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**Novel Technology for Sustainable Petroleum Oily Sludge Management:
Bio-Neutralization by Indigenous Fungal-Bacterial Co-Cultures**

By Mahmoud Said

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In
The Department
of
Building, Civil, and Environmental Engineering

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ABSTRACT

Novel Technology for Sustainable Petroleum Oily Sludge Management: Bio-Neutralization by Indigenous Fungal-Bacterial Co-Cultures

Mahmoud Said

The treatment and disposal of petroleum oily sludges represent major challenges for petroleum industries. The oily sludges consume a high portion of a refiner's budget and pose a serious threat to the environment. Montreal and Kyoto protocols introduced significant restrictions on the disposal of petroleum wastes and reduced the options available for treating this type of hazardous wastes. This research considered the application of bioremediation principles to petroleum oily sludge using a new testing technique. Approximately, 35% of the total petroleum hydrocarbons, and 81% of the aliphatic hydrocarbons in the sludge were degraded using a fungal-bacterial co-culture. To my knowledge, this was the first time fungal-bacterial co-cultures have been used in the treatment of petroleum oily sludge. Prior to treatment with the co-cultures, the sludge was subjected to a special electrokinetic separation technology that reduced its oil and water contents. The cultures were isolated from petroleum oily sludge taken from the bottom of crude oil storage tanks. The fungal and bacterial strains, used in the co-culture, were identified as *Paecilomyces variotii* and *Bacillus cereus*, respectively. The cultures were inoculated on 0.22 μm filters laid over the sludge. Reduction in aliphatic hydrocarbons was estimated using Fourier transform infrared spectrometry (FTIR). Total Petroleum Hydrocarbons (TPH) were estimated using solvent extraction. An amphoteric surfactant was used in the studies but it did not improve biodegradation rates. The testing technique gave a comprehensive indication of the efficiency of the process and the toxicity of the sludge for defined cultures of the microorganisms.

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Table of Contents

CHAPTER 1 INTRODUCTION.....	1
1.1. Statement of the Problem	1
1.2. Objectives	2
CHAPTER 2 LITERATURE REVIEW.....	3
2.1. Petroleum Oily Sludge	3
2.2. Environmental Concerns about the Petroleum Oily Sludge	6
2.3. Petroleum Hydrocarbons and their Fate in the Environment	7
2.3.1. Abiotic Mechanisms	9
2.3.2. Biotic Mechanisms	11
2.3.2.1. Microbial Metabolism of Petroleum Hydrocarbons	12
2.3.2.2. Factors Affecting Petroleum Hydrocarbons Biodegradation	16
2.3.2.3. Enhancement of Petroleum Hydrocarbons Biodegradation	19
2.4. Treatment and Disposal of Petroleum Oily Sludges.....	22
2.4.1. Incineration.....	22
2.4.2. Treatment with Fly-Ash.....	23
2.4.3. Lime Stabilization.....	23
2.4.4. Solvent Extraction	24
2.4.5. Coking	24
2.4.6. Thermal Desorption.....	24

2.4.7. Biological Treatment	25
2.4.7.1. Landfarming	25
2.4.7.2. Composting	27
2.4.7.3. Bioaugmentation and Biostimulation	28
2.5. Conclusions	30
CHAPTER 3 MATERIALS AND METHODS.....	31
3.1. Research Methodology.....	31
3.2. Materials	32
3.2.1. Petroleum Oily Sludge.....	32
3.2.2. Chemicals	34
3.2.3. Microbiological Media	34
3.3. Isolation of Indigenous Microbial Cultures	35
3.4. Screening the Capability of the Microorganisms to Degrade Petroleum Hydrocarbons.....	35
3.5. Identification of the Isolated Microorganisms.....	36
3.5.1. Identification of the Fungi	36
3.5.2. Identification of the Bacteria	36
3.6. Assessment of Sludge Bio-Neutralization.....	39
3.6.1. Development of the Testing Technique.....	39
3.6.2. Experimental Protocol for Oily Sludge Bio-Neutralization	40
3.6.3. Growth Assessment	42

3.6.4. Assessment of Sludge Degradation	42
3.6.4.1. Fourier Transform Infrared Spectrometry (FTIR)	42
3.6.4.2. Hexane Extraction	45
CHAPTER 4 RESULTS AND DISCUSSION.....	47
4.1. Isolation of Indigenous Petroleum Hydrocarbons Degrading Microorganisms	47
4.2. Screening the Potential of the Isolated Strains to Degrade Petroleum Hydrocarbons	49
4.3. Identification of the Bacteria and the Fungi	50
4.4. Oily Sludge Degradation and Cultures Growth.....	52
4.5. Discussion.....	64
CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS	70
REFERENCES.....	72
APPENDICES	79
Appendix A Bushnnel-Hass Solution	80
Appendix B Photos from the expereimnts	81
Appendix C API® Strips for Biochemical Identification of Bacteria	91
Appendix D Results of Bacteria Identification tests	97
Appendix E Terminology used in Describing the Morphology of Bacterial Colonies	100
Appendix F Calculations	101
Appendix G Microbiologica Media Preparation	102
Appendix H Microbial Inocula Preparation	103

List of Tables

Table 2-1 : Some sources of oily sludges in a petroleum refinery.....	3
Table 2-2: Typical compositions of petroleum oily sludges.....	4
Table 2-3: Main constituents in petroleum oily sludge	5
Table 2-4: Hydrocarbons in petroleum products	8
Table 2-5: Cost comparison of sludge disposal methods.....	27
Table 3-1: Absorption regions of selected bonds	43
Table 4-1 Characteristics of isolated bacterial colonies	47
Table 4-2 Characteristics of isolated fungal strains.....	48
Table 4-3 The influence of the amphoteric surfactant on the biodegradation of petroleum hydrocarbons	66

List of Figuers

Figure 2-1: Fate of petroleum hydrocarbons in a soil system.....	12
Figure 3-1 Research methodology	31
Figure 3-2 Petroleum oily sludge after and before electrokinetics	33
Figure 3-3 Petroleum oily sludge after the application of the electrokinetics separation technology	34
Figure 3-4 The cups and filters used in the experiments	41
Figure 4-1 Bacterial strains isolated from petroleum refinery sludge	48
Figure 4-2 Fungal strains isolated from petroleum refinery sludge.....	49
Figure 4-3 Screening test results.....	50
Figure 4-4 Carbohydrate metabolism by <i>Bacillus cereus</i>	51
Figure 4-5 Decrease in TPH content of the oily sludge when treated with the bacterial- fungal co-culture	52
Figure 4-6 Decrease in FTIR relative absorbance in oily sludge with and without surfactant when treated with the bacterial-fungal co-culture.....	53
Figure 4-7 FTIR spectra of oily sludge with surfactant before and after treatment with the fungal-bacterial co-culture	54
Figure 4-8 FTIR spectra for oily sludge before and after treatment with the fungal- bacterial co-culture.....	54
Figure 4-9 Plate counts of the bacterial strain (<i>B. cereus</i>) on oily sludge treated by the fungal-bacterial co-culture.....	55

Figure 4-10 Plate counts of the fungal strain <i>P. variotii</i> on oily sludge treated by fungal-bacterial co-culture.....	56
Figure 4-11 <i>P. variotii</i> and <i>B. cereus</i> growing on the oily sludge	56
Figure 4-12 Decrease in TPH of oily sludge when treated by bacterial inoculum.	57
Figure 4-13 Decrease in FTIR relative absorbance of oily sludge when treated by bacterial inoculum.....	58
Figure 4-14 FTIR spectra of the oil sludge with surfactant before and after treatment with <i>B. cereus</i>	58
Figure 4-15 FTIR spectra of the oil sludge before and after treatment with <i>B. cereus</i> ...	59
Figure 4-16 The growth of the bacterial strain <i>B. cereus</i> on sludge	59
Figure 4-17 Plate counts of the bacterial strain <i>B. cereus</i> on oily sludge treated with bacterial inoculum.....	60
Figure 4-18 Decrease in TPH of the oily sludge when treated by fungal inoculum.....	61
Figure 4-19 Decrease in FTIR relative absorbance in oily sludge when treated by fungal inoculum.....	61
Figure 4-20 FTIR spectra of the oil sludge before and after treatment with the fungus <i>P. variotii</i>	62
Figure 4-21 FTIR spectra of the oil sludge with surfactant before and after treatment with the fungus <i>P. variotii</i>	62
Figure 4-22 Fungal inoculum plate counts on oily sludge.....	63
Figure 4-23 Growth of the fungal strain <i>P. variotii</i> on the oily sludge	64
Figure 4-24 The decrease in petroleum hydrocarbons of the oily sludge by the three types of inocula.....	65

Abbreviations

CMC: Critical Micelle Concentration

DC: Direct Current

DCPIP: 2,6-Dichlorophenol Indophenol

EPA: United States Environmental Protection Agency

FTIR: Fourier Transform Infrared Spectrometry

RCRA: Resource Conservation and Recovery Act

TPH: Total Petroleum Hydrocarbons

VOC: Volatile Organic Carbons

CHAPTER 1 INTRODUCTION

1.1. Statement of the Problem

More than 28 000 tones of petroleum oily sludges are generated annually per refinery (Jean *et al.* 2001). This complex sludge is designated as hazardous waste in Resource Conservation and Recovery Act (RCRA) (EPA 1998), and represents a major source of several contaminants that pollute the soil and the ground water (e.g. petroleum hydrocarbons, metals), and the air (e.g. volatile organic carbons). Environmental regulations, emerging from Kyoto protocol, have stressed the necessity to decrease the emissions of volatile organic carbons (VOC) and have placed more restrictions on land disposal of the hazardous wastes (Riser-Roberts 1998). Several technologies have been implemented for the treatment and disposal off petroleum oily sludges. However, no technology has reached a compromising solution that could balance between the environmental regulations and treatment costs. Consequently, there is a need for sustainable technologies capable to neutralize the oily sludge and reduce its adverse impacts on the environment.

Bioremediation techniques have been successfully used to clean up sites contaminated with petroleum hydrocarbons. However, little has been done to remediate petroleum oily sludge. As a result, there is a lack of information available concerning the capability of microorganisms to grow in this habitat. Analytical studies have shown that oily sludge contains 52% alkanes and 30% aromatic compounds (Marks *et al.* 1992, Mishra *et al.* 1999, Van Hamme *et al.* 2000, Wright and Noordhuis 1999); these compounds are known to be degradable by microorganism (Atlas 1984, Cerniglia 1981, Evans 1977).

Bench-scale testing techniques in bioremediation studies employ long and tedious protocols. However, the results tend to have a high margin of error, especially in assessing the growth of microorganisms. There is need for new, simple, and more accurate techniques that facilitates the research of engineers, microbiologists and chemists on petroleum oily sludge, and encourage scientists from all environmental related domains to contribute to the research on this type of hazardous wastes.

1.2. Objectives

The main objective of this research was to neutralize petroleum oily sludge after the application of separation technologies. To achieve this, the following secondary objectives were formulated:

- a. Investigation of indigenous fungal-bacterial co-existing in the petroleum oil sludge.
- b. Development of novel testing technique to study the potential of the microbial cultures to grow on the oily sludge and neutralize it after applying the electrokinetic separation technology.

CHAPTER 2 LITERATURE REVIEW

2.1. Petroleum Oily Sludge

Oily sludges are produced from the first dig searching for the oil until the petroleum products are being delivered to the consumers. Generally, oily sludge is produced from two distinct sources; first source is the process of extracting and storing crude oil, the second source is the refining process. The sludge generated from the first source could be residues at oil wells (i.e. slop oil), sludge accumulated at the bottom of the storage tanks, and drilling mud residues. Refining process produces much more quantities of wastes; because each process in crude oil refining generates residue, which is a complex mixture of solid and liquid phases. The liquid phase contains oil and wastewater. EPA, in Resource Conservation and Recovery Act (RCRA), has identified the wastes produced by petroleum industry under subtitle K (EPA 1998). Table 2-2 shows some sources of petroleum oily sludges.

Table 2-1 : Some sources of oily sludges in a petroleum refinery

RCRA Code	Sludge Source
K049	Slop oil emulsion solids
K050	Heat exchange bundle cleaning sludge
K051	API separator residue
K052	Leaded tank bottoms
K169	Crude oily storage tanks sediments

Adapted from (EPA 1998, Schleck 1990)

There are no official statistics to give quantities of oily sludge that are generated by refiners. However, Mishra *et al.* (1999) has mentioned that in India an oil refinery generates approximately 20 000 tones of oily sludge per year, Jean *et al.* (2001) reported an average of 28 000 tones of oily sludges produced annually by each refinery. In another study done by Shell company more than 1500 m³ /year of oily sludge was collected from storage tanks bottoms, 800 m³ generated by drilling operations, and 550 m³ oil contaminated soil which results form exploration and extraction activities (Wright and Noordhuis 1991). All the mentioned numbers are increasing as a result of the ascending demand on crude oil.

The composition of the oily sludge is complex and depends on its source. Generally, it contains oil, water, and solids in disparate ranges. Table 2-2 shows typical composition of some types of petroleum oily sludges; the table shows that waste oil may form 30-60% of the generated sludge, this oil is a mixture of compounds mainly hydrocarbons that could be substrate for microorganisms.

Table 2-2: Typical compositions of petroleum oily sludges

Sludge type	Composition (w/w%)		
	Waste oil	Waste water	Solids
Tank bottoms	30-60	40-70	sediment
Desalter bottoms	5-40	30-85	10-40
API separator bottoms	10-40	50-90	5-30

Adapted from (Francis and Les Stehmeier 1991)

Advanced analyses on oily sludge showed that it is composed of 40-52% alkanes, 28-31% aromatics, 8-10% asphaltenes, and 7-22.4% resins (Marks *et al.* 1992, Mishra *et al.* 1999, Wright and Noordhuis 1991, and Van Hamm *et al.* 2000). Table 2-3 demonstrates the main constituents in oily sludge. All the studies mentioned above have found the pH of the oily sludge to be in a range between 6.5 and 7.5. Also, they have agreed that the sludge contains low concentrations of nitrogen and phosphorous.

Table 2-3: Main constituents in petroleum oily sludge

Constitute	Concentration
Carbon	69.7%
Water	28.1%
Nickel	13.9 mg/l
Vanadium	117 ppm

Adapted from (Rocha and Infant 1997)

From the previous discussion it is obvious that the oily sludge would possess high potential for being a suitable environment for microorganisms' growth. Alkanes and aromatics have been shown to be degradable if certain conditions are applied (Atlas 1984, Cerniglia 1981, Evans 1977).

2.2. Environmental Concerns about the Petroleum Oily Sludge

The treatment, storage, and disposal of hazardous wastes are regulated by EPA under subtitle (C) of Resource Conservation and Recovery Act (RCRA). In RCRA §1004 (5) EPA defined the hazardous waste as a “solid waste, or combination of solid wastes, because of its quantity, concentration, or physical, chemical, or infectious characteristics may possess a substantial present or potential hazard for human health or the environment when improperly treated, stored, transported, or disposed of, or otherwise managed” . In addition, EPA has defined what is called a Characteristic wastes “that are wastes which exhibit measurable properties that indicate that a waste possesses enough of a threat to deserve regulation as hazardous waste”, so that, EPA established four hazardous characteristics: Ignitability, Reactivity, Corrosivity, and Toxicity to be key measures in the assessment of wastes (LaGrega *et al.* 1994). The previous definitions are applicable to refinery sludge, since it is a mixture of different compounds that are listed as hazardous toxic materials such as heavy metals (e.g. arsenic) and polyaromatic hydrocarbons, besides the fact that refinery sludge contains oil which is an ignitable material. The transportation, storage, and disposal of refinery sludge generate various dangerous gases mainly volatile organic carbons (VOCs) (Atlas 1984, Riser-Roberts 1998).

EPA and Environmental Defense Fund have defined 14 petroleum refining waste streams, seven of them are toxic streams (EPA 1998). The following are examples for the toxic streams: crude oil storage tank sediment from petroleum refining operations (K169), clarified slurry oil storage tank sediment (K170), Spent hydro-treating catalyst (K171), spent hydro-refining catalyst (K172). According to EPA (1998), the reason for considering these wastes hazardous is that “the risk assessment results show that certain

concentrations of chemicals contained in these wastes may possess potential hazards to human health and the environment when disposed of in landfill treatment units.

The EPA has presented guidelines for control and monitoring any air pollution from-land disposal units. Wind-dispersion controls are required to minimize airborne particulates contamination of the environment. When they first applied, these regulations were not associated with the land disposal of petroleum wastes. However, as more research has proved that some volatile components of these wastes will express a serious risk for the environment; these compounds have been included in the guidelines (Atlas 1984).

2.3. Petroleum Hydrocarbons and their Fate in the Environment

Petroleum can be any mixture of natural gas and crude oil, the composition of crude oil can vary significantly depending on its origin, age, and history. Relatively, it is composed of 83 to 87% (by weight) carbon and 11 to 14% hydrogen, with lesser amounts of nitrogen (0.05-0.08%), sulfur (0.1-5.5%), and oxygen (0.1-4%), in addition to trace amounts (i.e. less than 1%) of phosphorus and heavy metals among them nickel and vanadium (Lehr 2002).

Basically, three structural groups of hydrocarbons exist in the petroleum: paraffins, naphthenes, and aromatics (OSHA 2002). Paraffins are saturated hydrocarbons (i.e. Alkanes) that have the general formula C_nH_{2n+2} and can be either straight chains (n-paraffins) or branched chains (isoparaffins). Naphthenes are saturated hydrocarbons that have a general formula C_nH_{2n} , arranged in the form of closed rings (cyclic-alkanes), they are found in all fractions of crude oil except the very lightest ones. Single-ring

naphthenes (mono-cycloparaffins) with five and six carbon atoms and two-ring naphthenes (dicycloparaffins) are the most common among this group.

Aromatics are unsaturated ring type (cyclic) compounds, which react readily because they have carbon atoms that are deficient in hydrogen. All aromatics have at least one benzene ring as part of their molecular structure. Complex aromatics (i.e. polynuclear of three or more fused aromatic rings) are found in heavy fractions crude oil. Table 2-4 lists hydrocarbons in some petroleum products.

Table 2-4: Hydrocarbons in petroleum products

Product	Major Hydrocarbons
Gas	Straight-chain and branched-chain paraffins, one to five carbons (e.g. ethane, propane)
Gasoline	Straight-chain, branched-chain paraffins of 6 and 10 carbons, and cycloalkanes
Kerosene/diesel fuel no.1	Straight-chain, branched-chain paraffins of 11 and 12 carbons, cycloalkanes, aromatics and mixed cycloalkanes and aromatics.
Light gas oils	Straight-chain, branched-chain paraffins with lower percentage in n-paraffins than kerosene, 12-18 carbons, and cycloalkanes, aromatics
Heavy gas oils	Hydrocarbons between 18 and 25 carbons long
Lubricants	Hydrocarbons between 26 and 38 carbons long
Asphalts	Heavy polycyclic compounds

Adapted from (Baker and Herson 1994)

Oxygen compounds such as phenols, ketones, and carboxylic acids occur in crude oils in varying amounts. Trace amounts of metals including nickel, iron, and vanadium

are often found in crude oils and are removed during the refining process. In addition to these groups, crude oil may contain small concentrations of other compounds like alkynes and alkenes.

As petroleum hydrocarbons enter the environment lighter compounds will be volatilized and heavier ones will remain in the environment. The fate of petroleum hydrocarbons in the environment is determined by their individual characteristics (i.e. concentration, distribution function, structure, and solubility), environment conditions (i.e. temperature, pH, moisture content, and wind), and microorganisms available (i.e. physiology and genetics). The solubility of hydrocarbons is a key factor in their fate. Generally, the solubility of organic compounds is disparate, some are infinitely soluble polar compounds (e.g. methanol), others are of low solubility (e.g. polyaromatic hydrocarbons), and these will be mostly sorbed on solid particles. Fractions that are soluble will be transported by advection, diffusion and will be more available for hydrocarbon degraders. Two mechanisms would contribute to the fate of petroleum hydrocarbons; abiotic mechanisms and/or biotic mechanisms.

2.3.1. Abiotic Mechanisms

Volatilization

Light compounds would volatile from solid phase or aqueous phase depending on the compound and environment conditions such as compound molecular weight, vapor pressure, concentration, Henry's constant, temperature, turbulence, wind, and moisture content (Riser-Roberts 1998). However, in soil ecosystems this process seems to be complicated due to sorptive and diffusive capacity of the hydrocarbons. In addition

volatile hydrocarbons are known to be toxic for microorganisms; lag phase of bacterial growth could be increased due to the existence of such compounds (Atlas 1984).

Sorption

It involves two mechanisms: adsorption and absorption, in adsorption the solute in the solution is attached to the surface of the solid material. While in absorption it is absorbed within the mass of the solid rather than its surface. Hydrophobic hydrocarbons such as polyaromatics will be adsorbed to the particles of surrounding environment such as soil or sediment particles in terrestrial ecosystems. Adsorption capacity of the soil depends on factors such as soil type and moisture content. Hydrocarbons could be adsorbed to the mineral surface of the soil or to the organic matter depending on the available adsorbing sites. If the soil is wet most of the mineral surface would be occupied by water and so organic matter would be the adsorption surface (Boonchan 1998, Harold and Fechner 2000). Generally, organic matter has high surface areas and exchange capacities, if hydrophobic compound were absorbed to the micropores of the soil they would be held by physical or chemical forces, which may reduce their accessibility to microorganisms but could reduce their toxicity in some cases (Kostecki and Calabrese 1991).

Dispersion and diffusion

Dissolved contaminants spread as they move with groundwater as a result of molecular diffusion and mechanical mixing that cause a net flux of the solutes from a zone of high concentration to a zone of lower concentration. As a result, the concentration of contaminant is expected to diminish with increasing distance. However,

the dispersion in the direction of flow is much greater than that of transverse direction (Mackay *et al.* 1985).

Advection

This process involves bulk movement of contaminants that percolate to the groundwater. The groundwater tends to move the contaminants from high to low water levels due to the hydraulic gradient. Advection is a dominant mechanism in migrating dissolved contaminants in sand and gravel aquifers. In most cases flow velocities under natural gradient conditions are probably between 10-100 m/yr (Riser-Roberts 1998), this rate would increase substantially in groundwater artificial-well fields.

2.3.2. Biotic Mechanisms

These mechanisms take place in the top layer of the soil where microbial cultures are concentrated. Several families of microorganisms have evolved metabolic capabilities to utilize petroleum hydrocarbons. Hydrocarbons exist in the environment as a result of human activities and via natural seeps (Spormann and Widdel 2000). Figure 2-1 demonstrates the fate of the degradable petroleum hydrocarbons in a soil system. The rate of biodegradation is a function of different variables including the solubility of hydrocarbon. The type and quantity of the existing microorganisms are, also, important factors in the biotic fate of petroleum hydrocarbons (Atlas 1984, Blakebrough 1977, Bollag 2000, Bonnier *et al.* 1980, Hughes 2000).

