Evaluation of the microbial adhesion to hydrocarbon assay applied to a hydrocarbon degrading bacterium

by Lawrence Otto Schmidt

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
Montana State University
© Copyright by Lawrence Otto Schmidt (1995)

Abstract:
The outer surface of microbial cells contain a variety of chemical compounds which may be involved in the attachment of cells to surfaces. Hydrophobic/hydrophilic interactions play a large role in attachment, leading to the development of the concept of cell surface hydrophobic!ty as a measure of the tendency of a cell to attach to a surface. One of the most popular tests of this tendency is the Microbial Adhesion to Hydrocarbons (MATH) test. In a typical assay, a cell suspension is contacted with a small volume of hydrocarbon, and the removal of cells by the hydrocarbon is determined. The goal of this project was to evaluate the effects of test conditions and bacterial growth conditions on the results and precision of the MATH test using a single species of hydrocarbon degrading bacteria. Cells were grown under either carbon limited (C:N = 5:1) or nitrogen limited (C:N = 15:1) conditions, and MATH contact assays were performed at various agitation intensities, with various hydrocarbon volumes, and for various mixing times. In addition, the results of the assay were evaluated using light absorbance and using viable plate counts to determine cell removal due to the hydrocarbon. Appropriate negative controls were also run in the absence of hydrocarbon to account for wall effects in the test vessels.

The results of the study were analyzed according to traditional methods, using percent removal and rate of removal, and compared to the literature. Additional statistical methods were used to evaluate the variance in results and to determine how much of the variance could be attributed to experimental test factor values versus how much was due to inherent variability in the assay. In general, reproducibility was found to be poor, owing perhaps to the strong tendency of the test organism to form clumps in aqueous solution. Multiple linear regression models and analysis of variance (ANOVA) produced p-values for the factors that often showed statistical significance, but the actual effects of the test conditions were found to be negligible from a practical standpoint. Only the agitation rate was found to be significant according to all test methods: at low agitation rates, significantly lower removals from aqueous phase were observed than at high rates.

EVALUATION OF THE MICROBIAL ADHESION TO HYDROCARBON ASSAY APPLIED TO A HYDROCARBON DEGRADING BACTERIUM

by

Lawrence Otto Schmidt

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Environmental Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 1995

© COPYRIGHT

by

Lawrence Otto Schmidt

1995

All Rights Reserved

N378
Sch547

APPROVAL

of a thesis submitted by

Lawrence Otto Schmidt

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

April 21, 1995
Date

Chairperson, Graduate Committee

Approved for the Major Department

21 April 1995
Date

Theodore E. Lang
Head, Major Department

Approved for the College of Graduate Studies

6/27/95
Date

R L Brown
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Lawrence Otto Schmidt

Date ______________________

TABLE OF CONTENTS

PAGE

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT ix

INTRODUCTION 1
- Surface Characteristics of Cells 1
- MATH test 3
  - Consistency of the MATH test 3
  - Physical Factors 4
  - Physiological Factors 5
  - Importance of the MATH test 5
- Goal of Research 6
- Hypotheses 6

BACKGROUND 8
- Cell Surface Hydrophobicity 8
- Previous Research 11
- Measurement of Cell Surface Hydrophobicity 12
- Hydrophobicity Assays 13
  - BATH and MATH Assays 13
  - Contact Angle Measurement 14
  - Hydrophobic Interaction Chromatography 14
  - Salt Aggregation Test 15
  - Two Phase Partition 15
  - Binding of Molecular Probes 16
  - Adhesion to Hydrophobic Surfaces 16
  - Direction of Spreading 16
  - Image Analysis 17
- Physiological Effects on Cell Surface Hydrophobicity 18
  - Growth Phase 18
  - Growth Temperature 19
  - Cell Generation 19
  - Cell Type and Preparation 19
- Physical Factors Effecting the MATH test 20
- Continued Research 21
  - Importance of Research 21
  - Comparable Tests 22

TABLE OF CONTENTS (continued)

METHODS 24
- Preliminary Work 24
  - Isolation 24
  - Substrate 25
    - C:N ratio calculations 26
  - Nutrient Medium 26
- Experimental Design 27
  - MATH Assay 27
    - C:N Ratio 27
    - Experimental Conditions 28
  - Agitation Rate 28
  - Hydrocarbon Volume 29
  - Hydrocarbon Screening 29
  - Time of Mixing 30
- Experimental Procedure 30
  - Inoculation 30
  - Washing Procedure 31
  - Resuspension of Cells 32
  - Addition to Test Tubes 32
  - Agitation 33
  - Cell Enumeration Techniques 33
  - Cell Enumeration 34
  - Absorbance Measurements 34

RESULTS 36
- Test Tube Observations 36
- Percent Removal Data 42
- Results from Statistical Analysis 48
  - Regression Analysis 48

DISCUSSION 50
- Analysis of Data 50
  - Percent Removal 50
  - Kinetic Approach 51
  - Graphical Analysis 57
  - Statistical Analysis 59
  - Regression 59
    - Data Inclusion 60
    - Significant Factors 61
    - p Values 61
    - Significant Coefficients 63
  - Variation Between Experiments 64
  - Means and Standard Error 65

TABLE OF CONTENTS (continued)

CONCLUSION 70

REFERENCES 71

APPENDICES 75
Appendix A.......... 76
Experimental Results 77
Appendix B.......... 95
Image Analysis Research 96

LIST OF TABLES

Table | Page

1. Composition of nutrient media 27
2. Settings for Agitaiton rate, hydrocarbon volume and mixing time 28
3 Absorbance and Percent Removal for Three Hydrocarbons 29
4. Composition of buffer solution 32
5. Example of a Typical Experiment 33
6. Absorbance Responses for Cells Cultured at a C:N ratio of 5:1 43
7. Cell Count Responses for Cells Cultured at a C:N ratio of 15:1 44
8. Absorbance Responses for Cells Cultured at a C:N ratio of 5:1 45
9. Cell Count Responses for Cells Cultured at a C:N ratio of 15:1 46
10. Absorbance and Cell Count Data for Cells Cultured at a C:N ratio of 5:1 for Experiments with Interference 47
11. Constant Terms and Coefficients for Responses 49
12. Percent absorbance data showing the change from the lowest settings to the highest settings for absorbance and cell counts........... 51
13. The p values for six responses 62
14. Constant terms and coefficients for responses 64
15. Values for Interexperimental and Intraexperimental Error................. 65

