography*, Vol. 648 (1993).

Desai, Jitendra D., "Microbial Surfactants: Evaluation, Types, Production and Future Applications," *Journal of Scientific & Industrial Research*, Vol. 46 (1987): 443-444.

"Detergents '93," *Chemical Marketing Reporter* (January 1993): SR3-34.

Dutkiewicz, et. al., "Occupational Biohazards: A Review," *American Journal of Industrial Medicine*, Vol.14 (1988): 605-623.

Elliott, L., et. al., "Perspectives on Opportunities Toward a Hazard-Free Bioprocessing Environment," in W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations*, Philadelphia: American Society for Testing and Materials, 1990.

Emulsan Biotechnologies, *Material Safety Data Sheet for Emulsan™ Brand Polysaccharide Biopolymer*, Greens Farms, CT, 1995.

Fiechter, Armin., "Biosurfactants: Moving Towards Industrial Application," *TIBTECH,* Vol. 10 (1992).

Flam, F., "EPA Campaigns for Safer Chemicals," *SCIENCE*, Vol. 265 (September, 1994).

Flick, Ernest W., *Advanced Cleaning Product Formulations: Vol. 2*, New Jersey: Noyes Publications, 1994.

Frantz, et al., "Toxics Use in Biotechnology: Capstone III Project," Medford, Mass: Tufts University School of Engineering, 1992.

Fuchs, John F., "Ultrasonic Cleaning - Fundamental Theory and Application," *Precision Cleaning '95 Proceedings*, 1995.

Gôbbert, U., S. Lang and F. Wagner, "Sophorose Lipid Formation by Resting Cells of *Torulopsis bombicola*," *Biotechnology Letters*, Vol. 6, No. 4 (1984).

General Services Administration, U.S. Environmental Protection Agency, "Guidance Document For Reporting Information on Environmental Attributes of Cleaning Products and Their Components," August, 1995.

Georgiou, George, Sung-Chyr Lin and Mukul M. Sharma, "Surface-Active Compounds From Microorganisms," *Bio/Technology*, Vol. 10 ( January 1992).

Ghurye, G., 1993, "Production and Characterization of Biosurfactant and its effect on the Biodegradation of Toluene," Master Thesis, University of Houston.

Ghurye, G.L., C. Vipulanandan and R.C. Wilson, "A Practical Approach to Biosurfactant Production Using Nonaseptic Fermentation of Mixed Cultures," *Biotechnology and Bioengineering,* Vol.44 (1994).

Giamporcaro, D., Section Chief, TSCA Biotechnology Program, U.S. Environmental Protection Agency, Personal communication, August 29 and October 17, 1995.

Giannos, Steven A., D. Shah, Richard A. Gross, (University of Lowell, MA), David L. Kaplan, S. Arcidiacono and Jean M. Mayer, (U.S. Army Natick Research, MA), "The Biosynthesis of Unusual Polyamides Containing Glutamic Acid."

Goldman, S., C. Shabtai, C. Rubinovitz, E. Rosenburg and D.L. Gutnick, "Emulsan in *Acinetobacter calcoaceticus* RAG-1: Distribution of Cell-Free and Cell-Associated Cross Reacting Material," *Applied and Environmental Microbiology*, Vol. 44, No. 1 (July 1982).

Gross, Richard A., "Control of Side Chain Fatty Acid Composition for the Natural Bioemulsifier Emulsan Produced by *Acinetobacter Calcoaceticus* Strain." In the *Proceedings of 208th ACS National Meeting Held in Washington, D.C., 21-25 August 1994, Vol. 34 No. 2* , American Chemical Society, Division of Environmental Chemistry, 1994.

Gutnick, David L., and Yossef Shabtai, "Exopolysaccharide Bioemulsifiers,"in Kosaric, N., W.L. Cairns and N. Gray, eds., *Biosurfactants and Biotechnology*, New York: Marcel Dekker, Inc, 1987.

Hayes, Michael E., E. Nestaas and Kevin R. Hrebenar, "Microbial Surfactants," *CHEMTECH*, (April 1996).

Herd, M.D., G.D. Lassahn, C.P. Thomas, G.A. Bala and S.L. Eastman, EG&G Idaho Inc., "Interfacial Tensions of Microbial Surfactants Determined by Real-Time Video Imaging of Pendant Drops," Society of Petroleum Engineers/ U.S. Department of Energy (SPE/DOE), 24206.

