UNIVERSITI PUTRA MALAYSIA

CRUDE OIL DEGRADING BACTERIA: ISOLATION, GROWTH AND BIODEGRADATION STUDIES

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CRUDE OIL DEGRADING BACTERIA: ISOLATION, GROWTH AND BIODEGRADATION STUDIES

By

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I Dedicate This Work To My Mother -

KADEGA A. BUBAKAR

She who has over prayed for my success ! ! !

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In the name of ALLAH the most Benevolent and Merciful.

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LIST OF ABBREVIATIONS

BHA	Bushnell Hass agar
BH	Bushnell Hass
BMA	basal media agar
BM	basal media
CFU	colony forming unit
FID	flame ionization detector
FT-IR	Fourier transform infra red
GC	gas chromatograph
HC	hydrocarbons
nm	nanometer
NA	nutrient agar
NP	nitrogen and phosphorus
OD	optical density
rpm	round per minute
TSB	trypticase soy broth

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

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Faculty : Science and Environmental Studies

A total of 61 bacteria isolates were isolated from the four soil samples during the primary screening using 0.1% crude oil (v/v) as sole carbon source by the direct plating and enrichment method. However, only 23 isolates gave good growth when grown in 1% crude oil out of which 9 isolates were able to grow in crude oil up to 50% concentration.

Substrate specificity studies done on 5 of the 9 isolates, showed that all the 5 isolates (170, 181, 183, 217 and 224) were able to grow on all the 10 different hydrocarbon substrates tested with varying preferences. Optimum growth for all the 5 isolates were observed at 30 ^{o}C, pH 7.5, with 1% nitrogen and phosphorous supplements and shaken at 150 rpm. They were able to grow in media containing up to 2.3 % NaCl concentration.

Among the 5 isolates, isolate 170 gave the highest OD_{540} reading (0.469) and viability count ($1.9x10^7$) after 5 days. Isolates 217 and 183 gave the lowest growth with OD_{540} and viability count after 5 days, respectively. Infrared spectrometry (IR) analysis showed that all the 5 isolates caused some peaks reduction, disappearance, appearance and intensification after 7 days incubation.

Biochemical and morphological studies on the 5 isolates revealed that isolate 170, 183 and 217 belong to *Bacillus* sp, while, isolates 181 and 224 are *Pseudomonas* sp. Detailed biochemical identification indicate that isolate 170 is *Bacillus subtilis* and isolate 181 is *Pseudomonas aeruginosa*.

The bioremediation potential of the isolate 170 studied using liquid media and soil containing 0.5% crude oil showed that the isolate was able to degrade more then 80% of the hydrocarbons compounds of crude oil. The GC profile on day 7^{th}, 14^{th}, 21^{st} and 30^{th} day; showed that 26, 48, 78 and 81% respectively, of the total hydrocarbon were degraded in liquid media. Similarly with the soil sample, GC analysis showed that 21, 57, 71 and 86% reduction of total hydrocarbon in the samples occurred after 15^{th}, 30^{th}, 45^{th} and 60^{th} day incubation. Most of the degradation occurred during the exponential phase of the growth.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia bagi memenuhi keperluan Ijazah Master Sains.

BAKTERIA PENDEGRADASI MINYAK MENTAH: KAJIAN KE ATAS PEMENCILAN, PERTUMBUHAN DAN BIODEGRADASI

Oleh

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April 1998

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Sejumlah 61 pencilan bakteria telah dipencilkan daripada 4 sampel tanah semasa penyaringan awal, menggunakan 0.1% minyak mentah (i/i) sebagai sumber karbon tunggal, melalui kaedah pemplatan secara terus dan kaedah pengkayaan. Walaubagaimanapun, hanya 23 pencilan sahaja yang memberikan pertumbuhan yang baik apabila ditumbuhkan didalam 1% minyak mentah. Daripada jumlah tersebut, 9 pencilan berupaya untuk tumbuh didalam minyak mentah sehingga kepekatan 50%.

