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Feasibility Study of Fungal Bioremediation of a Flare Pit Soil Using White Rot Fungi

by

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ABSTRACT

One of the major concerns in any bioremediation work related to petroleum hydrocarbons is the presence of heavy compounds. These heavy hydrocarbon constituents are hard to bioremediate and are considered to be a potential health risk (possible carcinogenic and mutagenic activities). An example seen throughout Alberta are the flare pits used for incineration of waste fluids produced at oil and gas well sites. The heavy hydrocarbon constituents have very low water solubility and are often bound to soil particles. Both factors severely reduce the bacterial biodegradation rates. The biodegradation rate has also been shown to decrease with increasing the number of fused molecules, branches or length of hydrocarbon chains. In many cases, bacterial biodegradation of such compounds to acceptable levels of 100-1000 ppm is impossible.

The primary goal of this study was to investigate the biodegradation potential of *lignin degrading fungi* or *white rot fungi* in bioremediation of the heavy hydrocarbon compounds. One of the advantages of using lignin-degrading fungi in bioremediation of heavy hydrocarbons is that the fungi extracellular enzyme directly attacks the hydrocarbons, whereas oil-degrading bacteria have only intracellular enzymes, which can only attack the water-soluble portions. Further more, some fungi species have shown the ability to degrade high molecular weight compounds (with 4-6 fused rings), whereas bacterial biodegradation is often limited to low molecular weight hydrocarbons (3-4 fused rings).

In this research, twelve different white rot fungi strains have been prescreened for the best biodegradation potential (best growth & enzyme activity in the soil). A *fractional factorial experiment* was conducted to assess the effectiveness of the fungi applications and the effects of moisture content and bulking agent in remediation of a flare pit soil. The results of the experiments were statistically analyzed and the effect of each variable was assessed.

The statistical analysis of the results suggested that the application of white rot fungi had no significant effect on the hydrocarbon loss. In other words, the biodegradation might or might not be caused by white rot fungi activity in the soil, but application of extra fungal inocula into the soil did not have any significant effect on hydrocarbon biodegradation. It was also showed that the change in moisture content from 30% to 50% (wt/wt of dry soil) didn't have any significant effect on the hydrocarbon loss. However the analysis did show that the increase in bulking agent content from 6% to 12% (wt/wt of dry soil) had a marginally significant effect on the hydrocarbon loss in contaminated soil.

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DEDICATION

Dedicated to My Mother, The Memory of My Father, and My Husband:

Manijeh Rabeie, Seyed Morteza Meysami, Amir Darvishi

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Chapter One: Introduction

1.1 General Introduction

Flare pits have been a commonly used for the incineration of oilfield wastes. Flare pits way contain a variety of hydrocarbon wastes from waxes to resins, produced water, process chemicals, site waste and even acids. According to the studies conducted by the government of Alberta, the estimated cost of cleaning up the flare pit sites in the province is about $4 billion, or an average of $50,000 per site (Alberta Environmental Protection, 1992 & Canadian Association of Petroleum Producers, 1997).

The pits are generally simple excavations that rarely have special construction or are lined. The soil permeability will lead to the migration of peat contents to the groundwater and the surrounding soil, which will impact vegetation at the site. Also, since the pits are often unfenced, wildlife can access the site (Speer, 1999). The potential hazards of these sites require the development of a fast and at the same time effective remediation method.

In recent years, the possibilities of using white rot fungi for bioremediation strategies have initiated considerable research effort in academia and industry. The ability of white rot fungi to degrade an exceptionally diverse group of very resistant or toxic environmental pollutants has raised the interest in use using white rot fungi in bioremediation research projects (Aust & Barr, 1994).

Some of the hazardous organic compounds that are degradable by white rot fungi are soil contaminants, thus methods for using these fungi to decontaminate soil are recently being developed. The use of white-rot fungi for bioremediation of soils presents a number of challenges, since white rot fungi normally colonize plants or plant residues and do not grow well in unamended soil particularly if the soil is not sterilized, (Boyle, 1995). In different studies, the degree of degradation found in lab scale and in field studies shows considerable variation (Anderson et al., 1995). Different factors such as competition and inhibition from natural micro flora of soil, the type of soil, soil moisture, temperature, oxygen concentration, pH, salinity, light intensity, sediment type, nutrients, and co-substrates, can affect the performance of white rot fungi in the degradation of soil contaminants (Azadpour et al.1997).

1.2 Research Goals

There is a large demand for remediation of flare pit sites by both regulatory agencies and upstream oil and gas companies. Due to the complex nature of flare pit sites, development of an effective and fast bioremediation method seems challenging. White rot fungi have been successful in degradation of an extremely diverse range of persistent chemicals mainly in liquid medias and in completely controlled and sterilized environments. In general, the control of various conditions required for growth and activity of white rot fungi compared to other systems is more difficult in open and non-sterilized soil systems. Despite the

challenges involved with application of white rot fungi in flare pit sludge bioremediation, the method possesses strong potential.

This research project is the first step in a multiphase project. The objective of this study is to evaluate the feasibility of application of white rot in bioremediation of a flare pit soil. In this study the impact of major soil variables such as moisture and organic amendments on the effectiveness of fungal bioremediation of flare pit soil is investigated. The optimum conditions for fungal growth and activity are also studied, which can be used in future application of field scale fungal bioremediation of flare pit sites.

1.3 Thesis Organization

In the course of this research, there were four main stages. The first step was conducting preliminary tests to determine the optimum soil condition and moisture content for the growth and activity of white rot fungi and to screen fungi strains for main bioremediation experiments. Peat moss, pine wood shavings, bran flakes or a mixture of them were studied for the selection of an effective bulking agent. The best fungal strain from twelve available fungi strains was chosen based on the fungi enzyme activity and growth rate. Based on the results of the preliminary tests, the second step, which was the design of main experiments, was conducted. The last step after finishing the main experiments, was statistical analysis of the results and assessment of the effect of each variable.

The thesis contains five main chapters. The first chapter briefly reviews the problem of flare pit sites, the objectives of this research and the thesis organization. The Literature Review chapter (Chapter 2) briefly discusses the basics of white-rot fungal metabolism and enzymology. It also discusses the conditions required for effective fungal bioremediation. The Materials and Methods chapter (Chapter 3) discusses the materials, experimental procedure, and the statistical methods used in this research project. The results are presented and discussed in the Results and Discussion chapter (Chapter 4), and the final

chapter (Chapter 5) looks at the main conclusions and consequent recommendations from this research project.

Chapter Two: Literature Review

2.1 Characteristics of Environmental Contaminants

It is essential to have a fundamental understanding of the classes of contaminants and their properties, to understand the behavior of contaminants in the environment. Generally contaminants can be divided into to major categories: organic and inorganic contaminants. The compounds that contain organic carbon are considered as organic and others are considered as inorganic (Suthersan, 1996).

A simplified classification tree of the potential environmental contaminants is shown in Figure 1.1.

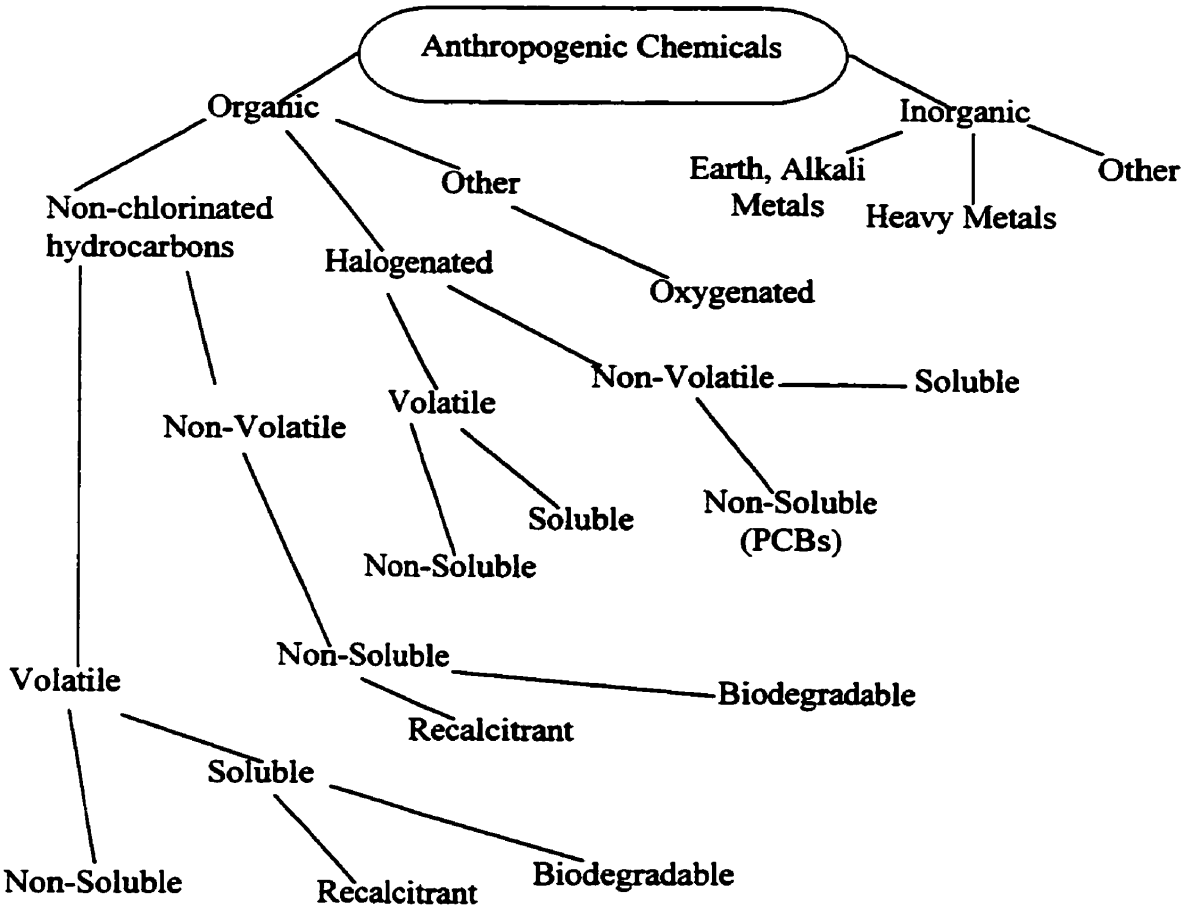


Figure 2.1) Contaminants classification tree (Taken from Suthersan, 1996)

2.1.1 Organic Contaminants

More than 1600 organic compounds have been identified to be present in both natural and polluted environments. The organic compounds, which are often associated with petroleum products or combustion of fossil fuels, typically are of more interest to subsurface contamination. Other chemicals, which are of environmental concern, are chlorinated and nonchlorinated solvents and degreasers, and organic compounds used as raw materials in various manufacturing processes. Petroleum hydrocarbons are generally complex mixtures of a variety of organic compounds with minor fractions of organic and inorganic additives (Suthersan, 1996). For the most part, the mixtures are less dense than water and therefore float on top of the ground water table. These compounds are scarcely soluble in water and relatively volatile.

2.1.2 Inorganic Contaminants

The most important group of inorganic contaminants is metals. Metals are natural constituents of the soil. In addition to the natural constituents, metals enter the soil via agricultural additives such as lime, fertilizer, manure, herbicides, and fungicides.

Metal containing wastes that may impact soil and groundwater pollution include municipal solid wastes, sewage sludge, storm water run-off, wastes from mining and smelting operations and ashes from burning of coal and oil.

2.1.3 Flare Pit Sites

Flare pits or earthen pits have been used by the upstream oil and gas industry to contain or burn produced fluids from either gas or oil production operations. Produced fluids are

defined as, burned oils, crude bitumen, liquid hydrocarbons, process chemicals, products from acidification and dewaxing operations, and water that is produced from oil wells, gas wells, and batteries and processing facilities (Green, 1997). Everything from herbicides to acids, waxes and resins has been found in flare pit sites. Flare pits were common at older well sites, compressor stations and pumping batteries throughout Alberta. Most of the pits are simple excavations lacking liners or other containment features. Flare pits are known as the primary source of environmental contamination at older oil and gas well sites (Speer, 1999). A report from the Alberta oil patch indicates that smaller independent operators have begun, over the last two or three years, to place waste effluent from their wells directly into barrels for recycling.

An accepted approach to bioremediation of flare pit sites often involves *in situ* remediation using such methods as nutrient stimulation, tilling and irrigation to optimize the moisture content of the soil. In many of these operations, no microbial culture is added into the soil and the bioremediation process is based on the activity of indigenous microorganisms.

Bioremediation costs are estimated to run from $35 to $85 per cubic meter of soil depending on the complexity of the underground plume of contamination, the type of soil and the depth of pollutants. The risk of pollutant migration to the level of the groundwater is minimized in many Alberta sites by the presence of heavy clay soils.

2.2 Bioremediation

Bioremediation is the ability of certain microorganisms, hetrotrophic bacteria and fungi, to degrade hazardous organic materials to innocuous compounds such as carbon dioxide, methane, water, inorganic salts, and biomass (Anderson, 1995). Microorganisms may use the organic contaminants as their carbon or energy source, and through biodegradation of organic contaminants derive the carbon and energy required for their growth, or, they might

live on other carbon and energy sources than organic contaminants and just transform more complex, synthetic chemicals through co-metabolism.

Bioremediation processes can be classified into two categories: natural bioremediation and enhanced bioremediation. Natural bioremediation depends on indigenous microflora to degrade contaminants using only nutrients and electron acceptors available in situ. However, if the nutritional and physiological requirements of microorganisms are not met, biodegradation rates will be less than optimal. Enhanced bioremediation technologies increase biodegradation rates by supplying those nutrients, electron acceptors, or other factors that are rate limiting. Enhanced bioremediation can be used to degrade contaminants in situ or exsitu. Some examples of in situ processes include land treatment, bioventing, liquid delivery, and air sparging. Ex situ technologies include slurry reactors, composting, biopiles, and biofilters.

The optimization of the environmental conditions will not be possible without a comprehensive understanding of the biological principles under which these compounds will be degraded. The "biodegradation triangle" (Figure 1.2) shows three important factors, which include the environmental conditions, the chemical and structural properties of the contaminant, and the microorganisms. Environmental conditions are different conditions that can affect microbial activity as pH, redox potential in the remediation environment, presence of nutrients, environment temperature, and moisture content. The presence of all these three factors at optimum level leads to a successful bioremediation project.

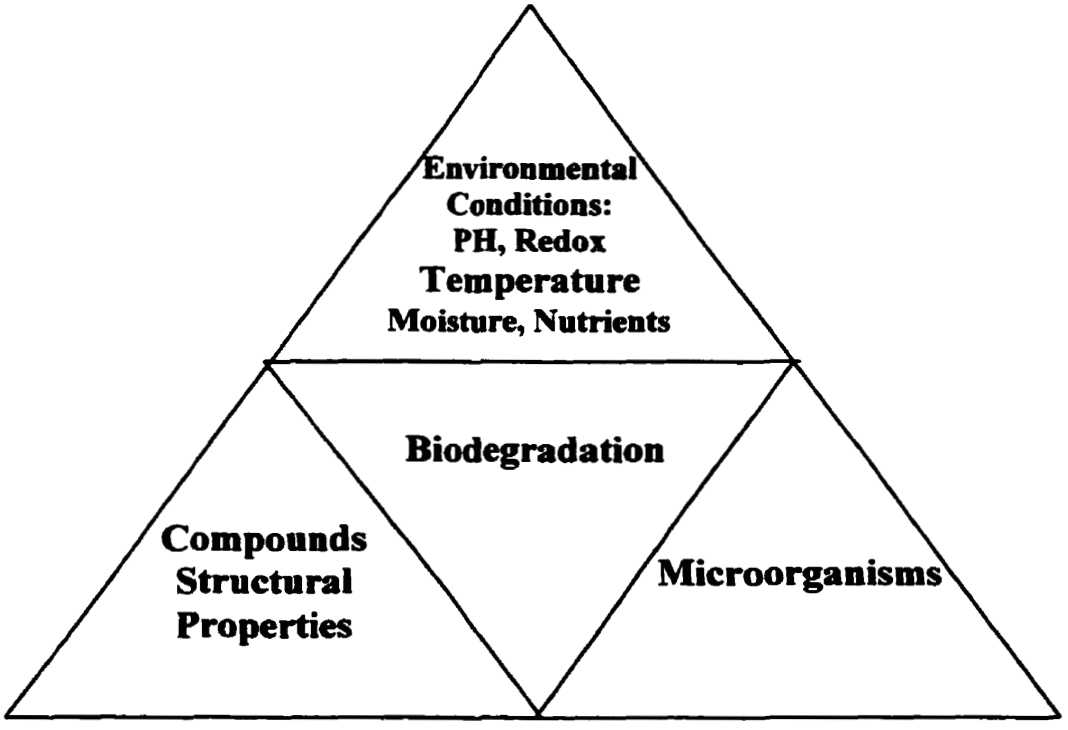


Figure 2.2) Biological Triangles (Adapted from Suthersan, 1996)

2.2.1 Bioremedial Systems

Bioremediation can be used to treat soil, water, and gases that contain biodegradable organic compounds. There are many different bioremediation processes, but some common factors are very important in all bioremediation processes and affect them all. In all Bioremediation systems there two major inputs and one output. The inputs include operational conditions and organic contaminants. The operational conditions include all the environmental conditions and other factors such as toxicity, bioavailability and degradation

rate of contaminants, and also the delivery system for amendments. The major factors are shown in Figure 1.3.

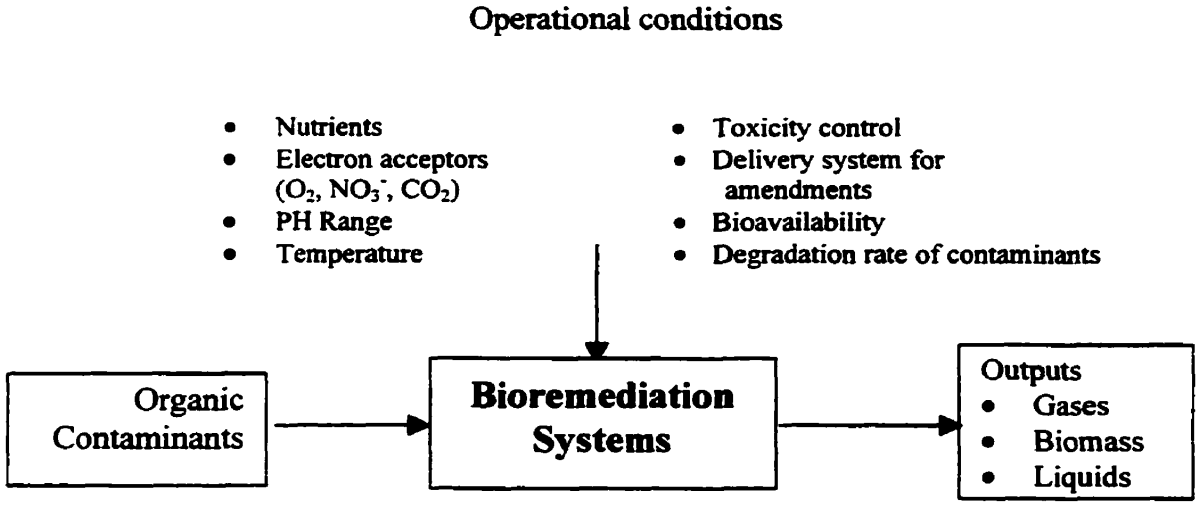


Figure 2.3) Bioremedial Systems (Adapted from Anderson, 1995)

2.2.2 Microbial Growth and Metabolism Requirements

2.2.2.1 Temperature

Microorganisms can be categorized into three major groups based on their optimum temperature range. The first group are called *psychrophilic* microorganisms and their optimum temperature rage is between 0° to 10°C. The next group is *mesophilic* microorganisms and their optimum growth temperature range is between 10°C to 40°C. The last group is called *thermophilic* microorganisms and they live in high temperature environments. Thermophiles can survive in temperatures as high as 60°C. Biodegradation can be detected at temperatures as low as 0° to 10°C (*psychrophiles*) and as high as 40° to

60°C (*thermopiles*). Even though biodegradation rates are slower at low temperatures, successful bioremediation at low temperatures has been reported (Gibb, 1999). The use of thermophilic microorganisms has been primarily limited to composting applications. Temperatures in mesophilic range, between 10° and 40°C, are more practical for field applications, and in some instances, contaminated materials have been heated artificially to this range as a prerequisite for biotreatment (Anderson, 1995).

In bioremediation reactions the bioremedial agent is often a complex community of microorganisms. Thus a shift in temperature of a few degrees can cause dramatic changes in the composition and function of the community. The specific population, which is able to degrade the contaminant, may function just over a narrow range of temperatures or may be replaced by populations with different degradation kinetics or mechanisms.

2.2.2.2 Moisture Content

Moisture has a very critical role in bioremediation processes. The moisture content of soil affects many other parameters in bioremediational systems such as the bioavailability of contaminants, the transfer of gases, the effective toxicity level of contaminants, the movement and growth of microorganism, and species distribution.

Soil moisture content is usually measured as a gravimetric percentage or reported as the percentage of field capacity. However, calculating the *"Water Activity"* factor (a_w) gives us more information about the water availability for microbial metabolism. Water activity factor is calculated as follow:

$$a_w = P_w / P_W^0$$

P_W^0 = Vapor pressure of pure water at the temperature of the system

P_w = Vapor pressure at equilibrium with water in the system.

In a simple term, the water activity is the ratio of the system's vapor pressure to that of pure water at the same temperature (Suthersan, 1996).

2.2.2.3 Nutrients

In addition to carbon, which is the main nutrient for all biological processes, inorganic nutrients, mainly nitrogen and phosphorus, are essentials. Nitrogen can be provided in a variety of forms such as nitrate, ammonium salts, and organic compounds, such as urea. Phosphorus can also be provided in several inorganic forms. Treatability studies are usually conducted to determine nutrient requirements, and results are specific to the particular sites and processes.

2.2.2.4 pH

Most natural environments possess pH values in the range between 5 and 9. Therefore, it is not surprising to find that most microorganisms have evolved with pH tolerance within this range. For bioremediation processes, the optimum pH is site and process-specific and must be determined during feasibility studies. Most microorganisms tolerate pH 5 to 9 but prefer pH 6.5 to 7.5. There are acidophilic bacteria, which have pH optimum near 2.5. Also, there are alkalophilic bacteria that can function at pH 10 to 12.

Since many bioremediation processes produce acids or bases, the buffering capacity of the system must be sufficient, or neutralizing agents must be added to maintain an optimum pH range. For example, the degradation of chlorinated solvents produces hydrochloric acids, and the fermentation of sugars produces organic acids.

2.2.2.5 Electron Acceptors

Much of the energy for growth of microorganisms is obtained during the transfer of electrons from organic substrates to inorganic electron acceptors. Therefore, appropriate electron acceptors are absolute requirements for biodegradation, and provision of these electron acceptors often constitutes the greatest challenge in the design of in situ bioremediation systems. The common electron acceptors are carbon dioxide, sulfate, nitrate, and oxygen. In some instances, halogenated organic contaminants can serve as electron acceptors when they are used as substrates for reductive dehalogenation. It is important to note that the electron acceptors listed above are not interchangeable. This means, even though some bacteria can use either oxygen or nitrate as the terminal electron acceptor, the microbial communities are distinctly different for each type of electron acceptors. Their metabolic processes and their potentials for biodegradation of pollutants are also very different.

2.2.2.6 Contaminant Bioavailability

Microorganisms may have the metabolic capability to mineralize a substrate and yet fail to do so because it is insoluble, sorbed or otherwise unavailable to the cell. For example the biodegradation of polycyclic aromatic hydrocarbons (PAHs) is often limited because the contaminants are not available to the biomass due to their low solubility. Bioavailability is an important consideration in the design of bioremediation systems.

Microorganisms can produce surfactants to aid in the solubilization of poorly soluble or immiscible substrates. Synthetic surfactants have also been used to aid the process of bioremediation in some cases (Bogan et al. 1999).

Other aspects of substrate availability have also taken considerations. Generally all organic contaminants that are candidates for bioremediation are toxic at high concentrations. But, many of higher molecular weight hydrocarbons are very little soluble in water and at their solubility limits in water they are not toxic.

Another limitation of bioremediation is inherent in the nature of the enzyme reactions involved. The kinetics of enzyme induction and substrate binding and transport dictates that there will be a threshold substrate concentration below which biodegradation rates will be negligible. Contaminants initially present at very low concentrations may not be biodegraded at all (Anderson, 1995).

2.2.3 Bioaugmentation

The natural occurring microbial community may not have the capability to degrade specific synthetic chemicals of concern at a particular site. If treatability studies show no degradation, or an extended delay before significant degradation is achieved, inoculating with strains known to be capable of degrading the contaminant may be helpful. This method has been successful in several laboratory applications while a few field trails have been documented (Anderson, 1995).

Many scientists believe that simply amending soils with appropriate substrates may suffice in encouraging growth of the soil indigenous microbial populations resulting in reduction of time and cost involved in the production and application of bacterial or fungal inoculants. In fact, recent studies have shown that both amended and non-amended systems may have substantial number of microorganisms at the end of the treatment period. On the other hand, it has been reported that, during a long-term soil treatment process, bioaugmentation has enhanced nutrient uptake and thus increased microbial metabolism. Thus bioaugmentation is an important design consideration in designing bioremediation systems (Azadpour, 1667).

2.3 White rot fungi and their role in waste decontamination

2.3.1 Fungi physiology

Fungi in general are heterotrophic (non-photosynthesizing) eukaryotes (they posses a nuclear membrane) (Glaser & Lamar, 1995). They absorb their food, typically at the many growing points of their diffuse and indefinite body, which is often called thallus or mycelium. Mycelium is made up of fine branching tubes called hyphae. The wall of these tubes is mainly composed of chitin or cellulose, and within these walls the cytoplasm and nuclei live and move. The fungi reproduce by means of spores (Kendrick, 1992).

Fungi are heterotrophic, and therefore depend on energy rich carbon compounds produced by other organisms. Fungi have evolved enzymes that can digest some extremely recalcitrant substrates such as chitin (insects exoskeleton), keratin (skin, hair, horn, feather), cellulose and lignin (Kendrick, 1992).

The fungi enzymes are secreted at the tips of hypha, which has a strong and waterproof chitinous wall. The hydrostatic pressure of enzymes at the tips of hypha is ideally suited for actively penetrating, exploring and exploiting solid substrates in a manner that no bacteria, chief competitors of fungi, can compete.

The fungi microscopic spores which have very diverse shapes to suit their specific functions, are often produced very quickly even in a matter of days or even hours after the initial colonization of the substrate. The spores are produced in enormous numbers and are dispersed by wind, water, or animals. Spores can often survive long periods, sometimes even years of unfavorable conditions such as freezing, starvation or dry out.

Fungi can survive in extreme environmental conditions. They can grow at temperatures as low as –5°C and as high as 60°C. Certain fungi can grow under extremely acidic conditions to the pH of as low as 1; others can tolerate extreme alkaline conditions up to pH 9.

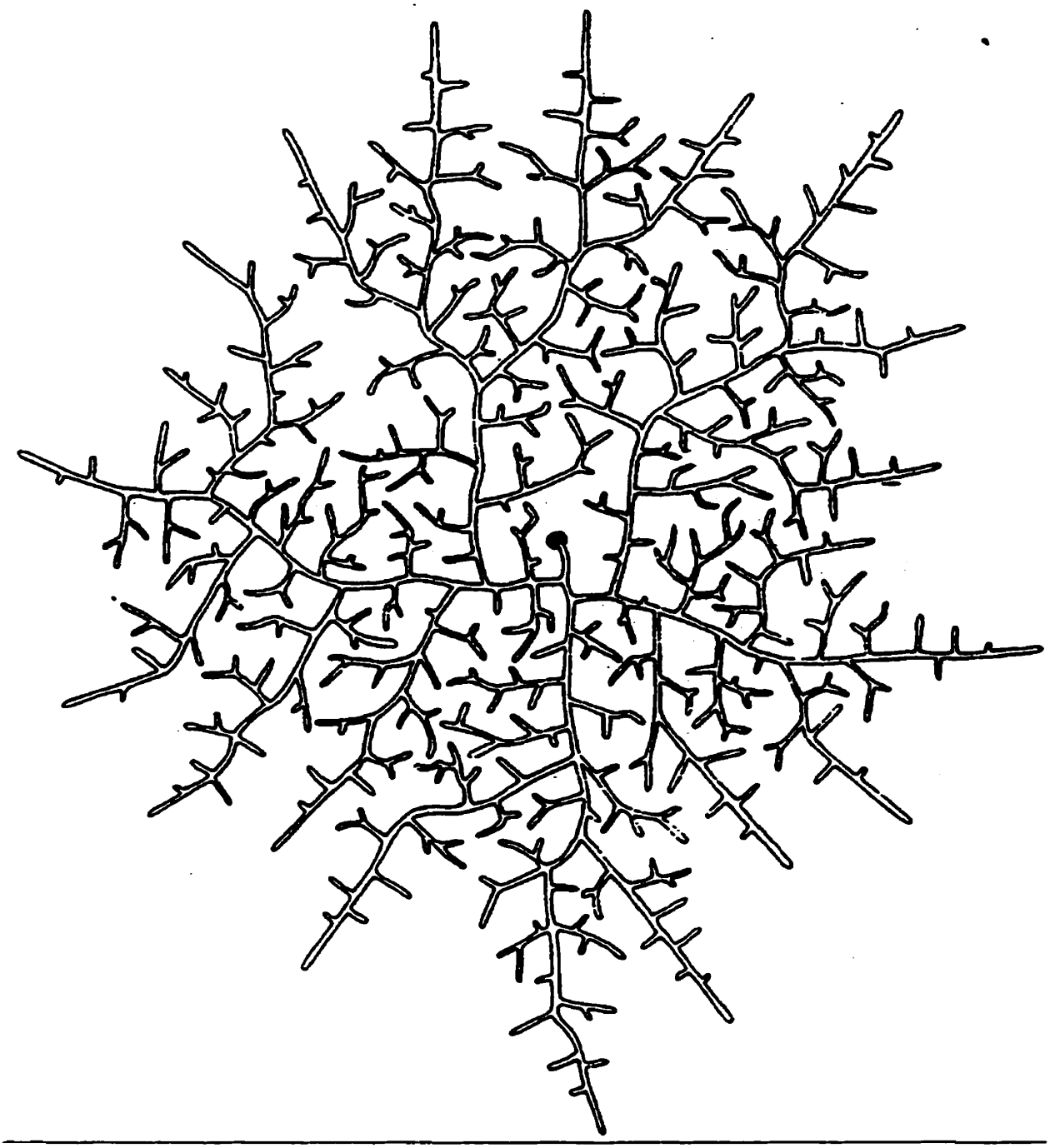


Figure 2.4) Young colony of fungi arising from a spore (Note the large number of hyphal tips) (Taken from Kendrick, 1992).

2.3.2 White rot fungi

Wood rotting fungi are classified into three major categories based on the type of wood decay caused by these organisms: white rot fungi, brown rot fungi, and soft rot fungi. From these three groups, white rot fungi, have been the most extensively studied group. White rot fungi primarily include a heterogeneous collection of hundreds of species of basidiomycetes (Gilbertson, 1980 & Pelaez et al. 1995).

White rot fungi are known for their variety and their remarkable ability to degrade complex and persistent natural materials. They produce a nonspecific, extracellular enzymatic system, which is capable of degrading lignin, one of the most resistant materials found in nature. Because of the similarities between chemical structural of lignin and some organic pollutants these fungi have been used widely as pollutant degraders. In contrast to bacteria, fungi are capable of extending the location of their biomass through hyphal growth. These features distinguish fungi as organisms having great potential for use in bioremediation of soils contaminated with some of persistent organic pollutants (Aust & Barr, 1994). The potential targets for white rot fungi include (i) sorbed contaminants, (ii) high molecular weight contaminants, and (iii) complex mixtures of chemicals typical of a contaminated site (Azadpour, 1997).

2.3.3 Wood rot fungi in nature

The basic role of wood-rotting fungi, basidiomycetes, in the world ecosystem is the recycling of carbon removed from atmosphere during photosynthesis by autotrophic organisms. If it wasn't for wood rotting fungi most of the earth's carbon would be locked in the form of lignin. Wood rotting fungi, in addition to their basic role in recycling carbon, have other ecologically beneficial effects. By weakening older trees, wood rotting fungi make them weak to wind throw and natural removal from the stand. This permits the

growth of young, vigorous trees and thereby plays an integral role in maintaining the dynamic and ever changing nature of our forests. Partially decayed wood residues are important components in forest soils and increase water-holding capacity and improve other physical and chemical characteristics essential for establishment and growth of native tree species (Gilbertson 1980).

Wood rotting fungi are also important in providing shelter and breeding sites for wildlife. The large number of cavity nesting birds in North America are mainly dependent on wood rotting fungi for development of their nesting sites (Gilbertson, 1980).

The number of North America wood rotting basidiomycetes is estimated to be between 1600 and 1700 species. Wood rotting basidiomycetes have become established in all environments in North America where woody plants are present from southern deserts to the arctic tundra (Gilbertson, 1980).

2.3.4 Optimum Environmental Condition for White-Rot Fungi

Temperature plays the primary role in lignin degradation by white rot fungi. The optimum temperature range for these fungi is from 20°C to 40°C. Lignin degrading fungi are aerobic microorganisms and cannot survive, grow or metabolize under low levels of oxygen (Azadpour, 1997). The optimum pH for white rot fungi is acidic and can vary from pH of 4 to 7.0 (Baker Lee, et al., 1995).