LIST OF FIGURES

Figure | Page

1. Cells Within the Circle are at the Hydrocarbon-water Interface.................. 37
2. Test tube containing unmixed hydrocarbon and cell suspension 38
3. Control test tube after agitation.(Agitation at a setting of 60 for 40 sec.)...... 38
4. Test tube agitated with carbon 39
5. Control test tube. (Agitated at a setting of 60 for 20 sec.) 39
6. Control test tube. (Agitated at a setting of 140 for 20 sec.) 40
7. Test tube containing .2 ml of hydrocarbon. (Agitated at a setting of 60 for 20 seconds) 40
8. Test tube containing .2 ml of hydrocarbon. (Agitated at a setting of 140 for 20 seconds)... 41
9. Test tube containing 1 ml of hydrocarbon. (Agitated at a setting of 60 for 20 seconds) 41
10. Plots used to determine the removal rate (k) and removal coefficient (K) for cells grown in a culture containing a 5:1 C:N ratio......................... 52
11. Plots used to determine the removal rate (k) and removal coefficient (K) for cells raised with a 15:1 C:N ratio 54
12. Effects of agitation setting, hydrocarbon volume and time of mixing on the percent removal (ideal) for isolate growth at a 15:1 C:N ratio for absorbance 57
13. Percent removal (ideal) at two mixing times with variation of agitation rate and hydrocarbon volume 59
14. Initial cell counts for cultures raised at 5:1 and 15:1 C:N ratio including the cell counts for interference data 61
15. Responses ln (Ao/A) and ln(Co/C) vs hydrocarbon volume including the standard error of the averages 67
16. Responses ln(Ao/A) and ln(Co/C) vs agitation setting including the standard error of the averages 68
17. Responses in (Ao/A) and ln(Co/C) vs time of mixing including the standard error of the averages 69

ABSTRACT

The outer surface of microbial cells contain a variety of chemical compounds which may be involved in the attachment of cells to surfaces. Hydrophobic/hydrophilic interactions play a large role in attachment, leading to the development of the concept of cell surface hydrophobicity as a measure of the tendency of a cell to attach to a surface. One of the most popular tests of this tendency is the Microbial Adhesion to Hydrocarbons (MATH) test. In a typical assay, a cell suspension is contacted with a small volume of hydrocarbon, and the removal of cells by the hydrocarbon is determined. The goal of this project was to evaluate the effects of test conditions and bacterial growth conditions on the results and precision of the MATH test using a single species of hydrocarbon degrading bacteria. Cells were grown under either carbon limited (C:N = 5:1) or nitrogen limited (C:N = 15:1) conditions, and MATH contact assays were performed at various agitation intensities, with various hydrocarbon volumes, and for various mixing times. In addition, the results of the assay were evaluated using light absorbance and using viable plate counts to determine cell removal due to the hydrocarbon. Appropriate negative controls were also run in the absence of hydrocarbon to account for wall effects in the test vessels.

The results of the study were analyzed according to traditional methods, using percent removal and rate of removal, and compared to the literature. Additional statistical methods were used to evaluate the variance in results and to determine how much of the variance could be attributed to experimental test factor values versus how much was due to inherent variability in the assay. In general, reproducibility was found to be poor, owing perhaps to the strong tendency of the test organism to form clumps in aqueous solution. Multiple linear regression models and analysis of variance (ANOVA) produced p-values for the factors that often showed statistical significance, but the actual effects of the test conditions were found to be negligible from a practical standpoint. Only the agitation rate was found to be significant according to all test methods: at low agitation rates, significantly lower removals from aqueous phase were observed than at high rates.

INTRODUCTION

Measurements of cell surface hydrophobicity are made by a variety of methods. The MATH (microbial adhesion to hydrocarbon) test is potentially the most useful of the techniques. The MATH test is simple, fast and reasonably reproducible. The main drawback with the method is its lack of quantification. The MATH test needs to be tested for reliability and further the factors which influence the test the most should be determined.

Surface Characteristics of Cells

The biochemical composition of the outer surfaces of microorganisms strongly affects the nature and extent of their interaction with and attachment to substrata. Once a cell contacts a surface, a variety of processes maybe involved in microbial attachment (Kjellberg 1984).

Structure-mediated processes can be initiated by extracellular polymers which project from the cell surface. These extracellular polymers attach to surfaces by two mechanisms: specific and nonspecific binding. Specific binding requires a complementary site on the binding surface to the extracellular polymer of the bacteria. Nonspecific binding involves macromolecules or extracellular polymers that interact with the surface of the substratum or macromolecules present on the surface (Group Report 1984). A number of possible nonspecific binding processes can occur including: ionic, dipolar, hydrogen bonding, and hydrophobic/hydrophilic surface interactions.

Cell surface hydrophobicity describes cells with hydrophobic surface characteristics. The hydrophobic nature of these cells promotes attachment to substrates which may provide access to essential nutrients. Attachment of bacteria at the hydrocarbon-

water interface is a good example of a hydrophobic interaction. These bacteria are able to colonize surfaces that are unavailable to other cells, thus providing a competitive advantage in nutrient availability. The hydrocarbon-water interface is a unique environment which may offer protection for the bacteria from predators (Marshall 1976). These bacteria need to be able to detach from oil once all the long-chain n-alkanes they grow on have been utilized. *Acinobacter calcoaceticus* RAG-1 releases encapsulated emulsans which cause the bacteria to desorb from the hydrocarbon. The emulsan forms a hydrophilic polymeric film around the depleted oil droplet that hydrophobic bacteria cannot attach to (Rosenberg and Kaplan 1987).

Hydrophobic organisms have been found in a variety of environments. In the medical field, studies on adhesion of bacteria to host tissues indicate that hydrophobicity is an important factor for the infection process. (Enzer and Douglas 1992). Another example in medicine is the persistence of bacteria which are able to adhere to plastics used in medical devices (Rosenberg and Doyle 1992). In the petroleum industry methods are needed for quick and cost effective removal of insoluble hydrocarbons. Bacteria that are able to adhere to the hydrocarbon-water interface may play an integral part in development of these methods (Goswami and Singh 1990). Chemical surfactants are used extensively in industry, agriculture and medicine. Surface-active compounds that aid in emulsification of the oil phase into the aqueous phase increase the interfacial area available for microbial contact (Atlas 1984, 672). Advantages of natural surfactants over manufactured surfactants are their ease of biodegradation and their selectivity for a specific surface. Emulsan, for instance, is specific for a mixture of aliphatic and aromatic hydrocarbons. The use of *Acinobacter calcoaceticus* RAG-1 to produce emulsan and other extracellular polysaccharides by fermentation processes is another area of research incorporating bacteria with hydrophobic surfaces (Shabtai and Wang 1989).