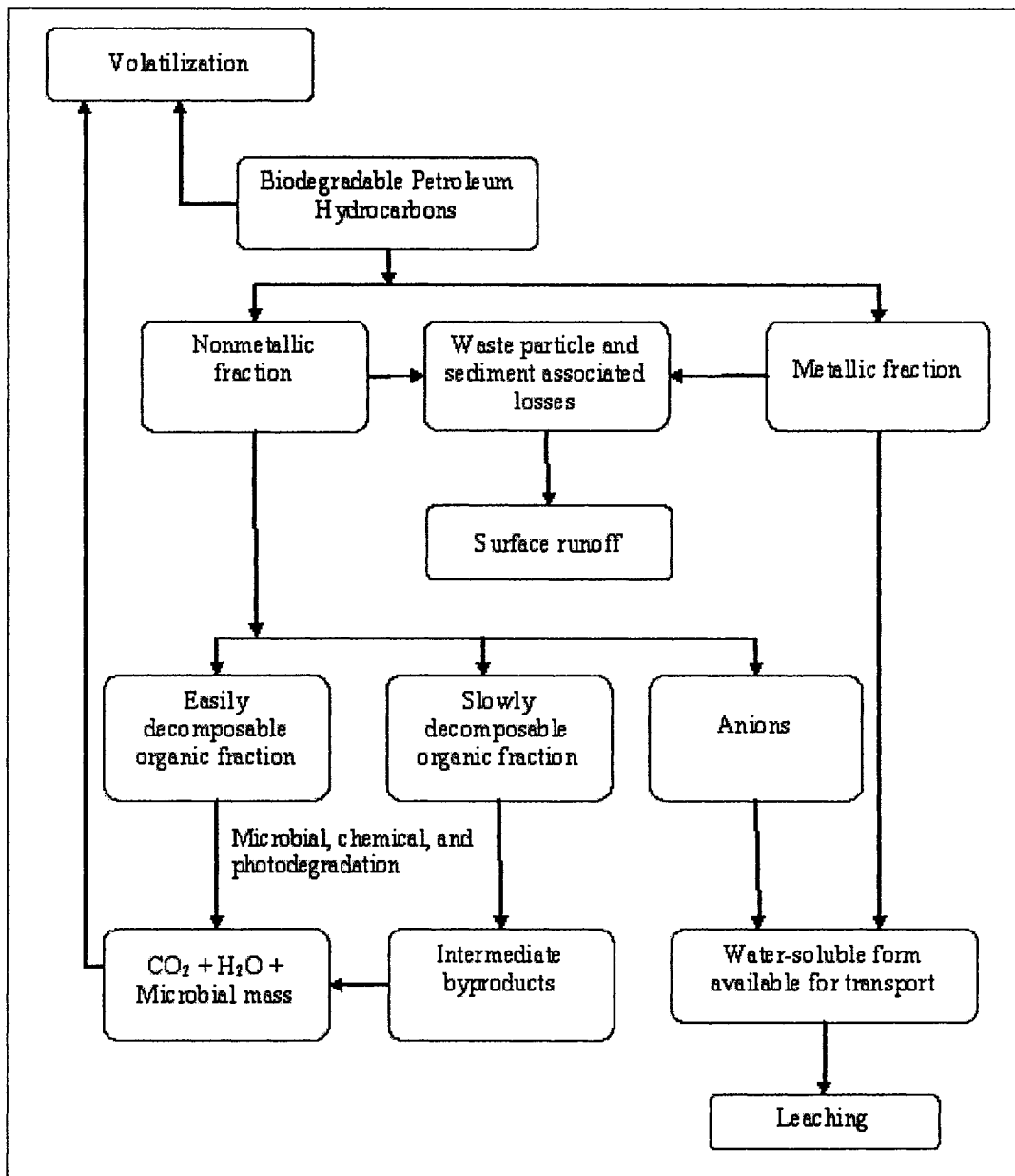


Figure 2-1 Fate of petroleum hydrocarbons in a soil system (Riser-Roberts 1998)

2.3.2.1. Microbial Metabolism of Petroleum Hydrocarbons

The metabolism of hydrocarbons in the environment is carried out mainly by bacteria and fungi; the fraction of total heterotrophic hydrocarbon utilizing community is highly variable: 6-80% for soil fungi, 0.13% to 50% for soil bacteria, and 0.003 to 90% for marine bacteria (Leahy and Colwell 1990). Various families of bacteria and fungi

revealed their ability to degrade petroleum hydrocarbons. In fact, 22 bacterial hydrocarbon-degrading genera and 31 fungal genera were isolated from soil contaminated with petroleum hydrocarbons. On the same regard, 25 bacterial genera and 27 fungal genera were isolated from marine environment (Leahy and Colwell 1990).

The following bacterial species were reported to grow on petroleum oily sludge as a part of bacterial consortia: *Pseudomonas spp.*, and *Alcaligenes spp.*, *Stenotrophomonas maltophilia*, *Yersinia spp.*, *Bacillus spp.*, *Micrococcus*, and *Enterobacteriaceae* (Lazar *et al.* 1999, Van Hamm 2000). However, *Pseudomonas spp.* are the most often reported especially *P. aeruginosa*, which is believed to produce biosurfactants that emulsify the oily sludge which helps enhancing biodegradation by other available microflora (Rocha and Infant 1997). Other bacterial strains like *Acinetobacter*, *Ocherobactrum*, *Achromobacter*, *Arthrobacter*, *Flavobacterium*, *Nocardia* were found to grow on petroleum hydrocarbons from crude oil or contaminated soils. (Atlas and Cerniglia 1995, Bento and Gaylarde 2001, Diegor 2000, Lazar *et al.* 1995a, Riser-Roberts 1998).

Fungal strains like *Aspergillus spp.*, *Penicilium spp.*, *Mortierella spp.*, *Paecilomyces spp.*, and *Trichoderma spp.* were shown the ability to grow on petroleum hydrocarbons in contaminated soils. Other fungi strains like: *Aureobasidium*, *Candida*, *Rodotorula* and *Sporobolomyces* where also isolated from soils contaminated with petroleum hydrocarbons (Atlas and Cerniglia 1995, Bento and Gaylarde 2001, Leahy and Colwell 1990, Riser-Roberts 1998, Rojas-Avelizapa *et al.* 1999). However, little information is available about the growth of fungi on petroleum oily sludge.

It is assumed that microorganisms utilize petroleum hydrocarbons by direct contact to the oil or by utilizing the compounds available in the aqueous medium, in both cases the process was found to take place in water-oil interface; since cells need to be in contact with water for other vital needs (i.e. oxygen, minerals, and CO₂ disposal) (Blakebrough 1977, Brown 1987). Bacterial metabolism of hydrocarbons starts by the oxidation of the compound, this involves enzymes to catalyze oxidation reactions (i.e. oxygenases), and these enzymes are divided into two types' Mono-oxygenases and Di-oxygenases. Usually, bacteria produce the second type that incorporates two oxygen atoms to oxidize the compound (Boonchan 1998). Fungi were found to produce special enzymes capable of initiating the oxidation process. However, these enzymes are different from bacterial enzymes (i.e. mono-oxygenases and lignin-degrading enzymatic systems). Fungi were not reported to be able to utilize polyaromatic hydrocarbons as their sole source of carbon and energy. Studies have shown that wide spectra of fungi have enzymatic capacities to transform these compounds co-metabolically when grown on an alternative carbon source (Cerniglia 1981, Juhasz and Naidu 2000, Ye *et al.* 1996).

Several researches have studied the ability of various microorganisms to degrade hydrocarbons, general rules were suggested to justify the interaction of microorganisms with hydrocarbons, the following points were adapted from Baker and Herson (1994):

- a. Aliphatic hydrocarbons are generally easier to degrade than aromatic compounds.
- b. Straight-chain aliphatic hydrocarbons are easier to degrade than branched-chain hydrocarbons.

- c. Long chain aliphatic hydrocarbons are more easily degraded than short chain hydrocarbons. Hydrocarbons with chain length less than 9 carbon atoms are difficult to degrade because of their toxicity to microorganisms. However, the optimal chain length for biodegradation is between 10 and 20 carbon atoms.

The most common aerobic mechanism for straight chain alkanes is through the oxidation of the terminal methyl group to a fatty acid which results from three steps (Juhasz and Naidu 2000). In the first step, the hydrocarbon is oxidized by oxygen (O_2) to alcohol then alcohol is oxidized to an aldehyde which is oxidized to fatty acid. Afterwards, the fatty acid is degraded by cleaving two carbon units from the molecule (i.e. β -oxidation) to form fatty Acyl-CoA and Acetyl-CoA, the last could be further metabolized via Krebs's cycle while the fatty Acyl-CoA, will be used as substrate for another (β -oxidation).

Generally, the metabolism of aromatic hydrocarbons has been reported to pass through four main phases (Evans 1977):

- a. Entry into the cell by passive diffusion or with specific transport mechanism.
- b. Manipulations of side chains and formation of substrate for ring cleavage.
- c. Ring cleavage.
- d. Conversion of the products of ring cleavage into useful intermediates that can be utilized.

The degradation of polyaromatic hydrocarbons (PAHs) was verified by several studies especially for two and three ring aromatic compound. As the number of rings increase the ability of microorganism to degrade these compounds decrease due to the toxicity and low solubility of the compound.

It is well known that not all petroleum hydrocarbons may be attacked simultaneously (Brown 1987). Normally the more easily degraded compounds and those in greatest concentration are degraded first. Basically, the greater the chain length (i.e. greater than 20 carbon atoms) and the greater the branching the more resistant the compound is to microbial attack.

2.3.2.2. Factors Affecting Petroleum Hydrocarbons Biodegradation

The main principle is to create conditions under which microorganisms can utilize hydrocarbons as substrate. The microorganisms may be indigenous or selectively isolated from an ecosystem exposed to hydrocarbons. These conditions have been mentioned as early as 1928 (Brown 1987), they include temperature, moisture, pH, nutrients and substrate characteristics.

Temperature

The temperature affects both the microorganisms and the characteristics of the substrate, at low temperatures the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced and their water solubility is increased delaying the biodegradation process (Leahy and Colwell 1990). Moreover, rates of enzymatic activity declined at low temperatures. Hydrocarbon degrading bacteria were found to be mesophilic, yet thermophilic alkane-utilizing bacteria do exist (Brown 1987). The best working temperature is in the range 22-40°C (Castaldi and Ford 1992, Hahn and Loehr

1992, Lazar *et al.* 1999, Van Hamme *et al.* 2000, Ward and Singh 2001), however, 30°C was found to be the optimal.

Moisture

Water is the battle field of the biodegradation process, it contains the nutrients and oxygen. It suspends the microorganisms, and hydrolyzes the substrate to ease its oxidation by the degraders. In soil land-farmed with oily sludge hydrocarbons optimal biodegradation rates were found to be between 30-90% moisture content (Bonnier *et al.* 1980).

pH

Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant to acidic conditions. It has been shown that hydrocarbon degradation rates are being doubled when the pH rose from low values (less than 4.5) to pH around 7. On the other hand, a decrease occurred when the pH was above 8.5 (Hahn and Loehr 1992). Controlling the pH is important since microbial degradation yields oxidized compounds that could cause a drop in pH (Boonchan *et al.* 2000). In some studies the addition of CaCO₃, to raise the pH of the soil, has caused increase in biodegradation rates of hydrocarbons in landfarms (Bonnier *et al.* 1980). Several studies have reported pH 7.0-7.5 as an optimal pH value. (Marks *et al.* 1992, Van Hamme *et al.* 2000, Ward and Singh 2001).

Nutrients

Oily sludge lacks main nutrients like phosphorus, nitrogen and metals (i.e. K, Fe, Mn, Zn) that are essential for microorganisms to grow. Any biological treatment process

should supply such essential ingredients to support the growth of microorganisms (Margesin and Schinner 2001, Mishra *et al.* 2001).

Oxygen

Aerobic conditions are more favorable for the biodegradation of the majority of petroleum hydrocarbons. Anaerobic microorganisms are very sensitive to most petroleum hydrocarbons. In addition, their degradation may produce toxic compounds and reduce the pH, which generates inconvenient environment for the process to continue (Zwolinski 2000). In an aerobic system, oxygen concentration should be maintained above specific levels, because the rate of oxidation can be reduced by lack of oxygen. Stoichiometrically, 3-4 mg of O₂ is required per mg of saturated hydrocarbon for complete oxidation to CO₂ and H₂O (Bonnier *et al.* 1980). Also, special measures should be taken to maintain uniform distribution of the oxygen within the system.

Substrate characteristics

The composition of the sludge is an important factor in the biological treatment. The microbial activity in crude oil is found to be highest for aliphatic compounds (i.e. alkanes, alkenes and alkynes) and lighter aromatics, higher aromatics and polar compounds come in the second place, while asphaltenes are the hardest to degrade (Brown 1987). However, studies on crude oil reported that identical compounds in different crude oils showed major differences in susceptibility to degradation (Brown 1987, Hahn and Loehr 1992). Another important parameter related to the sludge is the solubility of its compounds. Since hydrocarbon degrading microorganisms act mainly at the oil-water interface (Connan 1984) the degree of biodegradation was found to be linked to the available surface area of hydrocarbon. Therefore, biodegradation of some

hydrocarbons like polyaromatics (e.g. phenanthrene and naphthalene) depends on their solubility and bioavailability rather than substrate concentration. Consequently, it is possible to use a surfactant to increase the accessibility of the compounds for microbial degradation, but the surfactant should not be toxic or a possible substrate. However, substrate concentration is an important factor since some compounds are in such low concentration that they fail to induce the enzymatic systems necessary for their utilization.

2.3.2.3. Enhancement of Petroleum Hydrocarbons Biodegradation

Co-Metabolism

It is defined as “the condition where non-growth hydrocarbons are oxidized when present as co-substrate in a medium in which one or more different hydrocarbons are furnished for growth” (Boonchan 1998). The interactions between the microorganisms and heterogeneous mixture of hydrocarbons in natural environment are complex, as a rule of thumb, microorganisms will favor readily degradable hydrocarbons that are easy to degrade, and ignore other available molecules. However, certain compounds may only be metabolized in presence of others. These co-metabolic reactions are due to non-target molecules being attacked to some extent by degradative enzymes. Studies on PAHs mixture have shown that *Mycobacterium spp.* were able to metabolize Fluorene when yeast extract and peptone or acetate were used as growth substrate (Tiehm and Fritzsche 1995).

Stimulation

In this case the degradation of one compound is *enhanced* by the presence of another compound. The increased growth of microorganism on degradation enhancing compounds will increase the biomass, which will stimulate the degradation of the other compound. It is different from co-metabolism since degradation enhancing compound is not growth-supporting substrate. (Baker and Herson 1994, Boonchan 1998).

Adaptation

That implies prior exposure of the microbial community to petroleum hydrocarbons as a result of natural or man made sources such as waste oil disposal, accidental oil spill, oil exploration, storage, and transportation activities. Adaptation has been reported to enhance degradation rates, increase the numbers of hydrocarbon-utilizing microorganisms and their proportion in the heterotrophic community. In general the level of hydrocarbon utilizing microorganisms reflects the degree of contamination of ecosystem. Adaptation can be achieved by three interrelated mechanisms (Leahy and Colwell 1990):

- a. Induction and/or depression of specific enzymes.
- b. Genetic changes which result in new metabolic capabilities.
- c. Selective enrichment of organisms to transform the compound or compounds of interest, which is widely used in recent studies on petroleum hydrocarbons.

Additives

Surfactants have been introduced in petroleum hydrocarbons studies to increase the bioavailability of hydrocarbons, because they increase the dispersion of non-aqueous phase liquid-hydrocarbon in the aqueous phase, and facilitate the transport of the hydrocarbons from the solid phase to the aqueous phase. Mass transfer of hydrocarbons from solid phase to aqueous phase proved to be rate limiting for growth especially for higher molecular weight polyaromatic hydrocarbons (Atlas and Cerniglia 1995). Moreover, surfactants enhance the contact between the organism and the substrate.

The term surfactant is an abbreviated form of “Surface Active Agent”; they are large molecules consisting of two groups, a hydrophobic non-polar group, and a hydrophilic polar group. Based on the charge on the second group, surfactants are classified into four major types: cationic, anionic, nonionic, and amphoteric surfactants, the last type produces either anions or cations depending on the environment; they are cationic in acidic media and anionic in basic media. Properties of chemical surfactants that influence their performance include charge; hydrophile-lipophile balance (HLB); and critical micelle concentration (CMC).

Biological and chemical surfactants demonstrated disparate effects (i.e. beneficial, neutral, or inhibitory). Van Hamme and Ward (1999) studied the impacts of various anionic, cationic, and nonionic surfactants on crude oil degradation, among them Igepal CO-630, which is a nonionic surfactant, was the best to enhance total petroleum hydrocarbons degradation. In another research, Rocha and Infant (1997) have found that an anionic synthetic surfactant did not improve the biodegradation of oily sludge, though the surfactant did emulsify the sludge. However in the same research, the biodegradation

rate was enhanced when a biosurfactant isolated from *Pseudomonas aeruginosa* USB-CS1 (Rocha and Infant 1997) was added.

2.4. Treatment and Disposal of Petroleum Oily Sludges

Petroleum wastes must be treated before they could be released to the environment. In the past, refinery sludges have, commonly, been landfilled without being treated properly (Francis and Les Stehmeier 1991). The ban on land disposal of hazardous wastes has placed new extreme restrictions on such practices. Consequently, refineries have developed various methods to treat their wastes. Environmental legislations have promoted the incorporation of microbial activities in treatment methods (Baker and Herson 1994). The following sections describe in brief some common treatment technologies.

2.4.1. Incineration

It is a common technology especially in large refineries. Rotary kiln and fluidized bed incinerator are the most common incinerators. In rotary kiln incinerator, the combustion temperature is 980-1200°C and the residence time is around 30 mins. While in fluidized bed incinerators the combustion temperature is 732-760°C, and the residence time can be in order of days for solids entrapped by the bed (Schleck 1990). The incineration process requires sophisticated equipment and experienced operation to achieve adequate combustion of oily solids. Recent RCRA regulations require that the destruction and removal efficiency of hazardous organics in an incineration facility be greater than or equal to 99.9%.

Incineration requires high capital and operating costs because the oily sludge contains high concentrations of hazardous organics including those compounds most resistant to incineration. Main parameters that should be controlled are: waste feed rates, oxygen: air ratio, residence time, combustion temperature, and gas emissions. The residue remaining after petroleum-waste incineration is also considered hazardous; these include ash, scrubber waters, and scrubber sludges. Ash containing metals needs further treatment before being disposed off.

2.4.2. Treatment with Fly-Ash

Sludge containing oil, solids, emulsions and other impurities is treated with aqueous slurry of fly-ash and a small amount of polymer. Light sludges are mixed before thickening in small tank equipped with a mixer. Sludges having higher oil and solids contents after de-watering in a centrifuge are treated with ash slurry in a screw mixer.

The settled product from the thickener and the product from the mixer are transported in closed truck containers directly to a landfill, which must be well drained to minimize leaching. During the dry season the deposit quickly hardens to be hard enough to be carried in trucks and then it can be used for roadbeds, or after covering with a layer of soil, the deposit area can be used for growing grass or trees (Atlas 1984).

2.4.3. Lime Stabilization

Stabilization involves mixing a solid additive material to the oily solids in order to produce a matrix within which the oil and metals are “fixed” and will not leach out. Using lime for this purpose is well established in the literature, the addition of lime produces physical and chemical changes in the sludge, which facilitate hydrocarbon

adsorption and immobilization of metals as insoluble salts (Wright and Noordhuis 1991). The higher pH provided by adding the lime is essential in this process, some additives can be added to produce hydrophobic matrix to prevent contaminants from becoming acidic due to rainfall percolation in the landfill.

2.4.4. Solvent Extraction

In this method the sludge is extracted with a solvent to remove oil and other organics, the solvent is recovered and recycled. These solvents include propane, kerosene, and tri-ethyl amine. Though, the best type of solvent is likely to be kerosene, other lighter solvents might be used to reduce the costs and possibly giving lower final oil content. The oil content in the final product must be around 1% (Bonnier *et al.* 1980). Solvent extraction will not remove heavy metals such as arsenic, lead and selenium; these must be treated using other methods.

2.4.5. Coking

Coking is a process for thermally converting heavy residual into lighter products. It is used in petroleum refinery to produce gasoline and some petrochemical feedstock. As a disposal technique, it is commonly used for disposal of free metal oil recovered from the sludge (Schleck 1990). However, it is currently used for disposal of refinery sludges, it has shown to be a cost effective method in some cases depending on sludge composition.

2.4.6. Thermal Desorption

In thermal desorption (also called drying) the sludge is not combusted but heated to remove organics and water from solids, the water is converted to steam to help

stripping off high boiling point semi volatile compounds, which can be condensed for recovery and disposal (Schleck 1990). Thermal desorption is different from incineration since it is conducted in the absence of oxygen and under much lower temperatures.

2.4.7. Biological Treatment

Biological treatment systems were first introduced to treat wastewater effluents from the refinery to reduce the BOD of these effluents and to remove specific toxic substances (e.g. phenols). Because of the high sensitivity of anaerobic treatment systems to such toxicants; only aerobic systems are used in treatment plants. Common wastewater treatment systems like activated sludge, trickling filters, rotating biological contactors, and oxidation ponds are being used. Several studies have demonstrated the capability of certain biological treatment systems to treat oily sludges produced by refining processes. Marks *et al.* (1992) found that the concentration of some toxic hydrocarbons was reduced when an aerobic CSTR reactor was used. In this research, Benzo(a)pyrene concentration was reduced by 97%, and several parameters were controlled to optimize the results (i.e. pH, feeding rate). Oolman *et al.* (1992) and Castaldi and Ford (1992) used slurry reactor to treat oily sludges and they had succeeded in reducing the concentrations of hazardous hydrocarbons in the oily sludges to the acceptable limits. Ward and Singh (2001) described a method to treat petroleum oily sludge using a batch reactor, by forming oil-in-water emulsion and supplying essential nutrients.

2.4.7.1. Landfarming

Landfarming, also known as land treatment or land application, is an above-ground treatment technology in which refinery sludges are spread over and mixed with the surface soil. The method relies on the soil microflora to degrade and stabilize

hydrocarbon wastes. Consequently the soil should be able to degrade, transform, or immobilize hazardous organic wastes (Leher 2002). Unlike landfilling and other land disposal methods land farming does not require physical barrier (i.e. liner) to isolate hazardous constituents from the environment. Instead migration or leaching of these constituents is minimized by soil processes.

Extensive studies have been conducted since suggesting this method as disposal alternative for refinery wastes in mid 70s. It has been demonstrated that waste oil is degradable and/or immobilized in soil under appropriate environmental conditions. Hazardous inorganic constituents such as heavy metals and some organic fractions of petroleum wastes, which are not readily biodegraded, tend to be immobilized in the soil by physical and chemical forces including precipitation, complexation, cation exchange reaction and incorporation into the soil organic matter (Atlas 1984, Wright and Noordhuis 1991).

Landfarming is an acceptable land-disposal under RCRA as long as it is within EPA guidelines that aims to minimize the possibility of wash out and groundwater contamination (Riser-Roberts 1998). To ensure that, a landfarm should be located in 10-year flood zone, the depth of sludge incorporation should not be more than 1.5 m or not be closer than 1 m to highest seasonal water table level (Atlas 1984). In addition, operating parameters should maximize the efficiency of the process through aeration and fertilization of treated soil to enhance microbial activity and controlling the application rate, moisture content and pH. The optimum pH for biological activity and for metal insolubility is 6.5-7.5; above 6.5 the mobility of most heavy metals is very low, due to their low water solubility. Landfarm project must include monitoring schedule for the

surface soil, subsurface ground water quality, and air to ensure the safety of the process (Bonnier *et al.* 1980).

