UNIVERSITI PUTRA MALAYSIA

RHIZODEGRADATION OF PETROLEUM OILY SLUDGE- CONTAMINATED SOIL BY HETERO-RHIZOSPHERIC BACTERIA FROM Cajanus cajan (L.) MILLSP. (Pigeon Pea)

ALLAMIN IBRAHIM ALKALI

FBSB 2018 19

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ALLAMIN IBRAHIM ALKALI

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

June 2018

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DEDICATION

This thesis is dedicated to my beloved parents Alkali Allamin Kachalla and Hajja Halima Abba Kaka

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

RHIZODEGRADATION OF PETROLEUM OILY SLUDGE- CONTAMINATED SOIL BY HETERO-RHIZOSPHERIC BACTERIA FROM *Cajanus cajan* (L.) MILLSP. (Pigeon Pea)

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June 2018

Chairman : Associate Professor Mohd Yunus Abd Shukor, PhD
Faculty : Biotechnology and Biomolecular Sciences

A significant amount of oily sludge is generated from the petroleum industry during exploration, production, transportation, storage, and refining processes. However, due to the recalcitrant persistent nature of oily sludge, the search for effective treatment method is being intensively sought. Bioremediation has been touted as the most cost-effective method in remediating oily sludge pollution with phytoremediation being singled out as the best method. Of all plant types, legume plants are being explored as efficient remediation agents based on their ability to fix nitrogen and harboring numerous xenobiotic-degrading microorganisms in their rhizome. *Cajanus cajan* a legume plant, has been previously demonstrated on its ability to remediate soil spiked with spent engine oil and even light petroleum crude oil. In this study, the plant was experimented on its ability to remediate the petroleum oily sludge in soil. The plant's tolerance to the contaminant was monitored through the determination of parameters such as plant height, number of leaves and dry biomass. Culture-dependent and independent methods were used to determine the rhizosphere microorganisms. For culture dependent soil was sampled to determine (total heterotrophic bacteria (THB) using nutrient agar, nitrogen-fixing bacteria (NFB) using yeast extract mannitol agar, and hydrocarbon utilizing bacteria (HUB) using oil agar. Similarly, metagenomics 16s RNA sequencing was used to determine the bacterial community. Degradation rates were estimated gravimetrically and quantified on GC-FID. Accumulation of heavy metals by *C. cajan* was determined using AAS. The results for the effect of oily sludge to seed germination show a decrease in germination at higher concentrations of oily sludge, especially at 5% oily sludge, which could be due to the reported toxicity of oily sludge to plant germination. The next sets of experiments studied the effect of oily sludge to plant growth parameters compared to control plant with no oily sludge. The plant height at the lower concentrations CR1%, CR2% and CR3% were 28, 26.2 and 25.6 cm, respectively, while at the comparatively higher concentrations at CR4%