2.3.5 Lignin

Lignin (Latin lignum = wood) is the most abundant and widely distributed renewable aromatic polymer in the biosphere. Lignin, cellulose, and hemicellulose are the major

structural components of woody tissues. About 20-30% of dry mass of woody plants is made up of lignin. Lignin plays a number of important functional roles (Kirk & Farrell 1987). These include giving the desired strength, rigidity and elasticity to plant tissue and minimizing the water permeation across cell walls of xylem tissue. Because of its recalcitrance to biodegradation, lignin definitely plays a role in providing protection against microbial attacks to the plant.

About 70-90% of the lignin in wood fiber is to be found in the primary and secondary cell wall layers. The middle lamella contains the rest with 10-30% of the lignin. In the plant cell wall lignin is well inter-spread with hemicellulose and forms a matrix around the cellulose micro fibrils. Lignin also function as an adhesive cement binding cells together.

Lignin biodegradation is essential to the carbon cycle of earth because lignin is the second most abundant carbon source after cellulose and because lignin physically protects most of the world's cellulose and hemicellulose from enzymatic hydrolysis. Research on lignin biodegradation has been accelerated greatly, mainly because of the significant potentials of its application in pulp and paper industry, wood bleaching, converting lignin to useful products and treating wastes.

Lignin is actually not biodegraded anaerobically. Neither rapid nor extensive bacterial degradation of lignin has been reported, even under highly aerobic conditions (Kirk & Farrell 1987). White-rot fungi, only in aerobic conditions, cause the most rapid and extensive degradation described to date.

2.3.6 Lignin Structure

Lignin is a unique biopolymer. Unlike other naturally occurring biopolymers lignin does not contain identical, easily hydrolysable, repeating linkages that occur at regular intervals. Instead lignin is a highly irregular, three-dimensional polymer that has no precise chemical

structure but has a series of substructures, which occur at a random basis and they differ in lignin from various sources (Kirk & Farrell, 1987). Because of these structural features and also the large size of the lignin polymer no microorganism can intake lignin and thus all lignin-degrading enzymes are extracellular and relatively nonspecific.

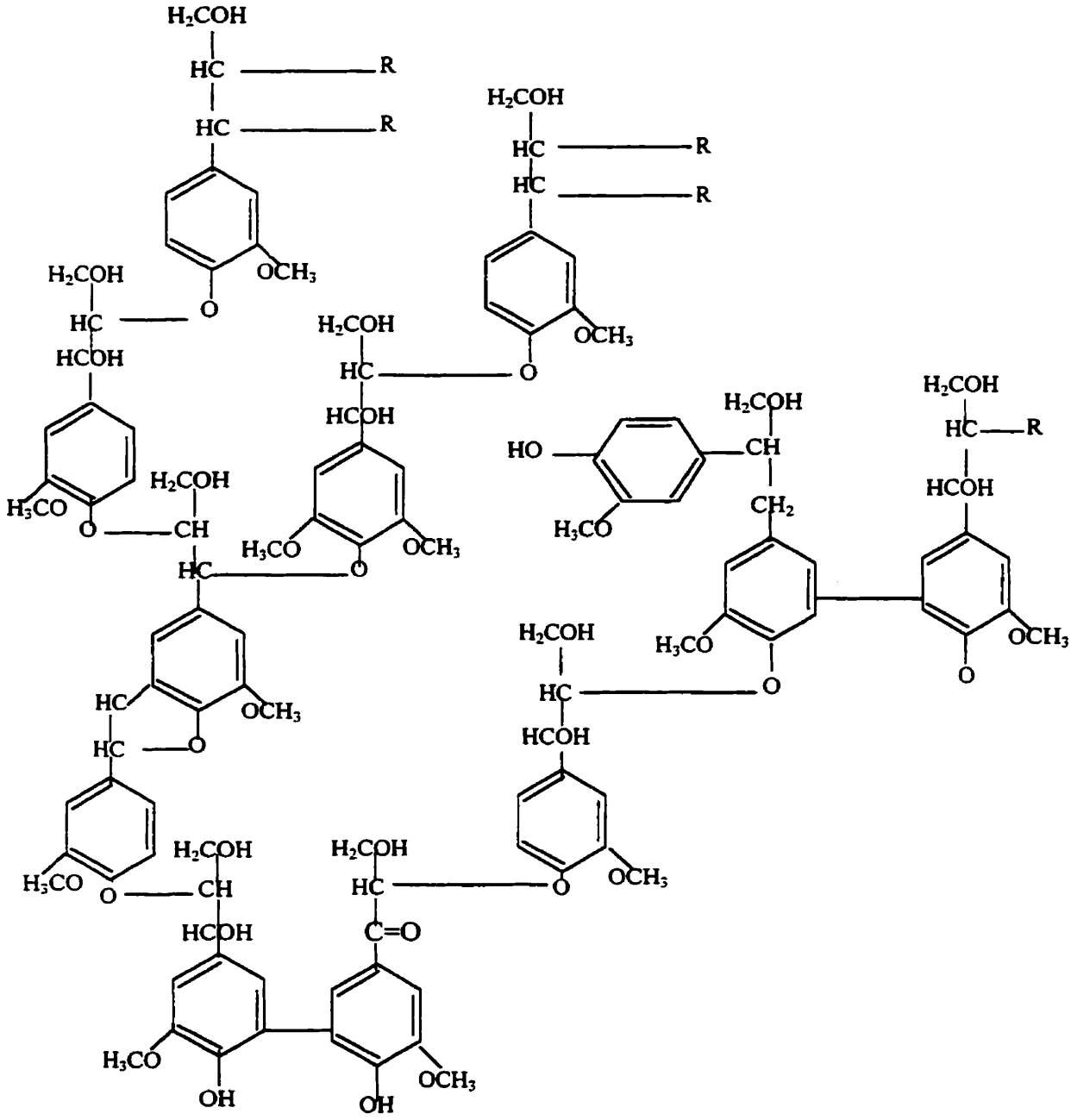


Figure 2.5) Representative structure for a part of lignin polymer (adapted from Aust & Barr, 1994)

2.3.7 Lignin Degradation by white rot fungi

Even though lignin is rich in carbon, it is not a growth substrate for microorganisms, which are reported to degrade lignin. Lignin degrading fungi, only in the presence of an alternate energy/carbon source, metabolize various lignin preparations. Thus, lignin degradation is co-metabolized when white rot fungi utilizes other carbon/ energy substrates such as cellulose, hemicellulose, simple carbohydrates, and glycerol. This is because fungi strains degrading lignin require an easy metabolizable growth substrate and the oxidative degradation of lignin by white rot fungi does not provide sufficient energy for growth. Degradation of substrates such as cellulose provides glucose for the fungus and when the amount of these substrates decrease, the fungus starves and thus it transfers fungus from a primary metabolism to a secondary metabolism mode. Therefore, to degrade lignin, the fungus needs easily metabolizable nutrients such as sugars from the polysaccharides of wood. These sugars are also necessary for the production of hydrogen peroxide, which is needed for the organisms to degrade lignin.

Lignin degradation by white rot fungi stops when excess carbon or nitrogen is added to the cultures limiting for the nutrients, and the fungus shifts to the primary growth mode. In nitrogen-limited cultures, ligninolytic activity is associated with the formation of new secondary mycelia.

2.3.8 Enzymology of White Rot Fungi

According to Hatakka (Hatakka, 1994) different white rot fungi produce different lignin degrading enzymes. The best characterized of these enzymes are laccase, lignin peroxidases (LiPs) and manganese peroxidases (MnPs) (Plaez et al. 1995, & Hatakka, 1994). Based on their production pattern of their extracellular, ligninolytic enzyme systems, white rot fungi may be divided into four main groups including: (i) LiP-laccase group, (ii) LiP-MnP group

(iii) MnP-laccase group (iv) Laccase-AAO group, AAO denoting aryl alcohol oxidase. Certainly there are some overlaps and exceptions in this categorizing (Hatakka, 1994).

Ligninase or lignin peroxidase (LiP) and manganese peroxidase or Mn-dependent peroxidase (MnP) require hydrogen peroxide as an oxidant. By abstracting one electron and generating cation radicals, lignin peroxidase oxidizes non-phenolic substructures. The produced cation radicals will then be decomposed chemically. Mn-dependent peroxidase oxidizes Mn (II) to Mn (III) then the produced Mn (III) oxidizes phenol rings to phenoxy radicals. Laccase is a copper-containing oxidase. As the oxidant laccase utilizes molecular oxygen and oxidizes phenolic rings to phenoxy radical. The role of veratryl alcohol oxidase (VAO) and other aromatic alcohol oxidases (AAOs) in lignin degradation is not known. They may play a role in the production of hydrogen peroxide for peroxidase enzymes. However, AAO activities have been found also in fungi that do not show strong peroxidase activities. Either glyoxal oxidase or AAO, but usually not both, seem to be produced by an individual fungus.

It has been reported that LiP is not a major requirement for lignin degradation, but it has been suggested that LiP may accelerate the conversion of lignin-derived preparation for CO_2. Also it has been concluded that MnP seems to have a role in initial depolymerization of lignin, mediated by a Mn (II)-Mn (III)-organic acid complex, and then more extensive degradation of the lignin oligomers is achieved by LiP. Data also indicate that the fungi, which produce both LiP and MnP, are very efficient lignin degraders.

The LiP-laccase group fungi seem to possess the most inefficient system for lignin biodegradation. Some recent results indicate that if the fungus does not produce MnP, lignin is only poorly degraded, although the fungus readily produces LiP. When the fungus does not produce LiP but only MnP and Laccase, lignin biodegradation is moderate to good.

The practical importance of fungi apparently lacking MnP may be low. These fungi have not been the best candidates in screening programs carried out for potential applications.

The first fungi belonging to this group have only been described recently. The ecological significance of this group is also difficult to assess (Hatakka, 1994).

2.3.9 The Advantages of White Rot Fungi

As described in previous sections, white rot fungi produce extracellular enzymes, which are able to co-metabolize complex molecules such as lignin. The same unique and nonspecific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants.

Along with their ability to degrade complex chemicals, white rot fungi possess a number of different advantages that are not associated with other bioremediation systems. Because the key component of the lignin degrading system of white rot fungi are extracellular enzymes, the fungi are able to degrade very insoluble components as lignin and many of the hazardous environmental pollutants such as Poly Aromatic Hydrocarbons (PAHs).

Many of the pollutants in hazardous waste sites are toxic to the organisms that may be employed to degrade them. For example, intake of cyanide by bacteria inhibits growth because cyanide is known to be a potent inhibitor of respiratory oxidase enzymes. Yet, in order to metabolize cyanide, bacteria must take up the pollutant because the enzymes are located inside the cell. As a result cyanide concentrations as low as 4ppm could inhibit microbial growth in a municipal sewage treatments system. However the extracellular system of the white rot fungi enables the fungi to tolerate considerably higher concentrations of a toxic pollutant such as cyanide (Aust & Barr 1994).

The other advantage of white rot fungi is the very nonspecific nature of the mechanism used by these fungi allows them to degrade even complex mixtures of pollutants, such as creosote, all the way to carbon dioxide. In contrast, different groups of bacteria may be needed to successfully and completely degrade these same mixtures. For example, it has

been found that anaerobic bacteria can effectively dehalogenate polychlorinated biphenyls (PCBs) to monochlorinated biphenyls. However, aerobic bacteria are then required to degrade these monochlorinated biphenyls to carbon dioxide (Aust & Barr 1994).

Another advantage of white rot fungi is that they do not require preconditioning to a particular pollutant. Because the degradation system of white rot fungi is induced by nutrient deprivation, limiting the nutrient source can initiate degradation. Thus, because the induction of the degradative enzymes is not dependent on the presence of the chemical, the fungi can effectively degrade very low concentrations of a pollutant to non-detectable or nearly non-detectable levels. Furthermore, repression of enzyme synthesis does not occur when the concentration of a chemical level is reduced to a level that is inefficient for enzyme induction. The effective decontamination of very low concentrations of chemicals is important because government regulatory agencies are continually lowering the maximum permissible levels of these hazardous environmental pollutants (Aust & Barr 1994).

Very inexpensive growth substrates such as corncobs or other crop residues, wood chips, or surplus grains can be used to cultivate white rot fungi in soil. The lignin degrading system allows the fungi to access the limited carbon source from such substrates. Other soil microbes are less able to utilize these substrates. Therefore the fungi maintain a competitive advantage (Aust & Barr 1994).

2.3.10 The Compounds Degradable by White Rot Fungi

A wide variety of components including color pigments, pesticides, wood preserving wastes, munitions, heavy hydrocarbon and plastics can be degraded by lignin degrading fungi. Table 1.1 shows a list of components, which can be degraded using white rot fungi (Azadpour, 1997).

Table 2.1) Compounds that may be degraded by white rot fungi (Taken from Azadpour, 1997)

Chlorinated phenols
Trichlorophenol (TCP)
Pentachlorophenol (PCP)
Dioxin
Dyes
Pesticides
Artazine
3-Amino-1, 2, 4-triazole (AT)
Chlordane, Lindane
2, 4-Dichlorophenoxyacetic acid
2, 4, 5-Trichlorophenoxyacetic acid
Hydramethylnon
1, 1, 1-Trichloro-2, 2-bis (4-chlorophenyl) ethane
Polychlorinated Biphenyls (PCBs)
Polycyclic Aromatic Hydrocarbons (PAHs)
Anthracene
Benzo[a]pyrene (B[a]P)
Phenanthrene
Trinitrotoleune (TNT)
Other Compounds
Cyanide
Humic compounds
Hydrocarbons/Chlorinated Hydrocarbons
Plastics

2.3.10.1 Biological Bleaching and wood pulping industry

Usual bleaching of Kraft pulps with chlorine is becoming unpopular, because of the generation of chlorinated organic by-products. Thus, there is a high demand for alternative pulp decolorization techniques. Generally use of lignin degradation fungi as decolorization agents has not gained a wide acceptance for two reasons: first, the fungal attack to the cellulose molecules during the bleaching process is inevitable, and second, in comparison

with chemical bleaching, biological bleaching is still rather slow and inefficient (the procedure takes days instead of hours) (Azadpour, 1997).

In order to protect cellulose from fungal attacks during the bleaching process, an experiment was conducted using a membrane filter as a physical insulator that prevent direct contact between hyphae and kraft pulp. These membranes were permeable to extracellular enzymes required for the bleaching process. During this process, pulp brightness increased 54.0% after five days of treatment (Azadpour, 1997).

Despite practical difficulties encountered with the use of a biological decolorizer in bleaching of the Kraft pulp, these systems have been more effective for bleaching of the wastewater pulps. For example rotating biological contractors have been successful in dechlorinating chlorolignin from pulp bleaching wastewater and during one day of treatment with a lignin degrading fungus, the total organic chlorine content of chlorolignin decreased approximately 50 percent.

Some literatures also indicate that immobilized fungus used in fluidized bioreactor systems may serve as a promising technique in simultaneous reduction of color, chlorophenolics, and adsorbable halo-organic from bleach plant effluents (Pallerla & Chambers, 1995).

2.3.10.2 Dyes

Synthetic dyes are used extensively for textile dying, paper printing, color photography, and as additives in petroleum products. It's been demonstrated that lignin degrading cultures could decolorized several polymeric dyes, and now these dyes (e.g., Poly R-478) are routinely used as indicators of secondary fungal metabolisms (Glenn & Gold, 1988).

Most biodegradation studies using dyes involve azo dyes, which are the largest class of dyes in terms of color and therefore, structural versatility. Their chemical structures are

based on azobenzene and the azo naphthol derivatives (Cookson, 1995). Because of the structural differences, azo dyes exhibit different molecular complexities, and they do not show uniform results to microbial attack. According to the literature, nitrogen limited cultures of *Phanerochaete chrysosporium* are the most extensively used lignin-degrading fungi in dye biodegradative studies. Also cultures of *Pycnoporus cinnabarinus* were used in a packed bed bioreactor to rapidly decolorize and clarify a pigment plant wastewater sample (Azadpour, 1997).

2.3.10.3 Pesticides

The biodegradation of pesticides in the environment is a necessity for environmental quality. Unlike herbicides and insecticides that usually do not cause damaging effects on microorganisms, effective fungicides can cause extensive damages to microbial and specifically fungal population. Thus, in addition to eliminating harmful fungi, these compounds harm beneficial fungi and bacteria. Microbial degradation of pesticides occurs by a series of enzymatic mediated step-wise reactions. These reactions may include oxidation, reduction, and/or hydrolysis (Azadpour, 1997).

Lignin degrading fungus may have good potentials in remediating and reducing toxicity of pesticide contaminated soils. As an example it has been reported that lindane, dieldrin, and polychlorinated Biphenyls (PCB) have been mineralized by *P. chrysosporium* (Bumpus et al., 1985 & Kennedy et al., 1990).

2.3.10.4 Wood Preserving Wastes (Pentachlorophenol)

Pentachlorophenol is one of the most widespread chemicals in the environment and has had the most varied use. US Environmental protection agency had registered over 500 products

containing PCP as active agent. Of the PCP production, 80 percent was used for the preserving of wood. PCP was normally carried in a solvent such as diesel fuel. Significant PCP accumulation in the food chain has resulted from its years of use.

Bacteria and fungi are active degraders of PCP. Fungal species include *Phanerochaete chrysosporium, P. sordida* and *Trametes versicolor* (Cookson, 1995). Pentachlorophenol is readily mineralized to carbon dioxide by *Phanerochaete chrysosporium,* although the transformation product of 1,4-tetrachlorobenzoquinone is also produced. Pentachloroanisole (PCA) is a major by product of PCP transformation in soil by both *P. chrysosporium,* and *P. sordida* (Lamar, 1990). The transformation of PCP in soil is a two step reaction. The first step yields rapid transformation of PCP and accumulation of PCA. This step represents a 60 to 70 percent conversion of PCP to PCA. In the second step, both PCP and PCA are reduced. The first transformation step for *P. chrysosporium* was significantly faster than that of *P. sordida.* The second step was much slower (Cookson, 1995).

The highest PCP degradation was reported in an experiment conducted in a fluidized bed bioreactor Using white rot fungi *P. chrysosporium.* In this experiment, PCP degradations of as high as 72% have been reported (Azadpour, 1997).

2.3.10.5 Poly Aromatic Hydrocarbon

Poly Aromatic Hydrocarbons (PAHs) are a group of important priority pollutants originating from coal gasification, cooking, wood preservation facilities and petroleum hydrocarbons. Low molecular weight (MW) PAHs are usually easily degraded but high molecular weight PAHs of five rings or more are highly resistant to bacterial degradation in soil and sediment media. This recalcitrant behavior can be caused by big molecular size of these PAHs, the limited boiavailability of PAHs due to their strong tendency to adsorb onto the soil organic matter (Azadpour, 1997).

A diverse group of fungi, including *Aspergillus ochraceus*, *Cunninghamella elegans*, *P. chrysosporium*, *Sacchromyces cerevisiae*, and *Syncephalastrum racemosum*, as well as *Bjerkandera sp.* strain *BoS55* have the ability to degrade PAHs.

Bjerkandera sp. strain *BoS55* is capable of degrading benzo(a)pyrene (B(a)P), a five ring PAH compound, in a sterile sandy loam soil artificially contaminated with B(a)P. The elimination of this compound was as high as 80% within 22 days (Field & Feiken, 1995).

On the other hand, experiments have shown that biodegradation of PAHs by fungi is incomplete, and intermediate products generated during the process may even exceed concentrations that are legally authorized. The transformation products of PAHs may include compounds such as transdihydrodiols, phenols and quinones in addition to other metabolites, depending on the type of the PAH structure (Azadpour, 1997).

2.3.10.6 Munitions Wastes

There are many old military installments all over the world and specially United States that were involved in manufacturing and processing of explosive materials. These installments might be major sources of many environmental problems such as soil and groundwater contamination. Explosive-contaminated soil and groundwater must be remediated in a manner that ensures environmental safety.

At the present time, incineration is commonly used for cleanup of explosive contaminated soils. Incineration is cost-prohibitive, unsuitable for aquifer remediation, and difficult to apply because of regulatory constraints. Therefore, the use of biological processes has initiated considerable research effort. In particular, according to the studies taken place, composting shows promise in treating explosive-contaminated soils (Bennett et al., 1995).

Because most explosives used in past, contained 2, 4, 6-trinitrotolouene (TNT) cyclotrimethylenetrinitramine, cyclonite, hexogene, and Royal Demolition Explosive (RDX) in varying proportions, most of soil contaminations result from the mixture of these compounds (Rosenblatt & Burrows, 1991). Although the biological treatment of RDX-contaminated soils seems not very effective, results from biological treatment of TNT by white rot fungi and particularly by *P. chrysosporium* in both water and soil has been promising.

2.3.11 Factors affecting the soil bioremediation using white rot fungi

The major limiting factor, affecting the application of white rot fungi in bioremediational processes is currently identified as the preparation and cost of inoculum (Anderson et al. 1995).

Biotic features of the soil may suppress the effectiveness of bioremediation in the field. The production of antibiotics by some bacteria in the soil can also cause inhibition of fungal growth. The competition for nutrients may be another contributing factor.

In addition to microbial component, the soil composition could affect the effectiveness of white rot fungi in pollutant degradation. The physical components, e.g. the clays or silt composition of the soil structure might be inhibitory. It has been shown that the fungal bioremediation is more effective in sandy soils than in silty or clayey soils. The partitioning of the pollutant into the clay may also be involved in reducing its availability to fungi.

The soil pH is another important feature in restricting fungal general metabolism and more specifically reducing fungal enzymatic activity because the enzyme has an acidic pH optimum.

Chapter Three: Materials and Methods

The first part of this chapter covers the materials and analytical methods, used during the course of this project. The preliminary experiments, main experiments and the experimental set up are described in details afterward. At the end of this chapter the statistical methods used for analysis of results are reviewed.

3.1 Materials

3.1.1 Fungi

Twelve strains of white rot fungi were obtained from Dr. Michael Pickard, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, and are shown in Table 3.1. Fungi were grown on potato dextrose agar plates (PDA, Difco) at 28°C for 5-7 days before being stored at 4°C and were transferred every 3 months.

Table3.1) The list of fungi used in the experiments.

Name of Fungi	Collection Number	
Coriolopsis gallica	UAHM*	8260
Bjerkandera adusta	BOS 55	
Bjerkandera adusta	UAMH	7308
Bjerkandera adusta	UAMH	8258
Pleurotus pilmanarius	UAMH	7989
Pleurotus ostreatus	UAMH	7988
Pleurotus ostreatus	ATCC **	58053
Pleurotus ostreatus	ATCC	44309
Phanerochaete chrysosporium	ATCC	24725
Trametes vervicolor	UAMH	8272
Trametes vervicolor	UAMH	8273
Trametes vervicolor	UAMH	7331

* University of Alberta Microfungus Collection Herbarium, Devonian Botanic Garden, Edmonton, Canada.

** American Type Culture Collection

3.1.2 Bulking Agents

Kellogg Bran Flakes, Peat Moss and pine wood shavings or a mixture of them were used as bulking agents. The bulking agents were readily available. Peat moss and wood shavings were purchased from Sunnyside garden stores, Calgary, AB, and Kellogg Bran Flakes were obtained from domestic grocery stores.

3.1.3 Soil

Three different types of soil were used in the experiments including clean soil, artificially contaminated soil and flare pit soil. Clean soil was provided from University of Calgary Grounds Department and was used for dilution of flare pit soil. Artificially contaminated soil was produced from clean soil and was used for preliminary tests. Flare pit soil was provided by Imperial Oil Research Center and was used for main experiments.

Artificially contaminated soil was prepared by contaminating the clean soil with weathered Alberta Federated Sweet Mixed crude oil at a concentration of 6000 ppm (w/w). To make a homogenous mixture, the oil was first dissolved in methylene chloride and the solution was then sprayed over the soil. The methylene chloride solvent also partially sterilized the soil. The soil was then kept under fume hood for 24 h for the solvent to evaporate.

The flare pit soil used for the main experiments was originated from one of the Albertan flare pit sites of Imperial Oil. This soil was heavily contaminated with hydrocarbons with an initial concentration of 15.9% (wt of hydrocarbons/wt of dry soil). The soil contained 63% Silt, 37% clay and 0% sand with a silty loam texture and with very low amounts of salt and heavy metals. More detailed information on characterization of the soil can be found in Appendix A.1. The "Saturates, Aromatics, Resins, and Ashphaltenes" (SARA) fractionation method was also used to characterize the hydrocarbon content of the soil (Peramanu, Pruden & Rahimi, 1999). The results are presented in chapter 4.

3.1.4 Buffer Solution

The buffer solution was used to add the required moisture to the soil in the main experiments and to keep the soil pH constant and slightly acidic. The buffer solution used for this purpose was 60 mM sodium phosphate buffer solution at pH 6. To prepare the sodium phosphate buffer solution, 500 ml of 0.6 M NaH_2PO_4 solution was added to 175 ml

of 0.6 M Na2HPO4. This solution was further diluted 10 times to 60mM before any application.

3.2 Experimental Methods

3.2.1 Preparation of PDA Plates

In order to prepare the fungi plates, Potato Dextrose Agar (Difco) was used as the plating media. To prepare the plates the procedure provided by the manufacturer was used. In this procedure, 39 g of the dry media was dissolved into 1 L of distilled water and was boiled to dissolve completely. Then the mixture was sterilized at 121-124°C for 15 to 20 min in an autoclave. The hot media was then poured into petri plates and the plates were left overnight for media to set.

3.2.2 Liquid Growth Media

Before applying the fungi to the soil or bulking agents, it was grown in glucose-malt extract-yeast extract (GYM) liquid growth media (Pickard et al. 1999a). The medium contained glucose: 10(g/L), malt extract (Difco): 3.5(g/L), yeast extract (Difco): 2.5(g/L), KH_2PO_4: 2.0(g/L) and $MgSO_4.7H_2O$: 0.5(g/L). The media was prepared in 500 mL batches in 1 L flasks and then was sterilized at 121-124°C for 15 to 20 min before being stored in the fridge for future use.

3.2.3 Measuring Fungal Ligninolytic Enzyme Activity Using Dye Decolorization Method

The poly R-478 decolorization assay was used in order to study the ligninolytic enzyme activity of fungi in both preliminary and main experiments (Glenn & Gold, 1988). This method was chosen because poly R-478 decolorization is a good indicator of the extracellular ligninolytic activity. The polymeric dyes remain extracellular and could not be taken into the cells and thus will provide a good model for lignin degradation. Glenn and Gold showed that the growing body of fungi in the media indicates that the dyes serve as substrates for at least some components of the lignin degradative system and that dye decolorization is correlated with the onset of secondary metabolism and ligninolytic activity.

In liquid media, the assay was based on monitoring the dye decolorization in GYM medium, placed in 125 ml loosely capped flasks. Fungi were incubated statically in these flasks at 28°C. The dye was added to the medium at the time of incubation (time zero). The initial concentration of dye added to the medium was 0.2 g/L. Decolorization was measured by removing 0.4 ml of extra cellular culture medium and diluting it up to 10-fold in water. The absorbance ratio (A520/A350) of the decanted supernatant solution was then measured by a UV-VIS spectrophotometer (Cary 4E, Varian Inc. Canada).

Poly R-478 decolorization assay was slightly modified to study the ligninolytic enzyme activity of fungi in the soil. To verify overall ligninolytic activity in the soil, 5 ml of dye poly R-478 aqueous solution (0.02 g/L) was added to 1 g of soil. The solution was kept under light for 24 h for the enzyme reaction to take place. Then the solution was centrifuged for 4 min at 5000 rpm (5804 Eppendorf Centrifuge) to separate the soil particles from the aqueous solution of dye and extracted enzymes. The absorbance ratio (A520/A350) of the decanted supernatant solution was then measured by the UV-VIS spectrophotometer (Cary 4E, Varian Inc. Canada). The reason for measuring the absorbance ratio is that adsorption of dye to fungal mycelia reduces the intensity of color and thus the measured absorbance, however under the same conditions the absorbance ratio A520/A350 remains essentially unchanged with time.

3.2.4 Moisture Content Measurement

Moisture content of the soil was determined by the EPA method No. 3540-7. Porcelain evaporating dishes were used to measure the moisture content of the soil samples. The dishes were washed with liquid detergent and water and left in the oven at 110°C over night to dry out. They were then kept in the desiccator until they were cooled to room temperature. Soil samples were pulverized in a clean mortar and pestle. The empty dishes were weighed and a known weight of soil (approximately 5 g) were added to the dishes and dried in the oven at 105°C for 24 h. The samples were then allowed to cool in desiccator and were reweighed with a Sartorius analytical balance with 0.1 mg accuracy.

3.2.5 Soxhlet Extraction of Hydrocarbons from the Soil

The Soxhlet extraction method was used to extract hydrocarbons from flare pit soil for SARA fractionation analysis. Soxhlet extraction was performed according to EPA SW846 Method No. 3540 (Method 3540C, 1985, and Ducatel, 1999). Anhydrous sodium sulfate (grade 99% from BDH Analar) was used as the soil-drying agent and Dichloromethane (DCM) (GC grade 99.9+ purity from Omni Solve) was chosen as the extraction solvent. The solvent is capable of extracting up to C_{60} (Gibb, 1999). Anhydrous sodium sulfate was placed in a furnace at 450°C for one hour to remove any moisture and organic material and then it was cooled in a desiccator to room temperature.

Approximately 25 g of soil sample was used for each extraction. An approximate equal mass of sodium sulfate was mixed with the soil and the mixture was pulverized to a free flowing powder using a mortar and pestle. The mixture was then added to a Cellulose Whatman Extraction Thimble with an inner diameter of 25 mm, a height of 80 mm, and a double thickness of 2 mm (VWR Can Lab).

All glassware were first cleansed by washing with liquid detergent and were rinsed first with tap water and then with distilled water. The glassware were then placed in a muffle furnace at 450°C for 12 h in order to burn down any organic compound. A 500 ml round bottom reservoir flask was filled with 250 ml of DCM. The soxhlet was assembled in a way that the reservoir was three quarters immersed in a water bath. The temperature of water bath was adjusted to 40°C, which is the boiling temperature of DCM. Each extraction was continued for 48 h or until the solvent ran completely clear.

After each extraction, the system was cooled down and the solvent from the extraction was evaporated in a rotary vapor extractor. The oil residue in the round bottom flask was washed with DCM and transferred into a 40 ml beaker. This solution was left under fume hood for 12-24 h until the solvent evaporated or the total weight of the beaker remained constant. The extracted oils were used later in SARA fractionation analysis.

3.2.6 Direct Extraction of Hydrocarbons from the Soil

Due to the large number of samples and the lengthy procedure of Soxhlet extraction, it was not practical to extract all samples using Soxhlet method. For the hydrocarbon extraction of the samples taken regularly an alternative extraction method based on the modified EPA 3550B method was used. To validate the method, ten soil samples were taken from contaminated soil and five of them were extracted using Soxhlet method and five of them were extracted using the modified EPA 3550B method. The results of two extraction methods were compared and the average difference between the results was calculated. The maximum difference found between the results of two methods was less than 2%.

In the direct method approximately 10 g of the soil samples were dried using anhydrous sodium sulfate (as described in previous section). The dried soil then was mixed with approximately 40 ml DCM in a 60 ml VWR Canlab Trace Clean short wide mouth glass jar with Teflon lined caps (VWR Canlab). The jar contents were mixed for about 1 minute and

were left to settle overnight, then the supernatant was decanted. The extraction procedure was repeated three times and the supernatant solutions were poured in a 250 ml round bottom flask. The solvent in the collected supernatant was then evaporated in a Buchi Rotavapor Extractor. After evaporating the solvent the oil residue in the round bottom flask was then washed with DCM and transferred to a 40 ml beaker. This solution was left under fume hood for 12-24 h to evaporate the solvent.

3.2.7 SARA Fractionation Method

SARA fractionation was carried out on the extracted oil from the flare pit soil based on the method described in Peramanu, et al. paper (Peramanu, Pruden & Rahimi, 1999). The oil was first de-asphaltenated with a 40:1 n-pentane to sample ratio (volume/mass). Approximately 5 g of extracted oil was added to 200 ml of n-pentane in a tightly sealed flask. The flask was placed in a 3510 Bran Son ultrasonic bath for 45 min and then left to settle overnight. The precipitated asphaltenes were filtered using a medium porosity (10-15 μm) Whatman filter paper. The collected asphaltenes were washed twice with 100 ml of n-pentane to ensure the complete removal of maltenes. The asphaltenes were dried at 45°C in a VWR Scientific vacuum oven for 3 h. After filtration, the maltenes were recovered by evaporating the solvent using a rotary evaporator followed by placing the beakers in a vacuum oven at 40°C for 3 h. The asphaltenes and maltenes were allowed to cool in a desiccator to room temperature and then weighed.

