NOTE TO USERS

This reproduction is the best copy available.

UMI®

University of Alberta

Isolation of Bacteria Capable of Degrading Decalin, Decane and Heptamethylnonane

by

Paul Stephen Chernik

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of *Master of Science*

in

Chemical Engineering

Department of *Chemical and Materials Engineering*

Edmonton, Alberta
Fall 2005

ABSTRACT

Processing bitumen from the Athabasca oil sands of Alberta, Canada offers many unique challenges. The conversion of polycyclic aromatic hydrocarbons to more linear alkanes for diesel fuel is a significant challenge. Hydrogenation can convert these aromatics to saturated cyclic structures. This study investigates the potential of opening the resulting saturated ring structures using biocatalysts. Decalin was selected as the model compound for the study. Efforts were made to isolate bacteria capable of degrading this compound. This process was successful, as at least two bacterial types were identified capable of causing decalin degradation. In addition, these bacteria were able to cause removal of 2,2,4,4,6,8,8-heptamethylnonane (HMN) via cometabolism. Finally the bacteria were able to degrade the *n*-alkane decane. Due to the lack of selective attack towards cycloalkanes, the bacteria isolated are likely unsuitable for use as biocatalysts in upgrading processes without genetic modification. However, the results offer new opportunities for bioremediation applications.

Acknowledgements

Many people and groups provided tremendous support in the course of this study. The following individuals and organizations in particular have my thanks for their knowledge, encouragement and financial assistance over the past two years.

Mr. Peter Chernik
Mrs. Elizabeth Chernik
Ms. Sarah Chernik
Ms. Julie Chernik
Dr. Murray Gray
Dr. Julia Foght
Ms. Kathlyn Kirkwood
Ms. Sara Ebert
Mr. Jonathan Klassen
Ms. Martina Rusnacik
University of Alberta Environmental Microbiology Group
NSERC Industrial Research Chair in Upgrading Processes
NSERC Graduate Scholarship Program
University of Alberta Graduate Scholarship Program

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BH: Bushnell-Haas
DBT: Dibenzothiophene
DCM: Dichloromethane
HMN: 2,2,4,4,6,8,8-Heptamethylnonane
GC: Gas Chromatography
LGO: Light Gas Oil
PCA: Plate Count Agar

1.0 INTRODUCTION

The oilsands of Alberta are an enormous energy resource for the future, but the nature of the material and the current process technology present a number of challenges. Processing of oilsands is an energy intensive process. Therefore, development of new low-energy technologies is a high priority. The oil from the oilsands is rich in aromatic compounds, which is undesirable for fuel quality, particularly for manufacturing diesel fuels that produce low amounts of soot upon combustion.

Current catalysts are not able to deal with these aromatic compounds efficiently. The aromatics are easily hydrogenated at high pressure to give compounds with one to five rings of saturated carbons joined together. When these compounds are used in fuel, they tend to dehydrogenate and form soot. In order to avoid this environmental problem, opening of the rings is desirable to give straight chain alkanes with improved combustion qualities. Unfortunately, the available catalysts are very inefficient in this process and give low yields of the desired product, along with high yields of unwanted products such as butane.

Bacteria commonly synthesize and degrade ring compounds that are remarkably similar to the saturated ring compounds in products from the oilsands. These compounds, structurally similar to cholesterol and other steroids, are common components of the cell membranes and cell walls of some organisms. Consequently, microorganisms likely possess interesting biocatalytic activity towards compounds containing saturated rings of carbon compounds.

In this study, an attempt was made to develop a novel biocatalyst for opening saturated ring compounds, such as the structures found in products from the oilsands. The study began by determining what was required to develop a successful biocatalyst. Desired attributes as well as known limitations and considerations of biocatalysts were explored. The investigation indicated that no bacterial species had been identified that was suitable for use as a biocatalyst for opening multi-ring cycloalkanes. As a result, an effort was made to find one. Decahydronaphthalene

(decalin) was selected as the model compound for the investigation. Mixed cultures were obtained from hydrocarbon-contaminated sites around the globe with the potential to contain suitable bacteria. These mixed cultures were then studied to determine which had the possibility of containing bacteria capable of degrading decalin.

Once the active mixed cultures had been identified, enrichment cultures using liquid and vapour carbon sources were carried out and bacteria were isolated from the mixed cultures that had the ability to degrade decalin. Some of the isolates also had the capability to cause the removal of 2,2,4,4,6,8,8-heptamethylnonane (HMN), which was being used as a carrier solvent. This removal was significant because HMN had previously been thought to be completely recalcitrant to microbial attack. Not only did the isolates have the ability to attack cycloalkanes and HMN, but they also attacked the *n*-alkane decane. As a result, the bacteria isolates were considered unsuitable for use as biocatalysts for selectively opening multi-ring cycloalkanes. However, the research results offer exciting possibilities in the area of bioremediation.

2.0 LITERATURE REVIEW

2.1. Traditional Upgrading Methods in Oil Sands Refineries

The Alberta oil sands represent a vast energy resource. Approximately 1.7 to 2.5 trillion barrels of oil are trapped in the reservoir system (5). Between 1996 and 2002, roughly $24 billion dollars were invested in the development of oil sands extraction and refining facilities and over 95,000 people were directly employed by the upstream facilities (3). Unfortunately, the crude that comes from the tar sands is poor quality, with an API gravity index around 10. As a result, a tremendous amount of energy and effort must be spent to upgrade the oil to suitable quality for sale. During oil sands upgrading, the oil must undergo three main processes: distillation, coking and hydrotreating. In addition, catalytic conversion may also be utilized. Distillation involves the separation of the oil into various fractions for further upgrading or sale. Coking removes the high molecular weight material in the oil, as well as some of the heavy metals. Catalytic conversion cracks the oil into smaller hydrocarbons, lowering the molecular weight of the oil and increasing the usable fraction. Finally, hydrotreating removes the majority of sulphur and nitrogen in the oil, and adds hydrogen to the molecules in the oil (5, 25).

Although each company utilizes different technology to achieve these steps, the general concepts are consistent throughout the industry. Figure 1 depicts the process utilized by Syncrude Canada Ltd.

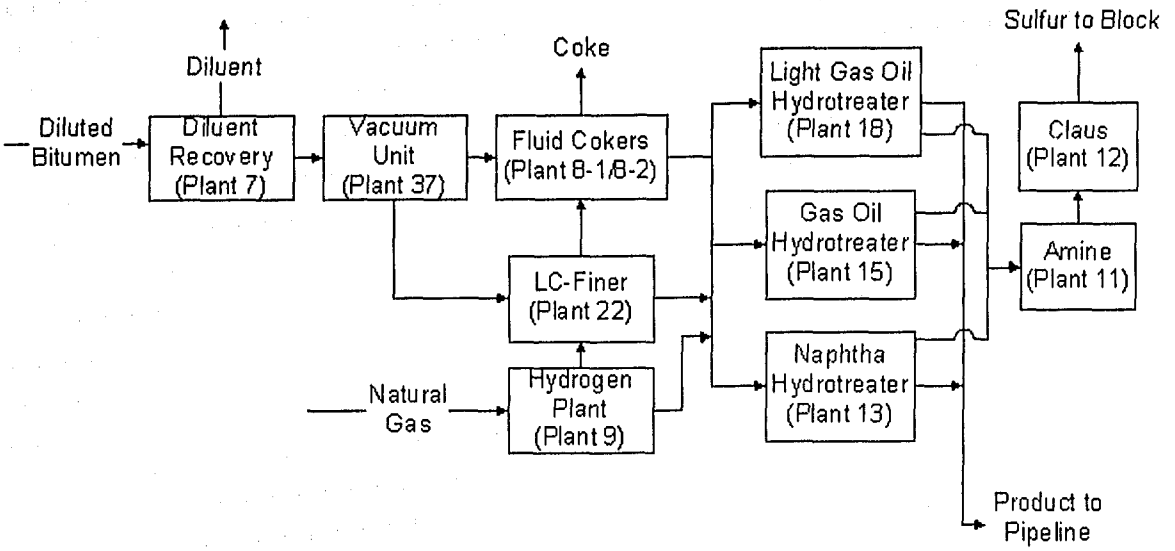

Figure 1: Syncrude Process Scheme
(25)

The impetus for biocatalyst research for cycloalkanes is the hydrotreating process. This is the stage in which olefins and polyaromatics in the oil are hydrogenated, and in which >80% of the sulphur and >70% of the nitrogen is removed from the oil. This removal is achieved with the use of catalysts such as NiS + MoS_2 at temperatures between 300°C and 410°C and H_2 pressures greater than 5MPa (25). Hydrogenation of the aromatics to cycloalkanes is desired as the density of the product is lowered. However, if the cycloalkanes remain in the process and end up in the fuel products they have the ability to dehydrogenate in combustion engines and form soot. Therefore, one would like to open the rings to form straight chain molecules. However, multi-ring saturated molecules are highly stable. Once they are formed, catalytic cracking is needed to break the bonds and open the rings. Current catalytic cracking methods are non-selective. When the Light Gas Oil (LGO) from the hydrotreater is sent to a catalytic cracker, not only can the bonds in the cycloalkanes be broken, bonds are also broken in other molecules. This leads to the formation of light end hydrocarbons such as butane, which are undesirable when the goal is to produce gasoline or diesel fuel. Much effort has been put into developing a selective inorganic catalyst.

Corma *et al.* (13) in 2001 specifically looked at the ability of zeolites to crack decalin and tetralin. In inorganic catalysis pore size and available active sites for catalysis play an important role in the selectivity. The experiments outlined by Corma *et al.* demonstrated that conversion of decalin in their reactor system (operating at 723 K and 1 atm) varied from 3% to 23%, using a variety of different zeolite catalysts. While this was an increase from thermal cracking conversions (without the presence of a catalyst) of 0.3%, the products were a major issue. The catalyst suggested by the authors for light gas oil quality improvement led to products that were mainly C4 and C5 alkanes and C6 naphthenics. These are undesirable as they represent the loss of carbon to light hydrocarbons (thus reducing the amount of product diesel) and the production of soot-forming aromatics. Combined with the low conversion yields, one can see that there is still much work needed to develop a cost effective selective inorganic catalyst. Therefore, opportunities exist for alternative catalysis techniques, including whole cell biocatalysts.

2.2. Development of Biocatalysts

In the natural environment, biological reactions are facilitated by enzymes. An enzyme is a protein that catalyzes biochemical reactions (33). Enzymes are also usually substrate-specific, according to the particular requirements of a given biological pathway. Because these reactions occur in living organisms, they most often occur at ambient temperatures and pressures. From an industrial standpoint, these naturally occurring reactions are of interest as they do not need the high temperatures and pressures often required when using inorganic catalysts and the reactions can be highly specific (7). This selectivity is ideal when attempting to open cycloalkanes after hydrotreating.

Enzymes can be used in two ways. The first method is by introducing enzymes directly to a system to facilitate a reaction (4). Unfortunately, many biochemical reactions involving hydrocarbons require multiple enzymes that must be utilized in a specific order and require organic cofactors (37). Thus difficulties can arise when attempting to use pure enzymes to achieve the desired reaction. The second method is to introduce whole cell biocatalysts (living,

active bacteria) to a system. The bacteria produce the enzymes which facilitate the desired reaction. The bacteria use the substrate as a nutrient source, and through their use of the substrate, initiate a desired reaction. An example is in biodegradation. Bacteria use a carbon-based contaminant, such as a hydrocarbon, as a carbon source. Through biochemical pathways, the carbon is used to produce energy in the cell, biomass and CO_2 as waste.

Whole cell biocatalysis has been an area of interest for many years. One area of interest has been the biodesulphurization of fossil fuels (43). In this area of research, a bacterium was discovered, called *Rhodococcus* sp. IGTS8, with the ability to selectively metabolize dibenzothiophene (DBT) and used the compound as a sole source of sulphur (24). The genes responsible for the removal of sulphur were identified and genetic manipulation was conducted to amplify the expression of the enzymes responsible for the degradation of DBT (43). With this completed, attempts were made to scale up the process and a 5bbl/d pilot plant was built. The plant used a combination of "separation schemes, including simple settling tanks, hydrocyclones, membranes, and low-speed centrifuges" (43), and in 1998 the total expenditure by the parent company on the project, Energy BioSystems Corporation, excluding partners was $50 million US. Process optimization was still an ongoing process as of 1998, suggesting that the issues of scaling up a laboratory process to an industrial level were proving to be challenging.

Another area of interest to researchers has been aromatic bio-processing. Biocatalysts have been studied for their ability to cleave the rings of di- and tricyclic homologues of crude oil (20). An example process is the two-stage "Biological Aromatic Ring Cleavage" (BioARC) process (21), which is shown schematically in Figure 2.

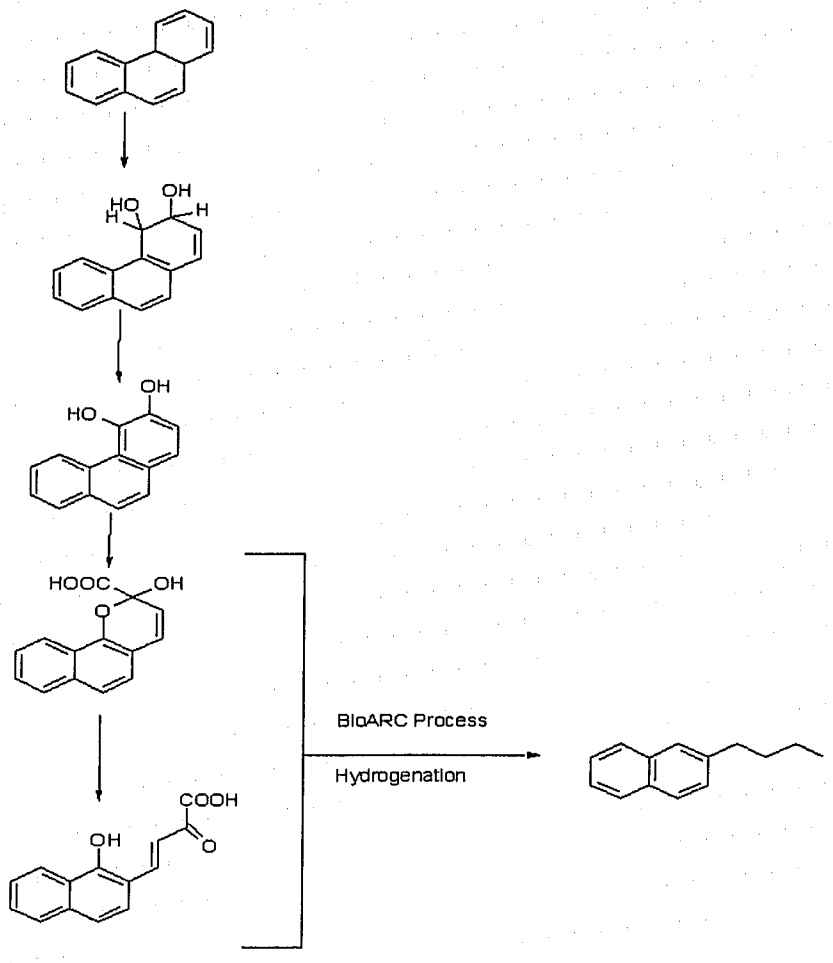

Figure 2: BioArc Ring Cleavage of Phenanthrene Ring
(20)

When bacteria are used as biocatalysts in a petrochemical process, complete recovery of the carbon is desired, without loss to either CO_2 or to biomass. Thus, in order to achieve the above reaction, enzymatic pathways were altered so that the petroleum hydrocarbon was not used for biomass, or oxidized to CO_2. The process used to develop a suitable biocatalyst involved isolating a bacterium, which would use phenanthrene as a carbon source and mineralize (completely degrade) it to CO_2. Genetic manipulation was conducted to halt the enzymatic pathway at the desired point. With this manipulation complete, a biocatalyst was created that produced the ring cleavage products (20). In theory, these oxygenated products will subsequently hydrogenated using conventional catalysis to yield the desired alkyl-aromatic products

The previous examples demonstrated that bacteria have the potential to be used as whole cell biocatalysts for petroleum upgrading. Several important lessons are also presented by the example. First, due to the need for genetic manipulation to achieve a desired product, preference is given to working with a pure culture (20). While some work has been conducted with mixed cultures in bioreactor systems (14, 44) these studies were concerned only with mineralization. Therefore, when looking for a biocatalyst to open saturated multi-ring compounds, a pure culture is desired. In addition to this requirement, the desired attributes of a biocatalyst for cycloalkane bio-processing can be summarized as follows:

1. "Rapid growth to a high cell count in an inexpensive medium without a hydrocarbon, generating biomass that either has constitutive or rapidly inducible biocatalytic activity.
2. Rapid, robust and sustained cycloalkane oxidation by pre-grown cells as a resting cell suspension in an aqueous phase.
3. A broad range of activity against cycloalkanes and alkyl substituted cycloalkanes.
4. Lack of activity against non-target substrates such as aliphatic hydrocarbons.
5. A genetic system that can be manipulated to allow the isolation of specific mutants blocked at the desired enzymatic step, thus preventing the loss of carbon and producing the desired oxidized product.
6. Stability and predictability of the phenotype and the mutation(s) regardless of whether the catabolic genes are plasmid borne or chromosomal.
7. Activity in two-liquid-phase reaction systems where the substrate is in the water-immiscible phase at a high volume ratio to the cell suspension
8. Tolerance towards any toxic effects of the feedstock or biocatalytic products
9. Non-pathogenicity of the biocatalyst for safe handling and disposal
10. The ability to scale up active biomass production to commercial scale operations" (20)

Therefore, one can see that in order to develop a biocatalyst for opening cycloalkanes based on existing pathways, one must understand how hydrocarbons are utilized by bacteria. Then, one must find an isolate which has the ability to use these rings as a sole carbon source.

2.3. General Information Regarding Hydrocarbon Degradation

Hydrocarbons are molecules which contain only carbon and hydrogen. The main groups are alkanes, alkenes, alkynes, aromatics and cycloalkanes. In aerobic degradation, the first step in

hydrocarbon degradation often involves oxidation of the hydrocarbon (38). This is achieved through a type of enzyme known as an oxygenase. There are two classes of oxygenases. The first is called a monooxygenase and this enzyme has the ability to take an O_2 molecule and transfer one of the oxygen atoms to an organic compound. In hydrocarbons, this leads to the formation of hydroxyl (OH) groups. The other type of oxygenase is called a dioxygenase and transfers both of the O_2 atoms to the hydrocarbon. The O_2 in both of these reactions operates as the electron acceptor (38).

Once this initial oxidation step occurs, further reactions are necessary to turn the hydrocarbon into a form that can be utilized by the bacteria as a source of cell mass and energy. End products of this process can be compounds such as acetate or pyruvate, which can then be used to form acetyl-CoA for biosynthesis (38). An example of a typical pathway is shown in Figure 3.

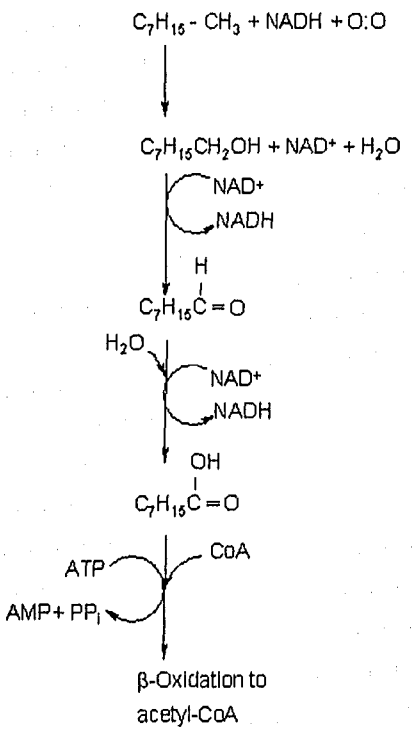

Figure 3: Oxidation of an Aliphatic Hydrocarbon, Catalyzed by a Monooxygenase (38)

Some hydrocarbons are more easily used by bacteria than others. The ease of use is dependent in part on the availability of sites on the hydrocarbon for the initial oxidation step. In the case of *n*-alkanes, usually the process involves oxidation of a terminal methyl group followed by β-oxidation (55). Branching in the hydrocarbons hinders biological attack. For example, if branching occurs at the β position, then additional steps such as α-oxidation, β-alkyl group removal or ω-oxidation may be required for oxidation to proceed (55). In addition terminal branching can inhibit the initial oxidation of the alkane. Research published by Schaeffer *et al.* (55) demonstrated that β-methyl substituted alkanes were highly recalcitrant towards microbial degradation. As a result chemicals such as 2,2,4,4,6,8,8-heptamethylnonane (HMN), which are branched at the β positions, have been known not to degrade when used as a sole carbon source (16, 18, 68). This resistance has led to the common use of HMN as an "inert" carrier liquid in laboratory studies of biodegradation (16).

2.4. Cyclic Hydrocarbon Biodegradation

Besides branching, cyclic structures can also inhibit hydrocarbon degradation. Cyclic structures have a lack of readily available sites for the initial oxidation attack. Cycloalkane biodegradation has long been an area of interest for researchers. In 1940, some of the first attempts were made to investigate the ability of bacteria to degrade decahydronaphthalene (decalin) (66). Reports were often mixed. Some researchers determined that cyclic hydrocarbons behaved as recalcitrant molecules, resisting all attempts at biodegradation. In one study in 1971 conducted by Pelz and Rehm, 250 different mixed cultures of bacteria were evaluated for their ability to degrade decalin (47). All met with failure. However, in 1973 Soli and Bens (65) contradicted this statement, reporting that two out of eight strains studied had the capability of degrading decalin, and five out of eight strains studied had the ability to degrade bicyclohexyl.

Until 1974, there seemed little consensus on how or why degradation of cyclic hydrocarbons occurred. Then, in 1974 Beam and Perry published a paper entitled "Microbial Degradation of

Cycloparaffinic Hydrocarbons via Co-metabolism and Commensalism" (9). In the paper, it was shown that bacteria can work in a consortium to degrade cycloalkanes through cooxidation and commensalism. Cooxidation occurs when growing microbes oxidize a compound, but do not utilize either carbon or energy from the oxidation (49). Commensalism is defined as an interaction between two organisms, where one is helped and the other is neither helped nor hindered (1). Beam and Perry demonstrated that in the biodegradation processes his group studied, the cycloalkane could not be used as the sole carbon source. Instead, another substrate such as a *n*-alkane had to be present. In the mixed culture, one bacterium used the *n*-alkane as a carbon source. During this process it cooxidized the cycloalkane. The oxidized cycloalkane was then utilized by another bacterial strain to produce cell material and CO_2 (8, 9, 49, 50). These findings were also independently proved by deKlerk and Van der Linden in 1974 (6). They demonstrated that hydroxylation of substituted cycloalkanes by *Pseudomonas aeruginosa* occurred in the presence of *n*-heptane. Their results are found in Figure 4.

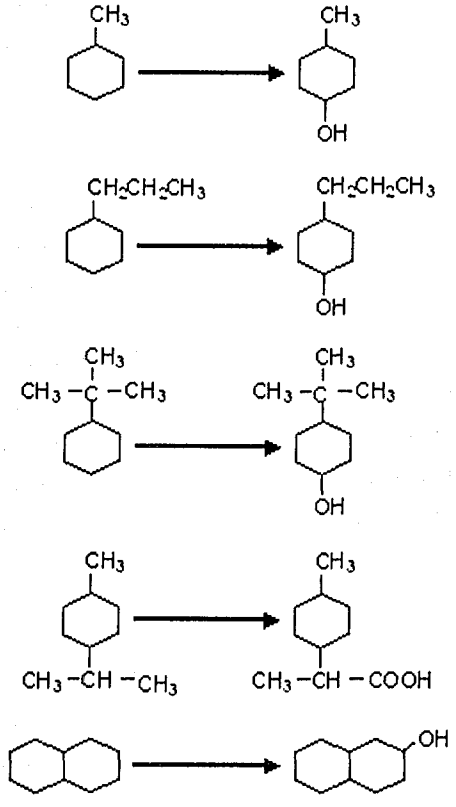

Figure 4: Hydroxylation of Substituted Alicyclic Hydrocarbons
(6)

Because researchers had now found a way to degrade previously recalcitrant molecules, efforts were made over subsequent years to determine the pathways of catabolism. The work conducted can be broken down into three categories: first, those researchers who chose to work with cycloalkanes with no branches; second, those researchers who chose to work with *n*-alkyl cycloalkanes; third, those researchers who chose to work with biomarkers.

2.4.1. Cycloalkane Biodegradation

In this field of study, researchers have been investigating the means by which direct ring attack occurs, and subsequent metabolic pathways. Most of the efforts were made in cyclohexane degradation. In 1977 Stirling *et al.* (6) published work describing their use of a *Nocardia* strain and proposed the pathway shown in Figure 5.

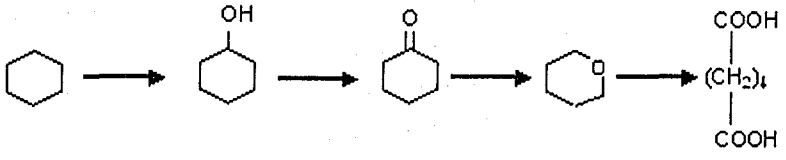

Figure 5: Pathway for Catabolism of Cyclohexane by *Nocardia* Strain
(6)

Further studies showed that a variety of organisms could convert oxidized cycloalkanes into cell material and CO_2. In 1976 Griffin *et al.* showed that *Pseudonomas* NCIB 9872 could utilize cyclopentanol in the following pathway (6):

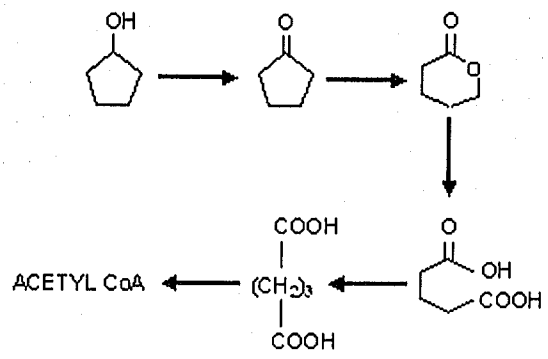

Figure 6: Biodegradative Pathway of Cyclopentanol by *Pseudomonas* NCIB 9872 (6)

After the work conducted by Griffin *et al.* there were continued efforts to fully define the mechanisms by which alcohols and carboxylic acids are broken down into usable molecules for catabolism (23). In addition, efforts have been made to understand how certain bacteria, such as *Acinetobacter* NCIMB 9871, use monooxygenase to produce cyclohexanol (67). In recent years, as cyclohexane has gained favour in use as an industrial solvent over benzene (58), there has been renewed interest in finding strains which can use cyclohexane as a pure carbon source. In addition, concerns with gasoline contamination in groundwater supplies and soils have spurred further research (39, 59, 61-63). Attempts to quantify the kinetics of cycloalkane degradation have been made in terms of O_2 and CO_2 consumption, and in terms of biomass production (64). There has also been some success at isolating bacteria strains which can use cyclohexane as the sole carbon source (53). As a general rule though, the research has shown that strains which can use cycloalkanes as a pure carbon source are rare (52), whereas cooxidation and commensalism can be an effective means of degrading cyclohexane (39).

A common finding throughout cyclohexane research has been that degradation utilizes several key steps. The ring must be hydroxylated by a monooxygenase to give cyclohexanol. Then the cyclohexanol is converted to a ketone cyclohexanone. The cyclohexanone serves as the substrate for the cyclohexanone monooxygenase, which catalyzes a Baeyer-Villiger oxidation to insert an oxygen atom into the ring. This creates a lactone, which is then cleaved by a lactone hydrolase (56).

In addition to cyclohexane studies, some work has been conducted with higher number carbon molecules. Ko and Lebeault (27) looked at the ability of mixed cultures to mineralize decalin and pristane. In their work, Ko and Lebeault demonstrated that a mixture of two strains, *Pseudomonas aeruginosa* K1 and *Rhodococcus equi* P1 were able to successfully break down decalin and pristane via cooxidation. However, no catabolic pathways were proposed. Schumacher and Fakoussa (56) investigated the degradation pathways of *Rhodococcus ruber* strain CD4 using cyclododecane as the sole carbon source. In their research, they found that degradation of cyclododecane follows a similar pathway to that of cyclohexane. First, the cyclododecane was converted to cyclododecanol and then to cyclododecanone. A cyclododecanone monooxygenase then converted the cyclododecanone to oxacyclotridecane-2-one and through further mechanisms eventually to 1,12-dodecandioic acid. The proposed pathway is shown in

Figure 7.

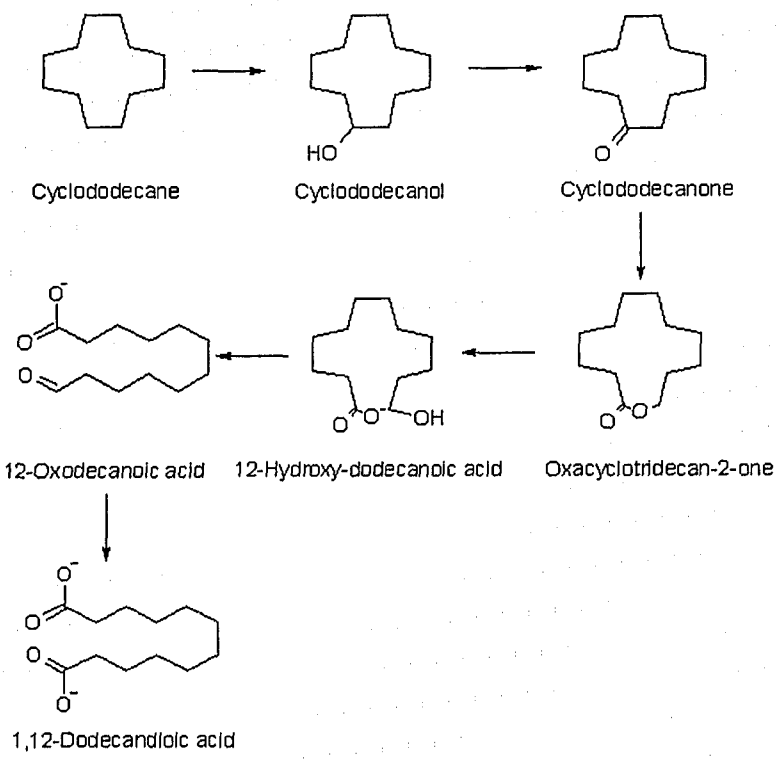

Figure 7: Proposed Pathway for degradation of cyclododecane by *R. ruber* CD4 (56)

Their research also showed that the cyclododecane monooxygenase was ring size specific, only being able to attack cycloketones with 10 carbons or more. Tests showed that *Rhodococcus ruber* CD4 had negligible abilities to oxidize 6 and 8 membered rings.

2.4.2. *n*-Alkyl Cycloalkane Biodegradation

Besides efforts to understand cycloalkane degradation, efforts have also been made to understand *n*-alkyl cyclohexane biodegradation. *n*-Alkyl cyclohexanes are compounds comprised of cyclohexane with an alkane substituent. Research in the 1970's showed that different intermediates are created, and vary due to the length of the *n*-alkyl branch. Molecules with odd-chain lengths are metabolized via β-oxidation to cyclohexane carboxyl CoA which is then easily degraded by other bacteria (23). Even length side chains are converted to cyclohexane acetic acid which is a more recalcitrant molecule but can be degraded by certain species of bacteria (6). A summary to 1984 is found in Table 1:

Table 1: Bacterial Species That Degrade *n*-Alkyl Cyclohexanes Prior to 1985

Researchers	Bacterial Species	Substrate	Produces	Year
Tonge and Higgins (6)	Nocardia petroleophila	Methyl cyclohexane acting as sole substrate	3-methyl-cyclohexanol, 3-methylcyclohexanone	1974
Arai and Yamada (6)	Alcaligenes faecalis	Ethylcyclohexane	Trans-4-ethylcyclohexanol	1969
Van Ravenswaay and van der Linden (6)	Pseudomonas aeruginosa	Ethylcyclohexane	Trans-4-ethylcyclohexanol	1971
Davis and Raymond (6)	Nocardia, strains 107-332 and M.O.	*n*-octadecane and *n*-butylcyclohexane	Cyclohexaneacetic acid	1961
Beam and Perry (6)	Mycobacterium vaccae, Mycobacterium concolutum and Mycobacterium rhodochrous	Dodecylcyclohexane and heptadecylcyclohexane	Cyclohexylacetic acid	1974

After this initial work, little research was conducted in this field. Possibly, this was due to a shift in the focus of research at the time towards aromatic hydrocarbons. However, there has been a resurgence of published works in the last decade. Most recently, this includes work conducted by Dutta and Harayama (17) and Koma *et al.* (28, 29). Dutta and Harayama investigated the

degradation of *n*-octadecylcyclohexane and *n*-nonadecylcyclohexane by *Alcanivorax sp.* strain MBIC 4326. When the *n*-alkyl cyclohexanes were used in conjunction with a BSM medium, *Alcanivorax sp.* strain MBIC 4326 degraded both chemicals to 4-cyclohexylbutanoic acid and 3-cyclohexylpentanoic acid. Unfortunately, at this point the bacterial strain was not able to use these compounds as sole carbon sources. To deal with this, the authors cultivated the bacteria on *n*-hexadecane and then added 4-cyclohexylbutanoic acid and 3-cyclohexylpentanoic acid to the cultures.

In this manner, they were able to continue the degradation process until cyclohexaneacetic acid and cyclohexanecarboxylic acid were detected as products from *n*-octadecylcyclohexane and *n*-nonadecylcyclohexane, respectively. These products were in part explained by β-oxidation. However, as Dutta and Harayama point out, this would not explain how both cyclohexaneacetic acid and cyclohexanecarboxylic acid were obtained from the two initial chemicals. The authors investigated further, and found other intermediates were formed that suggest that other pathways may be followed.

