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BIOREMEDIATION OF PETROLEUM HYDROCARBONS CONTAMINATED WASTEWATER FOR BAIJI THERMAL POWER STATION IN IRAQ

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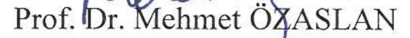

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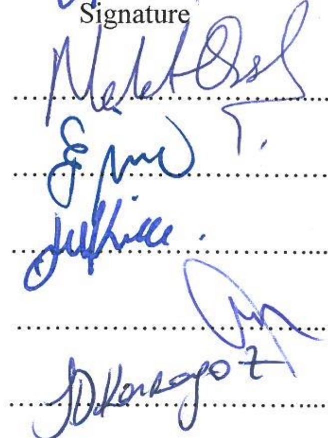

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Nawal AL-AAZI

" To My Family"

ABSTRACT

BIOREMEDIATION OF PETROLEUM HYDROCARBONS CONTAMINATED WASTEWATER FOR BAIJI THERMAL POWER STATION IN IRAQ

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M.Sc. in Biology Department
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3 selected yeasts - *Yarrowia lipolytica* NCAIM Y00591, *Candida albicans* ATCC 10231 and *Candida parapsilosis* ATCC 22019 and 2 bacteria isolated from Baiji thermal power plant wastewaters - *Stenotrophomonas maltophilia* and *Acromobacter sp.*, were used in order to investigate the microorganisms' feasibility for petroleum hydrocarbons biodegradation that contaminated wastewater for Baiji Thermal Power Station. The medium is prepared by adding 1 g of petroleum oil and inoculating 4 Mc Farland density of yeast separately while the wastewater is directly used for bacteria. The culture medium was incubated at 30 ºC with shaking speed of 150 rpm/min for two weeks; the bacteria and the yeast were counted once every two days for monitoring and recording the growth of the microorganisms. The experiment was terminated at the end of the second week and the samples were prepared for Gas chromatography analysis. The degraded hydrocarbon compounds were identified by GC-MS (Gas chromatography and mass spectrometry) analysis. The results showed that 80.95 % of the hydrocarbon compounds degraded by mixed indigenous bacteria, 66.6 % by Y. *lipolytica*, 47.6 % by C. *parapsilosis* and 38.1 % by *C. albicans*. This indicates that the bacteria have a better ability to use hydrocarbons than yeasts, and *Y. lipolytica* is the best among the three yeasts. So the removal of wastewater pollutants for Baiji power plant is possible by creating favorable conditions for the bacteria to carry out the biodegradation.

Keywords: Biodegradation, *C. albicans, C. parapsilosis*, Wastewater, *Y. lipolytica.*

ÖZET

İRAK BAİJİ TERMAL ENERJİ İSTASYONUNA AİT ATIK SUDAKI PETROL HIDROKARBON KİRLETİCİLERİNİN BİYOREMİDİASYONU

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Irak’taki Baiji termal santralinden alınan atık sulardaki petrol hidrokarbonlarının biodegradasyonu mikroorganizmalarla yapılabilirliği araştırılmıştır. Bu amaçla 3 maya ve ili bakteri kullanılmıştır. Mayalar ; (*Yarrowia lipolytica* NCAIM Y00591, *Candida albicans* ATCC 10231 ve *Candida parapsilosis* ATCC 22019), bakteriler ise Baji termal santral atık suyunda izole edilmiştir (*Stenotrophomonas maltophilia* ve *Acromobacter sp.*)

Hazırlanan ortamlara 1 g petrol hidrokarbonları ilave edilerek ayrı ayrı 3 maya 4 Mc farland yoğunluğunda inoküle edilmiştir. Bakteriler için doğrudan atık su kullanılmıştır. 30^0C’de 150 rpm/dk iki hafta inkübasyona bırakılmıştır. Gün aşırı maya ve bakteri sayımları yapılarak canlılıkları kontrol edilmiş olup kayıt altına alınmıştır. iki hafta sonunda deney sonlandırılarak örnekler GC analizi için hazırlanmıştır. Parçalanan hidrokarbon bileşikleri GC-MS (Gaz kromotografisi ve kütle spektrometresi) analizleri ile tanımlanmıştır. Yapılan çalışma sonucunda 80.95 % bakteriler, 66.6 % *Y. lipolytica*, 47.6 % *C. parapsilosis* ve 38.1 % *C. albicans* tarafından hidrokarbon parçalandığını göstermiştir. Bu sonuçlar, hidrokarbonların parçalanmasında bakterilerin mayalardan daha etkili olduğunu ve Y.lipolytica maya türünün diğer üç maya türleri arasında en iyi parçalayıcı olduğunu göstermiştir. Böylece bakterilerin biyodegredasyon yapması için uygun koşullar oluşturularak, Baiji elektrik santrali için atık su kirleticilerinin ortadan kaldırılması mümkündür.

Anahtar Kelimeler: Biyodegradasyon, *C. albicans*, *C. parapsilosis*, Atık su, *Y.lipolytica.*

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TABLE OF CONTENTS

ABSTRACT .... **IV**

ÖZET .... **V**

ACKNOWLEDEMENTS .... **VIII**

TABLE OF CONTENTS .... **IX**

LIST OF TABLES .... **XI**

LIST OF FIGURES .... **XII**

LIST OF SYMBOLS .... **XIV**

1. Introduction **1**

1.1. Chemical composition of the petroleum 5

1.2. Bioremediation and their applications 7

1.3. Petroleum hydrocarbons degrading microorganisms........................ 9

1.4. Biodegradation pathway for petroleum hydrocarbons 10

1.5. Factors affecting bioremediation 16

1.5.1 Microbial factors.. 16

1.5.2. Oxygen factors 17

1.5.3.Temperature factors 18

1.5.4.Nutrients factors 19

1.5.5. pH 19

1.6. Hydrocarbons effect of health 20

2. Literature abstructs **23**

3. Material and Method **25**

3.1. Equipments and Apparatus 25

3.2. Chemicals and biological materials.. 25

3.3. Prepared culture media 26

3.4. Sterilization methods 26

3.5. Wastewater samples collection 27

3.6. Petroleum oil extraction 27
3.7. Gas chromatographic analysis of crude oil 27
3.8. Isolation of bacteria and fungi from wastewater sample 28
3.9. Identification of bacterial isolates 28
3.10. Yeast growth medium preparation by using wastewater............................ 29
3.11. Culturing the wastewater prepared medium by yeast 29
3.12. Bacteria growth medium preparation 30
3.13. Growth monitoring 31
3.14. Extracting the remaining crude oil and determining their components 33
4. Results 35
4.1. Isolation and identification of wastewater samples bacteria 35
4.2. Detremination of petroleum oil components 35
4.3. Effect of indigenous bacteria on petroleum hydrocarbons 38
4.4. Effect of *Y. lipolytica* on petroleum hydrocarbons 39
4.5. Effect of *C. albicans* on petroleum hydrocarbons 41
4.6. Effect of *C. parapsilosis* on petroleum hydrocarbons 42
4.7. Test Results for the growth of bacteria and yeasts 44
5. Discussion and Conclusion... 45
6. Reference 49

LIST OF TABLES

Table 1.1. Examples of toxicity of hydrocarbons found in diesel 22

Table 2. Summary of biodegradation studies 23

Table 3.1. Equipments and apparatus were used throughout the study 25

Table 3.2. Chemicals and biological materials used throughout the study............. 25

Table 4.1. Colony characteristics of the isolated strain *Acromobacter sp.*
and *S. Maltophilia* 35

Table 4.2. Crude oil hydrocarbon compounds... 36

Table 4.3. Results of growth test of the yeasts and mixed bacteria 44

LIST OF FIGURES

Figure 1.1. Molecular structures of hydrocarbons 7

Figure 1.2. Metabolic path way of *n*-alkanes 12

Figure 1.3. Biodegradation of aromatic hydrocarbons 13

Figure 1.4. Proposed pathway for anaerobic degradation of *n*-alkanes................ 15

Figure 1.5. Proposed pathways of anaerobic degradation of aromatic Hydrocarbons.. 16

Figure 3.1. Oil extraction by separating funnel 27

Figure 3.2. *S.maltophilia* colony shape 31

Figure 3.3. *Acromobacter sp.* colony shape 32

Figure 3.4. *C. albicans* ATCC 10231 colony shape 32

Figure 3.5. *Y. lipolytica* NCAIM Y00591 colony shape 32

Figure 3.6. *C. parapislosis* ATCC 22019 colony shape 33

Figure 3.7. Microorganism's culture before biodegradation processe.................... 34

Figure 3.8. Microorganism's culture after biodegradation processe 34

Figure 4.1. Gas Chromatography (GC) tracing of crude oil compounds 37

Figure 4.2. Hydrocarbon compounds in crude oil sample extracted from wastewater 37

Figure 4.3. Gas Chromatography (GC) tracing of hydrocarbon compounds degrading by indigenous bacteria 38

Figure 4.4. Disappearance and reduction of hydrocarbon compounds through degradation by indigenous bacteria 39

Figure 4.5. Gas Chromatography (GC) tracing of hydrocarbon compounds degrading by *y.lipolytica* 40

Figure 4.6. Disappearance, and emergence of a difference in the percentage of hydrocarbon compounds through degradation by *Y.lipolytic* 40

Figure 4.7. Gas Chromatography (GC) tracing of hydrocarbon compounds degrading by *C. albicans*.. 41

Figure 4.8. Disappearance, and emergence of a difference in the percentage of hydrocarbon compounds through degradation by *C.albicans* 42

Figure 4.9. Gas Chromatography (GC) tracing of hydrocarbon compounds degrading by *C. parapsilosis*.. 43

Figure 4.10. Disappearance, and emergence of a difference in the percentage of hydrocarbon compounds through degradation by *C.parapsilosis* 43

Figure 4.11. The percentage of hydrocarbon compounds consumed by the microorganisms 44

LIST OF SYMBOLS/ABBREVIATIONS

TCA	Tricarboxylic Acid Cycle
BAB	Blood Agar Base
CFU	Colony Forming Units
DW	Distilled water
GC-MS	Gas Chromatography- Mass Spectrometry
MHA	Mueller-Hinton Agar
PAH	Polyaromatic Hydrocarbon
SDA	Sabouraud Dextrose Agar
UV	Ultraviolet ray

CHAPTER 1
INTRODUCTION

Oily wastewater is known as one of the mostly concerned pollution sources. Oil contaminated wastewater is the product of a wide variety of sources such as: crude oil production, petrochemical industry, metal processing and power plants. These sources are considered one of the most problematic environmental pollutants, especially in water and soil (Bujang et al., 2013).

Oily contaminated wastewater coming from industrial zones is considered one of the most hazardous causing great harm to the environment. These wastewaters contain toxic substances such as petroleum hydrocarbons where the polyaromatic hydrocarbons are considered to be mutagenic and carcinogenic to humans and to other living organisms; the inhibitory effect on the animal and plant growth is also expected (Bujang et al., 2013).

Oil pollution has steadily increased through the exploration, refining, transportation processes. The other pollution sources such as formalin waters, industrial effluents, urban run offs, cleaning residues, automobiles etc., crude oil also have a dangerous effect when it comes out through breakage of oil pipelines and leaks of petroleum plants or underground storage tanks (Eze and Eze, 2010).

Because of the increasing harmful effects of industrial wastewaters contaminated with petroleum hydrocarbons, many new techniques have continued to emerge for remediation of contaminated sites. One of the mainly used techniques is bioremediation. Bioremediation is a technology that uses microorganisms to treat the contaminated areas. This technology is used to treat the oily wastewater in order to reduce the toxicity of hydrocarbons into less toxic forms. Remediation of contaminated sites are made either by the indigenous microorganisms in contaminated site or by seeding the contaminated area with specific organisms isolated from other sites (Bujang et al., 2013). The enhancement of indigenous microorganisms or the addition of specific microorganisms (bacteria, cyanobacteria, algae, fungi, protozoa) can improve biodegradation efficiency in both *in-situ* and/or

ex-situ procedures (Plohl et al., 2002).
There are many methods for hydrocarbon-polluted remediation. The mechanical and chemical methods are among these methods but they are often expensive, technologically complex, and lack public acceptance. Thus, the bioremediation is the best method for effective removal of hydrocarbon pollutants from a variety of ecosystems (Speight and Arjoon, 2012).

Microorganisms reduce the toxicity of contaminants by transforming them into harmless products through metabolic or enzymatic processes. The products often are: carbon dioxide, water, and cell biomass. So, bioremediation technology is considered an alternative way to detoxify petroleum contaminants from the environment (Speight and Arjoon, 2012).

The microorganisms in the aquatic environment play an important role in hydrocarbons degradation. Several investigations have shown that the petroleum hydrocarbons biodegredation occurs according to the following priority scale: aliphatics>aromatics>polars>asphaltenes. In the biodegradation process the hydrocarbons are completely mineralized to carbon dioxide and water, with some biomass production. But this does not always occur because of the difference of effective factors of biodegradation. Biodegradation efficiency depends on the microorganism's ability to produce the degrading specific enzymes for target compound, in presence of important factors such as temperature, pH, and nutrient status. Oxygen is the rate-limiting factor in aerobic degradation of hydrocarbons in groundwater (Plohl et al., 2002).

This work was conducted on wastewaters contaminated with petroleum hydrocarbons for Baiji thermal power station in order to evaluate the effectiveness of indigenous bacteria isolated from the wastewater, comparative with a three selected degrading yeasts (*Yarrowia lipolytica* NCAIM Y00591*, Candida albicans* ATCC 10231 and *Candida parapsilosis* ATCC 22019) for cleaning up the contaminated wastewater in this site's.
Y.lipolytica dimorphism fungi, usually as single oval cells or as a filament, controls cell shape by environmental factors (Kawasse et al., 2003). The recommended temperature for growth is 25 – 30 °C and most strains prefer low pH levels well

(down to pH 3), so their growth is reduced above pH 7 and stops above pH 8. It is an obligate aerobe that cannot ferment in the absence of oxygen. Low O_2 reduces the growth rate strongly (Roth, 2008). *Y. lipolytica* is unique yeast that has the ability to degrade efficiently hydrophobic substrates such as *n*-alkanes, fatty acids, fats and oils through specific metabolic pathways. *Y. lipolytica* is considered to be nonpathogenic to humans (Coelho et al., 2010).

C. albicans dimorphism fungi generally grow at 37 °C. *C. albicans* Colonies (SDA) look white to cream-colored smooth, glabrous and yeast-like. *C. albicans* is a human pathogen (Ellis et al., 2007).

The genus Candida showed the ability to assimilate the hydrocarbons. *C.albicans* and *C. parapsilosis*, members of the genus Candida exhibited the ability to utilize *n*-alkanes of 9 to 18 carbon atoms and even-numbered 1-alkenes of 10 to 18 carbon atoms (Klug and Markovetz, 1967).

C. parapsilosis dimorphism fungi generally grow at 37 °C. Colonies (SDA) appear white to cream-coloured smooth, glabrous and yeast-like. Microscopy shows them as predominantly small, globose to ovoid budding blastoconidia, 2.0-3.5 x 3.0-4.5 µm, with some larger elongated forms present (Ellis et al., 2007). *C. parapsilosis* is known to be efficient in degradation of alkanes and alkenes and polycyclic aromatic hydrocarbons .The yeast is able to degrade oil both in seawater and freshwater media at 30 °C optimum temperature. *C.parapsilosis* is nonpathogenic to humans (Zinjarde and Pant, 2002).