Clay-gel adsorption chromatography based on the Peramanu, et al. procedure (Peramanu, Pruden & Rahimi, 1999) was used for the separation of maltenes into saturate, aromatic, and resin fractions. The adsorption column consisted of two identical glass sections assembled vertically. The upper adsorption column had 150 g of freshly activated attapulgus clay, and the lower column had 200 g of activated silica gel plus 50 g of attapulgus clay on top of the gel. A piece of glass wool was placed over the top surface of the clay in the upper column to prevent the agitation of the clay while charging the solvents. To improve the wetting and solvent flow characteristics, about 25 ml of n-

pentane was added to the top of the clay portion of the assembled column and allowed to penetrate into clay. The maltene fraction was dissolved in 300 ml of pentane and charged to the column. Resins were adsorbed onto attapulgus clay while the aromatics were adsorbed onto silica gel. The remaining saturates were eluted directly and were collected in a flask. 1.5 L Toluene and n-pentane (50:50) mixture was added to the column to remove any aromatics present in the attapulgus clay section, and eluted solution containing some aromatic fraction was collected in a flask. The silica gel column was carefully detached from the clay-gel column, and the balance of the aromatic fraction still adsorbed on the gel was removed by refluxing the silica gel column. Refluxing was carried out using 200 ml of toluene for 2 h at a rate of 10 ml/min. To collect the resins fraction, 500 ml of toluene and acetone (50:50) mixture was charged slowly to the top of the clay column and the effluent was collected. The saturate, aromatic, and resin fractions were recovered by evaporating the solvents using rotary evaporators followed by placing the beakers in a vacuum oven at 45°C until there was no change in their weights with time. Then the saturate, aromatic, resin, and asphaltenes fractions were weighed with a Sartorius analytical balance with 0.1 mg readability, and the percentage of each fraction was calculated.

3.2.8 Gravimetric Analysis of the Soil Hydrocarbon Content

Gravimetric analysis was one of the methods used to determine the rate of hydrocarbon degradation in the soil during the course of the experiments. Hydrocarbon content of the soil was extracted using direct method as explained in section 3.2.6. After recovering the extracts by a rotary vapor extractor, the hydrocarbon residues in the round bottom flasks were washed with DCM into 40 ml beakers. The beakers were previously washed once with toluene, and once with liquid detergent and de-ionized water and then they were dried in a furnace at 450°C for almost 24 h to burn out all the organic residues.

After transferring the extracts to the beakers they were left under fume hood to evaporate the solvent. The weight of hydrocarbons extracted is then calculated based on the

difference in the weight of beakers, measured to 4 decimal points using a Sartorius analytical balance.

To calculate the oil content in each soil sample, first the weight of dry soil in the soil samples was calculated using the following equation:

$$m_d = \frac{m_w}{1 + ba + mc}$$

where m_d represents the mass of dry soil, m_w represents mass of soil sample, ba represents bulking agent content of the soil and mc represents moisture content of the soil sample. After calculating the mass of dry soil, the oil content of dry soil was calculated using the following equation:

$$\%oil = 100 \times {}^{m_{oil}}\!/_{m_d}$$

where m_{oil} represents the mass of extracted oil, from soil sample.

3.2.9 Gas Chromatograph Analysis of Hydrocarbons

Since asphaltenes block the Gas Chromatograph (GC) column, the asphaltene fraction of the oil extracted from soil samples was removed (Wittaker & Pollard, 1994). The oil was first mixed with a 40:1 n-pentane to oil sample ratio. The solution was thoroughly mixed and then left overnight for asphaltenes to settle. The supernatant was separated using a pasture pipette, and placed in an 8 ml pre-cleaned amber jar under fume hood to evaporate the n-pentane. The precipitated asphaltenes were washed twice with the solvent to recover maltenes fraction. The collected maltenes were then dissolved in DCM (10 ml solvent: 1 g oil) to reduce the viscosity of the oil samples and then were kept in the freezer at –20°C before injecting into GC. 1 μL of the diluted hydrocarbon extract was used in GC injections.

A Hewlett-Packard 6880 Plus Series Gas Chromatograph was used for fingerprinting of the maltene fractions of extracted hydrocarbon samples. Extra high purity grade Helium (from Canadian Praxair) with a flow rate of 34.2 ml/min was used as the carrier gas. Ultra high purity grade hydrogen and breathing grade air, both obtained from Praxair Canada were used for the flame ionization detector (FID). The temperature of FID was set at 250°C. The split injection port was set at 200°C with a split ratio of 20/1. The column was a high temperature capillary column of a cross-linked methyl siloxane 30 m long with an inner diameter of 0.25 mm and a film thickness of 0.25 μm. The oven temperature profile was: initial temperature of 65°C with a holding time of 7 min, final temperature of 350°C, program rate 10°C/min, and final holding time of 10 min.

Hydrogen and air were mixed with the column effluent and burned. The organic compounds will produce ions and electrons when burned in the hydrogen/air flame. The current produced is then measured.

The calibration chromatograph was produced using two standard mixtures of normal alkanes. The first standard was a solution of C_7, C_9, C_{11}, C_{13}, C_{14}, C_{15}, C_{18}, and C_{19} in DCM, and the second one was a solution of C_{19}, C_{20}, C_{21}, C_{22}, C_{23}, C_{24}, C_{25}, C_{26}, C_{30}, C_{36}, C_{38} and C_{40} in DCM.

3.3 Preliminary Experiments

The goal of preliminary experiments was to prescreen fungi strains for optimum growth and activity in the soil. To reach this goal, the toxicity threshold for different fungi strains, the optimum conditions for fungal growth and activity, an effective bulking agent, and the most active fungal strain were determined. The preliminary experiments were conducted prior to the main experiments and in small scale and the results were further used in the design of main experiments.

3.3.1 Sensitivity Study of Fungi Strains to Hydrocarbons

The sensitivity assay was designed to determine the toxicity threshold of selected fungal strains to the hydrocarbon contaminants found in the soil. Differences in fungal growth rates were compared between test and control petri dishes. Test dishes were prepared by adding weathered crude oil (Alberta Sweet Federated Crude Oil) to warm potato-dextrose agar (PDA) solution. To have a uniform concentration of oil in all plates, the solution was thoroughly mixed with a magnetic stirrer, immediately before adding to the plates. Five concentrations of oil-PDA mixture (2000 ppm, 4000 ppm, 6000 ppm, 10000 ppm and 14000 ppm) were prepared. Pure Potato-Dextrose agar was used in control plates. All dishes were inoculated with 2-mm diameter plugs of fungal mycelia taken from agar inoculum plates. The dishes were incubated at 28°C in a Yamata IC 600 incubator. Fungi mycelia extension on the plates was measured using a measuring tape on a daily basis. The measured extension on the contaminated plates was compared to the measured extension on control plates, to determine any possible toxicity.

3.3.2 Fungal Growth on Different Bulking Agents

Pine wood shavings, peat moss and bran flakes were used for fungal growth assay on bulking agents. Bulking agents were sterilized for 30 minutes in the autoclave at 135°C. *T.versicolor* UAMH 8273; *P. ostreatus* ATCC 58053; *P. pilmanarius* UAMH 7989; *P. chrysosporium* ATCC 24725 and *B. adusta* Bos55, were grown for seven days in 50 ml of glucose-malt extract-yeast extract (GYM) media in a 250 ml flask, at 28°C (Pickard et al., 1999b). The cultures were then homogenized for 20 seconds using a homogenizer (SPER Scientific 460003 Homogenizer) and 15 ml of fungi homogenate was used to inoculate the bulking agents.

In another set of experiments, to minimize the *nutrients carry over* from the growth media, the fungi homogenate was centrifuged and the supernatant was discarded. Then the mycelia and buffer solution were mixed and added to the bulking agents.

During the experiments, the growth of fungi on different bulking agents was visually assessed and based on the results the best bulking agent was chosen.

3.3.3 Fungal Growth in Artificially Contaminated Soil

The main purpose of this growth study was to assess the ability of fungi to grow and penetrate into the contaminated soil. Two growth studies in the artificially contaminated soil were performed, one with a bulking agent and one without. *Bjerkandera adusta UAMH 7308* and *Bjerkandera adusta Boss55* inocula were prepared in GYM medium. The inocula were prepared by homogenizing 1 cm^2 of mycelium from a colony on a potato-dextrose agar plate in 50 ml of GMY medium for 20 s. After 7 days of growth in a 250 ml flask at 28°C, the cultures were homogenized and 15 ml of the fungi homogenate was centrifuged, then the supernatant was decanted and the remaining mycelia was used to inoculate the soil. Separation of the supernatant minimizes the nutrient carry over from the liquid media into the soil.

Approximately 100 g of artificially contaminated soil was used for each test. The soil was added into a 250 ml jar, with 2-3 g of peat moss and cereal bran (Kellogg's bran flakes). The fungi mycelia was then mixed with about 60 ml of buffer solution and added to the jars. The jars were kept in room temperature with loose caps until the fungi mycelia had grown and covered the bulking agent surface completely. The jar contents were then completely mixed and the jars were kept in room temperature for another 14 days. The experiments lasted for 21 days, and samples were taken at the beginning and the end of experiments for analysis of hydrocarbons. Later the soil samples were extracted according

to the EPA method no. 3540 for evaluation of solid waste (U.S. Environmental Protection Agency method No. 3540, 1996).

3.3.4 Measuring the Ligninolytic Enzyme Activity for Different Fungi Strains

To study the enzyme activity of each fungus, the poly R-478 decolorization assay was used (Glenn & Gold, 1988). The assay is based on monitoring the poly R-478 dye decolorization in 50 ml of GYM medium, placed in 125 ml loosely capped flasks. Fungi were incubated statically in 125 ml flasks at 28°C. The dye was added to the growth medium at the time of incubation (time zero). The initial concentration of the dye added to the medium was 0.2 g/l. Ligninolytic enzyme activity was measured by removing 0.4 ml of extra cellular culture medium and diluting it up to 10-fold in water and then measuring the absorbance ratio of A520/A350. The measured absorbance ratios of different extra cellular culture medium were compared with the absorbance ratio of a standard dye solution with a concentration of 0.02g/l.

3.4 Main Experiments

3.4.1 Experimental Design Based on Fractional Factorial Design

The main objective of this project was to study the impact of fungi application on hydrocarbon degradation in a controlled environment. In addition to the fungi application, the effect of moisture and bulking agent content on hydrocarbon degradation was also studied. The design of experiments was based on factorial design with three factors, each at two levels. The factors were 1) Fungi application, 2) moisture content, and 3) bulking agent. The effect of each factor was studied by applying them at different levels. The two levels of fungi were "no fungus application" and "fungi application". The moisture content was studied at two levels of 30 and 50% (w/w of dry soil) and the bulking agent was

studied at two levels of 6 and 12% (w/w of dry soil). More details of the design are presented in Tables 3.2 and 3.3. A total number of 13, 4-liter wide mouth glass jar were used for the main experiments.

In Tables 3.2 and 3.3 factors are shown with uppercase letters. A represents fungi, B represents moisture content and C represents bulking agent. The high level of each factor is designated with 1 and lower level with 0.

As can be seen from Table 3.3 application of single or mixed fungi strains were considered to have the same effect and further analysis of the results confirmed this assumption.

Table 3.2) Statistical Notations

Variables/ Levels	0	1
"A": Fungi	No Fungus	B. adusta or Mixture of 12 strains
"B": Moisture Content %(w/w of soil)	30	50
"C": Bulking Agent %(w/w of soil)	6	12

Table 3.3) Summary of Experimental Design

Test No.	A	B	C	Jar Description
1	1	1	1	Test (I): B.adusta
2	1	1	1	Replicate of 1
3	1	1	1	Test (I): with mixed fungi
4	0	1	1	No fungi (I)
5	1	0	1	Test (II): B.adusta
6	1	0	1	Replicate of 5
7	1	0	1	Test (II): mixed of fungi
8	1	0	1	Replicate of 7
9	0	0	1	No fungus (II)
10	1	1	0	Test (III): B.adusta
11	1	0	0	Test (IV): B.adusta
Extra jars served as controls for study of adsorption and evaporation				
12	0	*	**	Control
13	0	*	**	Control

*Moisture content in control test jars was 12%, the same value as original diluted soil.

** No treatment applied.

3.4.2 Fungi Applications

The experiments were conducted using two different fungal inocula. For one inoculum pure culture of *Bjerkandera adusta* UAMH 8258 was used and for the other inoculum a mixture of 12 fungi strains were used. *Bjerkandera adusta* UAMH 8258 was chosen based on previous experiments, where it showed a higher potential for degradation of poly aromatic dyes than other fungi strains. Fungal inocula were prepared by transferring 1 cm^2 of fungi mycelia from PDA plates to 50 ml of GYM media and homogenizing the mixtures for 20 s. The mixture was then incubated at 28°C. After 7 days of growth the cultures were homogenized for 20 s and centrifuged for 4 min at 5000 rpm to separate the fungal mycelia from the growth media. The mycelia were then used to inoculate every 125 g of sterilized mixture of bulking agent and nutrients. This mixture thereafter referred to as standard substrate, contained peat moss 60% (w/w) and Kellogg's bran flakes 40% (w/w). Sterilized standard substrate, fungal mycelia and the buffer solution (%45 w/w of standard substrate) were mixed completely and incubated at 28°C for 10 days, before being added to the soil.

3.4.3 Soil Dilution

The flare pit soil (provided by Imperial Oil) was mixed with clean soil to reduce the concentration of hydrocarbons to the final concentration of about 10,000 ppm. The clean soil was obtained from the Grounds Department of University of Calgary. The clean soil was excavated from 1.5 m below the surface and it was very low in organic material content. The larger particles in both clean and contaminated soils were separated using a sieve pan number 16 that had an opening size of 1.8 mm. This was done to have a uniform and homogeneous mixture.

The sieve pan was placed over a 60 L Rubbermaid garbage can. The soil was then scooped into the pan, and the larger pieces were separated out manually. Totally, 28 kg of clean soil and 2 kg of flare pit soil were sieved.

A small concrete mixer (from the concrete lab of the Civil Engineering Department of University of Calgary) was used to completely homogenize the mixture of the two soils. While the mixer was working, 1 kg of clean soil was added to the contaminated soil in the mixer, after 5 min another 1 kg of clean soil was added, and this process continued until the whole clean soil was added to the contaminated soils. The mixer was then left operating for another 20 min to ensure complete mixing of the soils.

3.4.4 Bulking Agent for the Main Tests

Based on the preliminary tests, a mixture of Kellogg Bran Flakes and peat moss was added to the soil as fungi nutrient and the bulking agent. Kellogg Bran flakes was added to the soil at a constant concentration of 4% to induce higher enzyme production and peat moss was added to the soil based on the values in the experimental design.

3.4.5 Experimental Setup

Thirteen 4 L jars with the dimension of 25 cm height and 16 cm diameter were used for the set up of the experiments. The jars were filled with a mixture of soil and other amendments, such as peat moss, bran flakes, fungi inocula, and buffer solution, and were kept at room temperature with loose caps. In each jar a plastic tube (0.7 m in length) was placed for future air sampling (Figure 3.1). Each individual jar was enclosed in a cardboard cylinder with an aluminum lid to prevent exposure of fungi to the light and to keep the moisture content of the soil constant. The picture of the cylinders can be seen in Figure 3.2.

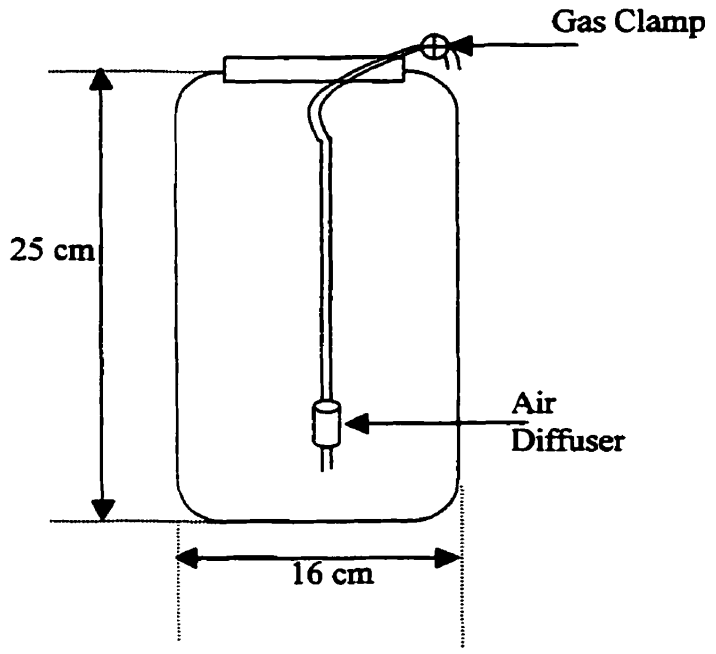


Figure 3.1) Schematic Diagram of the Bioreactors

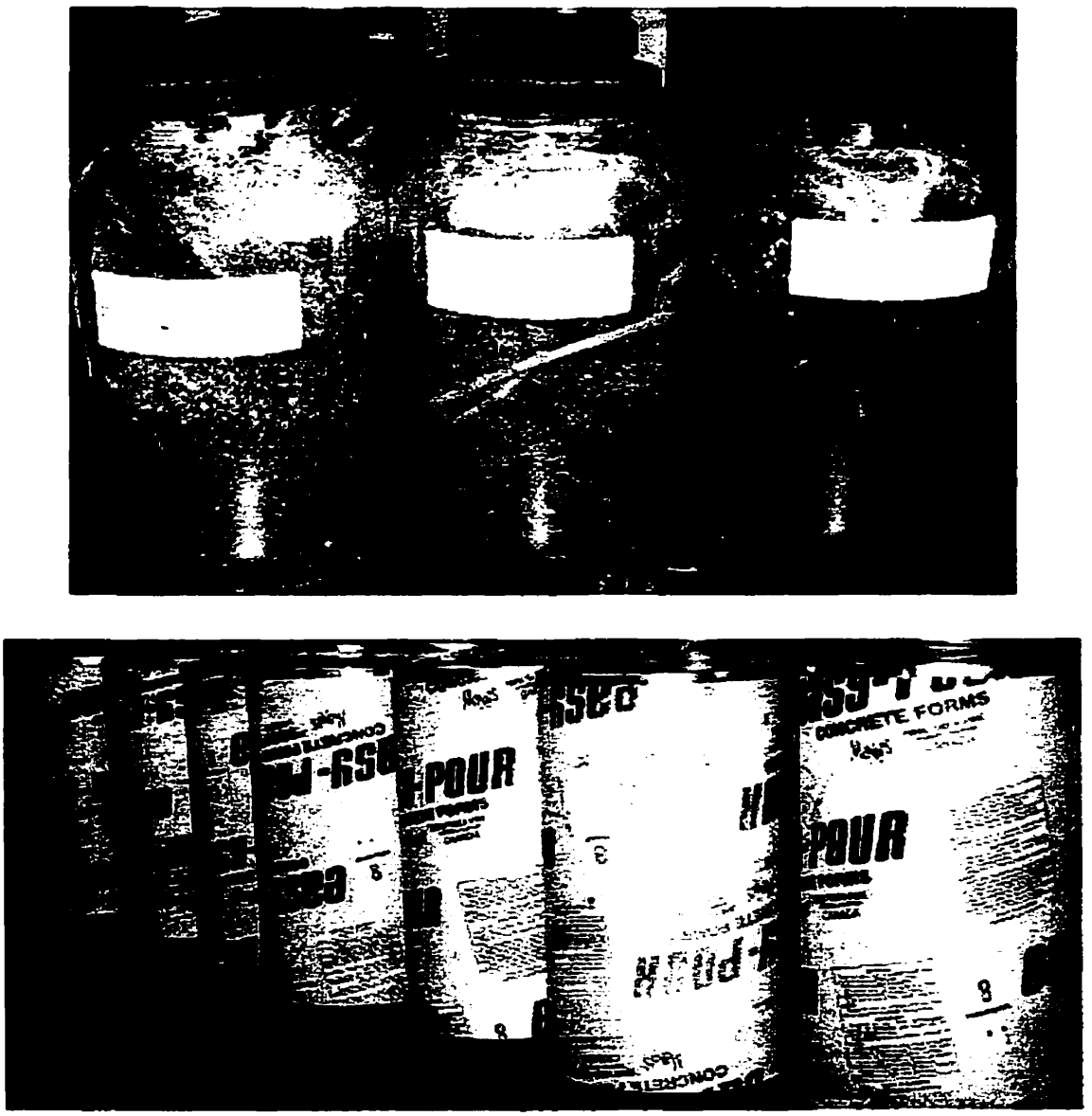


Figure 3.2) Bioreactors and Cylindrical cardboard chambers

Table 3.4 shows more details of the ingredients in each jar. Soil and other ingredients, such as fungi inocula, peat moss, bran flakes, and moisture content were mixed together thoroughly using the rolling method (Greenberg et al. 1985). In this method the soil was spread on a 4 ft by 4 ft plastic sheet and then each amendment was added to the soil in sequence. After adding each ingredient, the soil was mixed by lifting one corner of the plastic sheet, enough to roll the particles to the opposite corner. This operation was repeated up to 20 times, with the four corners of the sheet being lifted alternately until a homogeneous mixture was achieved. Bran flakes were first ground using a mortar and pestle to a free flowing powder and then were added to the soil. Buffer solution was the last ingredient that was added to the mixture. It was gradually sprayed to the soil in 3-4 stages, to prevent formation of mud lumps and to have a homogeneous mixture of the soil,. After each spraying period, the soil was rolled again for a few times and then more moisture was sprayed.

Table 3.4) Details of the Bioreactors Contents

Jar No.	Jar description	Mass of dry soil (g)	Buffer Solution (ml)	Peat Moss Mass (g)	Bran Flakes Mass (g)
1	Test (I): B.adusta	2183	1067	265	88
2	Replicate of 1	2183	1067	265	88
3	Test (I): mixed fungi	1746	880	230	70
4	No fungi (I)	1310	655	157	52
5	Test (II): B.adusta	1572	471	190	63
6	Replicate of 5	1572	471	190	63
7	Test (II): mixed of fungi	1572	471	190	63
8	Replicate of 7	1572	471	190	63
9	No fungus (II)	1572	471	190	63
10	Test (III): B.adusta	1572	900	95	63
11	Test (IV): B.adusta	1572	471	95	63
12	Control	1310	0	0	0
13	Control	1310	0	0	0

3.4.6 Soil Sampling Schedule in the Main Experiments

Soil sampling for analysis of moisture content, enzyme activity and hydrocarbon content was performed at 0, 8, 16, 22, 30, 46, 66, and 100 days. Soil was mixed completely before each sampling using the rolling method (Greenberg 1985). In this method a 4ft by 4ft plastic sheet was used for each individual test jar. The soil from each jar was placed in the center of the plastic sheet and was spread on the sheet using a scoop; the soil then was mixed by lifting one corner of the plastic sheet, enough to roll the particles of the sample to the opposite corner. This operation was repeated 8-10 times, with the four corners of the sheet being lifted alternately.

After thoroughly mixing the jar content, four 10 g samples, were collected from random locations on the plastic sheet. One of the samples was used for the analysis of enzyme activity and moisture content and the other three samples were used for hydrocarbon analysis. The three 10 g samples were kept in 60 ml glass vials with Teflon-lined screw caps and were stored in a freezer at –20°C for later analysis of hydrocarbon content.

3.5 Statistical Methods

The results from gravimetric analysis of hydrocarbon loss were further analyzed using two different statistical methods. The first method, used to analyze the results of the fractional factorial design (FFD), was the *Box Hunter* method (Box, Hunter, 1978). By this method, the effect of fungi application, moisture content and bulking agent content on biodegradation of hydrocarbons in the soil were studied (Box, Hunter, Hunter, 1978 & Montgomery 1997). The second method was based on *repeated measures analysis of variance* (RM-ANOVA), which provides additional information on any changes in the "effects" over time span of the experiments (Mead, 1988).

Due to complicated mathematical procedure for calculations of the *repeated measures analysis of variance*, the analyses were performed using the statistical analysis software, "Statview".

3.5.1 Statistical Analysis of Gravimetric Results Using Box Hunter Method

The main effects and interaction effects are calculated based on the Table of Contrast Coefficients (Table 3.5). In this table the high and low level of each factor is showed by + and – signs. This type of notation in factorial designs are called geometric notation. Table 3.5 shows the various combinations of levels of different factors, used in the experimental design of this research. In this table the $\bar{y}_i$'s are the average responses (%reduction in hydrocarbon content) obtained from different replicates of each combination of factors.

Table 3.5) Table of Contrast Coefficients for the Fractional Factorial Design Used in the Main Experiments of the Research

Test Condition	A	B	C	AB	AC	BC	ABC	Average of Different Replicates for Each Test
1	+	+	+	+	+	+	+	$\bar{y}_1$
2	-	+	+	-	-	+	-	$\bar{y}_2$
3	+	-	+	-	+	-	-	$\bar{y}_3$
4	-	-	+	+	-	-	+	$\bar{y}_4$
5	+	+	-	+	-	-	-	$\bar{y}_5$
6	+	-	-	-	-	+	-	$\bar{y}_6$

To calculate the main effect of each factor, the difference between two averages at two different levels of the factor was calculated:

$$\text{Main effect} = \bar{y}_+ - \bar{y}_-$$

Where $\bar{y}_+$ is the average response or here the average % reduction in oil content for the plus level of the factor and the $\bar{y}_-$ is the average response or here the average % reduction in oil content for the minus level. For example to calculate the fungi effect the following equation was used:

$$\text{Fungi Effect (A)} = (\bar{y}_1 + \bar{y}_3 + \bar{y}_5 + \bar{y}_6)/4 - (\bar{y}_2 + \bar{y}_4)/2$$

To derive this equation the information in Table 3.5 were used. The calculations for the rest of main effects were conducted similar to the fungi effect calculations.

To calculate the interaction effects between two factors, the interaction columns (columns AB, AC, BC, and ABC) in Table 3.5 were used. The interaction columns were obtained by multiplying the contents of columns relevant to main effect (column A, B, and C). For instance column AB is obtained by multiplying column A by column B. Similar to calculations of main effects, the interaction effects between two factors is the difference between two averages, but here the $\bar{y}_+$ is the average response for the time both factors are at the same level (plus or minus level) and $\bar{y}_-$ is the average response for the time both factors are at two different level (one at plus level and the other at minus level or vice versa). For example to calculate the interaction effect of factors A and B, the following equation is used. In this equation the $\bar{y}_+$ is the average of responses that have plus sign in the AB column and $\bar{y}_-$ is the average of responses that have a minus sign in the AB column.

$$\text{Fungi and Moisture Content Interaction (AB) Effect} = (y_1 + y_4 + y_5)/3 - (y_2 + y_3 + y_6)/3$$

The rest of Interaction effects were calculated similar to AB interaction effect. The standard error for effects were calculated using the variance of the effects:

$$\text{Standard Error} = \sqrt{V}$$

where V represents the *Variance* of each effect. Since each main effect and interaction effect is a statistic of the form $\bar{y}_+ - \bar{y}_-$, the variance of each effect is given by:

$$V(\text{effect}) = V(\bar{y}_+ - \bar{y}_-)$$

here $V(\bar{y}_+ - \bar{y}_-)$ can be calculated using the following equation:

$$V(\bar{y}_+ - \bar{y}_-) = \frac{\sigma^2}{n_+} + \frac{\sigma^2}{n_-}$$

where n_+ and n_- are the number of replicates for plus and minus level of every effect and σ is the average of estimated variance of replicates at every test condition. In general, if g sets of experimental conditions are genuinely replicated and the n_i replicate runs made at the *i*th set yield an estimate s_i^2 of σ^2 having $\nu_i = n_i - 1$ degree of freedom, the pooled estimate of run variance is:

$$s^2 = \frac{\nu_1 s_1^2 + \nu_2 s_2^2 + \ldots + \nu_g s_g^2}{\nu_1 + \nu_2 + \ldots + \nu_g}$$

with $\nu = \nu_1 + \nu_2 + \ldots + \nu_g$ degrees of freedom. In the previous equation every s_i^2 is calculated using the following equation:

$$s_i^2 = \frac{\Sigma(y_i - \bar{y}_i)^2}{n_i - 1}$$

3.5.2 Statistical Analysis of Gravimetric Results Using "Repeated Measures Analysis of Variance"(RM-ANOVA)

To study the effect of *Time* on the effectiveness of experimental factors, RM-ANOVA was used. In other words, different treatments ×time interactions were investigated using this method. For instance one factor might have a significant effect on the results during the first 30 days of the experiments but the same factor might not be effective over the three-month period. Such changes were studied using RM-ANOVA. Since the mathematical procedure of this analysis is complicated, it is usually performed using statistical softwares. In this research, the statistical analysis software, "Statview", was used. In these analysis the fungi, moisture content and bulking agents were considered as independent variables

and the percent of reduction in oil content was considered as the dependent variable. The reader is referred to Mead 1988 for more details of the method (Mead, 1988).

Chapter Four: Results and Discussions

The experimental and theoretical results obtained in the course of this study are presented in this chapter. The chapter first covers the results for the soil characterization, followed by the results of preliminary and main experiments. The last part of this chapter is dedicated to the results of the statistical analyses of hydrocarbon loss data.

4.1 Contaminated Soil Characteristics

The flare pit soil was provided from one of the Imperial Oil flare pit sites located in Alberta. The soil was characterized using gravimetric analysis and SARA fractionation. The flare pit soil was diluted to a non-toxic level using clean soil and the hydrocarbon content of diluted soil was measured using gravimetric analysis and *gas chromatography (GC)* finger printing.

4.1.1 General Soil Characterization of Flare Pit Site (Provided by Imperial Oil)

The analyses of the soil at the flare pit site showed that the soil is a mixture of hydrocarbons, trace amount of metals, and solids. The hydrocarbons include many compounds, principally aliphatic and aromatic hydrocarbons. A rough estimate of the break down of the hydrocarbons in the soil was provided by Imperial Oil Research Center and is shown in Table 4.1.

Table 4.1) General soil hydrocarbon characterization for the studied flare pit site.

	Moderate Hydrocarbon contamination (Layer3**)	Heavy Hydrocarbon Contamination (layer 2*)
Total PAH (mg/kg)	5.2	95
PAH (2-3 rings) %	100	82
PAH (4 rings) %	0	15
PAH (5-6 rings) %	0	3
Hydrocarbon Analysis TPH ($C_{10} - C_{50}$) (mg/kg)	5000	100,000
Aliphatic%	78	72
Aromatic%	20	19
Resin%	2	7
Asphaltenes%	0	2
Percent of Solids%	73	79

* Layer 2: The second level of soil below surface.

** Layer 3:The third level of soil below surface.

4.1.2 Soil Characterization Using SARA Fractionation Method

The data obtained from Imperial Oil showed the hydrocarbon content in different layers of the soil. The data are the average concentrations from different locations at the site. The flare pit soil provided by Imperial Oil for this research project, was a mixture of soils from level two and level three. To have more accurate data on the soil, it was analyzed in the lab using the SARA fractionation method.

The hydrocarbon contents of three randomly chosen soil samples were extracted using Soxhlet extraction method. The extracted hydrocarbons were then fractionated into saturates, aromatics, resins and asphaltenes using SARA fractionation method as explained in chapter 3. The SARA fractionation results are shown in Table 4.2.

Table 4.2) Hydrocarbon characterization of the soil using SARA fractionation method (Average of three replicates with ± 5.0% error).

Contaminant Class	Result of SARA Fractionation
Total Extractable Petroleum Hydrocarbons in Soil (%wt/wt of dry soil)	15.92
Saturates (%wt/wt of TPH)	45.85
Aromatic (%wt/wt of TPH)	28.52
Resin (%wt/wt of TPH)	17.3
Asphaltenes (%wt/wt of TPH)	8.33

4.1.3 Initial Characterization of Diluted Soil

4.1.3.1 Initial Characterization of Diluted Soil Using GC

After soil was diluted to the non-toxic level, a soil sample of known weight was treated to extract the hydrocarbons. Then the asphaltenes fraction was separated from the extracted hydrocarbons and the maltenes fraction was diluted in dichloromethane for analysis by a Gas Chromatograph equipped with a Flame Ionization Detector. The spectrum obtained for the extracted hydrocarbons is shown in Figure 4.1. The temperature ramping method was calibrated with normal alkanes between C_9 to C_{39} and the results of calibration are shown in Figures 4.2 and 4.3.