MATH Test

Many researchers are working with bacteria that have hydrophobic character and would like to be able to compare the cell surface hydrophobicity of their organism to others. A quantitative measure of hydrophobicity should be able to predict the tendency of bacteria to attach to a hydrophobic surface. The MATH (microbial adhesion to hydrocarbon) test has been used extensively to screen bacteria for hydrophobic character by measuring the tendency of organisms to attach to a hydrophobic surface. This test was first developed in 1980 by Rosenberg as a possible quick and easy method to determine the hydrophobic character of cells. The test has developed to the point where some researchers believe that it is a quantitative measure of cell surface hydrophobicity (Lichtenberg *et al.* 1985).

In a typical experiment, test tubes or cuvettes containing a cell suspension and hydrocarbon are agitated at a fixed rate for a given time period. The agitation of the solution allows the microorganisms to come into contact with the hydrocarbon. The phases are then allowed to separate, the lower aqueous phase is separated from the upper hydrocarbon phase and absorbance measurements are made on the aqueous phase. Removal of cells from the aqueous phase (as measured by change in absorbance) is then used as a measure of hydrophobicity.

Consistency of the MATH Test

In order for the MATH test to be useful, results and comparisons throughout a study must not be affected significantly by the results of error inherent in the MATH test. Correlations between the MATH test and other methods for determining hydrophobicity have been observed. A linear correlation was observed for *Pasteurella multiocida* when

percent adhesion to hydrocarbon was compared to percent retained on octyl-Sepharose (Darnell *et al.* 1987). The result of another method comparison was a direct correlation between cell-surface hydrophobicity and adhesion to epithelial cells (Ener and Douglas 1986). Epithelial cells make up the epithelium, the cellular tissue covering surfaces, forming glands and lining most of the cavities in the body. Evaluation of the hydrophobicity of the following organisms, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Saccharomyces carsbergensis* and *Kluyveromyces fragilis* varied widely depending on the measurement technique (Moses and Rouxhet 1987). The MATH test proved to be the most inconsistent of those techniques.

The MATH test does not always yield internally consistent results. For example, Sweet *et al.* (1987) state, "current methods of assessing bacterial hydrophobicity as a function of adherence to liquid hydrocarbons (especially hexadecane) do not always yield reproducible results." Sweet *et al.* determined in their study that p-xylene should be used instead of hexadecane. Factors which affect the internal consistency of the MATH test are both physical and physiological.

Physical Factors

Physical factors which affect the MATH test include agitation rate, hydrocarbon volume, time of mixing and test tube size. The hydrocarbon volume will affect the surface area available for attachment, as will the agitation rate. More vigorous agitation may also increase the transport of cells to the interface. Time may also affect the results since the longer the cell suspension is in motion the more likely it is that a microbe will come into contact with the hydrocarbon. If the MATH test is to be internally consistent, the effects of these factors on response should be minimized.

Physiological Factors

Physiological factors affect the growth of cells, subsequently causing changes in the chemistry of the cell surface. The carbon to nitrogen ratio is one such factor. A C:N ratio of 7.7:1 has been determined to support balanced growth and product synthesis (emulsan production) for *Acinetobacter calcoaceticus* RAG-1 (Shabtai and Wang 1989). Carbon limited media will typically limit the ability of organisms to produce extracellular, materials particularly exopolysaccharide. Conversely, a high C:N ratio should produce extracellular materials which could lower the hydrophobicity of the cells. Other nutrient conditions that affect the MATH test are phosphate limitation, inorganic ions and dissolved organic matter. Culture conditions such as temperature, pH, and ionic strength can also affect the results of the MATH test. Last, generation variation between cultures may affect the surface properties and thus the cell surface hydrophobicity of cells.

Importance of the MATH test

For the MATH test to be useful, results and comparisons throughout a study must not be affected significantly by errors inherent in the MATH test. As the number of comparisons between data from the MATH test increases, it is important to recognize the limitations of this test. This study will evaluate the effects of mixing time, agitation intensity, hydrocarbon volume and growth conditions on the performance and precision of the MATH test.

Goal of Research

The goal of this project is to evaluate the effects of test conditions and bacterial growth conditions on the results and precision of the MATH test. The specific objectives are:

1) Evaluate the reproducibility of the MATH test with a single hydrocarbon-degrading bacterial species.

2) Evaluate the effects of mixing intensity, mixing time and hydrocarbon volume on the test

3) Evaluate the effect of C:N ratio during growth of bacteria on the results of the MATH test.

The following null hypotheses were established as a basis for statistical evaluation of the experimental results. Each of these hypotheses were tested using viable cell counts and light absorbance as measures of the cell concentration in the suspension.

Hypotheses

1) For a given set of experimental and bacterial growth conditions, the MATH test yields reproducible results.

2) The time of mixing in the assay does not significantly affect the results.

3) The mixing intensity does not significantly affect the results.

4) The hydrocarbon volume does not significantly affect the results.

5) The C:N ratio during growth of the bacteria does not significantly affect the results.

6) Cells are not removed from suspension in the test when hydrocarbon is not present.

BACKGROUND

Cell Surface Hydrophobicity

The surface of many microbes can have a hydrophobic nature. This phenomenon promotes interactions such as partitioning of bacteria at liquid:liquid and liquid:air interfaces and adhesion of bacteria either to host tissue or to nonwettable solid surfaces (Marshall 1976). This property allows some bacteria to attach to hydrocarbons and use it as substrate, increasing cellular mass and population at the hydrocarbon:water interface (Rosenberg *et al.* 1982). Many other examples of microbial adhesion to hydrophobic surfaces exist, including adhesion to contact lenses, elemental sulfur, mineral particles, biomaterials and others thus emphasizing the importance of this phenomenon (Rosenberg and Doyle 1990). The term hydrophobicity is misleading, as "hydrophobicity" literally translates as "water aversion" and therefore contradicts the ability of many microbial cells to disperse readily in water. Dispersion in aqueous solutions allows microbes to come into contact with the desired surface, whether hydrophobic or hydrophilic in nature. Many hydrocarbon-degrading organisms have specifically adapted to surviving and interacting with the insoluble substrates they use as their carbon source.

The nature of bacterial attachment to hydrophobic surfaces or interfaces is a subject that has proved to be a source of dispute between physical chemists and microbiologists. At the Dahlem Konferenzen in 1984 a group of scientists made up of microbiologists and physical chemists formed a consensus on a number of definitions relating to mechanisms of attachment. The group agreed upon two basic mechanisms for bacterial attachment to hydrophobic surfaces: non-specific and specific binding (Marshall 1984). Non-specific binding involves electrostatic or hydrophobic interactions, while specific binding involves

complementary sites on the bacterial surface and the colonizable surface with high affinities for each other (Sweet *et al.* 1987). The complexity of defining and understanding the nature of microbial adhesion to surfaces demands further research.