Hikichi, K., H. Tanaka and A. Konno, "Nuclear Magnetic Resonance Study of Poly(γ-glutamic acid) Cu(II) and Mn(II) Complexes," *Polymer Journal*, Vol. 22, No. 2 (1990).

Hommel, R., O. Stüwer, W. Stuber, D. Haferburg and H.P. Kleber, "Production of Water-Soluble Surface-Active Exolipids by *Torulopsis apicola*," *Applied Microbiology and Biotechnology*, Vol. 26 (1987).

"Hot Prospects," *Chemical Week* (December 21/28, 1994).

Houghton, E., "Aqueous Parts Cleaning Solution Management," *Precision Cleaning '95 Proceedings*, 1995.

Javaheri, M., Gary E. Jenneman, Michael J. McInerney and Roy M. Knapp, "Anaerobic Production of a Biosurfactant by *Bacillus licheniformis* JF-2," *Applied and Environmental Microbiology*, Vol. 50, No. 3, September, 1985.

Kissa, Erik, "Kinetics of Soiling and Detergency," In Gale W. Culer and Erik Kissa, eds., *Detergency: Theory and Technology*, New York: Marcel Dekker, 1987.

Koelsch, James R., "Guilt-Free Parts Cleaning," *Manufacturing Engineering* (March 1995).

Kosaric, N., N. Gray and W.L. Cairns, "Introduction: Biotechnology and the Surfactant Industry," in N. Kosaric, W.L. Cairns and N. Gray, eds., *Biosurfactants and Biotechnology*, New York: Marcel Dekker, Inc, 1987.

Krivyakina, M., "ICI Unveils Two New Surfactant Products at Seminar," *Chemical Marketing Reporter* (March 1995).

Kubota, H., Y. Nambu,and T. Endo, "Convenient and Quantitative Esterification of Poly (γ-glutamic acid) Produced by Microorganisms," *Journal of Polymer Science:* Part A: Polymer Chemistry, Vol. 31 (1993).

Landrigan, P., et. al., "Medical Surveillance of Biotechnology Workers: Report of the CDC/NIOSH Working Group on Medical Surveillance for Industrial Applications of Biotechnology," *Recombinant DNA Technical Bulletin,* Vol. 5, No. 3 (publication no 82-99), U.S. Department of Health and Human Services, National Institute of Health, 1982.

Laughlin, Robert, "Solubilization by Solutions of Surfactants: Micellar Catalysis," *The Aqueous Phase Behavior of Surfactants*, London and San Diego: Academic Press, 1994.

Lewis, R., *Sax's Dangerous Properties of Industrial Materials*, New York:Van Nostrand Reinhold, 1992.

Magdich, P., *Oil-Water Separation Techniques: A Literature Review*, Minnesota Technical Assistance Program, University of Minnesota, July, 1986.

Martinez, K., et al., "General Considerations for Work Practices and Personal Protective Equipment in Biotechnology Industries," In W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations*, Philadelphia: American Society for Testing and Materials, 1990.

Marts, K., and J. Howard, "Alternative Cleaning Takes Flight in Aerospace Industry," *Precision Cleaning*, (November-December 1993).

McLaughlin, M., "Selecting an Aqueous Detergent Cleaner: Considerations in Metal-Part and Electronic-Component Manufacturing," *Precision Cleaning '95 Proceedings*, 1995.

Merchant, J., *Occupational Respiratory Diseases*, Cincinnati: National Institute for Occupational Safety and Health, Pub.86-102, 1988.

Millar, J.D., NIOSH Comments on OSHA Guidelines on Biotechnology, Docket number H-042, Cincinnati: National Institute for Occupational Safety and Health, 1985.

Miller, Clarence A., and Kirk H. Raney, "Solubilization - Emulsification Mechanisms of Detergency," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, Vol. 74, 1993.

Milmo, S., "A New Generation," *Chemical Marketing Reporter*, January, 1995.

MIT, NJIT, OSU, PSU, "Progress at the Emission Reduction Research Center," *ERRC Update*, September, 1994.

Mittal, K L., and P. Bothorel, *Surfactants in Solution,* New York: Plenum Press, 1984.

Mulligan, Catherine N., and Bernard F. Gibbs, "Recovery of Biosurfactants by Ultrafiltration," *Journal of Chem. Technology & Biotechnology*, Vol. 47 (1990).

Naude, Alice, "Family Resemblance - Detergents '93" *Chemical Marketing Reporter* (January 25, 1993): SR16.