In addition to determining that cyclohexaneacetic acid and cyclohexanecarboxylic acid were formed, the Dutta and Harayama noted that trace amounts of benzoic acid were found. Through further investigation, Dutta and Harayama discovered that cyclohexanecarboxylic acid was being broken down into 1-cyclohexane-1-carboxylic acid and then into benzoic acid. They also found that the bacteria could convert 3-cyclohexane-1-carboxylic acid into benzoic acid. A summary of the proposed pathways can be found below, in Figure 8. Note that solid arrows represent major metabolic routes following classic β-oxidation pathways. Open arrows represent minor metabolic routes following novel pathways.

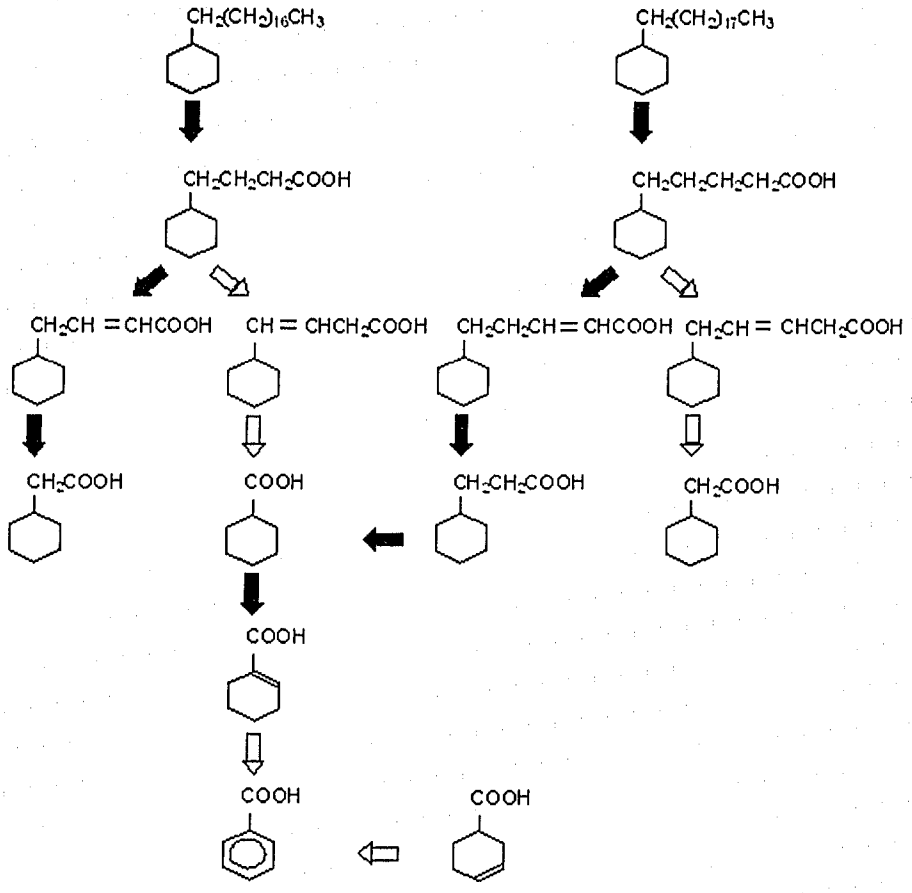

Figure 8: Proposed pathways for the biodegradation of *n*-alkyl cyclohexanes by *Alcanivorax* sp. strain MBIC 4326
(17)

Koma *et al.* also investigated the degradation of *n*-alkyl cycloalkanes. Two strains were isolated. The first, *Acinetobacter* sp. ODDK71 was unable to utilize *n*-alkyl cyclohexanes as a sole carbon source. However, it cooxidized dodecylcyclohexane and tetradecyclohexane in the presence of hexadecane (28). Of greater interest was their work with *Rhodococcus* sp. NDKK48, which used dodecylcyclohexane as a sole carbon source (29). Their research has indicated two mechanisms for the degradation of dodecylcyclohexane. The first follows the usual mechanism of alkyl side chain oxidation. The second involves direct ring oxidation. The products of the initial step of these two mechanisms are found in Figure 9.

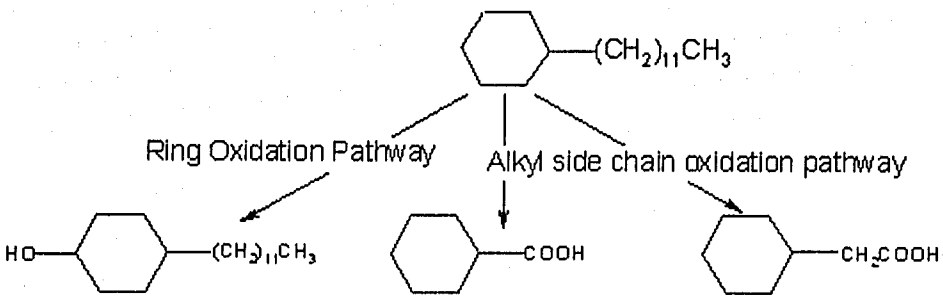

Figure 9: Putative degradation pathway of dodecylcyclohexane by *Rhodococcus* sp. NDKK48 (29)

Koma *et al.* (29) also investigated the ability of *Rhodococcus* sp. NDKK48 to use cycloalkanes with no side chains or with very short side chains as a sole carbon source. All results were negative, however cooxidation was observed in the presence of an *n*-alkane (29).

2.4.3. Biomarker Biodegradation

Biomarkers are defined as "structurally complex components of petroleum derived from biological molecular precursors, such as chlorophyll, sterols and hopanoids" (11). A group from Strasbourg, headed by Guy Ourisson (45) found that certain molecules, which he termed geohopanoids (see Figure 10) are ubiquitous in oils around the globe, (45, 46) can be used as geochemical markers to "correlate oils and sediments" (45) and can be used to determine the degree of biodegradation of a given oil sample (41). Understanding the degradation pathways of such compounds is valuable as they are naturally occurring saturated multi-ring molecules. Thus, if the pathways of degradation are understood for the complex compounds, extrapolation to simpler multi-ring compounds should be possible.

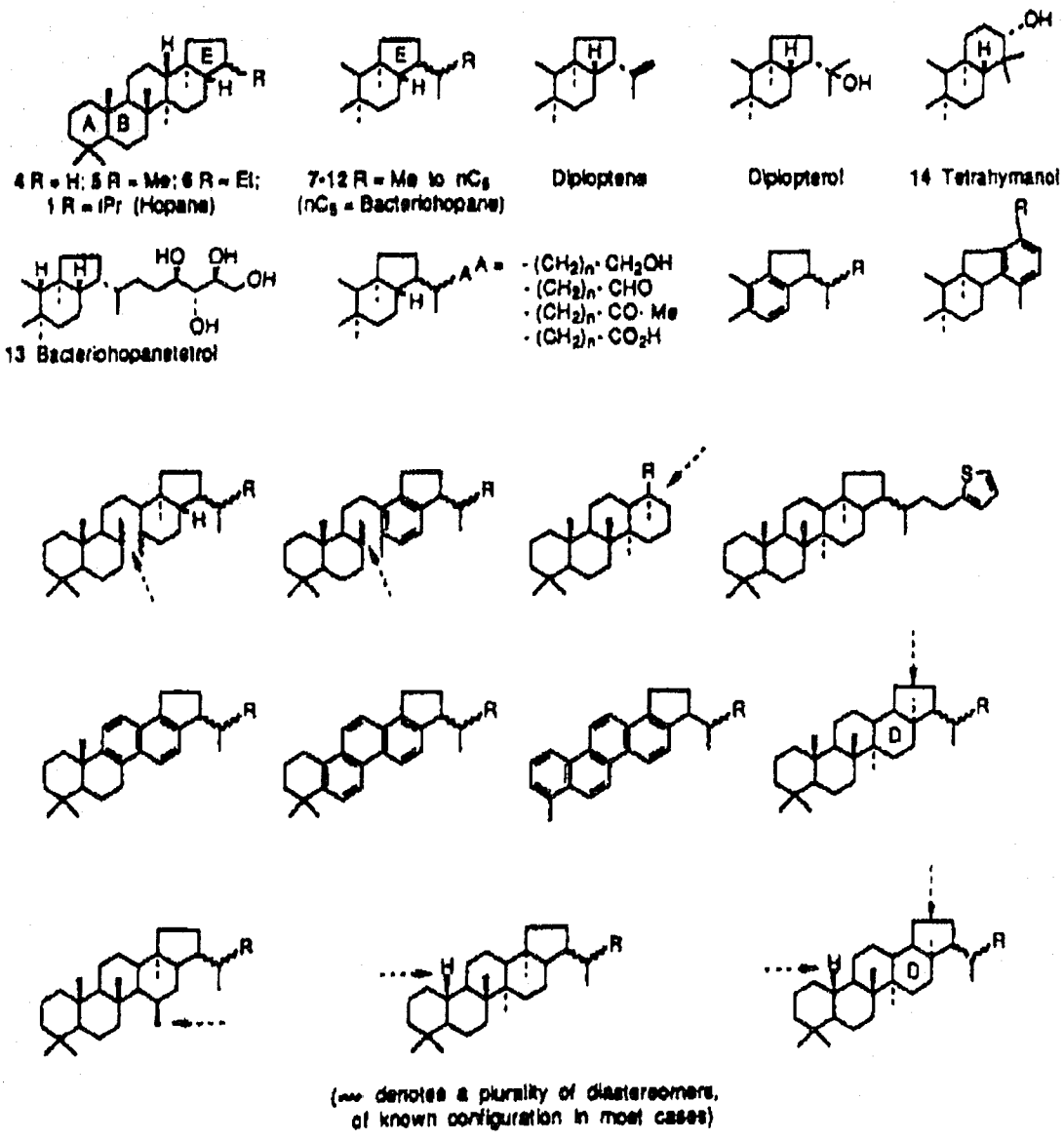

Figure 10: Geohopanoids of Established Structure
(Reprinted with permission from (45). Copyright 1992 American Chemical Society)

Since the initial work conducted by Ourisson several different families of biomarkers have been identified. Examples include steranes and triterpanes (11). Within these families are many molecules. For example hopanes and tricyclic triterpanes are both considered triterpane compounds with different molecular weights and structures. It has been found that biodegradation of hopanes and homohopanes does occur, while tricyclic terpanes can remain unaltered even

after hopanes have been degraded (11). Much work has been done to understand hopane biodegradation, but as yet there is no consensus on the pathways. Moldowan and McCaffrey (42) proposed that bacteria either degrade homohopanes by oxidizing the side chains on the molecules (similar to the attacks on simple *n*-alkyl cyclohexanes) or they directly attack the cyclic cores of the molecules and preferentially attack lower molecular weight compounds. Further research is required to validate either model. In addition, biodegradation pathways are known to be dependent on temperature, aeration, availability of nutrients and cosubstrates, the presence of simpler hydrocarbons for cometabolism and oil concentration (22).

2.5. Limitations of Biocatalysts

Every bacterial species is subject to limiting conditions, which control its ability to attack certain compounds. When utilizing bacteria as biocatalysts, there are several limiting criteria that must be considered.

2.5.1. Carbon Source Uptake by Cell

The first major consideration is uptake of the substrate by the cell. Uptake is often limited by solubility. There is a potential for mass transfer to limit the rate of carbon source biodegradation. If the carbon source has low polarity, then it will have low aqueous solubility. If the rate of dissolution is less than the rate of uptake by the biocatalyst, the process may be limited by the transfer of carbon source to the aqueous phase. This is of particular concern with saturated cyclic hydrocarbons, where water solubility is in the range of 0.683 mmol/l for cyclohexane to < 1ppm for decalin (2, 57). The situation is further complicated if the carbon source is dissolved in another nonpolar liquid and one must work with a multiphase system (12). As pointed out by Bressler and Gray (12), this leads to a host of other considerations including "mixing and viscosity of the hydrophobic phase, solute equilibrium between phases and interfacial properties of the two phases".

Besides solubility, one may also have to consider the ability of the carbon source to cross the cell membrane. For highly polar materials, this can be a significant issue, as the cytoplasmic membrane has a low permeability for charged and polar molecules (58). However, cyclic compounds are apolar. As a result they can readily pass through the lipid bilayer of the cytoplasmic membrane leading to the belief that the transfer of apolar molecules is probably a diffusion process (58). There is also a relationship between the permeability of the membrane to a specific substance, the hydrophobicity of the solutes and the size of the solutes. In addition, active transport of solutes in or out of the cell may also play a role (58). However, the relationship between a compound's apolar nature, hydrophobicity and size as well as the impact of active transport has not been quantified for cyclo-paraffins.

In general the proposed uptake mechanisms for attack on low solubility substrates by biocatalysts may be divided into three categories (16):

1. Substrates dissolved in the aqueous phase are used by the cells.
2. Cells directly come in contact with the substrate, often at the interface between the non-aqueous and aqueous phases. Uptake occurs by transfer near the point of contact between the cell and substrate directly through the membrane.
3. Microorganisms produce surface active compounds, which cause the substrate to form droplets or micelles, which are small enough to be assimilated by the cells.

2.5.2. Biological Limitations to Cell Activity

In addition to the concerns relating to the physical properties of the solute, there are also biological concerns. There are metabolic limitations, growth limitations and other process-specific limitations (12). Metabolic limitations deal with the kinetics of the biodegradation process within the cell. Cells must have the ability to produce enzymes which are active towards a given carbon source. In the presence of the carbon source, the cell must express the enzyme in sufficient quantities such that the carbon source can be used for cellular activity. The expression of enzymes is genetically regulated. In addition, there may be steric hindrance towards metabolic

activity (12). Steric hindrances are caused by functional groups on the substrates and may set the rate of metabolic activity.

In addition to metabolic limitations, growth limitations are an essential consideration when dealing with biocatalysts. As living organisms, biocatalysts thrive in specific environmental conditions. Temperature, salinity and oxygen levels are of particular concern (12). Another major area of concern is toxicity (12, 57, 58). The accumulation of lipophilic compounds (such as cyclic hydrocarbons) into the lipid bilayers of the cell membrane can enhance their availability to the cell but can also lead to toxic effects (57). The reason for these detrimental effects is that the cycloalkanes interact with the phospholipid bilayer of the cell membrane. The compounds interact with the acyl chains of the phospholipids, increasing the bilayer surface area, which in turn can change membrane fluidity, cause membrane swelling and alter membrane-embedded protein conformations (58). Therefore the ability to predict the partitioning of the solutes into the membrane is essential. Research has shown that there is a correlation between the octanol/water partition coefficient (log $P_{O/W}$) and the partition coefficient in the membrane/buffer. The relationship is described by the following formula, which is suitable for log P values between 1 and 4.5 (57):

$$\log P_{M/B} = 0.97 \times \log P_{O/W} - 0.64 \qquad \textbf{(1)}$$

where:

$P_{M/B}$ = Partition coefficient of membrane/buffer

$P_{O/W}$ = Partition coefficient of the octanol/water

Thus, the log $P_{O/W}$ can be used to estimate the toxicity of a given hydrocarbon. The lower the calculated value of the $P_{M/B}$ the greater the predicted toxic effect. Unfortunately, the results of the above equation are just an estimate, as there are many factors that change the degree of toxicity a given hydrocarbon will have to a given cell. If the cell has the capability of utilizing the hydrocarbon as a carbon source at a rate that is greater than the rate of dissolution of the

hydrocarbon to the aqueous phase, then even extremely toxic compounds can have no detrimental effects on the cell because the effective concentration is reduced. Conversely, if the rate of dissolution is greater than the rate of consumption by the cell processes, then even slightly toxic compounds can kill a cell. As a result one must be careful when evaluating toxic effects of a given cycloalkane.

2.6. Conclusion from the Literature Review

From the literature review one can make the following conclusions:

- No bacterium has been identified that can use multi-ring saturated cycloalkanes as a sole carbon source
- The work of Koma *et al.* (29) and Schumacher *et al.* (56) indicates that such bacteria may exist
- There is a possibility that either cooxidation or cometabolism may be necessary to degrade multi-ring saturated cycloalkanes
- When attempting to isolate a bacterium, one must take into consideration the numerous limiting factors listed in section 2.5.

3.0 MATERIALS AND METHODS

3.1. Materials

The following sections describe their source, preparation and use of chemicals and media utilized throughout the research.

3.1.1. Carbon Source Selection

There are several questions which must be asked when selecting a carbon source for a culture. These are:

- Is the compound toxic?
- If a multiphase system is utilized, is the carrier resistant to enzymatic attack?
- Is cometabolism and/or cooxidation necessary for a culture to attack the compound?

The objective of this project was to find a single bacterial species capable of opening the rings of multi-ring cycloalkanes. Decalin was chosen to be used as the cycloalkane carbon source. Decalin is a 10 carbon molecule, with *cis* and *trans* isomers. These isomers are important, for in a gas chromatograph, they come out at distinct intervals (see Appendix A) and there was a chance that they may have been degraded at different rates. A two dimensional representation of decalin in Figure 11 and three dimensional representations of the isomers are shown below in Figure 12.

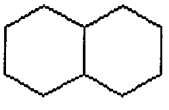

Figure 11: Decahydronaphthalene (Decalin) Structure

cis-isomer trans-isomer

Figure 12: *Cis* and *Trans* Isomer Configurations of Decalin
(http://web.umr.edu/~jstoffer/Solution/solparaexp.html, June 9, 2004)

Decalin was selected for several reasons. First, decalin has a relatively high membrane/buffer partition coefficient of approximately 11,000 (57) when compared to known highly toxic substances such as benzene, with a $P_{M/B}$ of 27 or cyclohexane with a $P_{M/B}$ of 498. The high $P_{M/B}$ would mean that decalin should be less toxic to bacteria, although this is a relative measure. Second, the molecule lacks side chains. Thus, any enzymatic attack would have to occur on the ring structure, which is the desired biocatalyst characteristic. One concern with the use of decalin is its low solubility in water. The solubility of decalin is so low as to be considered negligible when working at the mmol/l scale, at 25°C (57). However, this does not mean that no decalin enters the aqueous phase, as trace levels of the hydrocarbon would dissolve (58). Depending on the uptake mechanisms of the bacteria, this could have been an issue. If carbon source uptake occurs in the aqueous phase, the uptake of decalin by any bacteria would be mass-transfer limited.

Previous research conducted by Pelz and Rehm (47) found that decalin was not used as a carbon source by 250 different mixed cultures. However, Sikkema *et al.* (58) postulated that the concentration of decalin used in their experiments was enough to "kill any biological activity present in their enrichments". Even though the $P_{M/B}$ of decalin is large, toxicity would still be a major concern. In order to deal with this problem a two phase system was used in this study, where the carbon source was first dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN) before being added to the medium. By dissolving the decalin in HMN, the maximum concentration of decalin in the aqueous phase was reduced, hopefully lowering the toxic effects on the bacteria. Therefore, experiments were conducted to test for growth in both pure decalin, and decalin dissolved in HMN. Figure 13 depicts the structure of HMN.

Figure 13: 2,2,4,4,6,8,8-Heptamethylnonane (HMN) Structure

However, due to the use of a multiphase system, there was the concern of degradation of the carrier. To date, HMN has been found to be resistant to microbial attack (refer to section 2.3) and can serve as an effective nonpolar solvent for carbon sources when creating a multiphase system. However, when isolating bacteria using a carrier one must be ensure that a degrader for the carrier is not isolated (16). As both decalin and HMN are highly recalcitrant molecules, this was a possibility. In order to check for HMN degradation, a control culture was used with HMN as the sole carbon source.

In addition to these concerns, there was also the possibility that decalin may only be attacked through a cooxidation process. In order to test this possibility, equimolar amounts of decalin and decane were introduced to the culture as a carbon source and decalin and decane dissolved in HMN were used as a carbon source. Decane was added as it is readily degraded and could serve as an easily accessible carbon source. Figure 14 shows its structure:

Figure 14: Decane Structure

Summarizing the above discussion, one finds that five carbon source scenarios were to be investigated:

- Pure Decahydronaphthalene (Decalin)
- Equimolar Decane + Decalin
- 2,2,4,4,6,8,8-heptamethylnonane (HMN) + Decalin
- 2,2,4,4,6,8,8-heptamethylnonane (HMN) + Decalin + Decane
- 2,2,4,4,6,8,8-heptamethylnonane (HMN)

The details for each carbon source, including manufacturer and composition are listed in Table 2.

Table 2: Carbon Source Specifications

Hydrocarbon	Molecular Formula	Vendor	Purity
2,2,4,4,6,8,8-heptamethylnonane (HMN)	$C_{16}H_{34}$	Aldrich	98%
Decahydronaphthalene (Decalin)	$C_{10}H_{18}$	Aldrich	99+%
n-Decane	$C_{10}H_{22}$	Sigma	99+%

3.1.2. Bacterial Culture Selection

In 1973, Soli *et al.* (65) utilized marine bacteria which had the ability to degrade decalin. However from a biocatalyst perspective in oil sands processing one should not use marine species (halophiles). Halophiles require an environment high in salt content and a salt-water environment is highly undesirable when working with hydrotreated LGO. In order to meet product specifications, any salt that contacts the LGO must be removed utilizing expensive desalter equipment before the product can be sold. Therefore, the marine bacteria were not considered suitable as a biocatalyst for the purpose of this study.

Since 1973, only one paper by Ko *et al.* (27) has described bacteria with the ability to degrade decalin. The process involved cooxidation using a mixed culture of *Pseudomonas aeruginosa* K1 and *Rhodococcus equi* P1. Unfortunately, *R. equi* is considered to be an opportunistic pathogen, which can impact immunosuppressed individuals (30). As a result *R. equi* was not considered suitable for use as a biocatalyst in an industrial setting, as outlined in section 2.2.

At the beginning of this study no species of bacteria had been isolated which fit the criteria for a potentially successful biocatalyst in oil sands processing. Therefore one had to be isolated which would be suitable for selectively opening multi-ring cycloalkanes in Alberta, Canada. In particular the bacteria would find application in the Fort McMurray region. This region has a variable climate, where ambient temperatures can reach from -50°C to +40°C. Ideally, a whole cell biocatalyst for such a region would be a mesophile, which has temperature optima in the range of

8°C to 48°C (35). Heat tracing keeps industrial vessels above 5°C in the Fort McMurray region. Thus the biocatalyst could operate under most of the conditions experienced in the plant.

Given the criteria for a non-halophilic, mesophilic isolate, attempts were made to isolate bacteria from different mixed cultures obtained from cold climates. The name, source and growth environments of each culture are listed in Table 3. The preparation of the mineral salts medium can be found in section 3.1.3.

Table 3: Mixed Cultures From Hydrocarbon Contaminated Sites

Culture Name	Source Location of Culture	Mineral Salts Medium	Carbon Source Culture Grown In
Lodgepole	Lodgepole, Alberta, Canada, condensate well blow-out	220mL B+N8P	0.2mL Lodgepole Condensate
ErnBio 2	Refinery wastewater from Germany	220mL B+N8P	0.2mL Prudhoe Bay Crude Oil
Esso Ag	Refinery wastewater from Germany	220mL B+N8P	0.2mL Prudhoe Bay Crude Oil
Shell Lake	N.W. Territories, Canada freshwater lake sediment contaminated with aviation fuel	220mL B+N8P	0.2mL Prudhoe Bay Crude Oil
Vanda Contam	Lake Vanda Research Station, Antarctica, contaminated soil sample	220mL BH	0.2mL Prudhoe Bay Saturates
Scott Barrels	Scott Base, Antarctica, contaminated soil sample	220mL BH	0.2mL Prudhoe Bay Saturates
Marble Control	Marble Point, Antarctica, uncontaminated soil sample	220mL BH	0.2mL JP8 Jet Fuel
Crater Hill	Scott Base, Antarctica, uncontaminated soil sample	220mL BH	0.2mL JP8 Fuel

None of the mixed cultures required a salt water-based medium. In addition most of the mixed cultures came from hydrocarbon-contaminated sites in cold climates similar to Fort McMurray. Using the above cultures would hopefully allow for isolation of bacteria suitable for use as a biocatalyst in the environmental conditions experienced in the Fort McMurray region.

3.1.3. Media and Stock Solutions

3.1.3.1. Plate Count Agar (PCA)

Made by Difco Laboratories (Detroit, USA). Used to maintain cells on plates when a rich media was acceptable. Prepared using manufacturer's instructions.

3.1.3.2. Tryptic Soy Broth (TSB)

Made by Difco Laboratories (Detroit,USA). Used to grow cells in a liquid culture when a rich media was acceptable. Prepared using manufacturer's instructions.

3.1.3.3. R2A Agar

Made by Difco Laboratories (Detroit,USA). Used to grow cells on plates when a less rich media than PCA was required. Prepared using manufacturer's instructions.

3.1.3.4. Bushnell-Haas Agar

Noble Agar made by Difco Laboratories (Detroit, USA). Combination used to make plates which contained no carbon source. 15 grams of Noble agar added per litre of BH medium.

3.1.3.5. Phosphate Buffer:

Used to suspend cells. See below for preparation:

Phosphate Buffer

- Add 6.1g of Monopotassium Phosphate (KH_2PO_4) to 150mL of water (H_2O), creating a monobasic stock solution
- Add 13.05g of Dipotassium Phosphate (K_2HPO_4) to 250mL of water (H_2O), creating a dibasic stock solution
- Add the monobasic stock solution to the dibasic stock solution until a pH of 7.2 – 7.3 is reached. This mixture is now the phosphate buffer stock solution
- Add 10mL of phosphate buffer stock solution to 1L of water to produce the 3mM phosphate buffer used to wash cells

3.1.3.6. Ion Exchange Solvent:

Used to extract anions from resins which act as weak anion exchangers.

- 500mL Milli-Q® de-ionized water
- 29.25 g NaCl
- 500mL 95% Ethanol

3.1.3.7. B+N8P Minimal Salts Medium:

Contains no carbon source. Used to grow cells in a liquid culture when developing a carbon limited environment. See below for composition.

B+N8P Medium:

- Sodium Sulphate (Na_2SO_4) = 2.0g
- Potassium Nitrate (KNO_3) = 1.0g
- Ammonium Chloride (NH_4Cl) = 2.0g
- Iron Sulphate ($FeSO_4 \cdot H_2O$) = Trace
- Water (H_2O) = 900mL
- Phosphate Stock Solution for B+N8P = 100mL
- $MgSO_4$ Stock Solution = 1.0mL/200mL medium
- Fedorak and Grbic-Galic Trace Metals Solution = 1.0mL

Phosphate Stock Solution for B+N8P:

- Monopotassium Phosphate (KH_2PO_4) = 16.0g
- Dipotassium Phosphate (K_2HPO_4) = 24.0g
- Water (H_2O) = 1.0L

$MgSO_4$ Stock Solution:

- Magnesium Sulphate Hydrous ($MgSO_4 \cdot 7H_2O$) = 4.0 g
- Water (H_2O) = 100mL

Fedorak and Grbic-Galic Trace Metals Solution:

- ($CaCl_2 \cdot 2H_2O$) = 3.7g
- (H_3BO_3) = 2.5g
- ($FeCl_3$) = 0.65g
- ($CoCl_2$) = 0.01g
- ($ZnCl_2$) = 0.44g
- ($MnCl_2$) = 0.87g
- ($Na_2MoO_2 \cdot 2H_2O$) = 0.29g
- ($CuCl_2$) = 0.0001g
- (H_2O) = 1.0L

Note: The $MgSO_4$ Stock Solution was prepared and sterilized separately from the rest of the B+N8P solution. After the rest of the solution was autoclaved, the $MgSO_4$ Stock Solution was added. This prevented a precipitate from forming.

3.1.3.8. Bushnell-Haas (BH) minimal salts medium:

Contains no carbon source. Used to grow cells in a liquid culture when developing a carbon-limited environment.

Bushnell –Haas (BH) Medium:

- Magnesium Sulphate ($MgSO_4$) = 0.2g
- Calcium Chloride ($CaCl_2$) = 0.02g
- Monopotassium Phosphate (KH_2PO_4) = 1.0g
- Dipotassium Phosphate (K_2HPO_4) = 1.0g
- Ammonium Nitrate (NH_3NO_3) = 1.0g
- Ferric Chloride ($FeCl_3$) = 0.05g
- Water (H_2O) = 1.0 L
- Final pH = 7.0 ± 0.2 at 25°C

3.1.4. Other Compounds

Table 4 lists the other compounds were used during experimentation as well as their manufacturers and compositions.

Table 4: Chemicals Used for Purposes Other Than Culture Carbon Sources

Chemical	Molecular Formula	Manufacturer	Purity
Pentane	C_5H_{12}	Caledon	99%
Dodecane	$C_{12}H_{26}$	Aldrich	99+%
Hexadecane	$C_{16}H_{34}$	Aldrich	99%
Dichloromethane (DCM)	CH_2Cl_2	Fisher Scientific	HPLC Grade
BSTFA	N,O-bis(Trimethylsilyl)-trifluoroacetamide	Pierce	N/A
Stearic Acid	$C_{17}H_{35}COOH$	BDH	99%
Palmitic Acid	$C_{15}H_{31}COOH$	Aldrich	99%
Heptanoic Acid	$C_6H_{13}COOH$	Aldrich	99%
Oleyl Alcohol	$C_{18}H_{36}O$	Acros Organics	70%
Hydrochloric Acid	HCl	Fisher Scientific	36.5% - 38%
Anhydrous Sodium Sulphate	Na_2SO_4	Anachemia	N/A
Diazomethane	CH_2N_2	Created using Aldrich Dialzald kit	N/A
Dimethyl benzyl ammonium chloride (OstroSan)	N/A	OstroSan	0.8% (v/v) in H_2O
Amberlite IRA-92 Resin Anion exchanger with coarse particle size (~1mm)	N/A	Supelco	N/A

3.2. Methods

This section describes the experimental techniques and procedures used, as well as the means of qualitative and quantitative analysis.

3.2.1. Liquid Cultures

When experiments were run to quantify a culture's ability to cause the loss of a carbon source or to attempt to identify metabolites, liquid cultures were used. The liquid cultures were prepared in 500mL Erlenmeyer flasks. Attempts were made to use roller tubes, but these proved unsuccessful (see section 4.4). Depending on the experiment, BH or B+N8P minimal salts medium was used to provide all the necessary macro and micro nutrients except for carbon. Then, a hydrocarbon was added to the flask as a source of carbon. During the incubation phase, the flasks were placed on an orbital shaker at 200 rpm in the dark. Incubation temperatures ranged from room temperature (approximately 21°C) to 28°C, depending on the experiment. There were two types of liquid cultures, mixed and pure. Below are descriptions for the means of preparation of each type:

3.2.1.1. Mixed Cultures

In experiments involving mixed cultures, inocula were taken from maintenance cultures. Twenty millilitres of culture from the maintenance culture was transferred into 200mL of minimal salts medium. Then, appropriate carbon sources were added. When the maintenance cultures did not have a defined carbon source (as in the case of the original oil cultures) "carry-over" of the oil into the experiments was a concern. Therefore multiple transfers were conducted using a defined hydrocarbon before running the experiment to reduce the impact of residual oil on the carbon source experiments. The transfer process is depicted in Figure 15.

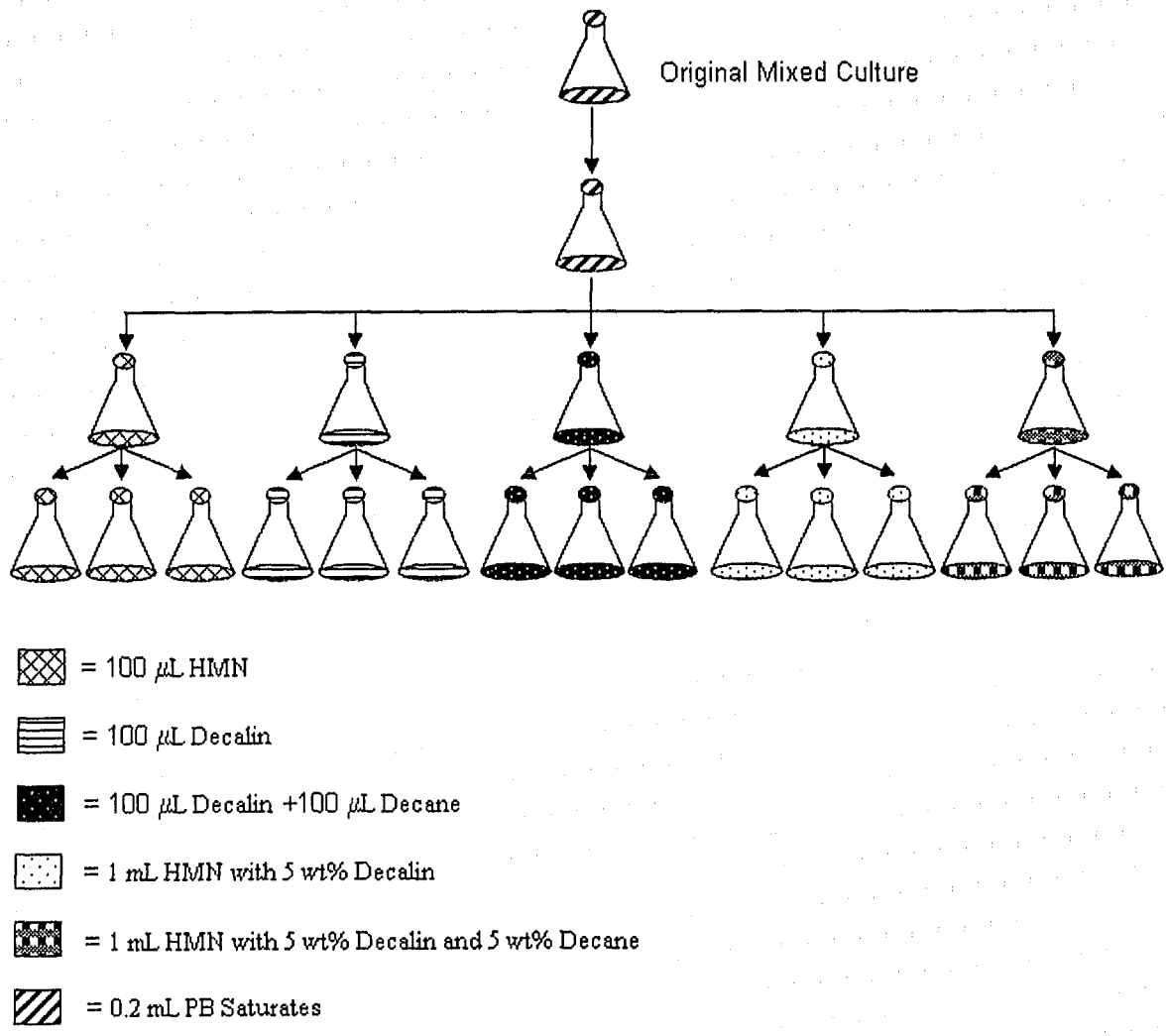

Figure 15: Series Transfers for Mixed Cultures

3.2.1.2. Pure Cultures

For experiments involving pure cultures a loopfull of the culture was taken from -70°C glycerol stock, and transferred into 10mL of TSB and incubated for 24h to 48h, depending on the culture. Then the cells were washed three times with phosphate buffer and suspended in 10mL of phosphate buffer. One millilitre of the cell/phosphate buffer suspension was used to inoculate flasks contained 220mL of BH medium and carbon sources were added.