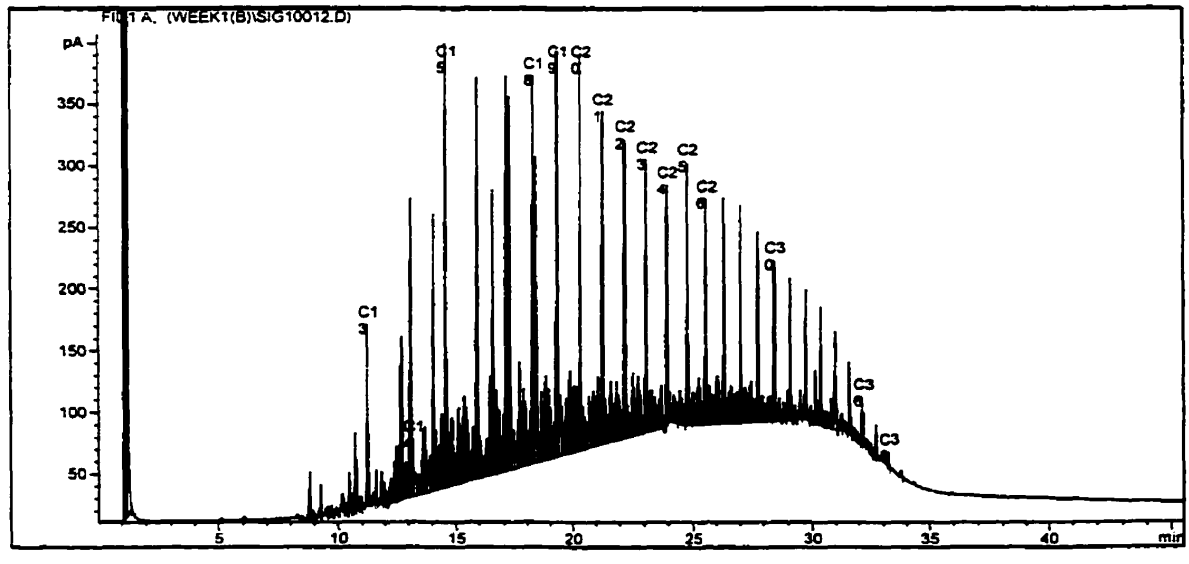


Figure 4.1) Initial GC fingerprint of the diluted soil

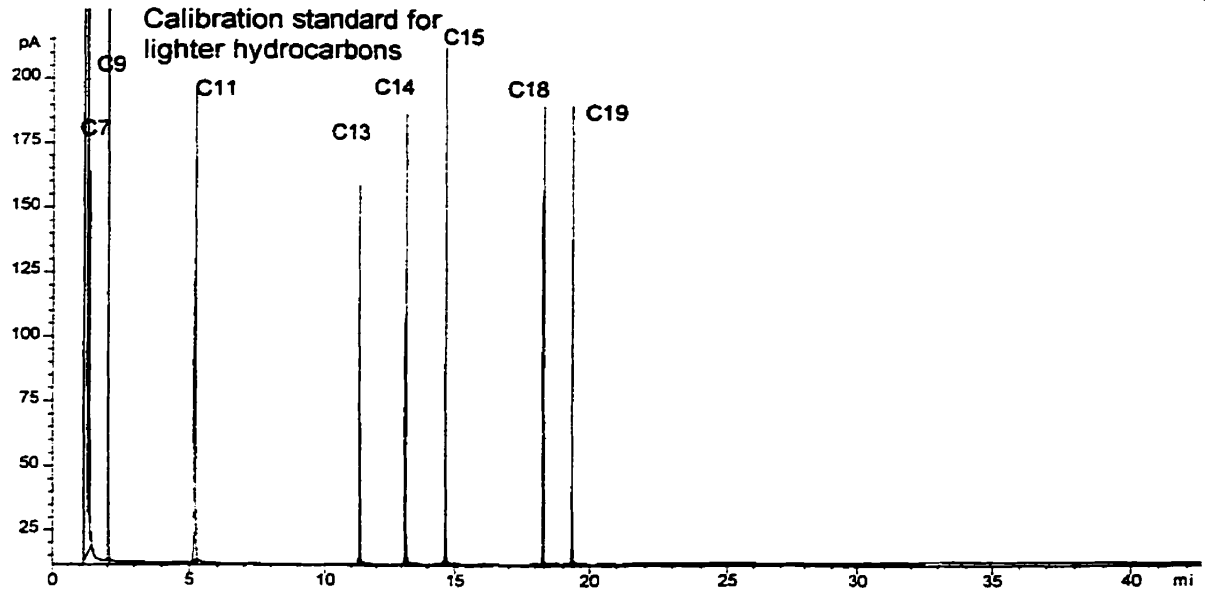


Figure 4.2) The GC calibration fingerprint for normal Alkanes between C7 to C19.

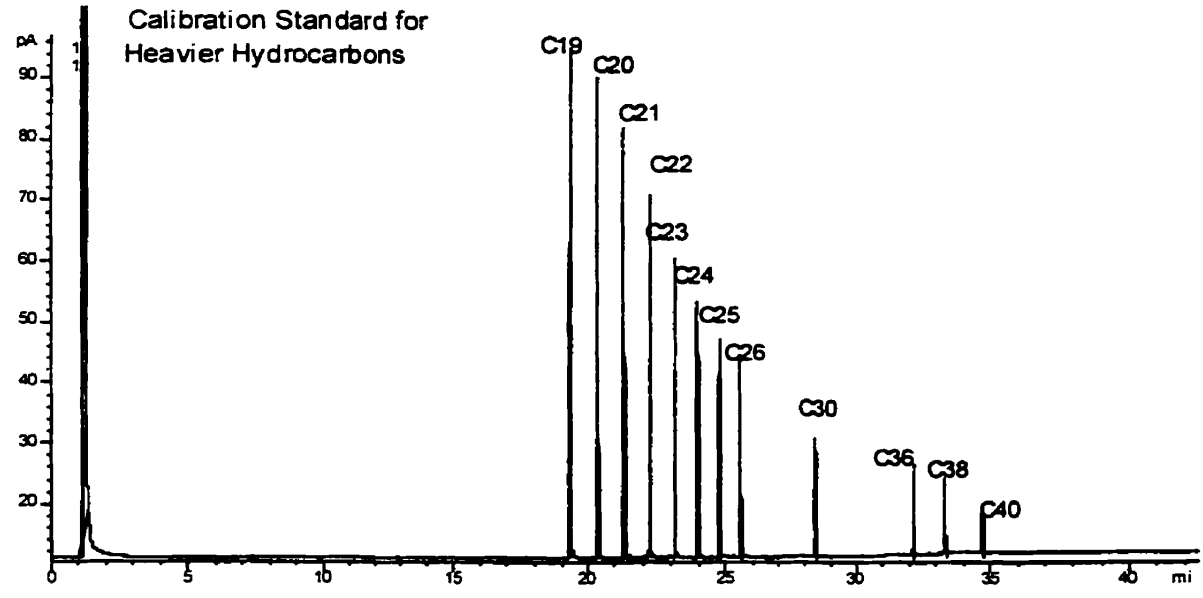


Figure 4.3) The GC calibration finger print for normal Alkanes between C19 to C40.

4.1.3.2 Initial Hydrocarbon Content of the Test Jars Using Gravimetric Analysis

After diluting the soil to the non-toxic level, it was mixed with the fungi inocula, buffer solution and bulking agent to the desired value for each test jar. Triplicate soil samples from each test jar were then further processed for hydrocarbon extraction and gravimetric analysis, as described in detail in chapter three. The average gravimetric analysis of the hydrocarbon content of each test jar is shown in Table 4.3. The amount of oil content is shown based on weight per weight of dry soil and thus all the corrections for moisture and bulking agent are taken into account.

Table 4.3) Initial gravimetric analysis of soil hydrocarbon content of test jars (Average of three replicates with ± 0.12% error)

Test no.	Jar Description	Weight % Per Dry Soil
1	Test (I): B.adusta	1.3
2	Replicate of 1	1.3
3	Test (I): mixture of fungi	1.33
4	No fungus (I)	1.37
5	Test (II): B.adusta	1.39
6	Replicate of 5	1.24
7	Test (II): mixture of fungi	1.19
8	Replicate of 7	1.24
9	No fungus (II)	1.35
10	Test (III): B.adusta	1.38
11	Test (IV): B.adusta	1.3
12	Control	1.22
13	Control	1.25

4.2 Preliminary Screening Test Results

4.2.1 Sensitivity Tests

The sensitivity tests were conducted to find out the hydrocarbon toxicity threshold for the fungi strains. Hydrocarbon amended Potato Dextrose Agar (PDA) plates were used to monitor the growth of different fungi strains over a 20-day period. The growth rate was measured as the amount of mycelia extension on PDA plates. All 12 fungi strains were used to inoculate the contaminated PDA plates, as described in chapter three. The results from measuring mycelia extension and visual observation of growth are summarized in Figure 4.4 and Tables 4.4 and 4.5. As can be seen in Figure 4.4, at the concentration of 10,000 ppm all the fungi strains covered the PDA plates within 5 to 10 days except for *Phanerochaete chrysosporium,* which covered the plate in 3 days. On eof the reasons for this difference is that this fungus forms spores in plate cultures. At the end of the experimental period all fungi strains grew completely, on the contaminated plates up to the concentration of 10,000 ppm; at higher concentration (14,000 ppm) the fungi started growing but they stopped growth after 2 to 3 days. Some of the strains showed a slight inhibition at 10,000 ppm in the first few days of the experiment, but not in the longer term of 10 to 15 days.

Figure 4.5 also shows typical growth of fungi on PDA plates and Table 4.5 shows the results of visual observation of the fungal growth on contaminated PDA plates.

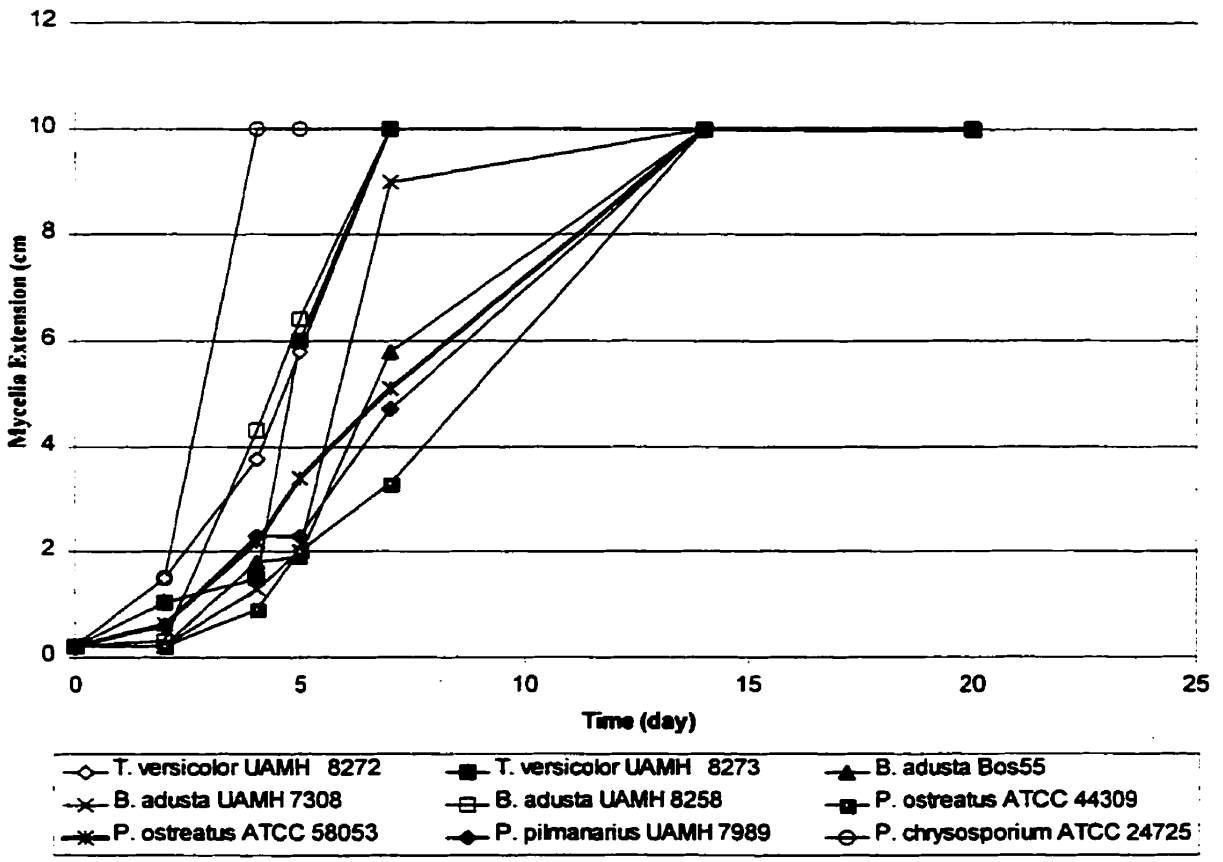


Figure 4.4) Typical mycelia extension on petroleum contaminated PDA plates (oil concentration of 10,000 ppm).

Table 4.4) Visual observance of mycelia extension on contaminated PDA plates.

	4000 ppm		6000 ppm		10,000 ppm		14,000 ppm	
	5days	**10days**	**5days**	**10days**	**5days**	**10days**	**5days**	**10days**
***Coriolopsis gallica* UAHM 8260**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Bjerkandera adusta* BOS 55**	No inhibition	No inhibition	Very little inh.	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Bjerkandera adusta* UAMH 7308**	Slight inhibition	No inhibition	Very little inh.	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Bjerkandera adusta* UAMH 8258**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Pleurotus pilmanarius* UAMH 7989**	No inhibition	No inhibition	Very little inh.	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Pleurotus ostreatus* UAMH 7988**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Pleurotus ostreatus* ATCC 58053**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Pleurotus ostreatus* ATCC 44309**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Phanerochaete chrysosporium* ATCC 24725**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Trametes vervicolor* UAMH 8272**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Trametes vervicolor* UAMH 8273**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition
***Trametes vervicolor* UAMH 7331**	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	Slight inhibition	Complete inhibition

Table 4.5) The results for measurement of mycelia extension on 1000 mg/kg oil contaminated PDA plates.

Time (Day)	0	2	4	5	7	14
Fungi Strain	Mycelia Extension (cm)					
T. versicolor UAMH 8272	0.2	1.5	3.75	5.8	10	10
T. versicolor UAMH 8273	0.2	1.03	1.5	6	10	10
B. adusta BOS55	0.2	0.2	1.8	1.9	5.8	10
B. adusta UAMH 7308	0.2	0.2	1.3	2	9	10
B. adusta UAMH 8258	0.2	0.3	4.3	6.4	10	10
P. ostreatus ATCC 44309	0.2	0.2	0.9	2	3.3	10
P. ostreatus ATCC 58053	0.2	0.61	2.2	3.4	5.1	10
P. pilmanarius UAMH 7989	0.2	0.6	2.3	2.3	4.7	10
P. chrysosporium ATCC 24725	0.2	1.5	10	10	10	10

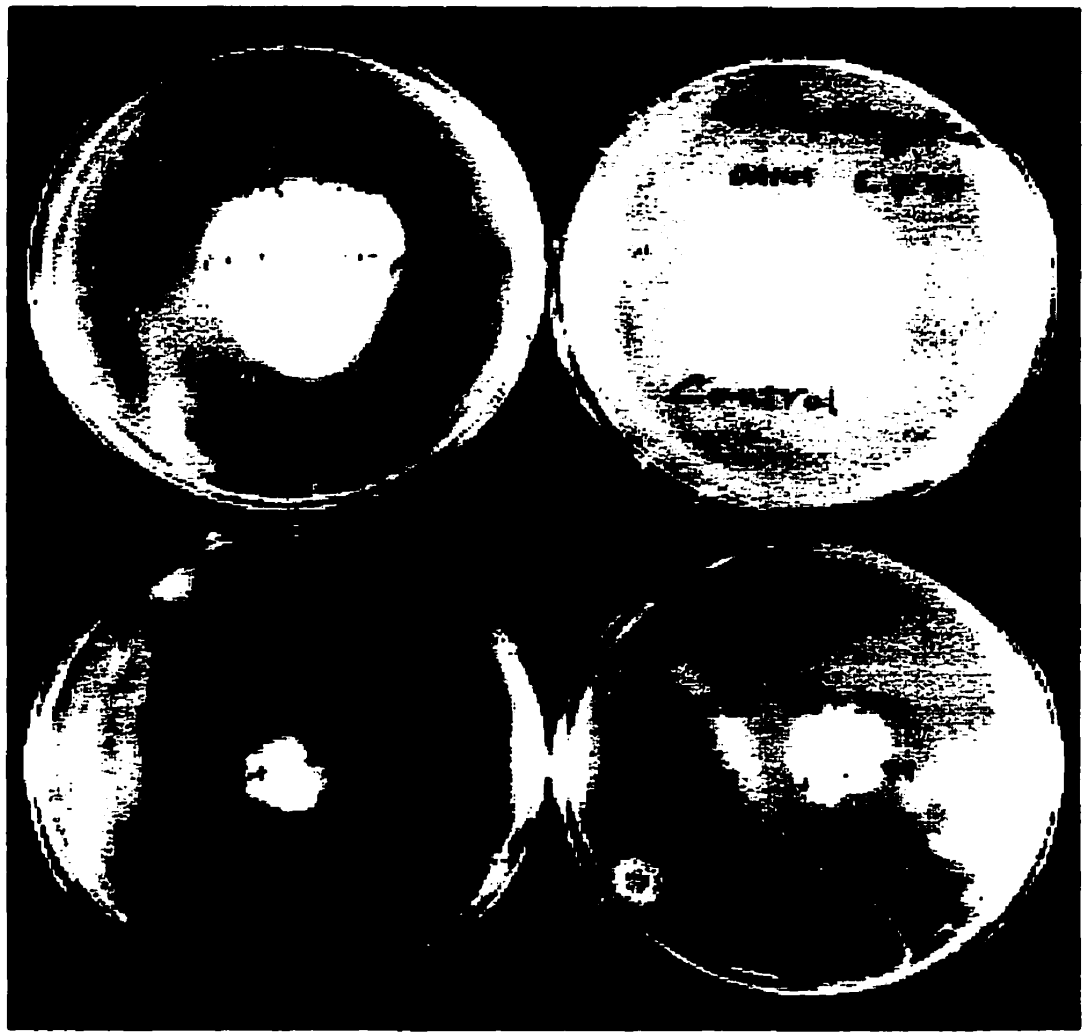


Figure 4.5) A typical growth rate difference on control plates (the plates on the right) and 10,000 ppm hydrocarbon contaminated plates (the plates on the left).

4.2.2 Fungal Growth Study on Bulking Agent

As mentioned before in chapter three, the growth of fungi on bulking agents was tested using two different methods. In the first method, the fungi mycelia were added to the bulking agent with the liquid media; in the second method the fungi mycelia were separated from liquid media before being added to the soil. The two methods were used to study the effect of nutrient carry over from the liquid media. Visual observation of the fungi growth on the bulking agent showed very similar results for both methods. Therefore it was concluded that the nutrients present in the bulking agents were enough for fungal growth and nutrient carry over from liquid medium doesn't have any significant effect on the growth of fungi.

All fungi strains showed very fast growth on bran flakes. Within one week, the whole bran flakes were covered with fungi mycelia and they had grown in all the pores between flakes. They also showed slower but complete growth on peat moss. After two weeks, the pores were filled with fungi mycelia. The growth on pine wood shavings wasn't as much as the growth on two other bulking agents and it was limited to the top surface.

4.2.3 Fungal Growth Study in the Artificially Contaminated Soil

The preliminary fungi growth study in the soil was conducted to ensure proper growth of fungi in the soil matrix. This was done by adjusting the moisture content of the soil and changing the type and percentage of the bulking agent.

The results showed that using a mixture of two bulking agents is more effective than using only one type of bulking agent. The best mixture of bulking agents for maximizing fungal growth in the soil was found to be a mixture of peat moss and bran flakes. Peat moss is a very lightweight bulking agent that enhances the void space and moisture retention in the

soil. Bran flakes contain bran and glucose, which makes fungi to grow faster and induce more enzymes (Pickard et al., 1999b).

The optimum moisture content for these solid-state bioreactors was found to be around 40% to 60% (wt/wt of dry soil), which is within the range mentioned in the literature (Okeke et al., 1996). At higher moisture content, the soil was too moist and no growth was started, in low moisture contents the growth was started but was stopped after a short period.

4.2.4 Fungi Selection Using Enzyme Activity Assay

The dye decolorization assay, described in chapter 3, was used to asses the level of extracellular ligninolytic enzymatic activity of different fungal strains, as the fungi bioremediation is often attributed to these enzymes.

The results for the dye poly R-478 decolorization by different fungal strains are shown in Figure 4.8. The graph shows the change of absorbance ratio, A520/A350, for the 12 fungi strains over a period of 35 days. The change in the absorbance ratio corresponds to the level of enzymatic activity of fungi; in the other word higher enzymatic activity leads to lower absorbance ratio (Glenn & Gold, 1988). All fungi strains showed decrease in the absorbance ratio, however, *B. adusta UAMH 7308* and *B. adusta UAMH 8258* showed the highest amount of reduction.

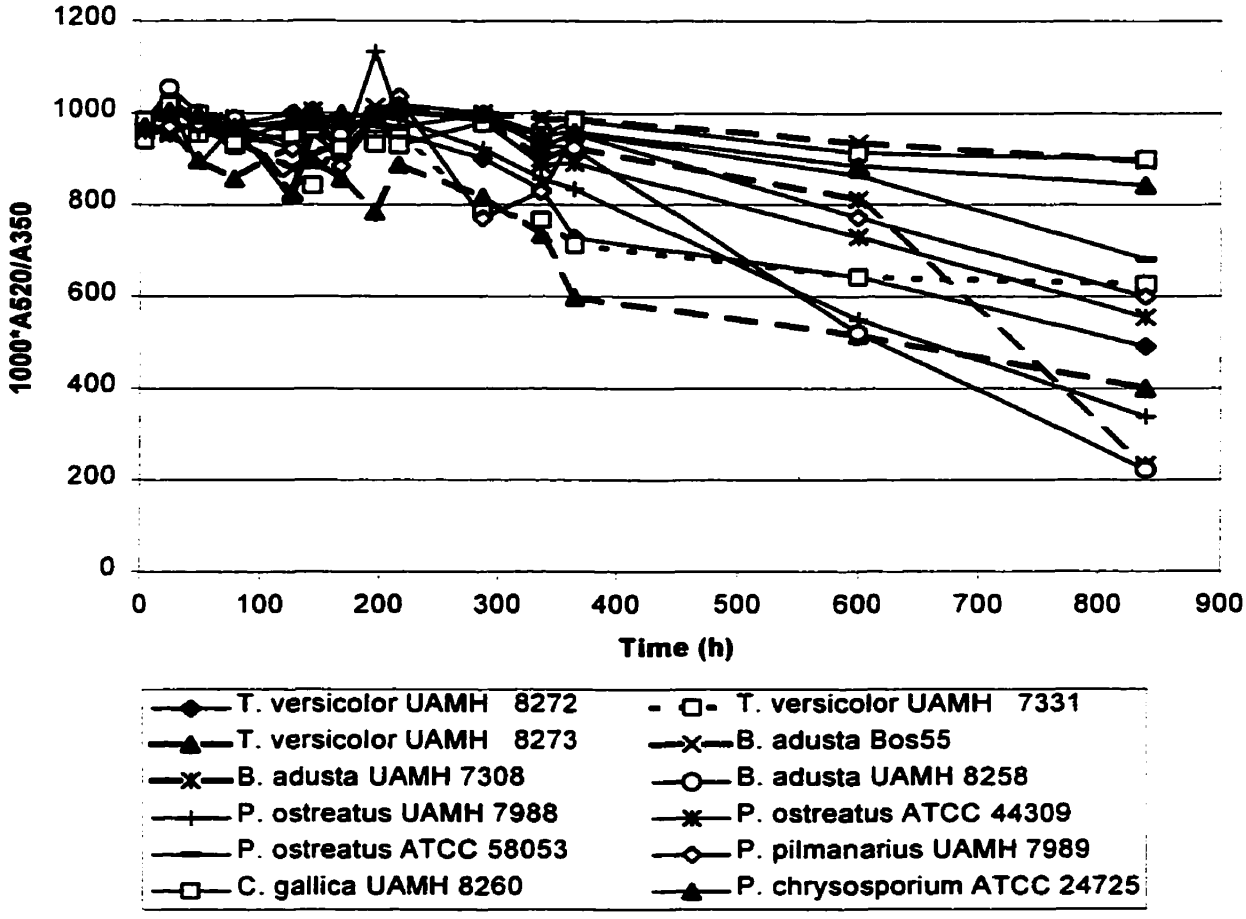


Figure 4.6) The results of dye decolorization test which corresponds to the fungal ligninolytic enzyme activity (change in absorbance ratio, A520/A350, versus time).

4.2.5 Oxygen Requirement

To decide if forced aeration of the main test jars is necessary, the experimental conditions were simulated in an airtight cylinder with the same height as the test jars. Air samples were then taken from a completely tight plastic tube placed inside the cylinder, once after one week and second time after two weeks of starting the experiment. The gas samples were analyzed for CO_2 and O_2 using a GC calibrated with standard air.

The results did not show any significant reduction in oxygen content in the cylinder even after a two weeks period. Therefore it was concluded that there was no need for forced aeration.

The same test was repeated during the main experiment. Plastic tubes were installed in the test jars both for air sampling and possible aeration. The upper openings of the tubes were kept air tight to prevent the diffusion of fresh air. Air in the jars was sampled twice, after one week and after two weeks of starting the experiments. Gas samples were analyzed with a GC and the result showed that there wasn't any significant amount of CO_2 accumulation or O_2 depletion in the bottom of the jars. The highest amount of CO_2 was found in the test jar number 6, which was 1.36% of the air volume. It was concluded that no aeration would be necessary, however the caps of the jars were kept loose to provide some fresh air for the fungi to grow.

Since the headspace of different test jars were not equal, the results of these experiments are not very conclusive for CO_2 content and oxygen content in different test jars.

The GC fingerprint of the standard air and the GC fingerprint of the air sample from the test jar number 6 after the 2nd week of experiment are shown in Figure 4.7 and Figure 4.8 respectively.

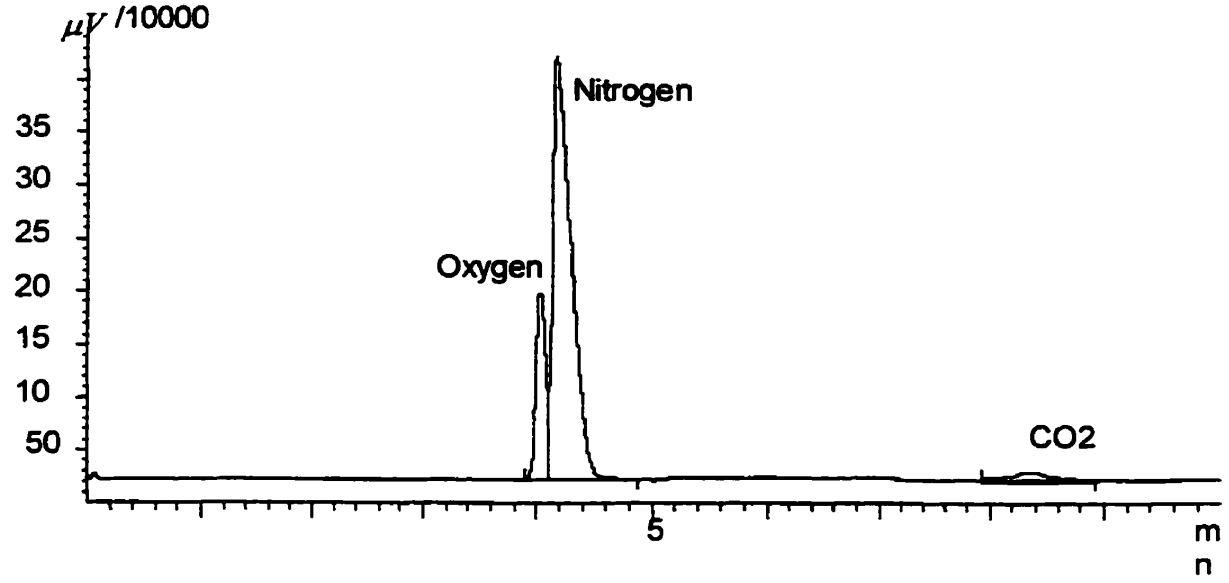


Figure 4.7) The Calibration Standard Finger Print

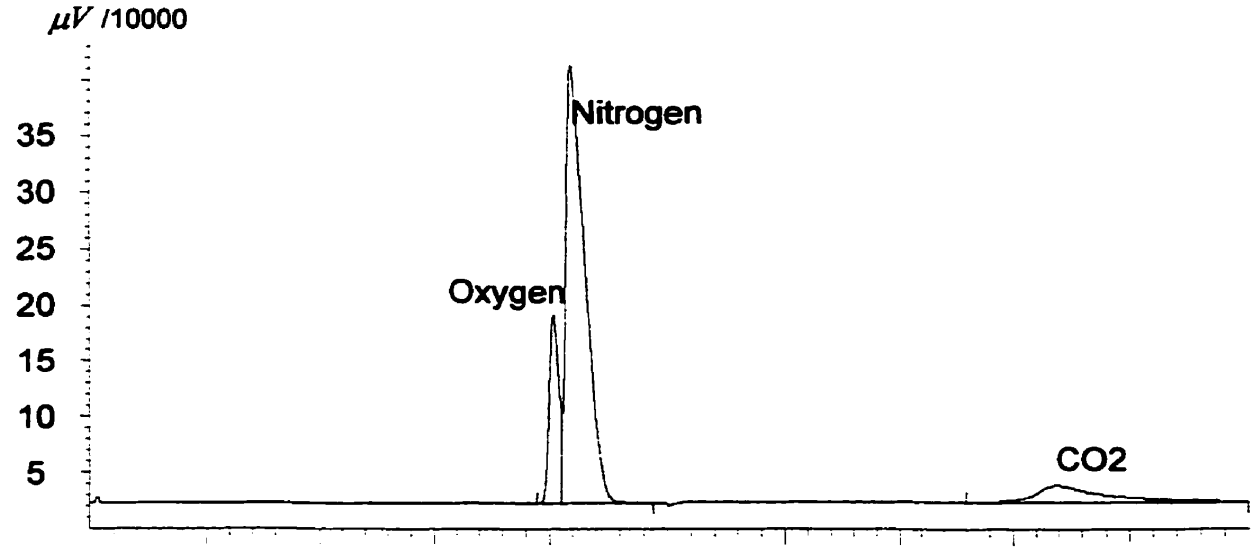


Figure 4.8) The GC Finger Print of Gas Sample from Test Jar Number 6.

4.3 Results for the Main Bioremediation Experiments (Fractional Factorial Design)

The preliminary tests were mainly conducted in small scale to obtain more information on fungal growth in the soil, optimum moisture content, the most active fungus strain, and the most efficient bulking agent and to screen the fungi strains and bulking agents for the main experiments. However, the main experiments were conducted to obtain accurate data for statistically analyzing the effect of various parameters. The main experiments, which were based on Fractional Factorial Design, were conducted in 13, 4 L glass jars, in which the moisture content, enzyme activity, and the hydrocarbon content were monitored over a 3-month period.

4.3.1 Moisture Content

The moisture content of each test jar was monitored on a regular basis, at every sampling time schedule. The results of moisture content analysis are showed in Figure 4.11. The results did not show any significant change in the moisture during the three months of the experiments. The highest change observed in the amount of moisture was related to the test jars number 1, 2, 3 and 10 after 8 days, which was 10%. After that there was not any significant change in the moisture content of the test jars. The moisture content of the test jars were adjusted by spraying the buffer solution whenever necessary.

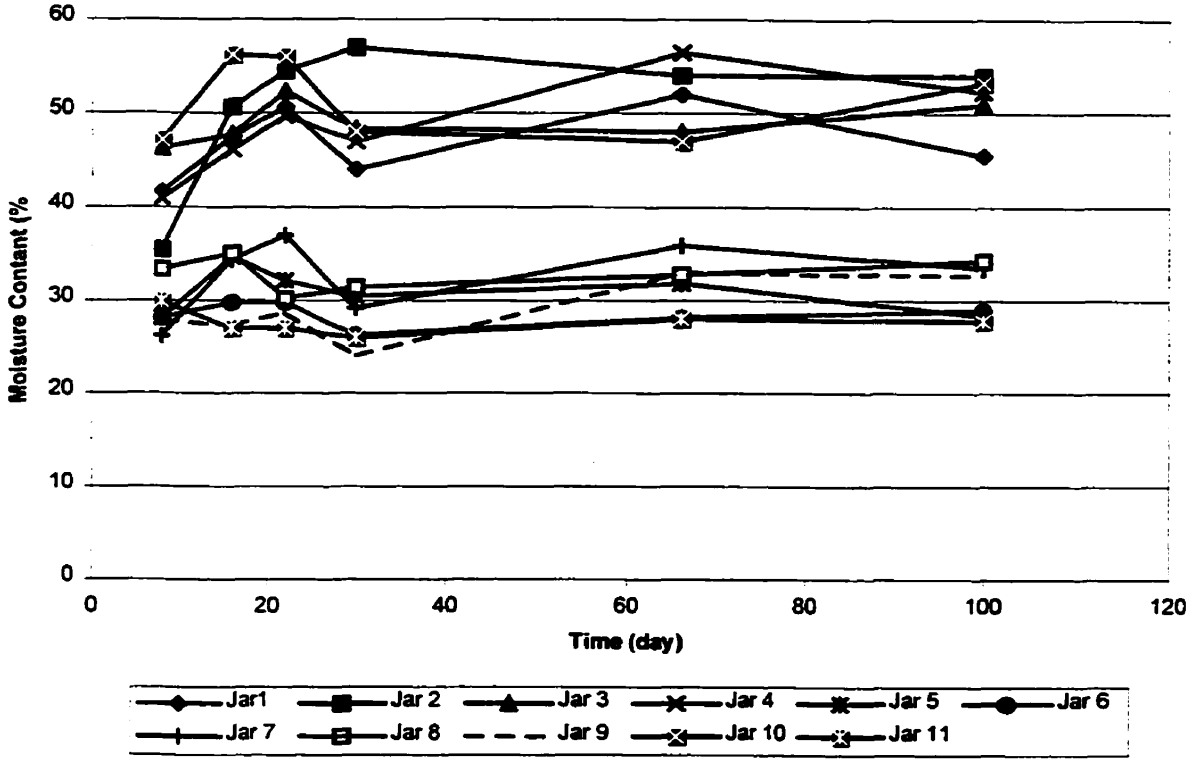


Figure 4.9) Results for the moisture content of the test jars for the 100-day period (Average of two replicates with 5.6% error).