Two bacteria were isolated from hydrocarbon-contaminated wastewater and were identified as *Achromobacter sp.*, and *Stenotrophomonas maltophilia*.

The genus *Achromobacter* is gram-negative bacilli, oxidase-positive, and attacks carbohydrates aerobically. *Achromobacter* species are straight rods 0.8–1.2 µm and 2.5–3.0 µm with rounded ends. They are widely distributed in nature and are found in aqueous environments frequently, especially water and moist soil (Liu, 2011).

S. maltophilia is a gram-negative non-fermentative bacillus that forms rod-shaped cells of 0.5–1.5 µm in length, straight to slightly curve. They are motile and carry many of polar flagella. It grows obligately aerobic at temperatures 5 °C – 37 °C on a wide variety of media [nutrient agar, lysogeny broth, MacConkey] and forms white-to-yellow, smooth colonies. *S. maltophilia* is widely distributed in nature and it has

been isolated from distribution systems of drinking water and bottled water, wastewater environments and natural water sources. It also has been found in soils. *S. maltophilia* is pathogenic to humans (Liu, 2011).
The petroleum hydrocarbon wastewater sample was taken from the oil separator system. The oily wastewater treated in this system depends on the gravity separation which is one of the used technologies for oily polluted water treatment. This technology takes advantage of the natural insolubility of most oil hydrocarbons and of the natural separation of organic and aqueous layers based on the different densities. In this treatment method the oil globules rise to the surface due to density of the oil which is less than that of water. A complete separation of the layers can not always be ensured only by the different densities of immiscible oil and water, thus often a complementary treatment such as biodegradation is necessary to obtain a higher efficiency in oil removal (Unipede and Eurelectric, 1997). In the power generation stations the wastewater that collected in the oil separator system coming from the oily areas such as storage area of the fuel oil tanks, transformer area, boiler area due to accidental losses of the equipment (oil storage tank and pipeline, transformer, pumps, compressor, etc.) or to scouring rainwater and the ongoing cleaning work in the electricity power plants.

However, Petroleum products are used in power generation stations for the following purposes:
1 – Combustion: Used for heating the boiler water such as (crude, heavy and diesel fuel oil)
2- Lubricating: Is used for heat transfer and lubrication of machines.

Crude Oil: Crude oil is a liquid in its unrefined state; its main chemical components are carbon and hydrogen elements (Salleh et al., 2003). Crude oils consists of carbon in the range 83-87 %, hydrogen 10 - 14% and small amounts of nitrogen, oxygen, sulfur and metals (Ni and V). Crude oils are classified in four main chemical classes: saturates aromatics, resins and the asphaltenes (Speight and Arjoon, 2012).

Heavy Oil: Heavy fuel oil is a residue from refinery distillation and cracking processes of crude oil. They are viscous liquids and require heating for storage and combustion. Used widely in industrial plants, marine applications, and power stations

in combustion equipment such as boilers, furnaces, and diesel engines. heavy fuel oils consists of hydrocarbons with a wide range of molecular weights, and the carbon numbers ranging from C 7 to > C 50 and boiling points between 120 to 600 °C. Fuel oil members are complex substances containing variable amounts of alkanes, cycloalkanes, aromatics, olefins, asphaltene constituents, and heteroatom molecules containing sulfur, oxygen, nitrogen, and organo-metals (Speight and Arjoon, 2012).

Diesel Oil: Diesel oil hydrocarbons are derived from crude oil refining. Diesel fuels vary from colourless to brown, they contain aliphatic hydrocarbons and polycyclic aromatic hydrocarbons such as naphthalene, fluorene and phenanthrene. Aliphatic hydrocarbons represent the most part of the diesel oil while aromatic compounds represent 5-30 %. Diesel oil solubility in the water is about 5 mg/ L at 20 °C. Therefore, diesel oil is partly soluble in water and possibly accumulative in tissues (Kauppi, 2011).

Lubricating oil: Lubricants used in the power generating industry can be divided into two categories. First is the one used for heat transfer such as the transformer oil and the second is a range of lubricants suitable for the lubrication of the machinery generating the energy (www.vps-international.nl).

Lubricants are one of the derivatives of crude oil. Lubricating oil is a complex mixture composed of straight and branched chain paraffinic, naphthenic, and aromatic hydrocarbons with more than fifteen carbon atoms. Lubricating oil boiling points range from 300 to 600 °C. The specific gravity of lubricating oil is 0.820 - 1.0. It's insoluble in water and alcohol, but it is soluble in benzene, ether, chloroform, carbon disulfide, and petroleum ether.

Lubricating oils are used in wide applications including engine oils, hydraulic fluids, automotive and industrial gear oils, transmission fluids, bearing oils, machine oils, steam engine oils and transformer oils (Speight and Arjoon, 2012).

1.1. Chemical Composition of the Petroleum

Petroleum is an extremely complex mixture of hydrocarbons. Hydrocarbons are (by definition) compounds containing carbon and hydrogen only, carbon atoms that bound to each other forming a backbone with hydrogen atoms attached to the

remaining sites on carbon. The petroleum hydrocarbons can be fractionated by silica gel chromatography into a saturate or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction (Atlas, 1981; Speight, 2002; Fallon, 1998).
Aliphatic hydrocarbons may be saturated or unsaturated. The saturated hydrocarbons include straight-chain and branched alkanes, as well as cycloalkanes (naphtenes). Unsaturated hydrocarbons known as olefins or alkenes contain one double bond, acetylenes or alkynes which contain one triple bond (Bingham and Cohrssen, 2012).

Classification of petroleum hydrocarbons based on their chemical structure:
1- Alkanes (also called paraffin hydrocarbons) characterized by unbranched (linear) or branched (non-linear) chains of carbon atoms with attached hydrogen atoms. Compounds of this family are also called "saturated hydrocarbons" because they contain no carbon-carbon double bonds, and are generally insoluble in cold water, having the general formula of CnH_{2n+2}; one example of alkanes is pentane (C_5H_{12}).

2- Cycloalkanes or cycloparaffins (also called naphthenes) characterized by the presence of simple closed rings of carbon atoms in their structure (such as the cyclopentane ring or the cyclohexane ring), are generally stable and insoluble in water - examples are cyclohexane and methyl cyclohexane.

3- Alkenes (also called olefins) characterized by the presence of a carbon-carbon double bond (>C=C<) can be unbranched (linear) or branched (non-linear) chains of carbon atoms. The general formula for the alkene family is C_nH_{2n}; an example for alkenes is ethylene ($CH_2 = CH_2$) (Speight and Arjoon. 2012).

4- Single-ring Aromatics.
Aromatic constituents are characterized by the presence of an aromatic ring with six carbon atoms. Aromatic hydrocarbons have low molecular weight and may be soluble in water. Aromatic hydrocarbons of crude oil are considered acutely toxic components because of their association with chronic and carcinogenic effects. (Speight and Arjoon. 2012).

5- Multi-ring Aromatics.

Polycyclic aromatic hydrocarbons (PAHs) consist of 2 to 13 aromatic rings. PAHs are insoluble in water and weakly volatile - solubility decreases with an increase of the aromatic rings number. All PAHs are solid and have high boiling and melting points (Skupinska et al., 2004).

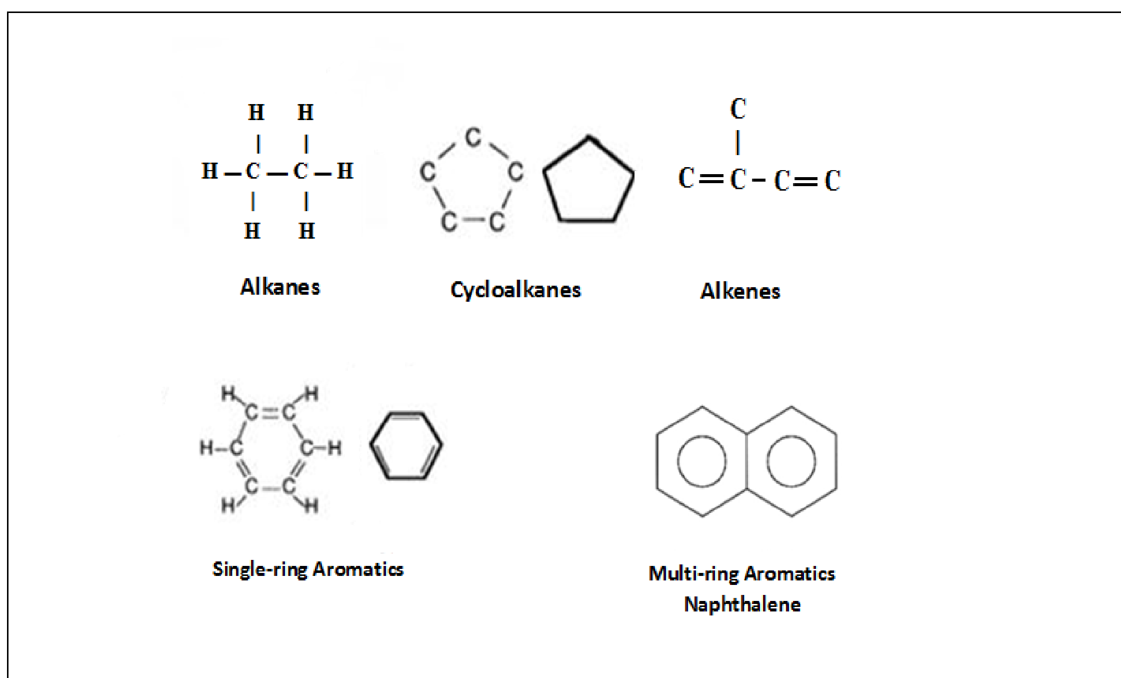


Figure 1.1 Molecular structures of hydrocarbons (Garapati, 2012).

1.2. **Bioremediation and Their Applications**

Bioremediation means to use biological organisms to solve an environmental problem such as contaminated water or contaminated soil. Bioremediation is considered one of the best technologies to deal with petroleum product contaminated environment (Thapa et al., 2012). In this technique the contaminants are removed from the environment thereby nature is being conserved and further pollution inhibited. Bioremediation may be employed to attack specific contaminants, such as chlorinated pesticides which are degraded by using bacteria, or it may be taken to a more general approach, such as oil contaminated wastewater that is broken down using multiple techniques including biostimulation to facilitate the degradation of oil by bacteria (Basharudin, 2008).

According to the application mode, bioremediation is classified into two types, namely: *In-situ* bioremediation and *Ex-situ* bioremediation

A. *In-situ* Bioremediation*:*

The method of *In-situ* bioremediation requires removing or scraping the contaminated subsoil. Treatment is carried out in subsoil either by biological methods such as hydrocarbons degradation by microorganisms, chemical processes, or by physical processes such as soil venting to extract contaminants from the soil as well as from the groundwater. Vertical and horizontal drilling processes may be required in order to do this treatment. Venting improves aerobic bacterial growth in the surface of the contaminated soil (Farhadian et al., 2008). Air suction is another method of contaminated soil treatment where suction of air helps to extract and remove volatile hydrocarbons from contaminated area. In-situ steam injection is another method where steam at high temperature and high pressure are injected into the contaminated soil. The temperature of the injected steam should be greater than the boiling points of volatile components in order to convert them into gaseous or volatile phases (Garapati, 2012).

Biological *in-situ* process includes two main methods applied in improving the microbial degradation. These are bioaugmentation and biostimulation. Bioaugmentation is addition of certain pre-grown microbes to enhance microbial populations at a contaminant site, in order to achieve the removal of contaminants at the lowest cost and time. Bioaugmentation technology is used in the case of the inability of the indigenous microbes to degrade a contaminant because of its presence in a small quantity insufficient to prevent the spread of contaminants (Speight and Arjoon 2012). Bioaugmentation technique uses microbes in order to remove the pollutants by converting them into CO_2, H_2O and biomass (Nopcharoenkul et al., 2012).

Biostimulation method involves identifying and adjusting some of the chemical and physical factors (such as the temperature, pH, nutrient content - such as nitrogen and phosphorus, etc) that may impact on the biodegradation rate of the contaminants by the indigenous microorganisms in the contaminant site. Appropriate adjustment of one of these factors may lead to significantly raise the biodegradation rates of pollutants through stimulation of the indigenous microorganisms' growth. In addition to these factors, the success of biostimulation technology depends on the properties

of the oil, type of the additive nutrients, and the contaminated environment nature (Abdulsalam et al., 2011; Speight and Arjoon, 2012).

B. *Ex-situ* Bioremediation:

The soil must be removed to an off - site remediation facility in this method. The treatment is carried out by burning the soil, chemical extraction or soil washing in order to remove the hydrocarbon components. The main methods of *ex-situ* treatment are combustion, steam stripping, chemical extraction and biological methods. The chemical extraction method involves the usage of different types of solvents. It can be applied for the soils contaminated with crude oil, where the contaminants are disolved in the solvent and separated from the soil. *Ex-situ* methods can be applied only if the amount of contaminated soil is small or if the contamination has occurred in a residential or industrial area where in-situ treatment cannot be applied. *Ex-situ* treatment leads to a higher degree of remediation because of controllability of many factors, like: temperature, pH, salinity, moisture etc (Garapati, 2012). Contaminated groundwater can be remediated by both *in situ* and *ex situ* methods. However, it is often not feasible to remediate contaminated groundwater *ex situ* because of the high costs of this treatment. So, bioaugmentation or biostimulation of organisms in this contaminated sites through *in situ* treatment is the best (Bamforth and Singleton, 2005).

1.3. Petroleum Hydrocarbons Degrading Microorganisms

The capability of microorganisms to degrade and transform contaminants provides benefits in the cleanup of petroleum polluted sites. Microorganisms used in bioremediation may be either natural microbes (indigenous) or cultivated in the laboratory. These microorganisms consisting of bacteria, yeasts, molds, fungi, algae, and to a lesser extent, plants consume oil pollutants as a source of energy or assimilate all harmful compounds such as heavy metals, which makes the contaminated area virtually free of contaminants (Speight and Arjoon, 2012). In the contaminated environments the hydrocarbons' degradation speed depends on the bacteria present and their quantities, petroleum compound, and on the environmental condition (Heul, 2009).

Acinetobacter sp., Actinomycete, Arthrobacter group, Bacillus sp., Micrococcus sp., Pseudomonass sp., Rhodococcus sp., and Streptomyces have been identified as

having the ability to degrade alkanes. *Pseudomonas sp., Rhodococcus sp., Ralstonia sp., Sphingomonas sp.* have the ability to degrade mono-aromatics, and the Alteromonas *sp., Arthrobacter sp. Bacillus, Mycobacterium sp., Pseudomonas sp.* have been identified as having the ability to degrade poly-aromatics (Salleh et al., 2003).

The yeast species, namely: *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum sp,* and *Trichosporon mucoides* which were isolated from contaminated water proved the ability to degrade the petroleum compounds (Das and Chandran, 2010). Some fungi such as (*Paecilomyces, Verticillium, and Beauveria* and *Penicillium* species) proved the ability to grow on long-chained alkylbenzenes as the sole source of carbon and energy. Moreover, studies proved that at least three of the four recognized fungal phyla, *Zygomycota, Ascomycota* and *Basidiomycotaic* have the ability to degrade the aromatic hydrocarbons such as benzene, naphthalene, and benzo[a]pyrene (Prenafeta-Boldu et al., 2006).