Table 2-5 compares the costs of disposing refinery sludges in three different methods. The table shows that landfilling is the most economic method, and landfarming costs are around one half those of incineration. But due to the restriction on landfilling hazardous wastes; landfarming is the most favorable for refineries. It is noted that, factors like land availability, soil characteristic, and VOC emission may limit the application of landfarming.

Table 2-5: Cost comparison of sludge disposal methods

Method	Cost (\$/tonne of sludge)
Direct land-filling	6-12
Incineration	30-40
Landfarming	13-24

Adapted from (Bonnier *et al.* 1980)

2.4.7.2. Composting

In this process the oily sludge is stabilized by reducing the concentration of hazardous compounds, so that, it can be considered as non-hazardous waste. Composting became a traditional way to stabilize solid or semi-solid organic matter such as municipal sewage solids, manure, agricultural corps and others, its products are carbon dioxide, water, stabilized material and sometimes odors. Bengtsson *et al.* (1998) has studied the efficiency of three types of compost to treat oily sludges. The three types were peat; peat and horse manure; and horse manure and straw mixed with agricultural composts. The

tests were conducted on both lab and pilot scale, the concentration of total and non-polar aliphatic hydrocarbons was determined over the test period. The maximum reduction was recorded when the third type of compost was used (85-90%) over 11 month period. Microbial studies, conducted to verify the results, demonstrated that oil degrading microorganisms originally dominating in the sludge were not necessarily responsible for the degradation since other bacterial strains were dominating during high growth rate phase. Similar results were reported by Kirchmann and Ewnetu (1998) who achieved 78-93% decomposition in oily sludges from a petroleum refinery using horse manure.

Compared to landfarming, composting has faster destruction rate and could treat more toxic compounds. Moreover, it is safer for the environment since the material is contained during the process. Heavy metals in the sludge could limit the extent of the degradation process. In addition, composting would increase the volume of the sludge due to amendment material addition.

2.4.7.3. Bioaugmentation and Biostimulation

Microorganisms were targeted towards the treatment and control of accidental oil spills since 1940's. After that, they were used to remediate contaminated shores and soils. Consequently, more knowledge became available about the ecological interactions between the microorganisms and soil, water and oil media. Recently, environmentalists are giving bioremediation high priority in hydrocarbon contamination problems, because bioremediation eliminates the hydrocarbons through mineralization into CO₂, H₂O, and biomass. Also, it is less expensive than other technologies. The main disadvantage of this process is the time, since, if compared to other technologies, bioremediation is a slow process (Baker and Herson 1994, Riser-Roberts 1998). Favorable conditions (e.g.

temperature, nutrients, and pH) and additives (e.g. surfactant) must be optimized to stimulate the growth of the microbial cultures.

Various bacterial consortia were used in petroleum refinery sludge treatment; the bacteria were selectively isolated from hydrocarbon-contaminated soil or from petroleum refinery sludge. Van Hamme *et al.* (2000) have studied the dynamics of growth of a bacterial consortium on a petroleum oily sludge. The results showed varying individual ability of the bacterial strains to grow on the sludge. The growth was improved by mixing the sludge with non-ionic surfactant. Petroleum hydrocarbons biodegradation was not studied; only the strains, which were able to grow on the sludge, were reported.

Lazar *et al.* (1999) studied the ability of six bacterial consortia to degrade the oily sludge. The consortia were isolated from contaminated soils. The experiments were conducted on bench-scale, and the samples were incubated under various conditions (i.e. temperature, nutrients, and additives). Petroleum hydrocarbons biodegradation rates varied from 16.75% to 95%. Mishra *et al.* 2001 have isolated a bacterial consortium from a soil contaminated with petroleum oily sludge. The consortium was called OILZAPPER, and was prepared in the form of powder. It was used along with fertilizers to treat land farms and petroleum oily sludge. 88% reduction in petroleum hydrocarbons was achieved when OILZAPPER was applied to a landfarm.

Very little is available about the use of microbial cultures to treat petroleum oily sludges on pilot scale and full-scale. It seems that refineries can not rely on bioremediation as a major treatment method. This could be due to the disparate results of bench-scale studies, which demonstrated that the performance of the microbial cultures is

dependent on the several factor including the composition of the sludge that varies depending on the type of crude oil and the source of the sludge inside the refinery.

Bioremediation of petroleum oily sludges is a slow process if compared to landfarming and composting, such that more research is to be conducted to stimulate the growth of microorganisms on the sludge and improve the performance of the bioremediation process.

2.5. Conclusions

Petroleum oily sludges have a complex composition. Their treatment should reduce the concentration of all the hazardous constituents contained in the sludge. The rising environmental awareness about the impacts of petroleum oily sludges on the environment has rendered most commonly used process uneconomical or inappropriate for managing this type of hazardous wastes.

More research is required on the biodegradation of petroleum oily sludges. This would lead to the improvement of sustainable and effective biological methods to neutralize petroleum oily sludges.

CHAPTER 3 MATERIALS AND METHODS

3.1. Research Methodology

The methodology applied in this research is demonstrated in Figure 3-1

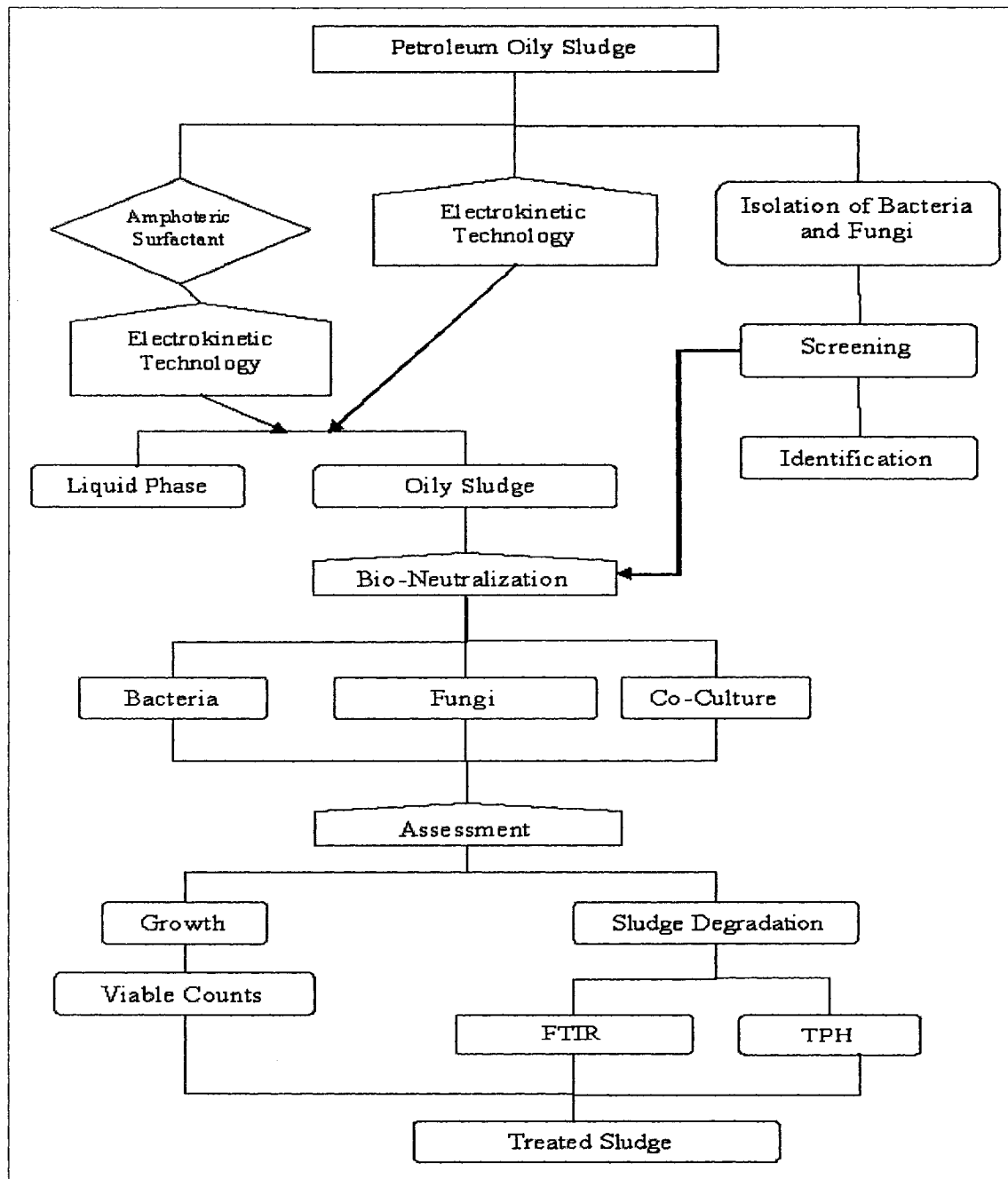


Figure 3-1 Research methodology

The methodology was designed to achieve the main objectives of the research, it involved the following stages:

- a. Investigate the indigenous cultures of microorganisms from the petroleum oily sludge collected from the bottom of crude oil storage tanks.
- b. Screen the capability of the isolated microorganisms to degrade petroleum hydrocarbons.
- c. Identify the microorganisms.
- d. Develop an experimental technique to grow the microbial cultures on the oily sludge that was subjected to the electrokinetic separation technology.
- e. Treat the oily sludge with the isolated microbial cultures.
- f. Assess the growth of the cultures.
- g. Assess the degradation of the oily sludge.

3.2. Materials

3.2.1. Petroleum Oily Sludge

The oily sludge was collected from the bottom of crude oil storage tanks in a Shell[®] Canada refinery located in Montréal. The sludge was collected and stored in sterile containers. Samples of the oily sludge were subjected to a new electrokinetic separation technology that used DC current to recover a portion of the liquid phase of the oily sludge. However, the liquid phase contained crude oil and water. As a result of this process, the density of the sludge increased as shown in Figure 3-2 and 3-3; solids weight

increased from 3% to 20% and the water content dropped from 18% to 7.5% (Elektorowicz *et al.* 2003a,b, Habibi and Elektorowicz 2004). This processed sludge is the material used in this research and will be referred to as “oily sludge” throughout the experiments.

Some samples of the oily sludge were mixed with an amphoteric surfactant before applying the electrokinetic separation. The concentration of the surfactant was 50 ml per liter of sludge (Elektorowicz *et al.* 2003a, b, Hatem 1999). This sludge was used to study the effect of chemical surfactant on biodegradation. It will be referred to as “oily sludge with surfactant” throughout the experiments.

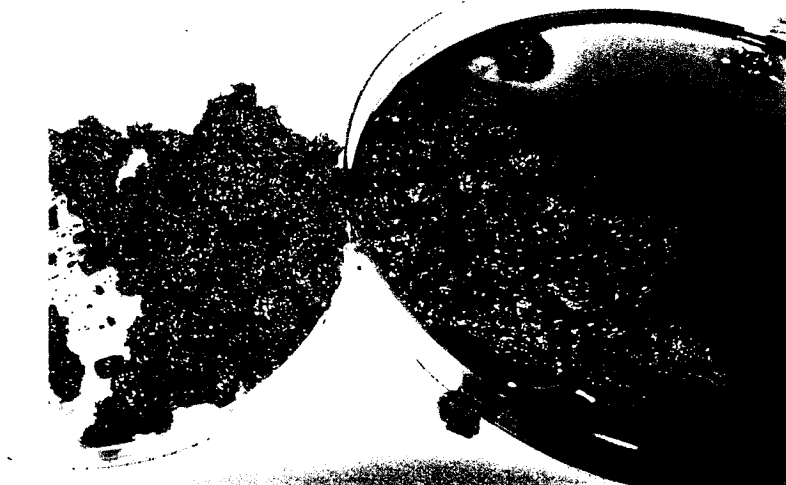


Figure 3-2 Petroleum oily sludge after electrokinetics (left) and before electrokinetics (right)



Figure 3-3 Petroleum oily sludge after the application of the electrokinetics separation technology

3.2.2. Chemicals

- The amphoteric surfactant used in this research was *Alkyl Dimethyl Betain* (CMC = 0.009 M).
- n-Hexane (C_6H_{14}) CAS# 110-54-3 was used in solvent extraction tests

3.2.3. Microbiological Media

Medium (A) was used for culturing the bacteria; the medium contained 3 g/l beef extract, and 5 g/l peptone (Atlas 1997). Agar plates were prepared from Medium (A) by adding 15 g/l Agar.

Medium (B) was used to culture the fungi; the medium contained 24 g/l Potato Dextrose (Atlas 1997). Agar plates were prepared from Medium (B) by adding 15 g/l Agar. The preparation of the media is explained in Appendix G.

3.3. Isolation of Indigenous Microbial Cultures

Microorganisms were isolated from oily sludge collected from the bottom of crude oil storage tanks. 1 gram of the sludge was transferred to 250 ml Erlenmeyer flask containing 90 ml sterile distilled water and was shaken for one hour at 175 rpm in an orbital shaker at room temperature to obtain an emulsion. 1 ml of the emulsion was serially diluted up to 10^{-8} . Then, 0.1 ml of the dilutions was spread onto agar plates with Medium (A) and Medium (B), the plates were incubated at 30°C. Bacterial colonies were observed on plates with Medium (A) after 24 hours. The colonies were classified based on their morphological characteristics and streaked onto new plates of the same medium to ensure their purity. Plates with Medium (B) were incubated for 48 hours before classifying the fungal colonies and streaking them onto new plates of the same medium to obtain single and pure colonies.

To store the isolated bacterial strains, pure cultures of each bacterial strain were prepared in liquid Medium (A) and supplemented with 15% glycerol before being frozen under -20°C. Fungi were grown on slants of Medium (B), and stored under 4°C. The fungal cultures were subcultured periodically.

3.4. Screening the Capability of the Microorganisms to Degrade Petroleum

Hydrocarbons

A special technique developed by Hanson *et al.* (1993) was adapted. The assay uses a redox indicator 2,6-dichlorophenol indophenol (DCPIP) which changes from blue to colorless when reduced. During the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as O₂, nitrates and sulfates. By using DCPIP as an electron acceptor, the ability of microorganisms to utilize hydrocarbons could be

ascertained by monitoring the color change of DCPIP. Microorganisms have different oxidation abilities that result in variable time for color changes.

The test was carried out in 16-well micro-plates, each well contained; 1 ml sterile Bushnell-Hass medium, and 0.1 ml of 0.15 g/l filter sterilized 2,6-dichlorophenol indophenol (DCPIP). The wells were inoculated by 0.5 ml of diluted bacterial culture prior to overlaying with 0.01 ml of filter sterilized Arabian light crude oil. Three replicates were prepared for each bacterial strain. Control wells were prepared in the following manner: crude oil and DCPIP, DCPIP and Bushnell-Hass medium, inoculum and DCPIP, and DCPIP alone. The plates were covered and incubated at 30°C and monitored to check the change in color.

3.5. Identification of the Isolated Microorganisms

3.5.1. Identification of the Fungi

To identify the fungal strain, the fungus was grown on agar plates containing Medium (B) for seven days. After that the fungus was observed under the microscope and identified using standard identification keys available in De Hoog *et al.* 2000 and Domsch and Grams 1993.

3.5.2. Identification of the Bacteria

To identify the bacteria, a series of biochemical tests were conducted, and the bacteria was identified from the resulting biochemical profile. The biochemical tests are described in the following sections.

3.5.2.1. Gram Coloration

A drop of 0.85% saline solution was transferred to a staining slide using a sterile loop. Few bacterial cells were transferred to the slide where the saline droplet was located. The droplet was left to dry. After that a series of colored solutions were applied to the slide (violet, iodine, decolorizer, pink), and each solution was maintained on the slide for a specific time which was dependent on solution type. Each colored solution was washed using tap water before applying the subsequent one. Finally, bacterial cells were observed under the microscope (i.e. 1000x magnification). Blue color is indicative of Gram +ve while a pink color represents Gram -ve. The theory behind this test originated from the fact that Gram +ve bacteria have a cellular membrane that is able to retain the violet color after applying the decolorizer, while Gram -ve cells do not. Therefore, application of the pink color would result in Gram -ve cells retaining the pink color.

3.5.2.2. Metabolism of Carbohydrates and other Substrates

Carbohydrate metabolism was tested using (API 50 CH) strip obtained from bioMérieux[®] Inc. The strip contained 49 types¹ of dehydrated carbohydrate; each type was in a separate micro-tube. To do the test, the inoculum of the bacterium under study was prepared in a nutrient broth², which contained phenol red as indicator. The turbidity of the medium was adjusted and the 49 micro-tubes were inoculated in addition to a control tube (i.e. did not contain any substrate). The strip was incubated at 37°C for 24 hours. During the incubation, metabolism of carbohydrates forms acids that decrease the pH of the medium and change the color of the indicator from red to yellow.

¹ The names of the substrates in this strip are listed in Appendix C

² bioMérieux[®] product no. API 50 CHB/E medium, ingredients of the medium are listed in Appendix C

A Supplementary strip (API 20 E) was used to verify the growth on substrates other than carbohydrates (e.g. sodium thiosulfate, urea, gelatin). A detailed list of the substrates is shown in Appendix C. The strip consisted of 20 micro-tubes and only 12 of them were used since the others contained substrates that were already in strip API 50CH. Each micro-tube was inoculated with a bacterial suspension and incubated at 37°C. During incubation, metabolism produced color changes that were either spontaneous or revealed by the addition of special reagents.

The results of both tests were recorded, thereby allowing for the formation of a biochemical profile related to the bacteria, this profile was entered to special computer software that interpreted the results, based on its built-in database, and gave out the genus of the bacteria.

3.5.2.3. Catalase and Oxidase Tests

These two tests verified the existence of specific enzymes responsible for aerobic respiration in the bacterial cells. They were conducted to complete the biochemical profile about the bacteria. In the catalase test, few drops of hydrogen peroxide (H₂O₂) were added to the bacterial cells. The formation of air bubbles indicates aerobic bacteria and test is considered positive; otherwise they are catalase negative. Catalase is an enzyme presents in the cells of aerobic bacteria, and it promotes the conversion of hydrogen peroxide to water and molecular O₂ (i.e. air bubbles). In the oxidase test, few drops of cytochrome were added over bacterial cells that were spread on a white filter paper. The filter was observed for the formation of purple color. If the spot where the bacteria were deposited turns purple within 15 seconds the test is positive.

3.6. Assessment of Sludge Bio-Neutralization

3.6.1. Development of the Testing Technique

The testing technique was designed to be simple and efficient for bench-scale studies. The main inspiration of this technique came from the nature of the oily sludge, which contained after applying the separation technology 73% organic carbon, 20% solids, and 7% water (Habibi and Elektorowicz 2004). By placing a filter paper on the sludge, the hydrocarbons would diffuse through the filter and be accessible for the microorganisms. Also, the sludge contained more than 50% alkanes, which is saturated aliphatic compounds that could be studied using Fourier Transform Infrared Spectrometry (FTIR). By studying the FTIR spectra of the sludge during the treatment process, the band which represents the carbon-hydrogen bond will give an indication of the aliphatic hydrocarbons that remained in the system (Griffiths and Rebhum 1986, Silverstein and Webster 1998). To obtain a comprehensive indication about the transformation of petroleum hydrocarbons as a result of the treatment process, solvent extraction with n-hexane was employed.

Microbial growth on the filters was quantified by counting the colonies on agar plates; since the filter will prevent the microorganisms from passing down to the oily sludge. This was ensured by using filter paper 0.22 μm (Hasegawa *et al.* 2003).

3.6.2. Experimental Protocol for Oily Sludge Bio-Neutralization

The protocol was conducted using the following procedure:

- 1- Samples of the oily sludge (i.e. average weight 0.18 g) were placed in special cups; 13 mm diameter and 3 mm depth (see Figure 3-4).
- 2- The cups were topped by 0.22 μm sterile filters (i.e. Millipore[®] brand, mixed ester cellulose, non-degradable filters), and left in contact with the sludge for 30 min before the deposition of the inoculum.
- 3- 0.05 ml of inoculum was placed on the top of the filter. The inoculum was prepared in Bushnell-Hass solution as described in Appendix H.
- 4- The cups were incubated in the dark at 30°C and moist conditions for 15 days.
- 5- Moisture was maintained over the cultures by adding sterile Bushnell-Hass medium every three days.
- 6- Periodic checks were conducted on day: 4, 10, and 15, to assess the growth of microorganism and sludge degradation.

The growth was assessed using the plate-counts technique as described in section 3.6.3. To assess sludge degradation, the sludge in the cups was scanned using the FTIR machine; the spectra were analyzed as described in section 3.6.4.1 and Appendix F. To estimate TPH in the sludge, the sludge was transferred to a 15 ml vial and extracted with 8 ml hexane as explained in section 3.6.4.2.

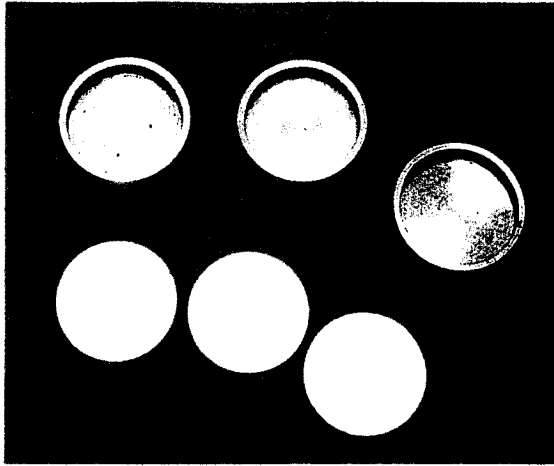


Figure 3-4 The cups and filters used in the experiments

The testing protocol was conducted in six separate experiments; two types of oily sludge and three types of inoculum were used as follows:

1. Oily sludge and fungal- bacterial inoculum
2. Oily sludge and bacterial inoculum
3. Oily sludge and fungal inoculum
4. Oily sludge with surfactant and fungal-bacterial inoculum
5. Oily sludge with surfactant and bacterial inoculum
6. Oily sludge with surfactant and fungal inoculum

Control cells containing oily sludge or oily sludge with surfactant (i.e. non-inoculated) were incubated under the same conditions as the inoculated cells. However, the sludge inside control cells was not sterile.

3.6.3. Growth Assessment

To assess the growth, at the end of each experimental period the filters were transferred to a sterile 15 ml vials, which contained 5 ml of 0.85% sterile saline solution. The vials were shaken gently to slip the cells and spores off the filter, and 1 ml of the mixture was serially diluted in a sterile saline solution. 0.1 ml of dilution was spread on agar plates containing Medium (A) and Medium (B). The plates were incubated under 30°C. Bacterial colonies were counted after 24 hours on plates with Medium (A), and fungal colonies were counted after 48 hours on plates with Medium (B).