4.3.2 Ligninolytic Enzymatic Activity

The enzymatic activity of fungi in each test jar was monitored regularly using the dye-decolorization method. The results for the enzyme activity measurements over 100 days are shown in Figure 4.12. All of the test jars showed high activity, except the control jars 12 and 13. The enzyme activity in jar 10 was not as high as others, possibly because the soil was wet and too adhesive due to its high moisture content and low bulking agent. The ligninolytic enzyme activity in the jars with no fungi could be mainly from the natural white rot fungi present in the bulking agent or in the soil. It could also be due to fungi cross contamination between jars.

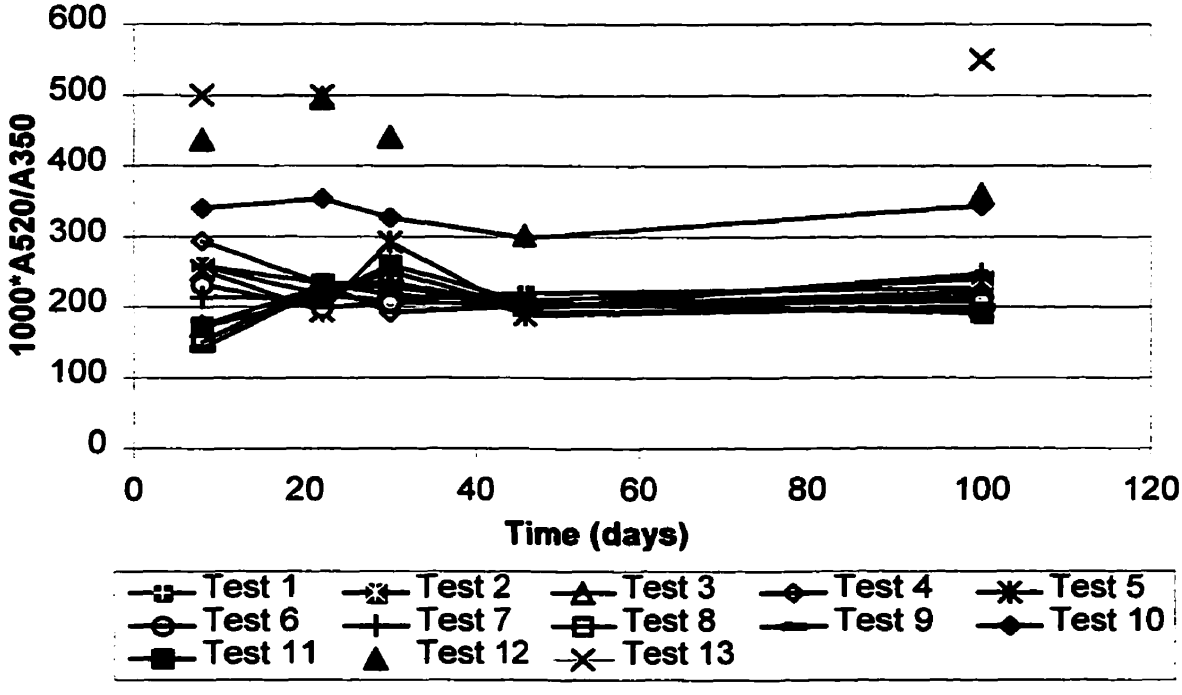


Figure 4.10) Results for the ligninolytic enzyme activity in test jars over a 100 dayperiod (note that lower absorbance ratio means higher enzyme activity)

4.4 Results for the Hydrocarbon Reduction in the Test Jars

Several factors can be responsible for the hydrocarbon reduction in the soil during the experiments. These include biological degradation, volatilization, and adsorption. In this research, even though the soil was excavated from an old site, volatilization could happen due to an increase in exposed surface area, or higher ambient temperature. More adsorption could happen due to the soil dilution or addition of bulking agents.

It was assumed that the hydrocarbon loss in the control jars was mainly due to volatilization and adsorption.

Two different methods were used to evaluate the hydrocarbon reduction in the soil samples. The first method was total extractable hydrocarbon loss measurement using gravimetric analysis. The second method was comparative qualitative analysis by GC fingerprinting.

4.4.1 Results for the Hydrocarbon Reduction in Test Jars Using Gravimetric Analysis

The soil in the test jars was sampled based on the time schedule of 0, 8, 16, 22, 30, 46, 66, and 100 days. A summary of gravimetric results is shown in Table 4.6 and Figure 4.13. As can be seen from the results all test jars showed some hydrocarbon reduction, but the reduction were minimum in controls. The control test jars showed a reduction of roughly 5% per month. Since no treatment was applied and the moisture content was low, this hydrocarbon reduction was assumed to be mainly caused by non-bioremediation methods. A more detailed analysis of the hydrocarbon loss results is presented in statistical analysis section.

Table 4.6) Results for oil loss from the test jars based on gravimetric analysis (Average of two samples with ± 4%)

		Hydrocarbon Loss (%)		Corrected hydrocarbon loss* (%)	
Test	Jar Description	After 28 days	After 100 days	After 28 days	After 100 days
1	Test (I): B.adusta	32.7	48.1	29.8	32.8
2	Duplicate of 1	37.0	46.8	34.1	31.6
3	Test (I): mixed fungi	23.8	45	21	29.7
4	No fungus (I)	31.6	47	28.7	31.3
5	Test (II): B.adusta	30.7	47	27.8	31.7
6	Duplicate of 5	28.2	42.4	25.3	27.1
7	Test (II): mixed fungi	29.7	43.3	26.8	28
8	Duplicate of 7	31.9	47.2	29	32
9	No fungus (II)	30.1	47.7	27	32
10	Test (III): B.adusta	18.3	40.5	15	25
11	Test (IV): B.adusta	32.6	38.2	29.7	29.1
12	Control	-1.7	21	----------	----------
13	Control	7.6	9.6	----------	----------

* Corrected for loss in controls by subtracting the average reduction of hydrocarbon content in controls from the loss obtained in different test jars.

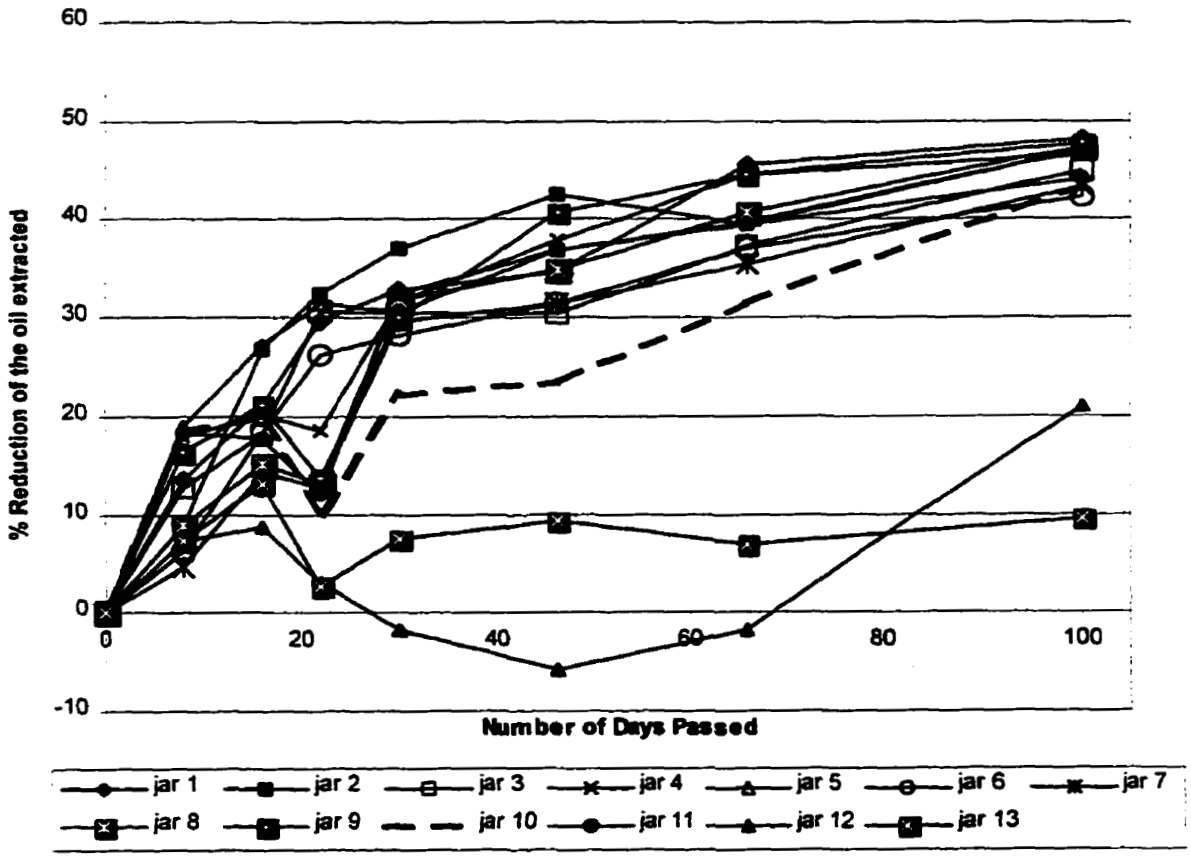


Figure 4.11) Gravimetric results for oil reduction in the 13 jars over 100 days (Average of two samples with ± 4% error)

4.4.2 Results for Hydrocarbon Loss in the Test Jars Using Gas Chromatography

In contrast to the gravimetric method, the GC analysis provides information on each individual hydrocarbon in the soil, except for asphaltenes fraction. At every sampling time three samples were taken and the hydrocarbons were extracted. The extracted hydrocarbons were then deasphaltenated and diluted with DCM. 1 μL of the solution was used for injection into the GC. The GC temperature ramping method used for this analysis was calibrated for normal C_7 to C_{40}. More details of the GC method can be found in chapter three. The changes in peak area under the peaks of calibrated components were studied. A summary of the results for the reduction of the peak areas in selected test jars can be seen in Figure 4.12. The reductions are based on the average from two samples and are calculated .from the following equation:

$$R = (1 - \frac{PA_{100}}{PA_0} \times \frac{m_{100}}{m_0}) \times 100$$

In this equation R represents the percent reduction in the peak area of each individual peak, PA_{100} and PA_0, represent the peak area of calibrated hydrocarbon peaks after 100 days of and at time zero and finally m_{100} and m_0 represent mass of extracted oil after 100 days and at time zero.

The mass ratio term in the above equation is to account for the dilution procedure used in preparing the GC samples.

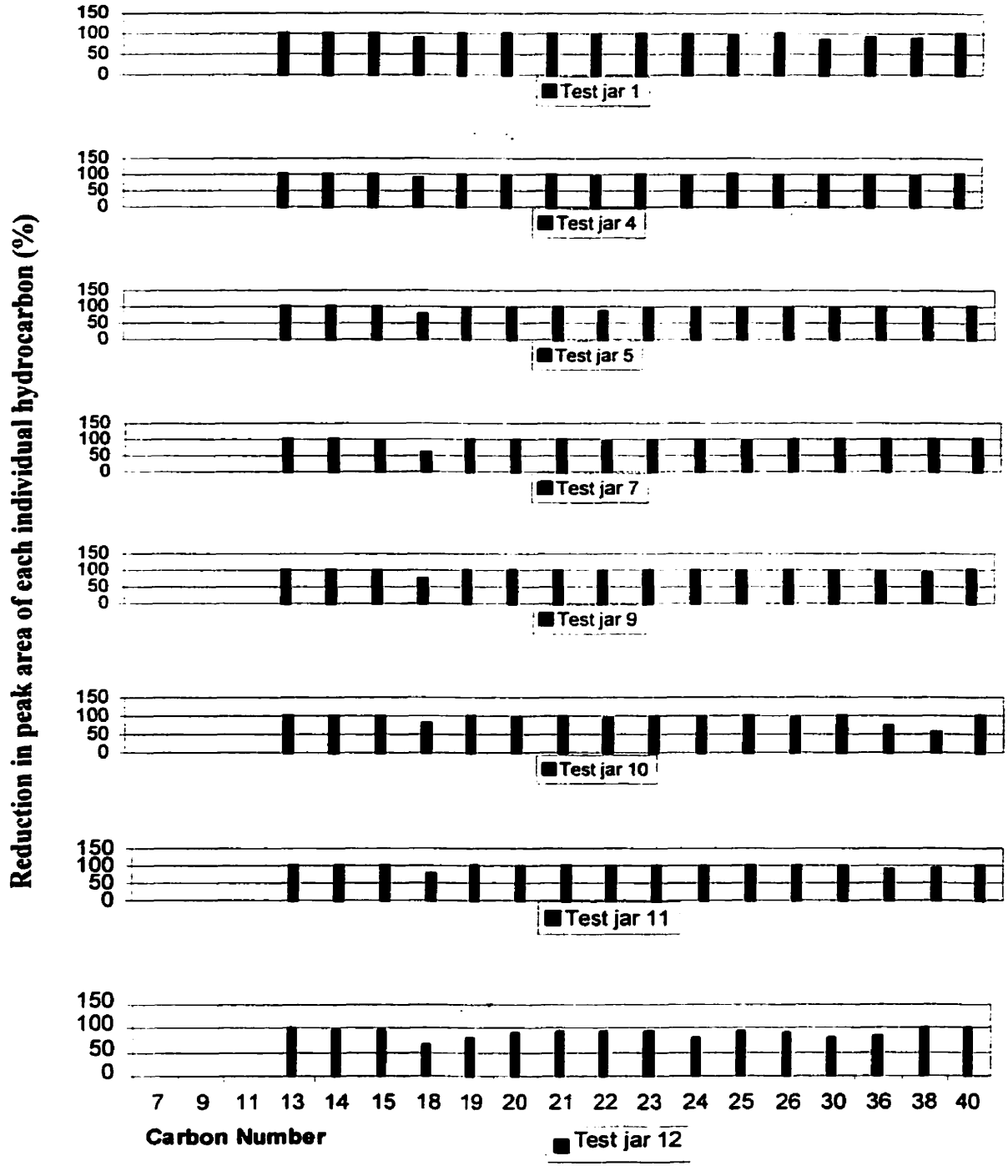


Figure 4.12) Change in the area of calibrated peaks of deasphaltenated hydrocarbons in the different test jars using GC analysis (Average of two samples with ± 10% error)

4.4.3 Reduction of Individual Hydrocarbons

In this section, normalized peak ratios were studied to determine if the hydrocarbon degradation was just limited to a certain fraction of hydrocarbons or it took place over the whole spectrum. In this study the ratio of the average area under calibrated peaks of injected samples of the different test jars to the area under calibrated peaks of injected samples of controls were calculated.

Figure 4.13 shows the normalized peaks ratio of the test jars to the control. A ratio equal to one indicates that there has been an equal reduction in the compound both in control and other test jars. Table 4.7 represents the data used to produce this graph. As can be seen from the data in Table 4.7, the ratio equal to one for C7 to C14 implies that total loss of the compound has happened both in controls and other test jars. Since no biodegradation has occurred in control test jars it has been assumed that the loss of the lighter compounds is mainly due vaporization. As can be seen in Figure 4.13 all of the other ratios are less than one. Figure 4.13 and Table 4.7 show that bioremediation has indeed taken place over the whole spectrum of GC fingerprint and is not limited to a specific hydrocarbon fraction.

The comparison between the tests with fungal application and without fungal application can be seen in Figure 4.13.

The data related to the jar number 10 show that minimum degradation has occurred in this jar. This is possibly caused by the low level of bulking agent and high level of moisture content and thus the adhesive nature of soil in this jar. Therefore the results of this jar were not used for the calculations of the peak ratio.

Table 4.7) The Peak Area under calibrated peaks. (Average of two replicates with10% error)

Test Jar No.	1	2	3	4	5	6	7	8	9	10	11	Average of 12 & 13
Hydrocarbon Peak Area ()												
C7	0	0	0	0	0	0	0	0	0	0	0	0
C9	0	0	0	0	0	0	0	0	0	0	0	0
C11	0	0	0	0	0	0	0	0	0	0	0	0
C13	0	0	0	0	0	0	0	0	0	0	0	0
C14	0	0	0	0	0	0	0	0	0	5.75	0	9.025
C15	1.8	3.6	7.6	4.95	12.1	8.95	13.85	17.05	11.6	22	7.2	29.55
C18	54.42	66.25	105.75	78.05	157.2	131.45	123.45	129.8	126.75	95.3	145.15	300.05
C19	6.55	9.4	7.2	9.15	28.45	18.25	11.565	12.95	14.55	4.65	6.755	131.2
C20	3.65	3.15	4.35	20.7	20.7	10.55	8.5	7.5	7.6	22.9	12.9	38.525
C21	3.9	4.95	38.65	7.35	10.75	7.165	9.55	8.1	9.35	17	6.45	29.75
C22	9.6	11.7	7.95	15.5	39	13.85	17.7	42.7	13.15	20.4	15.25	25.425
C23	3.15	3.95	9.05	4.9	10.5	13.7	14.85	6.7	6.2	17.35	6.25	25.475
C24	10.3	4.15	23.95	14.35	20.8	16.15	12.1	13.2	10.75	18.2	13.85	39.62
C26	2.55	8.2	18.8	8.25	8.4	10.3	10.85	14.05	7.85	24.3	9.3	31.875
C30	6	11.3	20.05	12.15	25.2	18.65	3.1	18.65	12.65	7.3	13	75.525
C36	11.2	7.3	6.8	5.6	8.3	4.3	2.35	2.4	7.15	13.8	9.8	12.05
C38	7.65	3.4	7.35	6.85	6.75	2.85	2.65	0	3.55	9.55	2.95	5.25
C40	0	0	0	0	0	0	0	0	0	4.25	0	0

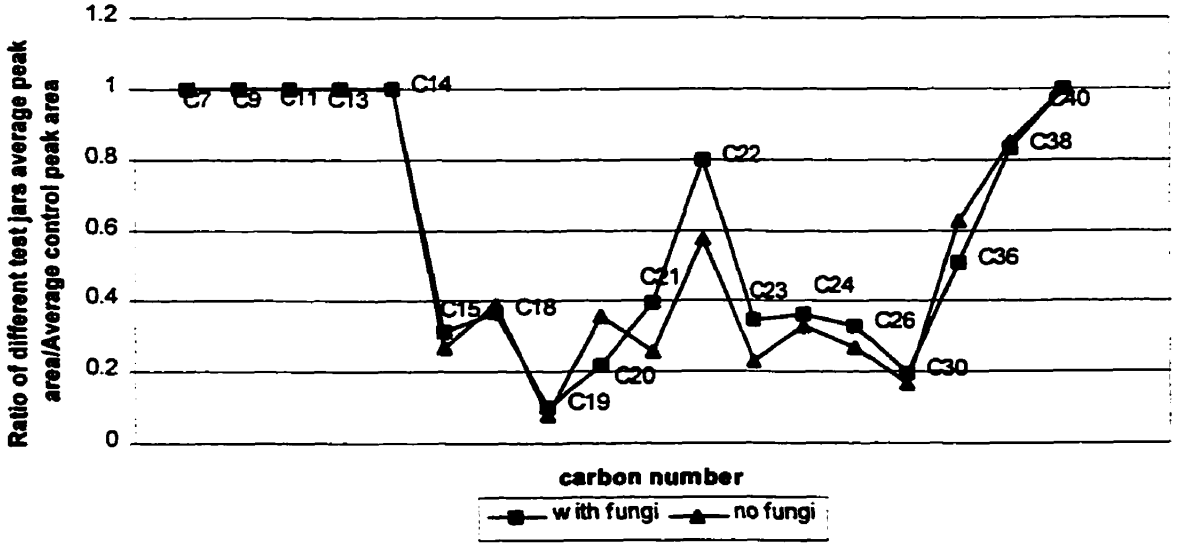


Figure 4.13) The ratio of average peak area under calibrated peaks of different test jars samples to peak area of control treated tests.

4.4.4) Comparison of the GC Fingerprint of Different Jars

In this section, the GC fingerprint for three different jars are studied. Figure 4.14 shows the GC fingerprinting of the extracted samples from jar 6 at 0 and 100 days. These fingerprints are a typical representative of the results for all jars with microbial activity and biodegradation. From the two graphs in Figure 4.14, it can be concluded that the height of most of the hydrocarbon peaks has decreased. This reduction has occurred across the whole spectrum and is not limited to certain fraction of hydrocarbons.

Figure 4.15 compares the GC fingerprints of extracted samples from jar 10 at the beginning and at the end of experimental period. As can be seen from this figure, the reduction in the height of the peaks in the 100-day sample from jar 10 is not as much as the reduction in height of the peaks in the 100-day sample from other jars with microbial activity. This can be caused by the improper conditions in the jar 10 for biodegradation and microbial growth and activity.

Figure 4.16 Compares the GC fingerprints of the extracted samples from control jars before and after experimental period. It can be concluded from this figure, that despite no significant biodegradation activity in the control jars; some hydrocarbon loss has occurred in these jars. The hydrocarbon loss in these jars has occurred due to vaporization and adsorption and is not as high as the loss in other jars with biodegradation activity.

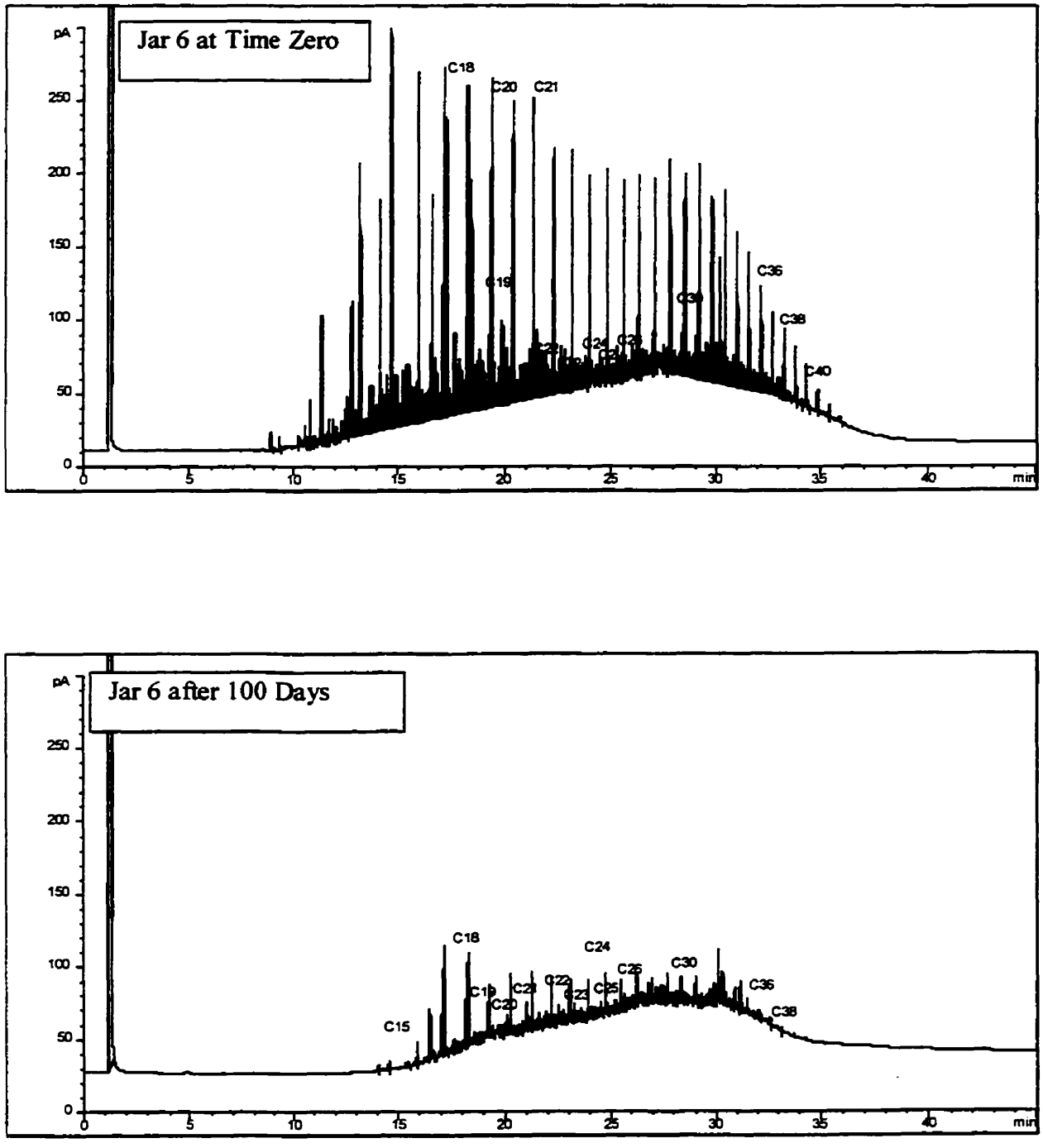
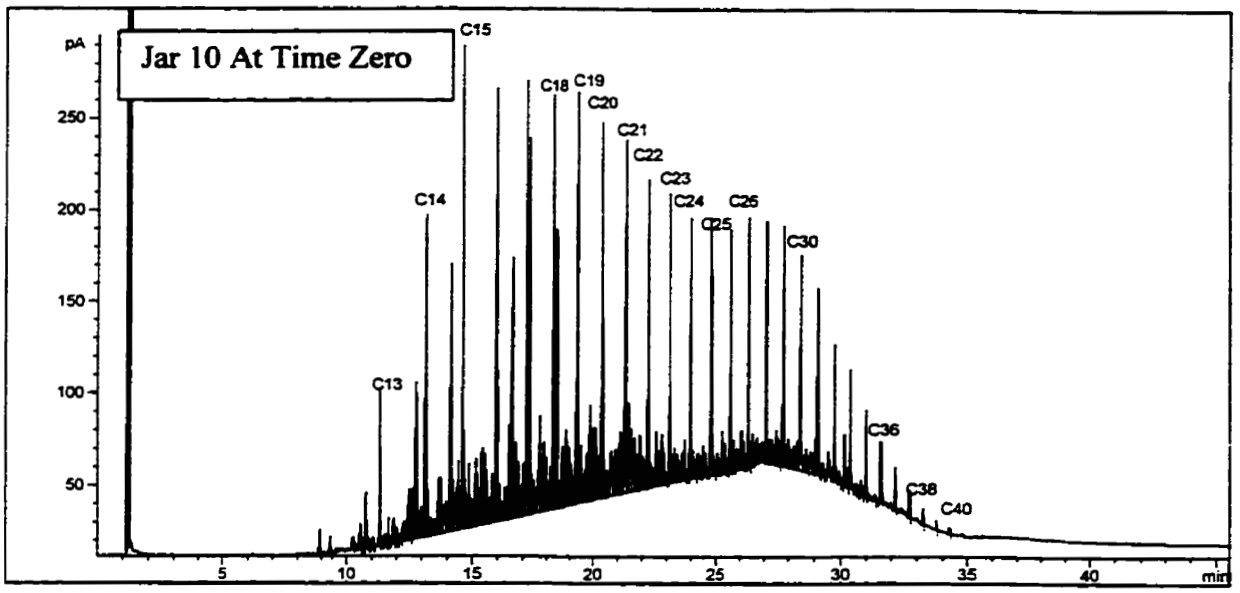


Figure 4.14a,b) GC fingerprint of the extracted oil from jar 6 before and after biodegradation.



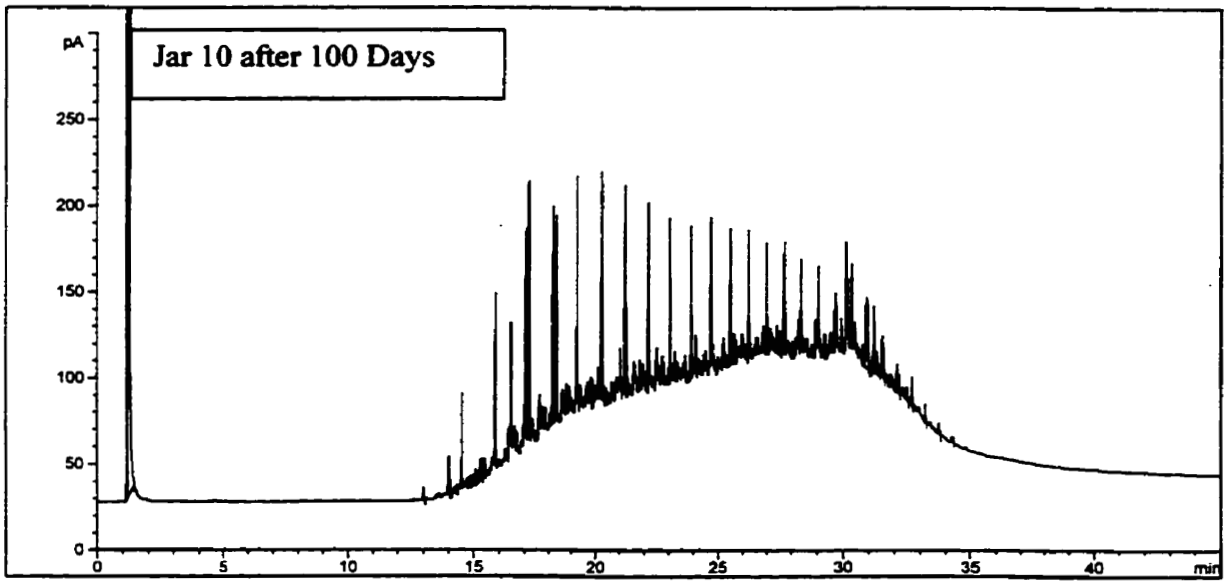
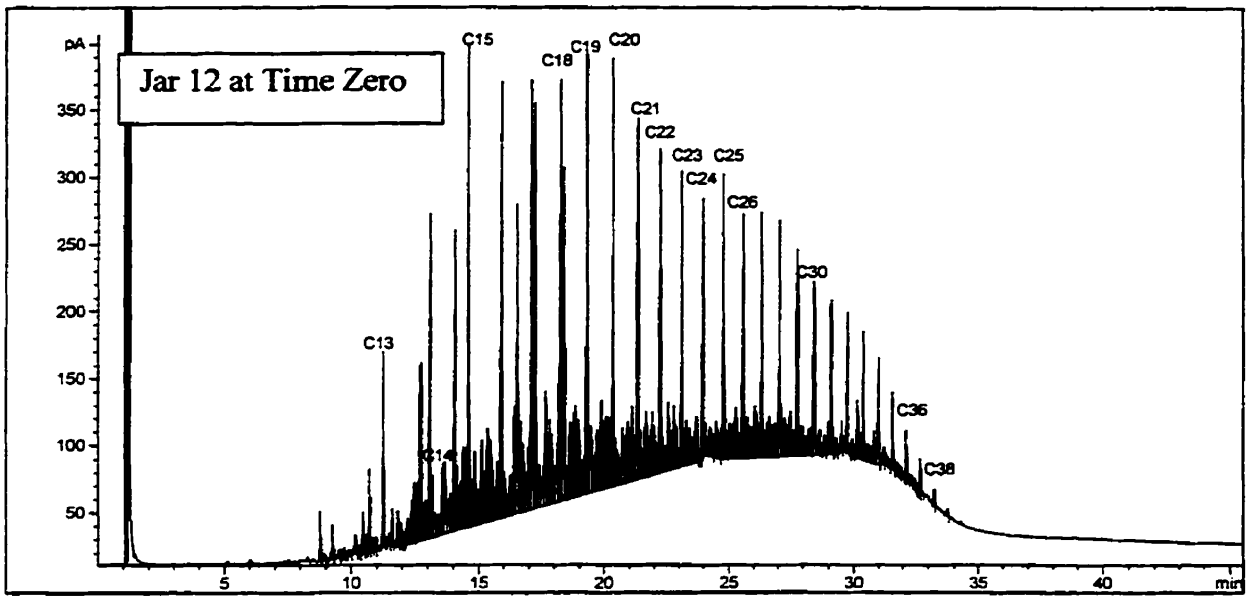


Figure 4.15a,b) GC fingerprint of the extracted oil from jar 10 before and after biodegradation (Test jar #6).



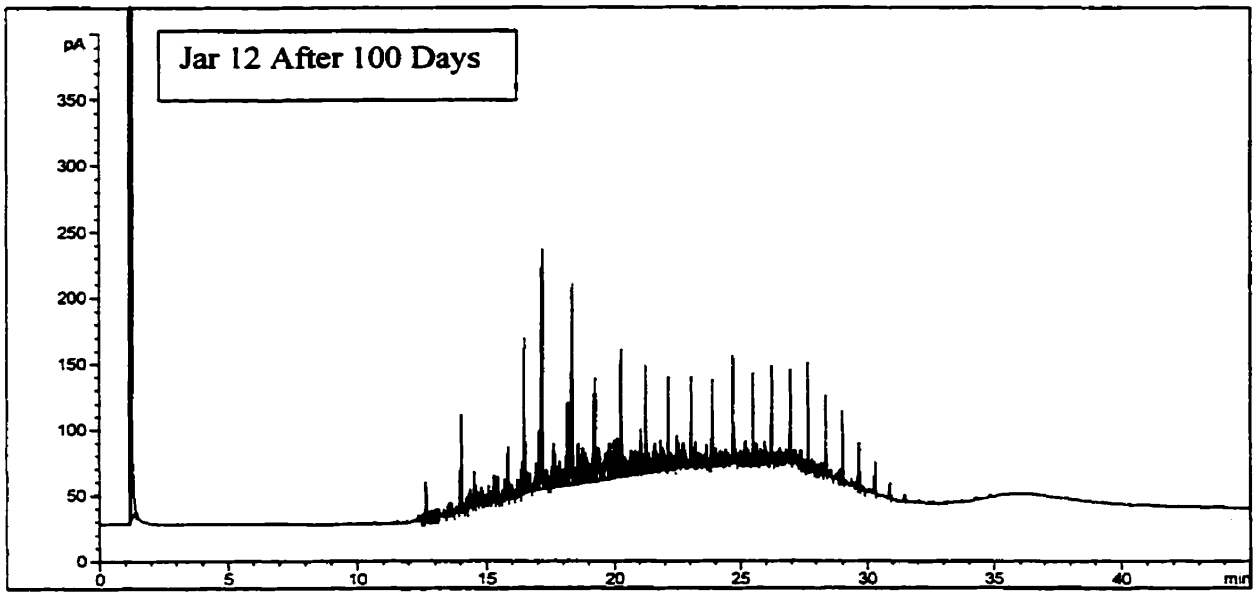


Figure 4.16a,b) GC fingerprint of the extracted oil from control test jar at zero days and 100 days.