1.4. Biodegradation Pathway for Petroleum Hydrocarbons

Biodegradation by microorganisms includes chemical transformation to remove toxic pollutants by using it as a source of food and energy, or may occur coincidently when the organism does not receive any benefit from these pollutants. The complete biodegradation of organic materials into inorganic products is called "Mineralization", which often occurs through the combined activities of microbial consortia rather than through a single microorganism.

Partial biodegradation (transforming organic compounds into simpler compounds rather than mineralization) is called "Co-metabolism"; it occurs fortuitously and it does not provide energy or cell biomass to the microorganisms. Co-metabolism can lead to partial transformation to an intermediate that can serve as a carbon and energy substrate for microorganisms, as with some hydrocarbons, or can lead to an intermediate that can be toxic to the transforming microbial cell, as the trichloroethylene and methanotrophs.

Petroleum biodegradation can occur under aerobic and anaerobic conditions.

A- Aerobic degradation involves the use of molecular oxygen (O_2) in the pathway where oxygen is gaining electron from an organic contaminant (Speight and Arjoon, 2012).

Organic substrate + O_2 $\longrightarrow$ biomass + CO_2+H_2O + other inorganic products

The first step of hydrocarbons degradation is the addition of one oxygen atom, in some cases, two oxygen atoms, to the hydrocarbon molecule, in order to convert it into an alkanol (in the case of aliphatic hydrocarbons) or to a phenol (in the case of aromatic molecules). In some species, the first intermediate is an epoxide. This activation makes the hydrocarbons more soluble in water; the first intermediate considers a reactive site, which introduces a reactive site for the next reactions.

The reaction requires energy, that is generally generated through the oxidation of a reduced biological intermediate such as NADH, which itself is re-oxidized by an electron acceptor. For the degradation of alkanes different enzyme systems participate in the primary attack. An omega-hydroxylase system that consists of three proteins (the rubredoxin reductase, a rubredoxin and an omega-hydroxylase) was isolated and characterized from *Pseudomonas*. In some bacterial or fungal species and in mammalian cells, enzyme systems which depend on cytochrome P450 are observed as a terminal oxidase. The main intermediates of the alkane degradation are fatty acid, which are produced from the alkanols via aldehydes.

Fatty acids can be further decomposed through the typical pathway of carboxylic acid degradation (fatty acids are converted to acetyl coenzyme (CoAst) through a process known as β-oxidation, acetyl-CoA then enter the TCA cycle). However, fatty acids can also be excreted by the cells and accumulated in the environment. Fatty acids can be considered as a source of carbon for bacteria which enhances the hydrocarbon degradation. On the other hand, fatty acids (chain length 14 C) may inhibit growth and hydrocarbon metabolism because they interfere with the cell membrane. This makes it a toxic effect and reduces growth (Mahmoudi, 2013; Chamy and Rosenkrans, 2013).

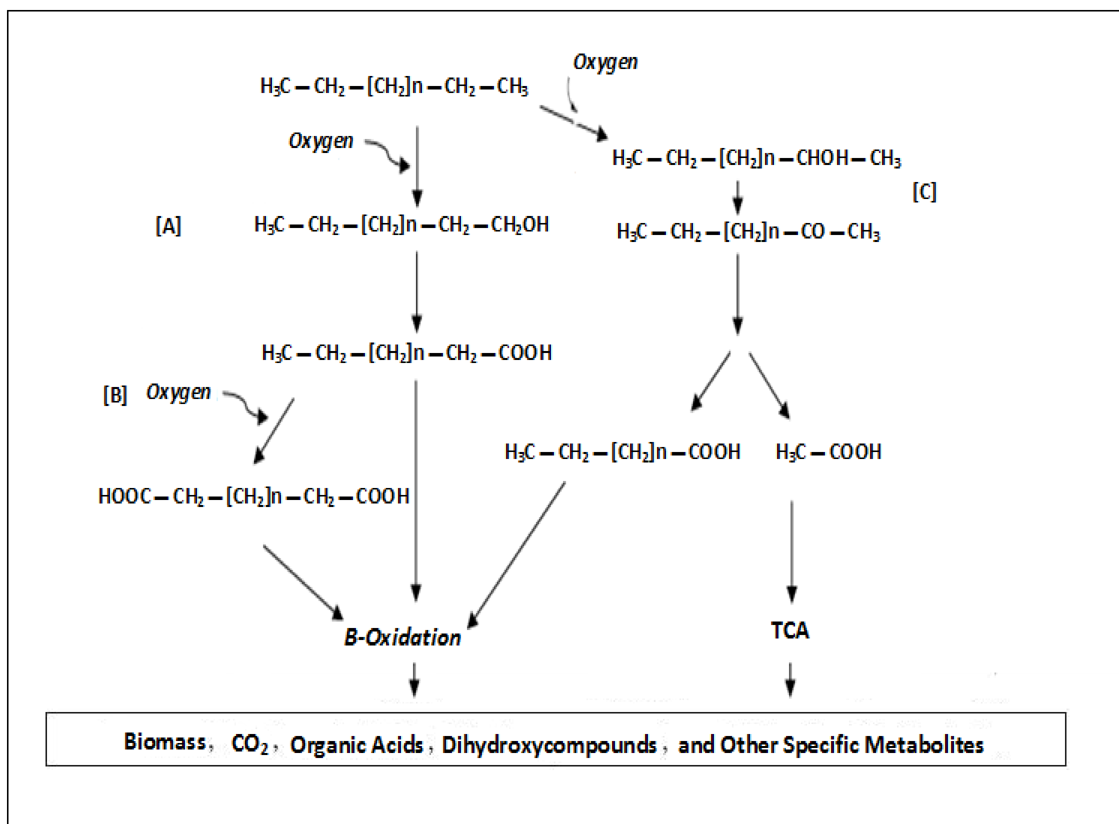


Figure 1.2. Metabolic path way of *n*-alkanes: metabolism begins with the activity of a monooxygenase which introduces a hydroxyl group into the aliphatic chain. [A]-monoterminal oxidation, [B]-biterminal oxidation, [C]- subterminal oxidation); TCA-tricarboxylic acid cycle (Chamy and Rosenkrans, 2013).

Different degradative pathways for aromatic substrates have been demonstrated .The choice of the pathway depends on the type of the organism and/or on the type of the aromatic molecule, and in the case of polyaromatic molecules (PAH) on the number of rings:

A - Complete mineralization or the dioxygenase pathway

In dioxygenase pathway (complete mineralization) which occurs mainly by bacteria, the monoaromatic molecule attacked by a dioxygenase is oxidized stepwise through formation of a diol and subsequent ring cleavage. Pyruvate is one of the main intermediates of the pathway. The products are biomass and carbon dioxide. In this pathway only ring systems of up to four rings are mineralized (Maneerat and Phetrong. 2007).

B- Co-metabolic transformation or the monooxygenase pathway

This pathway mainly occurs by yeasts and fungi, but it has also been demonstrated in bacteria and in some algae. The respective PAH-degrading species can carry out the degradation. If a compound is available that will be a source of carbon and energy. The enzymes which perform ring cleavage are monooxygenases (e.g., Cyt P450). Epoxides may also be transformed into trans-dihydrodiols. The latter have not been metabolized further by pure cultures in the laboratory and have to be regarded as dead-end products. However, these metabolites have not been detected in soil or in sediment (Chamy and Rosenkrans, 2013).

C- Unspecific oxidation via radical reactions

The wood-destroying white rot fungi, e.g., show the ability to destroy the structure of lignin via the activity of extracellular peroxidases and phenol oxidases. These fungi attack the phenolic molecule structure by a nonspecific action, thus attacking other aromatic structures such as PAH. Quinones are produced from frequent metabolism of PAHs (Chamy and Rosenkrans, 2013).

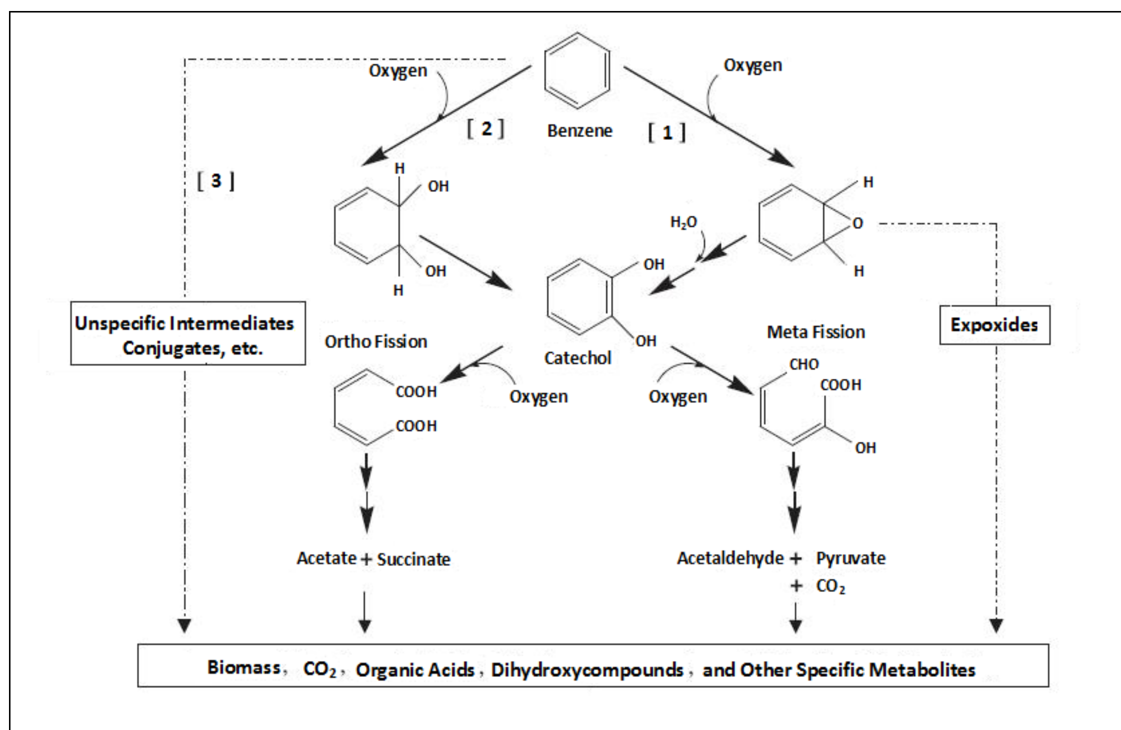
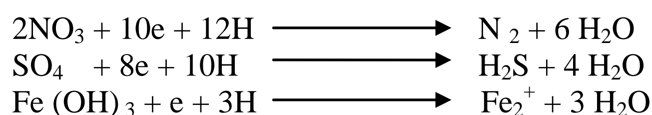


Figure 1.3. Biodegradation of aromatic hydrocarbons: metabolism begins with the activity of a monooxygenase [1] or a dioxygenase [2] which introduce one or two atoms of oxygen; it can also begin with unspecific reactions [3](Chamy R. and Rosenkrans F. 2013).

B- Anaerobic biodegradation is the organic substances degradation by microorganism in the case of oxygen absence. Degradation processes in anaerobic systems depend on sulfate, nitrate or iron ($Fe3^{+}$) as electron acceptor which in the end are being transformed into molecular nitrogen, hydrogen sulfide, iron(II) ion (Speight and Arjoon, 2012).



Anaerobic biodegradation of hydrocarbon is not uncommon in nature although, in most cases it is considerably slower than aerobic degradation. Hydrocarbons that can be degraded anaerobically include aliphatic alkanes and alkenes that have 6-20 carbon atoms, monocyclic alkylbenzenes, such as toluene, ethylbenzene, propylbenzene, as well as benzene and naphthalene (Chamy and Rosenkrans, 2013; Heider et al., 1999).

Alkanes and a lot of aromatics are degraded to form fat acids in bacteria by an anaerobic pathway, via addition of a one-carbon moiety or a fumarate molecule to terminal or sub terminal of alkane as an activation mechanism. For aromatic molecules, it has been demonstrated that alkyl benzenes that have a methyl group as a side chain undergo an enzyme's addition of fumarate. This becomes clear in toluene. Alkyl benzenes with side chains of two or more carbon atoms are activated by dehydrogenation of the side chain (Heul, 2009; Chamy and Rosenkrans, 2013).

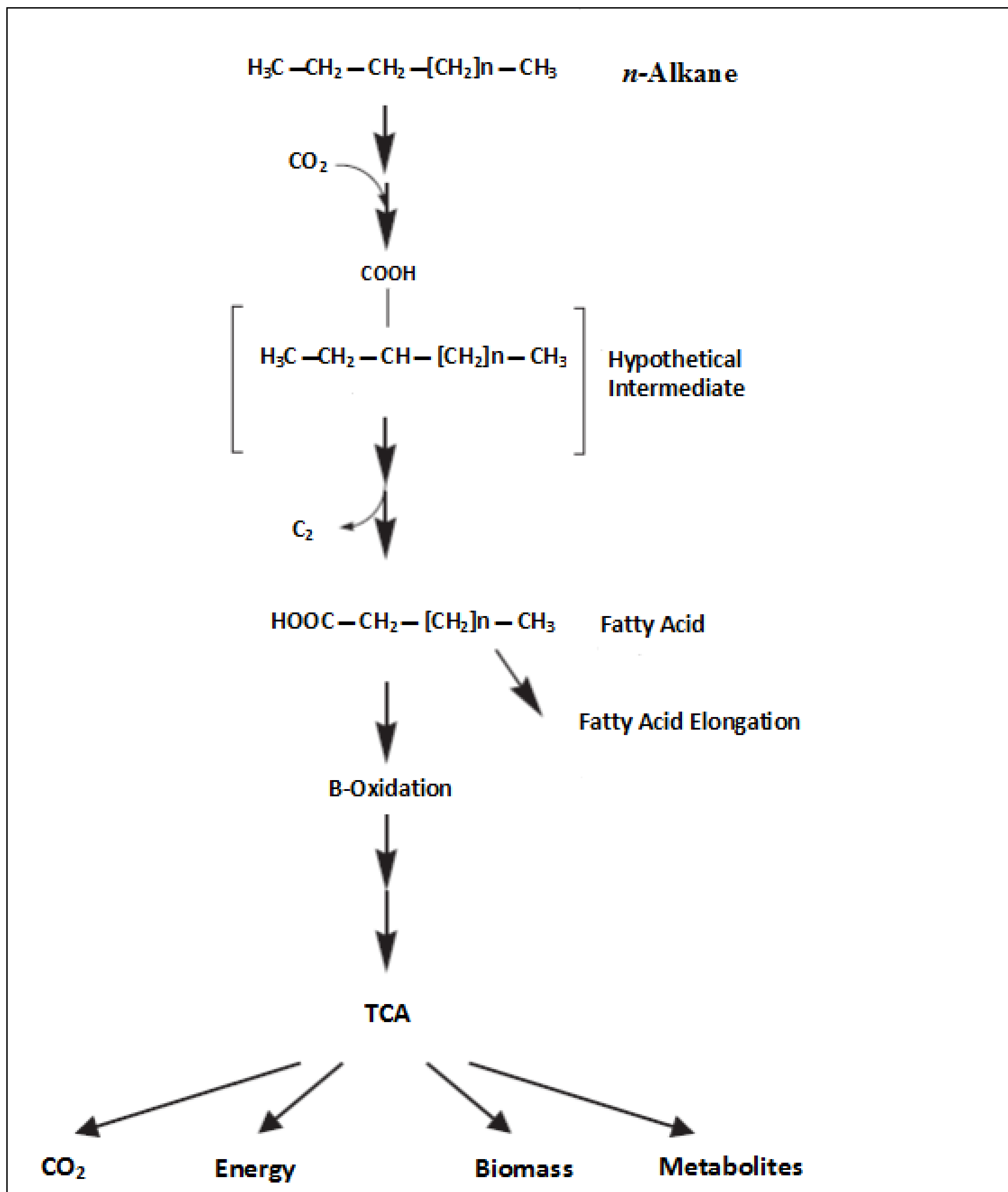


Figure 1.4. Proposed pathway for anaerobic degradation of *n*-alkanes; activation via addition of a C1-moiety (subterminal carboxylation at C_3); TCA tricarboxylic acid cycle (Chamy R. and Rosenkrans F. 2013).