Neufeld, Ronald J.,James E. Zajic and Donald F. Gerson, "Growth Characteristics and Cell Partitioning of *Acinetobacter* on Hydrocarbon Substrates," *Journal of Ferment. Technology*, Vol. 61, No. 3 (1983).

Office of Technology Assessment (OTA), U.S. Congress, *Biopolymers: Making Materials Nature's Way,* Washington, D.C., OTA-BP-E-102, 1993.

Office of Science and Technology Policy (OSTP), "Coordinated Framework for Regulation of Biotechnology; Announcement of Policy and Notice for Public Comment," 51 *Federal Register* No.123 (June 26, 1986): 23302-23350 .

Olenchock, S.A., "Health Effects of Biological Agents: The Role of Endotoxins," *Applied Occupational and Environmental Hygiene,* Vol. 9, No. 1, 1994.

Organization for Economic Co-Operation and Development (OECD), *Safety Considerations for Biotechnology*, Paris: OECD, 1992.

Parkinson, M., "Bio-Surfactants," *Biotechnology Advances*, Vol. 3 (1985).

Peterson, D., "An Introduction to Microfiltration of Aqueous Cleaner Solutions," 16th AESF Pollution Prevention and Control Conference Proceedings, 1995.

Pierce, S., and J. Ortego, "Recent Developments in Biotechnology Regulation," *Toxicology and Environmental Chemistry,* Vol. 40, 1993.

Pines, O., Edward A. Bayer and David L. Gutnick, "Localization of Emulsan-Like polymers Associated with the Cell Surface of *Acinetobacter calcoaceticus*," *Journal of Bacteriology*, Vol. 154, No. 2, May, 1983.

Pines, O., and D. Gutnick, "Role for Emulsan in Growth of *Acinetobacter calcoaceticus* RAG-1 on Crude Oil," *Applied and Environmental Microbiology*, Vol. 51, No. 3, March, 1986.

Quitmeyer, JoAnn, "The Aqueous Cleaning Handbook," Lexington, Mass.: W.R. Grace & Co - Conn., January 1986.

Raney, Kirk H., and H.L. Benson, "The Effect of Polar Soil Components on the Phase Inversion Temperature and Optimum Detergency Conditions," *JAOCS*, Vol. 67, No. 11 (November, 1990).

Reisfeld, A., E. Rosenburg and D. Gutnick, "Microbial Degradation of Crude Oil: Factors Affecting the Dispersion in Sea Water by Mixed and Pure Cultures," *Applied Microbiology*, Vol. 24, No. 3 (September, 1972).

Richards, David, "Going Natural - Detergents '93," *Chemical Marketing Reporter* (January 25, 1993) :SR20-24.

Rosen, Milton J., *Surfactants and Interfacial Phenomena,*New York: John Wiley and Sons, 1978.

Rosenberg, E., "Exploiting Microbial Growth on Hydrocarbons - New Markets," *TIBTECH*, Vol. 11 (October 1993).

Rosenberg, E., A. Perry, T. Gibson and D.L. Gutnick, "Emulsifier of *Arthrobacter* RAG-1: Specificity of Hydrocarbon Substrate," *Applied and Environmental Microbiology,* Vol.37, No. 3 (1979).

Rosenberg, E., Z. Schwartz, A. Tenenbaum, C. Rubinovitz, R. Legman and E.Z. Ron, "A Microbial Polymer That Changes the Surface Properties of Limestone: Effect of Biodispersan in Grinding Limestone and Making Paper," *Journal of Dispersion Science and Technology*, Vol. 10, No. 3 (1989).

Rosenberg, E. , A. Zuckerberg, C. Rubinovitz, and D.L. Gutnick, "Emulsifier of Arthrobacter RAG-1: Isolation and Emulsifying Properties," *Applied and Environmental Microbiology,* Vol. 37, No. 3 (1979).

Samdani, G., and A. Shanley, "Surfactants: Super Molecules," *Chemical Engineering* (March 1991).

Scamehorn, John F., ACS Symposium Series: An Overview of Phenomena Involving Surfactant Systems, Washington, D.C.: American Chemical Society, 1986.

Schramm, Laurier L., *Emulsions: Fundamentals and Applications in the Petroleum Industry*, Washington, D.C.: American Chemical Society, 1992.

Shabtai, Y., and D. L. Gutnick, "Enhanced Emulsan Production in Mutants of *Acinetobacter calcoaceticus* RAG-1 Selected for Resistance to Cetyltrimethylammonium Bromide," *Applied and Environmental Microbiology*, Vol. 52, No. 1 (July 1986): 146-51.

