UNIVERSITI PUTRA MALAYSIA

# *ISOLATION OF BACTERIA FROM CONTAMINATED SOIL AND THEIR BIODEGRADATION POTENTIAL*

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# ISOLATION OF BACTERIA FROM CONTAMINATED SOIL AND THEIR BIODEGRADATION POTENTIAL

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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**ISOLATION OF BACTERIA FROM CONTAMINATED SOIL AND THEIR BIODEGRADATION POTENTIAL**

By

**PARDIS KARIMI ALAVIJEH**

**September 2015**

**Chair : Normala bt Halimoon, PhD**
**Faculty : Environmental Studies**

Industrialization is accompanied by inevitable complications among which pollutions and global warming are of utmost importance. Oil spills are an important part of pollution and can be very hard to clean up, requiring weeks to even years. Among various different remediation methods, bioremediation is considered one of the best. Fundamental concern of this work was enhancing biodegradation capability of potential indigenous tropical soil bacteria without genetic mutations, changing soil mineral ratios or soil microbial community. Thus, in a new approach suitable bacterial isolates adapted sequentially to higher concentrations of crude oil at pH and temperature values simulating their natural habitat. Their biodegradation ability in terms of n-alkane removal analyzed both in liquid medium and soil within 28 days. In doing so, soil bacteria were isolated, enriched and selected based on their ability to grow on 5% (v/v) crude oil, leading to selection of 8 isolates. Later a simple mathematical model for their growth was calculated to monitor their growth easily and accurately. At later stages isolates were adapted sequentially to higher concentration of crude oil, i.e. 10, 20 and 50% (v/v) at the pH and temperature values similar to their natural habitat. Their growth monitored using their growth equations and exponential growth rate and doubling time of each isolate at each concentration was calculated. Only 5 out of 8 isolates could survive 50% (v/v) crude oil and the other 3 showed negative values for exponential growth indicateing lack of growth at 50% (v/v). To identify the bacteria, biochemical, morphological and molecular identification techniques were conducted; Top 5 oil degraders were identified *as P. putida, A. lwoffi, A. hydrophila, P. stutzeri*, and *A. johnsonii* by 16S rRNA sequencing; The other 3 isolates identified morphologically and biochemically only, and found to be 1 isolate from *Rhodococcus spp.* and 2 from Bacillus spp. Based on gas chromatography-mass spectrometry analysis adapted isolates and their consortium proved to be more efficient in n-alkane degradation as removal of C8-C33 was enhanced up to 93% in liquid cultures and 70% in artificially polluted soil after 28 days. *P. putida, A. lwoffi, A. hydrophila, P. stutzeri,* and *A. johnsonii* removed 74%, 56%, 76%, 61%, and 67%, respectively from a liquid medium amended with 50% (v/v) crude oil. A comparison between degradation ability of 5 bacterial mixture in soil before and after sequential adaptation showed that their biodegradation ability was enhanced up to 41%

without any exposure to biological mutation or addition of nutrients. In addition ratios of $n_{17}$ to pristane and $n_{18}$ to phytane before and after degradation supported the promising bioremediation ability of the consortiums and that removal was indeed happened as a result of their activity. In general result suggests preconditioning bacterial isolates to higher oil concentration can significantly enhance their biodegradation ability especially when the isolates are applied as a consortium.