3.6.4. Assessment of Sludge Degradation

3.6.4.1. Fourier Transform Infrared Spectrometry (FTIR)

Infrared (IR) radiation refers to the part of the electromagnetic spectrum of wavelength longer than the red end of visible light and shorter than microwaves, approximately between 1 and 100 microns. Any molecule even simple ones can give a complex spectrum and organic chemists take advantage of this complexity when matching the spectrum of an unknown compound against that of a known sample, since compounds are unlikely to give exactly the same IR spectrum. Chemists don't solely depend on IR spectra for identification of compounds. Therefore, detailed analysis of spectrum will not be required.

Infrared radiation of wavenumber¹ in the range from 10,000-100cm⁻¹ is absorbed and converted by an organic molecule into energy of molecular vibration. The vibrations are converted to spectra of bands from which the absorption can be quantified. The bands between 4000 and 400 cm⁻¹ are of particular interest in the analysis. Band intensities can be expressed as Absorbance (A) or transmittance (T). The absorbance is logarithm, to the base 10, of reciprocal of the transmittance. Carbon hydrogen stretching bond (C—H) in hydrocarbons can be identified using two bands; one in the region 3001-2700 cm⁻¹, and the other band is around 1435 cm⁻¹. However, absorption regions of other bonds are also reported in the literature as shown in Table 3-1.

Table 3-1: Absorption regions of selected bonds

Bond Type	Absorption Region (cm ⁻¹)
C=O ²	2390-2300
O—H	3800-3200
Si—O	1110-830

Adapted from (Silverstein and Webster 1998)

Fourier transform Infrared Spectrometer (FTIR) is an enhanced version of IR spectrometers. It splits the IR radiation into two beams; fixed length and variable length. The resulting interferogram is converted by fast Fourier transform software from time domain to frequency domain, such that, the data will be in the form of spectra which can

¹ Wavenumber (cm⁻¹)= 1x10⁴/wavelength(μm)

² in Carbon dioxide

be easily analyzed. Several techniques have been used to collect the radiation in an FTIR machine as demonstrated by Diem (1994) and Griffith (1986). In this research, the Diffuse Reflectance technique was employed, since it is the most suitable for the material under study. This method takes advantage of the fact that most substances, in their natural state (e.g. rough solid surface or powder), exhibit diffuse reflection. The incident light is scattered in all directions as opposed to reflection surface. The machine is equipped with optical devices to maximize the collection of this reflected radiation, which is then analyzed by the machine and transformed to common spectra.

The absorbance in IR spectra could be related to the concentration by Beer-Lambert Law (ASTM 1999)

$$A = k C l$$

Where:

A = absorbance of the sample at a specified wavenumber,

k = absorptivity of the component at this wavenumber,

C = concentration of the component,

l = sample path length.

In this research, the FTIR was used to examine the change in C-H bond, which represents the aliphatic hydrocarbons content of the oily sludge. The samples were scanned by the FTIR machine, and their spectra were analyzed for broad bands. Band in the range of (3001-2742 cm^{-1}) with a peak at 2945 cm^{-1} was chosen to track the change in C-H stretching bond. This band was broad and the changes due to biological treatment were notable in this range.

To make the absorbance values comparable, since the amount of the sludge in the cups are not identical, the absorbance value at the wavenumber 2945 cm⁻¹ was divided by the absorbance of a band in the range of (1100-1000 cm⁻¹) and a peak at wavenumber 1064 cm⁻¹. This band represents Si-O bond, which is independent of the biological oxidation reaction and depends only on the amount of the sludge in the cup. The results of FTIR test were presented as relative absorbance¹:

$$\text{Relative absorbance} = A_{2945} / A_{1064}$$

Since the absorbance is directly related to the concentration, the decrease in absorbance will indeed imply a decrease in the concentration of that bond and the compounds that contain this bond.

3.6.4.2. Hexane Extraction

Hexane extraction was used to measure TPH content of the oily sludge. Studies on petroleum have used the term Total Petroleum Hydrocarbons (TPH) to represent the hydrocarbon content in the material under study. Petroleum hydrocarbons are a family of several hundred chemical compounds. Therefore it is not practical to measure each one separately. The use of n-Hexane as a solvent has been promoted by EPA as replacement for Feron-113 a Class (I) Chlorofluorocarbon gas (CFC) as defined in Montréal Protocol (EPA 1999).

¹ See Appendix F for calculations

In this research the hexane was mixed with the oily sludge and allowed to settle. When two phases were formed, the hexane layer was transferred to a pre-weighed vial. The vial was left in the fume hood, thereby allowing for solvent volatilization. After reaching a constant weight, remaining non-volatile residue was calculated by subtracting the weight of the vial and residual TPH from the weight of the empty vial. The weight of the non-volatile TPH was divided by the weight of the sample¹. The resulting ratio represents the TPH content of the oily sludge.

¹ Sample calculations are shown in Appendix F

CHAPTER 4 RESULTS AND DISCUSSION

4.1. Isolation of Indigenous Petroleum Hydrocarbons Degrading Microorganisms

Six bacterial strains were isolated based on the color and the morphology of the colonies as shown in Table 4-1. Figure 4-1 shows the colonies before being purified. Streaks of each strain were made on new agar plates to obtain pure colonies.

Table 4-1 Characteristics of the isolated bacterial colonies¹

Morphology	Colony name					
	B01	B02	B03	B04	B05	B06
Shape	rhizoid	crenated	irregular	rounded	rounded	irregular
Elevation	flat	raised	flat	raised	raised	raised
Surface	ND ²	smooth	smooth	smooth	domed	ND
Color	white	white	bold whit	yellow	white	white
Number isolated (colonies/gram of sludge)	80	210	40	1	2	1

1 See Appendix E for the terminology used in colony description

2 ND= Not determinate

Two strains of fungi were isolated on potato dextrose plates. They were different in color and growth characteristics; the first strain had yellowish brown mycelia and spread quickly on the plate. However, the second strain had mixed white and green mycelia, and its growth remained centered on the inoculation point. Table 4-2 contains a detailed description of both fungi. The fungi were purified by growing them on separate plates. Then the fungal strains were stored on slant medium at 4°C, and subcultured periodically. Figure 4-2 shows fungi colonies on potato dextrose agar plates before

culturing them on separate plates (See Appendix B-2 for more pictures from this experiment).



Figure 4-1 Bacterial strains isolated from petroleum refinery sludge

Table 4-2 Characteristics of the isolated fungal strains

Strain name	Description	
	Color	Growth
SLG	yellowish- brown	spread broadly over the plate, it covers a 10 cm Petri dish plate in 9 days
FG	white mycelium, with a greenish nucleus	forms rounded colonies of confined margins, slow growth rate

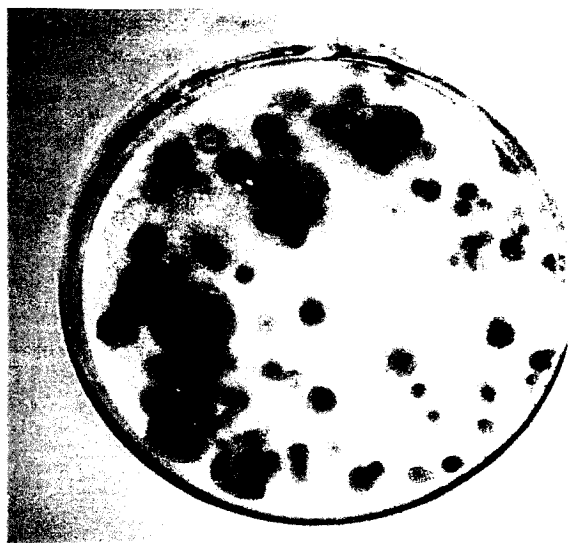


Figure 4-2 Fungal strains isolated from petroleum refinery sludge

4.2. Screening the Potential of the Isolated Strains to Degrade Petroleum

Hydrocarbons

Strain B01 was the first to change the color of the indicator; the blue color disappeared from three replicates of B01 after 18 hours. Other strains changed the color but after four weeks of incubation. The final chronological sequence of color change was (from first to last); B01, B04, B03, B02, B05, B06. Figure 4-3 shows the plate that contained strain B01 after 18 hours of incubation under 30°C, Additional photos from this experiment are shown in Appendix B-3. The same protocol was applied to the fungal strains; the fungus (SLG) was the first to change the color, but after one week. The fungus (FG) did not change the color of the indicator over a one month incubation period at 30°C.

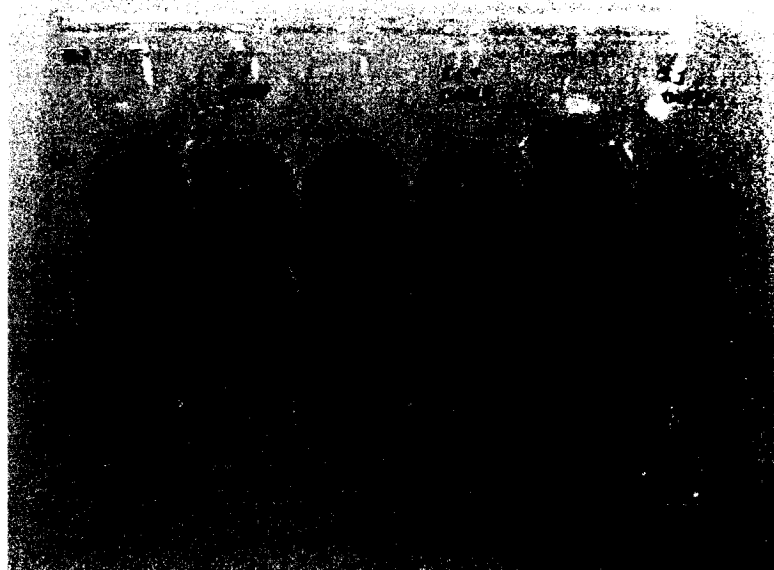


Figure 4-3 Screening test results (B01, DCPIP&B01, B02, DCPIP&B02, B02, DCPIP&B03 consequently from left to right)

4.3. Identification of the Bacteria and the Fungi

All identification experiments were done in INRS-Institut Armand-Frappier labs (Laval, Quebec). The bacterial strain (B01) was identified by applying the tests described in section 3.5.2. Microscopic examination demonstrated the strain B01 belongs to *Bacillus* genus, and gram coloration test revealed that B01 was Gram +ve. Catalase and oxidase tests results were both positive indicating aerobic bacteria. The biochemical profile for the bacteria was established using API 50 CH and API 20 E strips¹, the results obtained from the software demonstrated that B01 was the bacterial strain *Bacillus cereus*.

¹ More details about the strips are available in Appendix C

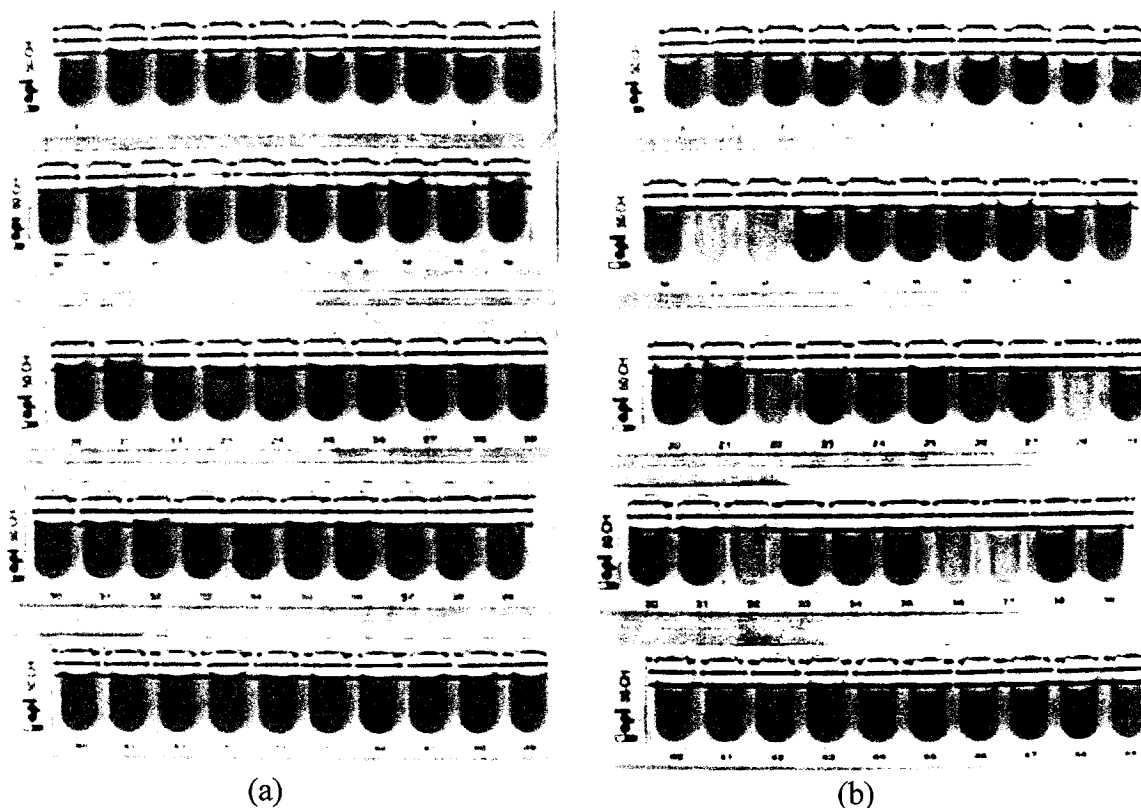


Figure 4-4 Carbohydrate metabolism by *Bacillus cereus* using API[®] 50 CH strip (a) before incubation (b) after 24hrs incubation at 37°C

Figure 4-4 shows the results of carbohydrate metabolism. The output of the software and detailed results are shown in Appendix D. More photos from this experiment are shown in Appendix B-4.

Fungus (SLG) was identified under microscope using standard identification keys in De Hoog *et al.* 2000 and Domsch and Grams 1993 as described in section 3.5.1. The fungus (SLG) was identified as *Paecilomyces variotii*.

4.4. Oily Sludge Degradation and Cultures Growth

The experiments on oily sludge were conducted as explained in section 3.6.2. The results are demonstrated below according to the type of inoculum used.

Fungal-Bacterial inoculum (*P. variotii* & *B. cereus*)

The fungal-bacterial co-culture of *P. variotii* and *B. cereus* decreased the TPH content of the sludge by 34% over a 15-day incubation period. Degradation rates were not improved when amphoteric surfactant was used as shown in Figure 4-5. TPH content of the sludge decreased from 50% to 25.3% during the first 4 days. However, in control samples (i.e. not sterile, not inoculated), TPH decreased by 9% during the same period. The controls did not show a significant decrease in TPH for the remainder of the incubation period.

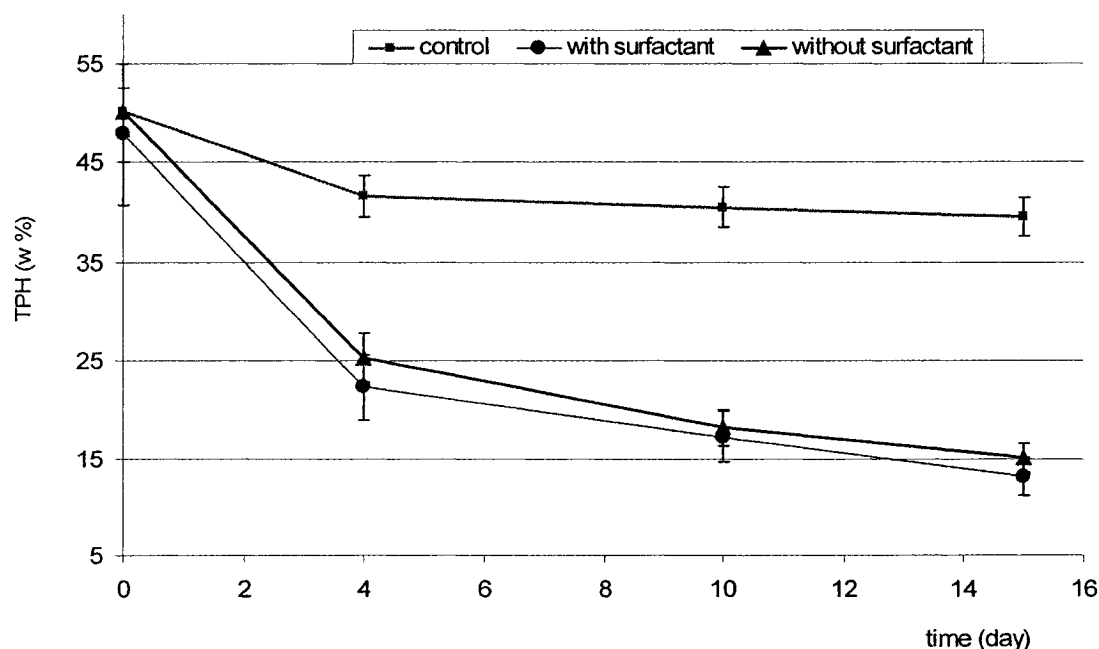


Figure 4-5 Decrease in TPH content of the oily sludge when treated with the fungal-bacterial co-culture. Error bars show the standard deviation of four replicates

FTIR spectra of oily sludge showed an 81.7% decrease in relative absorbance values over the 15-day incubation period. This can be used to represent the decrease in aliphatic hydrocarbons, because the band under study ($3001-2742\text{cm}^{-1}$) represents C-H bonding, which is in all aliphatic compounds. Figure 4-6 shows that degradation rates in oily sludge with surfactant were slightly lower than those of the oily sludge without surfactant. The overall decrease in aliphatic hydrocarbons in oily sludge with surfactant was 83.5%. The decrease in aliphatic compounds was obvious in the FTIR spectra of the oily sludge with and without surfactant respectively as shown in Figures 4-7 and 4-8

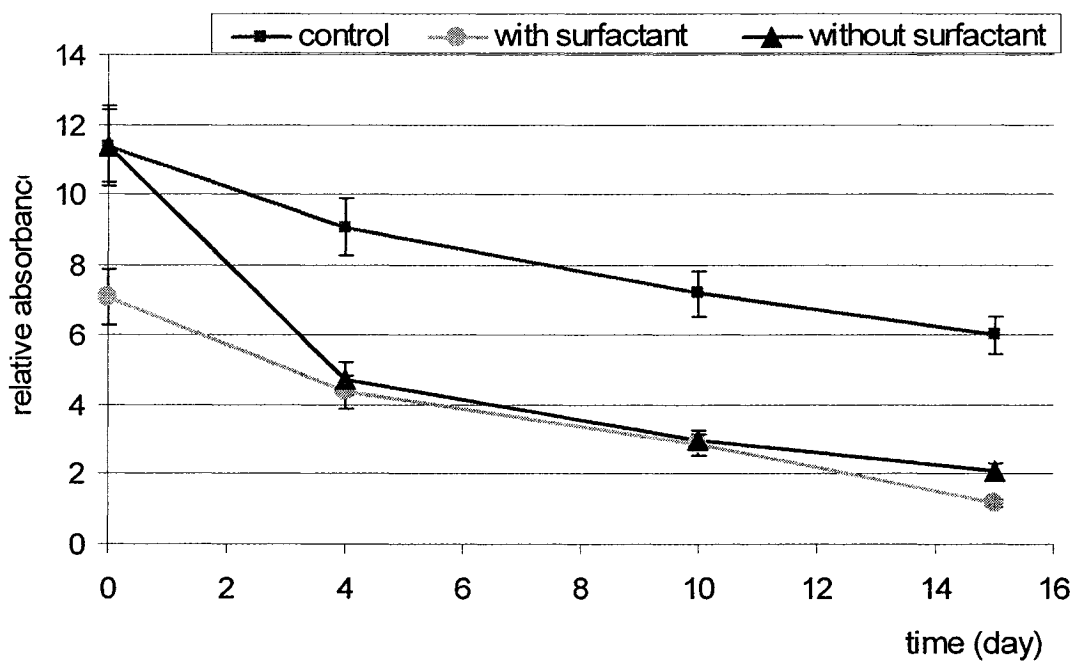


Figure 4-6 Decrease in FTIR relative absorbance in oily sludge with and without surfactant when treated with the fungal-bacterial co-culture. Error bars show the standard deviation of four replicates

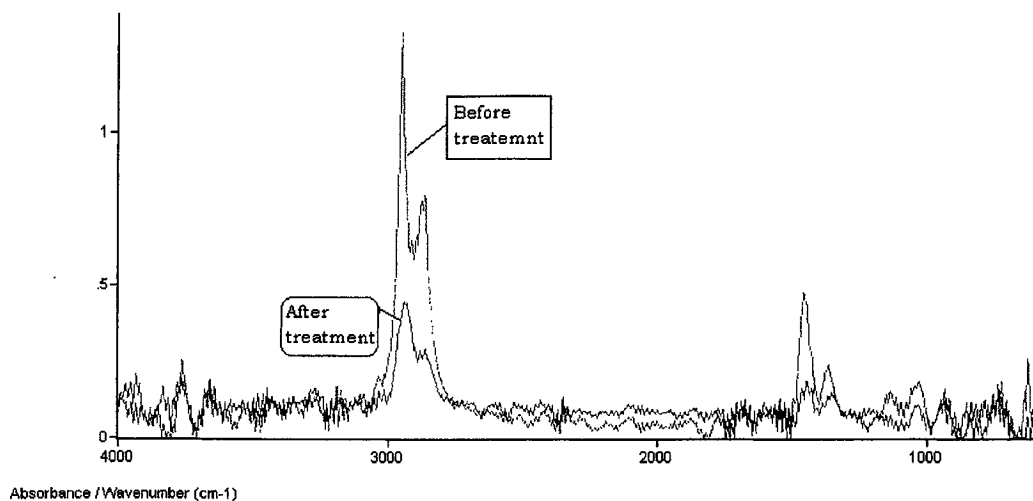


Figure 4-7 FTIR spectra of oily sludge with surfactant before and after treatment with the fungal-bacterial co-culture

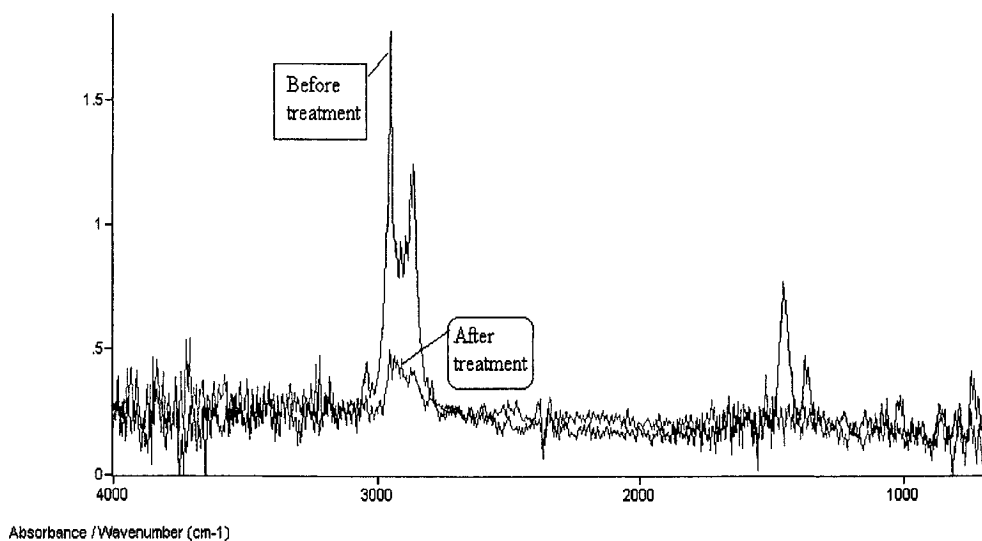


Figure 4-8 FTIR spectra for oily sludge before and after treatment with the fungal-bacterial co-culture

The results of plate counts test on the fungal-bacterial inoculum are presented in Figures 4-9 and 4-10. The surfactant seemed to reduce the adaptation time and helped the population of *B. cereus* to remain close to the initial level; because on oily sludge without surfactant, the population of *B. cereus* decreased by 50% in the first 4 days. Between day

5 and day 12, the bacterial culture seemed to enter a stationary growth phase as shown in Figure 4-9. In general, bacterial population did not increase significantly throughout the incubation period. On day 4, the population increased 1.6% on samples of oily sludge with surfactant. However, the highest population on oily sludge without surfactant was (2.63×10^4 CFU/ml) which represented 40% decrease from the initial population.