Shabtai,Y., and D. Wang, "Production of Emulsan in a Fermentation Process Using Soybean Oil (SBO) in a Carbon-Nitrogen Coordinated Feed," *Biotechnology and Bioengineering,* Vol. 35 (1990).

Shinoda, K., and S. Friberg, *Emulsions and Solubilization,* New York: John Wiley & Sons, 1986.

Shon, Melissa, "Green and Mild-Mannered - Detergents '94", *Chemical Marketing Reporter* (January 24, 1994): SR22-23.

Society of Manufacturing Engineers, *Tool and Manufacturing Engineers Handbook: Fourth Edition, Vol. 3, Materials, Finishing and Coatings*, 1985.

SRI International, *Chemical Economics Handbook,* 654.500G, May, 1993.

Swartz, Anthony M., and James W. Perry, *Surface Active Agents: Their Chemistry and Technology.* New York: Interscience Publishing, Inc., 1949.

Swarup, S., and Clifford K. Schoff, "A Survey of Surfactants in Coatings Technology," *Progress in Organic Coatings*, Vol. 23 ( 1993.)

Thomas, Karen B., and Michael Ellenbecker, "Evaluation of Alternatives to Chlorinated Solvents for Metal Cleaning," Report to the U.S. Environmental Protection Agency, National Risk Management Research Laboratory, November, 1995.

Toxics Use Reduction Institute, *Closed Loop Aqueous Cleaning, Technical Report No. 29*, Lowell, Mass.: Toxics Use Reduction Institute, 1995.

Tilton, H., "It's Not Pretty," *Chemical Marketing Reporter* (January, 1995).

U.S. Environmental Protection Agency, "Alternatives for CFC-113 and Methyl Chloroform in Metal Cleaning," EPA/400/1-91/019, June, 1991.

U.S. Environmental Protection Agency (EPA), Office of Air and Radiation, "Alternatives for CFC-113 and Methyl Chloroform in Metal Cleaning," June, 1991.

U.S. Environmental Protection Agency (EPA), "Microbial Products of Biotechnology", Proposed Regulation under the Toxic Substances Control Act, 59 Federal Register, No.169, September, 1994.

U.S. Environmental Protection Agency (EPA), Office of Research and Development, "Guide to Cleaner Technologies: Alternatives to Chlorinated Solvents for Cleaning and Degreasing," EPA/625/R-93/016, February, 1994.

U.S. Environmental Protection Agency (EPA), Office of Research and Development, "Guide to Cleaner Technologies: Cleaning and Degreasing Process Changes," EPA/625/R-93/017, February, 1994.

U.S. Environmental Protection Agency (EPA), *Solvent Alternative Guide, Version. 2.1*, 1995.

U.S. Congress, Office of Technology Assessment, *Biopolymers: Making Materials Nature's Way – Background Paper*, OTA-BP-E-102, Washington, D.C.: U.S. Government Printing Office, September, 1993.

United Nations Industrial Development Organization (UNIDO), *International Approach to Biotechnology Safety*, Vienna: UNIDO, 1990.

Van Dyke, Michele I., Susan L. Gulley, H. Lee, Jack T. Trevors, "Evaluation of Microbial Surfactants for Recovery of Hydrophobic Pollutants from Soil," *Journal of Industrial Microbiology,* Vol. 11 (1993).

Van Dyke, Michele I., Hung Lee and Jack T. Trevors, "Applications of Microbial Surfactants," *Biotech. Adv.*, Vol. 9 (1991).

Vranch, S.P., "Containment and Regulations for Safe Biotechnology," In W. Hyer, ed., *Bioprocessing Safety: Worker and Community Safety and Health Considerations*, Philadelphia: American Society for Testing and Materials, 1990.

Watts, Daniel J., "Emission Reduction Research Center Pollution Prevention in Batch Chemical Processing Through Selection of Synthesis Condition and Process Design," Preprint Extended Abstract, presented before the American Chemical Society Division of Environmental Chemistry at the 208th ACS National Meeting, Washington, D.C., August, 1994.

Webster, L., "Environmentally Benign Production of Commodity Chemicals Through Biotechnology: Recent Progress and Future Potential," Unpublished manuscript written as part of an EPA/AAAS Environmental Science and Engineering, 1994.

Wolf, K., "The Truths and Myths About Water-Based Cleaning - A Systems Approach to Choosing the Best Alternatives," *Pollution Prevention Review* (Spring 1994).