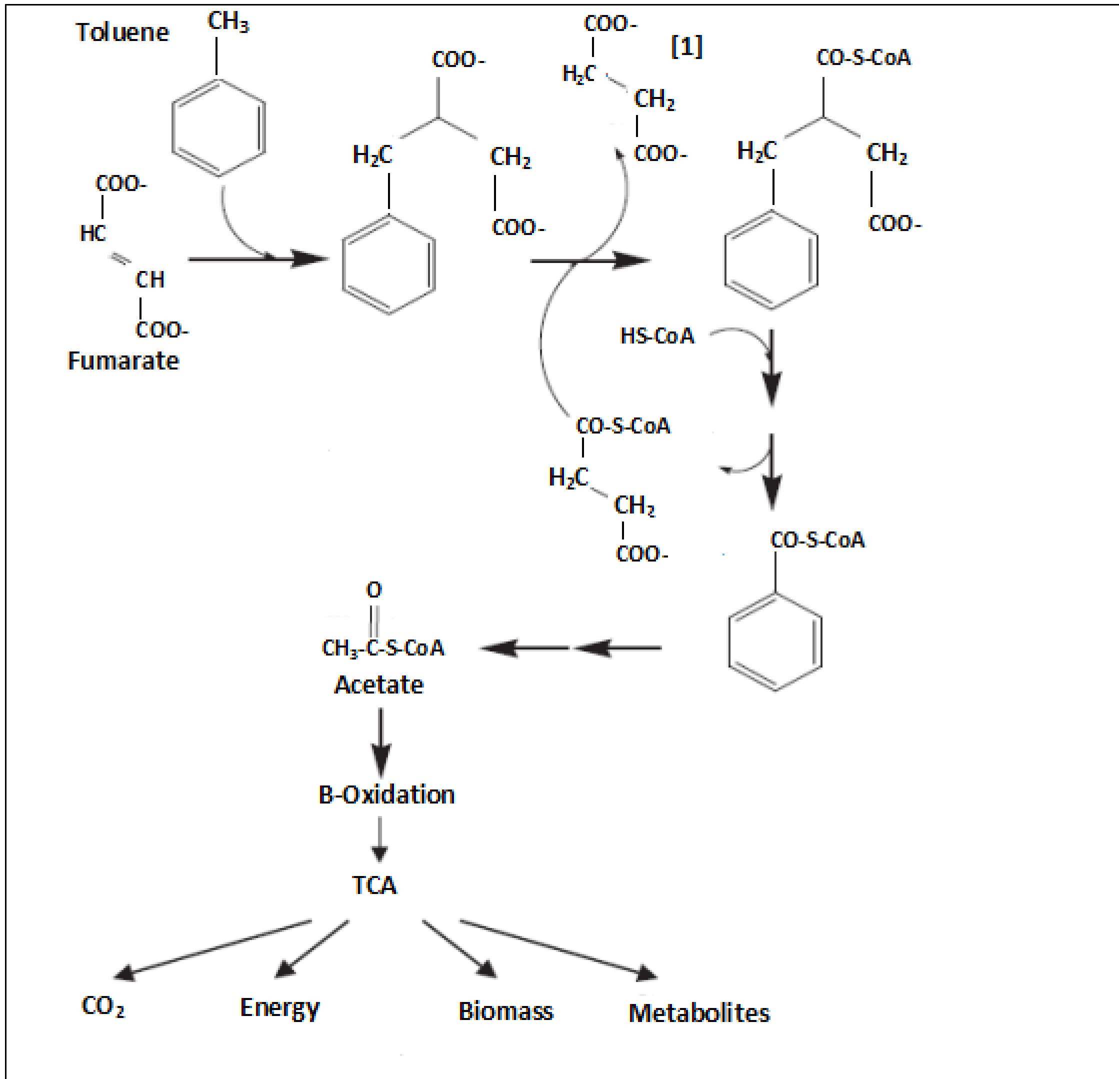


Figure 1.5. Proposed pathways of anaerobic degradation of aromatic hydrocarbons; activation via addition of fumarate. (Chamy R. and Rosenkrans F. 2013).

1.5. Factors Affecting Bioremediation

The success of the biodegradation process depends on the availability of several factors, the most important being: (1) Availability of suitable degrading microbes, (2) suitable environmental growth conditions, as oxygen presence, (3) optimal temperature, (4) availability of nutrients, (5) suitable pH(Speight and Arjoon, 2012).

1.5.1 Microbial Factors

Many microorganisms present in contaminated soil and water have the ability to degrade petroleum hydrocarbons. Most of them use the organic contaminants for

their growth. Occasionally, natural soil microorganisms may not have the metabolic capability to readily degrade certain compounds; therefore, microorganisms are seeding in the soil to enhance the process of bioremediation (Eng, 1998).

Seeding is the addition of not naturally existing microorganisms to the existing native oil degrading population in a polluted area. The purpose of seeding is to increase the population of microorganisms that can biodegrade the spilled oil (Speight and Arjoon, 2012).

Individual organisms can metabolize a limited range of hydrocarbon substrates. Therefore, it is necessary to assemble several bacteria that have the catabolic potential to break down complex organic molecules and release energy, in order to degrade the complex mixture of hydrocarbons that may affect a contaminated site. In a study, there were used pure and mixed *Pseudomonas* cultures to degrade toluene, chlorobenzene, ethanol and xylene. The results showed that a mixed culture made of three strains demonstrated more stable growth behavior and efficiency to contaminants degradation to much lower concentrations than pure cultures (Paramanik and Rajalakshmi, 2013; Eng 1998).

1.5.2. Oxygen Factors

A constant supply of oxygen for the microbes is necessary in aerobic biostimulation. Aerobic conditions are generally considered necessary for extensive degradation of petroleum hydrocarbon as their metabolic pathways involve oxygenases (Basu, 2005). The initial steps in the catabolism of aliphatic and aromatic hydrocarbons by bacteria and fungi required the molecular oxygen in order to oxidate the substrate by oxygenases (Leahy and Colwell, 1990).

During the aerobic biodegradation process, molecular oxygen is reduced to water while petroleum hydrocarbon is oxidized to create energy, cell mass, and carbon dioxide (Eng, 1998).

Aerobic degradation is much faster than anaerobic degradation. An experiment was done with three strains of bacteria isolated from polluted soils. The results showed that the aerobic bacteria is able to degrade 20-25% of the organic material and 90-95% of the alkanes during ten days time, while during an experiment that went on for 50 days, 15-18% of the organic material and only 20-25% of the alkenes were degraded. This proved that anaerobic degradation is far slower than degradation in the presence of oxygen (Heul, 2009).

In the water environment, bacteria using oxygen dissolved in the water biodegradates the oil hydrocarbons aerobically. The measurements of the deep plume of dissolved oxygen showed a rate of 30 percent oxygen depletion in the presence of the biodegrading microbes. The most part of the sediments on the ocean floor does not contain oxygen. Hydrocarbons that settle into sediments on the ocean floor undergo anaerobic biodegradation which is a much slower process. Therefore, these hydrocarbon contaminants become chronic (Speight and Arjoon, 2012).

Oxygen provided into the contaminant soil increases the activity of native bacteria and allows them to degrade the contaminants through the process called soil venting (Eng, 1998).

Anaerobic PAH degradation rates under denitrifying conditions showed comparable rates to those under aerobic conditions. This shows that anaerobic bioremediation benefits from hydrocarbons degradation. Generally, hydrogen peroxide, sodium nitrate and perchlorate are used for anaerobic degradation (Pawar, 2012).

1.5.3. Temperature Factors

Temperature plays a significant role in petroleum biodegradation because of its effect on the chemical composition and physical nature of the oil, rates of microbial hydrocarbon metabolism and composition of the microbial community (Leahy and Colwell, 1990).

At low temperatures, the viscosity of the oil increases, the evaporating of toxic short-chain alkanes is reduced, and their water solubility is increased, which causes lessenning metabolic rates and delaying the biodegradation. Decreasing temperature causes decreasing rates of enzymatic activity and as a result, decreasing of degradation rates (Heul, 2009; Leahy and Colwell, 1990).

Temperatures around 30 to 40 °C, which is the optimum temperatures for the enzymes of the bacteria, cause increasment of the rates of hydrocarbon metabolism. When the temperature becomes higher, the hydrocarbons become toxic to the bacterial membrane (Heul, 2009; Leahy and Colwell, 1990).

However, the microorganisms have adapted to metabolise PAHs at extreme temperatures; for example, naphthalene and phenanthrene degradation was reported in seawater at temperatures as low as 0 ºC. In comparison, PAHs degradation was reported by the laccase and manganese peroxidase enzymes of ligninolytic fungi at an optimum temperature of 50 ºC and >75 ◦C (Bamforth and Singleton, 2005).

1.5.4. Nutrients Factors

Nutrients such as nitrogen, phosphorus and iron play a much more critical role than oxygen in limiting the rate of biodegradation in marine waters. Nitrogen addition stimulates the biodegradation of alkane and polyaromatic hydrocarbons (PAHs), while phosphorus addition increases the biodegradation rate of alkane but not PAHs (Basharudin2008). Microorganisms require these mineral nutrients for cellular metabolism thereby for growth (Bamforth and Singleton 2005). These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants (Basharudin, 2008).

The native microorganism populations grow on limited supplies of the nutrients such as phosphorus and nitrogen in the nature. Nutrients are added to the contaminated environment to stimulate the microorganism to grow rapidly in order to increase the rate of biodegradation (Spieght and Arjoon, 2012). A number of studies indicated that lack of nutrients caused slow degradation rates of hydrocarbons (Fallon, 1998). An experiment was done with six both aliphatic and aromatic petroleum degrading bacteria. A mixture of nitrate, ammonium and phosphate was added to some cultures. The results showed clear difference in oil degradation. The degradation for total aliphatic hydrocarbons was 5-47 % in the cultures with the mix and 0-13 % in cultures without the mix. For the bigger alkanes, results is a change in degradation from 16-28 % to more than 90 % (Heul, 2009).

1.5.5. pH

Many contaminated sites with PAHs are not at the optimal pH for bioremediation. Because these pollutants change the pH of the contaminated sites less favourable conditions for microbial metabolism occured. Therefore, it is common practice to adjust the pH at these sites for a suitable range of microorganism growth (Bamforth and Singleton, 2005)

Soil pH is very variable, ranging from 2.5 to 11.0. Most heterotrophic bacteria and fungi favor a pH near neutrality (7). Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence to degrade hydrocarbons by microbial populations. Adjustment of the pH to the optimal range of microbes leads to increased hydrocarbons biodegradation (Leahy and Colwell, 1990).

Few investigations show phenanthrene degradation by consortium of both bacteria and fungi. Phenanthrene removal was only 40 % at pH 5.5 after 16 days, while at neutral pH values, phenanthrene removal was 80 % (Pawar, 2012).

1.6. Hydrocarbons Effect of Health

Living matter is exposed to petroleum in direct or indirect ways. Several of petroleum products have high toxicity effects. These toxic compounds are unintentionally released into the environment by natural oil seeps, accidental spills from oil tankers, oil storage wastes, refinery waste products and emissions, effluents of the petrochemical industries, etc. In spite of the large number of hydrocarbons compounds present in petroleum products, only a few of them are characterized by toxicity. Based on the level of exposure and susceptibility, petroleum hydrocarbons cause a wide impact on the people's health and on the wildlife where they can cause damage to any organic system in the human body, like: the respiratory, circulatory, immune, nervous, reproductive, endocrine, sensory systems, the kidney, the liver, etc). Thus, they can cause a large number of diseases (Romero-Zeron, 2012; Knox and Gilman, 1997).

The most susceptible cases of impact of toxic petroleum hydrocarbons are as follows:

1. Unborn babies, Infants and children.
2. Pregnant women.
3. People who have serious chronic diseases.
4. People living in stress conditions.

Past studies considered that water soluble fractions of the aromatic and polycyclic aromatic compounds are the most harmful because they are assumed to be mutagenic and carcinogenic. Especially the polyaromatic hydrocarbons with 4 or 5 rings are known to be carcinogens. The non aromatic substances in the petroleum were considered not very harmful. This is in fact not true, and alkanes and cycloalkanes are now also taken into account (Heul 2009).

The more toxic components of aromatic hydrocarbons to aquatic organisms include BTEX (i.e., benzene, toluene, ethylbenzene, and xylene) (Liu 2003). PAHs can have serious affects on human health. The researches proved that PAHs have acute carcinogenic, mutagenic and teratogenic properties (Bamforth and Singleton, 2005).

The volatile and soluble petroleum fractions can leak into groundwater quickly and can vaporize into the air. Thus, they may impact easily on living matter when breathing the air contaminated with vaporized fraction or drinking the contaminated water. (Romero-Zeron, 2012).

Volatile fractions of petrolum are one of the causes of childhood cancers. On the other hand, the non-volatile heavy fractions of crude oil absorbed by the soil and migrating to the spreading sites, may harm living beings by skin contact, or by intake of contaminated water and foods. The heavy fraction of crude oil consists mainly of naphthene-aromatics and polyaromatic compounds. The aromatic compounds are an important group of environmental pollutants where long exposure to these compounds leads to tumors, cancer, and failure of the nervous system. (Knox and Gilman, 1997).

Most components of petroleum hydrocarbons can enter the bloodstream rapidly when human breathe them in as a vapor or mist or when they swallow them. Some of these compounds that are distributed by the blood throughout the body, can quickly break down into more harmful chemicals while others may break down into harmless chemicals (U.S. Department of Health and Human Services 1999). The toxicity created by crude oil can show its impact on the human body systems immediately or it may take months or eve years. (Romero-Zeron, 2012).

Oil spills may affect the organisms in the marine environment by direct toxicity or by physical smothering. It reduces growth of the photosynthetic rate of the above ground biomass of Spartina alterniflora and S. Patens and may cause their death. Also, oil in water causes depletion of dissolved oxygen due to transformation of the organic component into inorganic compounds, which leads to losses of biodiversity through a decrease in amphipod population that is important in the food chain, and eutrophication. Toxicity in fishes includes lymphocytosis, hemorrhagic septicemia, and epidermal hyperplasia. In mammals, thousands of seabirds were killed as a result of spilled oil in the sea (Onwurah et al, 2007). Rats are often used to estimate toxicity to mammals (Table1.1).