Kajian kespesifikan substrat yang dijalankan keatas 5 daripada 9 pencilan, menunjukkan bahawa kelima-lima pencilan tersebut (170, 180, 183, 217 dan 224) berupaya tumbuh didalam kesemua sepuluh substrat

hidrokarbon yang diuji dengan kecenderungan yang berbeza. Pertumbuhan optima untuk kelima-lima pencilan dicerap pada 30^0C, pH 7.5, dengan pertambahan 1% nitrogen dan phosforus dan digoncang pada 150ppm. Mereka berupaya tumbuh dalam media yang mengandungi kepekatan NaCl hingga 2.3%. Diantara kelima-lima pencilan, pencilan 170 memberikan bacaan OD_{540} (0.469) dan bilangan viabiliti ($1.9x10^7$), tertinggi selepas 5 hari. Manakala pencilan 217 dan 183 memberikan pertumbuhan yang terendah dengan bacaan OD_{540} dan bilangan viabiliti, masing-masing. Analisis spektrometri inframerah (IR) menunjukkan bahawa kelima-lima pencilan menyebabkan berlaku penyusutan, kehilangan, kemunculan dan peningkatan keamatan keatas beberapa puncak selepas 7 hari pengeraman.

Kajian biokimia dan morphologi keatas kelima-lima pencilan menunjukkan bahawa pencilan 170, 183 dan 217 adalah dari kumpulan *Bacillus* sp., manakala pencilan 181 dan 224 adalah *Pseudomonas* sp. Identifikasi biokimia yang terperinci menunjukkan bahawa pencilan 170 adalah *Bacillus subtilis* dan pencilan 181 adalah *Pseudomonas aeruginosa*.

Potensi bioremediasi oleh pencilan 170 yang dikaji menggunakan media cecair dan tanah yang mengandungi 0.5% minyak mentah, menunjukkan bahawa pencilan tersebut berupaya mengdegradasikan lebih 80% dari sebatian hidrokarbon dalam minyak mentah. Profil GC pada hari 7, 14, 21 dan 30, menunjukkan bahawa 26, 48, 78 dan 81%, masing-masing daripada jumlah hidrokarbon telah didegradasikan, dalam media cecair.

Keputusan yang sama diperolehi dengan sampel tanah dimana analisis GC menunjukkan lebih 21, 57, 71 dan 86% penurunan jumlah hidrokarbon didalam sampel berlaku selepas 15, 30, 45 dan 60 hari eraman. Kebanyakkan degradasi berlaku semasa fasa eksponen pertumbuhan.

CHAPTER I

INTRODUCTION

Oil pollution is probably one of the most important subjects in any discussion on environmental pollution. The air we breathe, the water we drink and bathe in, the soil in which our crops are grown, and the environments in which animals and plants grow are continually contaminated by a variety of synthetic chemicals. The environmental contamination by petroleum and its derivatives is a problem of increasing magnitude with obvious ecological and economic implications. In fact, considerable amounts of oil and hydrocarbon materials have always found their way into the ecosystem. Hydrocarbon contamination in water and soil are for example a consequence of spills from oil tankers and activities carried out in oil-drilling sites, petroleum refineries and storage facilities.

The elimination of these pollutants occurs in part through the activity of some microorganisms which have the capacity to utilise different hydrocarbons as carbon and energy source (Heitkamp and Cerniglia, 1988). The role of microorganisms in the degradation of organic materials is well established. Certain microorganisms had been shown to be able to use petroleum and its derivatives as the sole source of carbon and energy

(Bossert and Burtha, 1984; Heitkamp and Cernigila, 1988). Thus, biodegradation of hydrocarbon by microorganisms represents one of the primary mechanisms through which hydrocarbons pollutants could be eliminated from the environment (Leahy and Colwell, 1990).

Practical applications for a controlled microbiological process of fossil hydrocarbon conversions are numerous, ranging from product formation to removal of harmful materials. Biodegradation of oils is by no means a simple subject since, in nature, hydrocarbon decomposition involves interactions of complex physical, chemical and biological processes, the rates of, which are interdependent. Thus, it is understandable that the enzymatic systems and responsible organisms involved in the complete biodegradation of crude oil are ill-defined. Moreover, since crude oils vary in constituency from well to well, the complexity of developing a microbial system for decomposing fossil hydrocarbons becomes even more difficult. Evidence suggests the need to adequately supplement crude oil-enriched waters with proper nutrients (i.e., nitrogen and phosphate) to stimulate and maintain the biodegradation process (Ahearn and Meyers, 1973).