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

**PENGASINGAN BAKTERIA DARI TANAH TERCEMAR DAN POTENSI BIODEGRADASI MEREKA**

Oleh

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Perindustrian disertai dengan komplikasi yang tidak dapat dielakkan diantara pencemaran dan pemanasan global adalah amat penting.Tumpahan minyak adalah salah satu bahagian penting dalam pencemaran dan boleh jadi sangat sukar untuk dibersihkan, yang memerlukan beberapa minggu hingga bertahun-tahun. Antara pelbagai kaedah pemulihan yang berbeza, bio-remediasi dianggap sebagai salah satu yang terbaik. Perhatian asas kerja ini telah meningkatkan keupayaan potensi bio-penguraian bakteria asli tanah tropika tanpa mutasi genetik, perubahan nisbah mineral tanah atau komuniti mikrob tanah. Oleh itu, dalam pendekatan baru bakteria asingan yang sesuai jujukan diadaptasikan pada kepekatan minyak mentah yang lebih tinggi pada nilai pH dan suhu yang disimulasikan habitat semulajadinya. Keupayaan biopenguraiannya dari segi analisis penyingkiran *n*-alkana kedua-duanya dalam medium cecair dan tanah dalam tempoh 28 hari. Dengan berbuat demikian, bakteria tanah dipencil, diperkaya dan dipilih berdasarkan kepada keupayaannya untuk tumbuh pada 5% (v/v) minyak mentah, yang membawa kepada pemilihan 8 asingan. Kemudian model matematik mudah untuk pertumbuhannya dikira bagi memantau pertumbuhannya dengan mudah dan tepat. Pada langkah seterusnya, asingan jujukan diadaptasikan pada kepekatan minyak mentah yang lebih tinggi, iaitu 10, 20 dan 50% (v/v) pada nilai pH dan suhu yang sama dengan habitat semulajadinya. Pertumbuhannya dipantau menggunakan persamaan pertumbuhan dan kadar pertumbuhan eksponen serta masa gandaan setiap asingan pada setiap kepekatan dikira. Hanya 5 daripada 8 asingan boleh terus hidup pada 50% (v/v) minyak mentah dan 3 yang lain menunjukkan nilai negatif pada pertumbuhan eksponen menunjukkan vi pengurangan pertumbuhan pada 50% (v/v). Untuk mengenalpasti bakteria tersebut, teknik biokimia, morfologi dan pengenalpastian molekul telah dijalankan; 5 pengurai minyak yang tertinggi telah dikenalpasti sebagai *P. putida, A. lwoffi, A. hydrophila, P. stutzeri,* dan *A. johnsonii* secara penjujukan rRNA 16S; 3 asingan lagi dikenalpasti morfologi dan biokimia sahaja, dan didapati 1 asingan dari *Rhodococcus spp.* dan 2 daripada *Bacillus spp.* Berdasarkan analisis gas kromatografi-spektrometri jisim asingan yang diadaptasi dan konsortiumnya terbukti lebih berkesan dalam penguraian *n*-alkana sebagai penyingkiran C8-C33 telah meningkat sehingga lebih dari 93% dalam kultur

cecair dan 70% di dalam tanah buatan tercemar selepas 28 hari. *P. putida, A. lwoffi, A. hydrophila, P. stutzeri,* dan *A. johnsonii* menyingkirkan 74%, 56%, 76%, 61%, dan 67%, masing-masing daripada media cecair yang dipinda dengan 50% (v/v) minyak mentah. Perbandingan diantara keupayaan penguraian 5 campuran bakteria di dalam tanah sebelum dan selepas adaptasi urutan menunjukkan bahawa keupayaan bio-penguraiannya telah meningkat sehingga lebih dari 41% tanpa sebarang pendedahan kepada mutase biologi atau penambahan nutrien. Tambahan pula, nisbah *n*17 pada pristane dan *n*18 pada phytane sebelum dan selepas penguraian menyokong keupayaan bio-remediasi yang dijanjikan bagi konsortium dan penyingkiran itu sememangnya berlaku hasil daripada aktivitinya. Secara umumnya keputusan mencadangkan keadaan awal bakteria yang diasingkan pada kepekatan minyak yang lebih tinggi secara ketara boleh meningkatkan keupayaan bio-penguraiannya terutama apabila asingan digunakan sebagai konsortium.