United Nation Environmental Program (UNEP 1995) introduced guidelines to identify the levels of harmful aliphatic hydrocarbons (>10 μg/g) and harmless ones (<10 μg/g) in the marine sedimentary environment (Nemr et al, 2012).

Table 1,1. Examples of toxicity of hydrocarbons found in diesel (Kauppi, 2011)

	Naphthalene	n-Decane	Anthracene
Rate			
LD50 Oral	>2000 mg/kg	5000 mg/kg	16000 mg/kg
LC50 Inhalation	>100 mg/L (4 h)	8,1 mg/L (8 h)	-
LD50 dermal	>2500 mg/kg	<2000 mg/kg	-

CHAPTER 2
LITERATURE ABSTRACTS

There are many studies in the biodegradation field that estimate the impact of the microorganisms (bacteria, yeasts, molds, fungi, algae) in order to reduce or limit the spreading of environmental pollution. A summary of some of these studies is presented in table 2.

Table 2. Summary of biodegradation studies

No	Authors name, year	studied sample	Studied Microorganism	Study Result
1	Csutak et al., 2010	many of petroleum components	*Yarrowia , Rhodotorula, Rhodosporidium, Trichosporon and Pichia*	Yeasts degradation. Small weight molecular *n*-alkanes are faster than high weight moleculars.
2	Ferreira et al., 2012	crude oil	*Y. lipolytica*	Initial cell concentration, agitation speed and temperature have the main impacts on biodegradations.
3	Martins et al., 2012	crude oil	*Y. lipolytica* IMUFRJ 50682	*y.lipolytica* have the ability to degrade crude oil component by 90- 97 %
4	Klug and Markovetz, 1967	n-alkanes	*Candida* species	Yeast species are able to consume some of hydrocarbons of the *n*-alkanes.
5	Zinjarde and Pant, 2002	crude oil	A group of yeasts, including *Y. lipolytica*, *C. albicane, C. parapsilosis* isolated from marine mud and water /Mumbai	Yeasts are unable to consume the aromatic fractions and asphalts.

6	Salleh et al., 2003 Vieira et al., 2009	Petroleum hydrocarbons	various populations of bacteria, inculding *Achromobacter* isolated from the environment	Creating the appropriate factors contributes greatly to the success of the deterioration process.
7	Zinjarde et al.,1998	crude oil	*Y. lipolytica* NCIM 3589 isolated from sea water, India	*Y. lipolytica* uses the yeast form to degrade the alkanes under aerobic conditions, and the mycelium form under partial anaerobic conditions.
8	De Felice et al., 1997	Olive oil	*Yarrowia lipolytica* ATCC 20255	Yeast utilizes most of the olive oil substances, except for the aromatic.
9	Ilori et al., 2008	Crude oil & Diesel oil	*C. albicans* isolated from lagoon water	Consumes the diesel oil better than crude oil.
10	Rushikesh Tasker., 1988	Diesel oil	A group of bacteria including types of *Achromobacter* and *Pseudomonas* species obtained from contaminated water and soil area	Mixed cultures bacteria showed more impact than the pure cultures.
11	Arulazhagan et al., 2010	polycyclic aromatic hydrocarbons	*S. maltophilia* isolated from marine water ,India	Increasing the salinity concentration reduces the degradation of polyaromatic hydrocarbons.
12	Erdoğan et al., 2011	crude oil	*S. maltophilia* isolated from soil , Turkey	Bacteria grows on crude oil
13	Juhasz et al., 2000	polycyclic aromatic hydrocarbons	*S. maltophilia* strain VUN10,003	Bacteria showed ability to consume and detoxify PAH

CHAPTER 3
MATERIALS AND METHODS

3.1. Equipments and Apparatuses

Table 3.1. Equipments and apparatuses used throughout the study

Equipment	Company / Origin
Autoclave	Hirayama / Japan
Sensitive balance	Denver instrument / Germany
Light microscope	Olympus, BX51/ Japan
Benchtop incubator shaker	New Brunswick scientific / USA
Gas Chromatography GC 6890N/ 5975 inert Mass selective detector	Agilent Technologies / USA
Incubator	Memmert / Germany
pH-meter	Martini instruments /Romania
Microbiological safty cabinet	Thermo scientific / Germany
Distillator	Microline / Turkey
UV Apparatus	BLX-254 / France
Rotary Evaporators	Heidolph /Germany
Optical density of microorganism suspension	DensiCHEK plus biomerieux / USA
Vortex mixer	Lab companion / Korea
Phoenix 100	Becton, Dickinson and Company/ USA
Refrigerator	Vestel / Turkey

3.2. Chemicals and Biological Materials

Table 3.2. Chemicals and biological materials used throughout the study:

Material	Company / Origin
Dichloromethane	Merck KGaA / Germany
Sodium nitrate $NaNO_3$	Merck KGaA / Germany
Dipotassium hydrogen phosphate K_2HPO_4	Carlo Erba / Italy
Hydrochloric acid HCl	Merck KGaA / Germany
Sodium sulfate Na_2SO_4	Alfa Aesar / Germany
Ethanol	J.T.Baker / Netherlands
Blood Agar Base	OXID CM0055/ England
Sabouraud Dextrose Agar	OXID CM0041 / England
Mueller-Hinton Agar	OXID CM0337 / England
Nuetrant Broth	MERCK / Germany

3.3. Prepared Culture Media:

- Blood agar Base (OXID CM0055/ England) used to prepare the culture media consists of the followings g/L :

Lab-Lemco powder 10.0

Peptone 10.0

Sodium chloride 5.0

Agar 15.0

Blood agar is enriched, differential media used to isolate fastidious organisms. It was prepared by dissolving 40g of blood agar base in 1 liter distilled water (DW), sterilized by autoclaving at 121 ◦C for 15 minutes. After cooling (45◦C), 70 mL of sterile, defibrinated human blood was added and mixed well.

- Sabouraud Dextrose Agar (OXID CM0041 /England) was used to prepare the culture media and consists of the followings g/L :

Mycological peptone 10.0

Glucose 40.0

Agar 15.0

It was prepared by dissolving 65 g of sabouraud agar base in 1 liter DW, sterilized by autoclaving at 121 ◦C for 15 minutes.

- Mueller-Hinton Agar (OXID CM0337 / England) used to prepare the culture media and consists of the followings g/L :

Beef, dehydrated infusion from 300.0

Casein hydrolysate 17.5

Starch 1.5

Agar 17.0

It was prepared by dissolving 38 g of Mueller-Hinton Agar base in 1 liter (DW), sterilized by autoclaving at 121 ◦C for 15 minutes.

3.4. Sterilization Methods

Culture media (liquid and solid) solutions (Hirayama / Japan) were sterilized by autoclaving for 20 minutes at 121ºC, the tubes being sterilized by UV Apparatus (BLX-254 /France).

3.5. Wastewater Samples Collection

Wastewater samples contaminated with petroleum products hydrocarbons (fuel oil and lubricating oil) were taken in sterilized bottles from the oil separator system in Baiji Thermal Power Station.

3.6. Petroleum Oil Extraction

Oil was extracted from wastewater by mixing 250 mL wastewater with 100 mL dichloromethane (Merck KGaA / Germany), mixing for 1 – 3 minutes three to four times opening the separating funnel valves to bring out the gase being left for 10-30 minutes for the oil layer separation from the water to take place. This oil was taken and anhydrous sodium sulfate (Na_2SO_4) (Alfa Aesar /Germany) was added as a drying agent for residual water absorption, then it was filtered and the evaporation of residual dichloromethane took place with the help of the rotary evaporator (Heidolph /Germany).

Figure 3.1. Oil extraction by separating funnel

3.7. Gas chromatographic analysis of crude oil:

0.02 g of extracted oil was diluted in 5 mL dichloromethane and analyzed by gas chromatography (Agilent Technologies, 6890N/ USA) in order to determine the components of petroleum hydrocarbons. Analysis performed with a Hewlett-Packard 6890 Series GC coupled to a 5975 inert Mass selective detector using a HP- 5MS capillary column (cross linked 5% phenylmethyl silixane, 30.0 m × 250 µm × 0.25 µm nominal) in a splitless injection mode. The carrier gas was helium with a flow

rate of 35 mL/min at a temperature of 280 °C. After sample injection, the oven temperature of the gas chromatograph was held at 40 ºC for 5.0 minutes then increased at a rate of 20 ºC/min to 280 °C and held for 15 minutes. Sample size used for injection was 2.0 μL.

3.8. Isolation of Bacteria and Fungi from Wastewater Sample

Waste water was filtered from oil. 100 μL of wastewater sample was cultured on Blood agar Base and Mueller-Hinton Agar plates, incubated at 37 ºC for 48 hrs (Memmert/ Germany). And 100 μL of wastewater sample was cultured on a SDA incubated at 30 ºC for 24 hrs. Isolated colonies were sub-cultured onto BAB plates to check the purity. Two colony types were obtained and were plated onto MHA plates for maintenance and identification.

3.9. Identification of Bacterial Isolates

Pure bacterial colony was identified by:

A - Morphological Tests

- **Gram's Stain**

The Gram Stain technique consists of five steps:

(1) One smear of each colony was prepared on a glass slide.

(2) Smear was stained with crystal violet solution for one minute then washed off with water.

(3) Gram's iodine solution was left on the smear for one minute, then washed with water and dried.

(4) Smear was decolorized with ethanol (95%) until free colour (approximately 30 seconds).

(5) Smear was flooded with safranine for 45 seconds, then washed and dried. The shapes of the cells were then observed through a microscope.

B- ID/AST combo

Bacterial strain identification was carried out by using BD Phoenix Automated Microbiology System (Becton, Dickinson and Company).

3.10. Yeast growth medium preparation by using wastewater

To prepare this medium, 1 g of petroleum oil, 1 g of Sodium nitrate ($NaNO_3$) and 0,5 g dipotassium hydrogen phosphate (K_2HPO_4) were weighted with a sensitive balance (Denver instrument/ Germany) and transferred to clean Erlenmyer flasks (250 ml). The mineral salts (MS) used as nutrients, then 100 mL of wastewater were added and the pH was adjusted to 7.4. The medium was sterilized by autoclaving at 121ºC for 20 minutes (Hirayama / Japan).

3.11. Culturing the Wastewater Prepared Medium by Yeast

Five mL (4 McF density) of activated yeast cell (*Y. lipolytica, C. albicans, C. parapsilosis)* was measured with DensiCHEK plus biomerieux / USA and inoculated into yeast growth medium mentioned above. Each yeast calture medium was incubated in a shaking flask (150 rpm/min) at 30°C for two weeks by an incubator shaker (New Brunswick scientific / USA).

Scientific classification of the used yeasts:

Kingdom: Fungi
Division: Ascomycota
Class: Saccharomycetes
Order: Saccharomycetales
Family: Dipodascaceae
Genus: *Yarrowia*
Species: *Y. lipolytica* (Van Der Walt and Von Arx., 1980)

Kingdom: Fungi
Phylum: Ascomycota
Subphylum: Saccharomycotina
Class: Saccharomycetes
Order: Saccharomycetales
Family: Saccharomycetaceae
Genus: *Candida*
Species: *C. albicans*
(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi)

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Saccharomycotina

Class: Saccharomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Candida*

Species: *C. parapsilosis*

(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=Candida+parapsilosis)

3.12. Bacteria Growth Medium Preparation

1 g of petroleum oil, 1 g of sodium nitrate ($NaNO_3$) and 0.5 g dipotassium hydrogen phosphate (K_2HPO_4) were weighted and transferred to sterilized Erlenmeyer flasks (250 mL) then 100 mL of wastewater was added and the pH was adjusted to 8.0. The prepared bacterial medium was incubated in a shaking flask (150 rpm/min) at 30°C for two weeks.

Scientific classification of the isolated bacteria

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: *Stenotrophomonas*

Species: *S. Maltophilia* (Palleroni And Bradbury., 1993)

Kingdom: Bacteria

Phylum: Proteobacteria

Class: BetaProteobacteria

Order: Burkholderiales

Family: Alcaligenaceae

Genus: Achromobacter

(http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=85698)

3.13. Growth Monitoring

Bacterial and yeast growth was monitored once every two days by plate count method. Each growth medium was taken and cultured in SDA, MHA under microbiological safety cabinet (Thermo scientific / Germany). Bacteria and yeast growth means that the colonies can use the petroleum oil as the sole carbon source for their metabolism and biodegradation is likely to happen.

Bacterial solution of 100 time dilution was inoculated on the MHA agar plate. The plates were incubated at 30 ^{0}C for 24 hours in the incubator. At the end of the incubation period, the cells on the petri plates were counted to estimate the number of cells per milliliter.

yeasts solution of 10 time dilution was inoculated on the SDA agar plate. The plates were incubated at 30 ^{0}C for 48 hours in the incubator. At the end of the incubation period, the cells on the petri plates were counted to estimate the number of cells per milliliter.

The number of live bacteria (or Colony Forming Units [CFU]) per mL of original culture was determined by using the following formula:

$$\text{CFU/mL} = \frac{\text{number of colonies per ml plate}}{\text{Total dilution factor}}$$

Figure 3.2. *S.maltophilia* colony shape

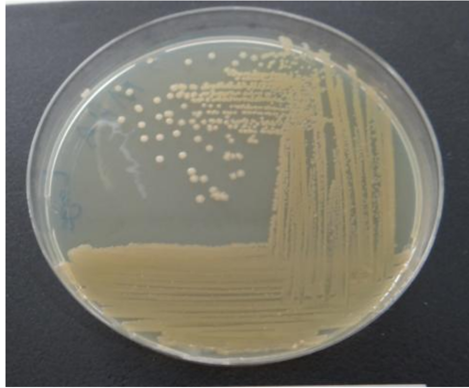


Figure 3.3*.* *Acromobacter sp.* colony shape

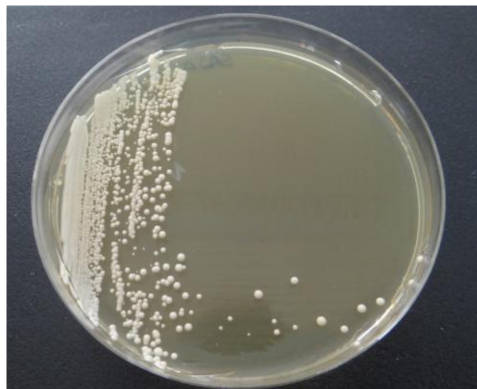


Figure 3.4. *C.albicans* ATCC 10231 colony shape

Figure 3.5. *Y. lipolytica* NCAIM Y00591 colony shape

Figure 3.6. *C.parapislosis* ATCC 22019 colony shape

1.14. Extracting the remaining crude oil and determining their components

After two weeks, the remaining oil was recovered by mixing the cultures medium with 50 ml dichloromethane, mixing for 1 – 3 minute, three to four times and opening the separating funnel valves to bring out the gase. It was left for 10 - 30 minutes for the oil layer separation from the water to take place.

This oil was taken and anhydrous sodium sulfate (Na_2SO_4) was added as a drying agent for residual water absorption, then it was filtered and the evaporation of residual dichloromethane took place with the help of the rotary evaporator; the remaining oil was analyzed by GC-Mass to determine the residual components after the biodegradation process.