3.2.2. Viable Count performed using R2A and Vapour Plates

In order to quantify the cell density and identify the colony morphologies of the bacteria in a culture, viable counts were conducted using the spread plate method (34). Cell suspensions were diluted from 10^{-1} to 10^{-6} in phosphate buffer and then plated on solid media. The plates were allowed to incubate and then were photographed and the colonies counted. Two different types of plate environments were used. The first was an R2A plate. The R2A medium was developed to cultivate heterotrophic bacteria, and contained complex carbon sources. The second was vapour plates. In these experiments, BH + Noble Agar solid medium was prepared. This medium contained no carbon source. Then carbon sources were separately provided. Decane and decalin were allowed to vaporize in closed containers. The plates were placed into the closed containers and exposed to decane and decalin in the vapour form. In order to expose the cultures to HMN as well, 50μL of HMN was placed directly on the plates.

3.2.3. Controls

In order to determine if degradation had occurred, a necessary part of every experiment was controls. There are four types of controls typically used in biological experiments:

1. Sterile Controls: Contain no living cells
2. Positive Controls: Known to achieve a positive result, prior to beginning the experiment
3. Negative Controls: Known to achieve a negative result, prior to beginning the experiment
4. Killed Controls: Contain killed cells

Comparing experimental results to controls allows a comparison for the results and gives a greater understanding of what was achieved in the experiment. Ideally, all four control types would be employed as often as possible. However, due to practical considerations this is not always feasible. For example, as no decalin degraders were available prior to beginning experiments, there was no possibility of including positive controls for decalin degradation. Therefore for each experiment choices were made regarding which controls would be used.

3.2.4. Qualitative Analysis

Qualitative analysis involved looking for visible indications of bacterial growth in a given culture. Possible visible indicators included:

1. Increased turbidity
2. Evidence of floc formation
3. Colour changes in the medium
4. Visible increase in cell mass

Qualitative means were used for initial screening of cultures. If no visible growth appeared, then the culture was considered inactive. However, if a culture appeared active, then quantitative analysis was used.

3.2.5. Pentane Extraction

This method was used to extract hydrocarbons from liquid cultures when hydrocarbon loss due to biotic activity was being measured. The method involved adding 50μL of a surrogate standard (dodecane) and 50mL of pentane to the liquid culture. The pentane floated on top of the aqueous phase, so that it could be removed directly from the surface using a Pasteur pipette. After mixing on an orbital shaker at 200rpm for 10 minutes to ensure homogeneity in the non-aqueous phase, a sample of the pentane/hydrocarbon mixture was removed using a Pasteur pipette for GC analysis. The benefit of this technique was that it involved no drying steps. Therefore, loss of volatile compounds such decalin and decane was kept to a minimum.

The pentane extraction technique did have one major limitation. When the pentane was added a rag layer formed at the interface between the aqueous and non-aqueous phase. The rag layer made it extremely difficult to remove all of the hydrocarbon phase without accidentally drawing up some of the aqueous phase. As a result, pentane extractions were only suitable for experiments where loss was being measured and the initial concentration of the carbon source was known. For experiments where attempts were made to find products of metabolic activity, an alternate extraction technique was needed and is described in section 3.2.6.

3.2.6. DCM Extraction

This method was used to extract metabolites from the aqueous phase in liquid cultures. A surrogate standard and dichloromethane (DCM) were added to the cultures. Because DCM has a higher density than water, it settled to the bottom the flasks. Therefore, 500mL separation funnels were used to draw the DCM layer from the aqueous phase.

Two types of extractions were conducted: neutral and acidified. In neutral extractions 50μL of dodecane was added as the surrogate standard and the samples were mixed with 30mL of DCM three times to ensure that all of the DCM-soluble material was removed from the culture. The DCM phase was passed through anhydrous sodium sulphate to remove any residual water. A roto-vaporizer operating at 45°C and nitrogen drying unit were then used to remove the DCM from the samples until the samples contained no DCM. The samples were then re-dissolved in 4mL of DCM, derivatized using BSTFA and analyzed using gas chromatography.

In acidified extractions the process was the same except for two changes. First, in some experiments neutral and acidified extractions were done in series. In these experiments a different surrogate standard was required for the acidified extraction. In these situations 10μL of hexadecane was added. The other change was that before adding DCM or surrogate standards, the pH of the samples was lowered to < 1 using hydrochloric acid. The benefit of this technique was that by drying down and re-dissolving the samples, any metabolites present could be concentrated so that they might be detected on the chromatograms. The problem was that volatile compounds would be lost during sample preparation.

3.2.7. Resin Bead Extraction

When metabolites could not be identified following the method outlined in section 3.2.6, the resin bead technique was used. Resin beads produced by Supelco (Table 4) were selected which could act as weak anion exchangers. They were added to the cultures prior to incubation. Anions produced during the metabolic activity of the cultures would be absorbed by the resin beads,

preventing their use by the culture. After incubation for two weeks the cultures/resin bead mixture was place in a glass chromatography column that had been previously packed with glass wool. 100mL of Milli-Q water was added to the column and the liquid phase removed, leaving the resin beads. The liquid culture + Milli-Q water mixture was acidified and 50μL of dodecane was added as a surrogate standard. The samples were then extracted using the method described in section 3.2.6. After this step, 250mL of ion exchange solvent, described in section 3.1.3.6, was added to the glass chromatography column containing the beads and then the ion exchange solvent was drained from the column. This step removed any anions present in the resin beads. The ion exchange solvent samples were acidified, 50μL of dodecane was added as the surrogate standard and the samples were extracted using the procedure in section 3.2.6. During the extraction process, the roto-vaporizer was operated at 80°C to remove the ethanol.

3.2.8. Gas Chromatography Program

Gas Chromatography (GC) was used to identify and quantify carbon sources and metabolites of carbon sources throughout the experiments. A program had to be developed, in order to produce chromatograms which would be able to identify the compounds of interest.

A Hewlett Packard 5890 GC unit with a HP-1 column from Agilent was utilized. The unit was equipped with a hydrogen flame ionization detector and the column specifications were 25m length x 0.32 mm ID x 17μm film thickness. Helium was used as the carrier gas for the system with a flow-rate of 19mL/min. The temperature at the injector and detector were set at 300°C. The unit also required a sample injection volume of 2μL. The initial temperature of the GC was set at 50°C. When decalin, decane and HMN degradation was being evaluated the temperature was raised at a rate of 6°C/min until a specified final temperature of 200°C. When attempts were made to identify metabolites the temperature was raised at a rate of 6°C/min until a specified final temperature of 300°C. Between samples the syringe was cleaned with a polar solvent (methanol) and a non-polar solvent (dichloromethane).

3.2.9. Concentration Correction for Hydrocarbons using Surrogate Standards

In addition to developing a program to run the GC unit, it was also necessary to develop standard curves for analysis of the mixtures. Due to the high volatility of pentane and DCM there was the chance of solvent loss throughout the extraction process. In addition, there was the possibility that 100% recovery of the material in the flask would not be achieved. In order to quantify losses in the extraction, a known mass of a chemical called a surrogate standard was added. This meant that the theoretical concentration of the surrogate standard added to the culture could be calculated. By tracking the concentration of the internal standard, the concentrations of the other compounds of interest could be corrected for losses. The following formula was used:

$$C_{cpd,culture} = \frac{C_{istd,culture}}{C_{istd,extract}} C_{cpd,extract} \qquad (2)$$

where: $C_{cpd,culture}$ = Concentration of compound in culture (mg/L)
$C_{istd,culture}$ = Concentration of surrogate standard in culture (mg/L)
$C_{istd,extract}$ = Concentration of surrogate standard in extract (mg/L)
$C_{cpd,extract}$ = Concentration of compound in extract (mg/L)

To follow this technique, standard curves for the hydrocarbons studied were developed. These standard curves allowed for the calculation of $C_{istd,extract}$ and $C_{cpd,extract}$. In order to make the standard curves, dilution series were conducted with decalin, decane and HMN in the range of 30 mg/L to 1500 mg/L. The solvent used was dichloromethane. These dilution series were then analyzed using gas chromatography, following the program outlined in section 3.2.8. Then a curve of peak area vs. concentration was plotted for each chemical. After this was complete two different chemicals were selected to act as surrogate standards: dodecane and hexadecane. These were selected because they had GC retention times that did not coincide with decalin, decane or HMN. Standard curves were also made for these compounds. Example chromatograms for all the above chemicals, as well as the standard curves developed can be viewed in Appendix A.

3.2.10. Sterile Techniques

Due to the nature of biocatalysis research, maintaining a sterile environment was essential. Several techniques were used to ensure sterility in materials. The first technique used was autoclaving. High pressure steam is injected into the autoclave chamber, raising the temperature to 121°C and killing the bacterial cultures. In order to ensure all heat resistant bacterial structures (such as endospores) were denatured, the autoclave was kept at 121°C for 20 minutes. All glassware, mineral media and other non-combustive materials that came in contact with the bacteria cultures were subject to autoclave treatment before use.

In addition to autoclaving, filter sterilizing techniques were also utilized. A Millipore Millex-FG filter with a pore diameter equal to 0.2μm was chosen. This type of filter was designed for hydrocarbon use, containing a PTFE membrane that would not degrade upon contact with solvents.

In order to limit the possibility of airborne contamination, all inoculations and transfers were conducted in a Class I bio-hood. An Edgegard Hood, model number EG6220, produced by The Baker Co., Inc. out of Sanford, Maine was used. Prior to use of the hood, all surfaces were cleaned with Ostro San™ disinfectant as specified in the manufacturer's instructions.

4.0 RESULTS

4.1. Screening Mixed Cultures

The eight mixed cultures, listed in section 3.1.2, were screened for their ability to degrade decalin. When designing the experiment, "carry over" of oil from the original mixed cultures into the controlled mixed cultures was a concern. Any residual oil present could have acted as a carbon source for the bacteria and skewed the results for decalin consumption. Another concern was decalin toxicity. Due to decalin's known toxic qualities (see section 3.1.1), the decalin was diluted in HMN and the mixture of hydrocarbons was added to the cultures as a carbon source. Finally, cometabolism needed to be considered. Therefore, an additional carbon source was also provided in the form of decane dissolved in HMN.

The experiment was conducted in 500mL Erlenmeyer flasks. Hydrocarbon volatility was not considered an issue at this point and foam plugs were used to stop the flasks. In order to ensure adequate oxygen availability and ensure good mixing in the samples, they were placed on an orbital shaker which operated at 200 rpm. To prevent photooxidation of aromatic metabolites and because biocatalysts for hydrocarbons would operate in vessels with no light, the shaker was enclosed to prevent light from reaching the cultures. Due to equipment limitations, the cultures were incubated at room temperature and the samples were not completed in triplicate. According to literature (65) 2 to 3 weeks was sufficient time for degradation of cyclic structures. Therefore, the cultures were allowed to incubate for 3 weeks.

In order to quantify loss, controls for the experiment needed to be selected. Because no bacteria were available that could degrade decalin, positive controls could not be used. Negative controls were created by inoculating the cultures in pure HMN, which according to literature was non-biodegradable. No GC analysis was conducted on the samples with pure HMN because the compound of study, decalin, was not present in those cultures. Qualitative means were instead used to check and ensure no growth was occurring. In order to quantify loss, sterile controls were

used. The sterile controls contained carbon source but no culture and all losses were due to abiotic causes. For each sterile control, two samples were run to achieve some level of statistical significance.

The eight mixed cultures were inoculated in 1mL of HMN + 1 wt% decalin and 1mL of HMN + 1 wt% decalin + 1 wt% decane following the method in outlined in section 3.2.1.1, with one important exception. In Figure 15, transfers were made in triplicate for the final step, to give greater statistical significance to the results. In this experiment, the transfers were not made in triplicate, so there was only one sample for extraction for all the biotic cultures. In all negative controls (those containing only HMN as a carbon source), no visible growth was noted. In all other cultures growth was noted. In particular, in the HMN + decalin + decane cultures heavy growth was noted. After qualitative observations were taken, the cultures were extracting using a pentane float. However, for this experiment an inferior method of extraction was utilized. Five microlitres of dodecane was added to 12mL of pentane and then this mixture was added to each flask to extract the remaining hydrocarbons. The rest of the method was identical to that described in section 3.2.5. The quantitative results for decalin degradation can be found in Figure 16 and Figure 17. The GC results showed that the *cis* and *trans* isomers of decalin were degraded at the same rate, indicating that there was no preference by the bacteria for one form or the other. Therefore, on the following graphs and all subsequent graphs, total decalin will be displayed.

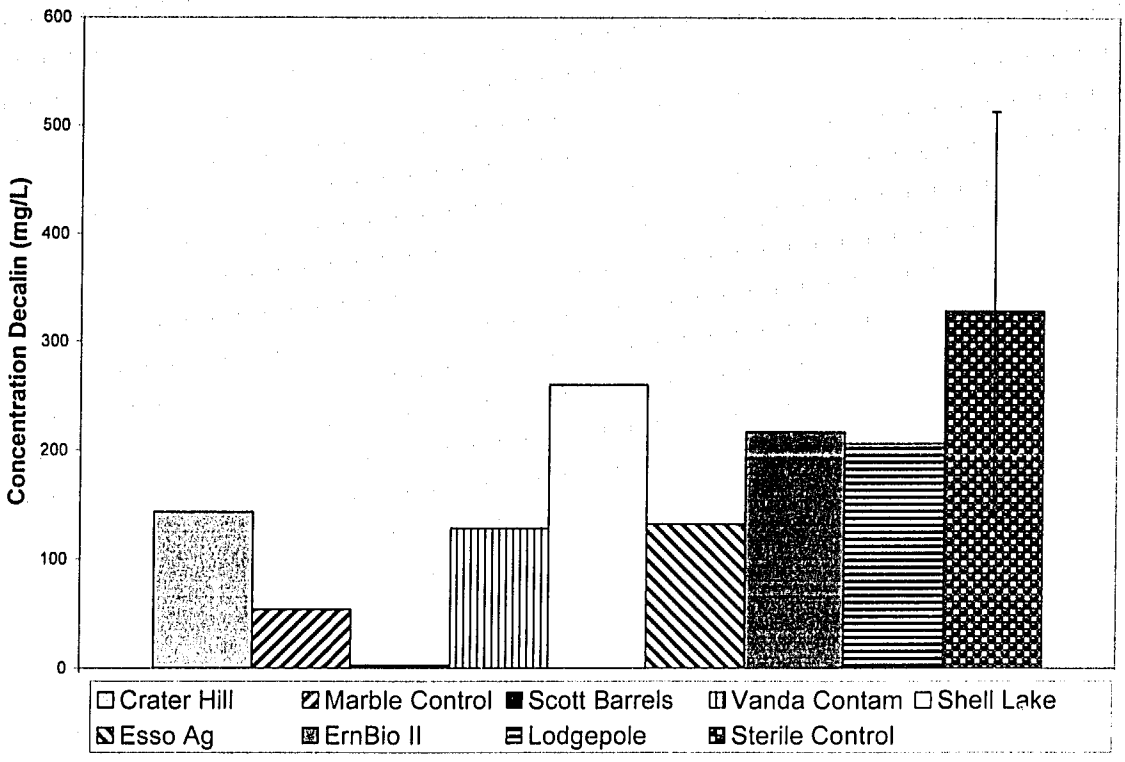

Figure 16: Total Decalin Recovery from Mixed Cultures in HMN + Decalin Environment
Note: Values represent analysis of single flasks except for the sterile controls (mean±1 stndev; n=x)

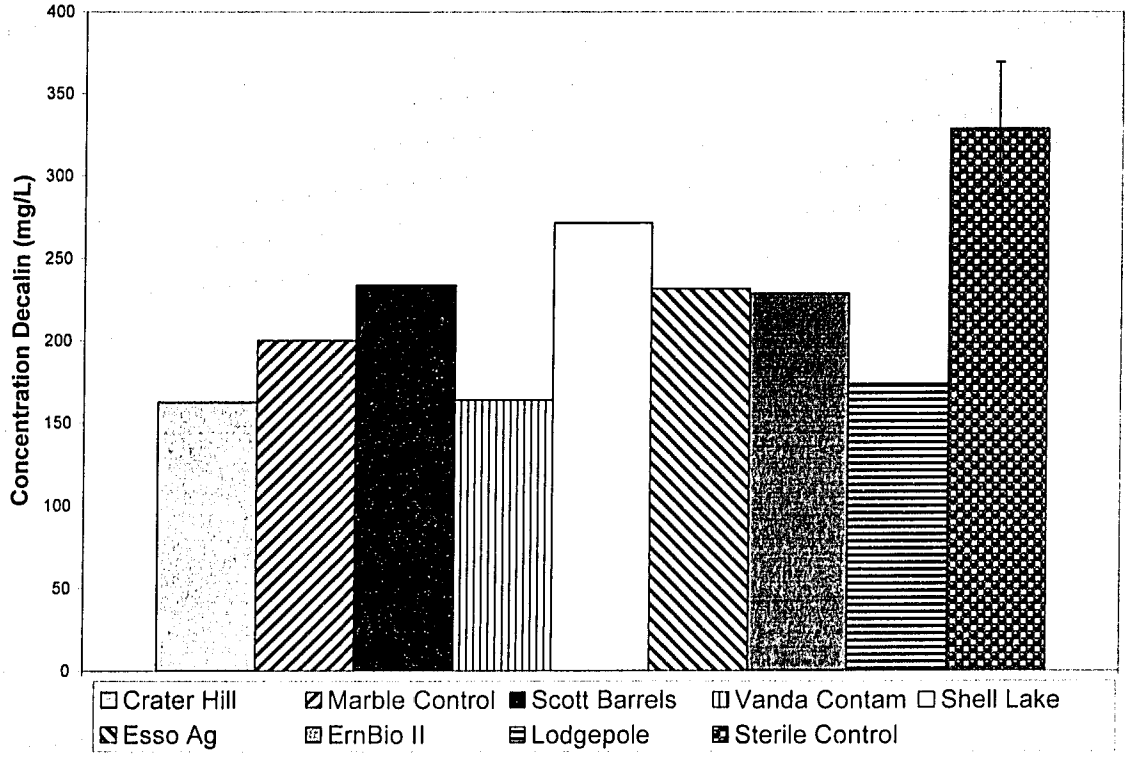

Figure 17: Total Decalin Recovery from Mixed Cultures in a HMN + Decalin + Decane Environment
Note: Values represent analysis of single flasks except for the sterile controls (mean±1 stndev; n=x)

Because all of the flasks contained the same amount of decalin initially, the amount of loss between the cultures could be directly compared. Table 5 contains the results:

Table 5: Percent Total Decalin Degradation by Mixed Cultures when compared to the Sterile Control in Screen Experiment

Mixed Culture	Percent Decalin Degradation from HMN + Decalin Carbon Source	Percent Decalin Degradation from HMN + Decalin + Decane Carbon Source
Crater Hill	51%	48%
Marble Control	39%	80%
Scott Barrels	29%	99%
Vanda Contam	50%	54%
Shell Lake	17%	6%
Esso Ag	30%	52%
ErnBio II	30%	21%
Lodgepole	47%	25%

The results above offered limited but important information. First, there was no preference for either the *cis* or *trans* isomers of decalin by the bacteria. This was expected as the *cis* and *trans* isomers differ only by the location of the hydrogen atoms on the bridge carbons between the rings (see Figure 12). Because attack was unlikely to occur at the bridge carbons of the decalin rings, one would not have expected a preferential selection by bacteria for one isomer over the other.

From Table 5 one can see that all the cultures had the ability to degrade decalin. However, the degree of degradation varied greatly with the maximum degradation achieved by the Crater Hill mixed culture at 51%. In addition to observing decalin degradation in all the cultures, in some of them the process was greatly enhanced by the addition of decane to the carbon sources. In the case of the Scott Barrels mixed culture, the process was enhanced to the point that 99% degradation was measured.

Besides offering insight into the ability of the cultures to degrade decalin, the experiment also revealed weaknesses in the experimental technique. The large error bars in Figure 16 and Figure 17 for the sterile control results indicated that there were flaws with the experimental process, either in the extraction technique or due to varying abiotic losses between samples. Because no replicates were performed for the biotic samples, the variability of the cultures could not be

evaluated. Due to the lack of confidence in the results, it was decided that in future experiments triplicate tests would be conducted. In addition, before follow up work was conducted on this experiment, the issue of experimental variability would have to be addressed.

Based on the results, two mixed cultures were chosen for further investigation. The first was the Vanda Contam mixed culture. The Vanda Contam culture gave one of the highest levels of decalin degradation when no decane was present. If experimental error and abiotic losses were not the cause of the decalin disappearance, it appeared to offer a good chance of containing a species of bacteria which could degrade decalin on its own. The other culture was the Scott Barrels mixed culture. The Scott Barrels culture had the highest degradation when both decalin and decane were present. If cometabolism ended up being necessary for decalin degradation, this culture had the best chance of containing bacteria which used enzymes highly active towards decalin.

4.2. Volatility of HMN

The goal of this experiment was to determine if the volatility of HMN led to significant abiotic losses during a 2 week shaker flask experiment. In this experiment screw cap Erlenmeyer flasks were utilized. There were two sample sets. In the first, HMN was added to 220mL of Bushnell-Haas medium and the flasks were sealed with screw caps with Teflon liners. The liners were added to reduce evaporative losses to an absolute minimum and to prevent absorption of the hydrocarbon by the cap. In the second, HMN was added to 220mL of Bushnell-Haas medium and the flasks were sealed with foam plugs, as used for the experiment in section 4.1. In both cases, the flasks were sterile because abiotic losses were the only variable in this experiment. Triplicate tests of each condition were conducted, in order to achieve statistical significance. Two hundred microlitres of HMN were added to each flask and the flasks were placed on a rotary shaker at 200 rpm at 28°C for 2 weeks.

The extraction procedure for this experiment differed from that used in section 4.1, as attempts were made to improve the extraction process. The internal standard was added directly to the culture, rather than being mixed in with the solvent first. In addition, more solvent was added. The larger volume of solvent served two purposes. The first was to decrease the concentration of HMN in the extraction sample, to prevent overloading of the GC column. The second was to reduce the relative impact of solvent losses. Because pentane is so volatile, there was continual loss of pentane due to evaporation during the extraction process. The impact of a loss of 0.5mL of pentane from an initial volume of 50mL would be less than the loss of 0.5mL from an initial volume of 12mL. The changes led to the method outlined in section 3.2.5. The results are as follows:

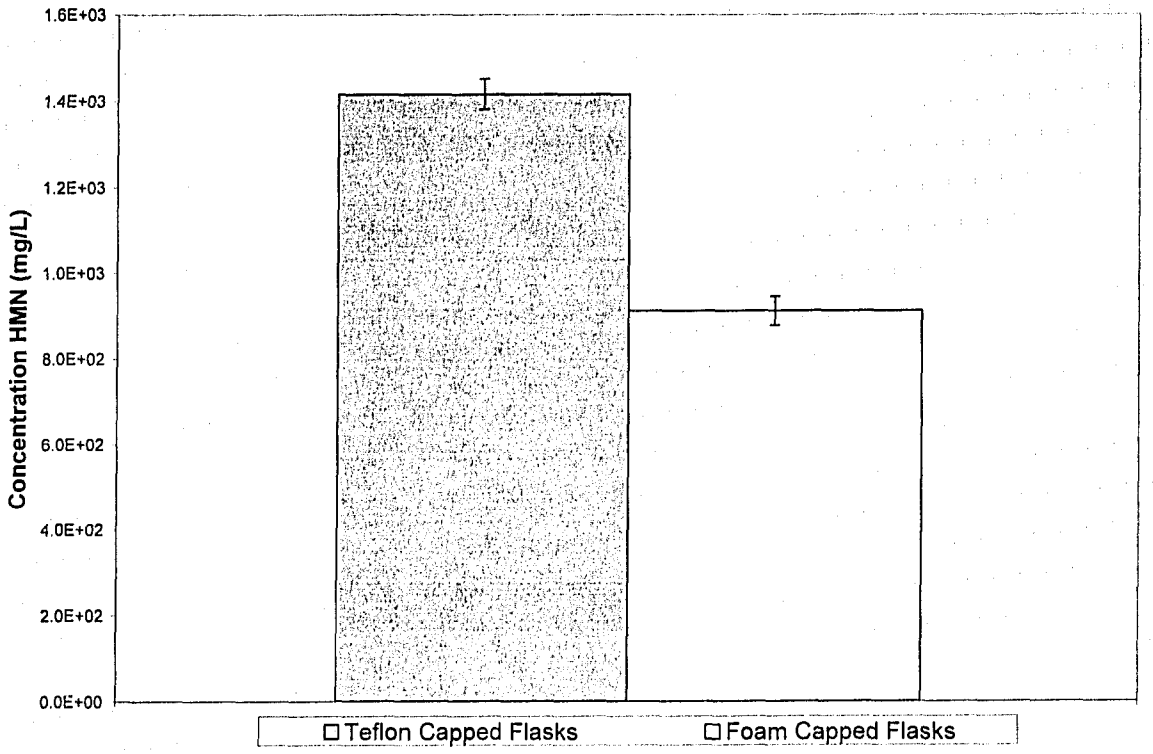

Figure 18: Concentrations of HMN Recovered from Abiotic Experiments

The data in Figure 18 show that by sealing the flasks with a Teflon lined cap, rather than a foam plug, the HMN losses were greatly reduced. As a result, in all subsequent experiments incubation containers were sealed with Teflon lined caps. In addition, the error bars in Figure 18 were greatly

reduced compared to those in Figures 15 and 16. Thus the changes in the extraction process were deemed successful.

4.3. Degradative Capabilities of "Scott Barrels" and "Vanda Contam" Mixed Cultures

The purpose of this experiment was to determine the degradative capabilities of the Vanda Contam and Scott Barrels mixed cultures. The cultures were incubated with either single carbon sources or multiple carbon sources to determine if cometabolism and/or toxicity played a role in decalin degradation.

In section 3.1.2 some criteria were listed for ideal bacterial biocatalysts. In this experiment the environmental conditions were chosen to encourage growth of bacteria that would thrive in the Fort MacMurray region. In order to select for mesophiles, cultures were incubated at a temperature of 28°C. The cultures were grown in 500mL screw-cap Erlenmeyer flasks with a Teflon liner to reduce evaporative losses during the incubation process. The flasks were placed in the dark and on orbital shakers operating at 200rpm. Carry over of the oil from the original cultures was again a concern, and a series of transfers was once more used to dilute any residual oil. In this experiment, 2 weeks was considered sufficient time for incubation. Both sterile and negative controls were used in this experiment, as outlined in section 4.1. However, HMN loss was also measured using GC analysis. In order to increase the statistical significance of the results, all the experiments were conducted in triplicate, including the biotic samples and sterile controls.

In this experiment the cultures were exposed to 5 different carbon limited environments as outlined in section 3.1.1. The two mixed cultures were inoculated in medium with the addition of one of the following:

1. 100μL of pure Decalin
2. 100μL of Decalin + 100 μL of Decane
3. 1mL of HMN + 5 wt% Decalin
4. 1mL of HMN + 5 wt% Decalin + 5 wt% Decane
5. 100μL of pure HMN

Not all of the cultures displayed visible signs of growth. Those grown on HMN or pure decalin had no visible cell mass. However, the cultures grown on decalin + decane, HMN + decalin and HMN + decalin + decane all had visible growth. The type of growth varied between the cultures. Table 6 contains the qualitative results:

Table 6: Qualitative Observations of Vanda Contam and Scott Barrels Mixed Cultures incubated with Various Hydrocarbons

Mixed Culture	Carbon Source Available	Visible Observations
Vanda Contam	Decalin	No visible growth when compared to sterile control
Vanda Contam	Decalin + Decane	Visible growth in the form of a relatively small number of large yellowish flocs apparent after 4-5 days. After two weeks flocs remained and had low turbidity except for flocs. One culture showed no growth
Vanda Contam	HMN + Decalin	Visible growth apparent after 3-5 days in the form of small white flocs. After two weeks flocs remained and culture had low turbidity except for flocs. Floc numbers did not increase significantly.
Vanda Contam	HMN + Decalin + Decane	After 24 hours the media turned a milky white color due to high cell mass. Some white flocs visible in the mass.
Vanda Contam	HMN	No visible growth when compared to sterile control
Scott Barrels	Decalin	No visible growth when compared to sterile control
Scott Barrels	Decalin + Decane	Visible growth in the form of high numbers of large white flocs apparent after 2-3 days. After two weeks flocs remained and culture had low turbidity except for flocs, but number of flocs increased significantly.
Scott Barrels	HMN + Decalin	No visible growth when compared to sterile control
Scott Barrels	HMN + Decalin + Decane	After 24 hours the media had large numbers of white flocs to the point that when shaken, the culture took on a milky appearance.
Scott Barrels	HMN	No visible growth when compared to sterile control

Quantitative results are found in Figures 18 to 23. Note that for the Vanda Contam in decalin + decane cultures, one of the three replicates did not grow. This led to the large error bars in Figures 18 and 20.