Zarcone, Camela, "Degradation Testing - Detergents '94", *Chemical Marketing Reporter* (January 24, 1994): SR8.

Zhou, Qing H., V. Klekner and N. Kosaric, "Production of Sophorose Lipids by *Torulopsis bombicola* from Safflower Oil and Glucose," *JAOCS.*, Vol. 69, No. 1 (January, 1992).

Zosim, Z., D. Gutnik and E. Rosenburg, "Uranium Binding by Emulsan and Emulsanosols," *Biotechnology and Bioengineering*, Vol XXV (1983).

Zosim, Z., E. Rosenberg and D.L. Gutnick, "Changes in Hydrocarbon Specificity of the Polymeric Bioemulsifier: Effects of Alkanols," *Colloid and Polymer Science*, Vol. 264, No. 3. (1986): 218-223.

Zuckerberg, A., A. Diver, Z. Peeri, D.L. Gutnick, and E. Rosenberg, "Emulsifier of Arthrobacter RAG-1: Chemical and Physical Properties," *Applied and Environmental Microbiology,* Vol. 37, No. 3 (1979.)

# APPENDIX A TSCA PREMANUFACTURE NOTIFICATION PROCESS FOR INTERGENERIC MICROORGANISMS

This appendix provides more detail on the definition of intergeneric and pathogenic as they relate to microorganisms. It also outlines PMN requirements for companies producing intergeneric microorganisms for commercial purposes.

Microorganisms can be either intergeneric or intrageneric. Either type can also be pathogenic or nonpathogenic.

- *Intergeneric* microorganisms are those organisms that contain genetic material from dissimilar source organisms (i.e., different genera). These organisms are deliberately formed to contain an intergeneric combination of genetic material. Excluded from this definition are organisms that have resulted from the addition of intergeneric materials that are well-characterized and contain only non-coding regulatory regions such as operators, promoters, origins of replication, terminators, and ribosome binding regions.

- A *pathogen* is a virus or microorganism that has the ability to cause disease in other living organisms (humans, animals, plants, microorganisms). Pathogens include microorganisms that belong to a pathogenic species (according to the Agency, producer, or scientific sources) or that contain genetic material from source organisms that are pathogenic. EPA states that where there is disagreement among sources about whether a microorganism strain belongs to a pathogenic species, the submitter must assume that it belongs to a pathogenic strain.[129]

Microorganisms that are intergeneric or pathogenic are regulated more strictly than intrageneric microorganisms. The requirements outlined below only apply to intergeneric microorganisms,[130] those organisms formed by combining genetic material from organisms in different genera. In addition to TSCA Section 8(e) reporting requirements in the case of identified hazards, these are the main requirements applicable to the commercial use of intergeneric microorganisms. Similar requirements apply to the chemical products of biosynthesis.

---

[129]The information in this appendix was obtained from an EPA document entitled "Points to consider in the preparation and submission of TSCA Premanufacture Notifications for Microorganisms". This document is based on requirements outlined in the 1986 EPA Policy. EPA will issue a guidance document for companies on filing PMNs once the 1994 proposed rule is finalized.

[130]Definitions from Office of Science and Technology Policy (OSTP). Coordinated framework for regulation of Biotechnology; announcement of policy and notice for public comment. 51 Federal Register, No. 123 (June 26, 1986): 23302-23350.

1. Determine whether the microorganism is subject to TSCA requirements. The manufacturer can contact EPA for this determination, as well as for any clarification on applicable regulatory requirements

2. Submit bona fide intent to manufacture. Companies may choose to submit a bona fide intent to manufacture to determine whether a microorganism or substance has previously been reviewed and is already on the TSCA Inventory of Chemical Substances. If the microorganism is on the TSCA Inventory, it is not necessary to submit a PMN. In submitting the bona fide intent to manufacture, the company must indicate: (1) whether a related organisms has been reviewed by the Agency; (2) taxonomic identity of recipient and source of the genetic material; (3) identification of the new recombinant microorganism; (4) methods used to develop the microorganism and its intended use; (5) description of research and development conducted with the microorganism; and (6) demonstration of intent and ability to produce the microorganism. The Agency will notify the submitter of the Inventory status of the microorganism within 30 days.

