

**BIODEGRADATION OF PETROLEUM HYDROCARBONS
BY MICROBIAL CONSORTIA**

By

FARINAZLEEN MOHAMAD GHAZALI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

March 2004

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

BIODEGRADATION OF PETROLEUM HYDROCARBONS BY MICROBIAL CONSORTIA

By

FARINAZLEEN MOHAMAD GHAZALI

March 2004

Chairman: Professor Abu Bakar Salleh, Ph.D.

Faculty: Science and Environmental Studies

This investigation consisted of a series of studies designed with the primary objective of constructing microbial formulations or consortia that could effectively bioremediate hydrocarbon pollutions by way of their metabolic capabilities. This objective was achieved first by studying 46 bacterial strains that were initially isolated from Malaysian soils and waters that were polluted with hydrocarbons. The screenings on varying concentrations of crude oil resulted in the identification of potential isolates with differing abilities to grow on crude oil or its individual hydrocarbon components. Six bacterial isolates were finally chosen for further investigation and for the construction of bacterial formulations. These isolates consisted of two strains of *Pseudomonas* sp., three strains of *Bacillus* sp., and one strain of *Micrococcus* sp. The *Pseudomonas* sp. and *Micrococcus* sp. strains were seen to be able to tolerate and grow in high concentrations (up to 50% v/v) of crude oil and were able to utilize compounds such as aliphatic and monoaromatic hydrocarbons as well as alcohols as substrates.

The second section of this study involved the construction of different formulations made up of the six isolates identified in the earlier screening section. The formulations of bacterial consortia consisted of between two and six of the above-mentioned strains. Formulations made up of *Pseudomonas aeruginosa* and *Bacillus* sp. strains resulted in the highest extent of bioremediation. Between 39.74 and 61.09% of the *n*-alkanes C₁₀ to C₂₈ was degraded after 30 days of incubation with the bacterial mixture consisting of the two *P. aeruginosa* sp. and one *Bacillus* sp. strains.

Following the construction of the hydrocarbon-degrading microbial consortia, bioremediation studies were performed which were to make up the third section of this investigation. The growth patterns and degradation of aliphatic components of crude oil were studied in differing concentrations of crude oil and media salinity. Consortium 1, which consisted of *P. aeruginosa* S4.1, *P. aeruginosa* S5 and *Bacillus* sp. S3.2, was more efficient at biodegrading crude oil compared to Consortium 2, which comprised of *P. aeruginosa* S4.1, *P. aeruginosa* S5, *Bacillus* sp. S3.2, *Bacillus* sp. O63, *Bacillus* sp. 113i and *Micrococcus* sp. S.

Further bioremediation studies were conducted using Consortium 1. The effects of incubation temperature, agitation rates, media pH, oil-medium interface area, bacterial adaptation, inoculum size, fertilizer addition and surfactant addition were investigated in liquid media. Bioremediation of crude oil by Consortium 1 was most extensive when artificial seawater with pH 7 was used as the culture medium. The addition of 0.1% of Triton X-100, a synthetic surfactant, into the culture medium improved bioremediation. Prior exposure to crude oil of the inoculum also enhanced bioremediation extent and rates. The starting inoculum size of 5% resulted in the

highest biodegradation of crude oil. An increased oil-medium interface area also resulted in enhanced bioremediation. The incubation conditions which lead to higher biodegradation extent were at 37°C and shaken on horizontal shaker at 100 rpm. When conducted under all the optimised parameters described above, Consortium 1 was seen to remove between 55.80 and 70.56% of *n*-alkanes following a 60-day bioremediation process.

In the fourth section of this investigation, the bacterial cells of Consortium 1 were immobilized using calcium alginate to study the possibility of reusing hydrocarbon-degrading bacteria in bioremediation processes. The entrapment of the bacterial cells did not impair the biodegradation activity of Consortium 1. The calcium alginate-entrapped cells could also be used repeatedly without loss of biodegradation capacity for up to 5 times or a total of 150 days of use.