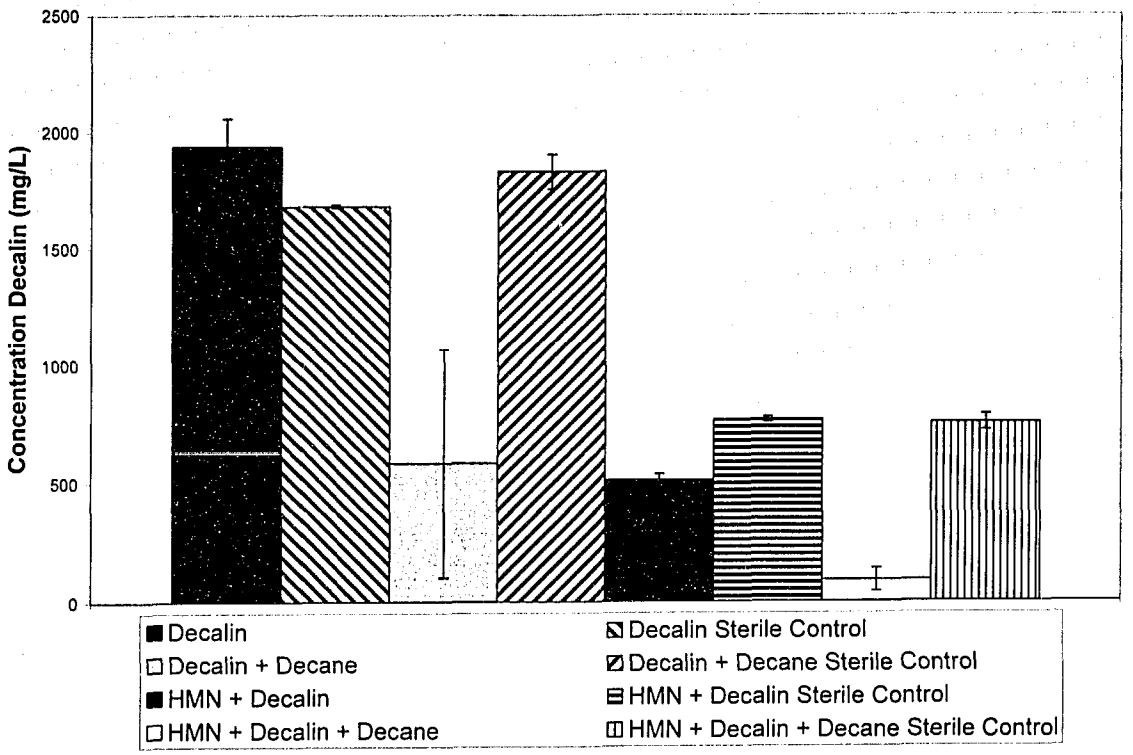

Figure 19: Total Decalin Recovery from Vanda Contam Mixed Culture

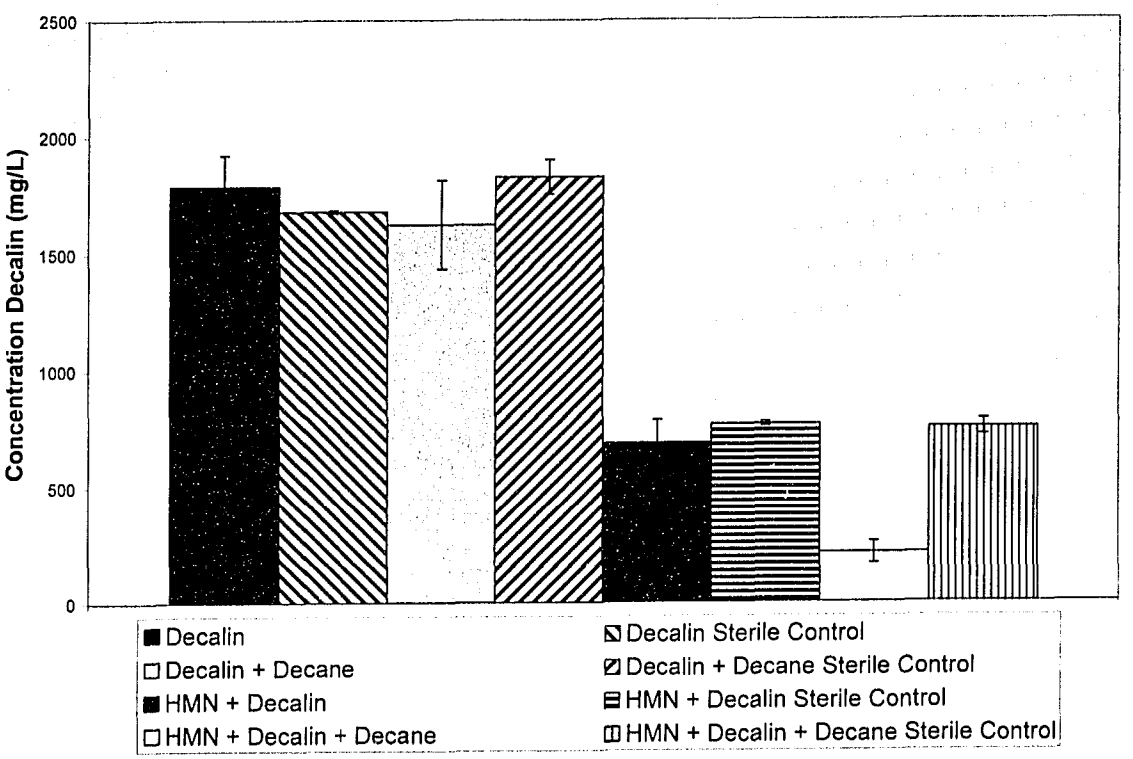

Figure 20: Total Decalin Recovery from Scott Barrels Mixed Culture

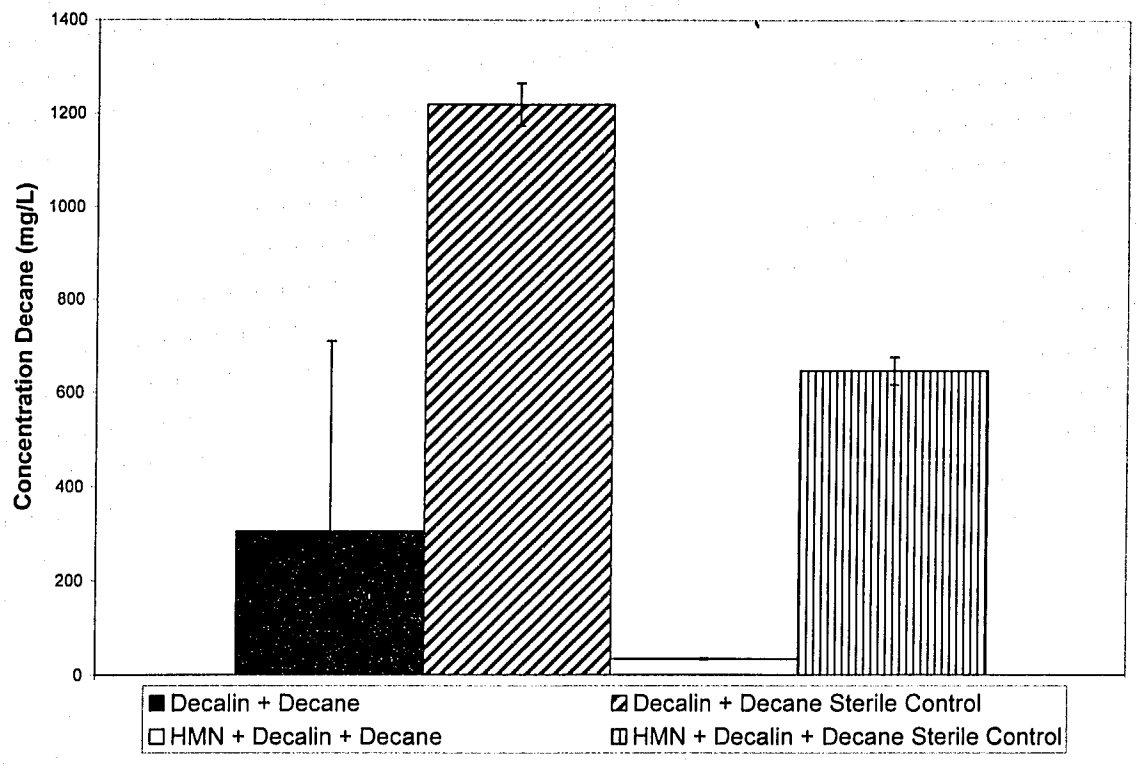

Figure 21: Decane Recovery from Vanda Contam Mixed Culture

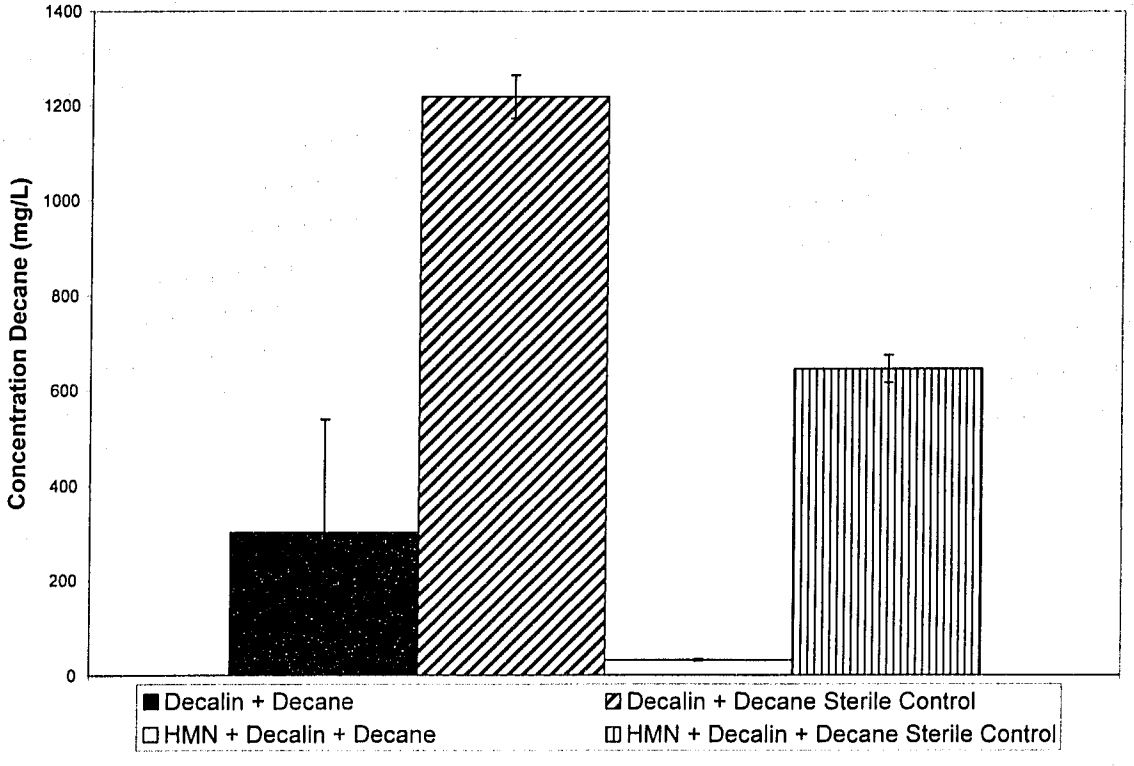

Figure 22: Decane Recovery from Scott Barrels Mixed Culture

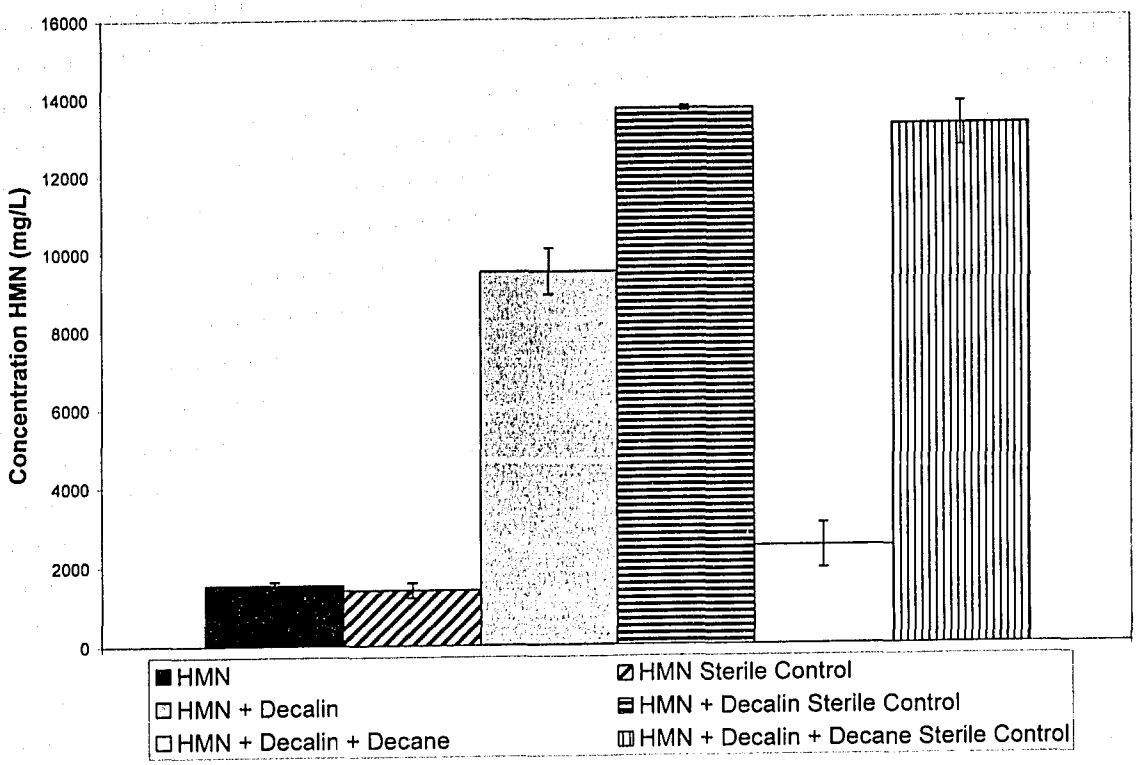

Figure 23: HMN Recovery from Vanda Contam Mixed Culture

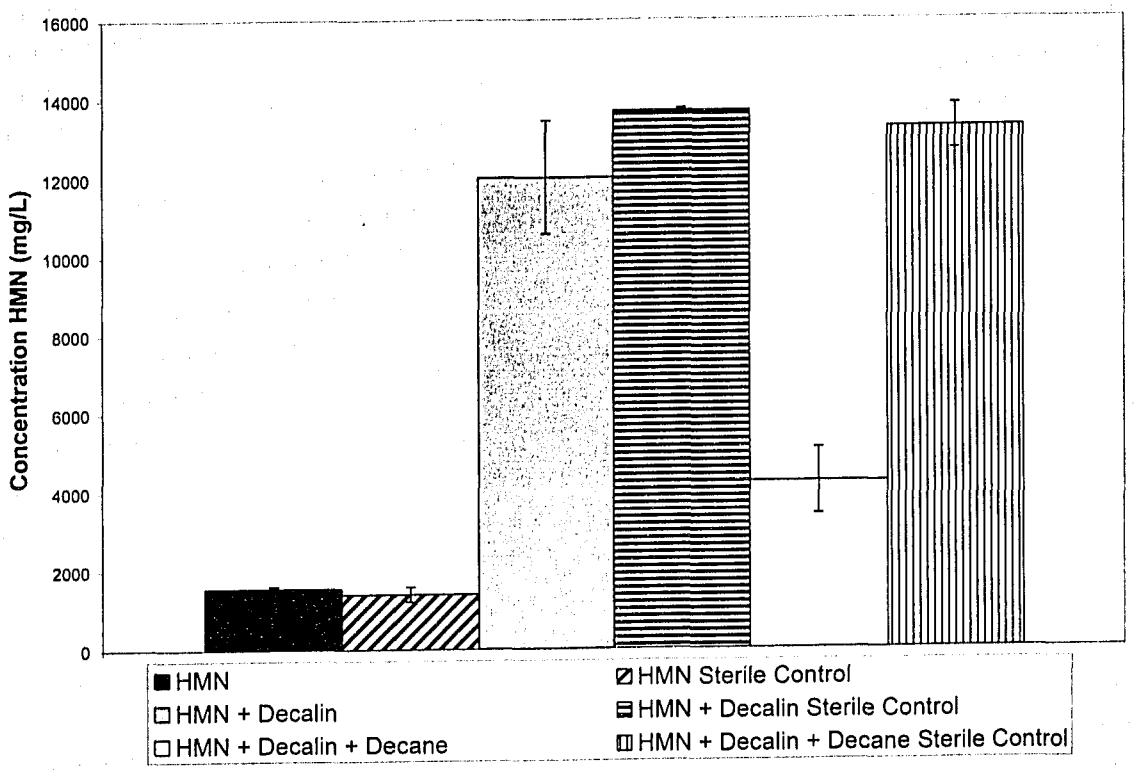

Figure 24: HMN Recovery from Scott Barrels Mixed Culture

T-Tests were then conducted to determine the probability that the sterile control and biotic sample were the same (no degradation had occurred). The "TTEST" function in Microsoft Excel™ was utilized and the biotic results were compared to the sterile results assuming unequal variances and one-tail distributions in the data. The results of the t-test are listed in Table 7, Table 8 and Table 9.

Table 7: Probability that the Biotic Sample is Equal to the Sterile Sample for Decalin Recovery

Culture	Carbon Source Available	Probability that biotic and sterile samples are the same (%)
Vanda Contam	Decalin	3.2
Vanda Contam	Decalin + Decane	2.2
Vanda Contam	HMN + Decalin	<0.1
Vanda Contam	HMN + Decalin + Decane	<0.1
Scott Barrels	Decalin	15.0
Scott Barrels	Decalin + Decane	9.8
Scott Barrels	HMN + Decalin	14.8
Scott Barrels	HMN + Decalin + Decane	<0.1

Table 8: Probability that the Biotic Sample is Equal to the Sterile Sample for Decane Recovery

Culture	Carbon Source Available	Probability that biotic and sterile samples are the same (%)
Vanda Contam	Decalin + Decane	2.7
Vanda Contam	HMN + Decalin + Decane	0.4
Scott Barrels	Decalin + Decane	0.8
Scott Barrels	HMN + Decalin + Decane	0.4

Table 9: Probability that the Biotic Sample is Equal to the Sterile Sample for HMN Recovery

Culture	Carbon Source Available	Probability that biotic and sterile samples are the same (%)
Vanda Contam	HMN	39.3
Vanda Contam	HMN + Decalin	<0.1
Vanda Contam	HMN + Decalin + Decane	<0.1
Scott Barrels	HMN	33.4
Scott Barrels	HMN + Decalin	9.0
Scott Barrels	HMN + Decalin + Decane	0.1

The GC results and the T-Test analysis demonstrated the ability of the mixed cultures to degrade the decalin and HMN. A summary of the statistically significant results is given in Table 10 and Table 11.

Table 10: Capabilities of Vanda Contam and Scott Barrels Mixed Cultures to Cause Decalin Degradation

Mixed Culture	Able To Degrade Pure Decalin in Liquid Culture	Able to Degrade Decalin in HMN + Decalin Environment	Able to Degrade Decalin in Decalin + Decane Environment	Able to Degrade Decalin in HMN+ Decalin+Decane Environment
Vanda Contam	No	Yes	Yes	Yes
Scott Barrels	No	No	No	Yes

Table 11: Capabilities of Vanda Contam and Scott Barrels Mixed Cultures to Cause HMN Removal

Mixed Culture	Able to Degrade Pure HMN in Liquid Culture	Able to Degrade HMN in HMN + Decalin Environment	Able to Degrade HMN in HMN + Decalin + Decane Environment
Vanda Contam	No	Yes	Yes
Scott Barrels	No	No	Yes

The above results offered a great deal of information as to the degradative capabilities of the two mixed cultures. The information in Table 10 showed that the Scott Barrels mixed culture was completely dependent on cometabolism to achieve decalin degradation, under the conditions given. The need for cometabolism was shown by the fact that no statistically significant decalin degradation was noted in any environment except that of HMN + decalin + decane. The Scott Barrels mixed culture also appeared to be sensitive to the toxic effects of decalin. If cometabolism was the only requirement for decalin degradation, then loss would have been noted in the decalin + decane environment. Although visual indications of growth were noted in Table 6, no quantitative loss of decalin was measured. Decalin toxicity might have been the reason why no growth was noted in the HMN + decalin culture as well. If the concentration of decalin had been reduced even further in the liquid cultures by lowering the wt% of decalin in the HMN, then decalin degradation may have occurred.

Another result of note was the removal of HMN described in Table 11. Although no HMN removal occurred when the culture was exposed to pure HMN, or when HMN and decalin were present, significant removal was noted in the HMN + decalin + decane culture. This removal suggested that HMN degradation was also possible via a cometabolic process.

The results from the Vanda Contam culture indicated that this culture was much more active towards decalin. Table 10 showed that the culture was able to degrade decalin in all cases except when exposed to pure decalin where the toxic effects of decalin may have been detrimental. Of great interest was the fact that decalin was degraded in the HMN + decalin environment. The loss of decalin confirmed the results found in section 4.1 and left the possibility open that the culture contained bacteria which could degrade pure decalin, if the decalin wasn't present at toxic concentrations.

The Vanda Contam mixed culture also possessed other interesting capabilities. The results for HMN degradation by the Vanda Contam culture, listed in Table 11, raised questions regarding the recalcitrance of HMN to biodegradation. HMN removal was noted in the HMN + decalin and HMN + decalin + decane cultures. However, no removal was noted for pure HMN. As HMN has no known toxic qualities, this implied that the HMN was being cometabolized in the presence of either decalin or decane. Decane though, acted as the best cometabolizing agent as greater losses occurred in that culture.

Based on the above results, the following cultures were kept for future investigation, and transferred once per month for the duration of the study:

- Vanda Contam + decalin + decane
- Vanda Contam + HMN + decalin
- Vanda Contam + HMN + decalin + decane
- Scott Barrels + HMN + decalin + decane

4.4. Roller Tube Liquid Culture Experiments

In an effort to increase the number of experimental runs that could be conducted at a given time, an attempt was made to determine if liquid culture experiments could be moved from 500mL Erlenmeyer flasks to 25mL roller tubes. Before conducting any biotic experiments, tests were done using abiotic samples, to ensure that the technique could be used reliably with repeatability in the results. The sterile samples for the experiment contained decalin, HMN + decalin and pure HMN. The carbon sources were placed in tubes containing 5mL of BH minimal salts media and

placed on a tube roller operating at 70rpm at 28°C for 2 weeks. Two techniques were attempted in the experiment to deliver carbon sources to the roller tubes. In the first, HMN and HMN + decalin were dissolved in DCM. Then the mixtures were placed in roller tubes and the DCM was allowed to evaporate overnight, leaving the HMN or HMN + decalin behind. The evaporation method was used in order to deliver the small amounts of HMN required for roller tube experiments. The other technique for carbon source delivery was to directly add decalin to the tubes. At the end of the two weeks the samples were extracted using an adaptation of the method outlined in section 3.2.5. The differences were that 10μL of dodecane was added, rather than 50μL and 2mL of pentane was added instead of 50mL.

After extraction and analysis using GC, extremely large variability was noted in the results, with error bars in the magnitude of 50%. Repeated attempts to reduce the size of the error bars associated with the experiments failed. Therefore, the main lesson learned from the experiment was that roller tubes could not be used for future studies. The extreme variability in the results showed that neither method of delivering carbon source worked. When the DCM was evaporating overnight there may have been some evaporation of the HMN as well. Another possibility was that a poor seal was being made on the tubes, allowing evaporation from some of the tubes over the course of the experiment. Without better equipment this second possibility could not be tested and as the source of the large error bars could not be determined the roller tube experiments were abandoned.

4.5. Isolating Bacteria Using R2A Plates

The Vanda Contam mixed cultures growing on decalin + decane, HMN + decalin and HMN + decalin + decane and Scott Barrels mixed culture growing on HMN + decalin + decane looked very different from each other when growing in liquid culture. This indicated that different strains of bacteria were able to thrive in the different carbon sources. Due to these variations in the appearance of the cultures this experiment was designed to determine how the bacterial species in the liquid cultures differed. Attempts were also made to quantify the cell densities of bacteria in the cultures.

In order to be able to isolate and view the various bacterial species in the cultures, solid media plates were used. However, there was the question of which medium to use. Typically, Plate Count Agar (PCA) is used to grow bacterial cultures. However, this is a rich and undefined medium that might have been too rich for cultures accustomed to relatively carbon limited environments. Therefore, instead of PCA plates, R2A plates were used which are less rich and designed for heterotrophic bacteria.

The experiment was conducted using the method outlined in section 3.2.2. Photographs of the cultures taken after they had been allowed to grow for 4 days at 4°C are found in Figures 25 to 28.

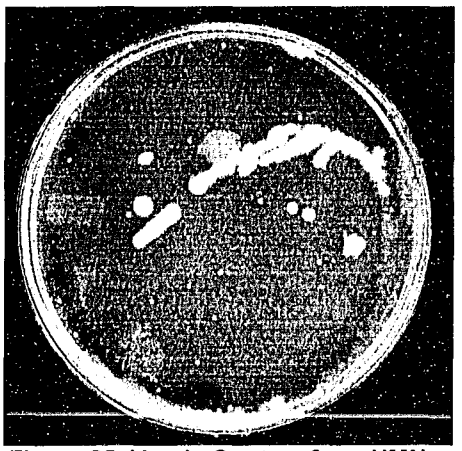

Figure 25: Vanda Contam from HMN + Decalin Flask on R2A Media

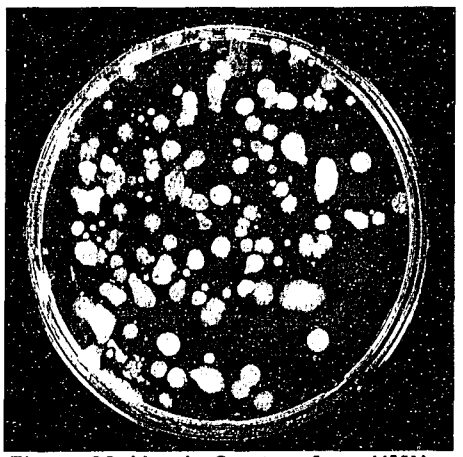

Figure 26: Vanda Contam from HMN + Decalin + Decane Flask on R2A Media

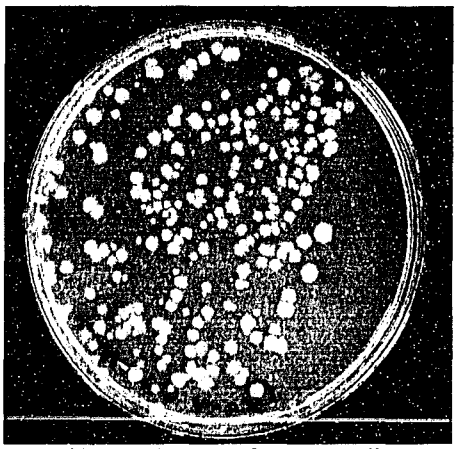

Figure 27: Vanda Contam from Decalin + Decane Flask on R2A Media

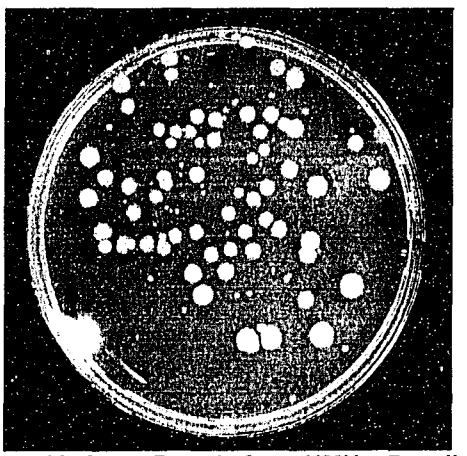

Figure 28: Scott Barrels from HMN + Decalin + Decane Flask on R2A Media

After counting the visible colonies, the cultures were found to have the following cell densities:

Table 12: Cell Densities of Mixed Cultures Using R2A Media Plate Counts

Culture	Dilution Level on R2A Plates	Mean Number of Colonies on Plates	Cell Density in Liquid Culture (CFU/mL)
Vanda Contam in HMN + Decalin	10^{-5}	46 ± 8	4.6×10^{6}
Vanda Contam in Decalin + Decane	10^{-6}	246 ± 38	2.5×10^{8}
Vanda Contam in HMN + Decalin + Decane	10^{-6}	161 ± 6	1.6×10^{8}
Scott Barrels in HMN + Decalin + Decane	10^{-6}	113 ± 15	1.1×10^{8}

Because so many species were present on the plates, determining which bacteria were responsible for decalin degradation was difficult. While all may not have been active towards decalin, they were at least able to survive to the point that when the R2A medium was provided they were able to actively grow. As a result, the R2A experiment was beneficial in that it helped characterize the mixed cultures. The plates showed that all the cultures contained a variety of bacterial types and that they were tolerant towards a variety of carbon source conditions. However, the experiment did little to further the isolation of a decalin degrader. A different technique would have to be developed to isolate the decalin degraders.

4.6. Metabolite Extraction

Both decalin and HMN loss had been noted in section 4.3. There was interest in determining what biochemical pathways were being used to cause the loss in the following cultures:

1. Vanda Contam growing in HMN + Decalin
2. Vanda Contam growing in HMN + Decalin + Decane
3. Vanda Contam growing in Decalin + Decane
4. Scott Barrels growing in HMN + Decalin + Decane

In order to gain an understanding of these pathways, an effort was made to find water soluble metabolites. First, the above cultures were transferred into fresh media with fresh carbon sources in triplicate and allowed to incubate at 28°C for 2 weeks. Then, two dichloromethane extractions were conducted in series. First a neutral extraction was conducted, then an acidified one. The first extraction was done at neutral pH to remove HMN. Due to the expected low concentration of metabolites in the aqueous phase, the extracts were concentrated by evaporating DCM. In the process the 1mL of HMN present in the flasks would also be concentrated. Unless the HMN was somehow removed, overloading of the column would have occurred and obscured any new metabolite peaks. However, with the HMN removed in the neutral extraction, identification of metabolites in the acidified extraction would be possible.

If carboxylic acids were present in the aqueous phase, they would have been present in the forms of carboxylate anions and hydronium ions. Thus, they would not have been efficiently extracted by the DCM at neutral pH. Therefore, the aqueous phase was then acidified using hydrochloric

acid to a pH of less than 1. By lowering the pH the carboxylate anions and hydronium ions would recombine to form carboxylic acids, which could then be extracted by DCM. Derivatization via BSTFA was then done to silylate the carboxylic acids so that they would be identifiable using gas chromatography. GC analysis was then conducted to determine if any new peaks were noted in the chromatograms from the biotic samples when compared to sterile samples. Before the experiment began, the location of these new peaks on the chromatograms was unknown. Therefore, samples were prepared for GC analysis using known carboxylic acids and alcohols and chromatograms were produced. The chemicals selected were oleyl alcohol, palmitic acid, stearic acid and heptanoic acid and the chromatograms can be found in section 9.1.

After extraction and analysis using GC, no metabolites were noted in either the neutral or acidified extracts. The lack of any metabolites suggested that all of the decalin, decane and HMN that underwent oxidation were being rapidly converted to CO_2 or biomass. If metabolites in the form of alcohols had been present, they would have been identified in the neutral extraction. If carboxylic acid metabolites had been present they would have been identified in the acidified extractions. Because the study was being conducted on a mixed culture that contained a variety of bacterial types, this was not surprising. If the rate limiting step in the process was the initial oxidation step, then as soon as this was completed the consortium of bacteria in the flask could have completely oxidized the metabolites, leaving no trace of metabolites to be found via GC analysis. A new technique was needed to remove the metabolites from the solution before being used by the mixed cultures in further metabolic processes.

4.7. Metabolite Adsorption on Resin

As no metabolites were detected in section 4.6 another attempt was made to find metabolites using an alternate technique. If the rate limiting step in the degradation process was the initial oxidation attack the mixed culture could have rapidly consumed and converted any metabolites to CO_2. The complete mineralization of metabolites would leave no chemicals to be identified during GC analysis. In order to combat this issue, an ion exchange resin was utilized. Resin beads were

added to each flask which had an ability to act as a weak anion exchanger. The idea was that the resin bead would adsorb any anions produced by the bacteria during metabolic processes, preventing them from being converted to CO_2. Then, during the extraction process an ion exchange step would be conducted to remove these anions from the beads for identification. The method outlined in section 3.2.7 was applied to the same cultures used in section 4.6. Again, an analysis was done to determine if any new peaks were noted in the chromatograms from the biotic samples when compared to sterile samples. One change was that in this experiment a new derivatizing agent, diazomethane, was used.

Surprisingly, despite the use of the resin beads, no metabolites were identified. This was an unexpected results as the resin beads should have removed any anion metabolites from the aqueous phase as soon as they were created, preventing their use by the mixed culture. Then, via the ion exchange technique and subsequent derivatization, these metabolites should have been visible on the chromatograms. Instead, the only new peak visible was that of a plasticizer contaminant.

An explanation for the lack of metabolites was errors in the experimental technique. Perhaps all the nitrogen drying steps in the process led to the loss of the metabolites. Perhaps the samples were re-suspended in too much DCM and the concentration of the metabolites was too low for identification using the GC unit. Due to lack of time, these issues could not be investigated. In any event, no useful information was gained from these experiments except to say that the process of metabolite identification from the mixed cultures was not a straightforward exercise.

4.8. Vapour Plates and "Vanda Contam" and "Scott Barrels" Mixed Cultures

The R2A plates illustrated in Figures 29 to 32 showed that the mixed cultures contained a variety of different bacterial strains. However, not all of these strains would necessarily be able to degrade decalin. As a result, the bacteria found on the R2A plates were not an ideal choice to begin the process of identifying an isolate. An alternate selective condition needed to be created

to select for bacterial species from the mixed cultures that were decalin degraders. Ideally, this alternate condition would still use solid medium so that individual colonies of bacteria could be identified and removed for further study. Unfortunately, the cultures could not be exposed to direct contact with liquid decalin. As shown in the results in section 4.3 the mixed cultures displayed no activity towards pure decalin in liquid form, probably due to its toxicity.

In order to deal with these issues, a vapour plate technique was utilized. Plates were made using Bushnell-Haas medium combined with Noble agar. This solid medium would theoretically contain no carbon source, but would provide all the other macro- and micronutrients required by the bacteria. Decalin and decane, which have relatively high volatility, would be presented to the bacteria in vapour form. The decalin in vapour form would have a low concentration, reducing toxicity concerns and hopefully allowing the bacteria to be grown on the plates. HMN was added directly to the plates, as it had no known toxicity issues and was not considered volatile enough to be accessed in sufficient quantities in the vapour phase. In addition, the HMN would provide a phase for the decalin to dissolve into from the vapour phase, further reducing the possibility of toxic effects.

Following the method outlined in section 3.2.2 the plates were prepared and the vapour environments created. Then the following cultures were used to provide inocula for the plates:

1. Vanda Contam growing in HMN + Decalin
2. Vanda Contam growing in HMN + Decalin + Decane
3. Vanda Contam growing in Decalin + Decane
4. Scott Barrels growing in HMN + Decalin + Decane

The cultures were allowed to incubate for four days at room temperature. In order to provide a basis of comparison, negative controls were made where cultures were placed on plates but no carbon source was provided. As an additional control, the cultures were also grown in pure decane vapours. These plates served as a positive control, as growth was expected on all cultures exposed to such an easily accessible carbon source. In total, the following five carbon source environments were provided:

1. Liquid HMN on Plates + Decalin Vapour
2. Liquid HMN on Plates + Decalin Vapour + Decane Vapour
3. Decalin Vapour + Decane Vapour
4. Decane Vapour
5. No Carbon Source

Growth of varying degrees was noted on every plate with every carbon source. The type and number of colonies varied depending on the source of inoculum. Of particular interest were the plates where the culture from the Vanda Contam grown in HMN + decalin was used. The HMN + decalin carbon source in the liquid culture would have served as an enrichment culture, hopefully selecting for bacteria able to use decalin as a carbon source. The belief was that plates would allow these bacterial species to be isolated. Photographs of these plates and the controls are found below in figures 29 to 34. Photographs of the plates from the other cultures can be found in section 11.1. The cell densities for the liquid cultures can be found in Table 13.