Microbial adhesion to hydrocarbons at the oil-water interface is a special case of microbial attachment to hydrophobic surfaces. In nature, microbial growth on water-insoluble hydrocarbons requires direct contact between bacterial cells and the oil phase. The first step in hydrocarbon degradation typically involves oxygenase an intracellular enzyme. With hydrocarbons being very nonpolar, and thus insoluble in water, it follows that the microbe must come into direct contact with the nonaqueous phase. Microorganisms cannot grow inside the nonaqueous phase as they require water as well as soluble nitrogen and phosphorus. As a result, hydrocarbon-degrading bacteria grow at the hydrocarbon-water interface.

There are four processes involved in biodegradation of hydrocarbons: adhesion, growth, desorption and surface renewal. Adhesion is the process by which the bacteria become attached to the water-petroleum interface. Hydrocarbon-degrading bacteria are able to adhere to the hydrocarbon through hydrophobic interactions. Thin fimbriae which extrude from the cell membrane allow hydrocarbon-degrading bacteria to adhere to the hydrocarbon. Three observations support this. First, mutants that lack thin fimbriae fail to adhere to the hydrocarbons, and grow poorly on the hydrocarbon substrates. Secondly, subjecting cells with thin fimbriae to high shear causes loss of cell surface hydrophobicity and the ability to adhere to hydrocarbons. Finally, revertants of nonadhering species regain the ability to adhere to hydrophobic surfaces (Rosenberg and Kaplan 1987).

Following adhesion/adsorption, growth occurs at the hydrocarbon-water interface. In this stage there are strong intercellular interactions, as supported by the observation that desorption of cells from oil yields cell clumps. It is important to note that in initial growth, cells multiply at an exponential rate until the surface is covered. When the surface cell

population reaches a monolayer, the surface area becomes the limiting factor, and the biomass increases at an arithmetic rate. This indicates why the amount of hydrocarbon surface area to which microbes can attach limits degradation of hydrocarbons.

There are two factors involved in extending the exponential growth phase: hydrocarbon emulsification, and the transfer to a new substrate. Hydrocarbon emulsification increases the surface area available for cell growth. When a 2 mm oil droplet is broken down to droplets of 10 μm diameter, a typical value for bacterial-induced emulsions, the surface area increases by 200 times. The interfacial tension between the bacterial-coated hydrocarbons and water is lower than between pure hydrocarbon and water. It is thought that emulsifying agents utilized by bacteria play a role in oil degradation at the microscopic level. Bacterial cells produce extracellular emulsans (polysaccharide-containing emulsifiers) which adhere to hydrophobic compounds. These encapsulated emulsans are present on cell surface. When the hydrocarbon droplets useful substrates have been utilized the encapsulated emulsan is released. The released emulsan forms a polymeric film which plays a key role in desorption and surface renewal. The depleted oil droplet is covered by a film which now has hydrophilic character restricting attachment of hyrophobic bacteria (Rosenberg and Kaplan 1987).

During the growth phase of the bacteria cells at the hydrocarbon-water interface, the emulsan is tightly bound to the cell. The emulsan capsule is released from the cell surface when starvation conditions occur. Then emulsan adsorbs avidly to the oil droplet, thereby displacing the cells to the aqueous phase. These "used" droplets are covered with a very stable monomolecular film of emulsan that prevents bacteria from reattaching to the depleted droplet (Inouye 1985). The fatty-acid ends are oriented towards the hydrophobic organic phase while the polar hydroxyl and carboxyl groups are faced towards the aqueous phase. The released capsule-deficient bacteria are free to attach to fresh substrate.

Previous Research

The earliest reference to cell surface hydrophobicity was in Mudd and Mudd's classic paper in 1924 in which experiments of bacterial attachment at the oil-water interface were studied. This research opened up a new field of research in microbial interaction with surfaces and interfaces. The study attempted to understand the physical-chemical factors involved in penetration of bacteria through epithelia cells of the animal body by first investigating the mechanisms of transport of a simpler system. Mudd and Mudd investigated the kinetic mechanism of bacterial transport at the interface between two imiscible fluids and postulated that the mechanism was dependent primarily upon the interfacial surface tension forces at the interface. Marshall and Cruickshank studied cell surface hydrophobicity and the orientation of certain bacteria (1972). Photographs portray *Flexibacter* CW7 cells at the hydrocarbon-water interface oriented perpendicular to the hydrocarbon surface (Marshall 1976). Another focus of the research was microbial oxidation of oil products, at first for development as a potential protein source, and later as a means of cleaning up after oil spills. One such study measured the interfacial area of hydrocarbon and its relationships to specific growth rates in yeast fermentors. This study (Wang and Ochoa 1972) determined that the specific growth rate is directly related to the specific hydrocarbon interfacial area.

The MATH assay was developed in 1980 and proposed bacterial adhesion to hydrocarbon as a simple, general method for measuring cell surface hydrophobicity. The method was based on the percentage of adherent cells to various liquid hydrocarbons subjected to agitation for brief periods. It was also discovered that hydrocarbon degraders were not the only bacteria with the ability to adhere to the bulk hydrocarbon (Rosenberg, Gutnick, and Rosenberg 1980). During this time many different hydrophobic interactions

were studied, including adhesion to mineral surfaces, fish surfaces, oral tissue and to inert surfaces such as polystyrene (Rosenberg and Doyle1990).

Research in the late 1980s and early 1990s continues to look at the adhesion of bacteria to different surfaces and the further development of quantitative measurement methods. During this time the MATH assay has been redesigned a number of times in an attempt to increase the reproducibility of the test results. A method that has proved to be successful in capturing the kinetics of surface colonization in dynamic systems is image analysis. Using the image analysis system Escher determined that sorption-related processes are a function of the bulk cell concentration and interface dynamics (Escher 1986). In 1990, Mueller looked at the adsorption process and the effects of different substrata on the sticking efficiencies of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* bacteria.

The importance of the MATH test in determining cell surface hydrophobicities of mutant and nonmutant strains of *Acinetobacter calcoaceticus* RAG-1 and the role of emulsan in surface renewal cannot be understated. Through the use of MATH, Rosenberg was able to determine the roles played by the extracellar thin fimbreae and encapsulated emulsan. From this work a novel method for comparing cell surface hydrophobicities was produced and has proved to be among the most useful methods to date.