4.5 Statistical Analysis of Results

The results from gravimetric analysis of hydrocarbon loss were further analyzed using two different statistical methods. The results were first analyzed using Box-Hunter method for analyzing fractional factorial design, to study the effect of different factors such as fungi application, moisture content and bulking agent. The second method was based on *repeated measures analysis of variance* (RM-ANOVA), which provides additional information on any changes in the "patterns "over the time span.

The calculations for the *repeated measures analysis of variance* were performed using the statistical analysis software, "Statview", as the method is much more complicated than the Box-Hunter analysis method.

4.5.1 Analysis of the Fractional Factorial Design Using Box-Hunter Method

Statistical analysis of the results is performed to determine if any of the factors (fungi, bulking agent and moisture content) has significant effect on the outcome of the experiment. The Box-Hunter method, which was explained in chapter 3, is one of the methods to analyze the fractional factorial design experiments. Table 4.8 shows the statistical notations of the test jars and a summary of the results for 28 and 100 days. In this table the combination of different treatments in the design are represented by lowercase letters. The high level of any factor in the treatment combination is denoted by the corresponding lowercase letter and the low level of a factor is denoted by the absence of the corresponding letter. The single and mixed strain fungi applications are considered in one category since the statistical analysis did not show any significant difference (Appendix C.1). The three different factors in the experiments are designated as fungi (a), moisture content (b), and bulking agent (c). The final results of the analysis are shown in Tables 4.9 and 4.10.

Figures 4.17 and 4.18 show the summary of the main effects for fungi, bulking agent and moisture content on the hydrocarbon loss in test jars, after 100 and 28 days.

Table 4.8) Summary of the results and statistical notations of the test jars.

Test no.	Jar Description	Statistical notations*	Reduction after 28 days (%)	Reduction after 100 days (%)
1	Test (I): B.adusta	abc	32.8	48.1
2	Duplicate of 1	abc	37.0	46.8
3	Test (I): mixture of fungi	abc	23.9	45
4	No fungus (I)	bc	31.6	46.6
5	Test (II): B.adusta	ac	30.7	47
6	Duplicate of 5	ac	28.2	42.4
7	Test (II): mixture of fungi	ac	29.7	43.3
8	Duplicate of 7	ac	31.9	47.2
9	No fungus (II)	c	30.1	47.7
10	Test (III): B.adusta	ab	18.3	40.5
11	Test (IV): B.adusta	a	32.6	44.3

* Notations are based on Box Hunter method

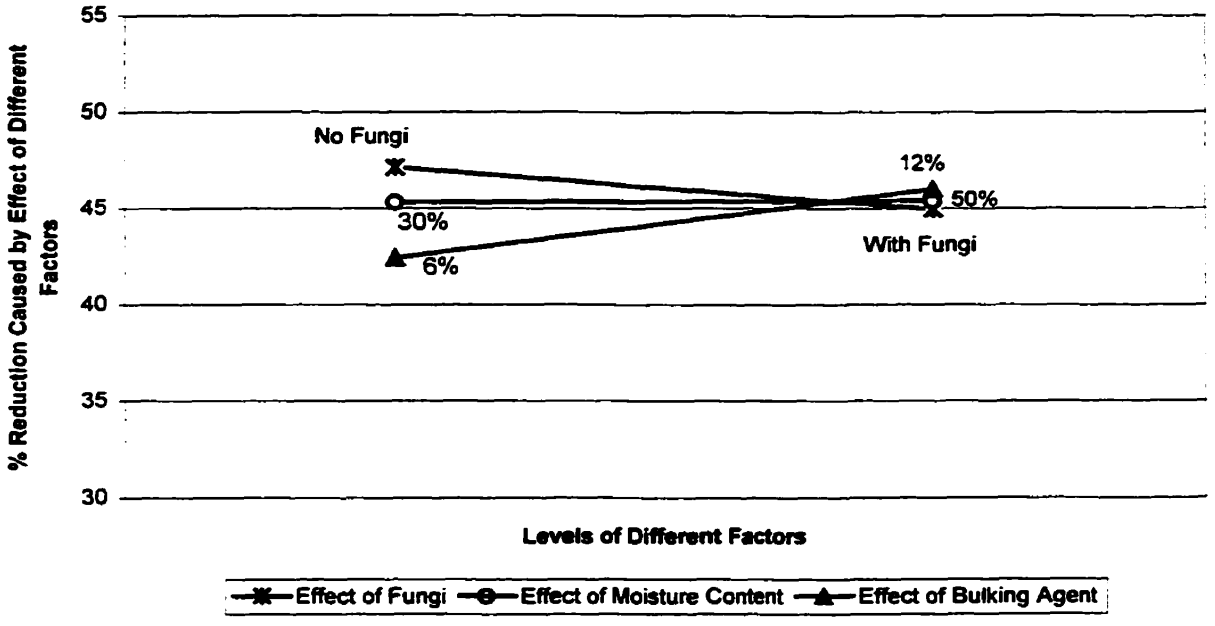


Figure 4.17) Graphic presentation of the main fungi, moisture content and bulking agent effects based on 100 days data (Note, the changes of the effects are within the range of experimental errors).

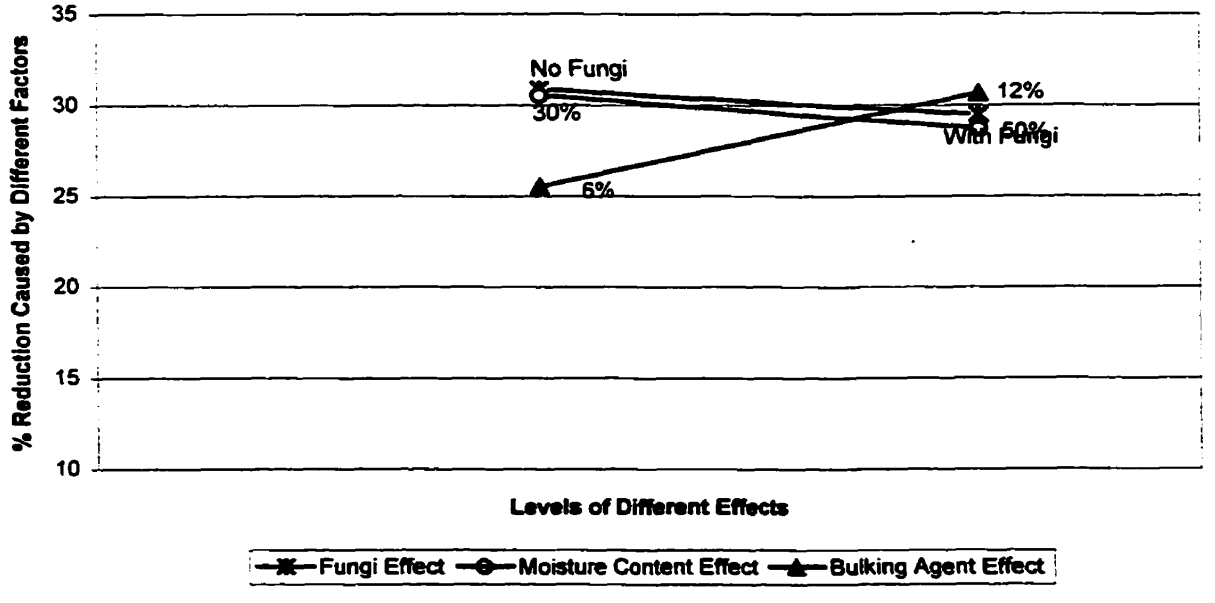


Figure 4.18) Graphic presentation of the main fungi, moisture content and bulking agent effects based on 28 days data (Note, the changes of the effects are within the range of experimental errors).

Table 4.9) The results for the main effects, interactions and standard error of the experimental design based on Box Hunter method after 28 days (More details on calculation of variance and average of effects can be found in Chapter 3, and Appendix C.2).

Factors and Interactions	Main Effect	2.0σ	Significant Effect
Fungi Effect, A	-1.69	5.66	No
Moisture content Effect, B	-1.42	4.38	No
Bulking agent content effect, C	4.93	5.66	No
Interaction: fungi × moisture content, A × B	3.46	4.38	No
Interaction: moisture content × bulking agent, B × C	2.09	4.38	No
Interaction: fungi × bulking agent content, A × C	3.81	4.54	No
Interaction: all three factors, A × B × C	3.3	4.38	No

Table 4.10) The results for the main effects, interactions and standard error of the experimental design based on Box Hunter method after 100 days of experiments (More details on calculation of variance and average of effects can be found in Chapter 3, and Appendix C.3).

Factors and Interactions	Main Effect	2.0σ	Significant Effect
Fungi Effect, A	-2.2	2.9	No
Moisture content Effect, B	0.09	2.26	No
Bulking agent content effect, C	3.63	2.9	Yes
Interaction: fungi × moisture content, A × B	0.514	2.26	No
Interaction: moisture content × bulking agent, B × C	1.49	2.26	No
Interaction: between fungi × bulking agent content, A × C	0.0919	2.34	No
Interaction: all three factors, A × B × C	1.91	2.26	No

4.5.1.1 The Effect of Fungi Application

As can be seen from Tables 4.9 and 4.10, both the fungi main effects and interaction effects are within the $\pm 2.0\sigma$ (2.0 times standard error of the experiments). This means that the fungi applications did not have any significant effect on the outcome of the experiments. This could be caused by several factors. One of the major factors can be caused because in these experiments neither the soil used for the experiments, nor the peat moss used as bulking agent were sterilized. Therefore there has been fungal growth and activity in all tests even the ones with no fungal inoculation, and that affects the results of fractional factorial design calculations. On the other hand since the soil and bulking agent were not sterilized the bacterial activity was present in all test jars. In many cases it has been reported that the competition between soil bacteria and white rot fungi for nutrients is an inhibitory factor for fungi. The production of antibiotics by bacteria, phenomena previously observed, may be another contributing factor (Anderson et al., 1995, Lang et al., 1997). As reported in previous researches, the degradative activity could be negatively influenced by these activities of soil microorganisms in the presence of fungi. The other possibility for fungi not having significant effect is that there was some chance of fungal cross contamination between different test jars.

4.5.1.2 The Effect of Moisture Content

The moisture content in the soil was 30% or 50% (w/w dry soil) in different test jars. The results from the analysis of the experiments using Box-Hunter method (Tables 4.9 and 4.10) showed that the change of moisture content from 30% to 50% did not have a significant effect on the hydrocarbon loss. The results of these calculations have been further confirmed using the Statview software, which will be presented in next section. Tables 4.10 and 4.11 show that the moisture main effect and interaction effects are within the $\pm 2.0\sigma$ (within 95% confidence interval).

4.5.1.3 The Effect of Bulking Agent

As can be seen from Table 4.10, the main effect of bulking agent after 100 days is not within $\pm 2\sigma$ (95% confidence interval), which means bulking agent had significant effect on the hydrocarbon loss of the test jars. In other words, the variations in the 100 day results from changing the percent of bulking agent are not merely caused by experimental errors. However, the bulking agent had no significant effect at 28 days of experiments.

The calculations showed that the change in bulking agent from 6% to 12% caused an increase of aproximately 4 to 6% in hydrocarbon loss during 100 days of experiments. The increase could be due to the higher microbial activity or higher adsorption rates in the high bulking agent jars.

4.5.1.4 The Interaction Effects

The Interactions between different factors was calculated using and Box Hunter method. As shown in Tables 4.9 and 4.10 the effect of interaction between different factors is within $\pm 2.0\sigma$ (95% confidence interval), which means that the interaction between different factors did not have any significant effect on hydrocarbon loss in test jars.

4.5.2 Analysis of Fractional Factorial Design Using "Repeated Measures Analysis of Variance"

To confirm the results from Box-Hunter analysis and to study the effect of time, *repeated measures analysis of variance* (RM-ANOVA) has been used. As with any ANOVA, repeated measures ANOVA tests the equality of means (Stat-38, 1997). This method is often used when all members of a sample are measured at different times. As the time passes, the measurement of the dependent variable, which in this case was the hydrocarbon loss, is repeated. In other words, this analysis gives more accurate information on the role of time as another factor effecting the hydrocarbon reduction in the soil. It generally shows if the effects of bioremediation factors (A, B, C) have changed their pattern over time. The *Statview* software was used for the analysis of RM-ANOVA. Software ran the analysis of variance on both 28 and 100 day data to determine the effect of "*Time*". The results are shown in Table 4.11 and Figures 4.19, 4.20 and 4.21, and in overall they confirm the Box-Hunter results. However, the P-value of the bulking agent effect is about 0.2, which considering the Box-Hunter results, can be interpreted as only a marginally significant effect.

The results of the RM-ANOVA also showed that the time interaction with other factors was insignificant. This means that the effectiveness of fungi application, bulking agent, and moisture content did not significantly change with time.

Table 4.11) Results from the "*Repeated Measures Analysis of Variance*" using Statview software.

	DF	Sum of Squares	Mean Square	F-value	P-Value	Significance of the Effect
Fungi	1	2.68	2.68	0.109	0.751	Not Significant
Moisture	1	3.066	3.066	0.125	0.735	Not Significant
Bulking Agent	1	53.52	53.52	2.173	0.184	Not Significant
Time	1	550.32	550.32	65.257	<0.0001	Significant
Time× Fungi	1	0.845	0.845	0.100	0.761	Not Significant
Time× Moisture	1	4.434	4.434	0.426	0.492	Not Significant
Time× Bulking Agent	1	2.246	2.246	0.266	0.622	Not Significant

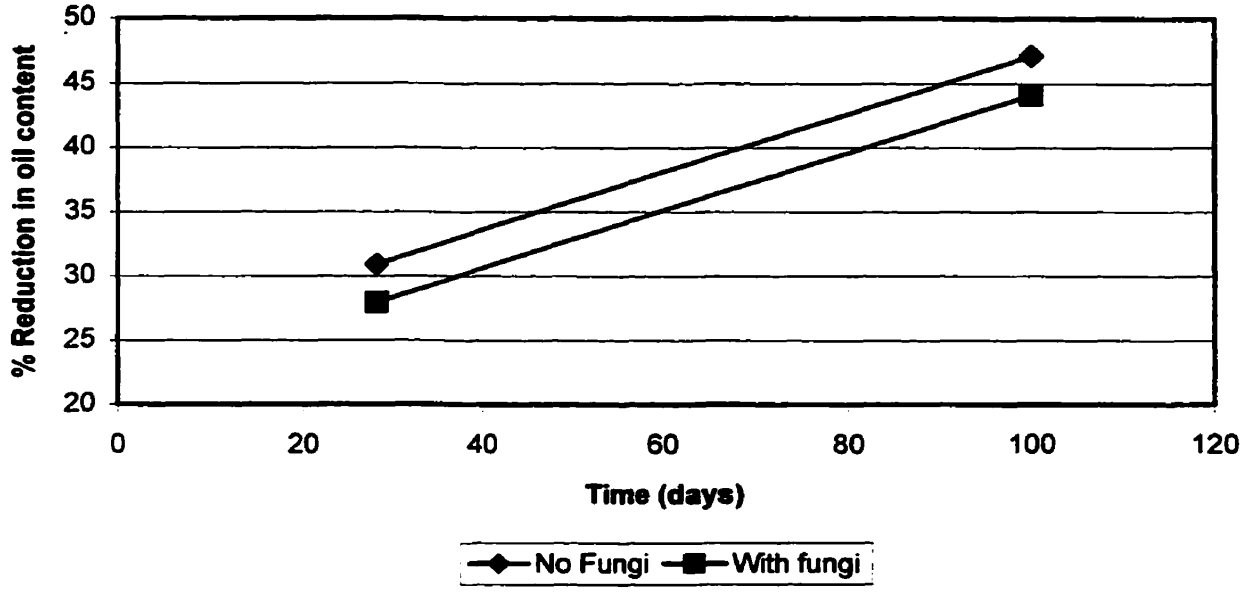


Figure4.19) The Effect of time on the pattern of hydrocarbon loss caused by fungi application (Time× Fungi).

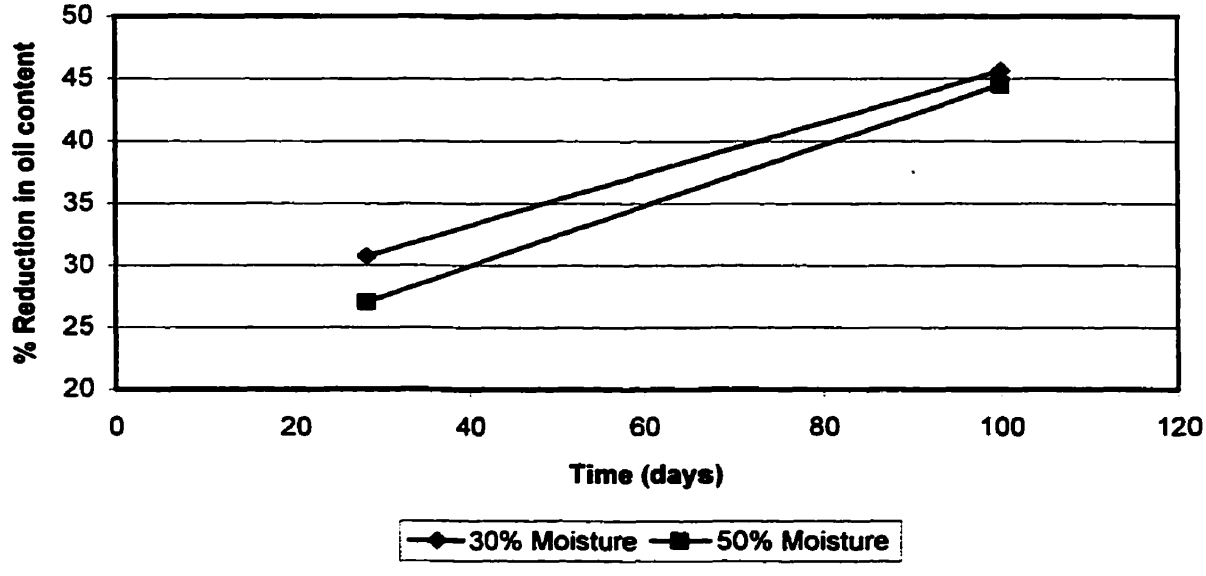


Figure 4.20) The effect of time on the pattern of hydrocarbon loss caused by increase in moisture content (Time× Moisture content).

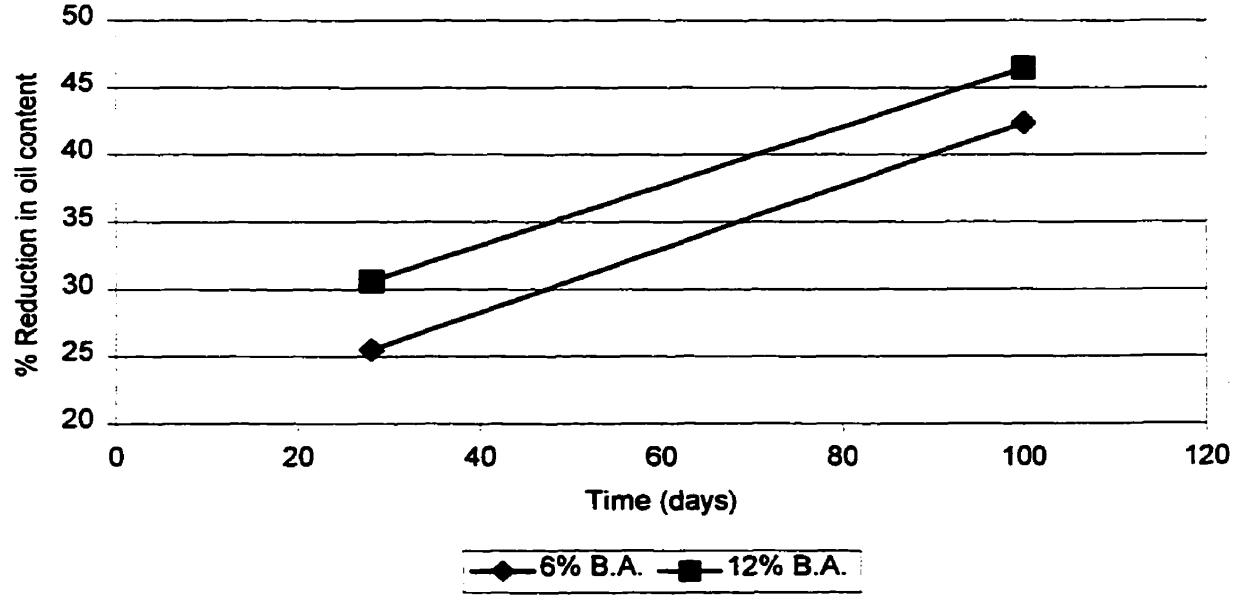


Figure 4.21) The effect of time on the pattern of hydrocarbon loss caused by increase in bulking agent content (Time× Bulking Agent).

Chapter Five: Conclusions and Recommendations

5.1 Conclusions

During this research, a detailed bench scale study of fungal bioremediation of flare pit soil was undertaken. The following conclusions can be made within the limits of this research project:

- Different bulking agents, including peat moss, pine wood shavings and Kellogg's bran flakes were tested during the preliminary tests, and fungi showed higher rate of growth on two bulking agents, peat moss and bran flakes. Peat moss, which is mainly composted woody residues, is a very light bulking agent that provides a considerable amount of void space in the soil. Bran Flakes is a breakfast cereal and mainly contains whole wheat and wheat bran is a very good source of nutrients for

fungi and it also increases the enzyme induction by fungi (Pickard et al., 1999b). A mixture of bran flakes and peat moss was found to be very effective bulking agent for the optimum fungal growth and enzyme activity.

- White rot fungi won't be able to grow on contaminated soil unless pre-grown on woody materials or bulking agent for at least 10 days to establish a solid and complete growth of mycelia (Holroyal & Caunt 1995). Otherwise the fungal mycelia will be very fragile and fungi won't be able to adapt to contaminated soil environment.

- The measurement of fungal enzyme activity using dye decolorization method in soil samples showed the presence of enzymatic activity in test jars with and without fungi application. This could be due to use of non-sterilized soil and bulking agents. White rot fungi are naturally present in peat moss and soil.

- The CO_2 and O_2 analysis of the air samples from different test jars showed that there is no need for aeration. Regular mixing at each sampling time will provide enough O_2 for fungal growth. The soil depth in the experiments was approximately 25 cm.

- The optimum moisture content for growth and optimum activity of white-rot fungi was found to be between 30 to 50% (wt/ wt of dry soil). This range of moisture content was for the systems in this research project and the free water content was not determined.

- The gravimetric analysis of hydrocarbon content in the control jars showed approximately 5% reduction of hydrocarbons per month, which could be generally attributed to vaporization and adsorption as there was little microbial activity.

- The toxicity threshold for the fungi strains was studied in the preliminary sensitivity tests. It was showed that all fungi strains grew completely on the concentration of up to 10,000 ppm; at higher concentrations (14,000 ppm) the fungi started growing but stopped growth after 2 to 3 days. Some of the strains showed a slight inhibition at 10,000 ppm in the first few days of the experiment, but not in the longer term of 10 to 15 days.

- It was concluded that *Bjerkandera adusta* UAMH 7308 and *B. adusta* UAMH 8258 showed the highest amount of enzyme activity, based on dye decolorization experiments that were conducted to measure enzymatic activity of different fungi strains.

- Based on the statistical analysis of the hydrocarbon loss in different test jars, it was concluded that application of white-rot fungi didn't have any significant effect on the hydrocarbon loss. In other words, the application of extra fungal inocula into the soil did not have any significant effect on hydrocarbon biodegradation. Dye decolorization assay also showed that fungi were naturally present in the bulking agent and soil.

- Statistical analysis of the hydrocarbon loss in different soil test jars Using Box Hunter method also showed that the change in moisture content from 30% to 50% (wt/wt of dry soil) didn't have any significant effect on the hydrocarbon loss. However the analysis show that the increase in bulking agent content from 6% to 12% (wt/wt of dry soil) had a significant effect on the hydrocarbon loss in contaminated soil after 100 days of experiments but not after 28 days of experiments (marginally significant).

- The results of the analysis of repeated measure ANOVA confirmed the results obtained by Box-Hunter method. The analyses also showed that the interaction

between time and other factors including fungi, moisture content and bulking agent were not significant.

5.2 Recommendations

- A full factorial design could provide more information. In a fractional factorial design, the interaction effects and main effects are often confounded. A full factorial design for two factors of fungi and bulking agent at two different levels with a whole replicate for the design could give more information.

- It is recommended that the oil samples be analyzed using a GC-MS at the end of experiments. That could provide more information on intermediate compounds and by-products produced by fungal activity.

- Sampling and mixing of the jar contents can cut fungi mycelia and even stop the fungal growth. To reduce damage to fungi, it is better to decrease the number of samplings. That will increase, the chances of fungi to survive and continue growth from weeks to months.

- To study the effect of soil natural micro flora on white-rot fungi, it is recommended to have a new design in which the hydrocarbon loss in a sterilized soil amended with white-rot fungi is compared with the hydrocarbon loss in a non-sterilized soil that is also amended with fungi.

- To obtain more information about the effect of each non-bioremedial factor such as adsorption or vaporization, it is recommended that in a new design each of these factors be individually studied. For this purpose the poisoned tests should be used and the results be compared with non-treated controls. The amount of loss in

poisoned tests shows the amount of loss caused by adsorption and the difference between hydrocarbon loss in poisoned tests and non-treated controls shows the amount of hydrocarbon vaporization. In these tests the poisoned tests must be kept in refrigerator to minimize the amount of hydrocarbon vaporization.

- It is recommended to conduct a series of experiments using other types of contaminated soils and flare pit sludge with different amounts of heavy metals and salt in order to study the fungal performance in other types of soil.

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Appendix A: Raw Data

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Appendix A.1) The Imperial Oil Characterization of Contaminated Soil

PAHs	Moderate Hydrocarbon Contamination (Layer 3)	Heavy Hydrocarbon Contamination (Layer 2)
Naphthalene, mg/ kg	2.1	28
Acenaphtylene, mg/ kg	<1.0	2.4
Acenaphthene, mg/ kg	<1.0	2.1
Fluorene, mg/ kg	1.1	16
Phenenthrene, mg/ kg	2	29
Antheracene, mg/ kg	<1.0	<1.0
Total 2-3 rings	5.2	77.5
Fluoranthene, mg/ kg	<1.0	<1.0
Pyrene, mg/ kg	<1.0	4.9
Benzo(a)anthercene, mg/ kg	<1.0	<1.0
Chrysene, mg/ kg	<1.0	9.2
Total 4 Rings	0	14.1
Benzo(b)fluoranthene, mg/ kg	<1.0	1.2
Benzo(k)fluoranthene, mg/ kg	<1.0	<1.0
Benzo(a)pyrene, mg/ kg	<1.0	<1.0
Indeno(1,2,3-c,d)pyrene, mg/ kg	<1.0	<1.0
Dibenzo(a,b)antheracene, mg/ kg	<1.0	<1.0
Benzo(g,b,i)perylene, mg/ kg	<1.0	1.7
Total 5-6 rings	0	2.9
Total PAH	5.2	95
PAH (2-3 rings), %	100	82
PAH (4 rings), %	0	15
PAH (5-6 rings), %	0	3

Hydrocarbon Analysis	Moderate Hydrocarbon Contamination (Layer 3)	Heavy Hydrocarbon Contamination (Layer 2)
TPH (C_{10}-C_{90}), mg/kg	**5000**	**100,000**
Contaminated Class Repartition		
Aliphatic, %	78	72
Aromatic, %	20	19
Resin, %	2	7
Asphalten, %	0	2

Biological and Chemical PArameters		
Percent Solids, %	73	79
PH	7.6	7.5
Total Microflora, CFU/g	2.9E+05	3.8E+05
Specific Microflora (extract), CFU/g	1.3E+04	5.0E+04
Total Organic Carbon, %	1.6	27.1

Total Sedimentometry		
Silt, %	63	n.d.
Clay, %	37	n.d.
Sand, %	0	n.d.
Textural Classes	Silty Loam	n.d.

Metals	Moderate Hydrocarbon Contamination, mg/kg (Layer 3)	Heavy Hydrocarbon Contamination, mg/kg (Layer 2)
Aluminium	1800	7200
Antimony	<10	10
Silver	38	<2
Arsenic	3	66
Barium	64	130
Berylium	<1	<1
Boron	4	16
Bismuth	<10	<10

Cadmium	<1	<1
Calcium	4300	18000
Chromium	42	10
Cobalt	3	7
Metals	**Moderate Hydrocarbon Contamination (Layer 3)**	**Heavy Hydrocarbon Contamination (Layer 2)**
Copper	42	28
Tin	<5	<5
Iron	8000	14000
Lithium	3	8
Magnesium	970	6300
Manganese	75	250
Mercury	<0.2	<0.03
Molybdenum	5	<2
Nickel	15	19
Lead	40	20
Potassium	350	1400
Selenium	<1	0.6
Silicon	70	783
Sodium	4100	2300
Titanium	23	78
Vanadium	25	22
Zinc	470	77

Appendix A.2) Table for Raw Data of Gravimetric Analyses (First Replicate)

Number of Days Passed	0	8	16	22	30	46	66	100
Test no.	Extracted Oil from Soil (%)							
1	1.377	1.194	1.062	0.868	0.886	0.898	0.723	0.667
2	1.377	1.256	0.932	0.868	0.851	0.757	0.779	0.705
3	1.415	1.236	1.083	0.868	0.895	0.951	0.791	0.735
4	1.442	1.183	1.065		0.972	0.874	0.732	0.696
5	1.485	1.205	1.022	0.937	0.932	0.921	0.815	0.744
6	1.227	1.150	0.994	0.865	0.876	0.844	0.805	0.73
7	1.208	1.150	1.017		0.88	0.805	0.807	0.689
8	1.263	1.150	1.037		0.861	0.830	0.728	0.670
9	1.392	1.164	1.079		0.953	0.822	0.703	0.693
10	1.524	1.241	1.2		1.103	1.095	0.984	0.83
11	1.283	1.047	1.033		0.917	0.834	0.779	0.69
12	1.359	1.260	1.053	1.195	1.179	1.302	1.152	1.094
13	1.359	1.260	1.031	1.263	1.119	1.018	1.083	1.199

Appendix A.3) Table for Raw Data of Gravimetric Analyses (Second Replicate)

Number of Days Passed	0	8	16	22	30	46	66	100
Test no.	Extracted Oil from Soil (%)							
1	1.224		0.985	0.95	0.859	0.803	0.692	0.677
2	1.224		0.962	0.881	0.785	0.734	0.787	0.675
3	1.243		1.084	0.962	0.942	0.895	0.864	0.722
4	1.288		1.104	0.808	0.892	0.818	0.773	0.755
5	1.304		0.999	0.967	0.989	0.836	0.856	0.730
6	1.252		1.020	0.966	0.904	0.856	0.754	0.699
7	1.174		1.024	0.877	0.795	0.827	0.732	0.662
8	1.233		1.070	0.904	0.833	0.792	0.746	0.641
9	1.306		1.047	0.947	0.931	0.777	0.790	0.716
10	1.347		1.103	1.074	1.120	1.095	0.974	0.796
11	1.081		1.092	0.986	0.828	0.797	0.784	0.754
12	1.076		1.131	1.14	1.256	1.245	1.275	0.835
13	1.33		1.108	1.153	1.161	1.205	1.205	1.05

Appendix A.4) Table for percent reduction in total peak area under GC fingerprints (Average of two Replicates)

Number Of days passed	0	28	66	100
Test Jar Number	Average %reduction in total peak area			
1	0	65.4	72.2	87.8
2	0	57.7	77.0	86.6
3	0	59.0	81.4	78.7
4	0	36.3	81.8	85.7
5	0	47.9	67.4	66.7
6	0	59.0	72.6	80.1
7	0	64.3	75.7	65.7
8	0	61.1	69.1	76.8
9	0	66.7	80.4	79.6
10	0	18.1	52.6	52.1
11	0	64.7	88.8	92.5
12	0	25.3	62.5	61.2
13	0	38.3	60.6	71.3

Appendix B: GC Fingerprints

FIGURE B1) THE GC FINGERPRINT OF THE EXTRACTED OIL FROM TEST JARS 1 BEFORE BIODEGRADATION. 128
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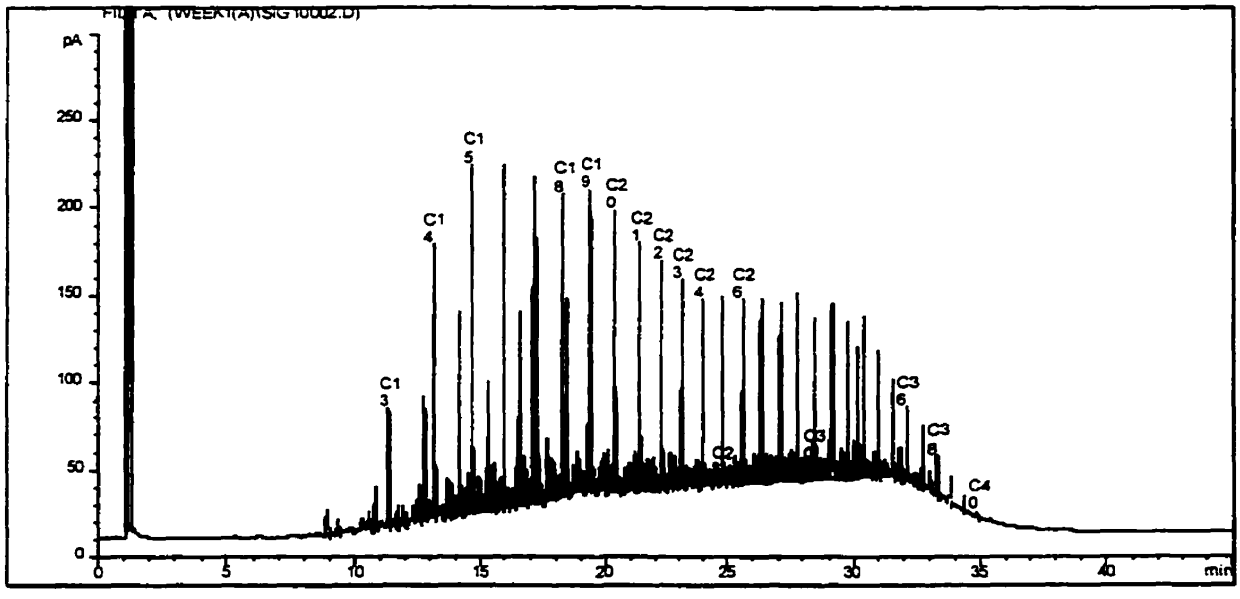

Figure B1) The GC fingerprint of the extracted oil from test jars 1 before biodegradation.

