BIODEGRADATION OF PETROLEUM HYDROCARBONS BY MICROBIAL CONSORTIA

By

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

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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 C_{10} to C_{28} 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 37ºC 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

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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 ditulenkan 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 mengandungi *Pseudomonas* sp. dan *Bacillus* sp. menghasilkan penguraian minyak mentah yang paling efektif. Di antara 39.91 dan 61.26% alkana C_{10} hingga C_{28} 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 37ºC 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 pengunaan 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 enjin, Konsortium 2 telah menghasilkan penguraian hidrokarbon dengan cepat di mana bahan alifatik tidak lagi dapat dikesan selepas 30 hari.

Secara kesuluruhan, 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.

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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 etroleum Hydrocarbon Pollution by Microbila 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:

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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

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