Measurement of Cell Surface Hydrophobicity

Historically, measurements of cell surface hydrophobicity have been used to compare the relative affinities of bacteria for different surfaces or to compare the influence of different experimental conditions such as nutrient, media or growth phase effects. Some of these methods can be very time-consuming and labor-intensive, prohibiting their usefulness in many laboratory situations. The development of the MATH assay was a

breakthrough in regard to the relative ease of collecting data indicating a certain affinity of the bacteria toward the hydrocarbon-water interface. Unfortunately, this assay does not always agree with some of the other methods. Even when comparing different experiments using the MATH assay, the results are often hard to interpret. One such example is the case of *A. viscous* cells that adhere to hexadecane only after vortexing and not when mixed with gentle agitation (Rosenberg 1991). Rosenberg's research in developing a simple test for cell surface hydrophobicity has undergone many alterations since he first published the method in 1980.

Hydrophobicity Assays

BATH and MATH Assays

In his first paper Rosenberg described a simple method for determining cell surface hydrophobicity using BATH (bacterial attachment to hydrocarbon). This was later changed to MATH (microbial attachment to hydrocarbon) in part because of extensive use of the assay in studies of eucaryotic organisms (Rosenberg and Doyle 1990). The MATH assay utilizes the fact that many hydrophobic microbes suspended in buffer media, when agitated, will adhere to hydrocarbon and form emulsions at the hydrocarbon-water interface. Microbes in a suspension of buffer solution are placed in a test tube to which the hydrocarbon (hexadecane, octane or xylene) is added in varying amounts. The test tubes are vortexed for 120 seconds, after which the mixture is allowed to separate and the absorbance of the aqueous layer is measured. The absorbance is plotted against the hydrocarbon volume, thus revealing the affinity the bacteria have towards the hydrocarbon as a function of hydrocarbon volume (Rosenberg *et al.* 1980). Lichtenberg *et al.* (1985) proposed a kinetic approach for the MATH assay. In this procedure varying amounts of

hydrocarbon are added to the cell suspensions and are agitated for fixed consecutive time periods. Following each agitation the phases are allowed to separate and the absorbance of the aqueous layer is measured. By plotting the logarithm of the percentage of cells remaining in the aqueous phase as a function of time, the results yielded a linear relationship. The authors determined, "the slope of the curve is a quantitative expression of the affinity of the cells tested for the water:oil interface, and denote it as the removal coefficient (K) of the cells from the bulk aqueous phase by the hydrocarbon" (Lichtenberg *et al.* 1985). The test was further developed with the use of polystyrene cuvettes as the experimental vessel instead of test tubes (Sharon *et al.* 1986). It is noteworthy that in a scientific citation in 1988, a paper in which E. Rosenberg is a co-author, the use of the kinetic method is not employed (Pines *et al.* 1988).

Contact Angle Measurement

Contact angle measurement (CAM) is a standard method for studying hydrophobic surfaces. The method measures the surface free-energy of solid surfaces. CAM was first used in measuring the hydrophobic surface properties of bacteria in 1972 (van Oss and Gillman). In order to measure the contact area the surface needs to be homogeneous, flat and dry. Therefore, conditions for using CAM on microbial studies requires that the cells be flat and dry. Microbial mats on agar plates is one method used for these studies. This method could prove useful in its own way, but it takes special equipment and technical experience to measure the contact angles.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) measures microbial adsorption to octyl-Sepharose or phenyl-Sepharose beads (Rosenberg and Doyle1990). A column

packed with an aqueous suspension of beads bearing either octyl or phenyl groups is treated with suspension of cells. As the cells pass through the column some of them stick to the beads. The percentage adhering is determined from the loss of turbidity or radioactivity in the elute compared to the initial level. Controls filled with untreated beads have had cells adhere to them . Another problem is that some researchers have used salting-out agents to increase the amount of adherence to the beads (Rosenberg and Doyle1990). The nature of suspension solution often plays a role in the results in this test method. The method has potential if all tests are performed under the same conditions.

Salt Aggregation Test

The salt aggregation test (SAT) is an extremely easy technique for studying the aggregate behavior of cells (Rozgonyi *et al.* 1985). The cells are challenged with increasing amounts of salting-out agents (ammonium sulfate), and as the salt concentration increases, cells start to clump together. Measurements are compared by observing at what concentration clumping began. This method will not work with bacteria that clump readily without added salt and also may be dependent on initial cell concentrations.

Two Phase Partition

Two phase partition (TPP) measures the distribution of cells between two immiscible fluids (Rosenberg and Doyle 1990). Usually one contains dextran and the other polyethylene glycol. The bacteria are added to the system and thoroughly mixed. After the mixture separates, samples are taken from each phase and the amount of cells in each phase is determined. In some cases cells may bind at the interface, causing problems for recovery and interpretation of the results. The test is also sensitive to changes in concentration, batches and molecular weight distribution of the two polymers.

Binding of Molecular Probes

This method uses hydrophobic molecular probes that bind at the outermost surface of cells to determine hydrophobicity. Kjellberg, Lagaercrantz, and Larson studied binding of radiolabled dodecenoic acid to bacterial cells to quantitatively determine the cell surface hydrophobicity (1980). Molecular probes often will aggregate without the addition of polar or charged groups, which can interfere with the results. Another potential problem is the internalization of some of the probes, which may cause cell damage (Rosenberg and Doyle 1990).

Adhesion to Hydrophobic Surfaces

Microbial adhesion to hydrophobic surfaces can be another very useful method. Study of bacterial adhesion to hydrophobic surfaces such as polystyrene employs a variety of techniques (Rosenberg and Doyle 1990). Some examples are measurements of radiolabeled dodecanoic acid to bacterial cells and microspheres of polystyrene in a aqueous suspension directly observed via microscope or dynamic observations using image analysis. One advantage to this method is the ability to use contact angle measurements to determine the hydrophobicity of the surface. One advantage of polystyrene is the transparency of the material, which allows direct observation of the surface with conventional microscopy.

Direction of Spreading

Direction of spreading (DOS) was developed to observe differences between the hydrophobicity of individual cells and the surfaces of bacterial colonies (Sar 1986). The direction in which a drop of water spreads when placed at the border between the bacterial

lawn and another surface determines the nature of the bacterial lawn surface. The border surfaces used to make the measurements are: agar/bacteria, glass/bacteria, polystyrene/ bacteria. The droplet tends to move away from the border of the microbial lawn towards the material surface if the microbial lawn is more hydrophobic. Therefore, the more hydrophobic the bacteria is the more the water droplet will move towards the hydrophobic material. Sar found that the method, when tested against the MATH assay, will sometimes gives higher values for cell surface hydrophobicity.