3. Submit Premanufacture Notification. If the microorganism is not on the TSCA Inventory and is subject to TSCA, the manufacturer is required to submit a PMN at least 90 days before manufacture. The PMN must include the following information:

    a. Taxonomic descriptions of recipient and PMN microorganism
    b. Construction of PMN microorganism
    c. Health and environmental effects of PMN microorganism
        i. genotypic and phenotypic characteristics of PMN microorganism, including pathogenicity, virulence, or toxicity to human, other animals plants, or microorganisms.
        ii toxicity testing on effects of PMN microorganism or its products on mammals.
        iii possible environmental effects, including involvement in biogeochemical processes, gene exchange, or predicted effects on other organisms
    d. Byproducts, production volume, and use information
    e. Worker exposure and environmental release
        i. worker exposure during process, including protection devices, monitoring, and hazard warnings
        ii. description of use and consumer exposure.
    f. Environmental release protocols, in the event of field tests

    The time frame for reviewing a PMN submission is 90 days although extensions can be arranged.

4. Companies may submit a confidential business information claim, including a "sanitized" PMN submission and generic microorganism information.

5. If the intergeneric microorganism is used as an intermediate in the production of a chemical substance, a process similar to that outlined above (with the exception of different information to be reported) will need to be repeated for the chemical product.

# APPENDIX B RESULTS OF THE SURFACE CLEANING TESTS

## Results of Coupon Screening Tests

*Cut 20*:

Emulsan: The drop of solution did not spread on the surface of the coupon. When the coupon was tipped the droplet fell off intact.

Valtron: The drop spread out on the surface of the coupon. It was observed that the solution lifted the soil from the point it had contacted.

Witconol SN-90: It was observed that the oil spread away from the point of contact of the droplet. One large drop of oil remained in the center of the surfactant droplet.

Witconol SN-90 and Emulsan: The oil spread away from the point of contact of the droplet. Small drops of oil formed in the droplet of solution. The small droplets then grouped, but did not coalesce.

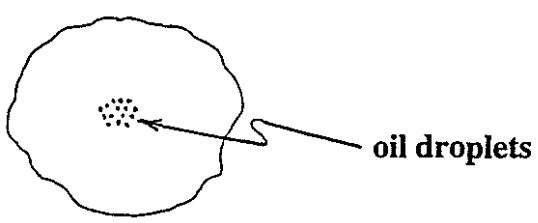
Deionized Water: Reacted identically to the emulsan solution.

*Hydraulic Oil 8-68*:

Emulsan: The drop of solution did not spread on the surface of the coupon. When the coupon was tipped the droplet fell off in one lump.

Valtron: The drop of solution spread out on the surface of the coupon more then it did on Cut 20. The solution appeared to push the oil more than it pushed Cut 20.

Witconol SN-90: The droplet spread on the surface of the coupon. Small oil droplets formed in the center of the droplet of solution. A sketch is given below.

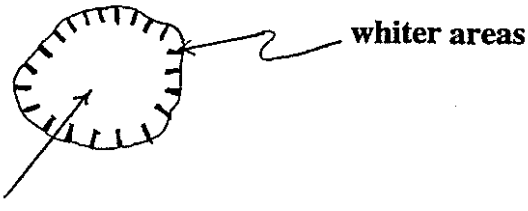


Witconol SN-90 and Emulsan: This solution reacted identically to the solution containing only Witconol SN-90.

Deionized Water: The pure deionized water reacted identically to the emulsan solution.

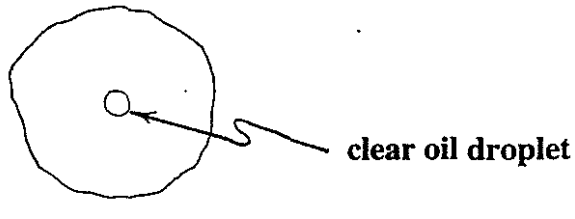
*Kutwell 40:*

Emulsan: The oil appeared to become emulsified. When the droplet of solution contacted the oil it became cloudy. After standing for one minute the droplet was creamy white throughout. At the edge of the droplet the creamy white solution oriented in sections.




Valtron: The oil became cloudy were the solution contacted it. When the coupon was tipped, a greater amount of the Valtron solution ran off the coupon than did the samples of emulsan or water.

Witconol SN-90: The droplet spread when it contacted the coupon. The area were the droplet was placed became cloudy and white. A small drop formed in the center of the cloudy area. A sketch is given below.

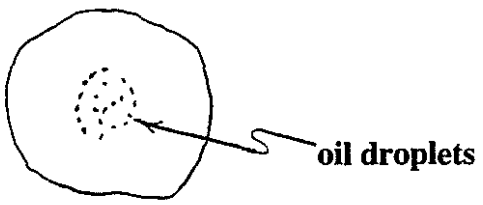



Witconol SN-90 and Emulsan: This solution reacted identically to the solution containing only Witconol SN-90.