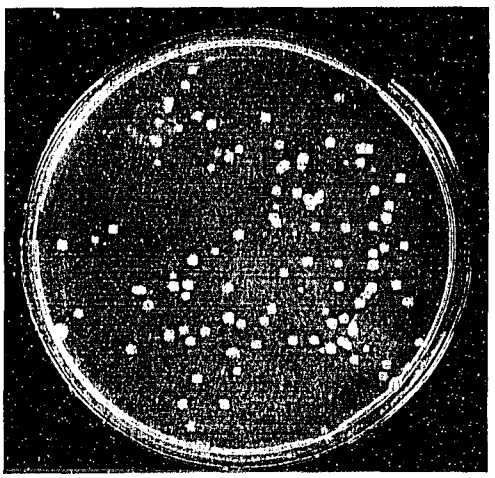

Figure 29: Vanda Contam from HMN + Decalin Flask (10^{-4}) in Decane Vapour

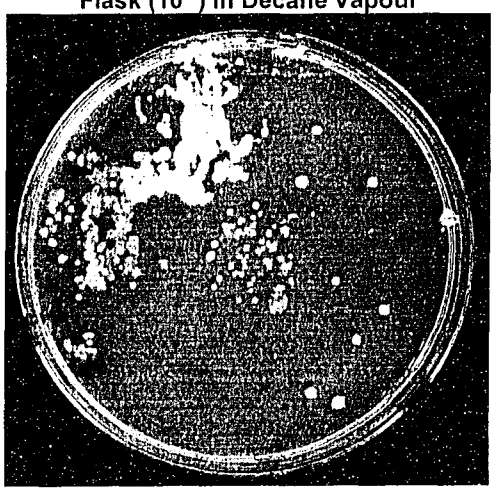

Figure 30: Vanda Contam from HMN + Decalin Flask (10^{-5}) in HMN + Decalin + Decane Vapour

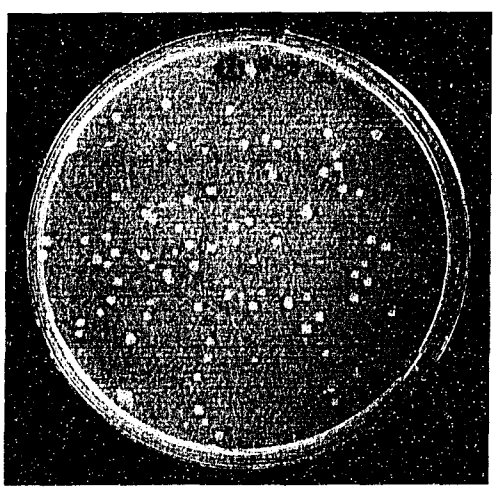

Figure 31: Vanda Contam from HMN + Decalin Flask (10^{-4}) in Decalin + Decane Vapour

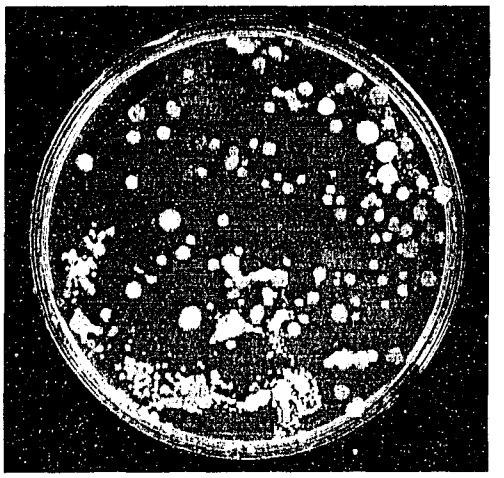

Figure 32: Vanda Contam from HMN + Decalin Flask (10^{-4}) in HMN + Decalin Vapour

Figure 33: Vanda Contam from HMN + Decalin Flask (10^{-4}) with No Carbon Source

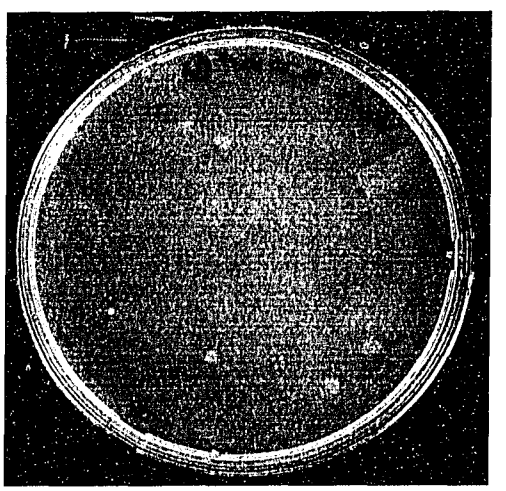

Figure 34: Vanda Contam from HMN + Decalin Flask (10^{-5}) with No Carbon Source

Table 13: Cell Densities of Mixed Cultures Calculated Using Vapour Plates

Culture Source	Carbon Source Environment	Dilution Level on Plates	Mean Number of Large Colonies on Plates	Cell Density in Liquid Culture (CFU/mL)
Vanda Contam in HMN + Decalin + Decane	HMN + Decalin + Decane	10^{-6}	198 ± 80	1.98×10^{8}
	HMN + Decalin	10^{-6}	175 ± 73	1.75×10^{8}
	Decalin + Decane	10^{-6}	113 ± 10	1.13×10^{8}
Vanda Contam in HMN + Decalin	HMN + Decalin + Decane	10^{-5}	154 ± 34	1.16×10^{7}
	HMN + Decalin	10^{-5}	158 ± 75	1.58×10^{7}
	Decalin + Decane	10^{-4}	92 ± 7	9.2×10^{5}
Vanda Contam in Decalin + Decane	HMN + Decalin + Decane	10^{-4}	0	No Large Colonies
	HMN + Decalin	10^{-4}	0	No Large Colonies
	Decalin + Decane	10^{-4}	0	No Large Colonies
Scott Barrels in HMN + Decalin + Decane	HMN + Decalin + Decane	10^{-5}	45 ± 12	4.5×10^{6}
	HMN + Decalin	10^{-5}	67 ± 23	6.7×10^{6}
	Decalin + Decane	10^{-5}	77 ± 77	7.7×10^{6}

Note: On the plates inoculated from the "Vanda Contam in Decalin + Decane" there were no large colonies (diameter > 1mm). However, on every plate there were hundreds of pinprick colonies with diameter < 1mm. In morphology, these colonies differed completely from those described in section 4.5. More than likely an error had occurred in the preparation of the dilution series, or the sample used from the Vanda Contam in Decalin + Decane flask to prepare the dilution series was a poor one and did not contain all of the bacteria available in the flask.

The results in Figures 29 to 34 and in Appendix C show that the cultures exposed to the hydrocarbons in the vapour form thrived. More importantly, the dominant colonies on the vapour plates differed from those on the R2A plates, and there appeared to be a lower number of different colony morphologies. The plates grown in HMN + decalin demonstrated that both Scott Barrels and Vanda Contam appeared to contain species of bacteria capable of utilizing pure decalin.

In the case of the Scott Barrels culture, this was an interesting finding as previous results had indicated that the culture lacked the ability to degrade decalin without decane present. The growth on the vapour plates indicated that the Scott Barrels culture did contain bacteria with some limited ability to degrade decalin, yet there were still questions as to how the Scott Barrels

culture handled decalin's toxicity. On the HMN + decalin plates, the decalin would first dissolve into the HMN, and then be available to the bacteria. Thus, decalin availability to the bacteria would be limited by mass transfer from the vapour phase into the HMN and subsequent mass transfer to the "aqueous phase" of the agar plates. Apparently, the bacteria had the ability to use the decalin when presented in this manner. Yet, when the results of section 4.3 were considered, the Scott Barrels culture was not capable of handling the toxicity of decalin in a liquid culture at a concentration of 5wt% decalin. Therefore, the colonies from the Vanda Contam plates warranted greater consideration than those from the Scott Barrel plates.

All of the plates inoculated from the Vanda Contam flasks had growth in all the vapour plate environments. The Vanda Contam plates inoculated from the HMN + Decalin flask (shown in Figures 29 to 34) had prolific growth, and based on visual inspection appeared to have a variety of species. One result that was unexpected was that on the plates that were grown in pure decane and decalin + decane, there appeared to be only one type of bacterium. The homogeneity of the growth suggested that one species of bacterium may have been responsible for decane degradation in the mixed culture, while several could attack decalin. Due to the proliferation of growth, only the Vanda Contam plates were used to attempt to find an isolate capable of degrading pure decalin.

4.9. Isolating a Decalin Degrader from "Vanda Contam" Vapour Plates

From previous experiments, there were several indications that the Vanda Contam mixed culture may have contained a single bacterial species capable of degrading decalin. The first indication was found in section 4.3 where decalin degradation occurred when the Vanda Contam mixed culture was given a carbon source of decalin + HMN, yet no HMN removal occurred when the culture was exposed to pure HMN. The second indication was in section 4.8 where individual colonies of bacteria were able to grow in the HMN + decalin environment. With this knowledge, an attempt was made to find an isolate which would have the capability of degrading pure decalin. The process involved 3 steps. First, the Vanda Contam plates illustrated in section 4.8

and Appendix C were examined. Colonies of similar appearance which were visible on plates grown in all the different carbon source environments were identified, and then these colonies were transferred from the vapour plates grown in HMN + decalin and streaked on R2A plates. In this process eight different colony morphologies were noted. These colonies were re-streaked several times, to ensure purity. The colonies were labelled as follows:

- Iso 1a
- Iso 1bY
- Iso 2aCl
- Iso 2aCr
- Iso 2bY
- Iso 2cO
- Iso 3a
- Iso 4aG

These eight different bacterial colonies were then streaked onto plates that contained Bushnell-Haas medium plus Noble agar and placed in the same vapour environments found in section 4.8. They were allowed to grow for four days, and then examined and photographed. Of particular interest were the results for the cultures grown in HMN + decalin. Without decane to support cometabolism, this environment offered the best chance of containing bacterial species which could use decalin as a sole carbon source. Photographs of the isolates grown in HMN + decalin can be found in Figure 35 to Figure 42. Photographs of the isolates in the other carbon source environments can be found in Appendix C. In order to ensure that no carbon source was contaminating the medium, the isolates were also streaked on Bushnell-Haas plates and then placed in incubators with no carbon source. An example photograph of a plate with no carbon source is shown in Figure 43 and served as a basis of comparison for bacterial growth.

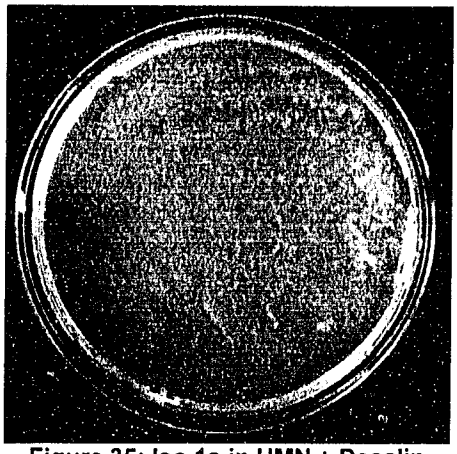

Figure 35: Iso 1a in HMN + Decalin

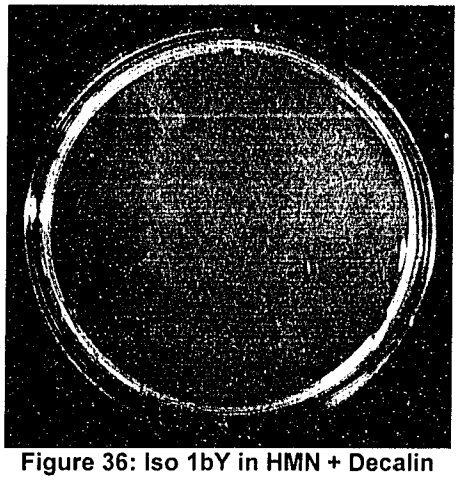

Figure 36: Iso 1bY in HMN + Decalin

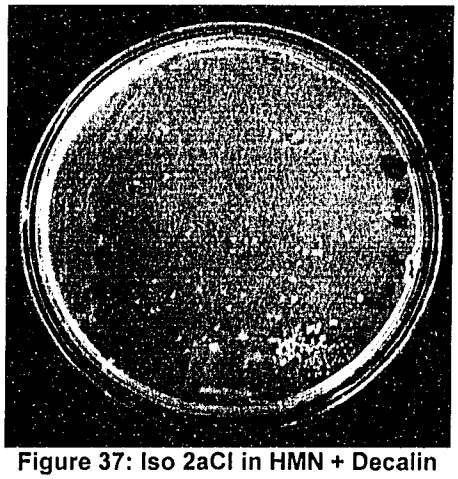

Figure 37: Iso 2aCl in HMN + Decalin

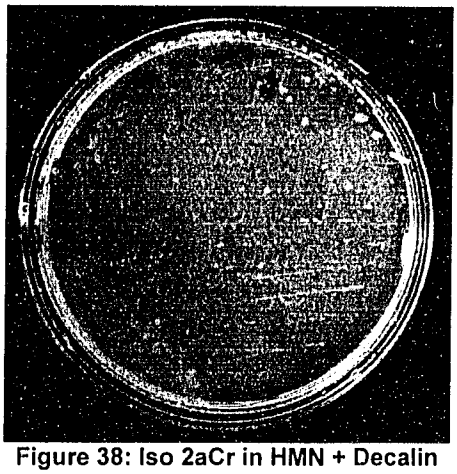

Figure 38: Iso 2aCr in HMN + Decalin

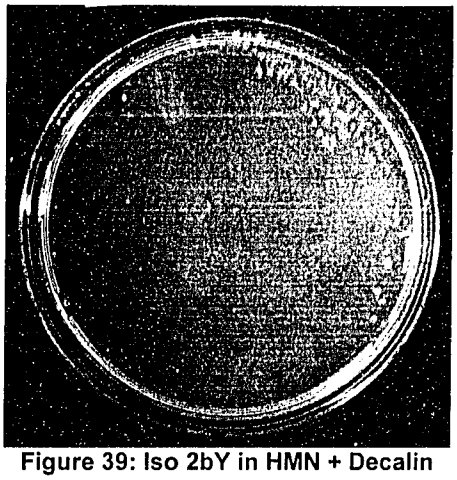

Figure 39: Iso 2bY in HMN + Decalin

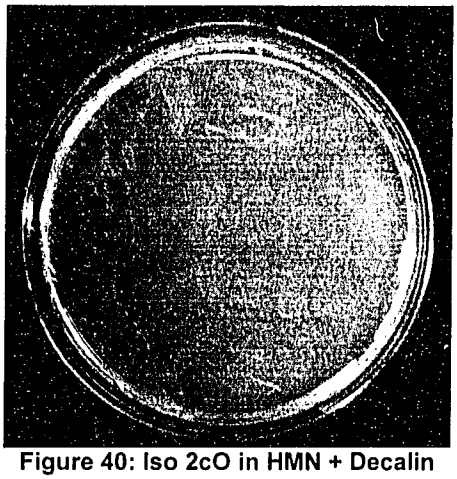

Figure 40: Iso 2cO in HMN + Decalin

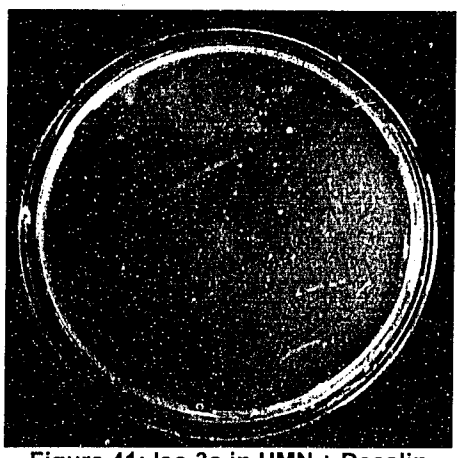

Figure 41: Iso 3a in HMN + Decalin

Figure 43: Iso 1a with No Carbon Source

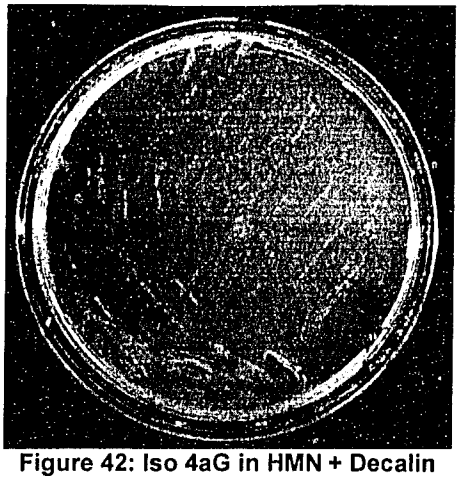

Figure 42: Iso 4aG in HMN + Decalin

From these plates, one can see that not all of the cultures had abundant growth. In particular Iso 1bY and Iso 2bY demonstrated limited to no growth when compared to the other isolates. In addition to this, a few of the plates contained bacteria with identical colony morphologies. Therefore, four of the eight isolates were selected for further study and streaked on fresh plates containing Bushnell-Haas + Noble agar. The isolates chosen were:

- Iso 1a
- Iso 2aCl
- Iso 2aCr
- Iso 2cO

Triplicate plates of each isolate were then placed in three carbon source environments of pure decalin, pure decane and pure HMN at room temperature.

In the case of the isolates placed in a pure HMN environment, no growth was noted. Isolates exposed to pure decane demonstrated prolific growth. When the isolates were placed in a pure decalin vapour environment, growth was also noted for all four isolates. Below are photographs of each isolate taken after incubating for seven days in decalin vapour.

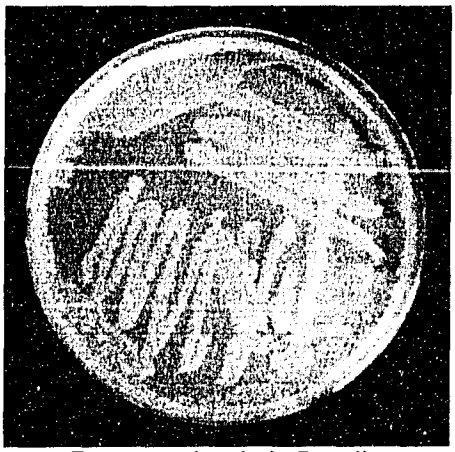

Figure 44: Iso 1a in Decalin

Figure 45: Iso 2aCl in Decalin

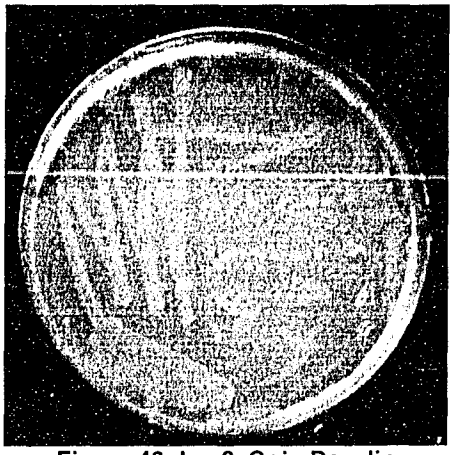

Figure 46: Iso 2aCr in Decalin

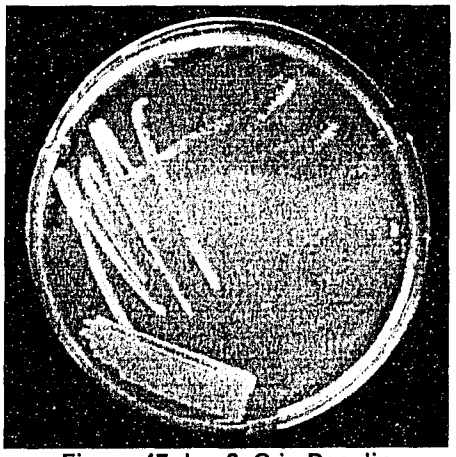

Figure 47: Iso 2cO in Decalin

Table 14: Morphologies of Decalin Degrading Isolates

Isolate Name	Colony Morphology
Iso 1a	Individual colonies grow in large irregular shapes, somewhat spherical but with defined edges. Whitish in color. Gooey slime layer visible.
Iso 2aCr	Individual colonies grow in large circular shapes, with smooth edges. Whitish in color. Colonies appear opaque at first, but with time take on a creamy appearance. Gooey slime layer is visible.
Iso 2aCl	Individual colonies grow in large circular shapes, with smooth edges. Whitish in color. Colonies appear opaque at first, but with time take on a creamy appearance. Gooey slime layer is visible.
Iso 2cO	Small round colonies form with smooth edges. Yellowish in color. No slime layer.

After obtaining these isolates and successfully transferring them to fresh plates, the bacteria were sent for 16S rRNA sequencing. After 16S rRNA sequencing only 300 base pairs were available for analysis. The results (courtesy of Jonathan Klassen) can be found in Appendix D. The sequencing indicated that all four bacteria were *Rhodococcus* spp. and that more than likely they were all *Rhodococcus erythropolis*. Iso 1a had an expectation value of 0.0 considering degeneracies, Iso 2aCl had one mismatched base and Iso 2aCr had two mismatched bases but both still had expectation values of approximately zero. Iso 2cO matched *R. erythropolis* and *Rhodococcus* sp. TM1 genBank accession #AY642543 with expectation values of approximately zero. Expectation values are a statistical method of evaluating the match between the 16S rRNA data for an unknown bacteria and the information available in microbiology databases. The closer the expectation value is to zero, the more likely the match. As the expectation values for all the database searches approached zero, the above results could be used with confidence.

While the species designation could not be guaranteed due to the low number of base pairs available, it was assumed for the remainder of the report that all four isolates were *R. erythropolis*. Although they were all the same species, they did not appear to all be the same strains. In terms of colony morphology, Iso 2aCl and Iso 2aCr were very similar. However, as noted in Table 14, three distinct morphologies were obvious when the four vapour plates were examined. The major visual difference was the mucoid colony morphology caused by the presence of a glycocalyx layer in Iso 1a, Iso 2aCl and Iso 2aCr.

4.10. Degradation of HMN + Decalin by Isolates in Liquid Culture

From the results in section 4.9, four bacterial isolates had been identified that had the capability to live on decalin vapours and there appeared to be three or four different strains. The purpose of this experiment was to determine if these bacteria were able to degrade decalin when presented in a HMN + decalin mixture in a liquid culture, similar to the experiment conducted in section 4.3. The method outlined in section 3.2.1.2 was utilized. Sterile controls were used to provide a basis of comparison. Qualitative results are found below in Table 17. Quantitative results are found in Figure 48 and Figure 49.

Table 15: Qualitative Observations for Isolates in HMN + Decalin Liquid Culture

Isolate Type	Qualitative Observations
Iso 1a	Large white flocs visible in culture in large numbers, when the flaks is shaken. If the flask is not shaken, the flocs float to the interface between the aqueous and non-aqueous phase.
Iso 2aCl	Culture has taken on a milky white color. Some turbidity. Some possible flocs apparent.
Iso 2aCr	Culture has taken on a milky white color. Some turbidity. Some possible flocs apparent.
Iso 2cO	Possible limited growth when compared to the sterile control. No floc formation. Flask contents may be taking on a slightly cloudy appearance.

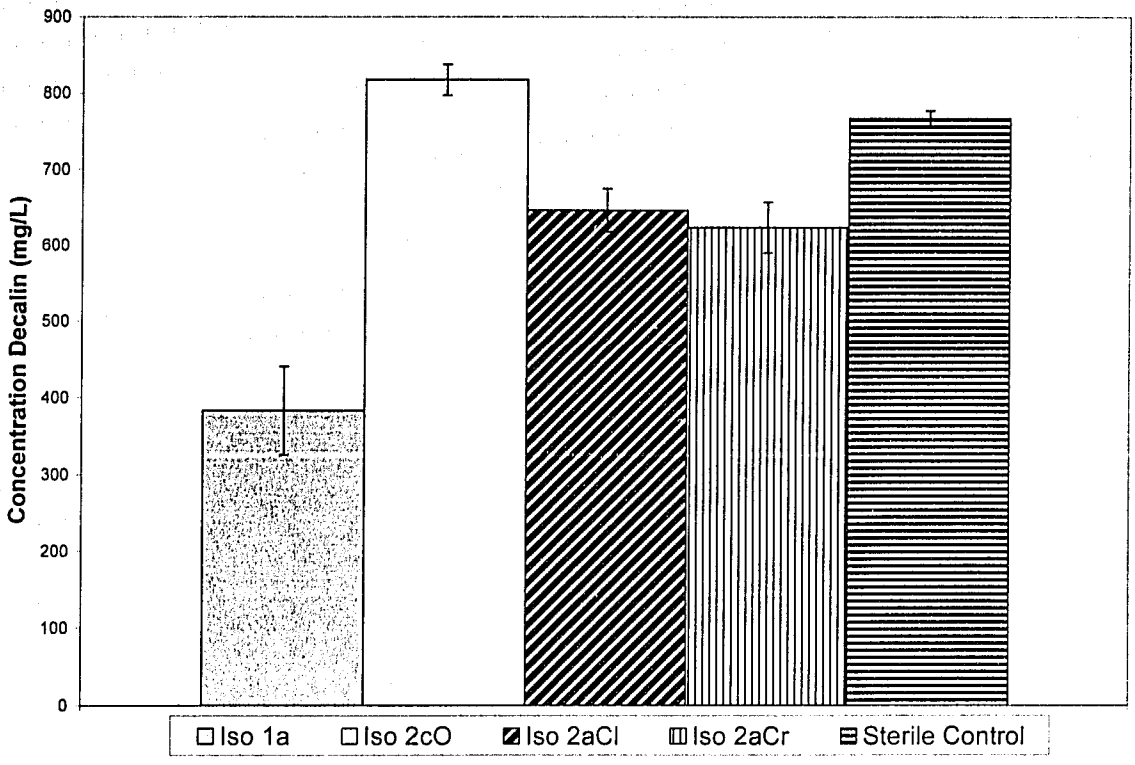

Figure 48: Recovery of decalin after incubation with isolates at 28°C for 14d

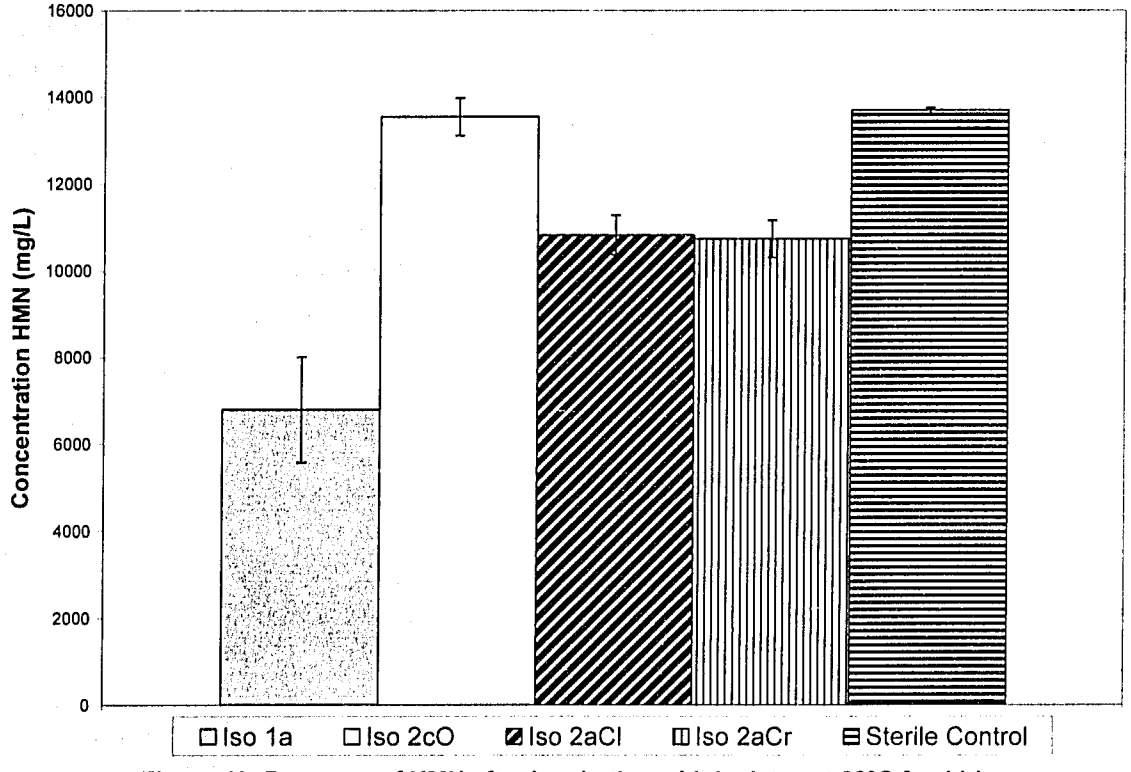

Figure 49: Recovery of HMN after incubation with isolates at 28°C for 14d

After the results in Figure 48 and Figure 49 were plotted, T-Tests were performed, as described in section 4.3. The results of the T-Tests are found in Table 16 and Table 17.

Table 16: Probability that the Biotic Sample is Equal to the Sterile Sample for Decalin Recovery from the Isolate Cultures

Isolate Type	Probability that biotic and sterile samples are the same (%)
Iso 1a	0.3
Iso 2cO	1.6
Iso 2aCl	0.6
Iso 2aCr	0.6

Table 17: Probability that the Biotic Sample is Equal to the Sterile Sample for HMN Recovery from the Isolate Cultures

Isolate Type	Probability that biotic and sterile samples are the same (%)
Iso 1a	0.5
Iso 2cO	31.0
Iso 2aCl	0.4
Iso 2aCr	0.3

From the results one can see that there were distinct differences in the behaviour of the isolates in liquid culture. Qualitatively they were different in appearance, with Iso 1a growing in flocs and the other isolates not. Also, Iso 1a, 2aCl and 2aCr grew well while Iso 2cO did not. Quantitatively, the isolates behaved differently as well. Iso 1a caused significant loss of both decalin and HMN while Iso 2cO had no impact within experimental error. Iso 2aCl and Iso 2aCr had intermediate capabilities. The percent of decalin degradation when compared to the sterile control is listed below in Table 18. In addition, three isolates caused HMN removal when compared to the sterile control (Table 19).

Table 18: Percentage of Decalin Degradation Caused by Isolates

Isolate Type	Percent Degradation of Decalin when Compared to Sterile Control
Iso 1a	50%
Iso 2cO	0%
Iso 2aCl	16%
Iso 2aCr	19%

Table 19: Percentage of HMN Removal Caused by Isolates

Isolate Type	Percent Removal of HMN when Compared to Sterile Control
Iso 1a	50%
Iso 2cO	1%
Iso 2aCl	21%
Iso 2aCr	22%

The results from this experiment indicated that there were indeed at least three different strains. Iso 2aCl and Iso 2aCr appeared to be the same bacterium, as their degradative capabilities were so similar, as well as the colony morphologies described in section 4.9. Iso 2cO was able to utilize pure decalin in vapour form, but displayed no activity towards decalin or HMN in the liquid culture in this experiment. Therefore, something was hindering the bacterium's activity. Iso 1a caused the highest levels of decalin and HMN loss indicating that the adaptations of that particular strain were best suited to the liquid culture environment.

5.0 DISCUSSION

5.1. Toxicity of Decalin

Prior to beginning experimental work, the toxicity of decalin was expected to have a major impact on culture activity, as described in section 3.1.1. This prediction turned out to be true. Toxic effects were first noticed from the results in section 4.3 when the mixed cultures were exposed to pure decalin (see Table 10). Sikkema *et al.* (58) proposed that the ratio of decalin to medium in a liquid culture was related to toxicity. In the experiment in section 4.3, only 100μL of decalin was present in 220mL of medium, yet no biological activity was observed. This suggested that the pure decalin had a high degree of toxicity towards the bacteria in the mixed cultures. When steps were taken to reduce the toxic effects, either by dissolving the decalin in HMN (as in sections 4.3 and 4.10) or presenting the decalin in vapour form (as in section 4.9), decalin degradation was observed. Thus, the prediction by Sikkema *et al.* (58) that decalin toxicity played a role in halting biological activity was proven to be correct. This could also explain why Pelz and Rehm (47) saw no activity towards decalin. Experimental technique and the means of presenting the decalin to the cultures obviously play an important role in biological activity.

An interesting finding was that there seemed to be a difference in the ability of the bacteria from the Vanda Contam and Scott Barrels mixed cultures to deal with the toxicity of decalin. In liquid cultures, the Scott Barrel culture only had activity towards decalin when decalin and decane were dissolved in HMN (see section 4.3). However, growth occurred on HMN + decalin vapour plates in the results from section 4.8. This difference in the culture's ability to degrade decalin when presented in the liquid and vapour forms indicated that toxicity may still have been playing a role in the activity of the Scott Barrels culture. Even when diluted in HMN this issue could only be overcome with the additional energy provided by decane as a cometabolic agent.

The Vanda Contam mixed culture had a different reaction to the decalin environments presented in section 4.3. As with the Scott Barrel mixed culture, decane seemed to aid the Vanda Contam mixed culture in dealing with the toxicity of decalin. However, the decalin did not need to be

dissolved in HMN for cometabolism to play a role. In Figure 19 one can see that decalin degradation was possible when decane was present. Only two of the three replicate cultures had activity though. The lack of growth in all three flasks indicated that the cultures were not particularly well suited to that environment. Then again, the activity that was noted suggested that with a source of energy the Vanda Contam mixed culture was able to deal with the toxic effects to a degree. The fact that Vanda Contam culture did not need to have the decalin dissolved in the HMN but the Scott Barrels did indicated that the two cultures had different mechanisms or abilities to handle the toxicity of decalin.

Possible toxic effects of decalin were also noted in the isolate studies. Iso 2cO was capable of growing using pure decalin in vapour form. However, the isolate showed no activity towards decalin when exposed to 5wt% decalin in HMN. There are many possible explanations for this (see section 5.4), but one possibility is that Iso 2cO was more sensitive to the toxic properties of decalin than the other isolates, and the 5wt% decalin inhibited metabolic activity.