Image Analysis

Image analysis enables dynamic systems to be studied. Bacterial adhesion to stationary surfaces can be studied by passing cell solutions through a flow cell and measuring directly the rates of attachment, detachment and growth on the surface. This method allows direct observation of the overall processes in a dynamic system through the use of microscopy and video techniques. The adhesion of *S. sanguis* 12 to FEP (fluoroethylenepropylene) and glass surfaces in real time using image analysis is described by Busscher *et al.* (1987). The adhesion kinetics of *S. sanguis* 12 during the first four to six hours of exposure to both substrata under various conditions yielded the initial rate of adhesion and the number of cells adhering in a stationary state. Escher in 1986 and Mueller in 1990 also used image analysis to determine the kinetics of bacterial attachment to surfaces. Escher's goal was to determine the influences of independent parameters such as the fluid dynamics, biomass concentration in the bulk fluid, surface characteristics and cell physiology on early colonization of surfaces. Mueller's goal was to determine the effects of different surfaces, environmental conditions, and microbial species on early bacterial colonization in continuous flow systems. Mueller used the MATH assay to determine the hydrophobicity of the bacteria in his study, but did not determine the hydrophobic character

of the surfaces tested. Comparisons between the hydrophobic surface character and the cell surface hydrophobicity may have helped to determine if a correlation between the two methods could be found.

Physiological Effects on Cell Surface Hydrophobicity

Growth Phase

Microbial populations in batch culture follow a typical growth cycle with three distinct phases: lag, exponential, and stationary. Lag phase is a time period just after innoculation of the media during which little or no growth occurs. During the exponential phase cells grow and replicate exponentially as long as nutrients are available in the culture media. Once the microbes have utilized most of the available nutrients they enter the stationary phase and growth of the cells slows down. Cells in an environment in which nutrients are limited begin to slow down the synthesis of cellular components. Stationary phase is not static. During this time, for example, *E. coli* cells will continue to produce DNA after protein synthesis has been reduced (Neidhardt *et al.* 1990). Physiological differences between cells harvested from different phases of growth have also affected the results of the MATH assay. Lichtenberg *et al.* (1985). report that *S. pyogenes* cells harvested during the logarithmic growth phase had a removal coefficient (K) several orders of magnitude lower then those harvested during stationary growth. Rosenberg reported similar results in 1980 when he compared the lag phase and stationary phase hydrophobicities of *S. marcescens* cells. For this reason, care has to be taken that the time of harvesting is constant, so that physiological characteristics of the cells do not change substantially between experiments.

Growth Temperature

Cultures incubated at different temperatures do not always have the same physical or chemical characteristics. These differences will often affect the MATH assay and other hydrophobicity tests. Sharon *et al.* 1986 grew *S. marcescens* at 30°C and 35°C, and a greater removal coefficient (more hydrophobic) for the cells grown at 30°C was observed. The temperature of culture solutions needs to be kept constant between experiments for valid results.

Cell Generation

Isolation of microbial cells for experiments may require cells to be removed from the original batch culture onto a more 'friendly' medium such as agar plates. In some cases, cells grown and subsequently transferred to another medium may lose some of their original characteristics (Rosenberg and Doyle 1990). Cells taken from cultures and then suspended should be of the same generation for each experiment to keep errors due to loss of genetic material at a minimum.

Cell Type and Preparation

Microbial cells when washed may lose some of their hydrophobic character. This could result from a loss of surface components when the bacteria are rinsed, agitated and resuspended. Microbes exist in a number of states in nature, such as in biofilms, microbial mats and cells in suspension. The microbes in the biofilm will not have the same characteristics as microbes in suspension or in a microbial mat. Sar found that bacteria in microbial mats had higher DOS and contact angle measurements correlating to higher hydrophobicity than readings on suspended cells from the MATH test (1987). Washed

cells and unwashed cells were prepared, measurements of cell surface hydrophobicity by DOS and MATH methods were made. The results for both methods were similar except that the washed cells in some cases had lower values for the MATH test (Sar and Rosenberg 1987). The authors suspect that removal of the hydrophobic slime from the cells decreases the hydrophobicity values obtained by the MATH test.

Physical Factors Effecting the MATH Test

The MATH test can be influenced by factors that do not depend on the physiological characteristics of the microbial cell as affected by growth conditions. Some outside influences such as adsorption to water films, properties of the aqueous phase, presence of amphipathic contaminants, cell concentration and cell clumping can affect bacterial adhesion to the hydrocarbon-water interface. These factors can have pronounced effects on the results by either increasing or decreasing the MATH measurement. Adhesion to the sides of glass vessels or other containers will likely cause higher MATH readings. Cells normally in the bulk liquid which adhere to the agitating vessel wall lower the final absorbance reading in the MATH test. The cells on the wall have not interacted with the hydrocarbon and should not be included in the test. A control vessel, a container treated the same without hydrocarbon, can be used to subtract the amount of cells adhering to the sides of the mixing vessel after agitation. Bacteria which are extremely hydrophobic have a tendency to adhere to one another. This tendency makes it difficult to get a well-mixed suspension of cells and consistent readings for initial absorbance of the cell suspension. Cell concentration may also prove to influence microbial adherence to hydrocarbons, so that cell concentrations must be constant between single experiments.

The ionic strength of the buffer solution affects the extent of adhesion of bacteria to hydrocarbons. Salting-out agents sometimes cause bacteria to aggregate. This causes more

of the bacteria to adhere to the hydrocarbon. Equipment used during the experiment must be clean and free of amphipathic contaminants. Glassware should be treated to remove all surfactants from the surface, otherwise interference with the MATH test will occur. Surfactants will interact with the cells in suspension and keep them from adhering to the hydrocarbon, reducing the hydrophobicity measurement. Even though there are a number of factors that influence the results, standard procedures and cleanliness can limit the effects on any one experiment.

Continued Research

Importance of Research

Hydrophobicity of hydrocarbon degraders is not the only area where the MATH test and other methods are used. Cell surface properties of bacteria isolated from fish have been studied using MATH and DOS methods Sar and Rosenberg 1987). This research was used to determine the surface character of the bacteria found on fish scales. The researchers found that the some of the bacteria have hydrophobic characteristics though the values for DOS were often greater then those for MATH. It is thought that the hydrophobic surfaces of the bacteria may aid the fish in motility by reducing the frictional force of water against the fish.

The development of mouthwash is another case where the MATH test has proved useful. In 1982 Weiss *et al.* found that a high proportion of oral bacteria isolated from extracted teeth and steel bands adhered to hexadecane. Other studies had earlier indicated that microbes which adhere to hydrocarbons will also behave similarly when treated with various nontoxic oils. Mouthwashes with an oil phase were tested against commercial

mouthwashes. The result was a significant reduction of oral microbial activity and bad breath when mouthwashes containing oil were used (Rosenberg 1991).