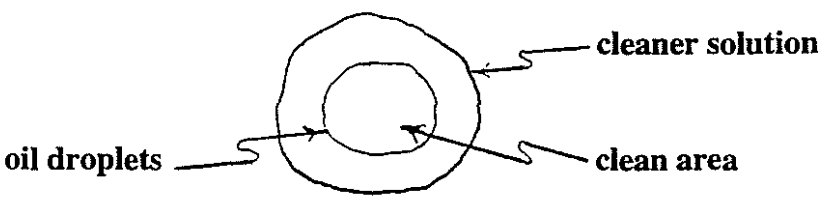
Deionized Water: The oil appeared to be emulsified. When the water contacted the coupon, the oil became creamy white.

*C-EBLIS Cutting Oil*:

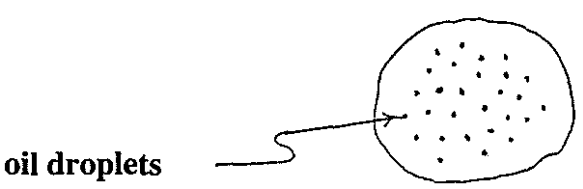
Emulsan: There was no spreading noted when the droplet was placed on the coupon. After one minute had passed, some gathering of small oil droplets in the center of the emulsan solution droplet was noticed. A sketch is given below.




Valtron: The droplet spread on the surface of the coupon. After standing for one minute it was noted that small oil droplets had oriented in a circle inside the droplet of solution. The area inside the circle of small oil droplets appeared to be clean. A sketch is given below.




Witconol SN-90: When the droplet of solution contacted the surface of the coupon it spread out. Small droplets of oil could be seen in the area were the solution was applied. A sketch is given below.




Witconol SN-90 and Emulsan: This solution reacted identically to the solution containing only Witconol SN-90.

Deionized Water: The droplet did not spread on the surface of the coupon. No oil droplets were formed in the solution droplet. No change was noted.

*Tap Magic Protap Cutting Fluid*:

Emulsan: There was no change noted when the solution contacted the contaminated coupon. There was still no change after one minute.

Valtron: Valtron did not spread on this surface as much as it did on the previous coupons.

Deionized Water: There was no difference noted from the emulsan solution.

The results from the test of Witconol SN-90 and Witconol SN-90 combined with emulsan were inconclusive because the oil was not distributed in an even layer over the surface of the coupon. When the droplets of the different solutions were placed on the coupon they ran together.

*Hydraulic Oil 8-32*:

Emulsan: The droplet of solution did not spread out. There was no change noted after one minute.

Valtron: The drop spread on the surface of the coupon. After one minute the coupon was tilted. The surface were the droplet of cleaner had been appeared to be clean.

Witconol SN-90: When the solution contacted the surface of the coupon, small droplets of oil formed in the area were the solution was applied. The oil droplets were oriented in the center of the surfactant droplet.

Witconol SN-90 and Emulsan: This solution reacted identically to the solution only containing Witconol SN-90.

Deionized Water: The pure deionized water acted identically to the emulsan solution.

The six sophorolipid concentrations evaluated (0.05, 0.06, 0.07, 0.08, 0.09, and 0.10%) reacted identically with the oil. Each droplet remained convex and no spreading of the oil took place. The oil was oriented in two or three large drops with numerous tiny drops all at the surface of the coupon.

**Results of Immersion Testing**

*875 mg/L of emulsan*: the surface of the bath was covered with large, clear droplets; oil on coupon appeared to be pushed to the top of the coupon but was not observed to be lifted.

*2% Witconol SN-90*: the oil was observed to form small drops on the surface of the coupon prior to floating to the top of the bath; coalescing of the oil droplets on the surface of the bath, but not

completely coalesced; very few droplets remaining on the coupon; droplets on the surface of the bath remained in contact with the coupon hanger.

*1% Rhodasurf DA-630*: the reaction was similar to the previous coupon; the droplets on surface of the bath were slightly larger; partial coalescing of the oil droplets on the surface of the bath.

*875 mg/L of emulsan and 2% Witconol SN-90*: the oil on the coupon was observed to form small drops on the surface of the coupon prior to lifting and floating to the top of the bath; the coupon appeared completely clean; a single, large coalesced oil drop was on the surface of the bath; three tiny drops of oil were on the surface of the bath at the side of the beaker.