and CR5%, the heights were 21.1 and 14.1 cm, respectively, while 25.6 cm was the UR (Uncontaminated control) at 28 days of plant growth. The shoot shows a similar pattern to that of the height as at lower concentrations of CR1%, CR2% and CR3% shows 11.3, 11.3, and 11 number of shoots, whereas at the higher concentrations CR4% and CR5%, the number of shoots were 6.3 and 5 after 28 days. The plant growth parameters plant height and number of shoots were not significantly affected by oily sludge at the concentrations of CR1, CR2 and CR3% petroleum oily sludge (w/w) while higher concentrations of CR4 and CR5% significantly reduce these plant growth parameters. The plant growth parameters wet and dry weights of the shoot and root of plant were found to be increased compared to control up to CR3% of oily sludge while higher concentrations were inhibitory as measured based on the wet weights after 60 and 90 days of growth. The relative growth rate (RGR) of the plant at the various treatments from 30 to 90 d shows a significant decrease in rates of growth at the higher concentrations (CR4 and CR5%) compared to control and at lower oily sludge concentrations (CR1, CR2 and CR3%). The RGR of lower concentrations (CR1, CR2 and CR3%) at 60 to 90 d show higher growth rates compared to control. In another sets of experiment, the microbial counts of various soil populations such as total heterotrophic rhizospheric bacterial (THRB), hydrocarbon-utilizing rhizospheric bacterial (HURB), nitrogen-fixing rhizospheric bacterial (NFRB), nitrogen-fixing endophytic bacterial (NFEB), hydrocarbon utilizing endophytic bacterial (HUEB) were determined for various soil treatments. Counts 1% oily sludge shows that the contaminated rhizosphere (CR) microorganism counts increased slightly but significantly ($p < 0.05$) from 125×10^7 to 148×10^7 CFU/g from 0 days to 90 days. The contaminated non-rhizosphere CN counts were 112×10^7 to 77.3×10^7 CFU/g whereas THRB for the 4 and 5% oily sludge concentrations in the treatments from 0 day to 90 days from 96.7×10^7 to 112×10^7 CFU/g and 57.7×10^7 to 45.4×10^7 CFU/g. The results indicate that the total heterotrophic rhizospheric bacterial (THRB) is significantly higher in contaminated rhizosphere compared to uncontaminated rhizosphere from day 0 to day 90 with a decrease in total count was observed at 4% oily sludge, which is the limit concentration of which the THRB can survive. For the hydrocarbon-utilizing rhizospheric bacterial (HURB), the result shows that for 1% oily sludge concentration, the increased microbial counts in all treatments were observed with the contaminated rhizosphere CR in microbial counts from 31.3×10^7 to 131×10^7 CFU/g, followed by uncontaminated rhizosphere UR from 30×10^7 to 86×10^7 CFU/g, uncontaminated non-rhizosphere UN from 25×10^7 to 58×10^7 CFU/g and the least was contaminated non-rhizosphere CN from 28×10^7 to 54×10^7 CFU/g, the results show that for 1 to 3% oily sludge concentration, the increased microbial counts for all treatments from day 0 to 90 d were observed with the contaminated rhizosphere CR showing the highest significant increase ($p < 0.05$) in microbial counts compared to other treatments. For the nitrogen-fixing endophytic bacterial (NFEB), the counts measured from 0 to 90 days show higher bacterial counts in the rhizosphere treatments than the non-rhizosphere. At the highest concentration of oily sludge tested (5%), a dramatic drop of NFRB count in the contaminated rhizosphere (CR) plot compared to the unaffected uncontaminated rhizosphere plot suggests that the NFRBs are sensitive to the presence of oily sludge. For the nitrogen-fixing endophytic bacterial (NFEB), the result indicates that the nitrogen-fixing endophytic bacteria were also affected by the high concentration of oily sludge but at the lower concentrations, appreciable number of endophytic nitrogen-fixing bacteria