The fifth and final section of this investigation involved the application of the bacterial consortia onto polluted soil samples in a lab-scale pilot study. Consortia 1 and 2 were tested using beach sand polluted by crude oil and soil polluted by a diesel spill. In contrast to the observations made in liquid media where Consortium 1 was most effective, Consortium 2 was seen to be more effective at removing hydrocarbon contamination in the polluted beach sand and soil. In the beach sand, between 21.01 and 80.19% of alkanes remained after 30 days with Consortium 1 whilst between 15.30 and 27.11% of these compounds remained when Consortium 2 was used. Repeated application of Consortium 2 onto the beach sand resulted in accelerated degradation of crude oil. In the engine-oil polluted soil, it was seen that the addition of Consortium 2 resulted in a rapid removal of hydrocarbons where the aliphatic compounds had fallen to undetectable levels within 30 days.

Overall, this investigation had achieved its primary objective of designing microbial formulations or consortia that could be employed in the bioremediation of petroleum hydrocarbon pollutions.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**PENGURAIAN PENCEMARAN TUMPAHAN MINYAK
OLEH FORMULASI BAKTERIA**

Oleh

FARINAZLEEN MOHAMAD GHAZALI

March 2004

Pengerusi: Profesor Abu Bakar Salleh, Ph.D.

Fakulti: Sains dan Pengajian Alam Sekitar

Matlamat utama kajian ini adalah untuk menghasilkan formulasi campuran mikroorganisma yang dapat digunakan dalam pemulihan pencemaran tumpahan minyak. Untuk mencapai matlamat ini, beberapa kajian telah dirancang bermula dengan mengkaji koleksi bakteria yang telah diasingkan dan dituliskan dari sampel tanah dan air yang tercemar dengan tumpahan minyak. Sebanyak 46 kultur tulen bakteria telah dikaji dalam bahagian pertama pengajian ini. Seterusnya enam kultur tulen telah dipilih untuk digunakan dalam menghasilkan campuran or konsortium bakteria. Kultur-kultur ini merupakan 2 *Pseudomonas* sp., 3 *Bacillus* sp., dan 1 *Micrococcus* sp. Kultur-kultur *Pseudomonas* sp. dan *Micrococcus* sp. dapat hidup di dalam kepekatan 50% minyak dan juga boleh menggunakan hidrokarbon jenis alifatik dan monoaromatik dan juga alkohol sebagai sumber makanan.

Di dalam bahagian kedua kajian ini, beberapa formulasi telah dihasilkan menggunakan kultur-kultur yang telah dipilih di dalam bahagian pertama pengajian. Campuran-campuran ini mengandungi antara 2 hingga 6 kultur yang tersebut di

atas. Formulasi yang mengandung *Pseudomonas* sp. dan *Bacillus* sp. menghasilkan penguraian minyak mentah yang paling efektif. Di antara 39.91 dan 61.26% alkana C₁₀ hingga C₂₈ telah diuraikan dalam 30 hari apabila campuran 2 *Pseudomonas* sp. dan satu *Bacillus* sp. digunakan.

Ujikaji dalam bahagian ketiga penyelidikan ini merupakan kajian untuk menentukan keadaan-keadaan optima bagi proses penguraian minyak mentah menggunakan formulasi-formulasi bakteria yang telah dihasilkan di dalam bahagian kedua pengajian. Kadar pertumbuhan dan tahap penguraian minyak mentah bagi formulasi bakteria di dalam kepekatan minyak dan kandungan garam yang berbeza-beza telah dikaji Konsortium 1, yang mengandungi *P. aeruginosa* S4.1, *P. aeruginosa* S5 dan *Bacillus* sp. S3.2 adalah lebih berkesan dalam penguraian minyak mentah berbanding dengan Konsortium 2, yang mengandungi *P. aeruginosa* S4.1, *P. aeruginosa* S5, *Bacillus* sp. S3.2, *Bacillus* sp. O63, *Bacillus* sp. 113i dan *Micrococcus* sp. S.