The fungus *P. variotii*, showed remarkable growth on the sludge as demonstrated in Figure 4-10. A rapid growth phase seemed to occur during the first 4 days, followed by a stationary phase that ended on day 12. After that the growth started to decline. The growth was better on oily sludge with surfactant. On day 10, the fungal counts were 5.25×10^4 (CFU/ml), which was 30 times higher than the initial population (1.7×10^3 CFU/ml). However, in oily sludge without surfactant, the day-10 culture had only (1.4×10^4 CFU/ml) that is 8 times higher than the initial culture (1.73×10^3 CFU/ml).

Figure 4-11 shows the growth of the co-culture on the sludge

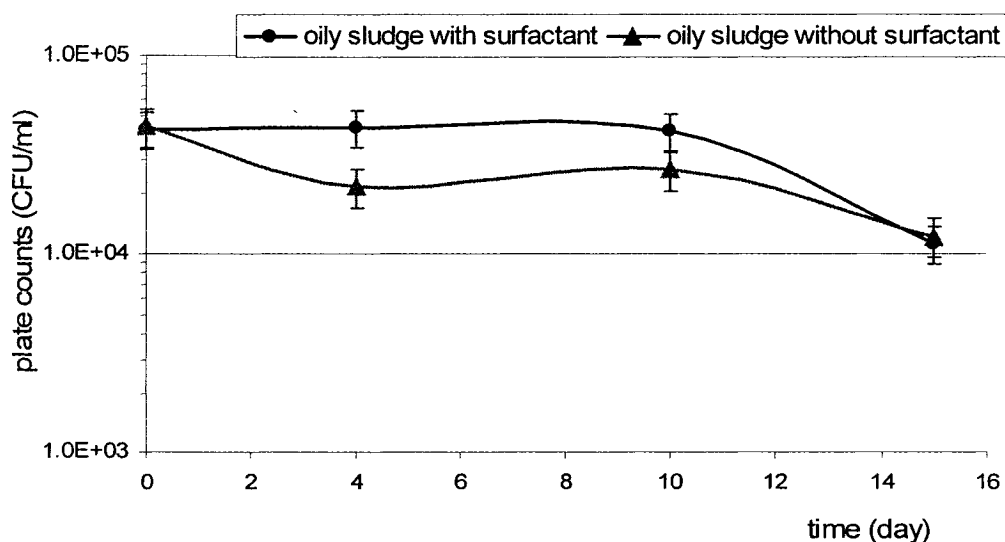


Figure 4-9 Plate counts of the bacterial strain (*B. cereus*) on oily sludge treated by the fungal-bacterial co-culture. Error bars show the standard deviation of four replicates

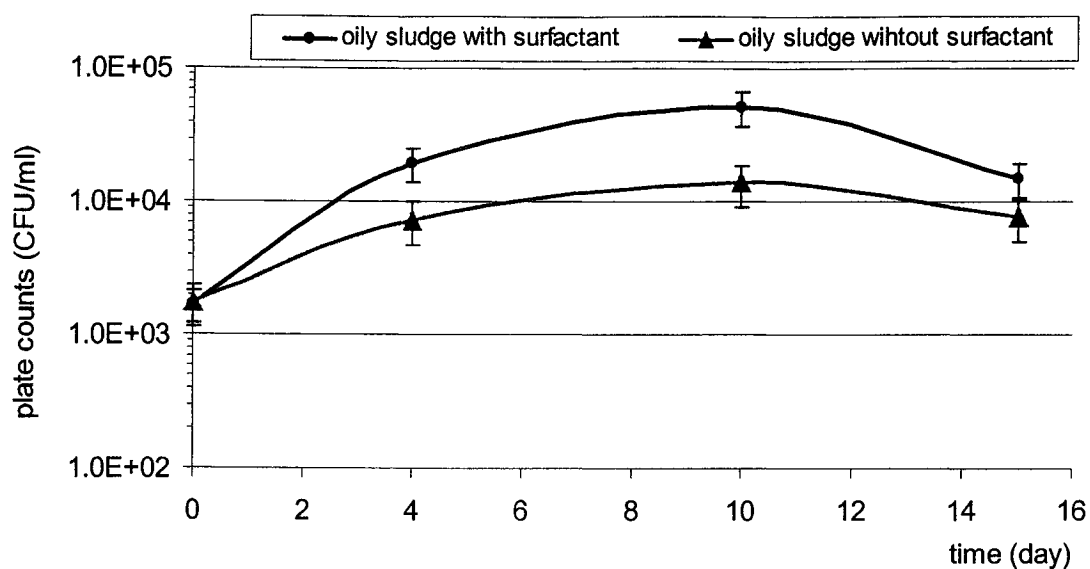


Figure 4-10 Plate counts of the fungal strain *P. variotii* on oily sludge treated by the fungal-bacterial co-culture. Error bars show the standard deviation of four replicates



Figure 4-11 *P. variotii* and *B. cereus* growing on the oily sludge

Bacterial inoculum (*B. cereus*)

The TPH content of the oily sludge decreased by 36.3% when bacterial inoculum was used, Figure 4-12 shows that 25.7% of the TPH decreased during the first 4 days. However, TPH in the control samples decreased by 9% over the same time period.

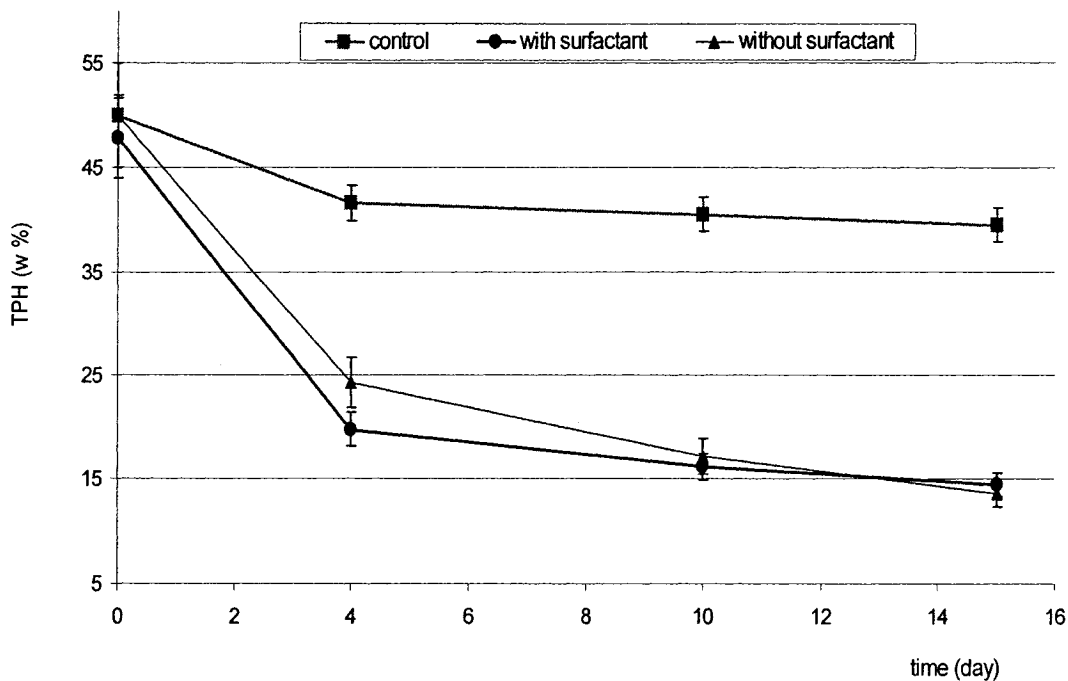


Figure 4-12 Decrease in TPH of oily sludge when treated by the bacterial inoculum. Error bars show standard deviation of four replicates

When bacterial inoculum was used, aliphatic hydrocarbons decreased by 90% over 15 days of incubation. The surfactant did not enhance the utilization of aliphatic hydrocarbons, since only an 82% reduction in the aliphatic compounds was achieved. Aliphatic hydrocarbons in control cells decreased by 47.4% as shown in Figure 4-13. The change in the FTIR spectra of the sludge before and after treatment with bacterial inoculum of *B. cereus* is shown in Figures 4-14 and 4-15.

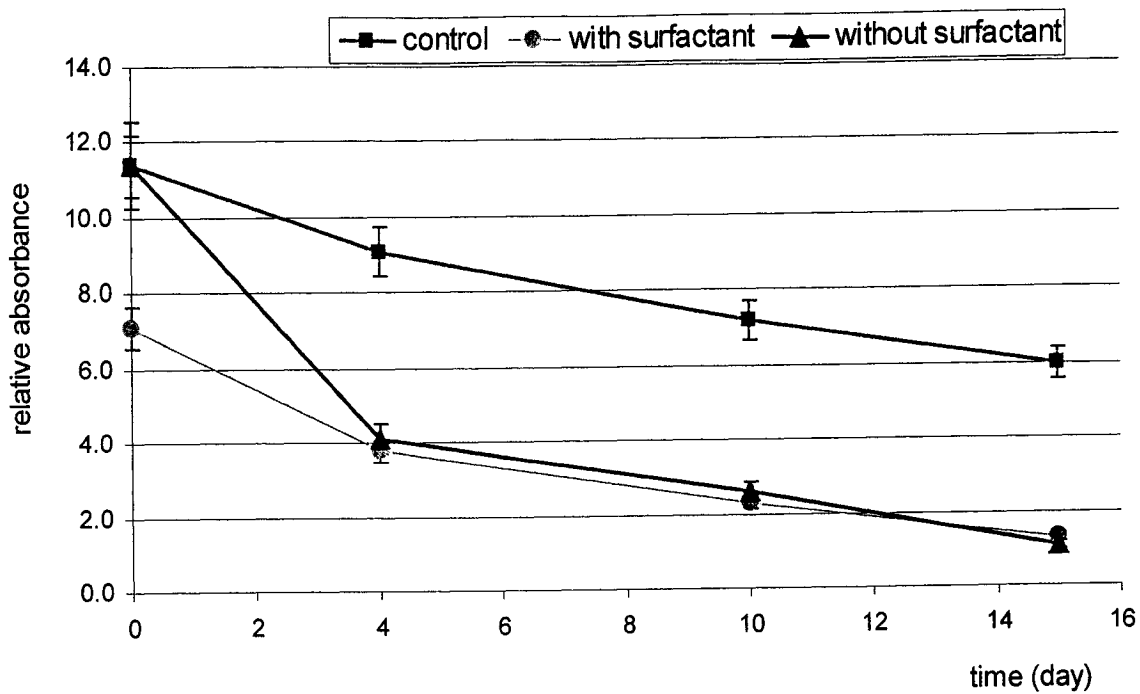


Figure 4-13 Decrease in FTIR relative absorbance of oily sludge when treated by the bacterial inoculum. Error bars show the standard deviation of four replicates

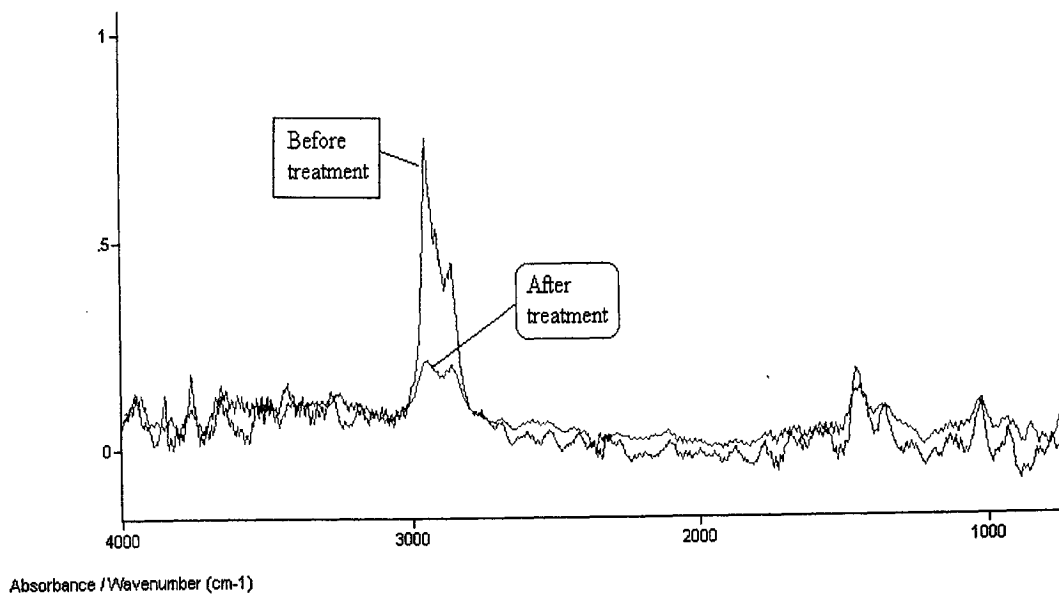


Figure 4-14 FTIR spectra of the oil sludge with surfactant before and after treatment with *B. cereus*

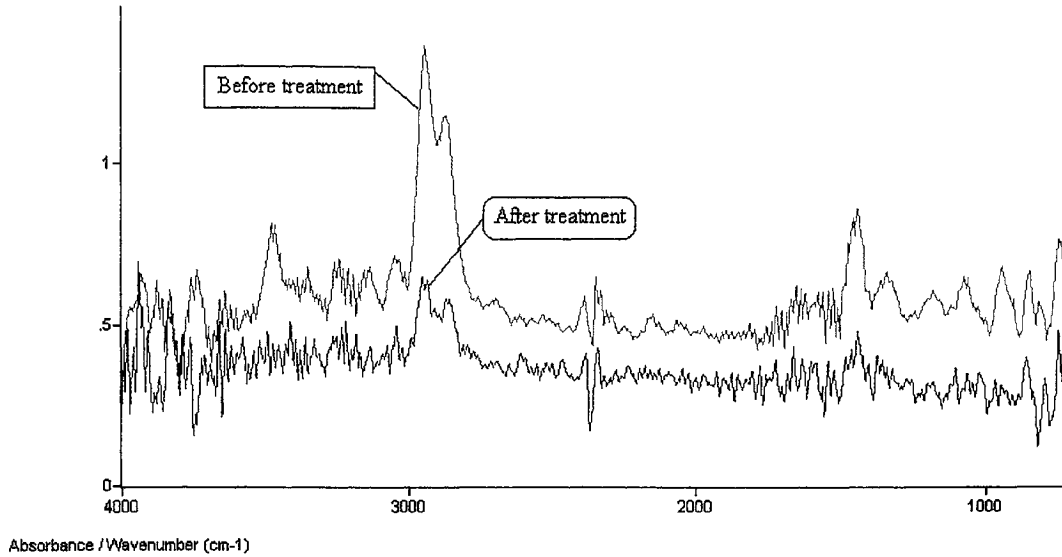


Figure 4-15 FTIR spectra of the oil sludge before and after treatment with *B. cereus*

Figure 4-16 shows a remarkable growth of *B. cereus* on the oily sludge; the population of bacteria increased significantly. On day 4, bacterial counts on oily sludge were (9.1×10^4 CFU/ml) that is 37% increase in the initial population. The surfactant seemed to increase the stationary growth phase of the bacteria as shown in Figure 4-17. The growth of the bacteria seemed to decline in the period between day 10 and day 15 on both types of sludge.

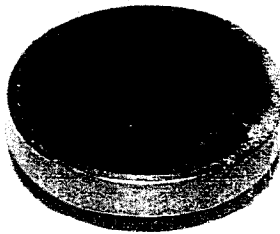


Figure 4-16 The growth of the bacterial strain *B. cereus* on sludge

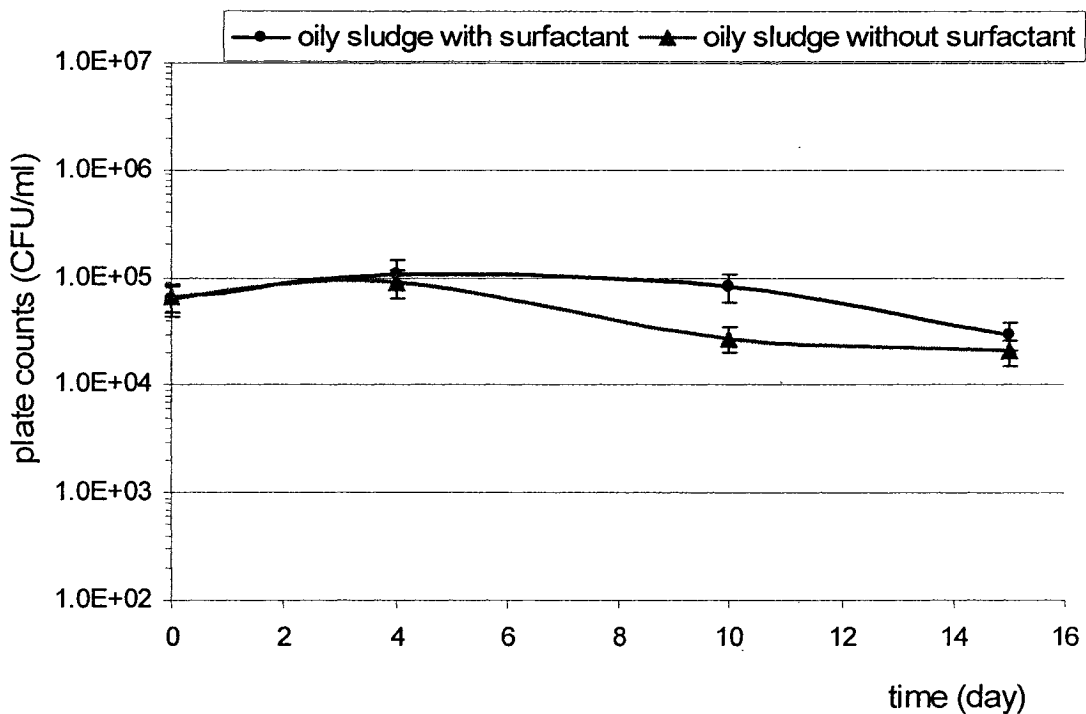


Figure 4-17 Plate counts of the bacterial strain *B. cereus* on oily sludge treated with the bacterial inoculum. Error bars show standard deviation of four replicates

Fungal Inoculum (*P. variotii*)

The fungal culture of *P. variotii* decreased the TPH content of the oily sludge by 26% over the 15-day incubation period. The amphoteric surfactant improved the degradation but slightly; TPH of oily sludge with surfactant decreased by 27% over the incubation period. Between day 4 and day 15, the decrease in TPH seemed to occur in a linear slope as shown in Figure 4-18. However, as in the results of the other inocula (i.e. fungal-bacterial and bacterial inocula), high drop in TPH occurred during the first 4 days.

Aliphatic hydrocarbons content of the oily sludge decreased by 74% over the 15-day incubation period as shown in Figure 4-19. The surfactant did not improve the degradation of aliphatic hydrocarbons; since relative absorbance values decreased by

only 67% throughout the incubation period. Figures 5-20 and 5-21 show the change in FTIR spectra of the oily sludge before and after treatment with fungal inoculum.