The previous examples illustrate the importance of cell surface hydrophobicity and the need for different adhesion tests. Hydrophobicity tests are used to elucidate the surface properties of microbial cells to help determine the possible interactions between the cells and a surface. Knowledge of this sort is useful, especially when one considers the vast number of systems that have colonizable surfaces. Understanding the mechanisms of bacterial degradation of hydrocarbons at the interface would also give valuable information about how bacteria are able to utilize this hydrophobic carbon source. If the kinetics of bacterial attachment, desorption, growth and surface renewal can be quantified the information obtained could help researchers develop methods to maximize degradation of oil in natural or man-made systems.

Comparable Tests

One of the flaws of the MATH test is its inability to allow for comparison between different experiments. Many of the factors involved in reproducibility have less to do with the overall experimental method than with standardization of factors influencing the test. Another factor that may prove to influence the results of the MATH test are "user errors". Some researchers may interpret a method differently then the author intended. For example, the method used to vortex the cells may have a pronounced effect on the results of the test. Even though there are better tests which allow comparisons between cell surface hydrophobicity, few of them are as fast and simple as MATH.

To make comparisons between experiments from many laboratories using MATH is, at this time, rather inconclusive. There are cases where MATH was compared to other methods of determining cell surface hydrophobicity, the bacteria came from one generation

of bacteria which were scraped from agar plates (Dillon and Koohmaraie 1986). The ability to compare a variety of microbes from a simple test would allow investigators to screen bacteria with unknown surface properties for desired or undesired traits with some assurance that their results are valid. Such a method would make it easier to distinguish nonadhering hydrocarbon degraders from adhering hydrocarbon degraders. A MATH test that is quantifiable would also allow results to be easily compared so that influencing factors can be more easily studied.

METHODS

The methods section is divided into four parts: preliminary work, experimental design, and experimental procedure. The preliminary work describes the isolation of the bacteria and background experiments and procedures. The next section describes experimental design followed by the experimental procedure portion which describes how each experiment was carried out.

Preliminary Work

Isolation

The bacteria used for this research project were isolated from a contaminated soil sample using a modification of technique designed to isolate acetonitrile-utilizing bacteria (Chapatwala *et al.* 1990). These isolates were transferred to silica slant tubes into which the hydrocarbon hexadecane was added as substrate (Funk and Krulwich 1964). The colonies on the slant tube that were able to degrade the hexadecane were further isolated to obtain single colonies. From the slant-tubes, colonies were selected for transfer to tryptone glucose extract agar plates. Transferred colonies were then streaked on the agar plates to isolate individual colonies once more. All of the slant tubes and agar plates were incubated at 35° C until enough growth occurred to allow for individual isolation of colonies. The colonies were transferred to 250 ml flasks containing PUM buffer nutrient media with hexadecane as the carbon source. These flasks were then placed on a shaker table that controlled the temperature and agitation rate for increased oxygen availability. The bacteria grew slowly, breaking down the hydrocarbon while creating a murky solution with a great

amount of bacterial clumping and emulsion production. From this procedure two isolates with the desired characteristics were cultivated for use in all experiments.

The characteristics of the two isolates were similar, although one isolate was pink in color while the other was clear. Both bacterial isolates degraded hexadecane, produced emulsions and collected at the hydrocarbon-water interface. Only the pink colonies were used in the MATH experiment. The pink isolates were chosen for two reasons; they were easier to spot at the hydrocarbon-water interface and individual colonies were easier to discern from groups of colonies. The isolates grew slowly on hexadecane and the suspension remained viable for over a month after which it became necessary tosubculture.

Substrate

The substrate desired for growth of cells in batch cultures had to have certain characteristics in order for the desired results to be achieved. Using an insoluble substrate such as hexadecane was not practical because the amount of substrate available to the cells is not easily quantified. Further, residual hydrocarbon adherence to cell surfaces could interfere with the MATH test results. It was also required that the substrate contain no nitrogen in the molecular formula. The first choice was fatty acids, such as formate, n-butyrate, iso-butyrate, propionate or acetate. These substrates dissolve readily in aqueous solutions and are often intermediates in the metabolism of straight-chain alkanes such as hexadecane. The isolate was able to metabolize all of the fatty acids. However, the inability to easily sterilize fatty acids decreased their desirability as the substrate. Ethanol was tried as a substrate because it is simple in chemical make-up and resembles the end of a fatty acid or alkane chain. Also, the alcohol did not have to be sterilized before addition to the nutrient media. The isolates were able to use ethanol, although the growth rates were extremely slow.

C:N ratio calculations Calculations for relative amounts of carbon in the form of ethanol and nitrogen as ammonium chloride were based on an article by Sykes (1975). Growth yield (Y_{ETOH}) and values for the carbon-to-nitrogen ratios for balanced growth were determined. The range varied from 6.26:1 to 4.94:1. The C:N ratio is important because it has been determined that the relative availability of carbon, nitrogen and phosphorus determines the cells reproduction and production of extracellular polymeric substances. When both carbon and nitrogen are plentiful, the cells grow and reproduce. As nitrogen is depleted, some cells will then switch to recycling of nitrogen. In this case carbon may be excreted from the cells as extracellular polymeric material (EPS). By picking a C:N ratio above that for balanced growth the cells should have enough carbon to grow readily. A C:N ratio of 15:1 is high enough above the minimum requirement that an excess of carbon will be available to the cells in solution. The physical and chemical surface properties should therefore be different for cells grown at a C:N ratio of 15:1 than for those grown at 5:1.

Nutrient Medium

The choice of medium in the growth experiments was determined from adjustments made to the media used to isolate hydrocarbon-degrading organisms. The components of the nutrient media passed through various changes until one that did not interfere with the absorbance experiments was found. Initial choices of media formed precipitates that were carried through the cell suspension preparation and contributed to the absorbance. Initial cell counts of these experiments were usually lower than those for experiments that did not have a precipitate left over from the nutrient medium. This interference was not noticed initially because the interference from the precipitate did not occur until low concentrations of cells in the solution occurred. When the precipitates were present, an initial drop in

absorbance was observed at low agitation rates and mixing times. When the higher agitation rates were used no further drop in absorbance was observed. At the point where the absorbance no longer changed cell numbers continued to decrease. The absorbance of the precipitate in the solution became a background that did not change with the continued removal of cells. For this reason a modified Buschnell-Haas broth was used for the culturing of the bacteria (Table 1).

Table 1. Composition of nutrient media.