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I certify that a Thesis Examination Committee has met on 29 September 2015 to conduct the final examination of Pardis Karimi Alavijeh on her thesis entitled "Isolation of Bacteria from Contaminated Soil and their Biodegradation Potential" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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| | |
|---|---|
| MSM | Mineral Salt Media |
| TSA | Tryptic Soy Agar |
| TSB | Tryptic Soy Broth |
| $MgSO_4$ | Magnesium sulfate |
| $CaCl_2$ | Cacium Chloride |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| $NH_4NO_3$ | Ammonium nitrate |
| $FeCl_3$ | Iron(III) chloride |
| $CaCl_2.2H_2O$ | Calcium Chloride Dihydrate |
| NaCl | Sodium chloride |
| $H_2SO_4$ | Sulfuric acids |
| $BaCl_2$ | Anhydrous barium chloride |
| OD | Optical Density |
| OD600 | Optical Density at 600 Nanometer |
| CFU | Colony Count Unit |
| CFU/mL | Colony Count Unit per milliliter |
| Cell/mL | Bacterial cell per milliliter |
| °C | Degree Centigrade |
| RPM | Revoltions per Minutes |
| %v/v | Volume per Volume percentage |
| mL | Mili Litre |
| g | Gram |
| g/L | Gram per Litre |
| cm | Centimeter |
| PCR | Polymerase Chain Reaction |
| UV | Ultra Violet |
| pr | Pristane |
| ph | Phytane |

**CHAPTER 1**

# INTRODUCTION

## 1.1 Introduction

In the past hundred years, a large number of ecosystems have been changed by the growing influence of human activity. As a result, we as humans become more and more aware of the need to protect our ecosystem as well as to control and reduce the extent of damage caused by various human activities. One of the most readily available examples of such risky activities are the ones that result in polluting soil, water and air. Due to insatiable hunger of humans for energy and the fact that crude oil is a relatively cheap and efficient source of energy and raw materials, oil pollutions as a result of its extraction, transportation and refining have been frequently affecting different environments and ecosystems all around the globe (Emami *et. al.,* 2014; Paíga *et. al.,* 2012).

Soil contamination with hydrocarbons causes extensive damage to local ecosystems as accumulation of pollutants in animal and plant tissues may cause infertility, unfavorable genetic mutations and catastrophic deaths (Arellano *et. al.,* 2015; Kriti and Chandra, 2014). By international and domestic laws and regulations of most countries release of petroleum into environment is strictly regulated due to its complex, non-aqueous, and hydrophobic nature of petroleum (Mandal, 2012). However in case of an inevitable accident, the pollution requires to be dealt with accordingly. Generally, various physiochemical cleanup techniques are costly, more energy demanding, and are not environmental sustainable (Liu, 2010). In some cases, their environmental impact like their adverse damage on soil structure and release of toxic elements have been reported (Sample, 2001).

Therefore it is of utmost importance to be able to constantly come up with innovative and effective measures to clean such contaminations effectively and permanently. One of the most effective and yet natural ways to clean these kinds of pollutions is with the help of biological organisms which is also known as bioremediation. The fact that petroleum is a good energy and electron source for microbes; makes use petroleum hydrocarbons an attractive food source which consequently results in complex hydrocarbons break down to the simpler forms. Hence, petroleum is both a product of microbes and also an energy source for them (Ehrlich, 1995).

The success of oil spill bioremediation depends on the ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment, meaning that the oil degraders should be selected in a way to suite the especial environment they are going to be applied to and at the same time should be provided with favorable conditions so they can fulfil degradation of hydrocarbon pollutants to the best of their potentials (Battikhi, 2014; Das and Chandran, 2011). There are limitations in taking both side into account however there are various methods currently being practiced and the search for better ones is ongoing.

Numerous scientific reviews have covered various factors that influence the rate of oil biodegradation. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities (Adams *et. al.,* 2015). If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that other requirements of biodegradation are at adequate level. Most existing studies have concentrated on evaluating the factors affecting oil bioremediation or testing favored conditions and external enhancers such as nutrients and supplements to act in combination with microbes to yield possible maximum hydrocarbon removal. Moreover the scope of current understanding of oil bioremediation is also limited because the emphasis of most of field studies and reviews have been on the evaluation of bioremediation technology rather than on whether these methods are environmental friendly themselves particularly in long run.