*Emulsification formulation alone*: the oil smeared toward top of coupon, but did not lift from the coupon; a few tiny, clear drops were observed on the surface of the bath; the oil on the coupon appeared much thicker at the top of the coupon.

*875 mg/L of emulsan and the emulsification formulation*: there were numerous clear drops of oil on the surface of the bath; the oil on the coupon appeared to be in the same condition as the coupon in the bath containing the emulsification formulation alone.

*1% Rhodasurf DA-630 and emulsification formulation*: small drops of oil were observed to form on the surface of the coupon and to float to the top of the bath; a few small scattered drops were left on the coupon; the small drops on surface of the bath coalesced after a short period of time; the drops on the surface of the bath remained in contact with the coupon hanger.

*1% Rhodasurf DA-630 and 2% Witconol SN-90*: a similar reaction to the previous; some coalescing of droplets; still a lot of small drops remaining on the surface of the bath; the oil on surface remained in contact with the coupon hanger.

*0.06% DA-630; 0.05% DA-630; 0.04% DA-630; 70 mg/L sophorolipid; and 50 mg/L sophorolipid*: no oil was observed to be removed from the coupons by these solutions.

*83 mg/L of emulsan and 0.05% DA-630*: large drops of oil on the surface of the bath. The coupon appeared to be clean.

*0.06% DA-630 and 70 mg/L of sophorolipid*: oil floating to the surface prior to removing coupons.

*0.05% DA-630 and 50 mg/L of sophorolipid*: small and large drops of oil on the surface of the bath during the immersion time.

*0.04% DA-630 and 50 mg/L sophorolipid*: oil floating to the surface prior to removing coupons.

*10% Inproclean 2500*: numerous tiny droplets of oil on the surface of the bath.

*Pure deionized water*: no oil floated to the top of the bath during the immersion time.

**Results of Immersion Testing with Mechanical Agitation**

*400 mg/L of emulsan*: Contaminant remained on one side of the coupon. This contaminated area decreased over the immersion time. At the end of the immersion time there were thin streaks of oil on surface of the bath. Oil was also around the edge of the surface, contacting the side of the beaker.

*400 mg/L of emulsan in the emulsification formulation*: The appearance of this coupon was identical to the coupon in emulsan in water. After the cleaning procedure the bath had tiny colored droplets of oil on surface. Also the surface of the bath appeared to be slightly tinted.

*Emulsification formulation alone*: Initially the coupon had a smear down one side of the coupon. At the end of the immersion time there were three droplets of oil left in this area, each about 1/8 of an inch in diameter. At the end of the cleaning procedure there were numerous oil droplets on the surface of the bath. The droplets were clear to slightly colored.

*Pure deionized water*: The contaminant remained on one side of the coupon throughout the immersion time. After the immersion time the bath had a slightly colored slick on the surface.

*0.04% DA-630 in water*: Initially the contaminant remained on one side of the contaminated surface. During the immersion time the oil was observed to lift from the coupon. At the end of the immersion time there were numerous small droplets remaining on the side of the coupon and tiny colored droplets of oil were on the surface of the bath in a swirled formation.

*10% Inproclean 2000 in water*: The contaminant had the same initial orientation as the coupon above. The coupon appeared to be completely clean within a couple of minutes. No oil was visible on the surface of the bath or dispersed throughout the bath at the end of the cleaning procedure. The surface was slightly tinted, but no oil slick was observed.

**Results of Fluorescing Analysis of Immersion Testing with Mechanical Agitation**

*Emulsan in water*: There was a contaminated area on the right side of the coupon. The contamination was thick at top, right corner. There were spots of contamination throughout the surface of the coupon.

*Emulsan in the emulsification formulation*: There was a contaminated area on the right side of the coupon. The contaminant was thick at the top near the center. There was spotting elsewhere on the surface of the coupon. The spotting was less dense than the previous coupon.

*Emulsification formulation alone*: There was a contaminated area on the right side of the coupon that was smaller than the previous two coupons. There were spots of contaminant on the other side of the coupon.

*Pure deionized water*: There was a scattered spotting of contaminant over the surface of the coupon. The contaminant was thick across the bottom of the coupon.

*0.04% DA-630 in water*: There were three large odd shaped drops of oil remaining on the coupon. There was some spots of contaminant in this area. The coupon appeared to be clean elsewhere.

*10% Inproclean 2000 in water*: Contaminant was in two of the identification numbers etched in the surface of the coupon. The coupon was clean everywhere else.