This difference in the ability of the two mixed cultures and isolates to deal with the toxicity of decalin is difficult to explain. In Gram negative bacteria, five mechanisms have been identified which bacteria utilize in response to toxic hydrophobic chemicals:

1. "Metabolism of toxic hydrocarbons, which can contribute to their transformation into non-toxic compounds
2. Rigidification of the cell membrane via alteration of the phospholipids composition
3. Alterations in the cell surface that make the cells less permeable
4. Efflux of the toxic compound in an energy-dependent process
5. Formation of vesicles that remove the solvent from the cell surface" (51)

Although rhodococci are Gram positive, the various bacterial species and strains in the mixed cultures and the various *R. erythropolis* isolate strains may have used different combinations of the above five mechanisms to handle the toxicity of decalin. For example, all the mixed cultures handled the toxicity of decalin better when decane was present. Therefore the bacteria in those cultures may have used efflux pumps to remove excess decalin. Energy derived from the metabolism of decane could be used to run the efflux pumps. Another possibility is that the bacteria were all able to use decalin at varying rates. This could explain the differences in the isolate's ability to degrade decalin in liquid culture. Because all four isolates thrived in the decalin vapour environment, one could assume that the rate of decalin transfer from the gas phase into the cells was lower than the rate of decalin consumption by the cells. However, the rate of decalin transfer from the 5wt% decalin in HMN into the cells of Iso 2cO was greater than the rate of consumption. The fact that Iso 2cO was the only isolate without a glycocalyx layer may indicate that the glycocalyx layer was necessary for cell survival. The hydrophilic surface could have hindered transport of decalin into Iso 1a, 2aCl and 2aCr to the point that they could metabolize the decalin at the rate of decalin transport into the cell. Iso 2cO lacked the protection of the glycocalyx layer and could not handle the toxic effects and no degradation was noted. Any of the above mechanisms could have played a role. An in depth investigation of toxicity will be required before any conclusions can be drawn.

5.2. Metabolism and Cometabolism of Decalin

Cycloalkane degradation had been noted many times due to cooxidation and cometabolism in reported literature (see section 2.4.1). Therefore it was assumed that decalin degradation would be possible in a mixed culture with decane present, if toxicity issues were dealt with. The mixed culture would provide the variety of bacteria needed for cooxidation and the decane would provide the primary carbon source to act as an inducer for oxidation of decalin. The results from section 4.3 validated the above assumption. For both the Vanda Contam and Scott Barrels mixed cultures, decalin degradation was noted when decane and decalin were dissolved in HMN and presented as a carbon source. Furthermore, these cultures showed the highest amount of

degradation of decalin, when compared to the other carbon source environments. These results showed that if decalin was presented below toxic levels with decane, decalin degradation was readily achievable. These results were interesting, yet not surprising. While biomarkers with more than two rings have been found in the natural environment (see section 2.4.3), no reports of decalin or similar structures accumulating in the natural environment have been published. Therefore, one would expect the ability to degrade decalin via cometabolic processes to be a common phenomenon. The cultures used in the screening experiment were taken from Europe, North America and Antarctica. Because degradation in the presence of decane happened in all the cultures (see section 4.1), this suggests that such degradation is a widespread occurrence.

By acting as the primary carbon source, the decane facilitated the degradation of decalin in the mixed cultures. Decane could have induced the production of broad spectrum enzymes, provided the energy to produce enzymes capable of specifically attacking decalin or provided energy to run efflux pumps. The cometabolic processes were not studied though, as they hold no interest to biocatalyst development. In section 2.2, criteria of a successful biocatalyst for cycloalkanes were listed. The criteria stated that a successful biocatalyst should target cycloalkanes and that it should not attack non-target substrates such as aliphatics. Therefore, species of bacteria that required cometabolism to operate were unsuitable.

The results from section 4.3 offered hope that the mixed cultures, particularly the Vanda Contam mixed culture, contained a bacterium capable of degrading pure decalin because degradation of decalin took place when only HMN and decalin were present. There was some question whether the HMN was acting as the primary carbon source in cometabolism of decalin. However, the Vanda Contam culture could not degrade pure HMN. As HMN was known to have no toxic qualities the HMN removal in Figure 23 and Figure 24 was assumed to be a result of cometabolism where the decalin was acting as the primary carbon source. By utilizing the vapour plate technique and the screening process outlined in section 4.9, four isolates were found capable of growing in a pure decalin environment. The ability of isolates to degrade decalin

confirms the results of the experiments by Soli and Bens (65) that have not been reported since 1973. As predicted in section 2.6, this result was not unexpected due to the published work of Koma *et al.* (29) and Schumacher and Fakoussa (56). In particular, the work of Schumacher and Fakoussa (56) with cyclododecane demonstrated that isolates have the capability of degrading cycloalkanes. Previously, failed studies reported in the literature were probably due to limitations caused by toxicity and mechanisms of hydrocarbon uptake. This is understandable, as much of the research into cycloalkane degradation was completed in the 1970's, when such issues were just being explored.

Similar to the biochemical pathway discovered by Schumacher and Fakoussa (56), one would expect the decalin to be oxidized to form an alcohol, and then follow the classic pathway of being converted to a ketone and then to a carboxylic acid. The attack may happen at both rings simultaneously, or one ring at a time. Without detecting the metabolites nothing can be confirmed, only postulated.

One question that might arise from the results is that without metabolites, how could one be sure that decalin degradation actually occurred? One could argue that without killed controls, the decalin loss noted in section 4.3 and 4.10 could have been due to absorption onto the cells, or uptake by the cells with no ensuing metabolic activity. However, when all the results are examined, decalin degradation can be confirmed. There was considerable growth on the pure decalin vapour plates for all four isolates when compared to the no-carbon source controls in the results for section 4.9. Qualitative observations in section 4.10 noted a visible increase in the biomass in the flasks for *R. erythropolis* Iso 1a, *R. erythropolis* Iso 2aCl and *R. erythropolis* Iso 2aCr, coinciding with decalin loss noted on the GC. These findings lead one to the conclusion that decalin degradation was occurring. However, now that isolates have been identified and cooxidation between species is not considered a requirement for degradation, killed controls should be utilized in all further experiments. In addition, the pure cultures should be used in an ion exchange experiment using resin beads, to attempt to find metabolites of decalin degradation,

to confirm that decalin degradation is occurring. Identification of CO_2 as a product of metabolism could also be used to confirm decalin mineralization. In order to complete such a study, ^{14}C-decalin would be required. Unfortunately, as this is not available commercially, it would have to custom manufactured.

5.3. Cometabolism of HMN

Throughout the experiments, no activity was noted towards pure HMN. The data in Table 11 show that the mixed cultures did not cause any HMN removal, when exposed to pure HMN. In section 4.9 the isolates were exposed to pure HMN on plates, and again no growth was noted. These results were consistent with literature, as described in section 2.3. However, when another hydrocarbon was available to be used as the primary carbon source, HMN removal was noted. The Scott Barrels and Vanda Contam mixed cultures caused the removal of HMN when decalin and decane were present in HMN, and the Vanda Contam mixed culture caused the removal of HMN when only decalin was present in HMN. In addition, *R. erythropolis* Iso 1a, *R. erythropolis* Iso 2aCl and *R. erythropolis* Iso 2aCr caused varying degrees of HMN removal when presented with a HMN + decalin mixture in liquid culture, as described in section 4.10. This removal of HMN has not been previously noted in literature and as a result questions arise as to the cause of this newly noted phenomenon.

No HMN metabolites were discovered in sections 4.6 and 4.7. Therefore, the cause of the HMN removal cannot be attributed to cometabolic degradation with a high degree of certainty. A possibility is that with a proper inducer, such as decalin, present to act as a primary carbon source the bacteria produce a broad spectrum enzyme or series of enzymes capable of attacking both decalin and HMN. There is cause to accept this concept because degradation of terminal branched alkanes by bacteria has been noted in literature. Pristane and phytane have been known to be biodegradable (27, 48, 54). In addition, isooctane (2,2,4-trimethylpentane) has been found to be degraded by *Mycobacterium austroafricanum* IFP 2173 (60). The proposed

biochemical pathway by Solano-Serena *et al.* (60) indicates that attack on a methylated structure is possible.

However, there is another possibility why the HMN is disappearing from the liquid cultures. *Rhodococcus* spp. are known to have selective uptake of hydrocarbons and to form inclusions of the hydrocarbons within the cell (26). Previous research has used HMN + *n*-hexadecane incubated with *Rhodococcus erythropolis* S+14He and found that selective uptake of the *n*-hexadecane occurred, while uptake of the HMN did not. However, *n*-hexadecane is a readily usable carbon source, whereas decalin is not. In order to access the decalin in liquid culture, *R. erythropolis* Iso 1a, *R. erythropolis* Iso 2aCl and *R. erythropolis* Iso 2aCr may be making inclusions of the hydrocarbons, but are unable to selectively transport decalin. Instead both HMN and decalin may be being brought into the cells. The cells may not be using the HMN as a carbon source though. This would account for the near equal removal of HMN and decalin in the cultures noted in Table 18 and Table 19.

5.4. Characteristics of the Rhodococcus Isolates

All four isolates identified in section 4.9 were *Rhodococcus* spp. This was not an unusual finding because *Rhodococcus* spp. have long been known to produce enzymes which are highly active towards a variety of hydrocarbons in both gas and liquid form (10, 15, 19). Although all the isolates were found to be *R. erythropolis*, the results from sections 4.9 and 4.10 indicated that there were at least three different strains. Because the morphologies and degradative capabilities of the strains in liquid culture varied so greatly, there appeared to be adaptations by the various strains that allowed some to have greater success than others when exposed to decalin + HMN in liquid culture.

Visually, the most obvious difference among the four isolates was the presence or lack of a glycocalyx layer. Glycocalyx layers (also known as capsule layers or slime layers) are known to play roles in cell attachment (32) and may have been necessary for successful function in the

multiphase system. Because loss of decalin and HMN only occurred with Iso 1a, Iso 2aCr and Iso 2aCl, all of which had visible slime layers, the slime layer may play an important role. Research has shown that a highly hydrophobic cell surface enhances the attachment of cells at the water/hydrocarbon interface and can increase the accessibility of the hydrocarbon phase to the cells (26). However, glycocalyx layers are hydrophilic and are often able to "bind a significant amount of water" (32) which would limit hydrocarbon uptake. As initially discussed in section 5.1, this inhibition may have been necessary to lower the rate of decalin transfer into the cells and allow the isolates to successfully degrade the decalin to non-toxic products.

To determine the cause of the differences between the isolates would be extremely difficult, due to the fact that plasmids may be playing a role. A plasmid is an "extrachromosomal genetic element that is not essential for growth and has no extracellular form" (31). They are known to play a significant role in conferring metabolic properties. Their role in changing the metabolic capabilities of *Pseudomonas* spp. towards hydrocarbons is well documented (36, 40). The issue with plasmids is that their presence cannot be detected by 16S rRNA sequencing. Meyer *et al.* (40) have shown that one cannot correlate metabolic properties of *Pseudomonas* bacteria with 16S phylogeny. *Rhodococcus* can also contain plasmids. If the *R. erythropolis* isolates discovered in section 4.9 contained different plasmids, then this would explain how three bacteria of the same species had different degradative capabilities and different physical appearance.

In section 2.5.1 three different mechanisms of uptake of hydrocarbons by bacteria were listed. Decalin has such a low solubility in water that one would not expect the primary means of access to be for cells to use decalin dissolved in the aqueous phase. Instead, the uptake probably occurred via direct contact with the hydrocarbon at the interface or by the bacteria producing surface active compounds which allow it to assimilate the substrate. Previous work has shown that a variety of rhodococci including *R. erythropolis* produce biosurfactants and bioflocculants (10). Plasmids could cause the production of different surface active compounds. They could also cause the production of different enzymes for attacking the decalin and HMN and could alter the

mechanisms the bacteria use for dealing with the toxicity of decalin. Thus plasmids could explain the possible differences in the ability of the isolates to handle the toxic effects of decalin, as discussed in section 5.1.

The strain Iso 2cO was sensitive to toxicity due to decalin and Iso 2aCr and 2aCl appear to be the same bacterium. Future studies should focus on Iso 1a and Iso2aCr. Because the metabolic capabilities of the isolates cannot be predicted using 16S RNA, experimental characterization of the isolates will need to be utilized to determine the enzymes and biochemical pathways used to attack cycloalkanes and isoprenoids and the limitations of these enzymes.

5.5. Suitability of Isolates for Biocatalysis

In section 2.2, a list of ten desired attributes for bacteria to be used as biocatalysts was given. The *R. erythropolis* isolates identified in this study had some of the attributes listed. The following are the positive characteristics of the isolates in terms of their use as a biocatalyst:

1. The bacteria could be grown to a high cell density in an inexpensive medium without a hydrocarbon. The medium used was TSB, and the process was conducted during the experimental procedure used for section 4.10.
2. The cells appeared to have a high level of activity against the model compound decalin, which suggests that they may also have activity against a broad spectrum of cycloalkanes.
3. Iso 1a, Iso 2aCl and Iso 2aCr appeared to be tolerant towards the toxic effects of decalin, when decalin was present in the vapour and liquid phase. However, the concentration of decalin could not be above an unknown toxic threshold in the liquid phase.

As the isolates were discovered at the latter stages of the research, several questions still remain unanswered. First is the issue of pathogenicity. There have been accounts that *R. erythropolis* may cause infections in immunosuppressed individuals, such as those with HIV (10). In addition

the kinetics of the decalin degradation have not been investigated, nor has the activity of the culture when the hydrocarbon is presented at a high volume ratio when compared to the initial cell suspension. Finally, the genetic issues have not even been considered, including the stability of the phenotype and genotype and the capability to manipulate the genetic system of the isolates.

Unfortunately, none of this may be necessary due to the fact that the bacteria seem to have non-selective enzymes, capable of attacking cycloalkanes, *n*-alkanes and highly branched alkanes. This is a major concern, as the main reason for considering the use of bacteria as biocatalysts in upgrading was the potential for selective attack on multi-ring cycloalkanes. If the *R. erythropolis* isolates identified produce several enzymes to attack hydrocarbons and if one of those enzymes specifically attacks cycloalkanes, then further research is warranted. Genetic manipulation could be conducted to halt the production of all the enzymes except the one involved in attacking cycloalkanes and a selective catalyst could still be developed. The more likely case however, is that the isolates produce a broad spectrum enzyme that attacks both cyclic and non-cyclic structures. If this is the case then the isolates cannot be used in oil sands upgrading. If they were exposed to the LGO out of the hydrotreater, they would consume the valuable fractions of the product, as well as the cycloalkanes, destroying the value of the product. Because of this, continued efforts with the isolates to turn them into biocatalysts for upgrading would be futile.

However, because the bacteria seem to be able to attack a broad spectrum of hydrocarbon structures, they would be ideal biocatalysts for bioremediation. There are many contaminated soil and water sites in the world that have cycloalkane contaminants. These are particularly prevalent at gasoline and diesel contaminated sites (59). Highly branched and cyclic hydrocarbons have long proven difficult to completely remove from contaminated sites due to the reasons outlined in sections 2.3 and 2.4. If the *R. erythropolis* isolates developed in this study can be used to remove such contaminants from the natural environment, they could prove to be a great boon to the

process of cleaning up industrial zones. Therefore, studies need to be conducted to determine the spectrum of compounds that the isolates can oxidize.

6.0 CONCLUSIONS

The goal of using bacteria as biocatalysts to open multi-ring cycloalkanes involves many research and development steps. These steps can be broken down into three main categories:

1. Identify a single species of bacterium capable of selectively attacking multi-ring cycloalkanes
2. Determine the biochemical process by which the degradation occurs and using genetic manipulation, halt the process at a desired point to produce opened structures
3. Develop a process to utilize the biocatalyst and optimize the conditions of the process

Due to the limited work conducted in this field, the focus of the research in this project was on step 1. Utilizing enrichment culture techniques with mixed cultures from hydrocarbon contaminated sites in Antarctica, two *R. erythropolis* isolates were identified. These isolates, named Iso 1a and Iso 2aCr used decalin as a carbon source when the decalin was presented as both a liquid and as a gas. While they are still susceptible to the toxic effects of decalin, when reasonable measures were taken to reduce the concentration of decalin, the isolates were able to maintain metabolic processes.

Unfortunately, the isolates had the ability to utilize cycloalkanes and *n*-alkanes as carbon sources. In addition, they caused the disappearance of the methylated hydrocarbon HMN. This occurrence had not been previously reported in the literature. The lack of selectivity towards cycloalkanes means that the isolates identified are more than likely not suitable for use as a biocatalyst in petroleum upgrading. However, the findings regarding decalin degradation and HMN removal offer valuable insights into our limited understanding of what the boundaries of biodegradation are, and offer exciting potential for highly robust bioremediation processes. Further investigations are required to determine the limits of these isolates in terms of their abilities to degrade cyclic and branched structures and what the mechanisms of these attacks are.

7.0 RECOMMENDATIONS FOR FUTURE WORK

With the discovery of several isolates capable of causing decalin and HMN loss, a variety of questions are raised. The following is a list of experiments that should be attempted to further qualify the degradative capabilities of the bacteria:

1. Conduct a study to determine what types of hydrocarbons the isolates can degrade. Use *n*-alkanes, branched alkanes, cycloalkanes and aromatics. For each class of hydrocarbon, studies should be done for a variety of chain lengths and ring sizes.
2. The effects of environmental conditions on bacteria activity should be investigated. As the bacteria come from Antarctica, the effect of low temperatures on activity is of particular interest.
3. An attempt should be made to identify metabolites of decalin degradation. To do so, bio-meter flasks should be used, where one flask will contain decalin and the other BH medium plus the inoculum of bacteria. Vaporized decalin will contact the liquid culture and hopefully be provided in sufficient amounts to allow growth without reaching toxic levels. In this way, decalin metabolites may be able to be identified using the resin bead technique outlined in section 3.2.7.
4. If ^{14}C-decalin can be manufactured, a study should be done to see if $^{14}CO_2$ can be identified as a product of mineralization of decalin.
5. The toxic threshold concentration of decalin in liquid cultures for each isolate should be identified if possible.
6. The mechanisms that the *R. erythropolis* isolates use to handle the toxicity of decalin should be investigated. Such a study should investigate if the glycocalyx layers play a significant role and if efflux pumps are used.

With these experiments completed a greater understanding of the biodegradation processes and limitations of the isolates should be reached.

8.0 REFERENCES

1. June 11, 2004, posting date. http://dictionary.reference.com/search?q=commensalism. [Online.]

2. June 9, 2004, posting date. http://home.flash.net/~defilip1/solubility.htm. [Online.]

3. June 18, 2004, posting date. http://www.energy.gov.ab.ca/com/Sands/Introduction/Oil_Sands.htm. [Online.]

4. August 25, 2004, posting date. http://www.irl.cri.nz/technology/bioprocessing.html. [Online.]

5. June 18, 2004, posting date. http://www.oilsandsdiscovery.com/oil_sands_story/resource.html. [Online.]

6. **Atlas, R. M. (ed.).** 1984. Petroleum Microbiology. Macmilland Publishing Company, New York.

7. **Ayala, M., and E. Torres.** 2004. Enzymatic activation of alkanes: constraints and prospective. Applied Catalysis A: General **272:**1-13.

8. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation and assimilation of alkyl-substituted cycloparaffins. Journal of Bacteriology **118:**394-399.

9. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. Journal of General Microbiology **82:**163-169.

10. **Bell, K. S., J. C. Philp, D. W. J. Aw, and N. Christofi.** 1998. The genus *Rhodococcus*. Journal of Applied Microbiology **85:**195-210.

11. **Bost, F. D., R. Frontera-Suau, T. J. McDonald, K. E. Peters, and P. J. Morris.** 2001. Aerobic biodegradation of hopanes and norhopanes in Venezuelan crude oils. Organic Geochemistry **32:**105-114.

12. **Bressler, D. C., and M. R. Gray.** 2003. Transport and reaction processes in bioremediation of organic contaminants. 1. Review of bacterial degradation and transport. International Journal of Chemical Reactor Engineering **1:**1-15.

13. **Corma, A., V. Gonzalez-Alfaro, and A. V. Orchilles.** 2001. Decalin and tetralin as probe molecules for cracking and hydrotreating the light cycle oil. Journal of Catalysis **200:**34-44.

14. **Daugulis, A. J., and C. M. McCracken.** 2003. Microbial degradation of high and low molecular weight polyaromatic hydrocarbons in a two-phase partitioning bioreactor by two strains of *Sphingomonas* sp. Biotechnology Letters **25:**1441-1444.

15. **de Carvalho, C. C. C. R., and M. M. R. da Fonseca.** 2005. Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. FEMS Microbiology Ecology **51:**389-399.

16. **Deziel, E., Y. Comeau, and R. Villemur.** 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation **10:**219-233.

17. **Dutta, T. K., and S. Harayama.** 2001. Biodegradation of n-alkylcycloalkanes and n-alkylbenzenes via new pathways in Alcanivorax sp strain MBIC 4326. Applied and Environmental Microbiology **67:**1970-1974.

18. **Efroymson, R. A., and M. Alexander.** 1991. Biodegradation of an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Applied and Environmental Microbiology **57:**1441-1447.

19. **Fairlee, J. R., B. L. Burback, and J. J. Perry.** 1997. Biodegradation of groundwater pollutants by a combined culture of *Mycobacterium vaccae* and a *Rhodococcus* sp. Canadian Journal of Microbiology **43:**841-846.

20. **Foght, J. M.** 2004. Whole-Cell bio-processing of aromatic compounds in crude oil and fuels. *In* R. Vazquez-Duhalt and R. Quintero-Ramirez (ed.), Petroleum Biotechnology. Developments and perspectives. Elsevier Science, New York.

21. **Foght, J. M., Q. Wu, P. M. Fedorak, M. A. Pickard, and M. R. Gray.** 1997. Paper 97-13. Process Joint Meeting Petroleum Society and BIOMINET.

22. **Frontera-Suau, R., F. D. Bost, T. J. McDonald, and P. J. Morris.** 2002. Aerobic biodegradation of hopanes and other biomarkers by crude oil-degrading enrichment cultures. Environmental Science & Technology **36:**4585-4592.

23. **Gibson, D. T. (ed.).** 1984. Microbial Degradation of Organic Compounds, 1 ed. Marcel Dekker Inc., New York.

24. **Gray, K.** 2004, posting date. http://umbbd.ahc.umn.edu/dbt/dbt_map.html. University of Minnesota. [Online.]

25. **Gray, M. R.** 2003. Course Notes - ChE 522 Hydrocarbon Properties and Processing, University of Alberta.

26. **Kim, S., J. M. Foght, and M. R. Gray.** 2002. Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S+14He. Biotechnology and Bioengineering **80:**650-659.

27. **Ko, S. H., and J. M. Lebeault.** 1999. Effect of a mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. Journal of Applied Microbiology **87:**72-79.

28. **Koma, D., F. Hasumi, S. Y. Chung, and M. Kubo.** 2003. Biodegradation of n-alkylcyclohexanes by co-oxidation via multiple pathways in *Acinetobacter* sp ODDK71. Journal of Bioscience and Bioengineering **95:**641-644.

29. **Koma, D., Y. Sakashita, K. Kubota, Y. Fujii, F. Hasumi, S. Y. Chung, and M. Kubo.** 2004. Degradation pathways of cyclic alkanes in *Rhodococcus* sp. NDKK48. Applied Microbiology and Biotechnology **66:**92-99.

30. **Linder, R.** 1997. *Rhodococcus equi* and *Arcanobacterium haemolyticum*: Two "Coryneform" bacteria increasingly recognized as agents of human infection. Emerging Infectious Diseases **3:**145-153.

31. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 2.2 Arrangement of DNA in Microbial Cells, p. 25, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

32. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 4.13 Bacterial Cell Surface Structures and Cell Inclusions, p. 91-92, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

33. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 5.5 Catalysis and Enzymes, p. 111-113, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

34. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 6.5 Direct Measurements of Microbial Growth: Total and Viable Cell Counts, p. 145-148, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

35. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 6.8 Effect of Temperature on Growth, p. 151-152, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

36. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 10.8 Plasmids, p. 288-289, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

37. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. 17.23 Hydrocarbon Oxidation, p. 597-599, Brock Biology of Microorganisms. Pearson Education Inc., Toronto.

38. **Madigan, M. T., J. M. Martinko, and J. Parker.** 2003. Chapter 17, Section IV: Hydrocarbon Oxidation and the Role of O_2 in the Catabolism of Organic Compounds, p. 597-601, Brock Biology of Microorganisms, 10 ed. Pearson Education Inc., Toronto.

39. **Marchal, R., S. Penet, F. Solano-Serena, and J. P. Vandecasteele.** 2003. Gasoline and diesel oil biodegradation. Oil & Gas Science and Technology-Revue De L'Institut Francais Du Petrole **58:**441-448.

40. **Meyer, A. F., D. A. Lipson, A. P. Martin, C. W. Schadt, and S. K. Schmidt.** 2004. Molecular and metabolic characterization of cold-tolerant alpine soil *Pseudomonas* sensu stricto. Applied and Environmental Microbiology **70:**483-489.

41. **Moldowan, J. M., J. Dahl, M. A. McCaffrey, W. J. Smith, and J. C. Fetzer.** 1995. Application of biological marker technology to bioremediation of refinery by-products. Energy & Fuels **9:**155-162.

42. **Moldowan, J. M., and M. A. McCaffrey.** 1995. A novel microbial hydrocarbon degradation pathway revealed by hopane demethylation in a petroleum reservoir. Geochimica Et Cosmochimica Acta **59:**1891-1894.

43. **Monticello, D. J.** 1998. Riding the fossil fuel biodesulfurization wave. CHEMTECH **28:**38-45.

44. **Munoz, R., B. Guieysse, and B. Mattiasson.** 2003. Phenanthrene biodegradation by an algal-bacterial consortium in two-phase partitioning bioreactors. Applied Microbiology and Biotechnology **61:**261-267.

45. **Ourisson, G., and P. Albrecht.** 1992. Hopanoids .1. Geohopanoids - the most abundant natural-products on earth. Accounts of Chemical Research **25:**398-402.

46. **Ourisson, G., P. Albrecht, and M. Rohmer.** 1984. The microbial origin of fossil-fuels. Scientific American **251:**44-51.

47. **Pelz, B., and H. J. Rehm.** 1971. Die Resistenz von Dekalin gegenuber mikrobiellem Abbau. Archiv für Mikrobiologie. **77:**288-290.

48. **Penet, S., R. Marchal, A. Sghir, and F. Monot.** 2004. Biodegradation of hydrocarbon cuts used for diesel oil formulation. Applied Microbiology and Biotechnology **66:**40-47.

49. **Perry, J. J.** 1979. Microbial cooxidations involving hydrocarbons. Microbiological Reviews **43:**59-72.

50. **Perry, J. J.** 1977. Microbial-metabolism of cyclic hydrocarbons and related compounds. CRC Critical Reviews in Microbiology **5:**387-412.

51. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-Gonzalez, A. Rojas, W. Teran, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Annual Review of Microbiology **56:**743 - 768.

52. **Ridgway, H. F., J. Safarik, D. Phipps, P. Carl, and D. Clark.** 1990. Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. Applied and Environmental Microbiology **56:**3565-3575.

53. **Rouviere, P. E., and M. W. Chen.** 2003. Isolation of *Brachymonas petroleovorans* CHX, a novel cyclohexane-degrading beta-proteobacterium. FEMS Microbiology Letters **227:**101-106.

54. **Ruaan, R. C., and D. J. Yu.** 2004. Bio-degradation of pristane in mixed alkanes by a mixed culture containing *Nocardia corynebacterioides*. Journal of the Chinese Institute of Chemical Engineers **35:**651-658.

55. **Schaeffer, T. L., S. G. Cantwell, J. L. Brown, D. S. Watt, and R. R. Fall.** 1979. Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. Applied and Environmental Microbiology **38:**742-746.

56. **Schumacher, J. D., and R. M. Fakoussa.** 1999. Degradation of alicyclic molecules by *Rhodococcus ruber* CD4. Applied Microbiology and Biotechnology **52:**85-90.

57. **Sikkema, J., J. A. M. Debont, and B. Poolman.** 1994. Interactions of cyclic hydrocarbons with biological-membranes. Journal of Biological Chemistry **269:**8022-8028.

58. **Sikkema, J., J. A. M. Debont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews **59:**201-222.

59. **Solano-Serena, F., R. Marchal, S. Casaregola, C. Vasnier, J. M. Lebeault, and J. P. Vandecasteele.** 2000. A *Mycobacterium* strain with extended capacities for degradation of gasoline hydrocarbons. Applied and Environmental Microbiology **66:**2392-2399.

60. **Solano-Serena, F., R. Marchal, S. Heiss, and J. P. Vandecasteele.** 2004. Degradation of isooctane by *Mycobacterium austroafricanum* IFP 2173: growth and catabolic pathway. Journal of Applied Microbiology **97:**629-639.

61. **Solano-Serena, F., R. Marchal, T. Huet, J. M. Lebeault, and J. P. Vandecasteele.** 2000. Biodegradability of volatile hydrocarbons of gasoline. Applied Microbiology and Biotechnology **54:**121-125.

62. **Solano-Serena, F., R. Marchal, J. M. Lebeault, and J. P. Vandecasteele.** 2000. Distribution in the environment of degradative capacities for gasoline attenuation. Biodegradation **11:**29-35.

63. **Solano-Serena, F., R. Marchal, J. M. Lebeault, and J. P. Vandecasteele.** 2000. Selection of microbial populations degrading recalcitrant hydrocarbons of gasoline by monitoring of culture-headspace composition. Letters in Applied Microbiology **30:**19-22.

64. **Solano-Serena, F., R. Marchal, M. Ropars, J. M. Lebeault, and J. P. Vandecasteele.** 1999. Biodegradation of gasoline: kinetics, mass balance and fate of individual hydrocarbons. Journal of Applied Microbiology **86:**1008-1016.

65. **Soli, G., and E. M. Bens.** 1973. Selective substrate utilization by marine hydrocaronoclastic bacteria. Biotechnology and Bioengineering **XV:**285-297.

66. **Strawinski, R. J., and R. W. Stone.** 1940. The utilization of hydrocarbons by bacteria. Journal of Bacteriology. **40:**461.

67. **Trudgill, P. W.** 1990. Cyclohexanone 1,2-monooxygenase from *Acinetobacter* NCIMB-9871. Methods in Enzymology **188:**70-77.

68. **Wodzinski, R. S., and D. Larocca.** 1977. Bacterial growth kinetics on diphenylmethane and naphthalene-heptamethylnonane mixtures. Applied and Environmental Microbiology **33:**660-665.