Berikutan pemerhatian Konsortium 1 adalah paling efektif dalam proses bioremediasi berbanding dengan formulasi yang lain, kajian-kajian pengoptimaan yang seterusnya telah dijalankan dengan menggunakan Konsortium 1. Kesan-kesan suhu pengeraman, kadar penggoncangan, pH media, keluasan permukaan antara minyak dan media, penyesuaian bakteria melalui pendedahan terhadap minyak mentah, saiz inoculum, pembajaan dan surfaktan telah dikaji. Keputusan keseluruhan bagi kajian pengoptimaan menunjukkan proses bioremediasi minyak oleh Konsortium 1 adalah paling berkesan di dalam media air laut dengan pH 7. Kehadiran surfaktan Triton X-100 dengan kepekatan akhir 0.1% juga merangsang bioremediasi. Pendedahan kepada minyak mentah bagi proses adaptasi sebelum

digunakan sebagai inokulum menghasilkan kadar penguraian lebih banyak minyak mentah dalam jangka masa yang singkat. Suhu pengeraman optima adalah 37°C manakala kadar goncangan optima ialah 100 putaran seminit. Apabila proses bioremediasi dijalankan di bawah kesemua parameter optima yang tersebut di atas, Konsortium 1 telah berjaya menguraikan di antara 55.80 sehingga 70.56% *n*-alkana di dalam masa 60 hari.

Di dalam bahagian keempat kajian ini, sel-sel Konsortium 1 telah disekatgerak menggunakan manik-manik kalsium alginat sebagai agen penyekat-gerak bagi mengkaji kesesuaiannya bagi penggunaan berulang-kali dalam proses bioremediasi. Proses sekat-gerak didapati tidak mendatangkan kesan negatif terhadap keupayaan Konsortium 1 dalam menguraikan minyak mentah. Manik-manik kalsium alginate yang mengandungi sel Konsortium 1 dapat digunakan sebanyak 5 kali ataupun selama sejumlah 150 hari tanpa menunjukkan kadar penurunan penguraian.

Bahagian kelima kajian ini melibatkan aplikasi formulasi bakteria ke atas sampel tanah yang tercemar dengan tumpahan minyak. Konsortium 1 dan 2 telah diuji dengan menggunakan sampel pasir yang dicemari dengan minyak mentah dan tanah yang dicemari dengan minyak diesel. Berbeza dengan pemerhatian di mana Konsortium 1 didapati paling berkesan bagi proses bioremediasi di dalam media cecair, di dalam sampel tanah tercemar pula didapati Konsortium 2 yang paling efektif dalam penguraian bahan tercemar. Di antara 47.40 dan 81.43% *n*-alkana dapat dikesan di dalam sampel tanah selepas 60 hari apabila Konsortium 2 digunakan sebagai agen bioremediasi berbanding di antara 74.50 dan 90.51% *n*-alkana masih terdapat di dalam sampel tanah apabila Konsortium 1 digunakan. Apabila digunakan di dalam kajian bioremediasi tanah yang tercemar dengan

minyak mesin, Konsortium 2 telah menghasilkan penguraian hidrokarbon dengan cepat di mana bahan alifatik tidak lagi dapat dikesan selepas 30 hari.

Secara keseluruhan, pengajian ini telah mencapai matlamatnya iaitu penghasilan formulasi-formulasi bakteria yang dapat digunakan bagi proses-proses pemulihan alam sekitar yang tercemar oleh tumpahan minyak dan bahan-bahan hidrokarbon lain.

ACKNOWLEDGEMENTS

All praises to the mighty Allah, the merciful and beneficent for the strength and blessings throughout this study.

I am deeply indebted to a number of people with whom I have worked and from whom I have learnt so much and have helped me in my task.

I wish most particularly to express my greatest gratitude to my supervisors, Professor Abu Bakar Salleh, Professor Mahiran Basri and Associate Professor Raja Noor Zaliha Abd Rahman, for their guidance, invaluable criticisms, suggestions and the great support demonstrated while seeing this thesis to its completion. I am also very much indebted to Associate Professor Che Nyonya Abd Razak for the supervision rendered in the earlier areas of my study.

I am also very grateful to the Malaysian Ministry of Science, Technology and Environment for the National Science Fellowship award, which has financed my study program. An exceptional mention also goes to the staff of the Faculty of Science and Environmental Studies, particularly those from the Department of Biochemistry and Microbiology.

Special thanks are also due to those with whom I have shared the Enzyme and Microbial Technology Research Laboratory, for their support and the friendship developed.