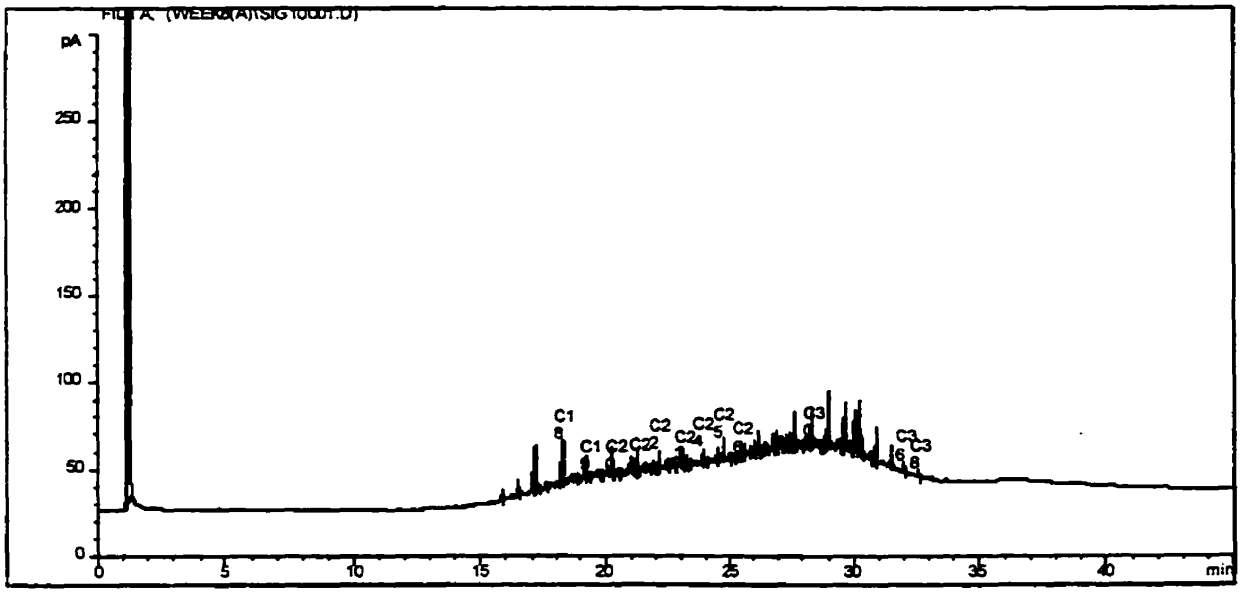

Figure B2) The GC fingerprint of the extracted oil from test jars 1 after biodegradation.

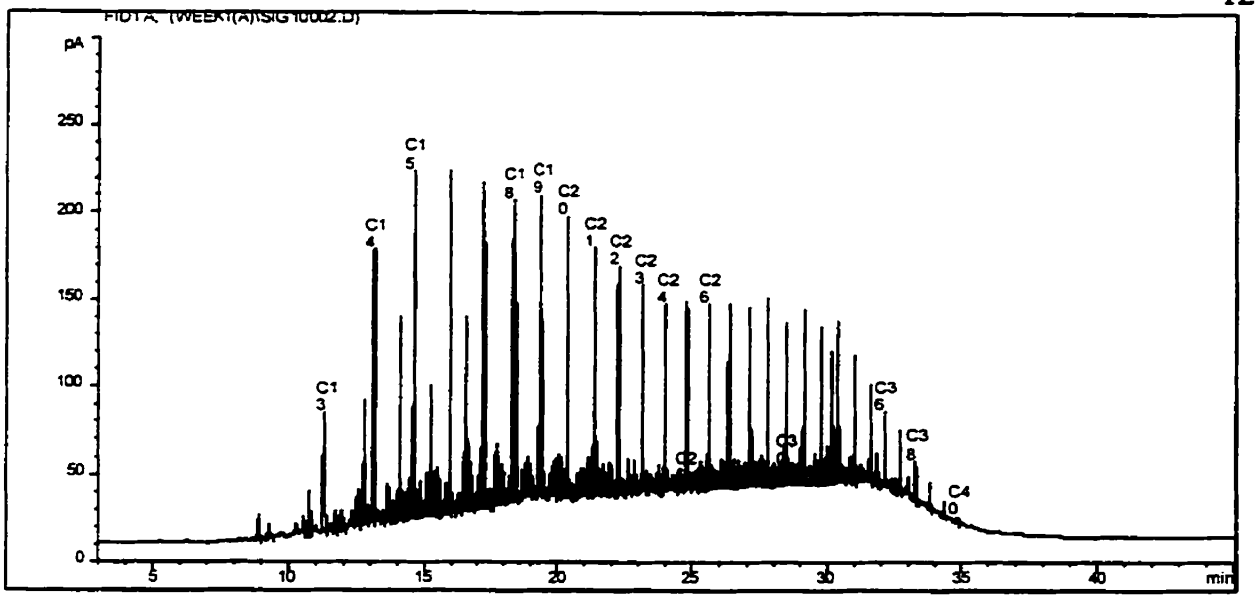

Figure B3) The GC fingerprint of the extracted oil from test jars 2 before biodegradation.

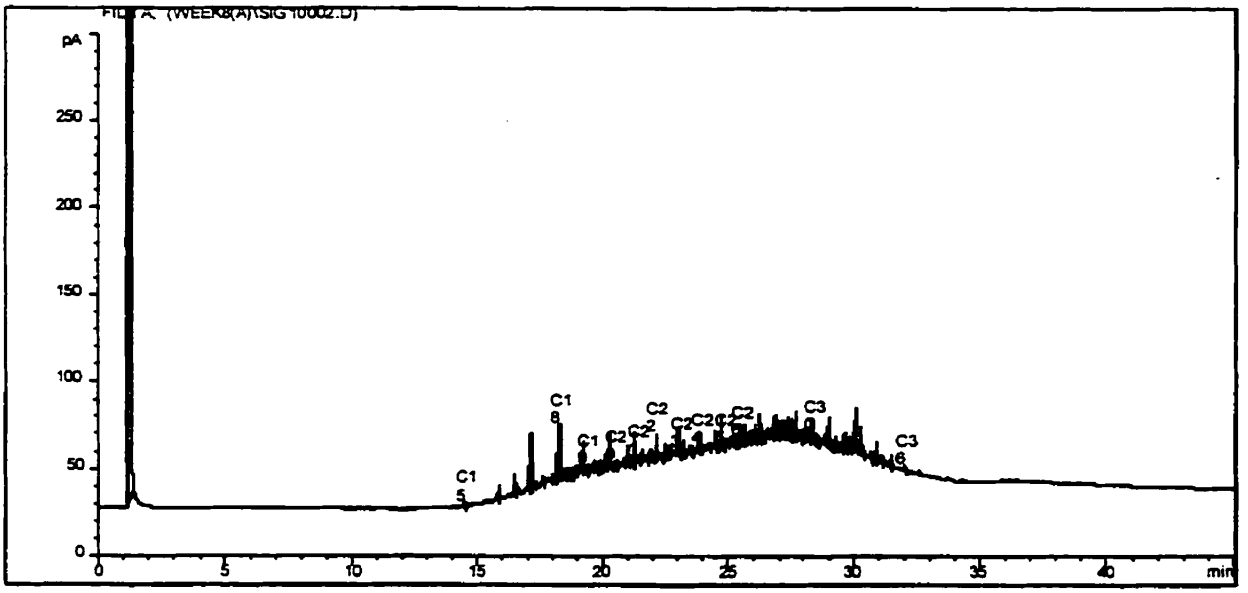

Figure B4) The GC fingerprint of the extracted oil from test jars 2 after biodegradation.

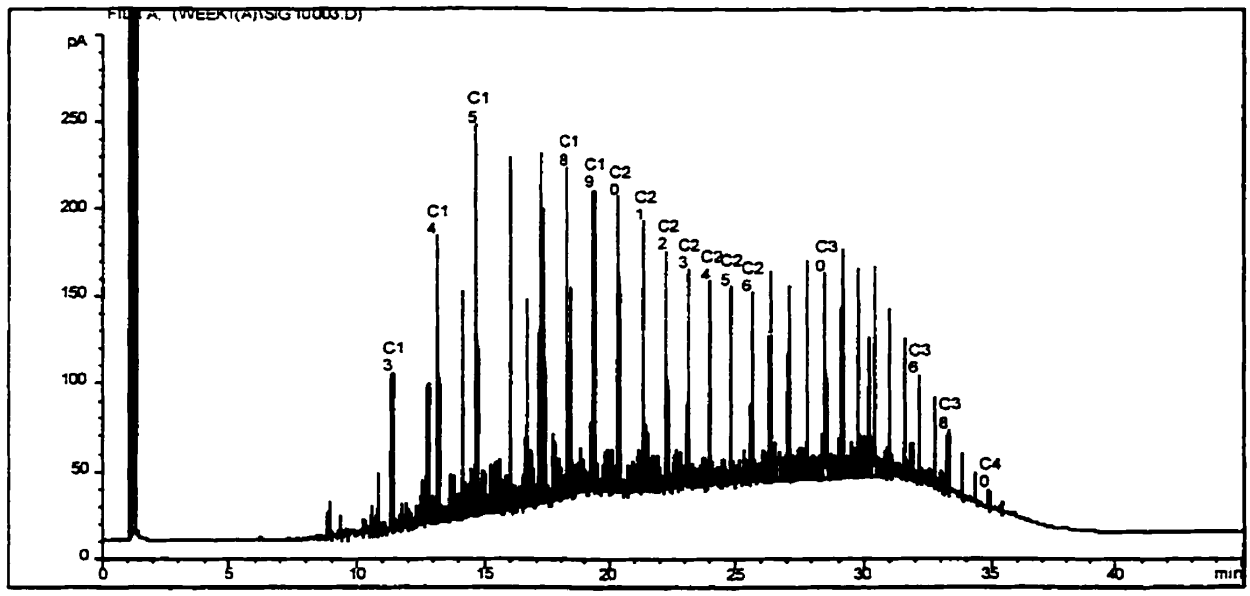

Figure B5) The GC fingerprint of the extracted oil from test jars 3 before biodegradation.

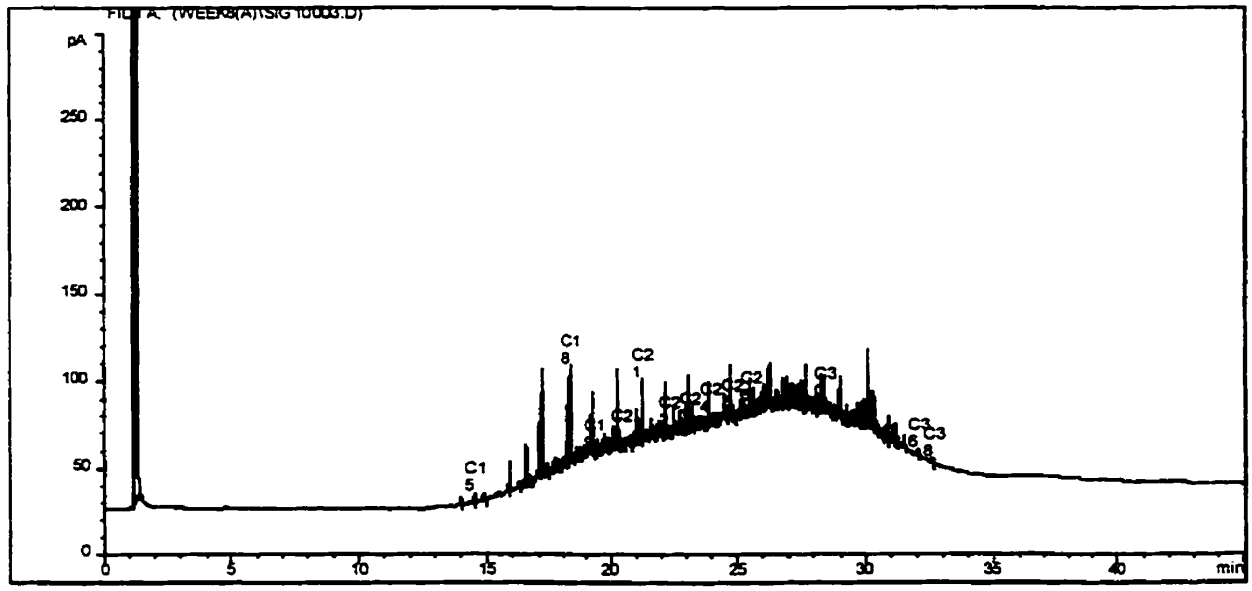

Figure B6) The GC fingerprint of the extracted oil from test jars 3 after biodegradation.

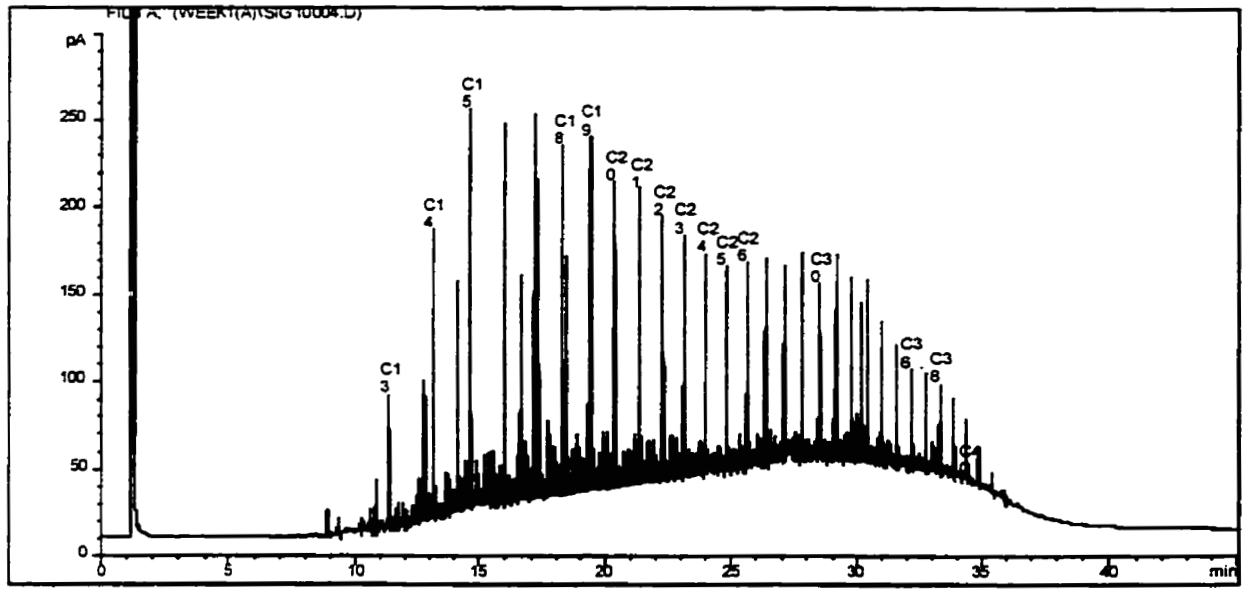

Figure B7) The GC fingerprint of the extracted oil from test jars 4 before biodegradation.

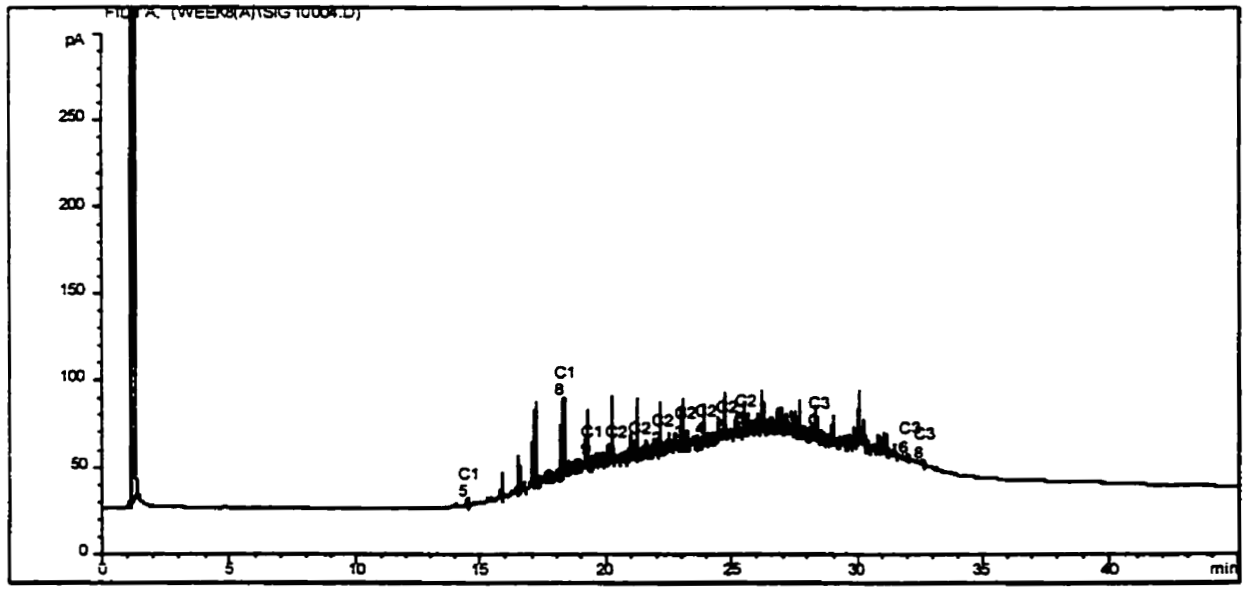

Figure B8) The GC fingerprint of the extracted oil from test jars 4 after biodegradation.

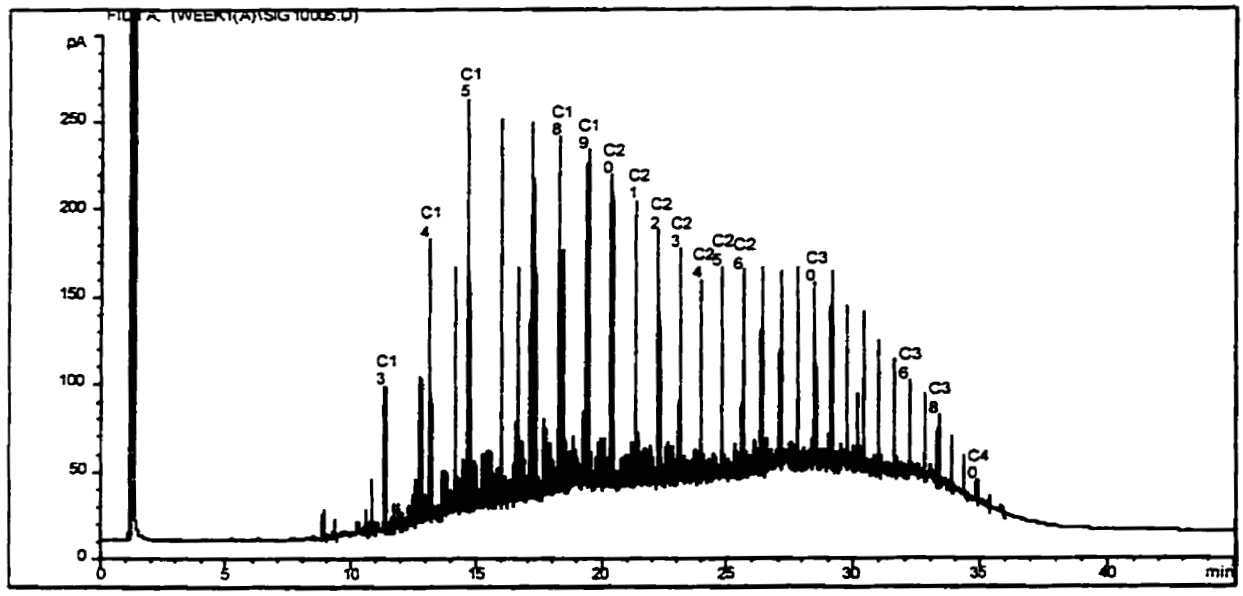

Figure B9) The GC fingerprint of the extracted oil from test jars 5 before biodegradation.

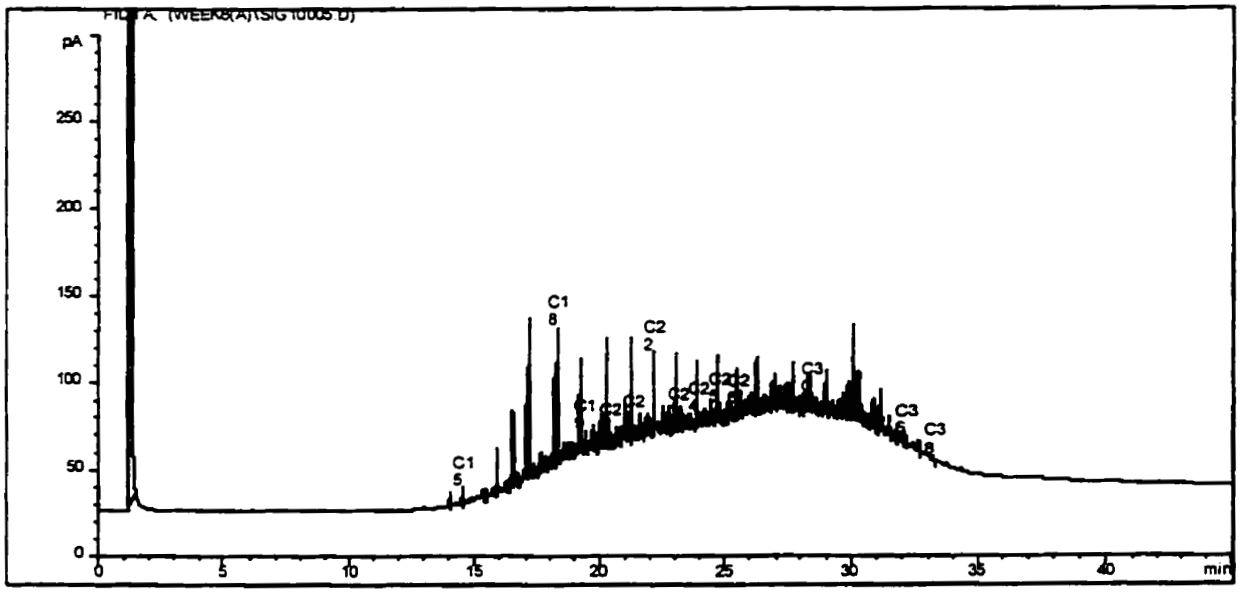

Figure B10) The GC fingerprint of the extracted oil from test jars 5 after biodegradation.

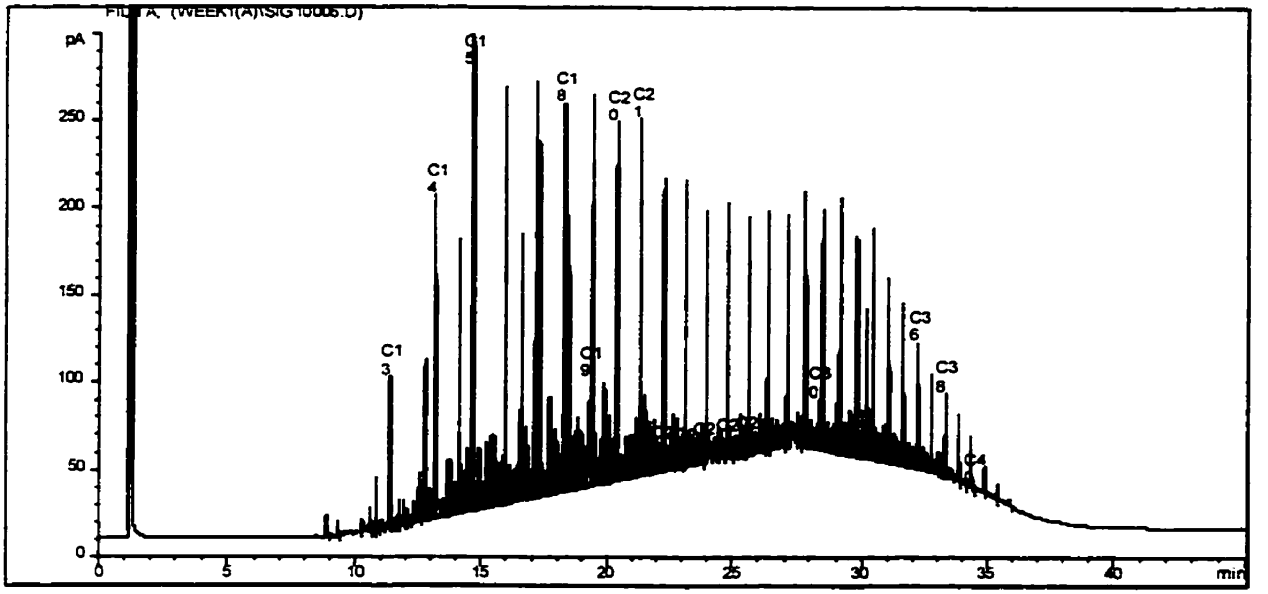

Figure B10) The GC fingerprint of the extracted oil from test jars 6 before biodegradation.

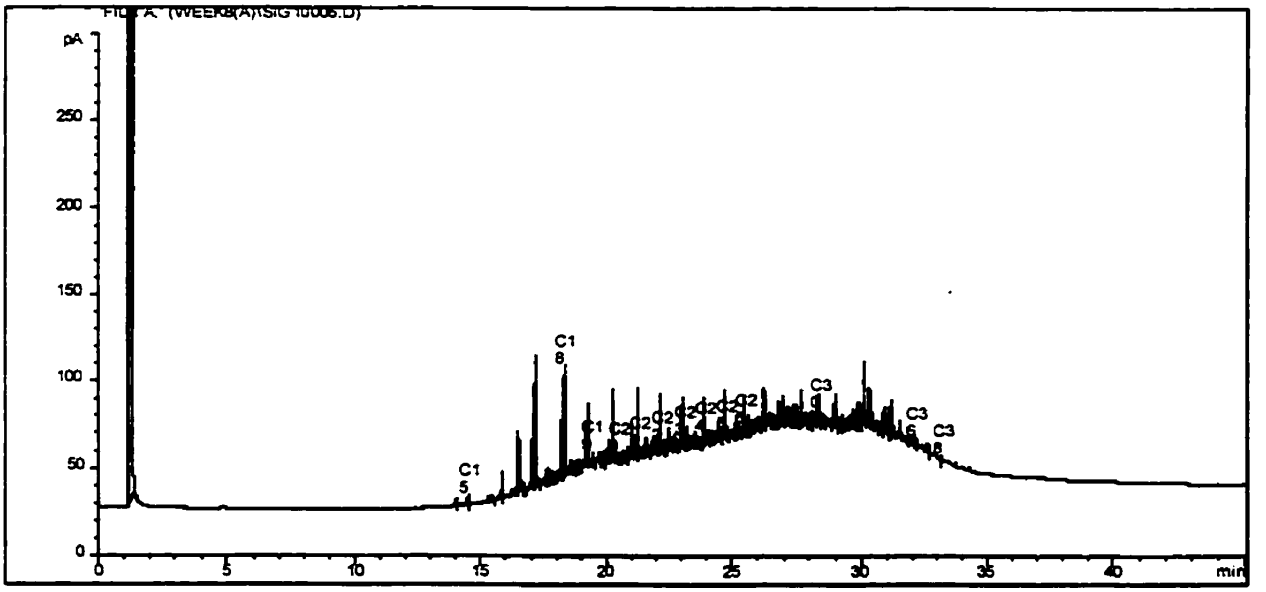

Figure B11) The GC fingerprint of the extracted oil from test jars 6 after biodegradation.

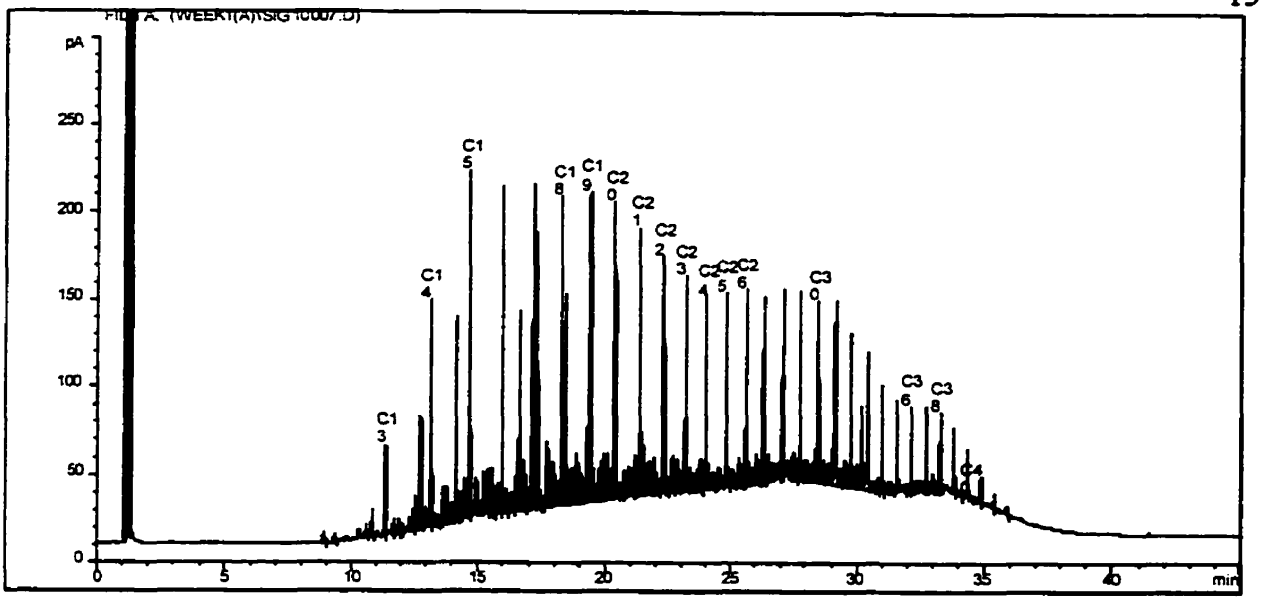

Figure B12) The GC fingerprint of the extracted oil from test jars 7 before biodegradation.

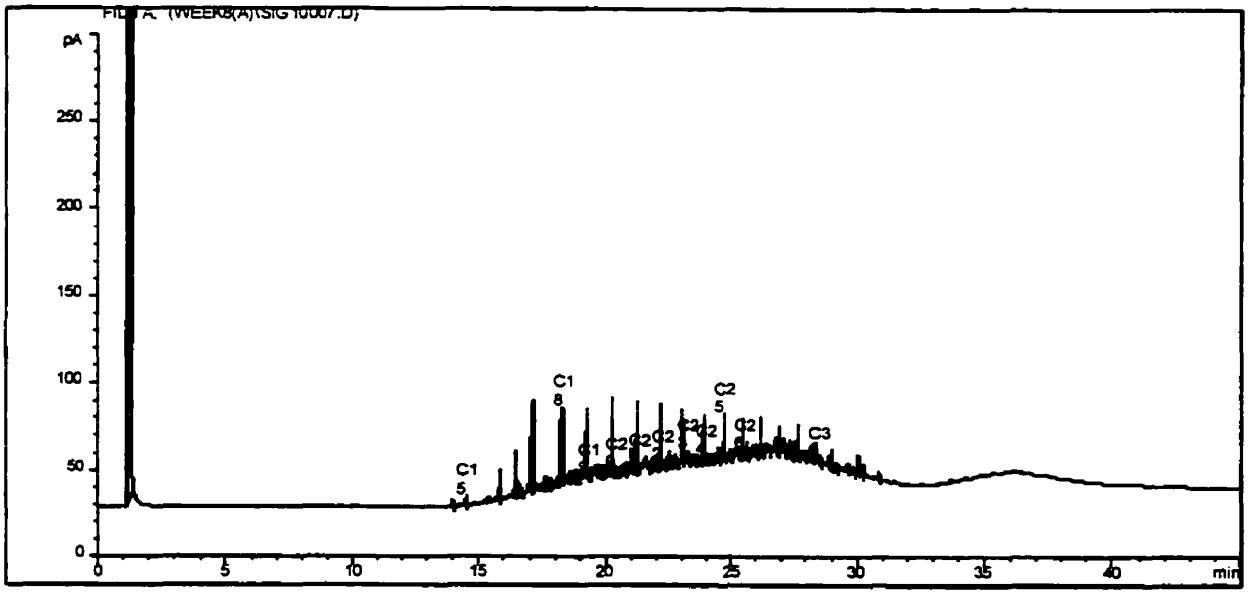

Figure B13) The GC fingerprint of the extracted oil from test jars 7 after biodegradation.