was observed. For the hydrocarbon-utilizing endophytic bacterial (HUEB), the HUEB counts were found to exhibit a similar pattern of higher bacterial counts in the presence of oily sludge (CR1 to CR3%) compared to control at all days from 30 to 90 d) and inhibition of counts at higher concentrations of oily sludge at 4 and 5%. A total of 30 hydrocarbon-utilizing rhizosphere and endophytic bacteria were isolated and characterized from the rhizosphere of *C. cajan*. Through morphological and biochemical identifications, 24 rhizospheric bacteria of which eight were nitrogen-fixing rhizospheric bacteria were identified whereas six endophytic bacteria were also identified. Of the 24 rhizospheric bacteria, 11 were Gram-positive bacteria whereas 13 were Gram-negative with *Bacillus* dominating the Gram-positive species. The calculated bacterial community abundance index showed a slight difference in the Ace, Cho, and Shannon indices. Nevertheless, the Simpson and coverage indices showed a significant difference between the two treatments. The principal component analysis (PCA) plot revealed community level differences between the contaminated non-rhizosphere control (CN3) and contaminated rhizosphere (CR3) microbiota. The component differentiated the two treatments based on the presence or absence of plant. The composition and taxonomic analysis of microbiota amplified sequences were categorized into eight phyla in the contaminated non-rhizosphere (CN3) and ten phyla in the contaminated rhizosphere (CR3). The overall bacterial composition of the two treatments varied, as the distribution show a similar variation between the two treatments in the phylum distribution. The removal rate of total petroleum hydrocarbon (TPH) from the soil after 90 days of treatments was inhibited at higher concentrations of oily sludge composition in the soil with CR1%, CR2% and CR3% (w/w) of oily sludge showed 92%, 90% and 89% removal rate, respectively, and 68.3% and 47.3% removal rate for the relatively higher concentrations of oily sludge of CR4% and CR5% (w/w) respectively. These results were further confirmed by the chromatographic peaks in the GC-FID profile of the treatments. The results of heavy metal shows Pb was accumulated in the CR1 to CR5% oily sludge root of *C. cajan* was 0.04 mg/kg to 0.18 mg/kg. Likewise Zn was accumulated in the root for the CR1% to CR5% was 2.13 mg/kg to 4.16 mg/kg. The accumulation of Ni was also similar with 1.3 mg/kg was accumulated in the root of *C. cajan* at CR1% oily sludge which increased to 2.06 mg/kg in CR5% oily sludge. Mn was accumulated in the root of *C. cajan* with CR1% oily sludge showing a value of 0.4 mg/kg that slightly increases to 0.5 mg/kg in CR5% oily sludge. Cu shows the highest accumulation at higher oily sludge concentrations while Zn was accumulated at higher concentration at the lower oily sludge treatment. Cu was accumulated in the treatment with CR1% oily sludge to a value of 1.9 mg/kg which increased to 6.8 mg/kg in CR5% oily sludge. Cr was slightly accumulated in the root with CR1% oily sludge showing a value of 0.03 mg/kg which slightly increased in CR5% to 0.09 mg/kg. The heavy metal analysis in *C. cajan* tissues indicated a considerable accumulation of the metals (Pb, Zn, Ni, Mn, Cu and Cr) in the root and stem of the plant, with negligible metal concentrations detected in the plant leaves suggesting a low translocation factor but indicating that *C. cajan* is resistant to heavy metals. As the search for more eco-friendly and sustainable remediating green plant continues, *C. cajan* shows a great potential in reclaiming petroleum oily sludge-contaminated soil due to the above properties including resistance to toxic heavy metals from oily sludge. These findings will provide solutions to polluted soils and their subsequent re-vegetation.

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

RIZODEGRADASI TANAH TERCEMAR DENGAN LUMPUR PETROLEUM BERMINYAK OLEH BAKTERIA HETERORIZOSFERIK DARI *Cajanus cajan* MILLSP. (Kacang Dhal)

Oleh

ALLAMIN IBRAHIM ALKALI

Jun 2018

Pengerusi : Profesor Madya Mohd Yunus Abd Shukor, PhD
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Banyak enapcemar berminyak telah dihasilkan dari industri petroleum semasa proses eksplorasi, pengeluaran, pengangkutan, penyimpanan, dan peninjauan semula. Walaubagaimanapun, disebabkan oleh sifat enapcemar berminyak ini yang susah dirawat menyebabkan pencarian kaedah rawatan yang berkesan sedang dicari secara intensif. Bioremediasi telah dikenalpasti sebagai kaedah yang paling kos efektif dalam memulihkan pencemaran enapcemar berminyak dengan kaedah fitoremediasi dipilih sebagai kaedah yang terbaik. Daripada semua jenis tumbuhan, tumbuh-tumbuhan kekacang sedang dinilai sebagai agen pemulihan yang cekap berdasarkan keupayaannya untuk mengikat nitrogen dan mengandungi banyak mikroorganisma pengurai xenobiotik di dalam rizom mereka. *Cajanus cajan* merupakan tumbuhan legum yang sebelum ini telah menunjukkan keupayaannya untuk meremediasi tanah yang dicemar dengan minyak enjin sisa dan juga minyak mentah. Dalam kajian ini, eksperimen ke atas keupayaan pokok ini untuk memulihkan enapcemar petroleum dalam tanah telah dijalankan. Toleransi tumbuhan ini terhadap bahan pencemar dipantau melalui penentuan parameter seperti ketinggian tumbuhan, bilangan daun dan berat biomas kering. Kaedah yang bergantung kepada pengkulturan dan kaedah bebas pengkulturan telah digunakan untuk menentukan mikroorganisma di dalam rizosfera. Bagi bakteria boleh dikultur, penentuan jumlah bakteria heterotropik (THB) telah dijalankan agar nutrien, jumlah bakteria pengikat nitrogen (NFB) menggunakan agar ekstrak yis manitol, dan jumlah bakteria pengurai hidrokarbon (HUB) menggunakan agar berminyak. Kadar degradasi dianggarkan secara gravimetrik dan juga menggunakan alatan kromatografi gas dengan pengesan pengionan api (GC-FID). Pengumpulan logam berat oleh *C. cajan* ditentukan dengan menggunakan kaedah spektrometri penyerapan atom (AAS).