9.0 APPENDIX A: Figures for GC Analysis

9.1. Chromatograms for Chemicals Used as Carbon Sources and Internal Standards

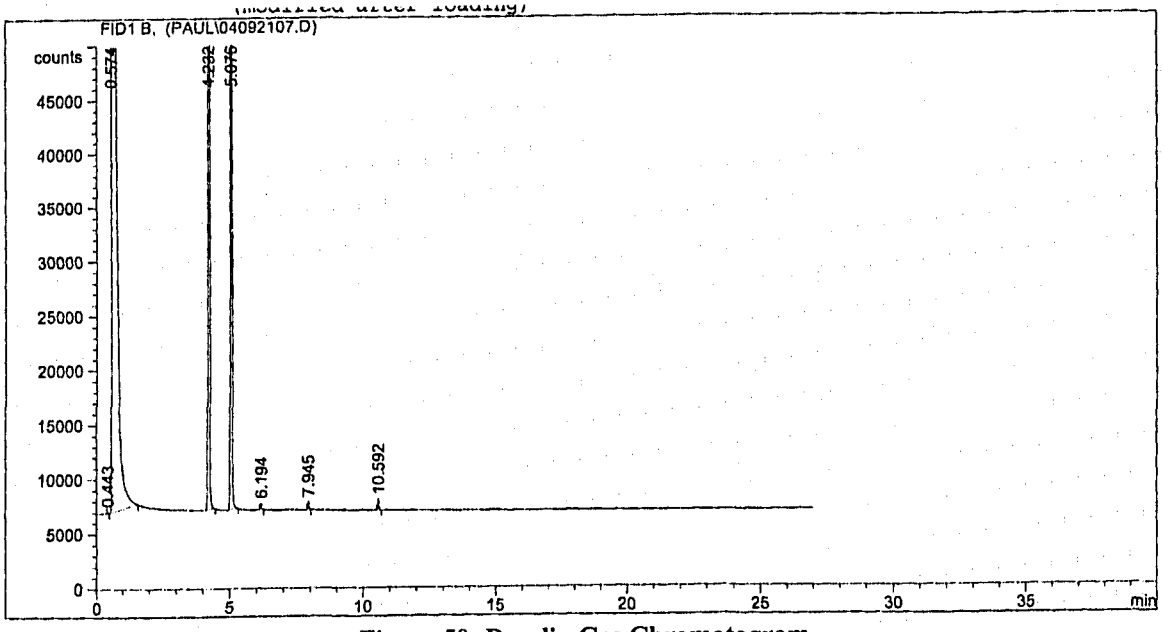

Figure 50: Decalin Gas Chromatogram

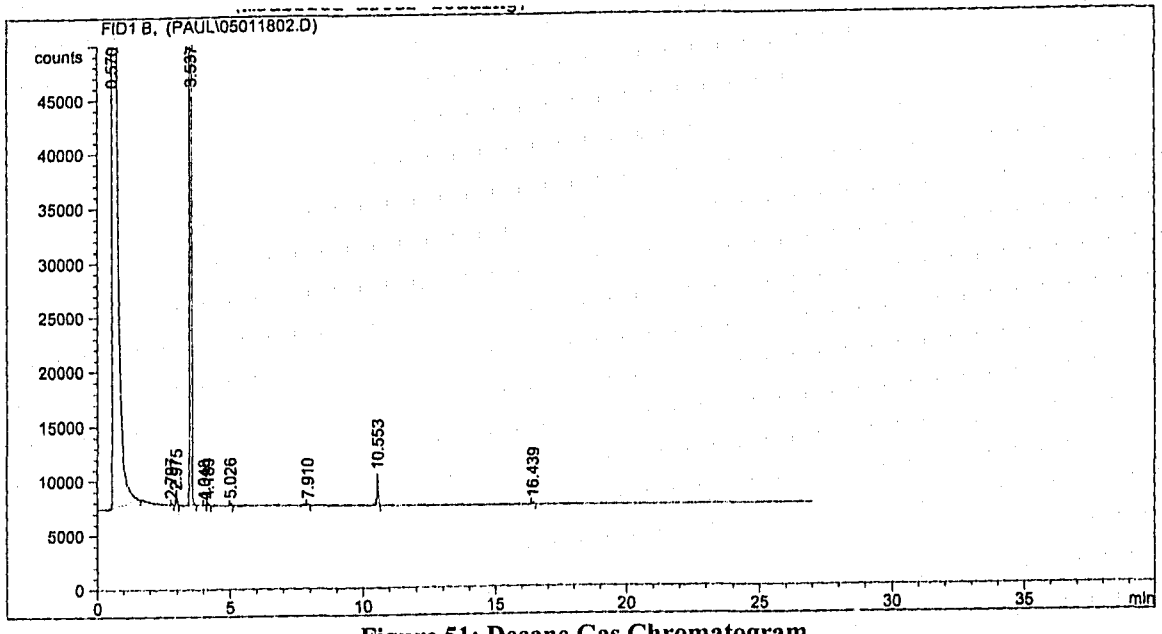

Figure 51: Decane Gas Chromatogram

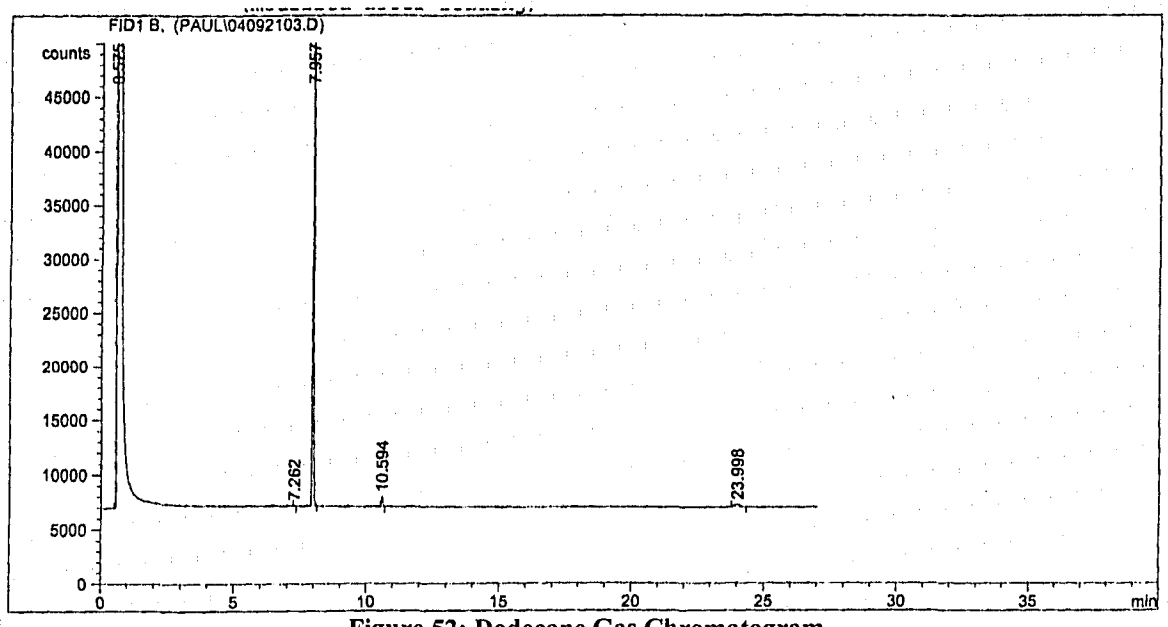

Figure 52: Dodecane Gas Chromatogram

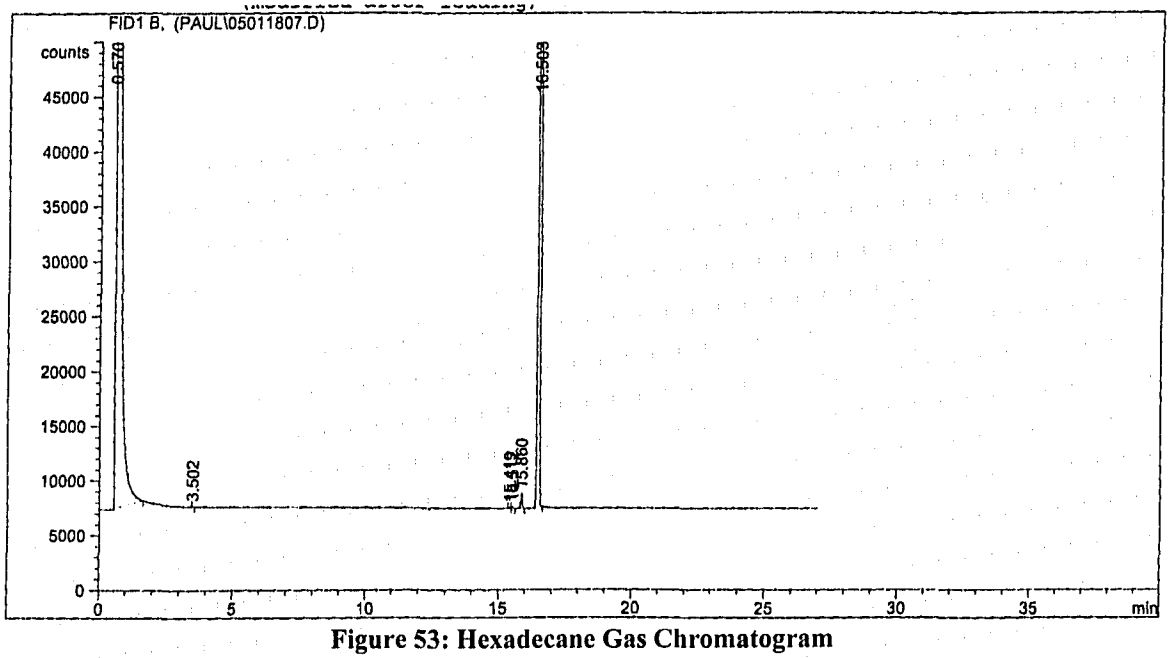

Figure 53: Hexadecane Gas Chromatogram

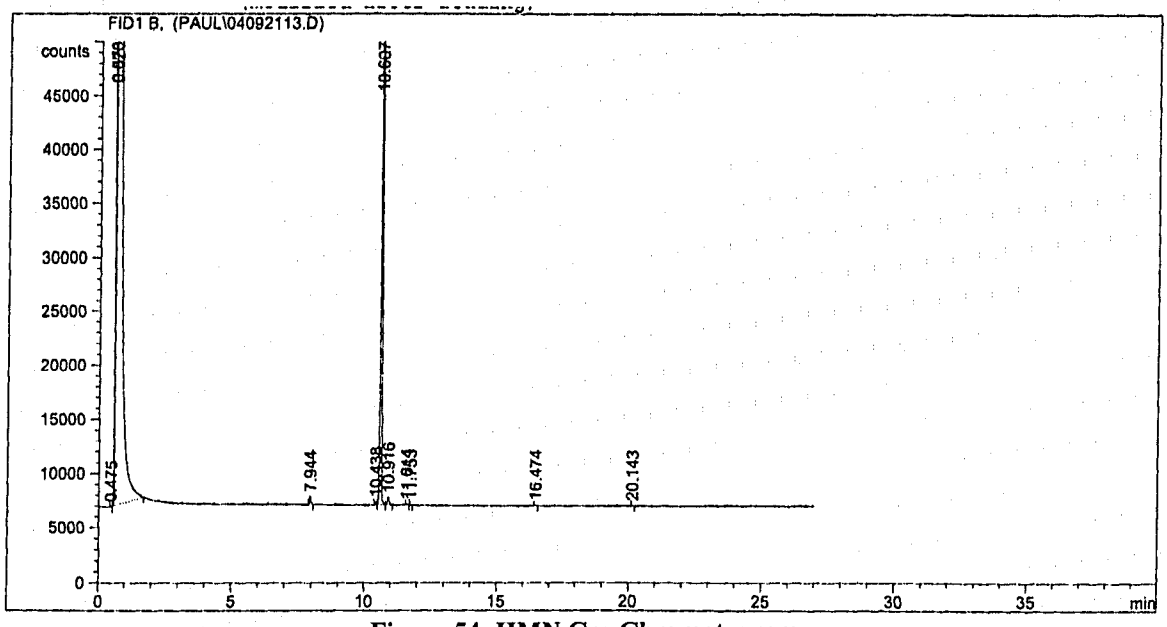

Figure 54: HMN Gas Chromatogram

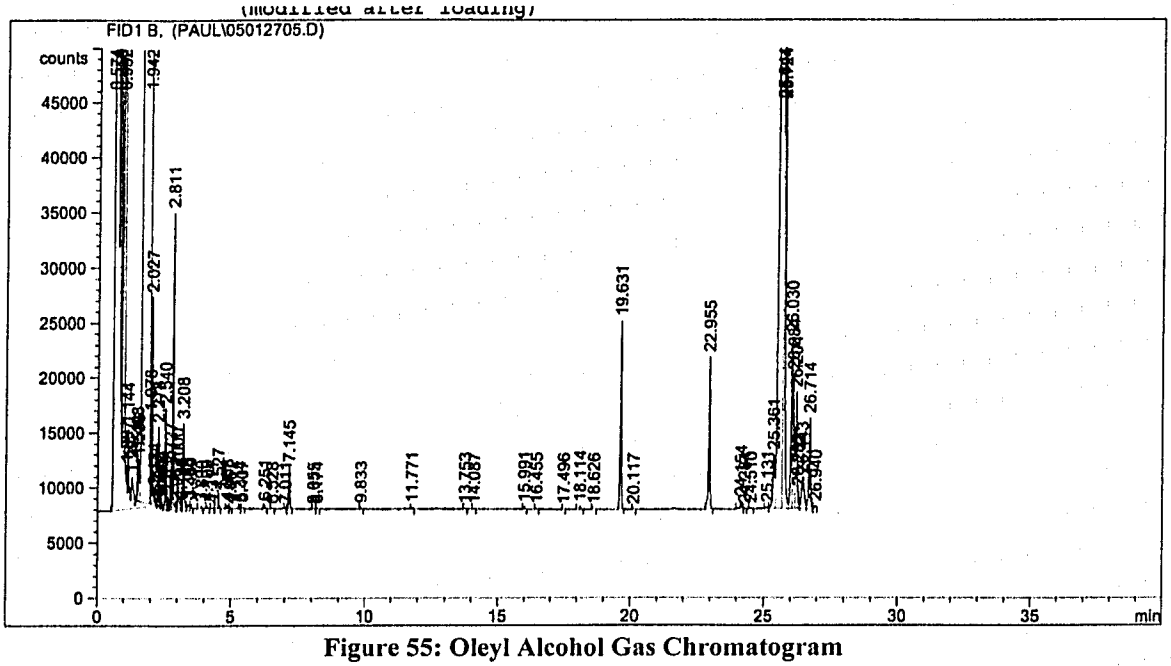

Figure 55: Oleyl Alcohol Gas Chromatogram

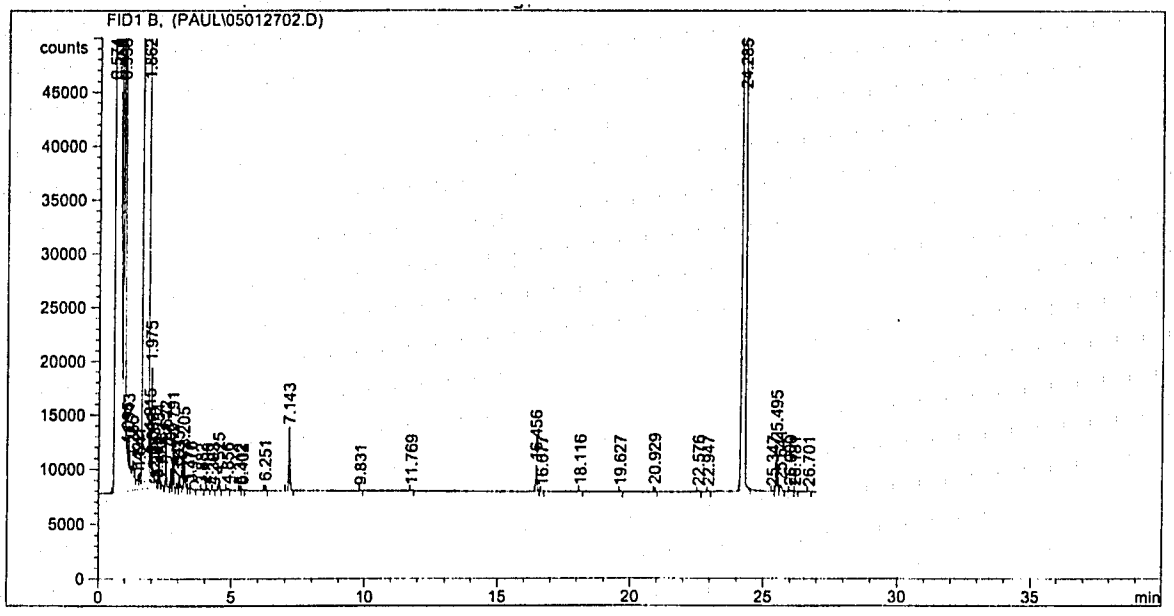

Figure 56: Palmitic Acid Gas Chromatogram

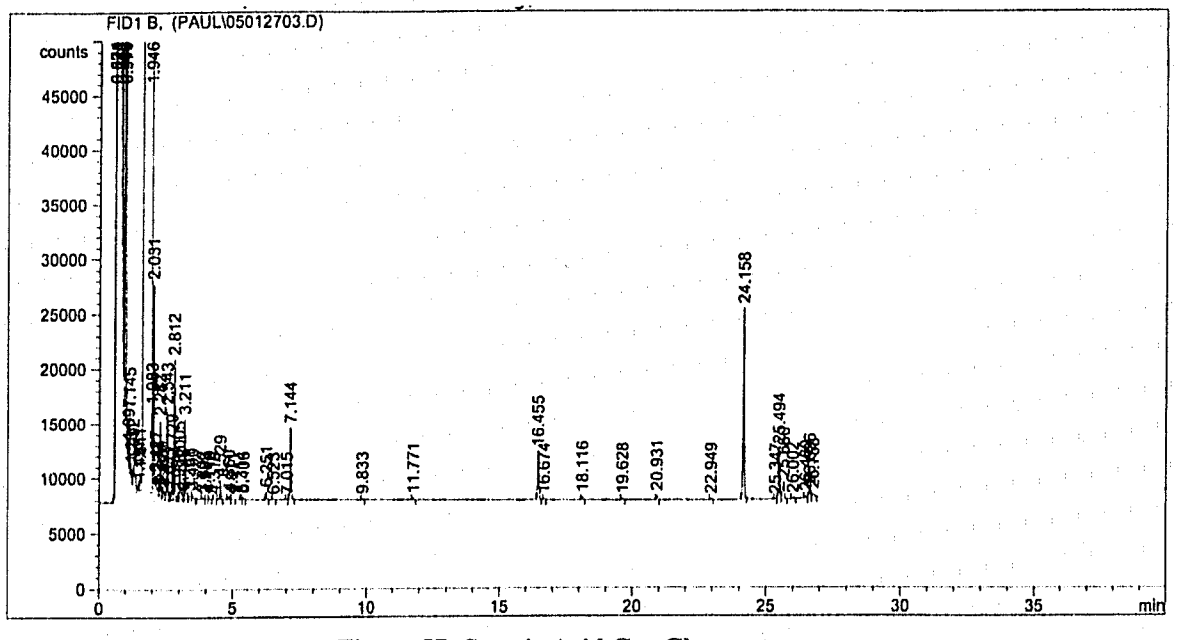

Figure 57: Stearic Acid Gas Chromatogram

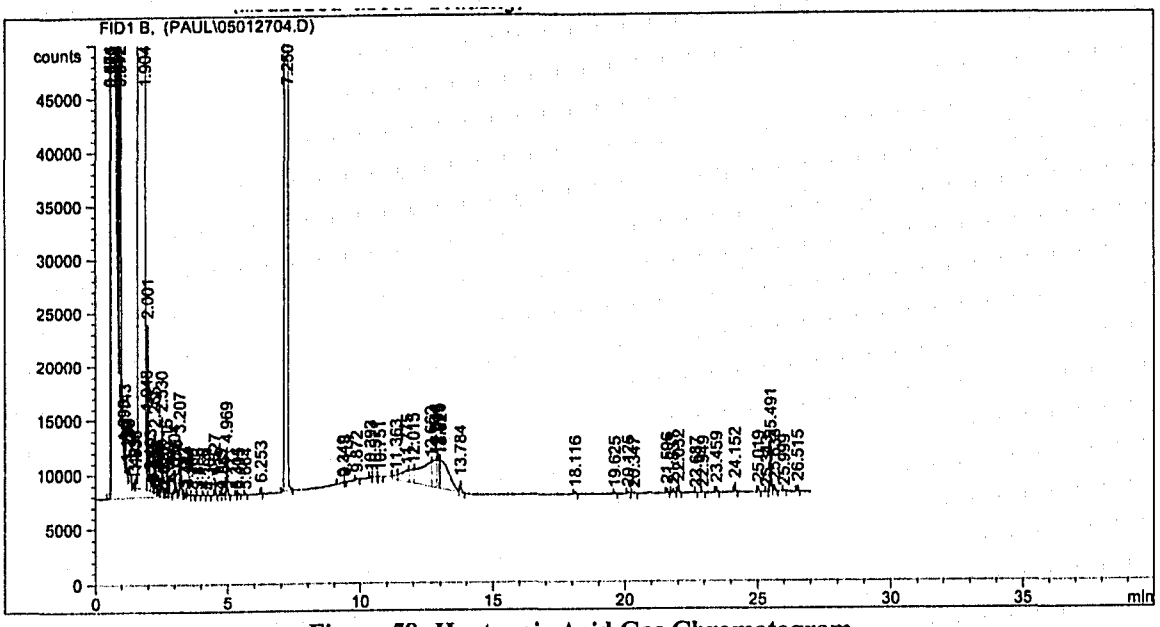

Figure 58: Heptanoic Acid Gas Chromatogram

9.2. Standard Curves for Chemicals Used as Carbon Sources and Internal Standards

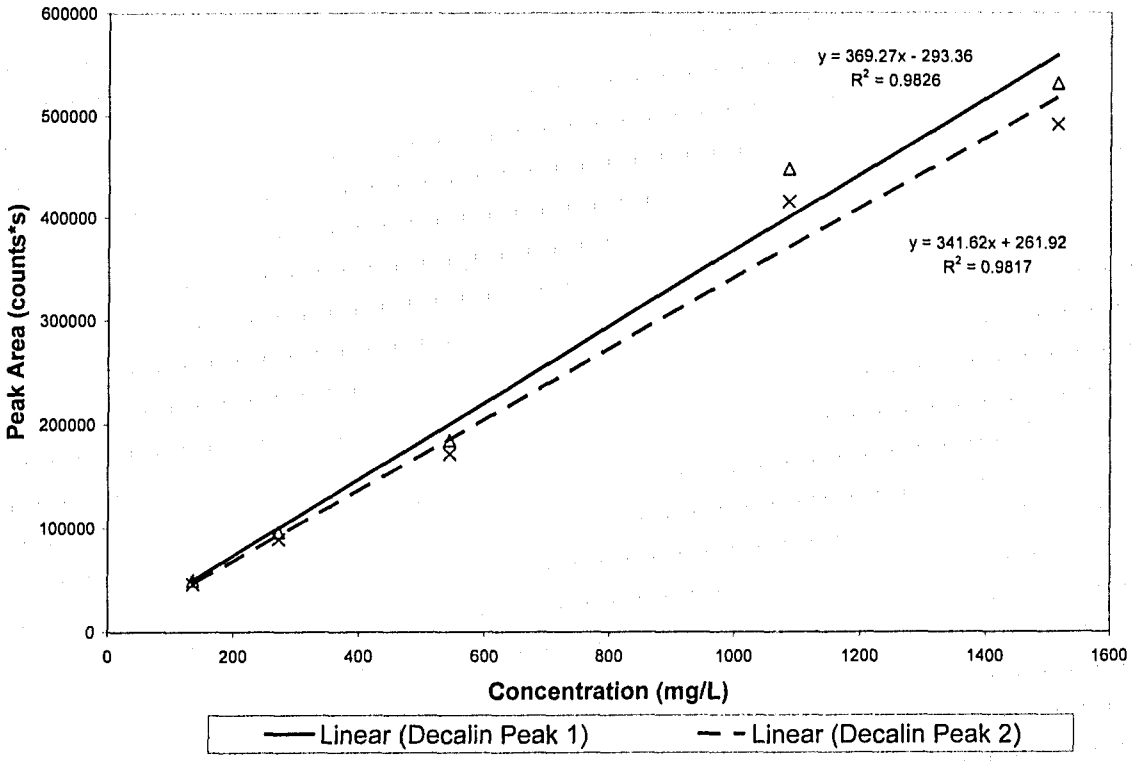

Figure 59: Decalin Standard Curve

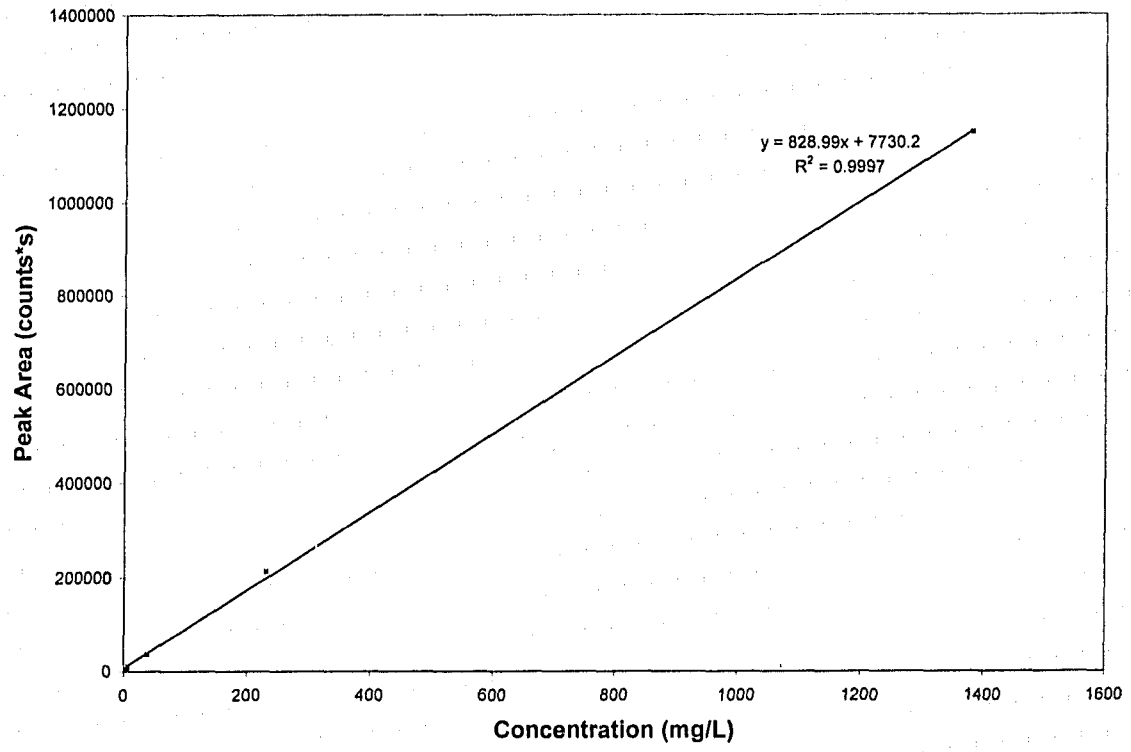

Figure 60: HMN Standard Curve

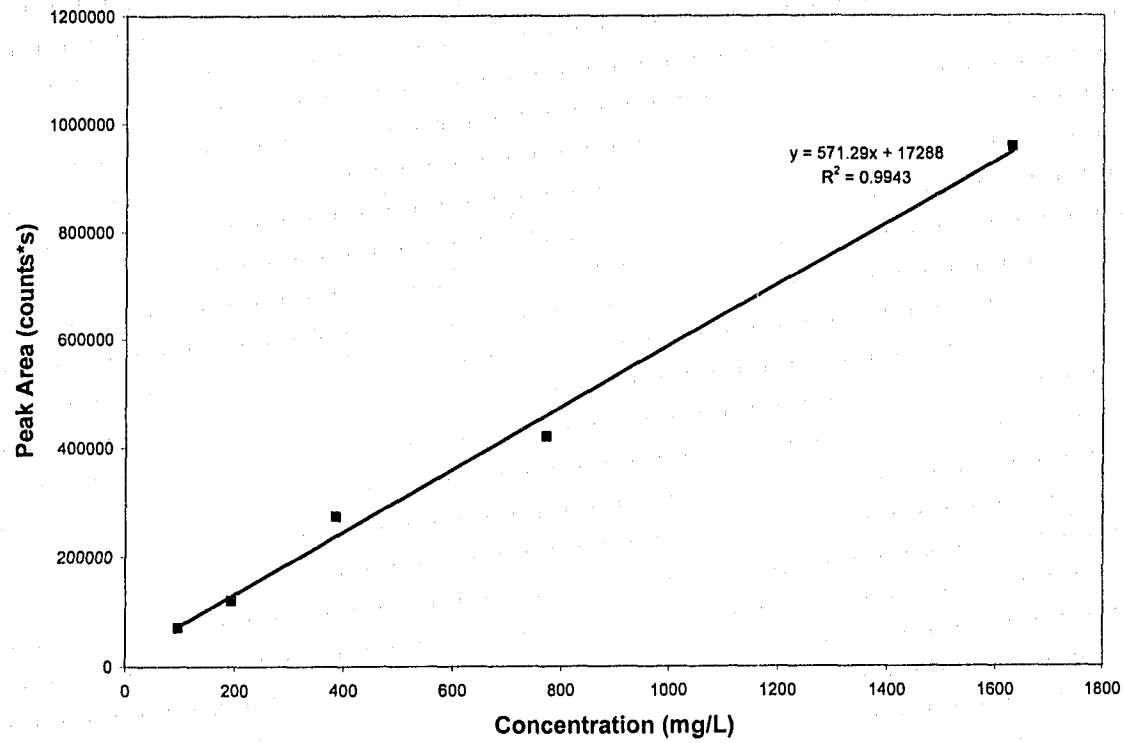

Figure 61: Decane Standard Curve

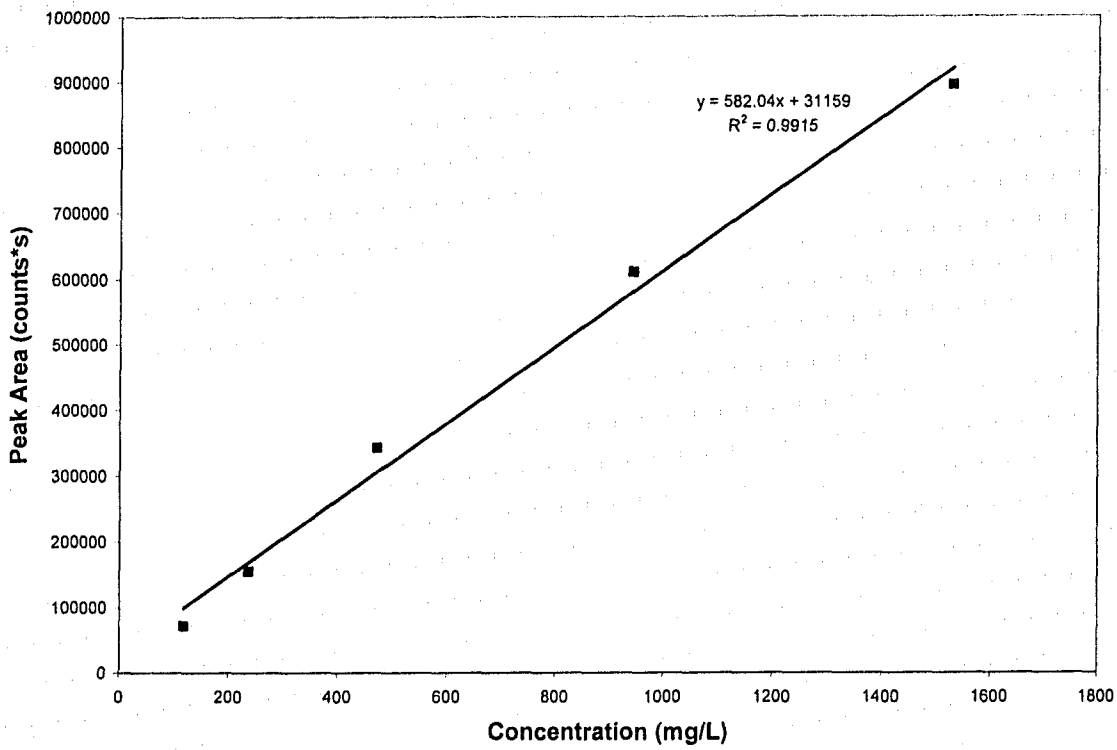

Figure 62: Dodecane Standard Curve

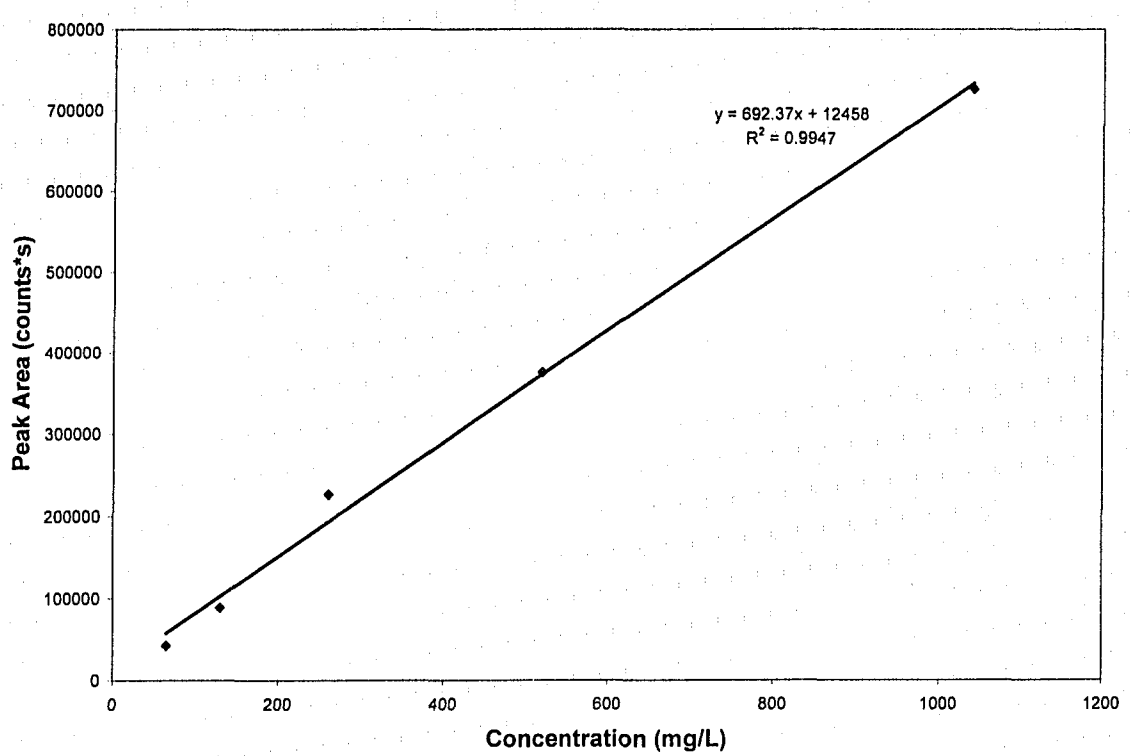

Figure 63: Hexadecane Standard Curve

10.0 Appendix B: Sample Calculations

10.1. Calculation for the Mass of Resin Beads Required per Flask

Calculation for the mass of beads required so that there is sufficient capacity for the anions from the medium plus anions produced by bacteria metabolic processes.

Step 1: Calculate the anion contribution from the medium

$MgSO_4 \cdot 7H_2O$

$MgSO_4 \rightarrow Mg^{2+} + SO_4^{2-}$ (2meq/mmol)

$$\frac{0.2g}{1L} \times \frac{1mol}{120.39g} \times \frac{1000mmol}{1mol} \times \frac{2meq}{mmol} \times 0.22L = 0.73 \frac{meq}{flask}$$

$CaCl_2$ (Anhydrous)

$CaCl_2 \rightarrow Ca^{2+} + 2Cl^-$ (2meq/mmol)

$$\frac{0.02g}{1L} \times \frac{1mol}{110.983g} \times \frac{1000mmol}{1mol} \times \frac{2meq}{mmol} \times 0.22L = 0.0793 \frac{meq}{flask}$$

KH_2PO_4

$KH_2PO_4 \rightarrow K^+ + H_2PO_4^-$ (1meq/mmol)

$$\frac{1.0g}{1L} \times \frac{1mol}{136.09g} \times \frac{1000mmol}{1mol} \times \frac{1meq}{mmol} \times 0.22L = 1.616 \frac{meq}{flask}$$

K_2HPO_4

$K_2HPO_4 \rightarrow 2K^+ + HPO_4^{2-}$ (2meq/mmol)

$$\frac{1.0g}{1L} \times \frac{1mol}{174.18g} \times \frac{1000mmol}{1mol} \times \frac{2meq}{mmol} \times 0.22L = 2.526 \frac{meq}{flask}$$

NH_3NO_3

$NH_3NO_3 \rightarrow NH_3^+ + NO_3^-$ (1meq/mmol)

$$\frac{1.0g}{1L} \times \frac{1mol}{79.05g} \times \frac{1000mmol}{1mol} \times \frac{1meq}{mmol} \times 0.22L = 2.78 \frac{meq}{flask}$$

$FeCl_3$

$FeCl_3 \rightarrow Fe^{3+} + 3Cl^-$ (3meq/mmol)

$$\frac{0.05g}{1L} \times \frac{1mol}{162.2g} \times \frac{1000mmol}{1mol} \times \frac{1meq}{mmol} \times 0.22L = 0.203 \frac{meq}{flask}$$

Total anions from medium = 0.73+0.0793+1.616+2.526+2.78+0.203 = 7.934 meq/flask

The number of anions from the culture cannot be determined, because it is not known what anions will be produced, or in what quantities.