Hydrocarbon removal by bacteria is extremely dependent on the environmental situation like pH, temperature, contamination concentration extent of pollution (Bossert, 1984). The complexity of the hydrocarbon is also important in the bioremediation rate and extent; the less the complexity, the higher the extent of bioremediation would be. Although majority of petroleum hydrocarbon are degradable by bacteria; however, low molecular weight hydrocarbons are easier to be degraded and more complex compounds of petroleum hydrocarbons; for example, long-chained alkanes are not degradable instantly and completely because of their extremely hydrophobic properties (Caumette, 2004); therefore, no matter how favorable the conditions are or how strong are the metabolism of the indigenous microorganism, complete cleanup is still a matter of time.

One possible and effective solution is to generate biotechnologically improved bacterial populations as a way to enhance degradation capacity; however, this method may cause yet other complications concerning introduction of genetically modified organisms (GMO) in the environment. The introduced GM bacteria may have an unknown adverse effect on the environment by influencing ecological factors or inflicting pathogenic effects on different members of the ecosystem (Megharaj, *et. al.* 2011).

In another approach it was tried to improve biodegradation ability of bacteria by exposing them to biological mutation by adding specific chemical substances (Alsulami *et. al.,* 2014; Wasify, 2014). In addition various biodegradation experiments have revealed that prior exposure of bacteria to hydrocarbon pollutants results in their genetic adaptation to hydrocarbon degradation (Das, 2015; Sangeetha and Thangadurai, 2014). Therefore it seems that for a fast, reliable, unhazardous cleaning of crude oil contamination through native bacterial bioremediation, having a native bacterial population with most adaptability to the environmental conditions and at the same time taking advantage of their collective biodegradation capability to increase their biodegradation power and efficiency can be a promising solution (Mishamandani *et. al.,* 2015; Kostka *et. al., 2011;* Swannell, 1996). Which can be enhanced further in extent and power by preconditioning selected bacterial isolates to gradual increase in stressful conditions that need to be overcome in order for a successful bioremediation to happen.

The purpose of this study was to ascertain if the predominant species of oil-degrading bacteria in oil contaminated soil are a potential oil-degraders and to determine how well the indigenous hydrocarbon-degrading bacteria are able to degrade crude oil. Specifically after being sequentially adapt to higher crude oil concentrations. Characterizing these microbial communities will provide the scientific community with valuable insights that can be used in the future to determine suitable remediation strategies after oil spills or determine microbial resilience in a given ecosystem.

In doing so following hypotheses were established:

- Exposing bacterial populations to higher crude oil concentrations will result in better adaptation ability in surviving through stressful environmental conditions caused by soil pollution.

- Gradual preconditioning will result in better adaptability and consequently better remediation of hydrocarbon pollutions of tropical soil.

- Combining adapted microorganism, in a consortium will help in using their collective metabolic ability which has already been enhanced through sequential adaptation, to remove crude oil hydrocarbons more extensively.

To examine these hypotheses following questions were tried to be answered in this study:

1. What are the best specific types of bacteria capable of degrading crude oil pollution in tropical soils?
2. Whether or not being sequentially preconditioned and adapted to higher crude oil concentrations will significantly enhance biodegradation ability of indigenous tropical soil bacteria?

3. Whether or not it will be a better approach if a collective set of adapted bacteria is used to remove crude oil pollution in tropical soils?

As such, following objectives were implemented:

1. Isolation, enrichment, characterization and identification of oil degrading bacteria from tropical soils.

2. Determination of pH and temperature at sampling site as well as determining the optimum pH and temperature for growth and survival of selected bacteria.

3. Sequentially adapting and preconditioning the selected isolates to higher crude oil concentrations at pH and temperature values simulating their natural habitat.

4. Biochemical and molecular identification (16S rRNA sequencing) of selected bacterial isolates.

5. Bioremediation evaluation after sequential adaptation using gas chromatography-mass spectrometry.

It is worth to mention that *n*-alkane fraction of crude oil used as a marker for evaluation of removal ability of selected potential bacterial isolates as *n*-alkanes comprise the main fraction of crude oil regardless of where crude oil is originated from.

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