Each species generally metabolizes only a narrow spectrum of homologous hydrocarbons. Certain species produce constitutive enzymes, but in a good many species the enzymes which catalyze the oxidation of hydrocarbons are adaptive. Quite commonly pure cultures of various species have been induced to attack hydrocarbons by cultivating them in

appropriate heterotrophic media enriched with one or more hydrocarbons (Ahearn and Meyers, 1973).

Bioremediation is the use of microorganisms to mitigate hazards of soils or groundwater contaminated with organic waste materials. The technology has been receiving increased attention over the past 6-8 years due to the following reasons, it is cheaper compared to physical and chemical methods since it involve minimum amount of equipment and space, it can be carried out *in situ* and does not have toxic effect, thus environmental friendly and has long term effect.

Biological degradation of organic wastes can and usually does, result in complete destruction of the contaminants and elimination. Bioremediation has become more popular and subsequently more widely used. However, as the popularity of using biological treatment increase, the pressures for performance beyond developed capabilities also increase. It has long been realised that microorganisms are capable of metabolising naturally occurring organic compounds. However, many problematic environmental sites also have xenobiotic or synthetic compounds, which may, or may not be biodegradable. This latter phase has been the focus of much microbiology research in the laboratory for the past few years. However, field developments have primarily focused on the application of naturally occurring organic compounds, such as petroleum waste products.

Bioremediation of petroleum in soil using bacteria can be carried out by two methods. Firstly, through addition of nutrients into soil to stimulate the endogenous bacteria already present in the soil. These bacteria are stimulated to grow by introducing nutrients into the soil and thereby enhancing the biodegradation. The second method is through addition of exogenous bacteria with petroleum degradability to the contaminated site.

However, there are conflicting conclusions in the literature concerning the ability of individual isolates to degrade both the aliphatic and aromatic components in crude oil, Bushnell and Haas (1940) reviewed the earlier literature on this subject which was supportive of the idea that there were isolates that could use aliphatic but not aromatic compounds for growth. Similarly, Austin *et al.*, (1977) showed that there was some degree of specificity in the types of hydrocarbons degraded by given bacterial species.

It is known that both extrinsic factors, that is, the physico-chemical environment, and intrinsic microbial factors influence the rate of biodegradation of a specific hydrocarbon compound. The environmental constraints on degradation of petroleum hydrocarbons were extensively reviewed by Bartha and Atlas (1977) and Atlas (1981). The more important environmental factors include availability of oxygen, presence of nutrients (nitrogen and phosphorus mainly), temperature, presence of particulate matter, creation of stable emulsion, cometabolising microbial community, and previous exposure to petroleum hydrocarbons. These factors will influence the values of 'hydrocarbon degradation activity'.

Therefore, the specific objectives of the research are :

1. To screen and isolate hydrocarbon degrading bacteria.
2. To determine the substrate specificity of the potential isolates.
3. To determine the growth parameters affecting the growth of selected isolates in 1% crude oil.
4. To identify the potential isolates to genus or species level.
5. To study laboratory scale bioremediation process using water and soil contaminated with crude oil by a potential isolate.

CHAPTER II

LITERATURE REVIEW

Oil Toxicity

Oil pollution, whether due to spilling crude oil or refined product, may damage the environment in many different ways. Many biological processes which are important for the survival of marine organisms are affected by the presence of low concentration of petroleum hydrocarbons in the seawater (National Academy of Science, 1985). Oil toxicity is caused by the soluble fraction, which consists of low boiling point aromatic hydrocarbon which include benzene, toluene, xylene, naphthalene and phenanthrene. These substances can cause organism damage and mortality (Bishop, 1983) and the following damages to;

a) Marine Plants

i) Inhibition of Photosynthesis

Little is known of the mechanisms by which petroleum affects either photosynthesis or metabolism in marine plants. However, studies with