Assume: Total anions from culture = Total anions from medium = 7.934 meq/flask

Capacity of Resin = 4.4meq/g

Therefore, mass of resin can be calculated as:

$$\frac{2 \times 7.934 meq / flask}{4.4 meq / g} = 3.6 \frac{g}{flask} \approx 4.0 \frac{g}{flask}$$

Therefore, 4g of resin will be added to each flask.

11.0 Appendix C: Photographs of Bacterial Colonies

11.1. Photographs of Vapour Plate Results for Section 4.8

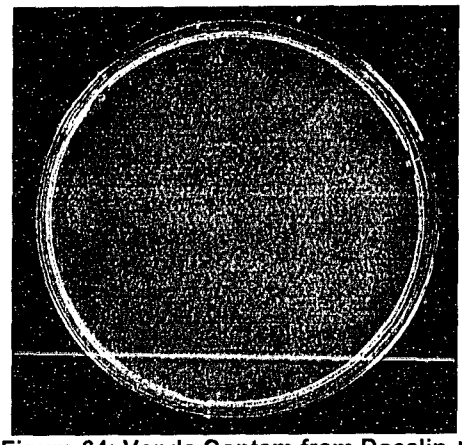

Figure 64: Vanda Contam from Decalin + Decane Flask (10^{-4}) in Decane Vapours

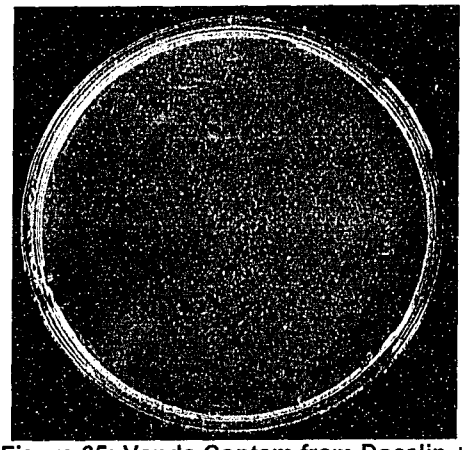

Figure 65: Vanda Contam from Decalin + Decane Flask (10^{-4}) In HMN + Decalin + Decane Vapours

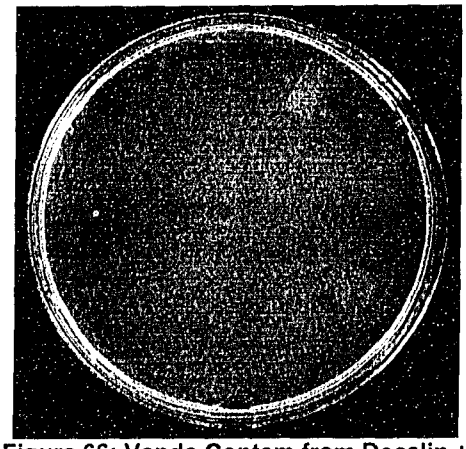

Figure 66: Vanda Contam from Decalin + Decane Flask (10^{-4}) In No Carbon Source

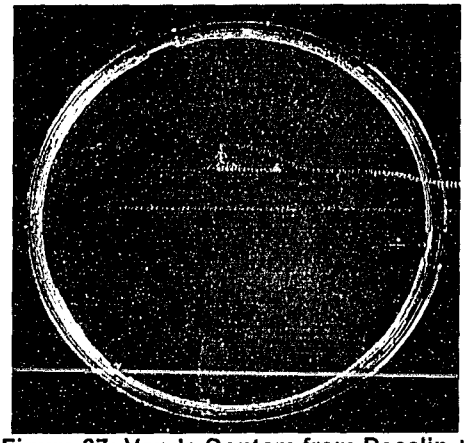

Figure 67: Vanda Contam from Decalin + Decane Flask (10^{-4}) in Decalin + Decane Vapours

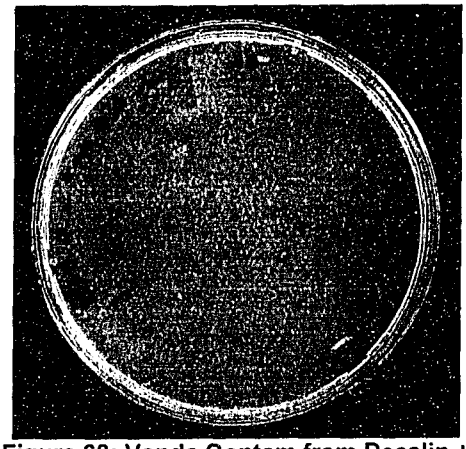

Figure 68: Vanda Contam from Decalin + Decane Flask (10^{-4}) In HMN + Decalin Vapours

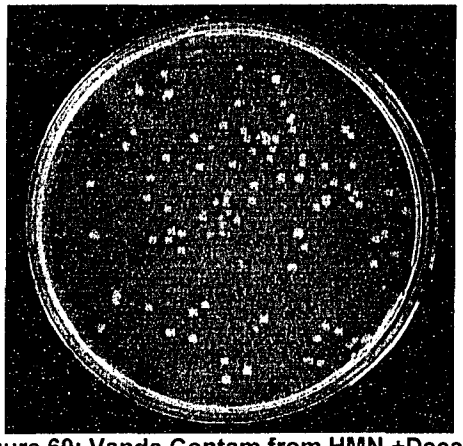

Figure 69: Vanda Contam from HMN +Decalin + Decane Flask (10^{-6}) in Decane Vapours

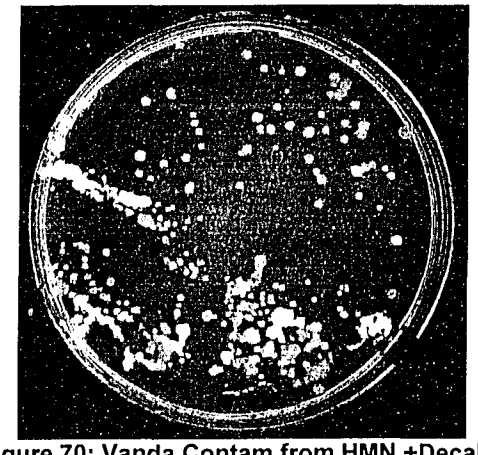

Figure 70: Vanda Contam from HMN +Decalin + Decane Flask (10^{-6}) in HMN + Decalin + Decane Vapours

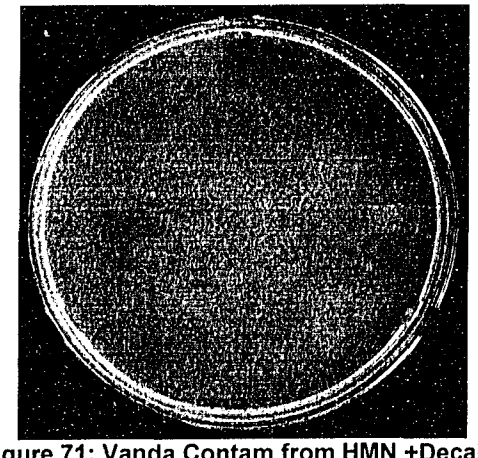

Figure 71: Vanda Contam from HMN +Decalin + Decane Flask (10^{-6}) *in No Carbon Source*

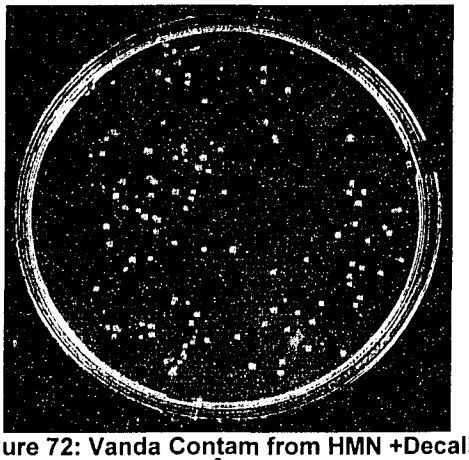

Figure 72: Vanda Contam from HMN +Decalin + Decane Flask (10^{-6}) in Decalin + Decane Vapours

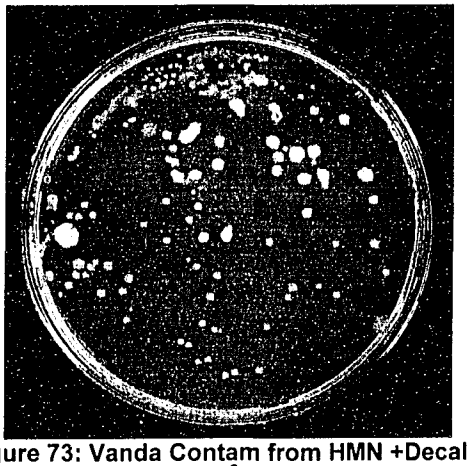

Figure 73: Vanda Contam from HMN +Decalin + Decane Flask (10^{-6}) in HMN + Decalin Vapours

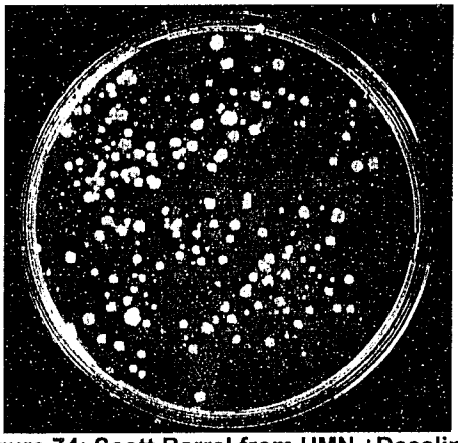

Figure 74: Scott Barrel from HMN +Decalin + Decane Flask (10^{-4}) in Decane Vapours

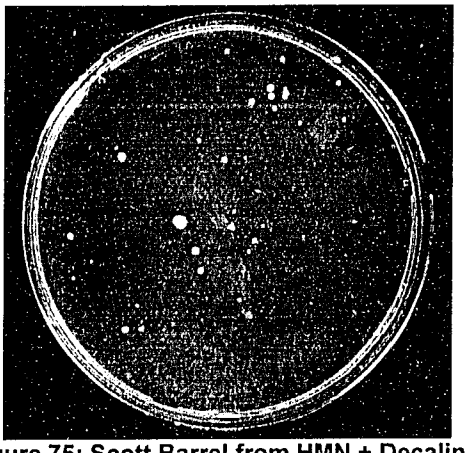

Figure 75: Scott Barrel from HMN + Decalin + Decane Flask (10^{-5}) In HMN + Decalin + Decane Vapours

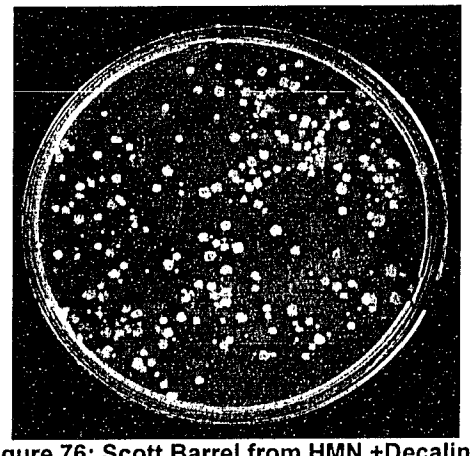

Figure 76: Scott Barrel from HMN +Decalin + Decane Flask (10^{-4}) in Decalin + Decane Vapours

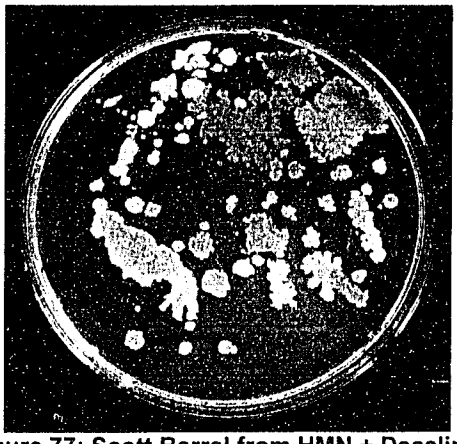

Figure 77: Scott Barrel from HMN + Decalin + Decane Flask (10^{-5}) In HMN + Decalin Vapours

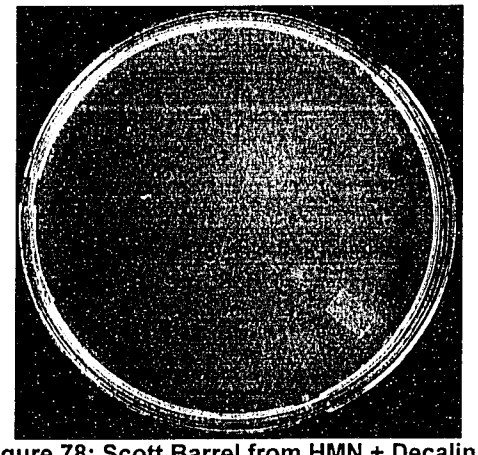

Figure 78: Scott Barrel from HMN + Decalin + Decane Flask (10^{-5}) In No Carbon Source

11.2. Photographs of Isolates in Various Carbon Sources for Section 4.9

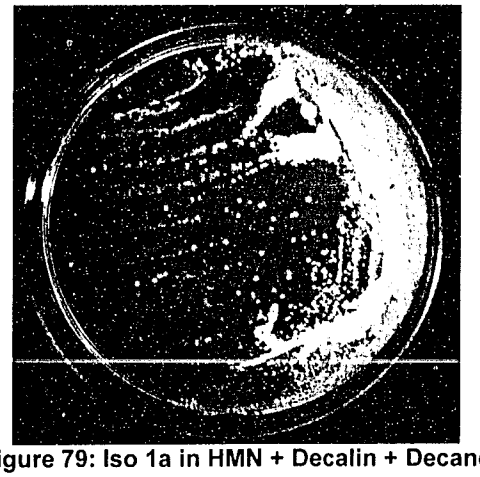

Figure 79: Iso 1a in HMN + Decalin + Decane

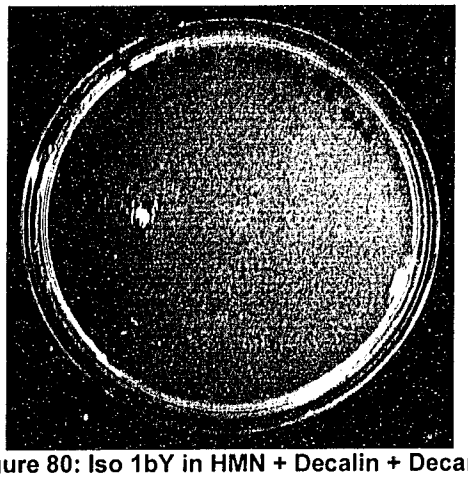

Figure 80: Iso 1bY in HMN + Decalin + Decane

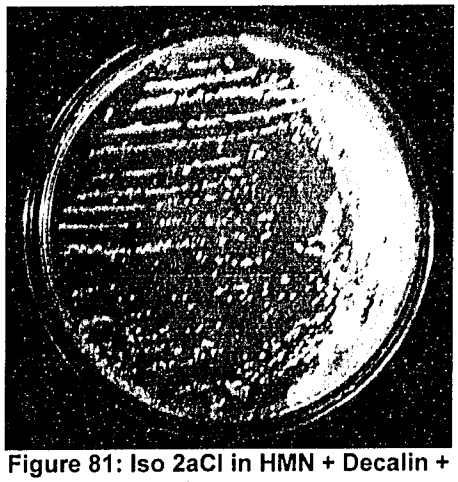

Figure 81: Iso 2aCl in HMN + Decalin + Decane

Figure 82: Iso 2aCr in HMN + Decalin + Decane

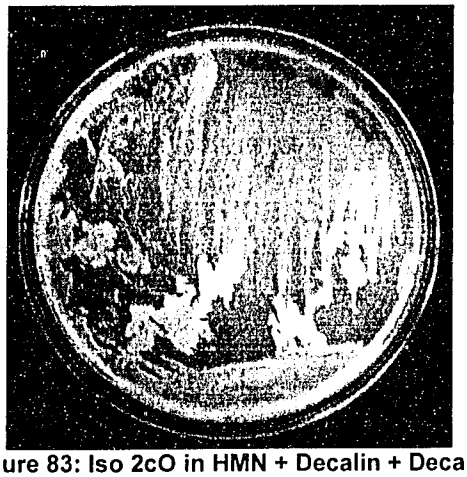

Figure 83: Iso 2cO in HMN + Decalin + Decane

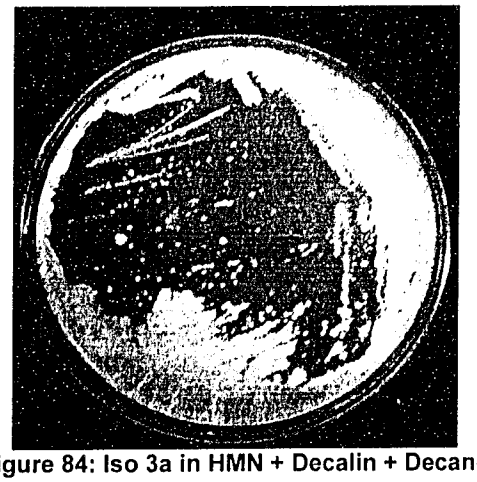

Figure 84: Iso 3a in HMN + Decalin + Decane

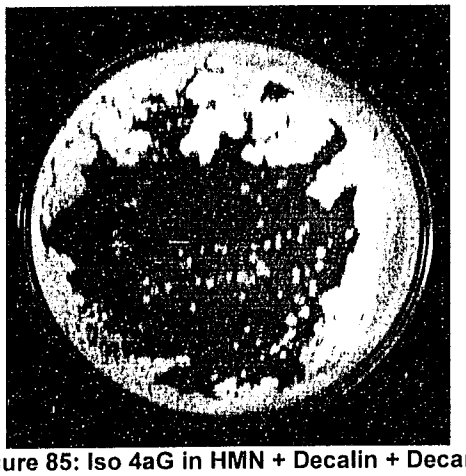

Figure 85: Iso 4aG in HMN + Decalin + Decane

Figure 86: Iso 1a in Decalin + Decane

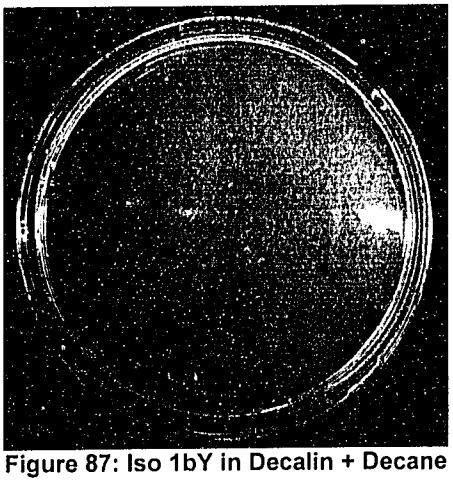

Figure 87: Iso 1bY in Decalin + Decane

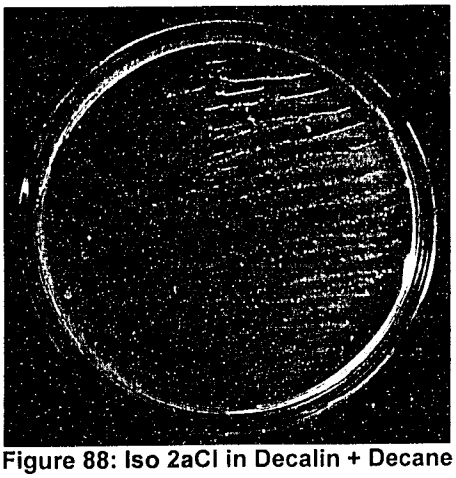

Figure 88: Iso 2aCl in Decalin + Decane

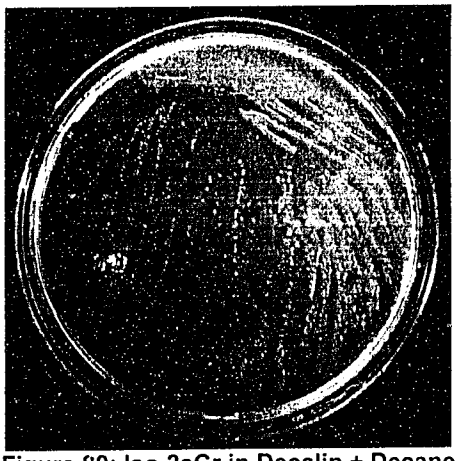

Figure 89: Iso 2aCr in Decalin + Decane

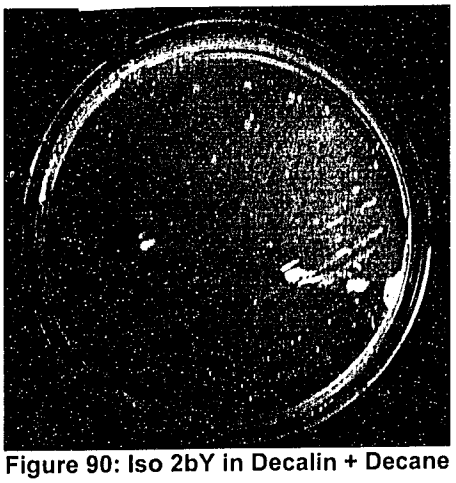

Figure 90: Iso 2bY in Decalin + Decane

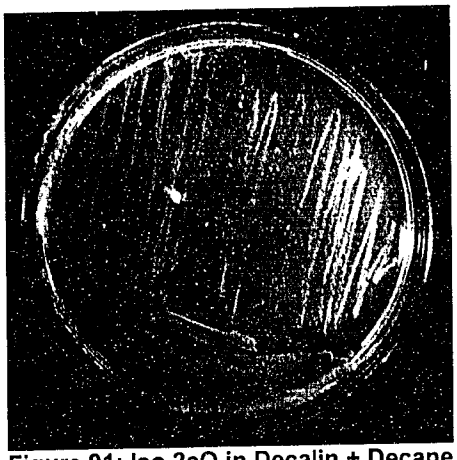

Figure 91: Iso 2cO in Decalin + Decane

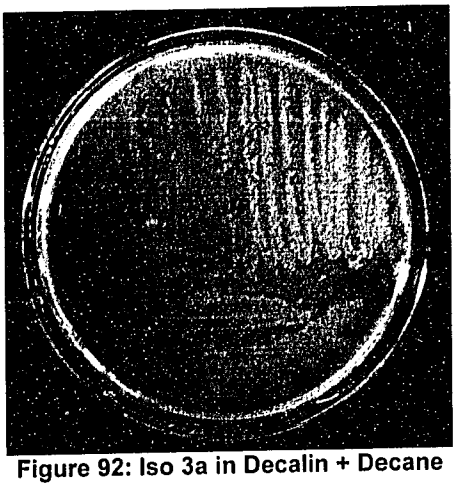

Figure 92: Iso 3a in Decalin + Decane

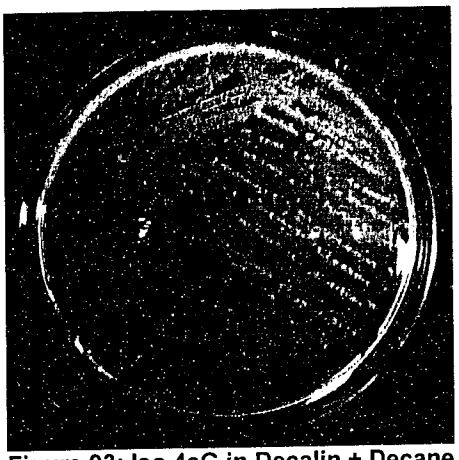

Figure 93: Iso 4aG in Decalin + Decane

12.0 Appendix D: Genetic Sequencing and Matching

12.1. Genetic Sequence Alignment

In the below information, Iso 1a = 1a_PB36F; Iso 2aCl = 2a_clear_PB36F; Iso 2cO = 2c_PB36F; Iso 2aCr = 2a_cream_PB36F

```
                1                                                        50
      1a_PB36F  .......... .......... .......... .......... ..........
      AY281123  .......... .......... .......... .......... ..........
      AY281124  .......... .......... .......... .......... ..........
      AY281125  .......... .......... .......... .......... ..........
      AY281121  .......... .......... .......... .......... ..........
      AF181691  .......... .......... .......... .......... ..........
2a_clear_PB36F  .......... .......... .......... .......... ..........
      AY642534  .......... .......... .......... .......... .....TGCTT
      2c_PB36F  .......... .......... .......... .......... ..........
2a_cream_PB36F  .......... .......... .......... .......... ..........
      AY822047  TGGAGAGTTT GATCCTGGCT CAGGACGAAC GCTGGCGGCG TGCTTAACAC

               51                                                       100
      1a_PB36F  .......... .......... .......... .......... .........T
      AY281123  .......... .....AAGGC CTTTCGGGGT ACCCAAACGG CCGAACGGGT
      AY281124  .......... ......AAGC CCTTTCGGGG TACACGAGCG GCGAACGGGT
      AY281125  .......... ......AAGC CCTTTCGGGG TACACGAGCN GCGAACGGGT
      AY281121  .......... ......AAGC CCTTTCGGGG TACCCGAGCG GCGAACGGGT
      AF181691  .......... .......AGG CCTTTCGGGG TACACGAGCG GCGAACGGGT
2a_clear_PB36F  .......... .......... .......... .......... ..........
      AY642534  AACACAGTCG AGCGGTAAGG CCTTTCGGGG TACACGAGCG GCGAACGGGT
      2c_PB36F  .......... .......... .......... .......... ...AACGGGT
2a_cream_PB36F  .......... .......... .......... .......... ..........
      AY822047  ATGCAAGTCG AGCGGTAAGG CCTTWCGGGG TACACGAGCG GCGAACGGGT

               101                                                      150
      1a_PB36F  GAGTAACACG TGGGTGATCT GCCCTGCNCT TCGGGATAAG CCTGGGAAAC
      AY281123  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      AY281124  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      AY281125  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      AY281121  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      AF181691  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
2a_clear_PB36F  .......... TGGGTGATCT GCCCTGCACT TCNGGATAAG CCTGGGAAAC
      AY642534  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      2c_PB36F  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
2a_cream_PB36F  ....AACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC
      AY822047  GAGTAACACG TGGGTGATCT GCCCTGCACT TCGGGATAAG CCTGGGAAAC

               151                                                      200
      1a_PB36F  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCNTGGTGG GTGGTGGAAA
      AY281123  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
      AY281124  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
      AY281125  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
      AY281121  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
      AF181691  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
2a_clear_PB36F  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCNTGGTGG GTGGTGGAAA
      AY642534  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
      2c_PB36F  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA
2a_cream_PB36F  TGGGTCTAAT ACCGGATATG ACCTCCTNTN CGCCTGGTGG GNGGTGGAAA
      AY822047  TGGGTCTAAT ACCGGATATG ACCTCCTATC .GCATGGTGG GTGGTGGAAA

               201                                                      250
      1a_PB36F  GATTTATCGG TGCAGGATGG GCCCNCGGCC TATCANCTTG TTGGTGGGGT
      AY281123  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      AY281124  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      AY281125  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      AY281121  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      AF181691  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
2a_clear_PB36F  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      AY642534  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
      2c_PB36F  GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT
2a_cream_PB36F  GATTTATCGG TGCCCGATGG GCCCGCGGC. .......... ..........
```

```
AY822047   GATTTATCGG TGCAGGATGG GCCCGCGGCC TATCAGCTTG TTGGTGGGGT

           251                                                300
1a_PB36F   AATGGCCTAC CAAGGCNACG ACGGGTAGCC N.ACCTGAGA GGGTGACCGG
AY281123   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
AY281124   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
AY281125   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
AY281121   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
AF181691   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
2a_clear_PB36F AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG
AY642534   AATGGCCTAC CAAGGCGACG ACGGGTAGCC GGACCTGAGA GGGTGACCGG
2c_PB36F   AATGGCCTAC CAAGGCGACG ACGGGTAGCC GCACCTGAGA GGGTGACCGG
2a_cream_PB36F ..........  ..........  ..........  ..........  ..........
AY822047   AATGGCCTAC CAAGGCGACG ACGGGTAGCC G.ACCTGAGA GGGTGACCGG

           301                                                350
1a_PB36F   CCACACTGGG ACTGAGACAC GGCCCATACT CCTACGGGAG GCAGCAGTGG
AY281123   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
AY281124   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
AY281125   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
AY281121   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
AF181691   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
2a_clear_PB36F CCACACTGGG ACTGAAACAC GGCCCANACT CCTACGGGAG GCAGCAGTGG
AY642534   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
2c_PB36F   CCACACTGGG ACTGAGACAC GGCCCAAACT CCTACGGGAG GCAGCAGTGG
2a_cream_PB36F ..........  ..........  ..........  ..........  ..........
AY822047   CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG

           351                                                400
1a_PB36F   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
AY281123   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
AY281124   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
AY281125   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
AY281121   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
AF181691   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
2a_clear_PB36F GGAATATTGC ACAATGGGCG AAAGCCTG.. ..........  ..........
AY642534   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG
2c_PB36F   GGAATATTGC ACAATGGGCG AAAGCCTGAT GC........  ..........
2a_cream_PB36F ..........  ..........  ..........  ..........  ..........
AY822047   GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCGACGC CGCGTGAGGG

           401                                                450
1a_PB36F   ATGACNGCCT TCNGGTTGTA AACCTCTTTC AGCAGGGACN AAGCNCAAGT
AY281123   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGCAAGT
AY281124   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGCAAGT
AY281125   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGCAAGT
AY281121   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGCAAGT
AF181691   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGCAAGT
2a_clear_PB36F ..........  ..........  ..........  ..........  ..........
AY642534   ATGACGGCCT TCGGGTTGTA AACCTCTTTC AGCAGGGACG AAGCGC....
2c_PB36F   ..........  ..........  ..........  ..........  ..........
2a_cream_PB36F ..........  ..........  ..........  ..........  ..........
AY822047   AT........  ..........  ..........  ..........  ..........

           451          469
1a_PB36F   GACGGTACCT GCA.....
AY281123   GACGGTACCT GCAGAAG..
AY281124   GACGGTACCT GCAGAAGA.
AY281125   GACGGTACCT GCAGAAGA.
AY281121   GACGGTACCT GCAGAAGA.
AF181691   GACGGTACCT GCAGAAGAA
2a_clear_PB36F ..........  .........
AY642534   ..........  .........
```

Page 2

12.2. Genetic Sequence Matching

		Isolate 1a (362 bp)		Isolate 2a-clear (266 bp)		Isolate 2a-cream (125 bp)		Isolate 2c (280 bp)	
Isolate/clone	Accession #	% match	E-value	% match	E-value	% match	E-value	% match	E-value
Rhodococcus erythropolis strain ATCC 53968 locus E	AY281125	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain ATCC 53968 locus D	AY281124	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain ATCC 53968 locus C	AY281123	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain ATCC 53968 locus A	AY281121	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. 7/1	AF181691	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain EPWF	AY822047	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain FSD-2	AY793377	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis isolate OUCZ20	AY785731	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured Rhodococcus sp.	AJ631300	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured Rhodococcus sp.	AJ631299	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured Rhodococcus sp.	AJ631298	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain NVI 00/50/6670	AY147846	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured actinobacterium	AJ888587	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured actinobacterium	AJ888540	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. BDC14	AY249053	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Uncultured bacterium	AJ867668	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis IAM 1399	AY017138	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Corynebacterium sp. WS2071	AY017134	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Arthrobacter hydrocarboglutamicus	AY017125	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis DSM 312	AY017124	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Corynebacterium humiferum	AY017121	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Brevibacterium paraffinoliticum	AY017118	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis DSM 20665	AY017116	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Arthrobacter oxamicetus subsp. propiophenicolus	AY017114	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Arthrobacter oxamicetus	AY017113	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis isolate CV71b	AJ717371	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. 871-AN053	AF420422	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. ENG-AN033	AF420419	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. ARG-AN025	AF420417	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. ARG-AN024	AF420416	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. 870-AN019	AF420415	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. IND-AN014	AF420414	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. ANT-AN007	AF420412	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}

		Isolate 1a (362 bp)		Isolate 2a-clear (266 bp)		Isolate 2a-cream (125 bp)		Isolate 2c (280 bp)	
Isolate/clone	Accession #	% match	E-value	% match	E-value	% match	E-value	% match	E-value
Rhodococcus sp. 67-BEN001	AY044096	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. 122-AN065	AY044095	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis specimen-voucher CNCM 2208	AF512839	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis specimen-voucher CNCM 2207	AF512838	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain HAMBI2390	AF501339	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain HAMBI2393	AF501338	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Antibiotic-resistant bacterium RO1	AB194590	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Antibiotic-resistant bacterium RJ2	AB194586	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. PG6	AF500326	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis strain TB2	AJ250924	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis isolate EK5	AJ237967	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Nocardia sp. H17-1	AF487704	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. 1awq	AY077846	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. R-20813	AJ786781	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. T1-1	AY904720	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus erythropolis isolate MR32W	AJ576250	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Actinobacterium TH-K22	AJ785987	96	0	98	e^{-139}	95	$8e^{-48}$	99	e^{-153}
Rhodococcus sp. TM1	AY642534							99	e^{-155}
Uncultured Rhodococcus sp.	AY394636							99	e^{-153}