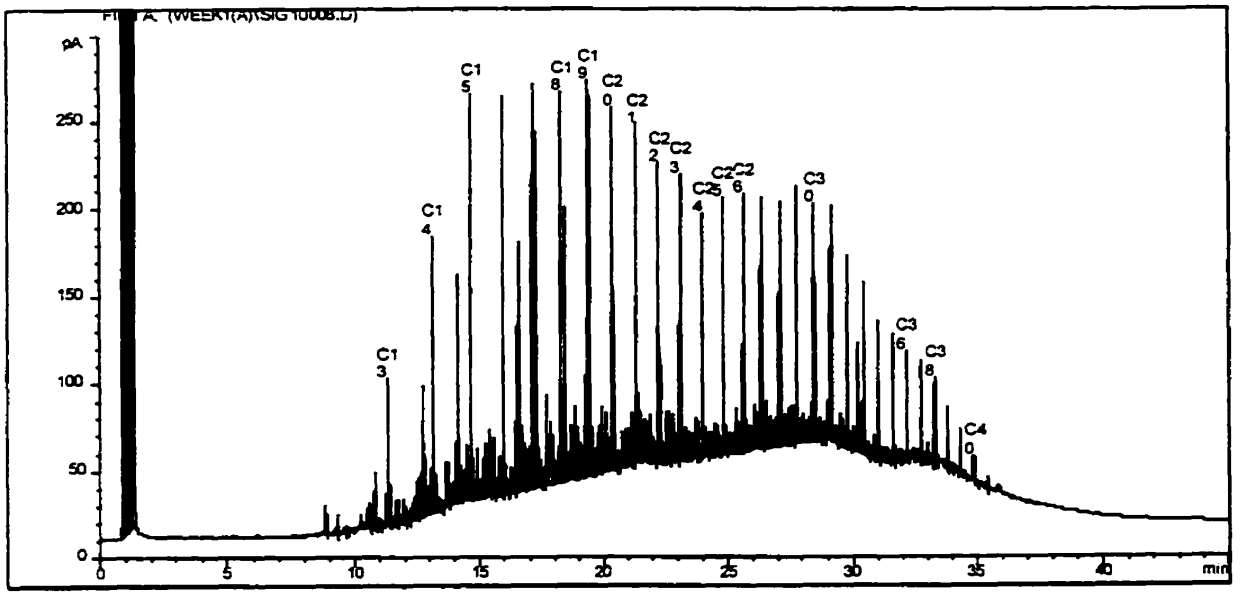

Figure B14) The GC fingerprint of the extracted oil from test jars 8 before biodegradation.

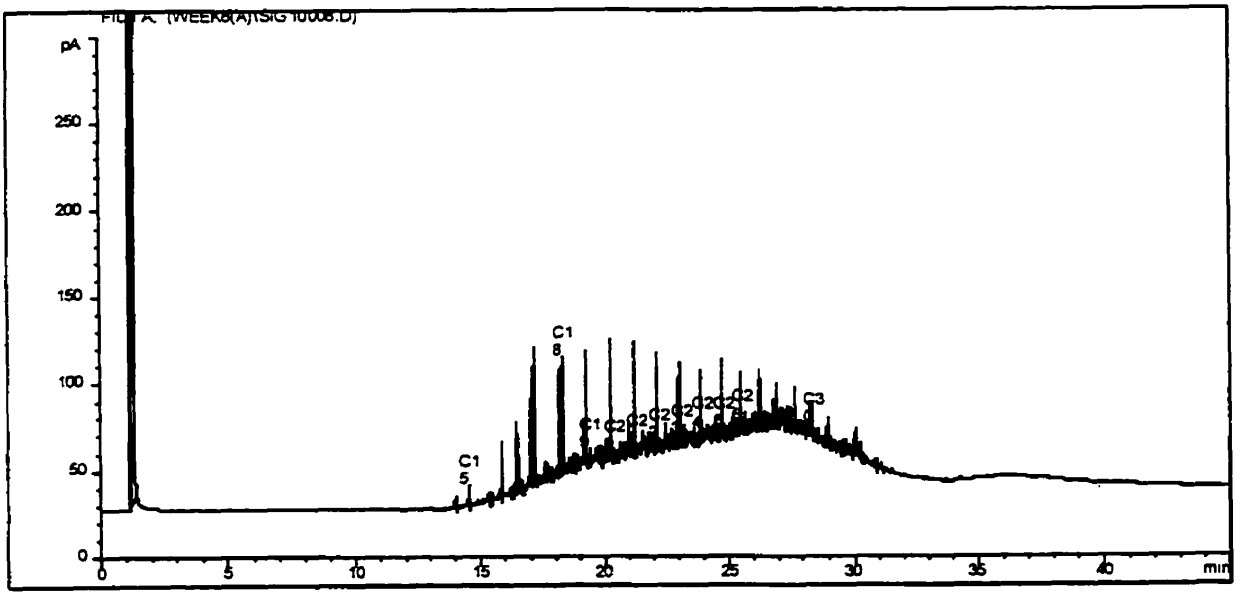

Figure B15) The GC fingerprint of the extracted oil from test jars 8after biodegradation.

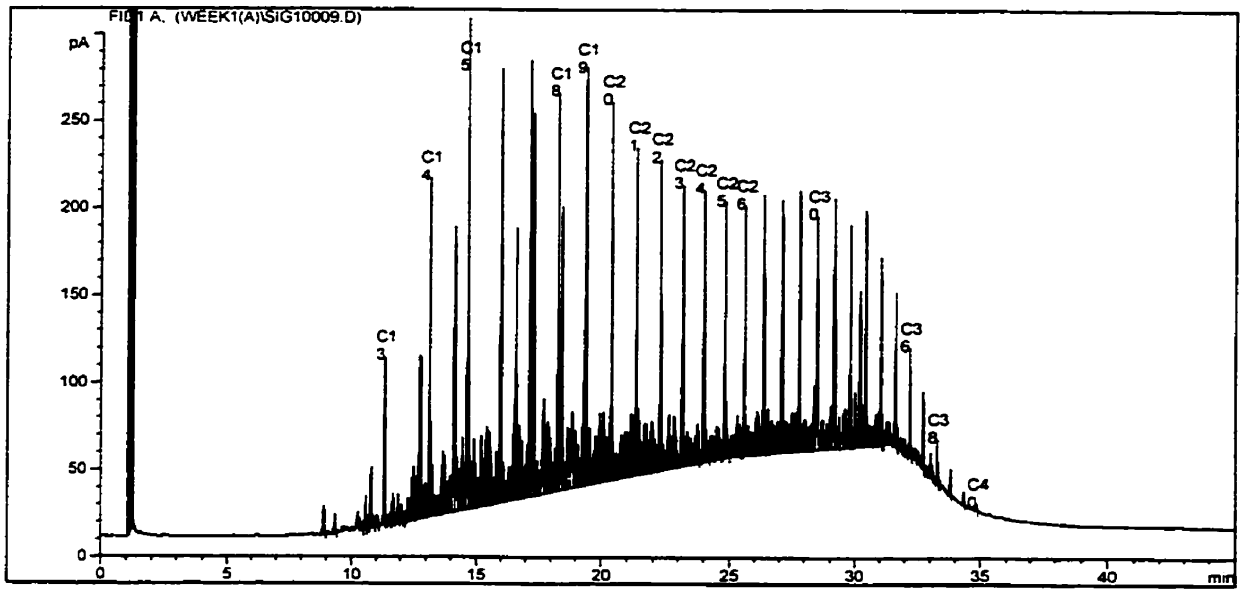

Figure B16) The GC fingerprint of the extracted oil from test jars 9 before biodegradation.

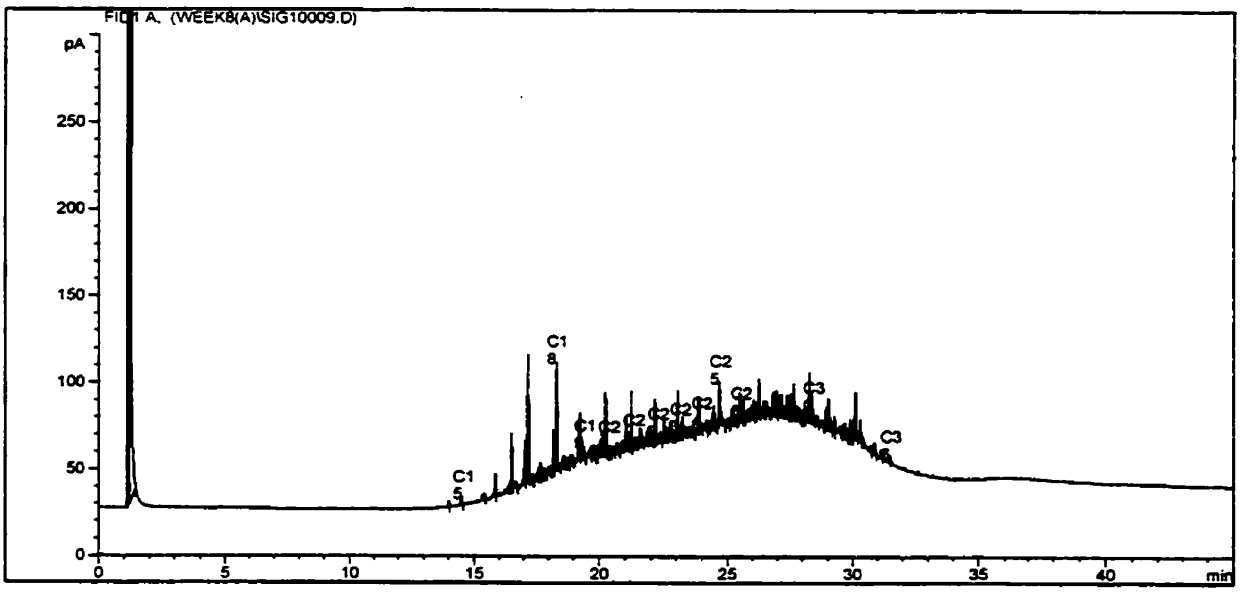

Figure B17) The GC fingerprint of the extracted oil from test jars 9 after biodegradation.

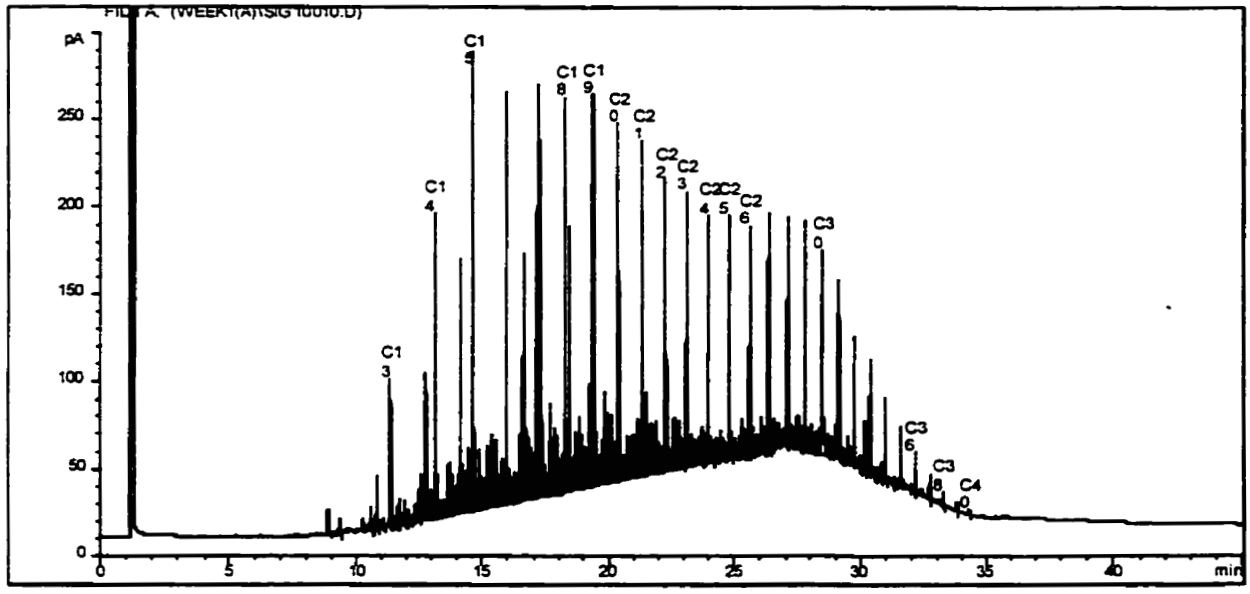

Figure B18) The GC fingerprint of the extracted oil from test jars 10 before biodegradation.

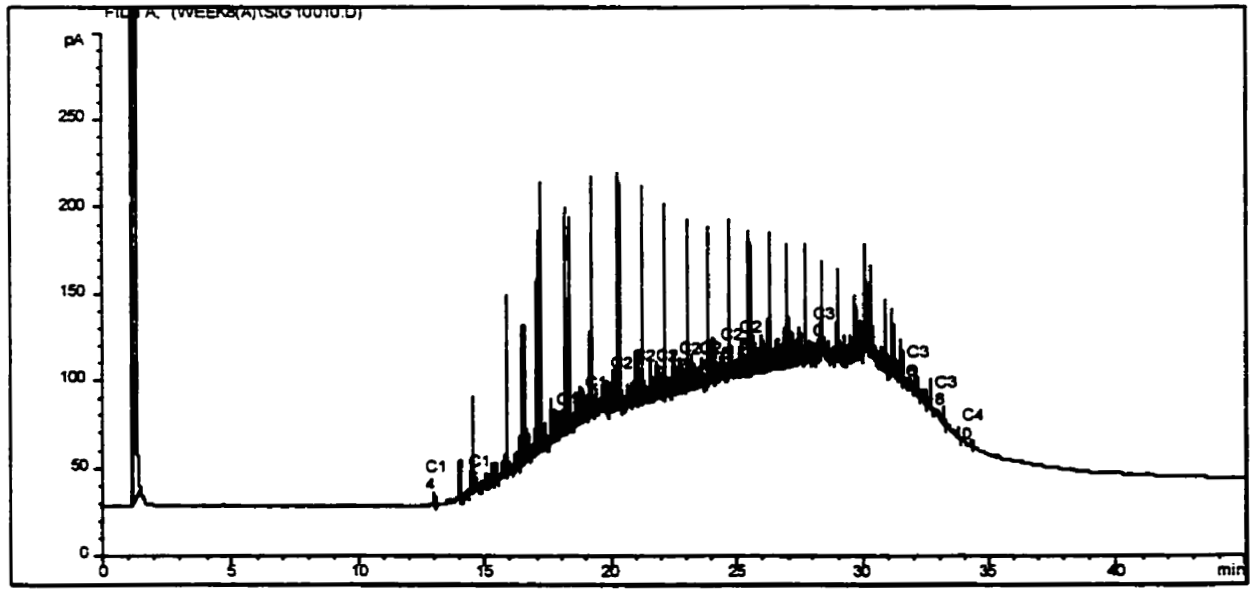

Figure B19) The GC fingerprint of the extracted oil from test jars 10 after biodegradation.

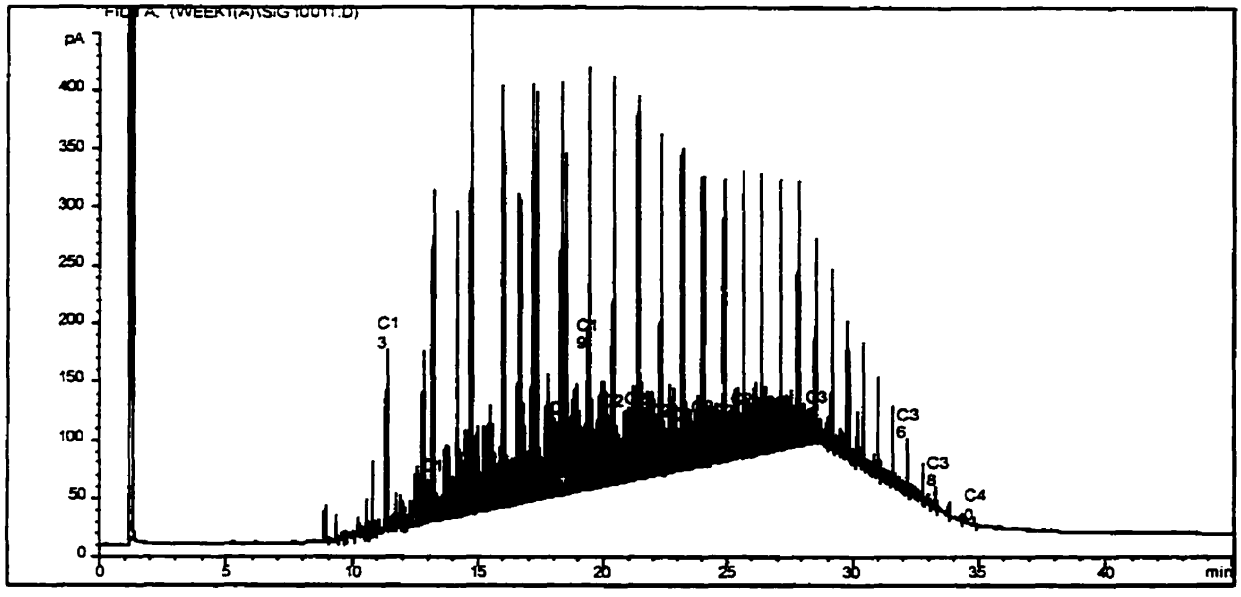

Figure B20) The GC fingerprint of the extracted oil from test jars 11 before biodegradation.

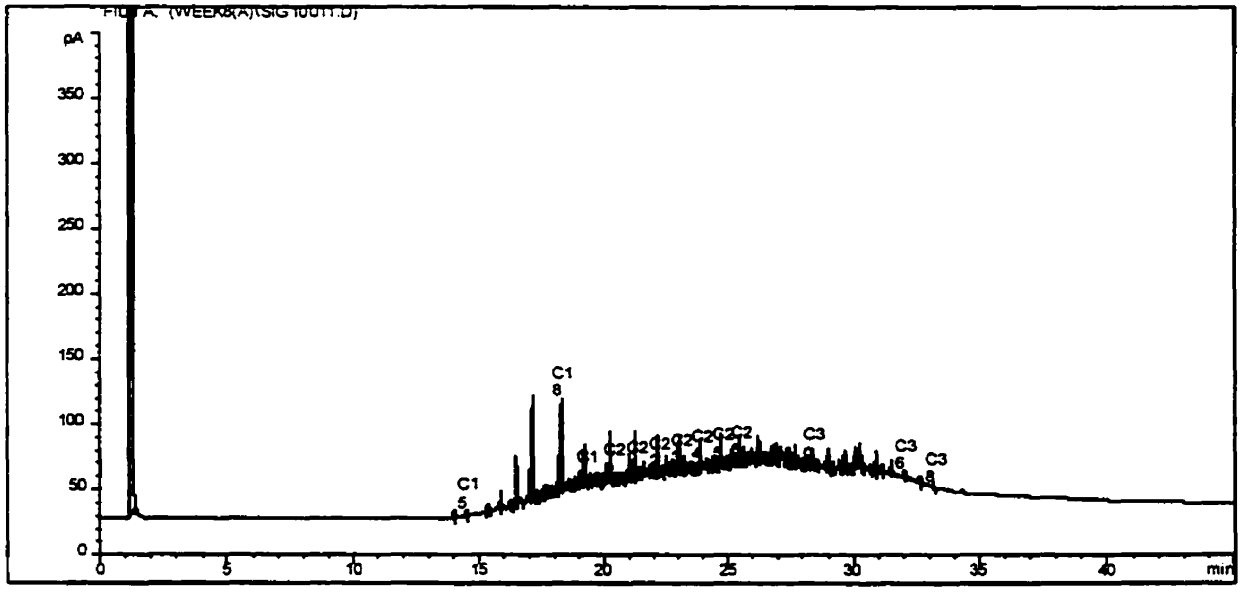

Figure B21) The GC fingerprint of the extracted oil from test jars 11 after biodegradation.

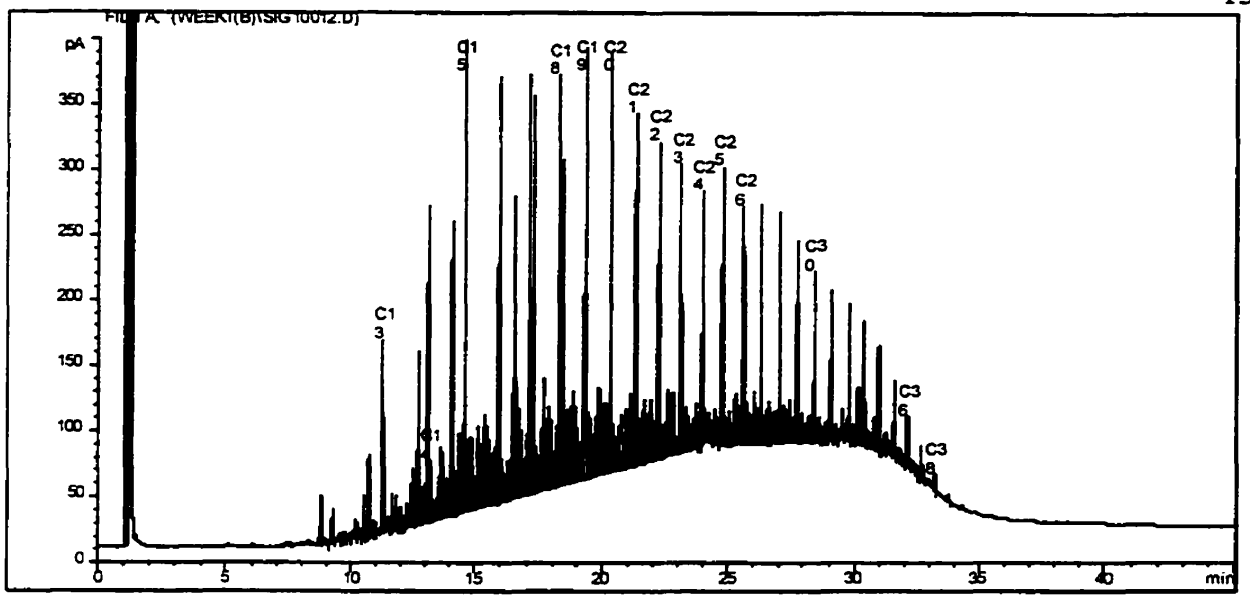

Figure B22) The GC fingerprint of the extracted oil from test jars 12 before experiments.

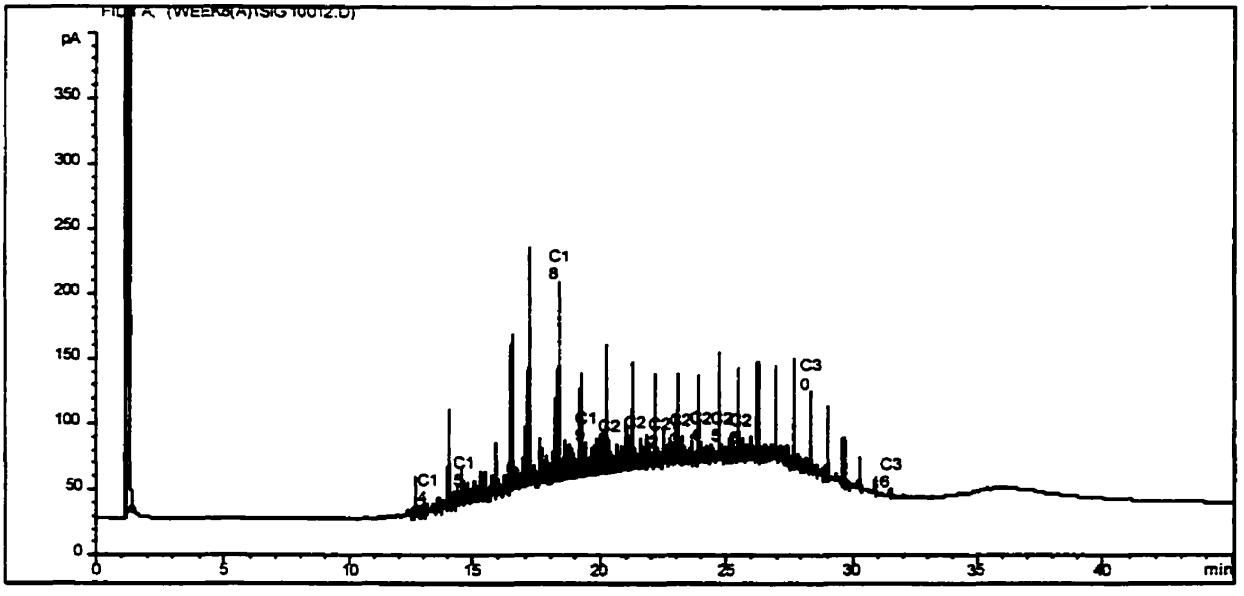

Figure B22) The GC fingerprint of the extracted oil from test jars 12 after experiments.

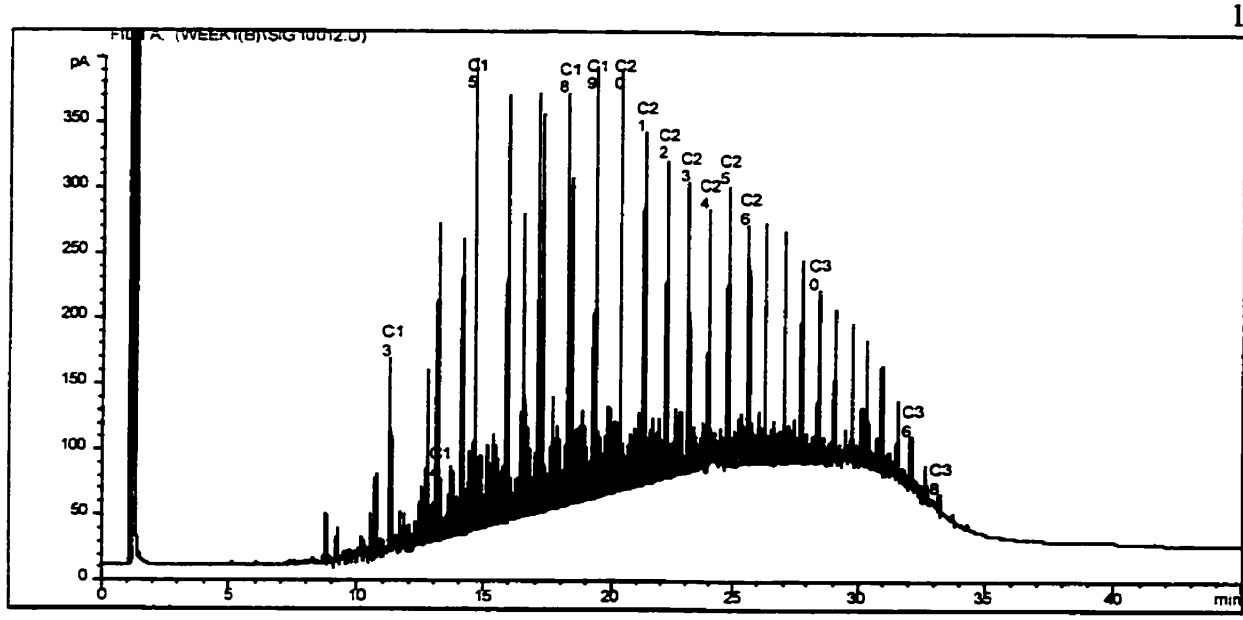

Figure B23) The GC fingerprint of the extracted oil from test jars 13 before the experiments.

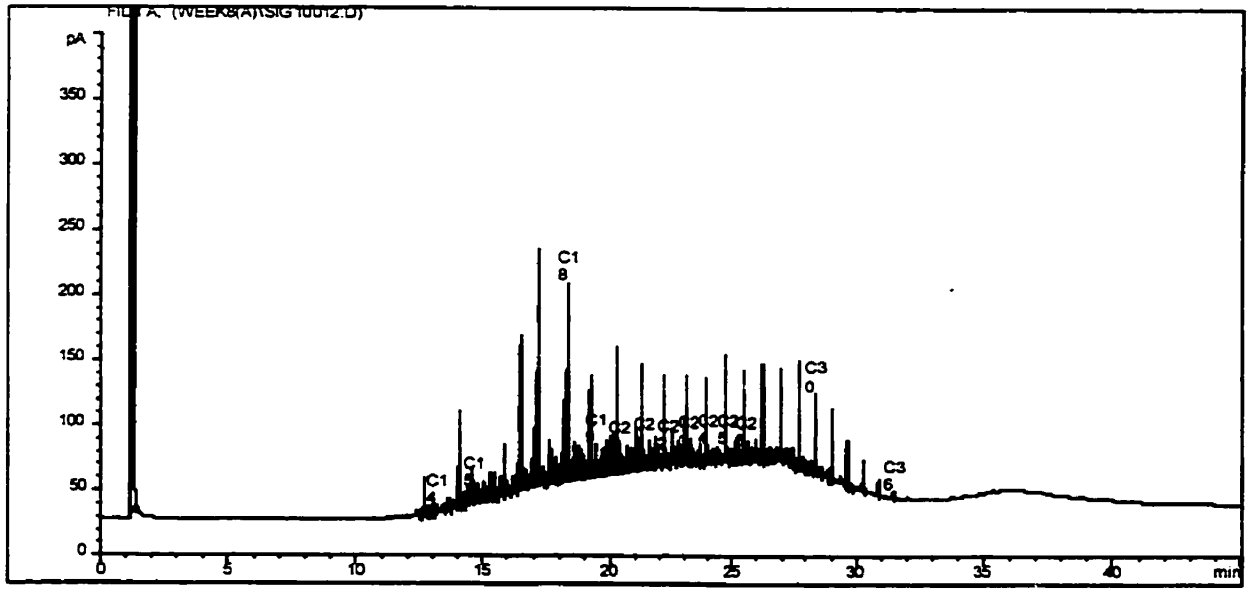

Figure B24) The GC fingerprint of the extracted oil from test jars 13 after the experiments.

Appendix C: Statistical Analysis

APPENDIX C.1) EXPERIMENTAL DESIGN WITH MIXED FUNGI APPLICATION AS THE THIRD LEVEL OF FUNGAL EFFECT. 142

APPENDIX C.2) AVERAGE EFFECT AND VARIANCES IN THE EXPERIMENTAL CALCULATIONS BASED ON THE BOX HUNTER METHOD FOR 28 DAYS OF EXPERIMENTS. 143

APPENDIX C.3) AVERAGE EFFECT AND VARIANCES IN THE EXPERIMENTAL CALCULATIONS BASED ON THE BOX HUNTER METHOD FOR 100 DAYS OF EXPERIMENTS 143

Appendix C.1) Experimental design with mixed fungi application as the third level of fungal effect.

Test no.	Jar Description	Soil	A	B	C
1	Test (I): B.adusta	X	1	1	1
2	Duplicate of 1	X	1	1	1
3	Test (I): Mixed fungi	X	2	1	1
4	No fungus (I)	X	0	1	1
5	Test (II): B.adusta	X	1	0	1
6	Duplicate of 5	X	1	0	1
7	Test (II): mixed fungi	X	2	0	1
8	No fungus (II)	X	0	0	1
9	Test (III): B.adusta	X	1	1	0
10	Test (III): B.adusta	X	1	0	0
11	Control	X	0	*	*

* No treatment applied

Variables	0	1	2
"A": Fungi	No Fungus	B. adusta	Mixture of Fungus
"B": Moisture Content %(w/w of dry soil)	30	50	NA
"C": Bulking Agent %(w/w of dry soil)	6	12	NA

Appendix C.2) Average effect and variances in the experimental calculations based on the Box Hunter method for 28 days of experiments.

	A	B	C	AB	AC	BC	Average Effect	Variance
1	+	+	+	+	+	+	46.6	1.65
2	-	+	+	-	-	+	46.6	0
3	+	-	+	-	+	-	45.0	4.7
4	-	-	+	+	-	-	47.7	0
5	+	+	-	+	-	-	40.5	0
6	+	-	-	-	-	+	38.2	0

Appendix C.3) Average effect and variances in the experimental calculations based on the Box Hunter method for 100 days of experiments

	A	B	C	AB	AC	BC	Average Effect	Variance
1	+	+	+	+	+	+	31.2	30.0
2	-	+	+	-	-	+	31.6	0
3	+	-	+	-	+	-	29.5	1.8
4	-	-	+	+	-	-	30.1	0
5	+	+	-	+	-	-	18.3	0
6	+	-	-	-	-	+	25.9	0