Hasil keputusan keatas kesan enapcemar berminyak pada percambahan benih menunjukkan penurunan percambahan pada kepekatan enapcemar berminyak yang tinggi, terutamanya pada kepekatan enapcemar berminyak pada 5%, yang bolehjadi disebabkan oleh ketoksikan enapcemar berminyak pada proses percambahan. Set percubaan berikutnya mengkaji kesan enapcemar berminyak pada parameter pertumbuhan berbanding dengan tumbuhan kawalan tanpa kehadiran enapcemar berminyak. Ketinggian tumbuhan pada kepekatan yang rendah iaitu CR1%, CR2% dan CR3% adalah 28, 26.2 and 25.6 cm, masing-masing, manakala pada kepekatan yang lebih tinggi iaitu pada CR4% dan CR5%, ketinggiannya adalah 21.1 dan 14.1 cm, masing-masing, manakala 25.6 cm adalah UR (kawalan tidak terkontaminasi) pada hari ke 28 pertumbuhan pokok. Jumlah bilangan pucuk menunjukkan corak yang sama dengan ketinggian tumbuhan iaitu didapati jumlahnya tidak terjejas dengan ketara dengan kehadiran enapcemar berminyak pada kepekatan CR1%, CR2% dan CR3% (w/w) dengan jumlah bilangan pucuk purata 11.3, 11.3, dan 11, masing-masing, manakala pada kepekatan yang lebih tinggi iaitu CR4% dan CR5% bilangan purata pucuk adalah 6.3 dan 5, masing-masing pada hari ke 28. Parameter pertumbuhan seperti berat basah dan berat kering pucuk dan akar tumbuhan didapati meningkat berbanding dengan kawalan sehingga kepekatan CR3% enapcemar berminyak manakala kepekatan yang lebih tinggi telah merencat nilai parameter seperti yang diukur berdasarkan berat basah selepas pertumbuhan pokok pada 60 dan 90 hari. Kadar pertumbuhan relatif (RGR) tumbuhan untuk pelbagai rawatan dari 30 hingga 90 d menunjukkan penurunan kadar pertumbuhan yang ketara pada kepekatan enapcemar berminyak yang tinggi (CR4 dan CR5%) berbanding dengan kawalan dan pada kepekatan enapcemar yang lebih rendah (CR1, CR2 dan CR3%). RGR pada kepekatan yang rendah (CR1, CR2 dan CR3%) pada hari pertumbuhan 60 dan 90 hari menunjukkan kadar pertumbuhan yang lebih tinggi berbanding kawalan. Dalam satu lagi set eksperimen, jumlah mikrob yang terdiri daripada pelbagai populasi tanah seperti bakteria heterotropik rhizospherik (THRB), bakteria pengurai hidrokarbon rhizospherik (HURB), bakteria pengikat nitrogen rhizospherik (NFRB), bakteris pengikat nitrogen endofitik (NFEB), bakteria pengurai hidrokarbon endofitik (HUEB) telah ditentukan pada pelbagai rawatan tanah.