Most of all, I am indebted to my family, for their immeasurable support, encouragement and understanding, and especially, my husband Shah, for his unfailing patience and faith, my children Anissa and Ameer for being the source of inspiration and my mother for her love and prayers.

I certify that an Examination Committee met on 11th of March, 2004 to conduct the final examination of Farinazleen Mohamad Ghazali on her Doctor of Philosophy thesis entitled "Biodegradation of petroleum Hydrocarbon Pollution by Microbial Consortia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Norhani Abdullah, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

Abu Bakar Saleh, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Mahiran Basri, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Raja Noor Zaliha Abdul Rahman, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Abd. Jalil Kadir, Ph.D.

Professor
School of Biosciences and Biotechnology
Universiti Kebangsaan Malaysia
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, Ph.D.

Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Abu Bakar Saleh, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

Mahiran Basri, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Raja Noor Zaliha Abdul Rahman, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or any other institutions.

FARINAZLEEN MOHAMAD GHAZALI

Date:

TABLE OF CONTENTS

	Page
ABSTRACT	2
ABSTRAK	6
ACKNOWLEDGEMENTS	10
APPROVAL	11
DECLARATION	13
LIST OF TABLES	18
LIST OF FIGURES	19
LIST OF ABBREVIATIONS	25
CHAPTER	
1 INTRODUCTION	26
2 LITERATURE REVIEW	28
2.1 Petroleum formation and accumulation	28
2.1.1 Crude oil	28
2.1.2 Sources of hydrocarbon contaminants in the environment	30
2.2 Behaviour and effects of hydrocarbon pollutants on microbial community in soils	31
2.3 Behaviour and effects of hydrocarbon pollutants on microbial community in the aquatic systems	32
2.4 Traditional cleanup techniques in mediating hydrocarbon contamination	34
2.5 Biodegradation and bioremediation	36
2.5.1 Microorganisms that metabolize crude oil and other petroleum products	38
2.5.2 Genetic encoding of hydrocarbon degradation	41
2.5.3 Microbial degradation of alkanes	42
2.5.4 Microbial degradation of monoaromatic hydrocarbons	45
2.5.5 Microbial degradation of polyaromatic hydrocarbons	47
2.6 Factors influencing rates of microbial degradation of hydrocarbons	52
2.6.1 Temperature	52
2.6.2 pH	55
2.6.3 Salinity	55
2.6.4 Oxygen	56
2.6.5 Nutrients	58
2.6.6 Chemical composition of petroleum	60
2.6.7 Physical state of the hydrocarbon	60
2.6.8 Concentration of the petroleum or hydrocarbons	61
2.6.9 Adaptation	62
2.7 Petroleum biodegradation by microbial consortia	63
2.8 Immobilized cells	66
2.8.1 Introduction	66

	2.8.2	Applications and advantages of immobilized cells	67
	2.8.3	Immobilized cells and bioremediation of petroleum hydrocarbons	69
3		MATERIALS AND METHODS	71
	3.1	Materials- Reagents and instruments	71
	3.2	Media preparation	73
	3.2.1	Artificial Seawater Medium	73
	3.2.2	Basal Medium	73
	3.2.3	Bushnell-Haas Broth	74
	3.2.4	Tryptic Soy Broth	74
	3.2.5	Nutrient Agar Plates	74
	3.3	Solutions and reagents preparation	74
	3.3.1	Nitrogen-Phosphorus (NP) stock solution	74
	3.3.2	Substrate stock solutions	75
	3.3.3	Bradford reagent	75
	3.3.4	NaOH solution	75
	3.3.5	Saline solution	76
	3.4	Monitoring bacterial growth	76
	3.4.1	Colony-forming unit counts	76
	3.4.2	Whole cell protein assays	76
	3.5	Bioremediation assay	77
	3.5.1	Extraction of residual crude oil	77
	3.5.2	Analyses of extract	78
	3.5.3	Evaluation of bioremediation	78
	3.6	Preparation of bacterial isolates	78
	3.6.1	Preparation of working and stock cultures	78
	3.6.2	Preparation of bacterial inoculum	79
	3.7	Preliminary screening of isolates on crude oil	80
	3.7.1	Selection of isolates	80
	3.7.2	Primary screening	80
	3.7.3	Secondary screening	81
	3.8	Substrate specificity study	81
	3.9	Construction of bacterial consortia	82
	3.9.1	Preparation of consortia inoculum	82
	3.10	Growth and degradation study in liquid medium	83
	3.10.1	Effects of salinity	83
	3.10.2	Effects of crude oil concentrations	83
	3.10.3	Effects of incubation temperature	84
	3.10.4	Effects of agitation	84
	3.10.5	Effects of pH	84
	3.10.6	Effects of contact area	85
	3.10.7	Effects of pre-conditioning	85
	3.10.8	Effects of replacing inorganic salts in medium with fertilizers	86
	3.10.9	Effects of inoculum size	86
	3.10.10	Effects of surfactants	87
	3.11	Biodegradation of hydrocarbons by immobilized bacterial cells	87
	3.11.1	Immobilization of bacterial cells in alginate	87
	3.11.2	Batch fermentations	88
	3.11.3	Repeated batch fermentations	88
	3.12	Biodegradation study in polluted soils	89