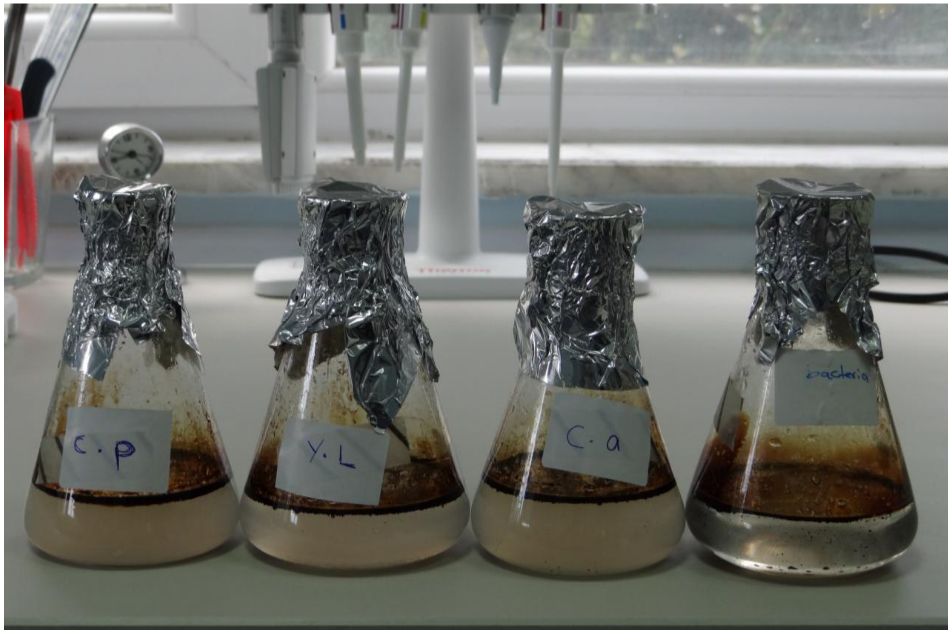


Figure 3.7. Microorganism’s culture before biodegradation processe

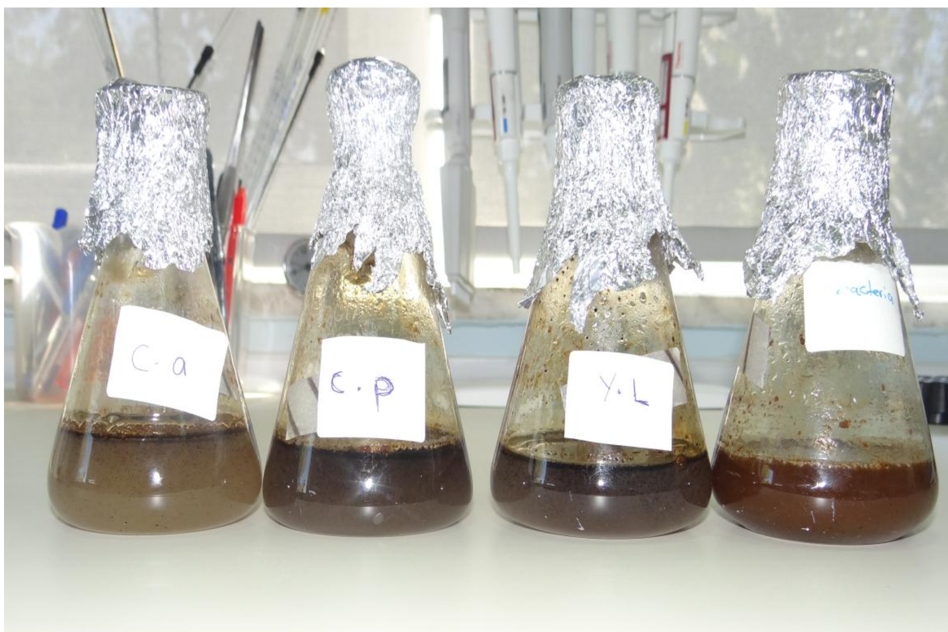


Figure3.8. Microorganism’s culture after biodegradation processe.

CHAPTER 4
RESULTS

4.1. Isolation and Identification of Wastewater Samples Bacteria:

Petroleum hydrocarbons contaminated wastewater samples were collected from Baiji Thermal Power Station in the month of April 2013.

Two bacterial isolates were isolated as shown in figure (3.2) (3.3). The isolates were identified as *Achromobacter sp.* and *Stenotrophomonas maltophilia* bacteria. *Achromobacter sp*. showed large, circular, cream colored, mucoid colonies, and S.maltophilia looked like small, circular, cream colored, mucoid colonies. The characteristics of colonies were detected and are listed in Table (4.1).

Table 4.1. Colony Characteristics of the isolated strain *Acromobacter sp.* and S. maltophilia

Colony	Colony characteristic	Cell shape	Gram stain	Cell colour after stain
Acromobacter sp.	Large, circular, cream Colour	straight rod	Negative	Pink
Stenotrophomonas maltophilia	Small, circular, cream colour	Small rod	Negative	Pink

4.2. Determination of Petroleum Oil Components

The results of GC-MS analysis were adopted in assessing the level of biodegradation. The analysis results of the crude oil (extracted oil from the wastewater) showed that Baiji power plant wastewaters contaminated with 21 of hydrocarbon compounds consist of aliphatic and aromatic fractions. Identification of the

compounds was obtained from NIST MS search library as shown in the Table (4.2) and Figure (4.1).

Table 4.2. Crude oil hydrocarbon compounds

No.	RT	Area %	Hydrocarbon Compound	Chemical Formula
1	8.917	0,7	Undecane	$C_{11}H_{24}$
2	9.553	1,06	2- methylundecane	$C_{12}H_{26}$
3	9.880	2,14	dodecane	$C_{12}H_{26}$
4	9.997	0,77	3,6 dimethylundecane	$C_{13}H_{28}$
5	10.404	2.05	nonadecane	$C_{19}H_{40}$
6	10.473	3.3	2,6,10 -trimethyldodecane	$C_{15}H_{32}$
7	10.682	4 ,390	tridecane	$C_{13}H_{28}$
8	10.912	1 ,39	Bicyclo(4.4.1)undeca-1,3,5,7,9-pentaene	$C_{11}H_{10}$
9	11.142	6.34	2,6,10 trimethyltetradecane	$C_{17}H_{36}$
10	11.393	5,27	Tetradecane	$C_{14}H_{30}$
11	11.575	0,78	2,6 - dimethylnaphtalene	$C_{12}H_{12}$
12	12.046	6 ,53	Pentadecane	$C_{15}H_{32}$
13	12.420	7.43	3-(2-methyl-propenyl)-1 H-indene	$C_{13}H_{14}$
14	12.709	5,20	Hexadecane	$C_{16}H_{34}$
15	12.768	3.02	*Tert*-Hexadecanethiol	$C_{16}H_{34}S$
16	13.249	1,91	2-methyl-1-Hexadecanol	$C_{17}H_{36}O$
17	17.501	2.87	Eicosane	$C_{20}H_{42}$
18	19.946	1,88	Octacosane	$C_{28}H_{58}$
19	23.048	2.56	Heptadecane	$C_{17}H_{36}$
20	25.877	0,87	Octadecane	$C_{18}H_{38}$
21	26.755	2.05	3-ethyl-5-[(2-ethyl)butyl] octadecane	$C_{26}H_{54}$

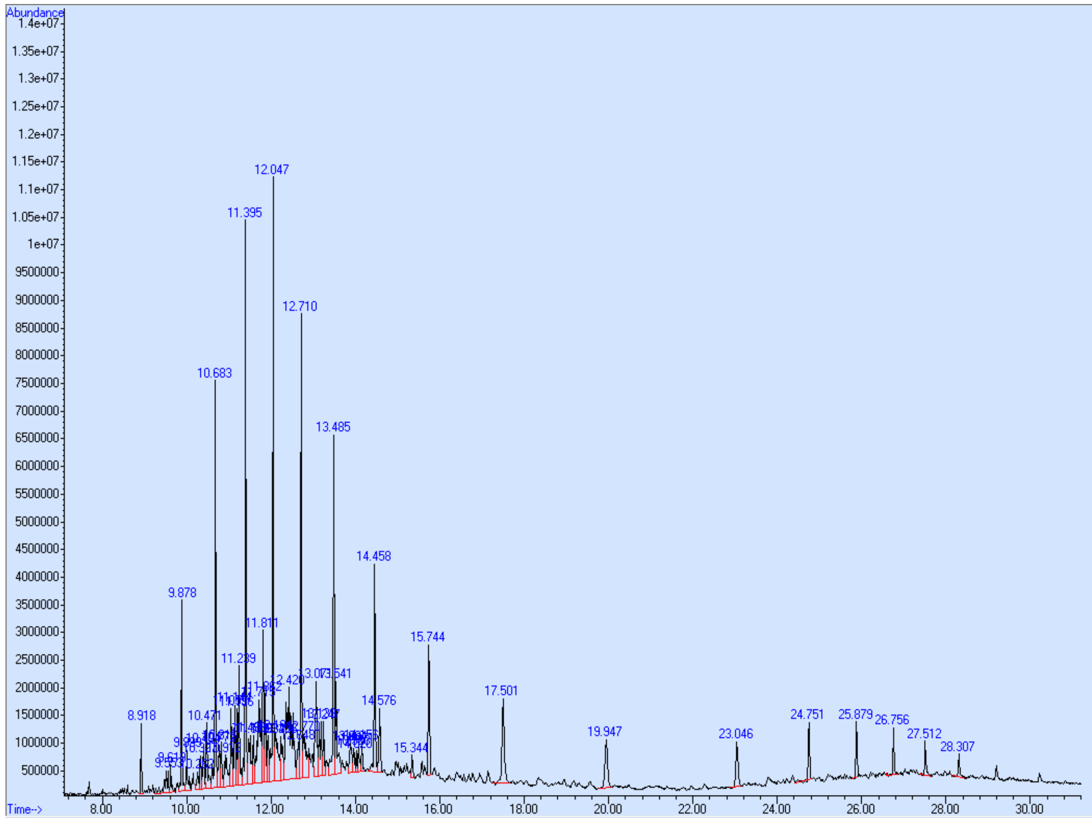


Figure 4.1. Gas chromatography (GC) tracing of the crude oil compounds, X-axis represents the retention time and Y-axis represents the hydrocarbon abundance.

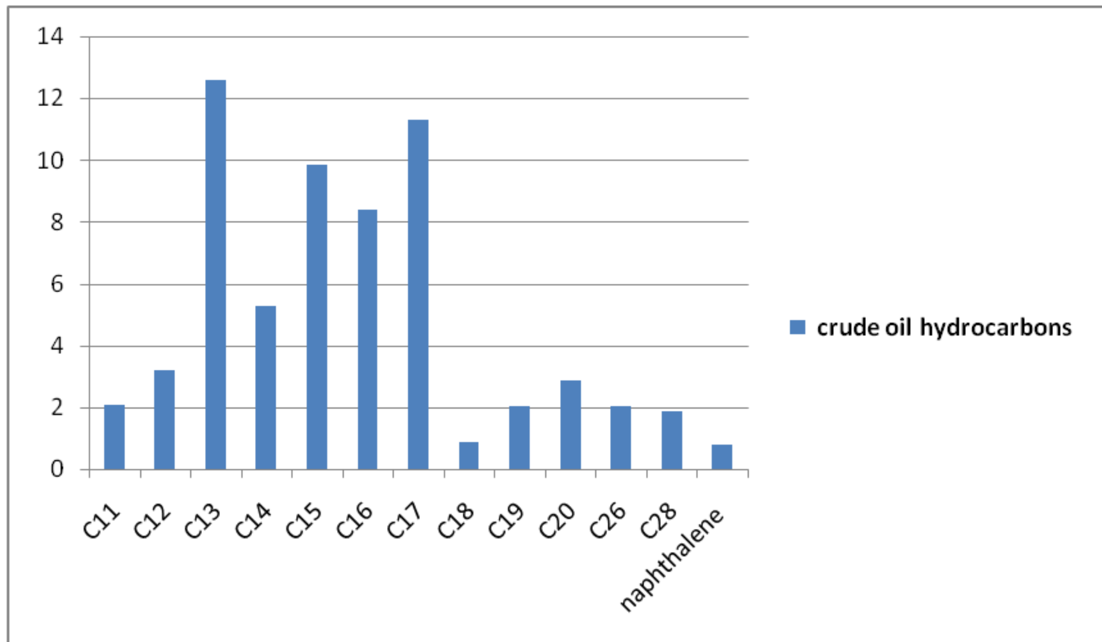


Figure 4.2. Hydrocarbon compounds in crude oil sample extracted from wastewater

4.3. Effect of Indigenous Bacteria on Petroleum Hydrocarbons:

The ability of the indigenous bacterial to degrade the petroleum hydrocarbons was examined at the end of the experiment, which continued for two weeks, through extraction of the remaining crude oil from bacterial cultures medium and analyzed by GC-MS device. The GC chromatogram showed a decrease in the abundance of hydrocarbon peaks, like from 21 to 17 as seen in Figure (4.3), (4.4).

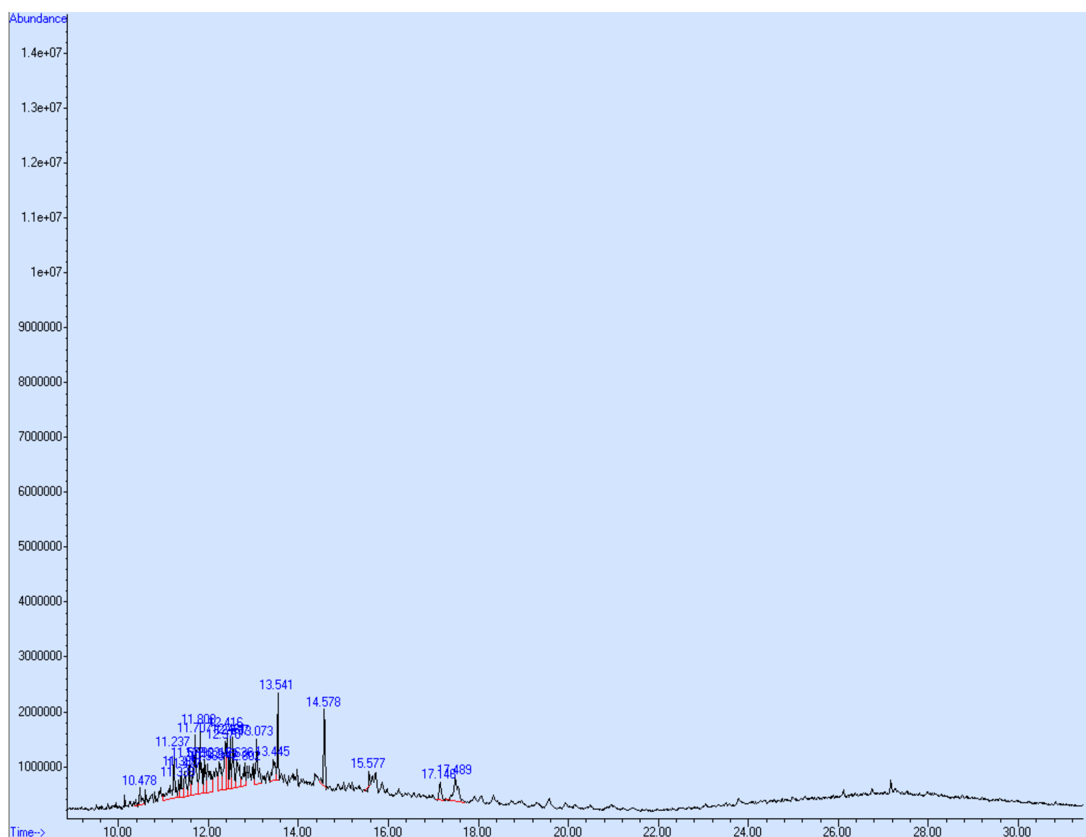


Figure 4.3. Gas chromatography (GC) tracing of the hydrocarbon compounds degrading by indigenous bacteria. X-axis represents the retention time and Y-axis represents the hydrocarbon abundance.