Jumlah kiraan bakteria pada CR1% kepekatan enapcemar berminyak menunjukkan bahawa kiraan mikroorganisma pada rizosfera tercemar (CR) meningkat sedikit tetapi signifikan ($p<0.05$) dari 125 x 10^7 ke 148 x 10^7 CFU/g dari hari 0 hingga ke hari 90. Jumlah kiraan bakteria bukan rizosfera (CN) adalah dari 112 x 10^7 ke 77.3 x 10^7 CFU/g, manakala untuk THRB pada CR4 dan CR5% kepekatan enapcemar berminyak pada hari 0 hingga hari 90 meningkat dari 96.7 x 10^7 ke 112 x 10^7 CFU/g dan 57.7 x 10^7 ke 45.4 x 10^7 CFU/g, masing-masing. Hasil kajian telah menunjukkan bahawa jumlah bakteria heterotropik rhizospherik (THRB) adalah jauh lebih tinggi dalam rizosfera yang tercemar berbanding rizosfera yang tidak tercemar dari hari 0 ke hari 90 dengan pengurangan jumlah bakteria telah didapati pada kepeakatan 4% enapcemar berminyak, iaitu kepekatan maksimum yang mana THRB boleh bertahan. Bagi bakteria pengurai hidrokarbon rhizospherik (HURB), keputusan menunjukkan bahawa untuk kepekatan enapcemar berminyak dari CR1 hingga CR3%, peningkatan jumlah mikrob untuk semua rawatan dari hari 0 hingga 90 d telah diperhatikan dengan rizosferea tercemar atau CR menunjukkan peningkatan yang paling tinggi dalam

jumlah mikrob berbanding dengan rawatan lain. Untuk bakteria pengikat nitrogen endofitik (NFEB), kiraan yang diukur dari 0 hingga 90 hari menunjukkan jumlah bakteria yang lebih tinggi dalam rawatan rizosfera daripada bukan rizosfera. Pada kepekatan tertinggi enapcemar berminyak paling tinggi (CR5%), penurunan dramatik NFRB dalam plot rizosferea tercemar (CR) telah berlaku dalam plot rizosferea tercemar berbanding dengan plot rizosfera yang tidak tercemar menunjukkan bahawa NFRB adalah sensitif terhadap kehadiran enapcemar berminyak. Bagi bakteria pengikat nitrogen endofitik (NFEB), hasilnya menunjukkan bahawa jumlah NFEB juga dipengaruhi oleh kepekatan enapcemar berminyak yang tinggi tetapi pada kepekatan yang lebih rendah. Untuk bakteria pengurai hidrokarbon endofitik (HUEB), kiraan HUEB didapati mempamerkan pola yang sama, dengan jumlah bakteria yang lebih tinggi terdapat pada rizosfera tercemar enapcemar berminyak (CR1 hingga CR3%) berbanding dengan kawalan pada setiap hari dari 30 hingga 90 hari) dan perencatan terjadi pada kepekatan enapcemar berminyak lebih tinggi iaitu pada CR4 dan CR5%.

Sejumlah 30 bakteria pengurai hidrokarbon rizosfera dan endofitik telah dapat diasingkan dan dicirikan dari rizosfera *C. cajan*. Melalui pengenalpastian morfologi dan biokimia, 24 adalah bakteria rizosferik yang mana lapan adalah bakteria rizosferik yang mengikat nitrogen telah dikenal pasti manakala enam bakteria endofitik juga dikenalpasti. Daripada 24 bakteria rizosferik, 11 adalah bakteria Gram-positif manakala 13 lagi adalah Gram-negatif dengan *Bacillus* mendominasi spesies Gram-positif. Indeks banyaknya masyarakat bakteria yang dikira menunjukkan sedikit perbezaan pada indeks Ace, Cho, dan Shannon. Walau bagaimanapun, indeks Simpson dan indeks lingkungan menunjukkan perbezaan yang signifikan di antara kedua-dua rawatan. Analisis komponen komponen utama (PCA) mendedahkan perbezaan tahap komuniti di antara kawalan rizosfera yang tidak tercemar (CN3) dan microbiota rizosfera (CR3) yang tercemar. Komponen ini membezakan dua rawatan berdasarkan kehadiran atau ketiadaan tumbuhan. Analisa komposisi dan taksonomi mengenai susunan mikrobiota yang diperkuatkan dapat dikategorikan kepada lapan phyla dalam rizosfera yang tidak tercemar (CN3) dan sepuluh phyla dalam rizosfera yang tercemar (CR3).