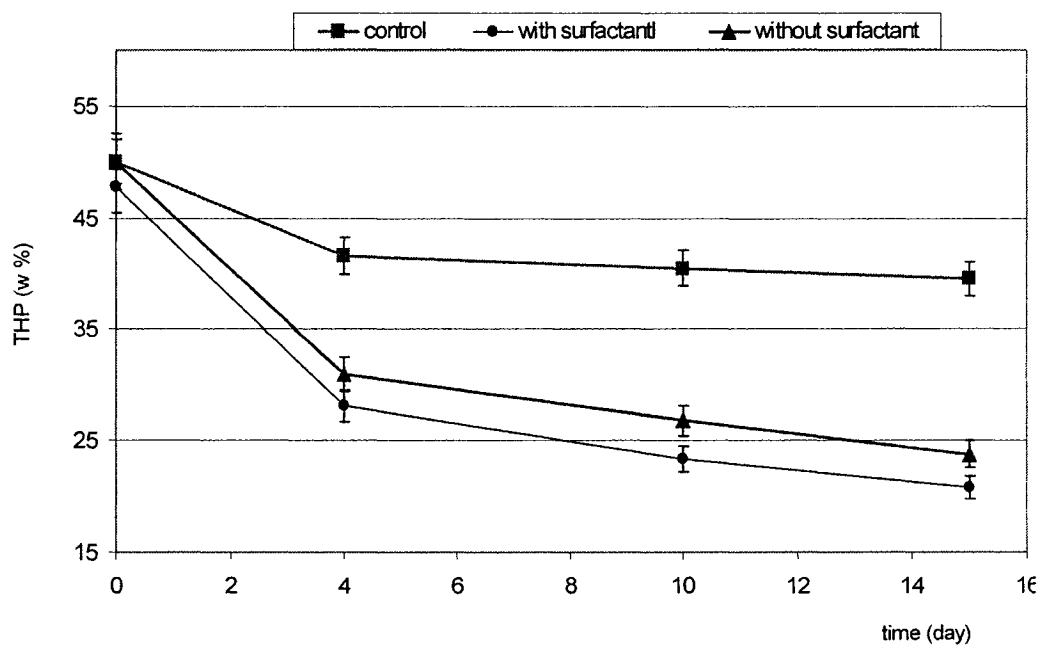


Figure 4-18 Decrease in TPH of the oily sludge when treated by the fungal inoculum. Error bars show standard deviation of four replicates

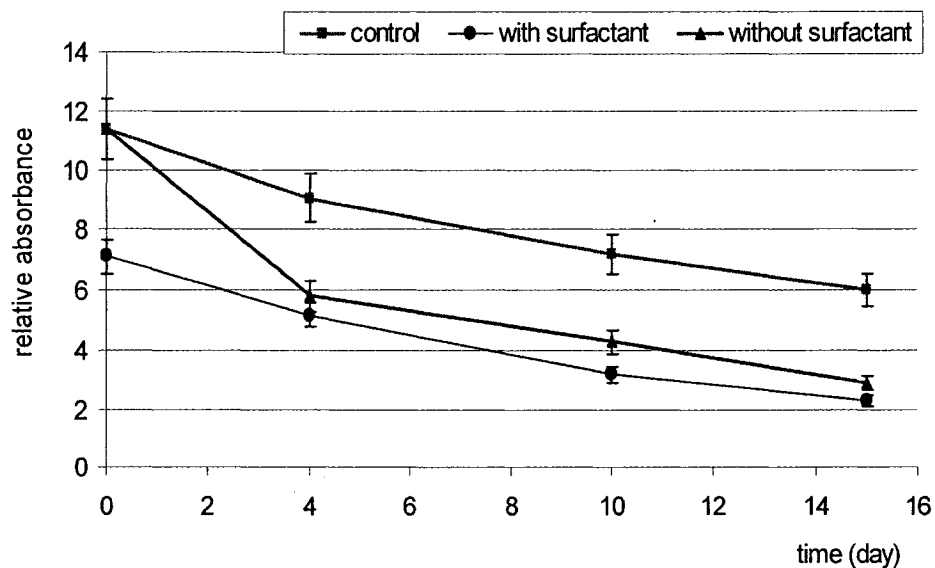


Figure 4-19 Decrease in FTIR relative absorbance in oily sludge when treated by the fungal inoculum. Error bars show standard deviation of four replicates

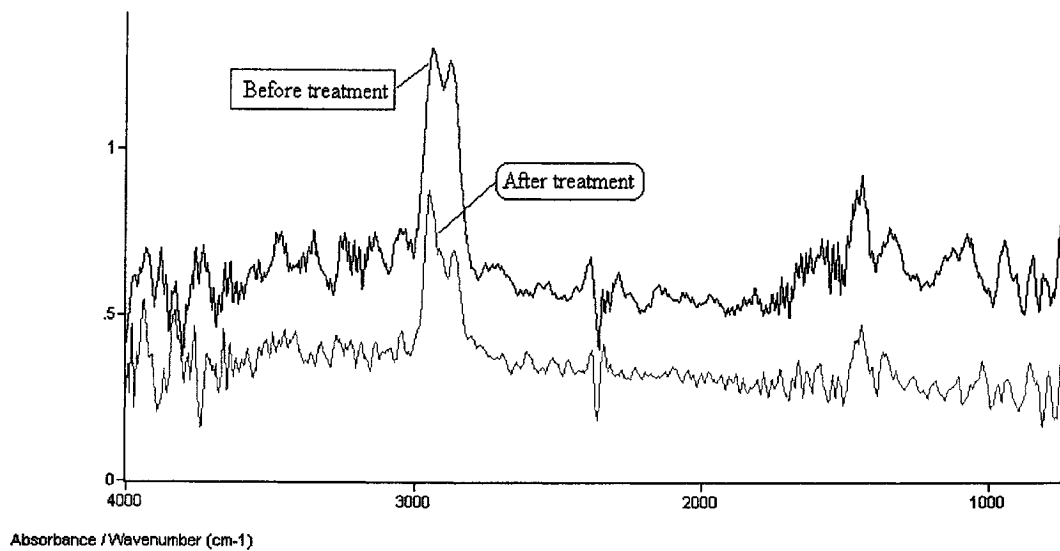


Figure 4-20 FTIR spectra of the oil sludge before and after treatment with the fungus *P. variotii*

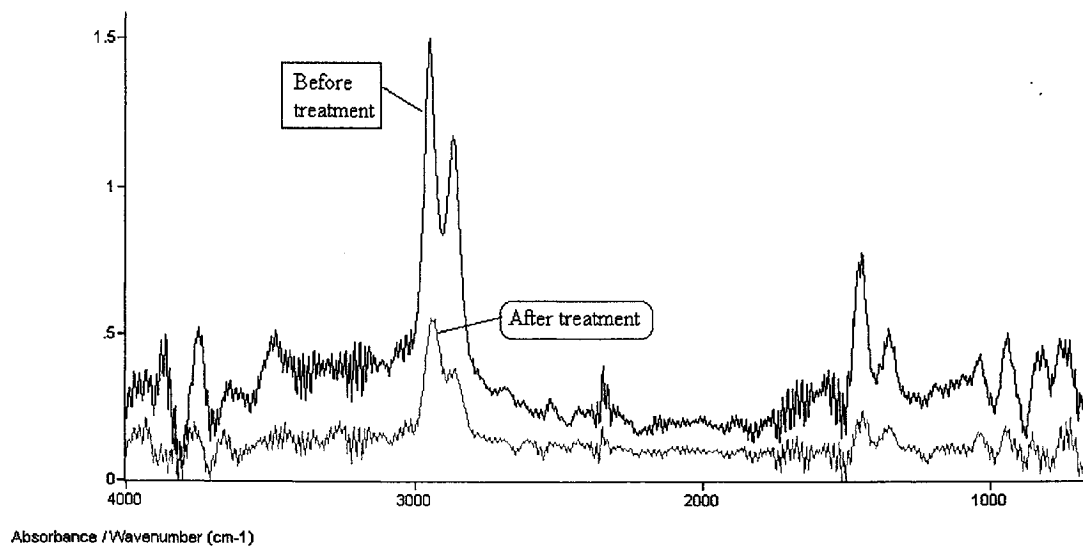


Figure 4-21 FTIR spectra of the oil sludge with surfactant before and after treatment with the fungus *P. variotii*

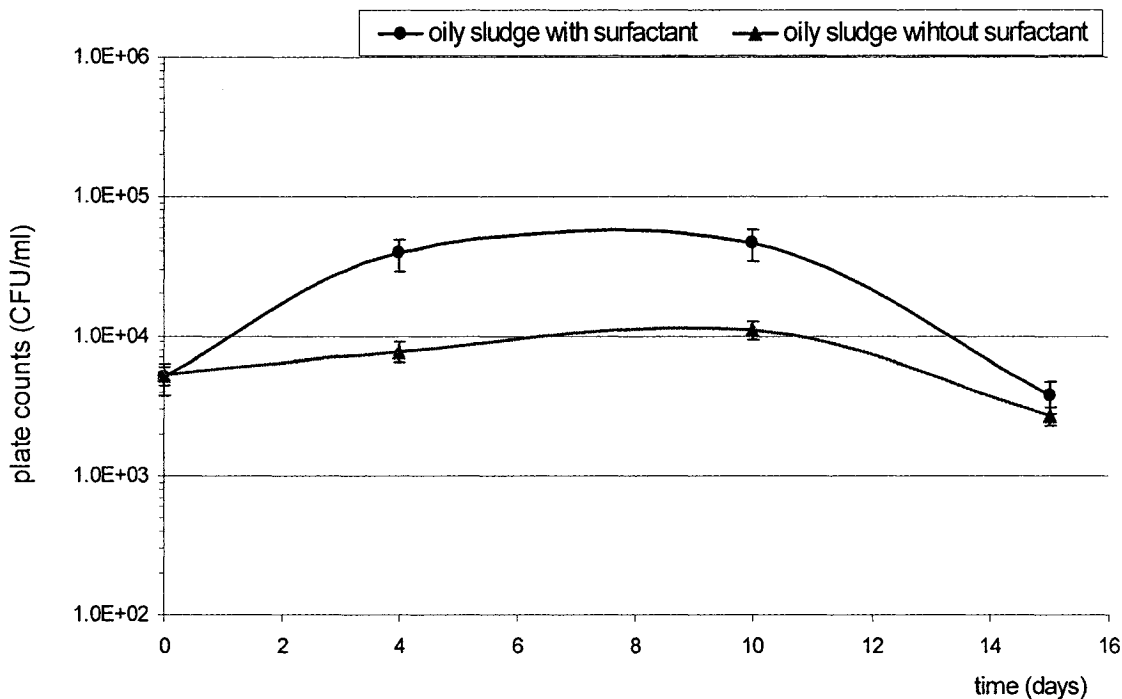


Figure 4-22 Fungal inoculum plate counts on oily sludge. Error bars show standard deviation of four replicates

The growth of *P. variotii* on oily sludge was notable; its population increased up to $(1.1 \times 10^4 \text{ CFU/ml})$ on day 10 this is 2 times the initial culture $(5.2 \times 10^3 \text{ CFU/ml})$. The growth was improved significantly when the surfactant was used; day 10 counts were $(4.61 \times 10^4 \text{ CFU/ml})$ that is 9 times the initial culture $(5.03 \times 10^4 \text{ CFU/ml})$, Figure 4-22 shows the change in *P. variotii* plate counts. Figure 4-23 shows the growth of this fungus on oily sludge with surfactant; it is notable that the fungus *P. variotii* did not develop the same color and intensity of mycelia it did on agar plates.

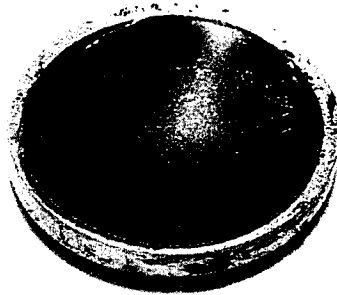


Figure 4-23 Growth of the fungal strain *P. variotii* on the oily sludge

4.5. Discussion

The efficiency of the fungal-bacterial co-culture in oily sludge biodegradation

The results showed that the fungal-bacterial co-culture was capable of degrading petroleum hydrocarbons in the oily sludge. Figure 4-24 compares the results of the three types of inocula used in this research, 35% of TPH¹ in the oily sludge were degraded, and aliphatic hydrocarbons content was decreased by 82% from its original level. Single cultures of the fungal strain *P. variotii* and the bacterial strain *B. cereus* grew on oily sludge but the degradation rates and their intensity of growth were disparate. Single culture of *P. variotii* degraded 26% of the TPH content and 74% of the aliphatic content. In terms of growth, the fungal population in the co-culture increased 8-fold from the initial culture. However, the growth of the single culture was slower as counts increased only 2-fold from the initial population.

¹ Wight ratio

The growth of *B. cereus* in single cultures seemed to be better than in co-cultures; TPH and aliphatic hydrocarbons degradation rates were slightly better when single bacterial cultures were applied. In addition, the growth of the bacteria was much better in single cultures than in co-cultures; bacterial population increased by 4% in the first 4 days, however, the population in the co-culture decreased by 50% during the same period. This drop in population could be related to the existence of the fungus, since the conditions in both cases were the same. Therefore, it is possible that the fungus did limit the access of the bacteria to some substrates. To the contrary, the bacterial strain *B. cereus* appeared to enhance the growth of the fungal strain *P. variotii*, since the fungal grew better in co-culture than in single cultures. As a result, the use of *P. variotii* and *B. cereus* as a fungal-bacterial co-culture did improve the capability of the fungal strain to degrade petroleum hydrocarbons in the oily sludge. This result adds to a previous work done by Boonchan *et al.* (2000); they found that the use of fungal bacterial co-cultures improved the capability of the bacteria to mineralize polyaromatic hydrocarbons.

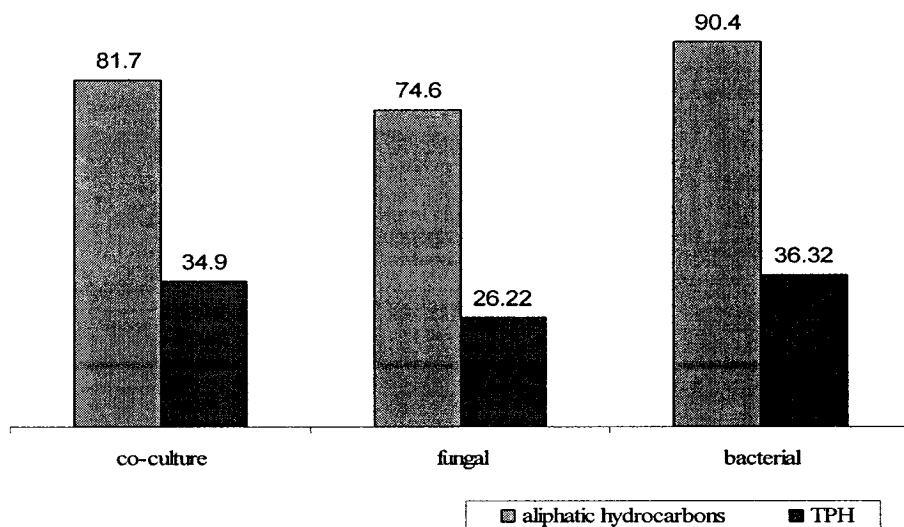


Figure 4-24 The decrease in petroleum hydrocarbons of the oily sludge by the three types of inocula

The effect of the amphoteric surfactant on sludge degradation and microbial growth

The amphoteric surfactant did not improve biodegradation rates as shown in Table 4-3, but it had an effect on the growth of the cultures. The growth of the fungus *P. variotii* was substantially improved when the surfactant was used. In addition, it improved the growth of the bacteria in single bacterial culture, and helped reduce the adaptation time of the bacteria in the fungal-bacterial co-culture. These results could be due to the nature of the surfactant, which helps increase the availability of the compounds for microorganisms. But this did not contribute substantially to the degradation rate of the hydrocarbons. This result was reported in previous works (Boonchan 1998, Rocha and Infant 1997, Van Hamme and Ward 1999).

Table 4-3 The influence of the amphoteric surfactant on the biodegradation of petroleum hydrocarbons

Inoculum type	Oily sludge		Oily sludge with surfactant	
	TPH (%) ¹	Aliphatic hydrocarbons (%) ²	TPH (%)	Aliphatic hydrocarbons (%)
Fungal-Bacterial	35	81.7	34.7	83.5
Bacterial	36.3	90.4	33.3	82
Fungal	26	74.6	27	67.6
No inoculum (control)	10.5	47.4	12	29.7

1. Weight percent

2. Decrease from initial level

The capability of *P. variotii* and *B. cereus* to biodegrade the petroleum oily sludge

Both microorganisms used in this research have shown a strong potential to grow on the petroleum oily sludge. This is an interesting result since the fungal genus *Paecilomyces spp.* was reported to grow on petroleum hydrocarbons or had been isolated from soils contaminated with petroleum hydrocarbons (Atlas and Cerniglia 1995, Bento and Gaylarde 2001, Rojas-Avelizapa *et al.* 1999), but no study has reported its ability to grow on the oily sludge. The bacterial genus *Bacillus spp.* is known for its ability to grow on petroleum hydrocarbons (Atlas and Cerniglia 1995, Leahy and Colwell 1990, Rahman *et al.* 2002, Riser-Roberts 1998). However, few researchers studied its growth on petroleum oily sludge (Lazar *et al.* 1999, Van Hamme *et al.* 2000). Moreover, these studies didn't mention the strain *B. cereus* itself in their results, but they mentioned the genus in general.

The suitability of the new technique for bioremediation studies

The new developed technique that involved growing microorganisms on filters seemed to be a successful in testing the capability of microorganisms to grow on the oily sludge. The growth of microorganisms was notable on the top of the filters. In addition plate counts test became much easier since there was no cross contamination, particularly, during the first 10 days of incubation. However, on day 15, colonies of a new microbial strain appeared on the plates. The colonies were on the filter side that was touching the sludge, and were not too many; this is expected since the samples were not sterile.

The new technique could be described as a simple, short-term, bench-scale, screening assay for examining the ability of defined cultures to grow on petroleum oily sludges. However the results are theoretical and further analysis should be conducted on a larger scale to verify the ability of these cultures to survive in a real environment.

The use of FTIR in this research was effective in detecting the decrease in one of the major constituents in the oily sludge, namely the aliphatic hydrocarbons. However, this is expected since 52% of the sludge is alkanes (Marks *et al.* 1992, Mishra *et al.* 1999, Van Hamme *et al.* 2000, Wright and Noordhuis 1999).

The results of the new technique gave integrated idea about the potential of selected cultures to grow on petroleum oily sludge. However, if a research aims to degrade particular groups of compounds, advanced analytical techniques should be used (e.g. GC, MS).

Advantage and disadvantages of the testing protocol

The use of filters reduced cross contamination substantially, and facilitated the use of plate counts technique for growth-quantification. In addition, growing microorganisms on filters allowed for better studying of the relationship between defined cultures of microorganisms in the absence of constraints from other microorganisms. Moreover, the growth was obvious on the surface of the filter. The FTIR spectra verified the decrease in aliphatic hydrocarbons, but it didn't specify the groups of the hydrocarbons that were eliminated or reduced.

Growing microorganisms on filters involves some inherent disadvantages. First, the filter could have formed a barrier between some hydrocarbons and the microorganisms and this might limit the range of substrates available. Another disadvantage is associated to impossible incubation of the samples under shaking conditions. Most studies have reported better results in moving conditions (Lazar *et al.* 1999, Ward and Singh 2001). Mixing the substrate and the aqueous medium maintains a good distribution of the substrate and oxygen. In this study, the surface area of the cup was 22 mm², which is small, such that the mixing was not vital to the biodegradation process.

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- Bio-neutralization of petroleum oily sludge using fungal-bacterial co-cultures was a useful tool in reducing the hydrocarbon content of the sludge. This would indeed reduce the emissions of the volatile organic carbons and green house gases, and ensure sustainable recycling of petroleum oily sludges.
- This first study on the oily sludge after the application of the electrokinetic separation technology showed that the sludge is a suitable substrate for microbial growth.
- The petroleum oily sludge from the bottom of crude oil storage tanks contained indigenous microbial cultures. This was the first time the fungal strain *Paecilomyces variotii* to be isolated from petroleum oily sludge.
- The research revealed the capability of the fungal strain *Paecilomyces variotii* and the bacterial strain *Bacillus cereus* to grow on the oily sludge as co-cultures and as individual cultures.
- According to my knowledge, this was the first research to study the growth of fungal-bacterial co-cultures on petroleum oily sludge.
- Fungal-bacterial co-culture of *Paecilomyces variotii* and *Bacillus cereus* decreased the TPH content of the oily sludge by 35% and the aliphatic hydrocarbons content by 81%.
- The presence of the bacterial strain *B. cereus* improved the growth of the fungal strain *P. variotii*

- The amphoteric surfactant didn't improve biodegradation rates.
- The growth of *P. variotii* could be stimulated by adding amphoteric surfactant.
- The amphoteric surfactant didn't improve the growth of *B. cereus*.
- The developed technique was efficient in studying the capability of defined microbial cultures to grow on the oily sludge. Also, it facilitated studying the ability of individual microorganisms to grow on petroleum oily sludges.
- FTIR spectrometry was an effective technique for examining the change in aliphatic compounds in the oily sludge.
- Growing microorganisms on filter membranes made growth assessment easier and more accurate. The growth was obvious on the surface of the filters.

Recommendations and Future Research

- The neutralized sludge could be used in further research for sustainable landfarming in combination with phytoremediation.
- Additional work is required to examine various combinations of fungal-bacterial co-cultures and bacterial co-cultures on petroleum oily sludge.
- A larger scale experiments should be conducted to verify the results and to optimize the performance.
- More work is required to quantify and evaluate the results from environmental and economical standpoints.

REFERENCES

- Al-Gounaim, M. Y., Diab, A., 1998, Ecological Distribution And Biodegradational Activities of Oil-Degrading Marine Bacteria in the Arab Gulf Water at Kuwait, Arabian Gulf Journal of Scientific Research, 16 (2): 359-377.
- ASTM, 1999, E168-99: Practices for General Techniques of Infrared Quantitative Analysis, American Society for Testing and Materials.
- Atlas R., 1984, Petroleum Microbiology, Macmillan Publishing Company, USA.
- Atlas, R. and Bartha R., 1987, Microbial Ecology: Fundamentals and Applications, 2nd Ed., The Benjamin/Cummings Publishing Company Inc., USA.
- Atlas, R., and Cerniglia, C., 1995, Bioremediation of Petroleum Pollutants, BioScience, 45(5):332-338.
- Atlas R., 1997, Handbook of Microbiological Media, 2nd Ed., CRC Press, USA.
- Baker, K. and Herson, D., 1994, Bioremediation, McGraw-Hill Inc., USA.
- Bengtsson, A., Quednau, M., Haska, G., Nilzén, P., and Persson, A., 1998, Composting of Oily Sludge-Degradation, Stabilized Residues, Volatiles and Microbial Activity, Waste Management and Research, 16:274-284.
- Bento, F. M., Gaylarde, C. C., 2001, Biodeterioration of Stored Diesel Oil: Studies in Brazil, International Biodeterioration and Biodegradation, 47:107-112.
- Blakebrough, N., 1977, Interactions of Oil and Microorganisms in Soil, *In*: Charter, K. and Somerville, H. (Eds.), 1977, The Oil Industry and Microbial Ecosystems, Heyden & Son Ltd., UK.
- Bollag, W., 2000, Biodegradation, *In*: Lederberg, J., 2000, Encyclopedia of Microbiology, 2nd, Vol. 1, Academic Press, USA.
- Bonnier, P. E., Akoun, G. L., Cardon, E. C., Edwards, E. D., Hocknell, W., Holtari U., Leygue, G., Long D. V., Moorse, J. H., Peterse, J. L., De Roocker, A., and Levi, J. D. , 1980, Sludge Farming: A Technique for the Disposal of Oily Refinery Wastes, The Oil Companies' European Association for Environment, Health, and Safety in Refining and Distribution (CONCAWE) report no. 3/80.

Boonchan, S., 1998, Biodegradation of Polycyclic Aromatic Hydrocarbons: Application of Fungal-Bacterial Co-Cultures and Surfactants, PhD. Thesis, Victoria University, Melbourne, Australia.

Boonchan, S., Britz, M., and Stanley, G., 2000, Degradation and Mineralization of High Molecular-Weight Polycyclic Aromatic Hydrocarbons by Fungal-Bacterial Co-Cultures, *Applied and Environmental Microbiology*, 66:1007-1019.

Brown, R., 1987, Oil Degradation, American Institute of Chemical Engineering, Spring national meeting, Mar. 20-Apr. 2.

Castaldi, F. J. and Ford, D. L., 1992, Slurry Bioremediation of Petrochemical Waste sludge, *Water Science and Technology*, 25(3):207-212.

Cerniglia, C., 1981, Aromatic hydrocarbons: metabolism by bacteria, fungi, and algae, *Reviews in Biochemical Toxicology*. 3:321-361.

Cerniglia, C., 1984, Microbial Metabolism of Polycyclic Aromatic Hydrocarbons. *Advances in Applied Microbiology*, 30:31-71.

Cerniglia, C., 1992, Biodegradation of Polycyclic Aromatic Hydrocarbons, *Biodegradation*, 3:351-356.

Collins; C. and Lyne; P., 1984, *Microbiological Methods*, 5th Ed., Butterwoths, UK.

Connan, J., 1984, Biodegradation of Crude Oils in Reservoirs, *Advances in Petroleum Geochemistry*, 1:300-332.

De Hoog, G. S., Guarro, J., Gené, and Figueras, M. J., 2000, *Atlas of Clinical Fungi*, 2nd ED., Centralbureau voor Schimmelcultures, Spain.

Diegor, E., 2000, Biodegradation of Aromatic Hydrocarbons: Microbial Isotopic Studies, *Journal of Conference Abstracts, Goldschmidt*, 5(2):349-350.

Domsch, K., and Gams, W., 1993, *Compendium of Soil Fungi*, vol. 1, IHW-Verlag, Germany.

Downes, F. and Keith, I., 2001, *Compendium of Methods for the Microbiological Examination of the Food*, 4th, American Public health Association, USA.

Eggen, T. and Majcherczyk, A., 1998, Removal of Polycyclic Aromatic Hydrocarbons in Contaminated Soil by White-Rot Fungus *Pleurotus Osetreatus*, International Biodeterioration and Biodegradation, 41:111-117.