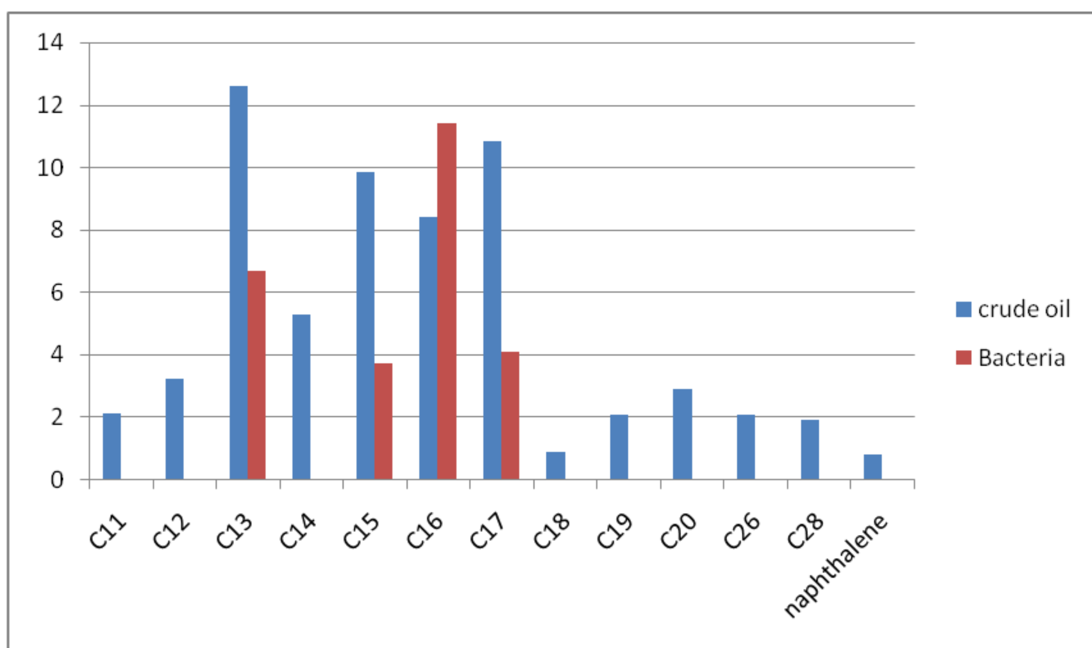


Figure 4.4. Disappearance of some hydrocarbon compounds and reduction in the percentage of others through degradation by indigenous bacteria.

4.4. Effect of *Y.lipolytica* on petroleum hydrocarbons:

GC-MS analysis results of remaining oil from biodegradation experiment of *Y.lipolytica* cultures showed the yeast's ability to degrade petroleum hydrocarbons during two weeks of incubation as shown in the following Figures (4.5),(4.6).

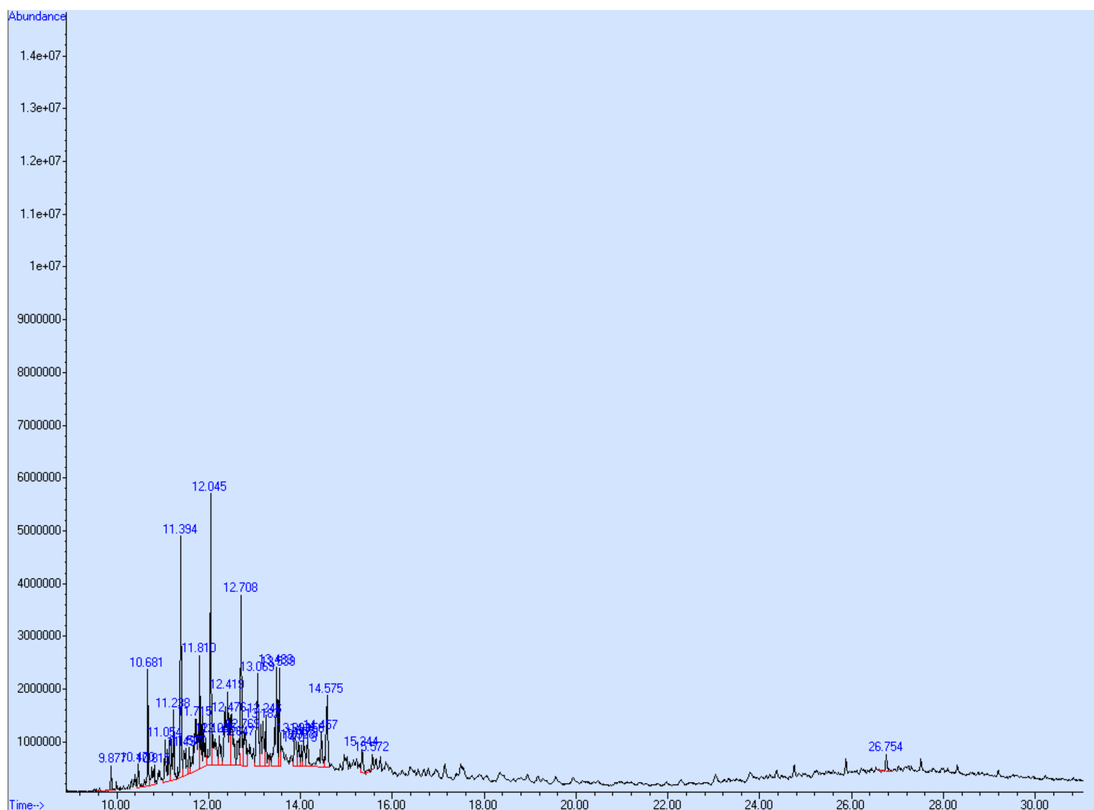


Figure 4.5. Gas Chromatography (GC) tracing of the hydrocarbon compounds degrading by *y.lipolytica*. X-axis represents the retention time and Y-axis represents the hydrocarbon abundance.

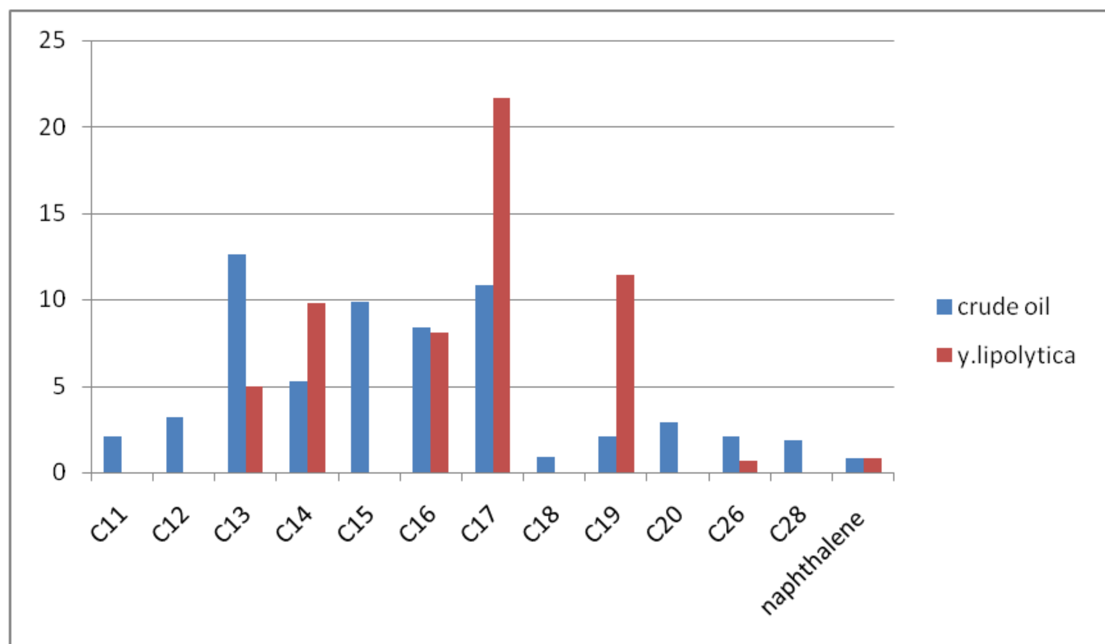


Figure 4.6. Disappearance of some hydrocarbon compounds and emergence of a difference in the percentage of others through degradation by *Y.lipolytica.*

4.5. Effect of *C.albicans* on petroleum hydrocarbons:

The result of the remaining oil from biodegradation experiment of *C. albicans* culturing by GC-Mass showed the yeast's ability to degrade the petroleum hydrocarbons as shown in the Figures (4.7),(4.8).

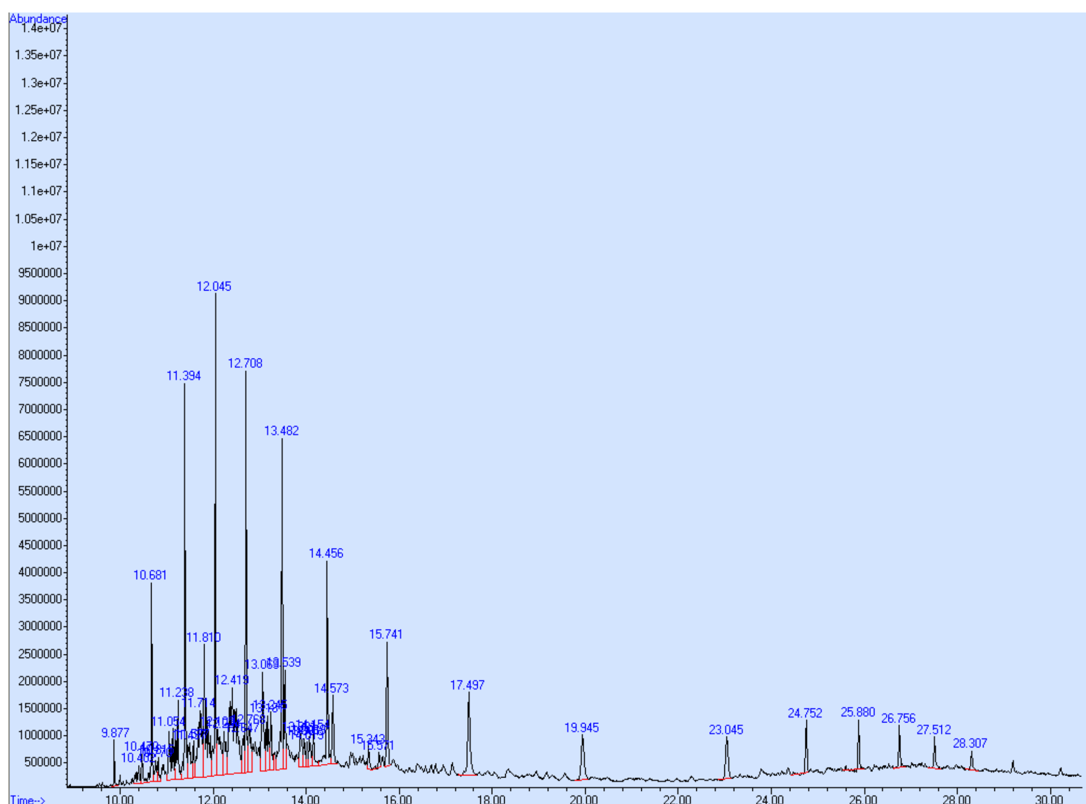


Figure 4.7. Gas Chromatography (GC) tracing of the hydrocarbon compounds degrading by *C.albicans.* X-axis represents the retention time and Y-axis represents the hydrocarbon abundance.

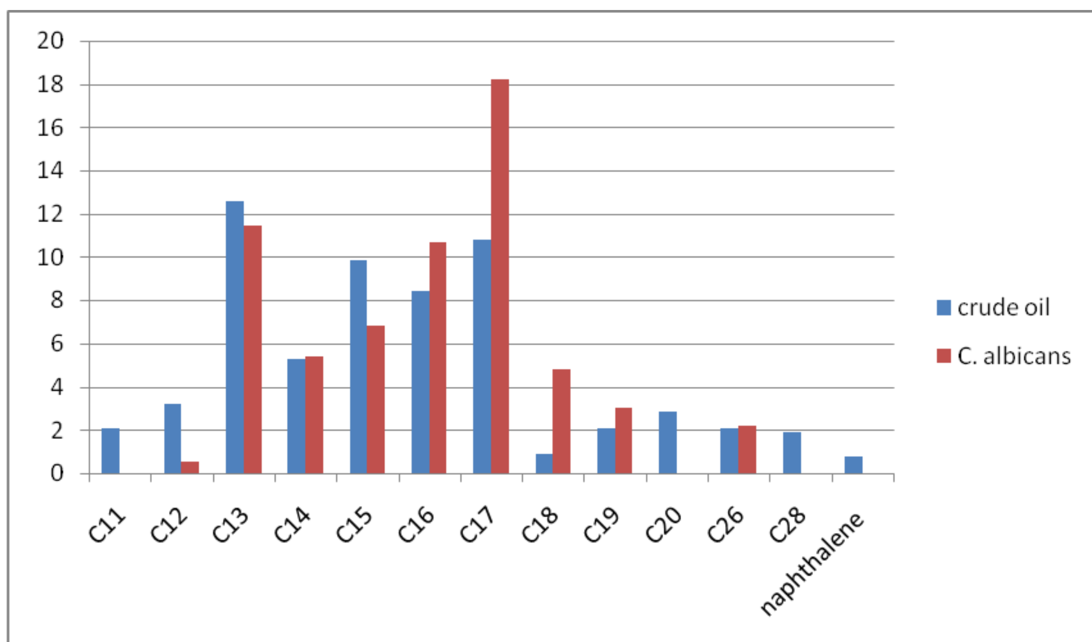


Figure 4.8. Disappearance of some hydrocarbon compounds and emergence of a difference in the percentage of others through degradation by *C.albicans*.

4.6. Effect of *C.parapsilosis* on petroleum hydrocarbons:

GC-MS analysis results of the remaining oil from biodegradation experiment of C. parapsilosis cultures showed that *C. parapsilosis* have the ability to degrade petroleum hydrocarbons as shown in the Figures (4.9),(4.10).