Komposisi bakterial pada keseluruhan kedua-dua rawatan adalah berbeza, kerana taburan menunjukkan variasi yang sama di antara kedua-dua rawatan dalam taburan filum. Kadar jumlah penyingkiran hidrokarbon petroleum (TPH) dari tanah selepas 90 hari rawatan direncat pada kepekatan enapcemar yang lebih tinggi dalam tanah dengan CR1,CR2, CR3, CR4 dan CR5% (w/w) kepekatan enapcemar berminyak menunjukkan kadar penyingkiran sebanyak 92, 90, 89, 68.3 dan 47.3, masing-masing. Keputusan ini disahkan lagi oleh puncak kromatografi dalam profil kromatografi gas GC-FID. Keputusan untuk logam berat menunjukkan bahawa kepekatan Pb terkumpul dari kepekatan enapcemar berminyak CR1 hingga CR5% pada akar *C. cajan* adalah di antara 0.04 mg/kg hingga 0.18 mg/kg. Begitu juga, Zn terkumpul di akar untuk rawatan enapcemar berminyak dari CR1% hingga CR5% adalah dari 2.13 mg/kg hingga 4.16 mg/kg. Pengumpulan Ni juga sama dengan 1.3 mg/kg terkumpul di akar *C. cajan* pada kepekatan enapcemar berminyak CR1% yang meningkat kepada 2.06

mg/kg pada kepekatan CR5%. Mn terkumpul di akar *C. cajan* pada rawatan kepekatan enapcemar berminyak CR1% adalah 0.4 mg/kg yang meningkat sedikit kepada 0.5 mg/kg pada kepekatan enapcemar CR5%. Cu menunjukkan pengumpulan tertinggi pada kepekatan enapcemar berminyak yang tinggi manakala Zn terkumpul pada kepekatan yang tinggi pada rawatan enapcemar yang lebih rendah. Cu terkumpul sebanyak 1.9 mg/kg pada rawatan enapcemar berminyak pada kepekatan CR1% yang meningkat kepada 6.8 mg/kg pada kepekatan CR5%. Pengumpulan Cr adalah sedikit pada akar dengan rawatan kepekatan CR1% menunjukkan pengumpulan sebanyak 0.03 mg/kg yang kemudiannya meningkat kepada 0.09 mg/kg pada kepekatan enapcemar berminyak CR5%. Hasil analisis logam berat dalam tisu *C. cajan* menunjukkan pengumpulan logam yang tinggi (Pb, Zn, Ni, Mn, Cu dan Cr) di akar dan batang tumbuhan, dengan kepekatan logam yang hampir tidak dikesan di daun tumbuhan yang menunjukkan faktor translokasi yang rendah tetapi menunjukkan bahawa *C. cajan* adalah rintang terhadap logam berat. Memandangkan pencarian untuk tumbuhan hijau yang lebih mesra alam dan mampan adalah berterusan, *C. cajan* menunjukkan potensi yang besar dalam menebus semula tanah tercemar lumpur bertenaga petroleum kerana ciri-ciri di atas termasuk rintangan terhadap kandungan logam berat toksik daripada enapcemar berminyak. Penemuan ini akan memberikan penyelesaian kepada rmediasi tanah yang tercemar dan penumbuhan semual tumbuhan.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, Most Merciful

All praise and total glory are due to Allah alone for his mercy and blessings throughout the period of my research. We praise him and seek his assistance, and we seek for his forgiveness.

I am most grateful to my major supervisor Prof. Madya. Dr. Mohd Yunus Abd Shukor for his genuine guidance, advice and total confidence in me, his long experience in teaching and research has been valuable to me. My co-supervisors, Dr. Siti Aqlima Ahmad, and Dr Nur Adeela Yasid and Prof. Dr. Siti Rozaimah Sheikh Abdullah I appreciate your effort in remodelling my thinking and orientation in the field of biremediation.