3.12.1	Crude oil-polluted beach sand	89
3.12.2	Diesel-polluted soil	90
3.12.3	Used-engine oil-polluted soil	91
3.12.4	Effects of hydrocarbon amounts in beach sand on biodegradation	92
4	RESULTS	93
4.1	Preliminary screening of isolates on crude oil	93
4.1.1	Selection of isolates	93
4.1.2	Primary screening	93
4.1.3	Secondary screening	96
4.2	Substrate specificity study	98
4.3	Construction of bacterial consortia	102
4.3.1	Selection and identification of suitable isolates	102
4.3.2	Formulation of bacterial consortia	108
4.4	Growth and degradation study in liquid medium	114
4.4.1	Effects of salinity	114
4.4.2	Effects of crude oil concentrations	122
4.4.3	Effects of temperature	127
4.4.4	Effects of agitation	130
4.4.5	Effects of pH	134
4.4.6	Effects of oil-medium interface area	137
4.4.7	Effects of pre-conditioning	139
4.4.8	Effects of replacing inorganic salts in medium with fertilizers	144
4.4.9	Effects of inoculum size	151
4.4.10	Effects of surfactants	154
4.4.11	Biodegradation of crude oil under combined optimized conditions	166
4.5	Biodegradation of hydrocarbons by immobilized bacterial cells	169
4.5.1	Batch fermentations	169
4.5.2	Repeated batch fermentations	172
4.6	Biodegradation study in polluted soils	175
4.6.1	Crude oil-polluted beach sand	175
4.6.2	Diesel-polluted soil	183
4.6.3	Used-engine oil-polluted soil	188
4.6.4	Effects of hydrocarbon amounts in beach sand on biodegradation	192
5	DISCUSSION	208
5.1	Selection and screening of isolates for investigation	208
	Primary screening	208
	Secondary screening	211
	Substrate specificity study	212
5.2	Selection of suitable isolates for the construction of bacterial consortia	217
	Identification of isolates	219
	Construction of consortia	221
	Analysis of residual hydrocarbons as an assessment of bioremediation	224
5.3	Biodegradation study in liquid medium	224

	Salinity	225
	Concentration of crude oil	225
	Incubation temperature	228
	Agitation	229
	pH	230
	Oil-medium interface area	231
	Adaptation	232
	Fertilizer addition	232
	Inoculum size	235
	Surfactants	236
	Overall conclusion of biodegradation study in liquid medium	240
5.4	Immobilization	241
	Batch fermentations	241
	Repeated batch fermentations	242
5.5	Biodegradation study in polluted soils	244
	Beach sand contaminated with crude oil	244
	Soil contaminated with diesel	246
	Soil contaminated with used engine oil	250
	Effects of oil concentration levels on hydrocarbon biodegradation in beach sand	253
6	CONCLUSIONS	255
7	RECOMMENDATIONS FOR FURTHER STUDY	257
	REFERENCES	258
	APPENDICES	280
	BIODATA OF THE AUTHOR	287
	PUBLICATIONS AND PROCEEDINGS	288