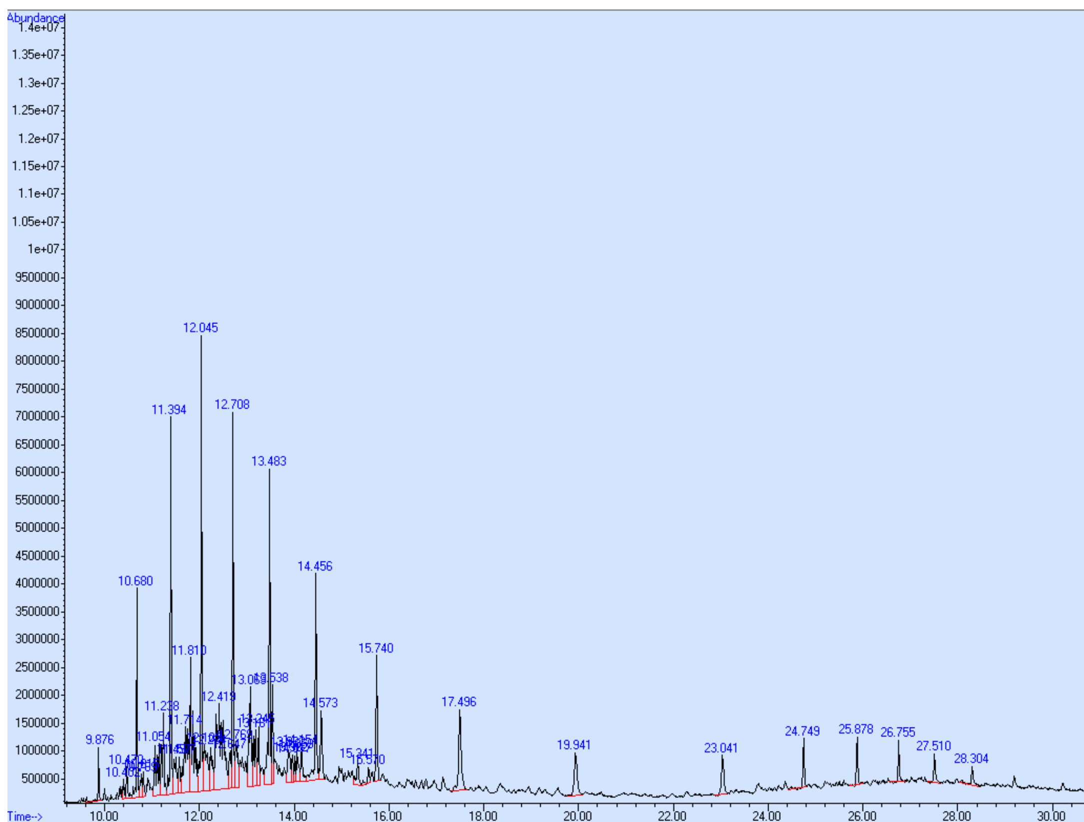


Figure 4.9. Gas Chromatography (GC) tracing of the hydrocarbon compounds degrading by *C.parapsilosis.* X-axis represents the retention time and Y-axis represents the hydrocarbon abundance.

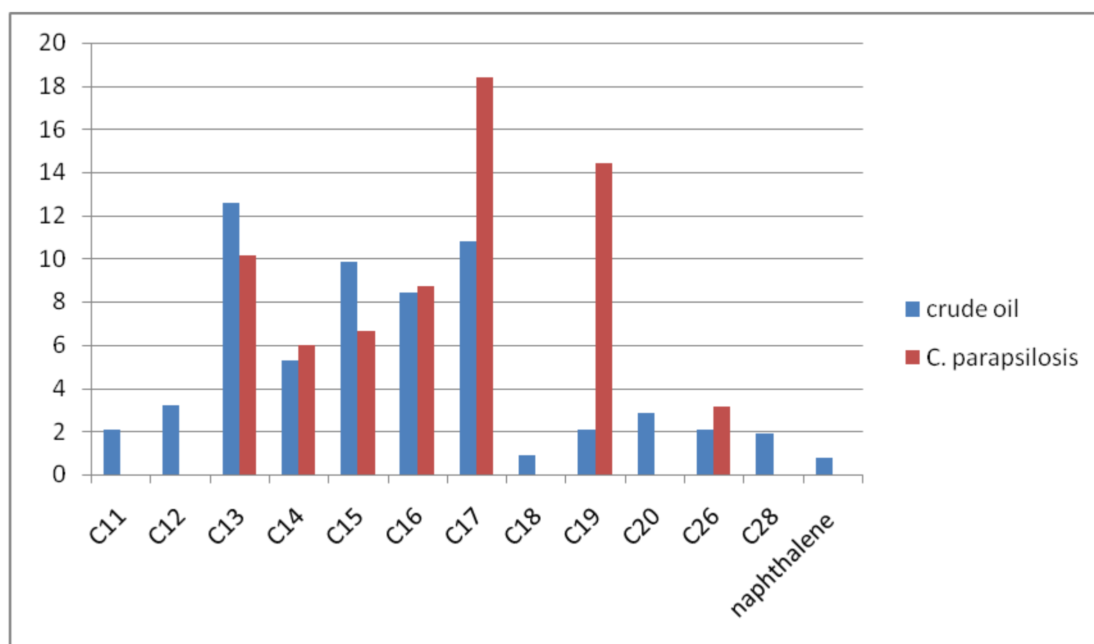


Figure 4.10. Disappearance of some hydrocarbon compounds and emergence of a difference in the percentage of others through degradation by *C.parapsilosis*.

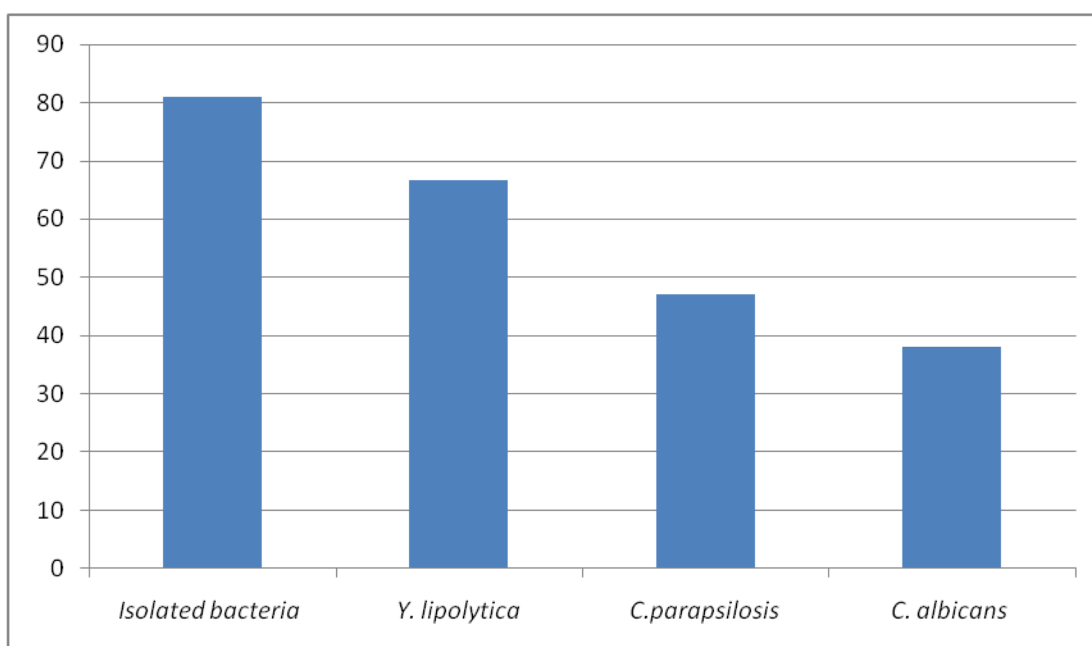


Figure 4.11. The percentage of hydrocarbon compounds consumed by the microorganisms used in the experiment after two weeks of incubation.

4.7. Test results for the growth of bacteria and yeasts:

Growth test results for bacteria and yeast showed their growing ability along the experiment as shown in table (4.3), which means that the colonies can use the petroleum oil as the sole carbon source for their metabolism and biodegradation.

Table 4.3. Results of growth test of the yeasts and mixed bacteria

Date	Mixed bacteria CFU/ mL	*Y.lipolytica* CFU/ mL	*C.albicans* CFU/ mL	*C. parapsilosis* CFU/ mL
24.09.2013	5×10^9	5.0×10^7	5×10^8	5×10^8
26.09.2013	5×10^9	1.5×10^7	5×10^8	5×10^8
28.09.2013	5×10^9	1.5×10^7	5×10^8	5×10^8
30.09. 2013	5×10^9	1.4×10^7	5×10^8	5×10^8
02.10. 2013	5×10^9	2.0×10^7	5×10^8	5×10^8
04.10. 2013	5×10^9	2.1×10^7	5×10^8	5×10^8
06.10. 2013	5×10^9	1.5×10^7	5×10^8	5×10^8

CHAPTER 5
DISCUSSIONS AND CONCLUSIONS

Due to their broad applications, petroleum products potentially contaminate soils and water, thereby they pose a serious threat to both the environment and human health. Power plants are one of the sites where petroleum products are used and therefore one of the sources of pollution of the environment. In this research work, it was found that the wastewater for Baiji power plant was contaminated with aliphatic hydrocarbons that contained n-alkane C11-C28 and aromatic hydrocarbons such as naphthalene as shown in table 4.2.

After two weeks of incubation of biotic samples, all microorganisms showed their ability to use the petroleum hydrocarbons, where GC-chromatogram of remaining crude oil clearly showed disappearance in the abundance of some hydrocarbon peaks by all tested microorganisms.

At the same time, some other organic compounds which were not originally present in the petroleum sample appeared. These compounds are considered to be a product of petroleum hydrocarbons degradation. In addition, a difference in the ratio of abundance of some hydrocarbon compounds has emerged; comparative with their ratio in original crude oil, the difference was either increase or decrease .The decrease in the ratio refers to degradation of hydrocarbon compounds by the microorganisms, while increase refers to the inability to use those compounds. It should be noted that the increase of ratio of the hydrocarbon compounds does not mean increase in their quantity, but means increase of abundance compared with remaining hydrocarbons after the biodegradation process.

Indigenous bacteria S. maltophilia and Achromobacter sp. showed their ability to degrade the petroleum hydrocarbons. While 17 from 21 of hydrocarbon compounds totally disappeared, 6 new hydrocarbon compounds appeared as a product of biodegradation after two weeks of incubation. This shows the possibility of the indigenous bacteria to degrade 80.95 % of the oil components.

In a similar study conducted by Arulazhagan et al.; 2010 *S. maltophilia* was isolated from the marine environment in Chennai, India. The bacteria could degrade (> 95%) polycyclic aromatic hydrocarbons at 30g/L of sodium chloride concentration in 4 days.

In another study conducted by Rushikesh Tasker B.S., 1998 *Achromobacter sp.* was isolated from a petroleum-contaminated water sample; the bacteria proved their ability to use diesel fuel as carbon source in an experiment that lasted for three weeks.

The yeast *Y.lipolytica* also showed the ability to use the petroleum hydrocarbons. Where 14 from 21 of hydrocarbon compounds disappeared totally, 4 new hydrocarbon compounds appeared as a product of biodegradation after two weeks of incubation. This shows the possibility of the *Y.lipolytica* yeast to degrade 66.6 % of the oil components.

Zinjarde et al., 1998 demonstrated the potential of the Y. lipolytica NCIM 3589 yeast to consume aliphatic fraction of crude oil and also pure alkanes (20-60% within 48 h).

The *C. albicans* yeast showed its ability to use the petroleum hydrocarbons through the disappearance of 8 of the 21 hydrocarbon components, and the emergence of 6 new hydrocarbon compounds as a product of biodegradation after two weeks of incubation. This shows the possibility of the *C. albicans* yeast to degrade 38.1 % of the oil components.

In a study conducted by Itah et al., 2009 the *C. albicans* yeast was isolated from aircraft fuel; the yeast could degrade 60.6 % of the same fuel after 14 days of incubation.

In a similar study, Ilori et al., 2008 demonstrated the potential of the yeast *C. albicans* to use the crude oil and diesel as sole sources of carbon and energy.

C.parapsilosis yeast also showed its ability to use the petroleum hydrocarbons, where 10 of the 21 of the hydrocarbon compounds disappeared totally, and 7 new hydrocarbon compounds appeared as a product of biodegradation after two weeks of incubation. This shows the possibility of the *C. parapsilosis* yeast to degrade 47.6 % of the oil components.

Klug and Markovetz, 1967 observed the ability of a high number of species of the genus *Candida,* including C. *albicans and C. parapsilosis,* to degrade alkanes and alkenes efficiently.

The yeast *Y. lipolytica* showed better ability to break down hydrocarbon compounds than the yeast *C. albicans* and *C. parapsilosis*. In a similar study conducted by Zinjarde and pant. 2002, *Y. lipolytica* was found to be the best among *C. albicans*, *C. parapsilosis*, *C. guilliermondii* , *C. intermedia* and *C. tropicalisto* to degrade the aliphatic fraction of Bombay High crude oil.

In this thesis work indigenous bacteria, the yeast *C. albicans* and *C. parapsilosis*, showed the ability to transform polycyclic aromatic hydrocarbons, while *C. albicans* and *C. parapsilosis* showed the inability to attack aromatic fractions of crude oil in a study conducted by Zinjarde and pant. 2002.

The bacterial growth is an indicator of hydrocarbon biodegradation in the incubation period. Increase and decrease of bacterial numbers are related with hydrocarbons and also the bacterial species, according to the results of viable cell count that were exhibited in the table (4.3). There wasn't any significant difference in bacterial growth through the first week of incubation, but decrease in growth was obtained in the twelfth day of the incubation period. Also, the yeasts *C. albicans*, *C.parapsilosis* showed no significant change in growth during the two weeks experiment period. *Y.lipolytica* growths fluctuated during the experiment; the experiment showed a decrease in growth during the first week of incubation, but showed an increase in growth in the second week**.**

Incubation usually causes increase of bacterial growth while, in some experiments, the number of microorganisms were reduced at the end of the incubation period, and in some other cases decrease in bacterial population occurs at the first step of the

biodegradation. This shows that the composition and concentration of hydrocarbons cause shock and stress on the bacteria and then the bacteria adapt gradually and incite some of the mechanisms that help re-growth and survival (Ebrahimi et al., 2012).

Klug and Markovetz, 1967 demonstrated that impurities of hydrocarbon substrates could cause growth limitation of genus Candida. That means that the purity of hydrocarbon substrates plays a role in the biodegradation process.

Toxicity of contaminants is one of the major reasons for biodegradation. According to the results, the reduction in hydrocarbons components concentration shows that all microorganisms used in this study have the ability to decrease the toxicity of these components. Similar results have been reached by Juhasz et al., 2000 showing that *s.maltophilia* strain VUN10,003 could be utilized for the detoxification of PAH-contaminated wastes.

Conclusion:

Comparing the results of biodegradation experiments through microorganisms, we see that the indigenous bacteria *S. maltophilia* and *Achromobacter sp.* have a better and greater ability to degrade hydrocarbon components than the three yeasts species. Also, *Y.lipolytica* was the best one between the yeasts. Therefore, the removal of wastewater pollutants for Baiji power plant is possible by creating favorable conditions for the bacteria to carry out the biodegradation, like adjusting the P_H values and the temperature, supplying the required nutrients and providing appropriate agitation.

Recommendations:

Future research could focus more on isolated degrading bacterial species (S. *maltophilia, Acromobacter sp*.) in order to identify their efficiency in biodegradation separately.

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