Magnesium Sulfate	($MgSO_4 * 7H_2O$)	0.2 g/l
Calcium Chloride	($CaCl_2 * 2H_2O$)	0.02 g/l
Monopotassium Phosphate	(KH_2PO_4)	1.0 g/l
Dipotassium Phosphate	(K_2HPO_4)	1.0 g/l
Ferric Chloride	($FeCl_3 * 6H_2O$)	0.625 ml of 0.01g/ml stock solution added to each 125 ml flask.
Ammonium Chloride for C:N 15:1	(NH_4Cl)	0.319 ml of 10 mg/ml stock solution added to each 125 ml flask.
Ammonium Chloride for C:N 5:1	(NH_4Cl)	0.955 ml of 10 mg/ml stock solution added to each 125 ml flask.

Experimental Design

MATH Assay

C:N Ratio The MATH test was used to determine if the carbon to nitrogen ratio (C:N) would affect the hydrophobicity of a hydrocarbon-degrading bacterium. C:N ratios of 5:1 and 15:1 were used, representing carbon-limited and carbon-rich environments respectively. In the breakdown of hydrocarbons by bacteria in sea-water the limiting nutrient is nitrogen (Atlas 1984). For carbon-limited cells the C:N ratio is 5:1, although there can be some variation (Characklis and Marshall 1990, 145). The carbon-rich C:N

ratio at 15:1 is higher then the ratio (7.7:1) needed for maximum production of emulsan for *Acinetobacter calcoaceticus* RAG-1 (Shabtai and Wang 1989).

Experimental Conditions Concurrent with testing the stated hypothesis on the effects of the C:N ratio, the experiments were designed to obtain statistical data on the accuracy and precision of the MATH test itself. The design of this part of the experiment involved three factors of the MATH test: agitation rate, hydrocarbon volume, and time of agitation (Table 2). Two different methods were used to obtain this data: absorbance measurements and viable cell counts. These methods were also compared to determine if there were significant differences in the results of the two cell enumeration techniques.

Table 2. Settings for agitation rate, hydrocarbon volume and mixing time.

Agitation Rate	Hydrocarbon Volume in ml.	Time of Mixing in seconds.
60	0.2	10
100	0.6	20
140	1.0	40

Agitation Rate

Three agitation rates were used in the test. A Maxi Mix II (vortexer source) was used at the highest setting and the rates were controlled using a Powerstat type 116B (voltage regulator) on three settings; 60, 100 and 140 (Rosenberg 1980). The rate of rotation was measured by strobe light for each of the settings. The rotation rate of the mixing plate and the fluid inside the test tubes for each agitation rate measured as rpm (rotations per minute) were: 1470 at 60, 2810 at 100, and 3100 at 140.

Hydrocarbon Volume

The amount of cell suspension in the test tubes was held constant at 5 ml. Three different hydrocarbon amounts (0.2 ml, 0.6 ml and 1.0 ml) were used in the experiments. In the initial MATH test, 1.2 ml of cell suspension was subjected to varying volumes of hexadecane, xylene and octane. The volume of hydrocarbon ranged from 0 to 0.2 ml. A later testing procedure used 5 ml of cell suspension to 1 ml of hydrocarbon. For the reported data, the larger volume technique was used because cell counts and absorbance measurements after agitation required the full 5 ml of cell suspension.

Hydrocarbon Screening

To determine which hydrocarbon would be used for the research the three hydrocarbons (hexadecane, xylene and octane) were tested. The experimental design compared the results of each hydrocarbon's efficiency and variability for removal of bacterial cells from the media. Initial results indicated the three methods had similar percent removals and reproducibility (Table 3). The experimental error associated with the three methods seemed to be similar, and in some cases the experimental error was large.

Table 3. Absorbance and Percent Removal for Three Hydrocarbons

		Time of Mixing				
Hydrocarbon	Init. Abs.	10 sec.	20 sec.	40 sec.	80 sec.	% Removal
Xylene	.301	.209	.196	.192	.191	36.5 %
Octane	.302	.213	.204	.198	.196	35.0 %
Hexadecane	.290	.215	.209	.204	.191	34.1 %

This finding was similar to results obtained by Sweet et al(1987), where it was determined that residual hydrocarbon can adhere to bacteria and increase the refractive index of the

bacteria. The residual could be removed by aerating the solution for one minute causing desorption from the bacterial surface. The results using this method were less reliable for hexadecane than for xylene.

One potential problem that can occur when using xylene as the hydrocarbon is lysis of the cells. This would cause lower absorbance measurements to be observed in the experiments. Sweet *et al.* determined that lysis of cells was not occurring in the presence of xylene (1987). By aerating a suspension of cells and xylene overnight, the xylene was removed and the original absorbance was recovered. Viable cell counts confirmed that the cells were not being lysed. This research followed the procedure of Sweet *et al.* by using xylene as the hydrocarbon and aerating the cell suspensions to remove xylene attached to the cells or dissolved in the water.

Time of Mixing

Researchers using the MATH test have used agitation times ranging between 5-120 seconds. To minimize the number of mixing periods the point where continued mixing no longer changed the absorbance was determined. After using three different hydrocarbons and mixing times running to 120 seconds, it was determined that no significant changes were taking place after 40 seconds. Table 3 also shows this trend, with absorbance values changing little after 40 seconds of mixing.

Experimental Procedure

Inoculation

Numerous authors cite growth phase as affecting the MATH test. It was for this reason that all cells were harvested during stationary phase at the same interval after

inoculation of the culture. The soil isolate had a slow growth rate and did not reach the stationary phase until the seventh day of incubation. To determine when stationary phase was reached, 5 ml samples were taken twice a day from the culture for nine days. Stationary phase was reached when the absorbance measurements no longer increased. Stationary phase was also confirmed from cell count data. Once the stationary phase was determined, a harvest time was picked that would be constant in all the experiments. All cells for each experiment were therefore harvested between 190-194 hours, or about 8 days after inoculation. The culture flasks were agitated at 110 rpm on a New Brunswick Scientific water bath shaker table at 37° C.

First generation cells, scraped from R2A agar plates, were suspended in 125 ml of Bacto Bushnell-Haas Broth nutrient medium (Table 3). For a 5:1 C:N, 0.955 ml of stock solution was added to the nutrient medium, while for a 15:1 C:N, 0.319 ml of solution was added. Filter sterilized ferric chloride (0.625 ml) was added separately because of its tendency to precipitate when autoclaved. 31.3 μl of ethanol, the substrate, was added to the nutrient medium. After 8 days, the cell suspension was transferred to centrifuge bottles and placed in the centrifuge.

Washing Procedure

The cell suspension was added to centrifuge tubes and placed in a RC5C Sorvall Instruments centrifuge set to run for 25 minutes at 12,000 xg. After centrifuging, the liquid was poured off and the pellet was washed with 50 ml of buffer solution (Table 4) which had been filtered through a .2 μm filter to remove any precipitate formed by the autoclaving process. The cells were vortexed to break up the pellet and placed in the centrifuge again. The same procedure was then performed twice more.