Elekrowicz M., Habibi, S., Chifrina, R., and Esmaeily, A., 2003, Development of a Sustainable Technology for Revitalisation of Petroleum Wastes, 8th CSCE Conference on Environmental and Sustainable Engineering, Moncton June 2003

Elekrowicz M. and Habibi S., 2003, A New Sustainable Technology for the Recovery of Fuel from Petroleum Wasted Sludge, CJCE Special Issue on Sustainable Engineering (submitted).

EPA, 1998, Final Standards Promulgated for Petroleum Refining Waste (EPA 530-F-98-014), United States Environmental Protection Agency, Office of Solid Waste.

EPA, 1999, Method 1664: N-Hexane Extractable Material and Silica Gel Treated N-Hexane Extractable Material by Extraction And Gravimetry, United States Environmental Protection Agency, office of water.

Evans, W., 1977, Biochemistry of the Bacterial Catabolism of Aromatic Compounds in Anaerobic Environments, Nature (London), 270:17-22.

Francis, M. and Les Stehmeier, 1991, Biotreatment of Waste Oil and Sludges, *In: Gould W. et al.* (Eds), Proceedings of the 8th Annual General Meeting of BIOMINET, 31 Oct. 1991, Calgary, Alberta, Canada, pp.55-74.

Galil, N. and Rebhum, M., 1992, Waste Management Solutions at an Integrated Oil Refinery Based on Recycling of Water, Oil, And Sludge, Water Science and Technology, 25(3):101-106.

Griffiths, P. and deHaseth, J., 1986. Fourier Transform Infrared Spectroscopy, Wiley Interscience, NY, USA.

Habibi, S. and Elekrowicz, M., 2004, A New Environmental-Friendly Technology for the Recovery of Fuel from Oily Sludge, 1st Water and Environment specialty Conference of the Canadian Society for Civil Engineering, June 2004, Saskatoon, Canada.

Hahn, N. and Loehr, R. C., 1992, Biological Treatment of Petroleum Oily Sludges, Proceedings of Permian Basin Oil and Gas Recovery Conference, March 18-20, pp. 519-530, Midland, Texas, USA.

Hanson, K. G., Desai, J. D., and Desai, A. J., 1993, A Rapid and Simple Screening Technique for Potential Crude Oil Degrading Microorganisms, *Biotechnology Techniques*, 7(10):745-748.

Harold, F. and Fechner-Levy, E., 2000, *Chemical Fate and Transport in the Environment*, 2nd Ed., Academic press, USA.

Hasegawa, H., Kouta, N., and Yoji, N., 2003, Membrane Filter (pore size 0.22-0.45 μ m) Passing-Through Activity of *Pseudomonas aeruginosa* and Other Bacterial Species with Indigenous Infiltration Ability, *Microbiology Letters*, 223(1): 41-46.

Hatem, G., 1999, Design of the Surfactant Enhanced Electrokinetic System for Hydrocarbons Removal from Clayey Soils in Pilot Scale Conditions, Master Thesis, Concordia University, Quebec, Canada

Higgins, I., 1977, The Biodegradation of Hydrocarbons, *In: Chater K. and Somerville, H. J. (Eds), 1977, The Oil Industry and Microbial Ecosystems*, Hyden & Son Ltd., UK.

Hughes, J., 2000, Bioremediation, *In: Lederberg, J., 2000, Encyclopedia of Microbiology*, 2nd, vol. 1, Academic Press, USA.

Jean, D., Chu, C. B., and Lee D. J., 2001, Freeze/thaw Treatment of Oily Sludge From Petroleum Refinery Plant, *Separation Science and Technology*, 36(12):2733-2746.

Juhasz, A., Britz, M. L., and Stanley, G. A., 1996, Degradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons by *Pseudomonas cepacia*, *Biotechnology Letters*, 18:577-582.

Juhasz, A. and Naidu, R., 2000, Bioremediation of High Molecular Weight Polycyclic Aromatic Hydrocarbons: A Review of The Microbial Degradation of Benzo[a]Pyrene, *International Biodeterioration and Biodegradation*, 45:57-88.

Kirchmann, H. and Ewnetu, W., 1998, Biodegradation of petroleum based oil wastes through composting, *Biodegradation*, 9:151-156.

Kostecki, P. T. and Calabrese, E. J., 1991, Hydrocarbon contaminated soils and ground water: analysis, fate, environmental and public health effects and remediation, vol. 1, Lewis Publisher Inc., USA.

LaGrega, M. L., Buckingham, P. L., and Evans, J. C., 1994, *Hazardous waste management*, McGraw-Hill, NY, USA.

Lazar, I., Voicu, A., Dobrota, S., Stefanesc, M., Sandulescu, L., Lazar, I. G., Mucenica, D., and Balalia, A., 1995a, Microbial Communities in Soils and Waters Polluted with Oil and Phenols and Their Role in Bioremediation of such Environments, Proceedings of the International Biohydrometallurgy Symposium, Nov. 19-22, Chile.

Lazar, I., Voicu, A., Dobrota, S., Stefanesc, M., Sandulescu, L., Lazar, I. G., Mucenica, D., Balalia, A., and Nicolescu, C., 1995b, Investigation on Potential Bacteria for the Bioremediation Treatment of Environment Contaminated with Hydrocarbons, *In*: Bryant, R. and Sublette, R. (Eds), The Fifth International Conference on Microbial Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems, pp. 535-547, Texas, USA.

Lazar, I., Voicu, A., Dobrota, S., Stefanesc, M., Sandulescu, and Petrisor, I. G., 1999, Microbial Degradation of Waste Hydrocarbons in Oily Sludge From Some Romanian Oil Fields, *Journal of Petroleum Science and Engineering*, 22:151-160.

Leahy, J. G. and Colwell, R. R., 1990, Microbial Degradation of Hydrocarbons in the Environment, *Microbiological Reviews*, 54(3):305-315.

Lehr, J., 2002, *Handbook of Complex Environmental Remediation Problems*, McGraw-Hill, USA.

Mackay, D., Roberts, P. V., and Cherry, J. A., 1985, Transport of Organic Contaminants in Groundwater, *Environmental Science and Technology*, 19:384-392.

Margesin, R. and Schinner, F., 2001, Biodegradation and Bioremediation of Hydrocarbons in Extreme Environments, *Applied Microbiology and Biotechnology*, 56:650-663.

Marks, R., Field, S. D., Wojtanowicz, A. K., and Britenbeck, G. A., 1992, Biological Treatment of Petrochemical Wastes for Removal of Hazardous Polynuclear Aromatic Hydrocarbon Constituents, *Water Science and Technology*, 25(3):213-220.

McFarland, M., Qiu, X. J., Sims, J. L., Randolph, M. E., and Sims, R. C., 1992, Remediation of Petroleum Impacted Soils in Fungal Compost Bioreactions, *Water Science and Technology*, 25(3): 197-206.

Mishra, S., Lal, B., Jyot, J., Rajan. S., Khanna, S., and Kuhad, R., 1999, Field Study: In Situ Bioremediation of Oily Sludge Contaminated Land Using "Oilzapper", *Hazardous and Industrial Wastes: proceedings of mid-atlantic industrial waste conference*, pp. 177-186.

Mishra S, Jyot J, Kuhad R C, Lal B., 2001, Evaluation of Inoculum Addition to Stimulate in Situ Bioremediation of Oily Sludge Contaminated Soil, *Applied and Environmental Microbiology* 67: 1675-1681

Oolman, T., Castaldi, F. J., Behrens, G. P., and Owen, M. L., 1992, Biotreat Oily Refinery Wastes, *Hydrocarbon Processing*, 71(8):67-69.

OSHA, 2003, OSHA Technical Manual, U.S. Department of Labor, Occupational Safety and Health Administration (OSHA).

Rahman, K., Thahira-Rahman, J., Lakshmanaperumalsamy, P., and Banat, I. M., 2002, Towards Efficient Crude Oil Degradation by A Mixed Bacterial Consortium, *Bioresource Technology*: 85:257-261.

Reeves, T., 1995, Petrochemicals, *Water Environment Research*, 67(4):529-531.

Riser-Roberts, E., 1998, Remediation of Petroleum Contaminated Soils: Biological, Physical, and Chemical Processes, CRC press LLC, USA.

Rocha, C., and Infant, C., 1997, Enhanced Oily Sludge Biodegradation by Tensio-Active Agent Isolated from *Pseudomonas aeruginosa* USB-CS1, *Applied Microbiology and Biotechnology*, 47:615-619.

Rojas-Avelizapa, N. G., Rodríguez-Vázquez, R., Enriquez-Villanueva, F., Martínez-Cruz, J., and Poggi-Varaldo, H. M., 1999, Transformer Oil Degradation by An Indigenous Microflora Isolated from A Contaminated Soil, *Resource, Conservation and Recycling*, 27:15-26.

Salanitro, Joshep, 2000, Oil Pollution, *In: Lederberg, J., 2000, Encyclopedia of Microbiology*, 2nd, vol. 3, Academic Press, USA.

Schleck, D., 1990, Treatment Technologies for Refinery Wastes and Waste Waters, National Petroleum Refiners Association (NPRA) annual meeting, USA.

Schmitt, J., and Flemming, H., 1998, FTIR-Spectroscopy in Microbial and Material Analysis, *International Biodeterioration and biodegradation*, 41:1-11.

Shie, J., Chang, C., Lin, J., Wu, C., and Lee, D., 2000, Resources Recovery of Oil Sludge by Pyrolysis: Kinetics Study, *Journal of chemical Technology and Biotechnology*, 75:443-450

Silverstein, R. and Webster, F., 1998, Spectrometric Identification of Organic Compounds, 6th, John Wiley and Sons Inc., USA.

Spormann, A. and Widdel, F., 2000, Metabolism Of Alkylbenzenes, Alkanes, and Other Hydrocarbons in Anaerobic Bacteria, *Biodegradation*, 11:85-105

Stiff, H., 1977, Biodegradation of Surfactants, *In: Chater K. and Somerville, H. (Eds), 1977, The Oil Industry and Microbial Ecosystems, Hyden & Son Ltd., UK.*

Tiehm, A. and Fritzsche, C., 1995, Utilization of Solubilized and Crystalline Mixtures of Polycyclic Aromatic Hydrocarbons by *Mycolacterium Sp.*, *Applied Microbiology and Biotechnology*, 42:964-968

Van Hamme, J. D. and Ward, O. P., 1999, Influence of Chemical Surfactants on the Biodegradation of Crude Oil by A Mixed Bacterial Culture, *Canadian Journal of Microbiology*, 45:130-137.

Van Hamme, J. D, Odumeru J. A., and Ward, O. P., 2000, Community Dynamics of a Mixed-Bacterial Culture Growing on Petroleum Hydrocarbons in Batch Culture, *Canadian Journal of Microbiology*, 46:441-450.

Ward, O. P. and Singh, A., 2001, Biodegradation of Oily Sludge, Patten No. CA 02229761, Canadian Intellectual Property Office, Industry Canada.

Wright, R. A. and Noordhuis, B. R., 1991, The Treatment and Disposal of Oily Solids, *Proceedings of The First International Conference on Health, Safety, and Environment In Oil and Gas Exploration And Production, part 2* , pp. 521-526.

Ye, B., Siddiqi, M. K., Maccubbin , A. E., Kumar, S., and Sikka, H. C., 1996, Degradation of Polynuclear Aromatic Hydrocarbons by *Sphingomonas Paucimobilis*, *Environmental Science and Technology*, 30:136-142.

Zwolinski, M., 2000, Microbial Consortia Involved in the Anaerobic Degradation of Hydrocarbons, *Biodegradation*, 11:141-158.

APPENDICES

Appendix A Bushnell-Hass Medium

The medium contained (g/l)

0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.002 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

1.0 KH_2PO_4

1.0 K_2HPO_4

1.0 NH_4NO_3

0.05 FeCl_3

Appendix B Photographs from Experiments

B.1. Incubation of samples

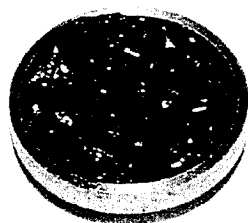


Figure B- 1 The oily sludge inside the cups before laying the filter

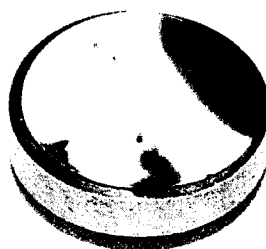


Figure B- 2 Membrane filter on the oily sludge before absorbing petroleum hydrocarbons

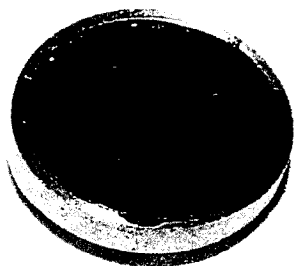


Figure B- 3 Membrane filter after absorbing petroleum hydrocarbons

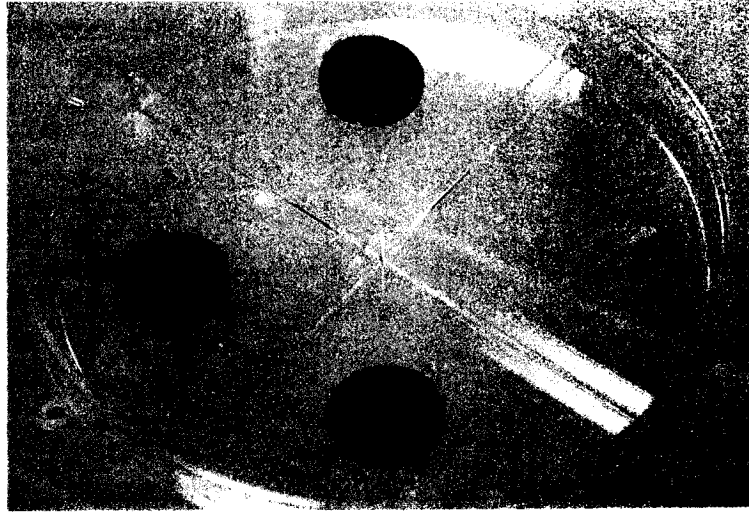


Figure B- 4 Cups inside Petri dish



Figure B- 5 Sludge samples inside the container

B.2. Isolation of potential Bacterial and Fungal strains:

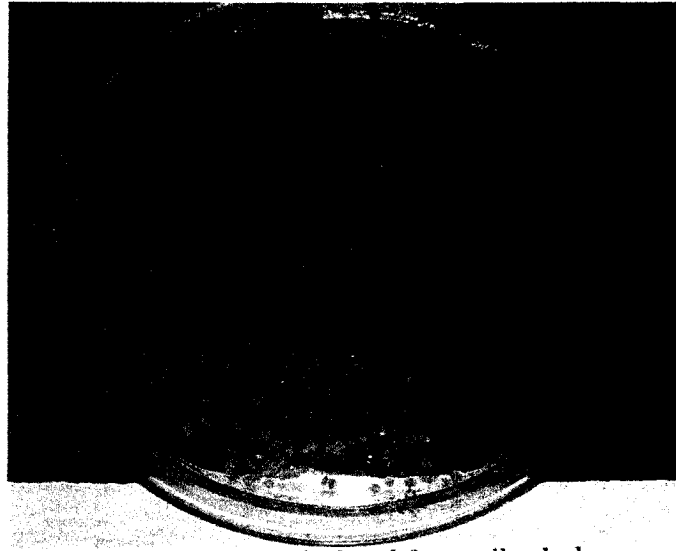


Figure B- 6 Bacterial colonies isolate from oily sludge



Figure B- 7 Pure bacterial colonies streaked on agar plate

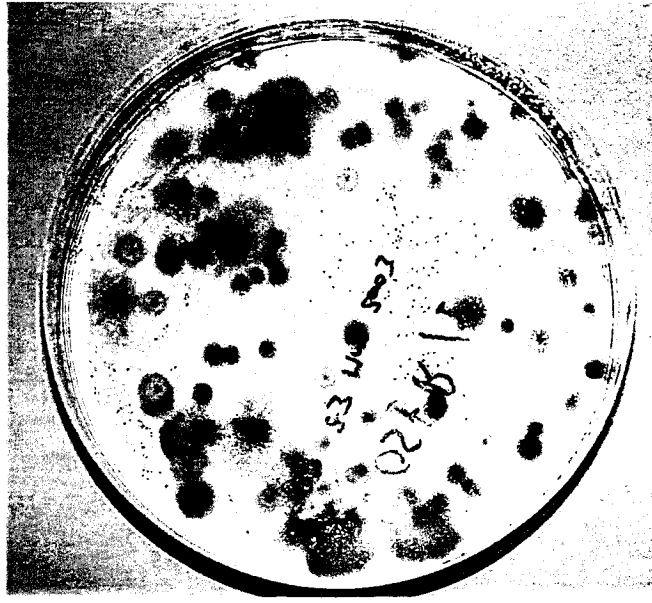


Figure B- 8 Fungal colonies isolated from Oily Sludge

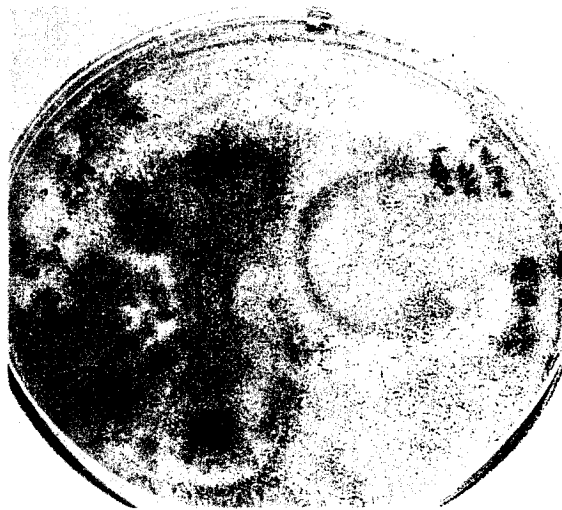


Figure B- 9 The fungal strain *Paecilomyces variotii* cultured on separate plate with potato dextrose agar



Figure B- 10 The fungal strain *Paecilomyces variotii* on Rose Bengal agar

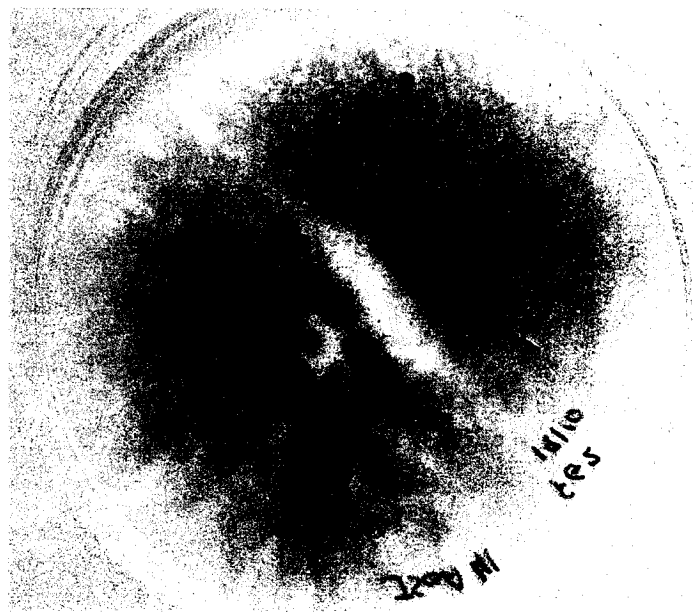


Figure B- 11 Second Fungal strain isolated from oily sludge culture on potato dextrose agar

B.3. Screening the Potential of the Isolated Colonies to Degrade Petroleum Hydrocarbons



Figure B- 12 Screening test (colonies B01, B02, B03)

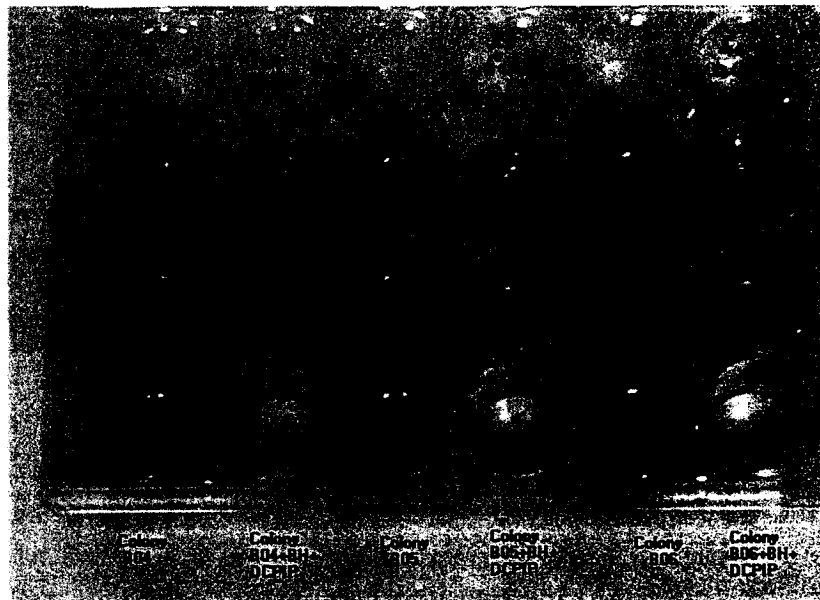


Figure B- 13 Screening test (colonies B04, B05, B06)

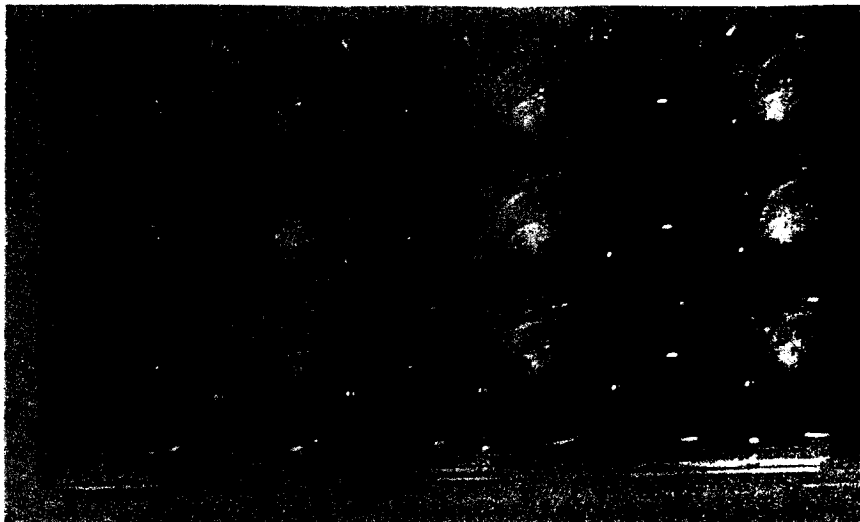


Figure B- 14 Controls of screening test, from left to right the contents of each column are BH+ DCPIP, DCPIP only, crude oil + DCPIP

B.4. Bacteria Identification:



Figure B- 15 Strip API 20 E before incubation



Figure B- 16 Strip 20 E after 24hrs incubation at 37°C

B.5. Samples results from plate counts

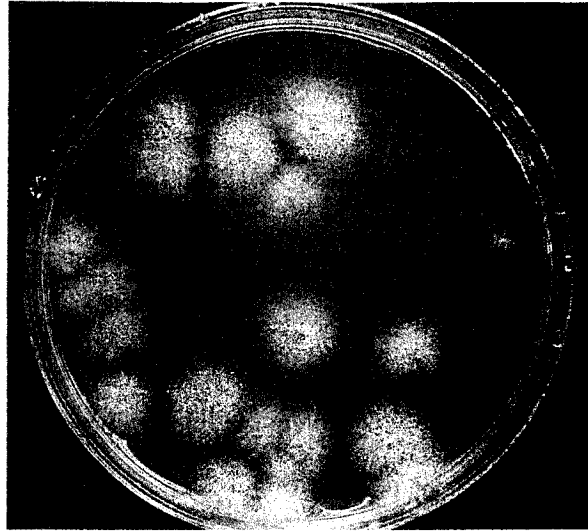


Figure B- 17 Plate counts of the fungal strain *Paecilomyces variotii*

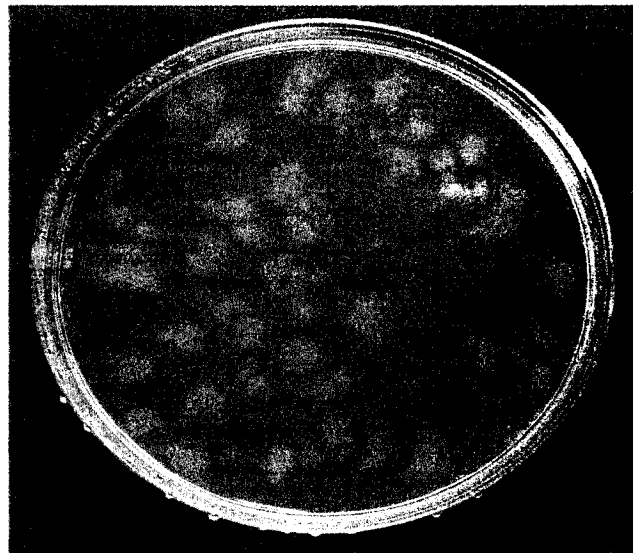


Figure B- 18 Colonies of *Bacillus cereus* on agar plate

B.6. FTIR Instrument and accessories:

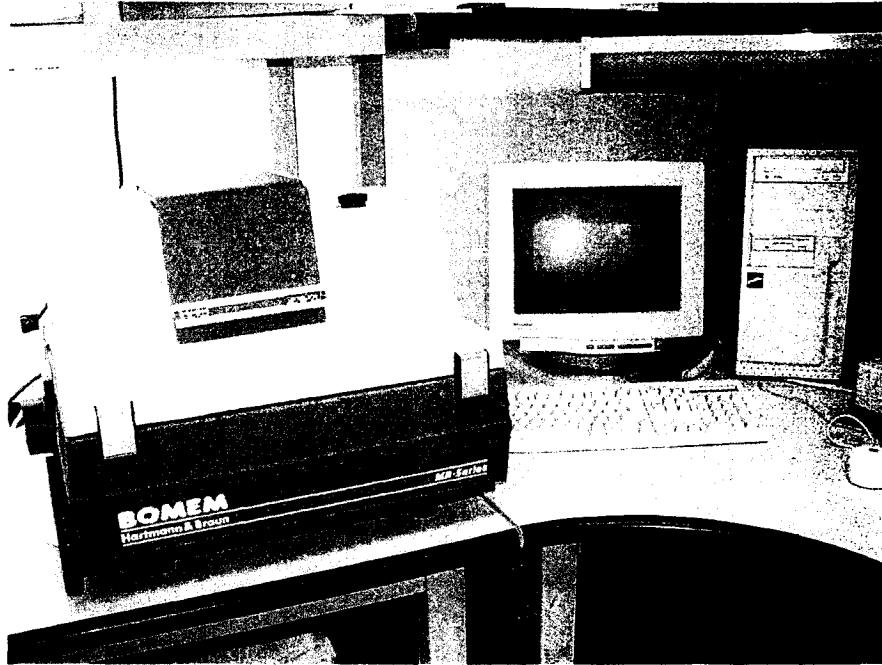


Figure B- 19 Mechelson FTIR Machine

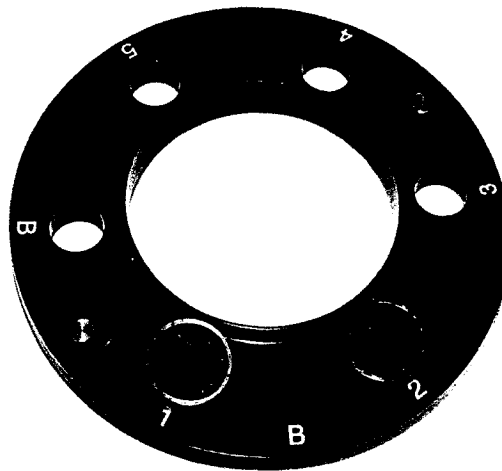


Figure B- 20 the cups fixed inside a special tray for FTIR test

Appendix C API® Strips for Biochemical Identification of Bacteria

C.1. API® 50 CHB/E Medium

(for identification of *Bacillus* and related genera/ *Enterobacteriaceae* and *Vibrionaceae*)

The table below shows the composition of API 50 CHB/E medium¹, which was used in carbohydrates metabolism test

Table C-1: Composition of the medium

Ingredient	Quantity
Ammonium sulfate	2g
Yeast extract	0.5g
Tryptone (bovine/porcine origin)	1g
Disodium phosphate	3.22g
Monopotassium phosphate	0.12g
Trace elements	10ml
Phenol red	0.17g
Demineralized water	1000ml
pH 7.4-7.8 at 20-25°C	

¹ As provided by the supplier (i.e. bioMérieux® Inc.)

C.2. API® 50 CH strip

(Carbohydrates)

The table below demonstrates the carbohydrates sullied in strip's micro-tubes²

Table C-2: Carbohydrates inside API 50 CH strip micro-tubes

Tube No.	Test	Active ingredients	Quantity (mg/cup.)
0		control	-
1	GLY	GLYcerol	1.64
2	ERY	ERYthritol	1.44
3	DARA	D-ARAbinose	1.4
4	LARA	L-ARAbinose	1.4
5	RIB	D-RIBose	1.4
6	DXYL	D-XYLose	1.4
7	LXYL	L-XYLose	1.4
8	ADO	D-ADOnitol	1.36
9	MDX	Methyl-βD-Xylopyranoside	1.28
10	GAL	D-GALactose	1.4
11	GLU	D-GLUcose	1.56
12	FRU	D-FRUctose	1.4
13	MNE	D-MaNnoseE	1.4
14	SBE	L-SorBosE	1.4
15	RHA	L-RHAMinose	1.36
16	DUL	DULcitol	1.36
17	INO	INOsitol	1.4
18	MAN	D-MANnitol	1.36
19	SOR	D-SORbitol	1.36
20	MDM	Methyl-αD-Mnnopyranoside	1.28
21	MDG	Methyl-αD-Glucopyranoside	1.28
22	NAG	N-AcetylGlucosamine	1.28
23	AMY	AMYgdalin	1.08
24	ARB	ARButin	1.08
25	ESC	ESCulin ferric citrate	1.16 0.152
26	SAL	SALicin	1.04
27	CEL	D-CELlobiose	1.32
28	MAL	D-MALtose	1.4
29	LAC	D-LACTose (bovine origin)	1.4

² As provided by the supplier (i.e. bioMérieux® Inc.)

(Continued) Table C-2 Carbohydrate inside API 50 CH strip micro-tubes

Tube No.	Test	Active ingredients	Quantity (mg/cup.)
30	MEL	D-MELibose	1.32
31	SAC	D-SACcharose (sucrose)	1.32
32	TRE	D-TREhalose	1.32
33	INU	INUlin	1.28
34	MLZ	D-MeLeZitose	1.32
35	RAF	D-RAFFinose	1.56
36	AMD	AmiDon (starch)	1.28
37	GLYG	GLYcoGen	1.28
38	XLT	XyLiTol	1.4
39	GEN	GENTIbiose	0.5
40	TUR	D-TURanose	1.32
41	LYX	D-LYXose	1.4
42	TAG	D-TAGatose	1.4
43	DFUC	D-FUCose	1.28
44	LFUC	L-FUCose	1.28
45	DARL	D-ARabitoL	1.4
46	LARL	L-ARabotolL	1.4
47	GNT	Potassium GlucoNaTe	1.84
48	2KG	potassium 2- KetoGluconate	2.12
49	5KG	potassium 5- KetoGluconate	1.8

C.3. API® 20 E strip

Table C-3 shows the contents to each micro-tube in API 20 E strip³

Table C-3 Contents of API 20 E strip

Tests	Substrates	Qty	Enzymatic activity Or reaction tested	Results	
				Negative	Positive
ONPG	ortho-nitrophenyl-, -D-galactopyranoside(ONPG)	0.2 mg	beta-galactosidase	colorless	yellow ⁽¹⁾
	isopropylthiogalactopyranoside (IPTG)	8.0 µg			
ADH	arginine	2.0 mg	arginine dihydrolase	yellow	red / orange ⁽²⁾
LDC	lysine	2.0 mg	lysine decarboxylase	yellow	red/orange ⁽²⁾
ODC	ornithine	2.0 mg	ornithine decarboxylase	yellow	red / orange ⁽²⁾
CIT	sodium citrate	0.8 mg	citrate utilization	pale green / yellow	blue-green / blue ⁽³⁾
H2S	sodium thiosulfate	80.0 µg	H ₂ S production	colorless / greyish	black deposit / thin line
URE	urea	0.8 mg	urease	yellow	red / orange ⁽²⁾
TDA	tryptophane	0.4 mg	Tryptophane deaminase	Ferric chloride (1 drop) / immediate	
				yellow	brown-red

³ As provided by the supplier (i.e. bioMérieux® Inc.)

(Continued) Table C-3 Contents of API 20 E strip

IND	tryptophane	0.2 mg	indole production	Kovacs' reagent (1 drop) / 2 min	
				IND yellow	IND red ring
VP	Creatine sodium pyruvate	0.9 mg 2.0 mg	acetoin production	VP1 (1 drop) + VP2 (1 drop) / 10 min	
				colorless	pink / red ⁽⁴⁾
GEL	Kohn's charcoal gelatin	0.6 mg	gelatinase	no diffusion of black pigment	diffusion of black pigment
GLU tube Nitrate Reduction	Potassium nitrate	80.0 µl	NO ₂ production reduction to N ₂ gas	NIT 1 (2 drops) + NIT 2 (2 drops) / 2-3 min	
				yellow	Red
				Zinc Dust / 5 min	
				orange-red	yellow

(1) A very pale yellow should also be considered positive.

(2) An orange color after 36-48 hours incubation must be considered negative.

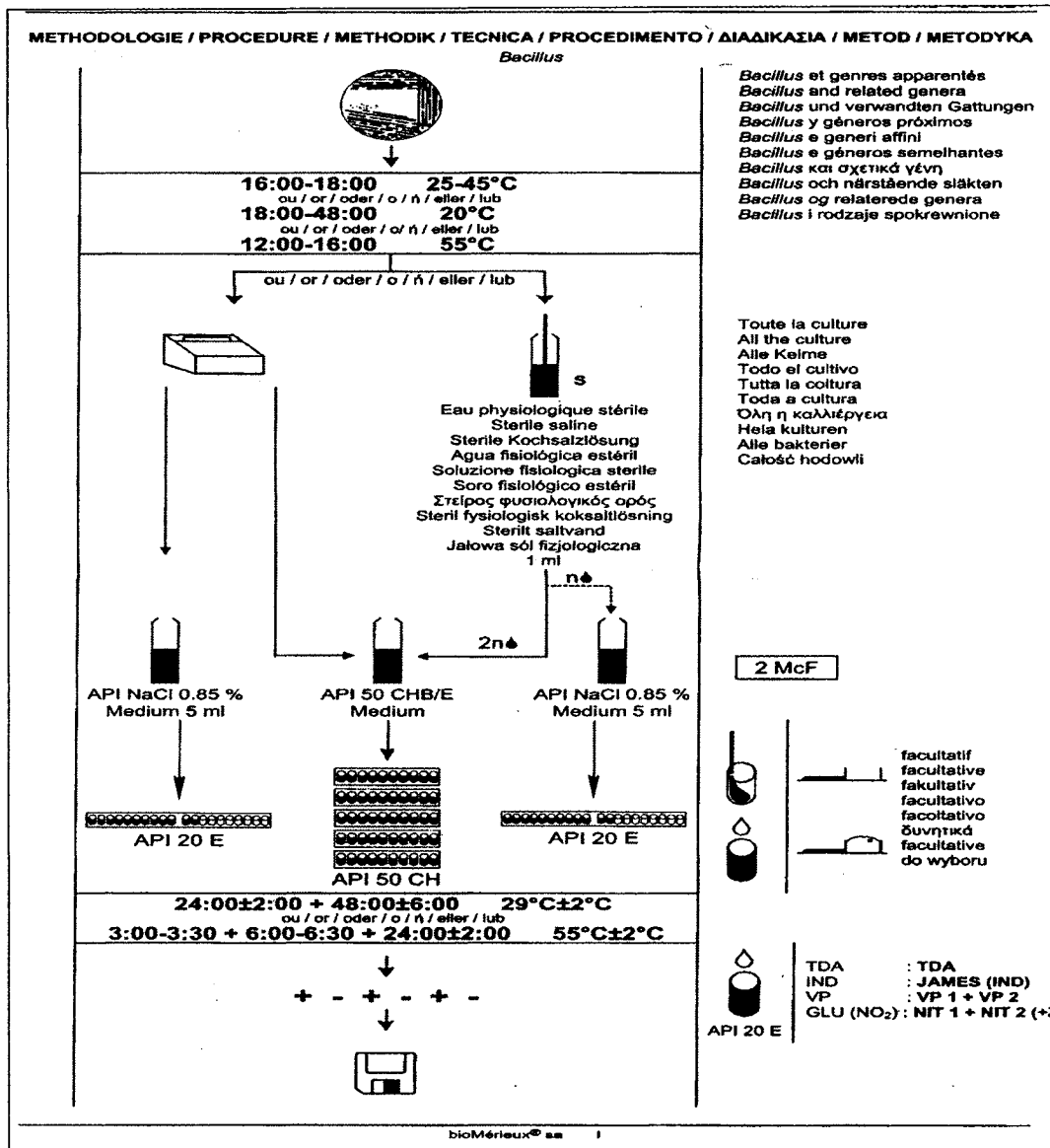
(3) Reading made in the cupule (aerobic).

(4) A slightly pink color after 10 minutes should be considered negative.

C.4. Test Procedure

Figure C-1 illustrates the steps followed to identify the bacteria using API 50 CH and API 20 E strips

Figure C- 1 Test procedure for biochemical Identification of bacteria using API 50CH and API 20 E strips



Adapted from API 50 CH strip instructions manual

Appendix D Results of Bacteria Identification Tests

D.1. Strip API 50 CH (Carbohydrates)

Table D-1: Results Of carbohydrates metabolism test

Tube No.	Test	Result
0		-
1	GLY	+
2	ERY	-
3	DARA	-
4	LARA	-
5	RIB	+
6	DXYL	-
7	LXYL	-
8	ADO	-
9	MDX	-
10	GAL	+
11	GLU	+
12	FRU	+
13	MNE	-
14	SBE	-
15	RHA	-
16	DUL	-
17	INO	-
18	MAN	-
19	SOR	-
20	MDM	-
21	MDG	-
22	NAG	+
23	AMY	-
24	ARB	+
25	ESC	+
26	SAL	+
27	CEL	-
28	MAL	+
29	LAC	-

Tube No.	Test	Result
30	MEL	-
31	SAC	-
32	TRE	+
33	INU	-
34	MLZ	-
35	RAF	-
36	AMD	+
37	GLYG	+
38	XLT	-
39	GEN	-
40	TUR	-
41	LYX	-
42	TAG	-
43	DFUC	-
44	LFUC	-
45	DARL	-
46	LARL	-
47	GNT	-
48	2KG	-
49	5KG	+

D.2. API 20E (other Substrates)

Table D-2: Metabolism test results of substrates other than carbohydrates

Tests	Result
ONPG	-
ADH	-
LDC	-
ODC	-
CIT	-
H ₂ S	-
URE	-
TDA	-
IND	-
VP	-
GEL	+
GLU tube Nitrate Reduction	-

D.3. Computer output sheet

Référence : 5293-4-Nutrexol	Date : 2004/02/11			
BONNE IDENTIFICATION AU GENRE				
Bacillus cereus	1			

Galerie : API 50 CHB	V3.0			
Lue le : 2004/02/11				
Profil : -+-----++-----+-----+-----+-----				

0 - GLY + ERY - DARA- LARA- RIB + DXYL- LXYL- ADO - MDX - GAL -				
GLU + FRU + MNE - SBE - RHA - DUL - INO - MAN - SOR - MDM - MDG -				
NAG + AMY - ARB + ESC + SAL + CEL - MAL + LAC - MEL - SAC - TRE +				
INU - MLZ - RAF - AMD + GLYG+ XLT - GEN - TUR - LYX - TAG - DFUC-				
LFUC- DARL- LARL- GNT - 2KG - 5KG - ONPG- ADH - LDC - ODC - CIT -				
H2S - URE - TDA - IND - VP - GEL + NIT -				
----- Taxons significatifs ----- % Id. --- T -- Tests à l'encontre -----				
Bacillus cereus	1	75.5	0.93	0
Bacillus mycoides		21.5	0.92	1
Taxon suivant				
Bacillus anthracis		2.4	0.76	2
POSSIBILITE DE Bacillus thuringiensis				

Bacillus cereus	1	: 0 test(s) à l'encontre		

Bacillus mycoides		: 1 test(s) à l'encontre		
GLYCEROL	(GLY)	15 %		

Taxon suivant				
Bacillus anthracis		: 2 test(s) à l'encontre		
SACCHAROSE	(SAC)	99 %	REDUCTION DU NITRATE	(NIT) 78 %

Figure D- 1 output of computer software after manipulating the results in sections D1 and D2 (the document is in French language)

Appendix E Terminology used in Describing the Morphology of Bacterial Colonies

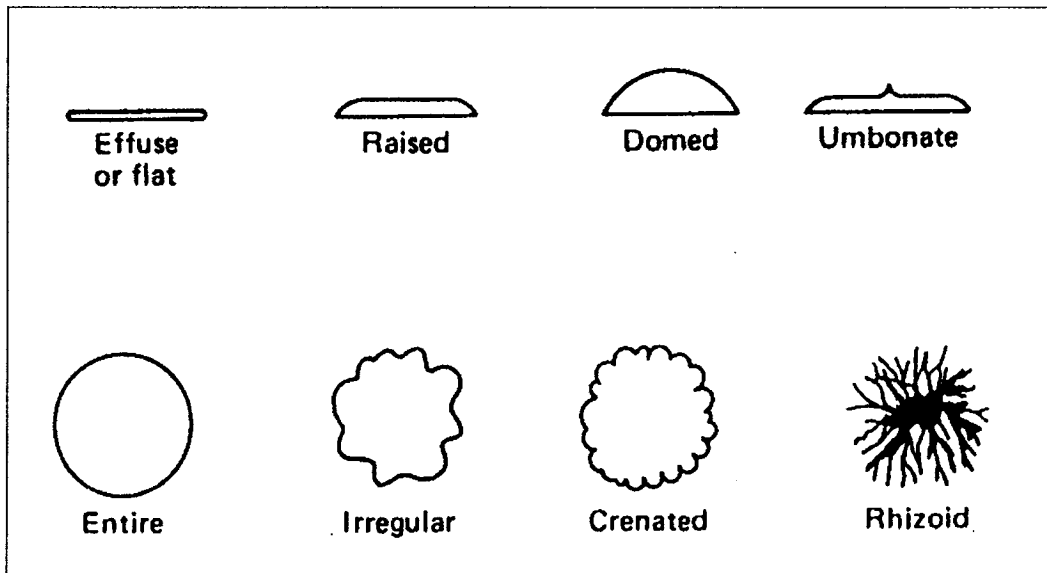


Figure E-1 Description of bacterial colonies (Collins and Lyne 1984)

Appendix F Calculation

F.1. Estimation of Total Petroleum Hydrocarbons (TPH)

All weights are in grams (g)

First vial empty weight = W_1

First vial weight +the sludge (Before hexane extraction) = W_2

Second vial empty weight = W_3

Second vial weight +the weight of residual TPH = W_4

Sludge weight (W_5) = $W_2 - W_1$

$$\text{TPH (\%)} = (W_4 - W_3) / W_5 * 100$$

Each point on the curves represents the average of four replicates

F.2. FTIR Relative Absorbance

Absorbance at the peak 2945 cm^{-1} (band range $3001.1-2742 \text{ cm}^{-1}$) = A_{2945}

Absorbance at the peak 1064 cm^{-1} (band range $1100-1000 \text{ cm}^{-1}$) = A_{1064}

Relative absorbance = A_{2945} / A_{1064}

Each point on the curves represents the average of four replicates

F.3. Plate Counts (CFU/ml)

The filter was immersed in 5 ml 0.85% saline solution

0.1 ml of diluted culture was spread on agar plates

Culture population (CFU/ml) = No. of counts on the plates* dilution factor*10

Each point on the curves represents the average of four replicates

Appendix G Microbiological Media Preparation

The ingredients of the media were dissolved in distilled water and stirred for about ten minutes until all contents dissolved. The pH of Medium (A) was adjusted to 7 , and the pH of Medium (B) was adjusted to 5.5. The media were sterilized by autoclaving at 121°C and 105 kPa pressure for 20 min.

Appendix H Inocula Preparation

H.1. Bacterial inoculum preparation

Bacterial colonies were first cultured in a Medium (A) inside Erlenmeyer flasks. The flasks were incubated on orbital shaker at 30°C and 175 rpm. To prepare the inoculum the broth culture was centrifuged at 4000 rpm for 10 min.. The broth was decanted, and the cells were re-suspended in a sterile saline solution (0.85% NaCl). The suspension was centrifuged as before and the cells were re-suspended in a new saline solution, the last step was repeated twice. A turbidometric standard curve was obtained by concurrent plate counts and turbidometric measurements of a serially diluted bacterial-cell suspension.

H.2. Fungal Inoculum Preparation

Fungus was grown on plates of Medium (B) for 7 days. 10 ml of a sterile solution containing 0.05 g/L of a nontoxic wetting agent (i.e. sodium dioctyl sulfosuccinate) was poured into the plate. Using a sterile nichrome-inoculating loop, the growth surface was scraped, and the contents of the plate were poured into a sterile screw-capped tube. Distilled water and 10 to 15 solid 5 mm glass beads were added. The tube was shaken vigorously to liberate the spores from fruiting bodies and to break the spore clumps. The shaken suspension was filtered through a thin layer of sterile glass wool in a glass funnel to remove mycelia fragments. After that, the filtered suspension was centrifuged, and the supernatant was decanted and the spores were re-suspended in sterile distilled water. The suspension was centrifuged, the spores were washed in this manner three times. The final washed residue was diluted in the experiment mineral medium. Spore numbers were determined by conducting concurrent turbidometric and spore counting measures, counting chamber was used to count the spore.