My appreciation also goes to my parents Alkali Allamin Kachalla and Hajja Halima Abba Kaka, brothers and sisters, for their support and prayers. I thank my wife Falmata Bukar Burim for her understanding and encouragement. To my daughter Maryam Ibrahim and son Allamin Ibrahim (whom was delivered in my absentia) thank you for your patience. My special appreciation goes to my friends and colleagues in Biremediation Lab, Faculty of Biotechnology and Biomolecular sciences and Prof. Siti Rozaimah's lab in UKM. I appreciate all those who contributed directly or indirectly towards the success of this research, may Allah reward them abundantly. Finally I acknowledge my employer University of Maiduguri for awarding me study fellowship.

Ibrahim Alkali Allamin, 2018

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

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%	Percent
Abs	Absorbance
ANOVA	Analysis of variance
CFU	Colony forming unit
cm	Centimetre
Cr	Chromium
Cu	Copper
dH_2O	Distilled water
d	Days
et al	and friends
Fe	Iron
G	Gram
h	Hour
kg	Kilogram
L	Liter
M	Molar
min	Minute
mL	Mililiter
MSM	Minimal salt medium
MW	Molecular weight
NA	Nutrient agar
Ni	Nickel
^oC	Degree Celcius
OD	Optical density
Pb	Lead
RPM	Rotation per minute

µL	Microlitre
µm	Micrometer
µM	Micromolar
Zn	Zinc

CHAPTER 1

GENERAL INTRODUCTION

1.1 Research background

The constant rise in number and area of contaminated soils and aquatic ecosystems due to oily petroleum sludge is a concern worldwide (Hu *et al*., 2013). Oily sludge is hazardous according to Environment Protection Act and Hazardous Wastes Handling Rules (Prakash *et al*., 2015). This sludge cannot be disposed of in a landfill, even if it is de-oiled unless they are remediated. Oily sludge is generated in petroleum industries as unprocessed waste in all the stages of the industry. It accumulates in crude oil tanks, in refined products tanks, and elsewhere during oil production and processing which are always discharged to the environment (Prakash *et al*., 2015). It is estimated that more than 50,000 tons of oily sludge are being generated annually in Malaysia due to a higher number of refinery and transportation of oil in the Strait of Malacca (Keshavarzifard *et al*., 2014; Thia-Eng *et al*., 2000). In addition, almost one billion tons of oily sludge is deposited worldwide (Hu *et al*., 2013). The uncontrolled disposal practices of this oily sludge have caused serious environmental pollution (Ubani *et al*., 2013). The ecological effects caused by all the processes in petroleum industry is a major concern in oil producing and consuming countries (Hou *et al.*, 2015; Shahsavari *et al*., 2015; Bauddha *et al.*, 2016), due to the significant amount of waste disposed (Islam, 2015). The oil destroys living organisms and alters development in the biosphere (Sangeetha and Thangadurai, 2014).

Physical and chemical methods used in remediation of oily sludge contaminated soils are costly and environmental unfriendly (Ubani *et al*., 2013). Meanwhile, a biological method is efficient, but had some limitation especially to heavy metal-contaminated soil, leading to the use of phytoremediation, which is more tolerant to heavy metals (Ijaz *et al*., 2016). Legumes are known to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen (Ugrinovic *et al*., 2014) and thus, do not have to compete with microorganisms and other plants for limited supplies of available soil nitrogen at oil-contaminated sites. Common desirable characteristics of these plants are the ability to fix nitrogen (a major limiting factor for effective degradation of pollutants) and drought tolerance (Vázquez-Luna, 2015). The synergistic approach of legume-bacterial interaction in which legume plant supplies the rhizosphere bacteria with vital organic nutrients and space, while the bacteria degrade hydrocarbons into less-toxic compounds (Bauddha *et al*., 2015). In addition, rhizo- and endophytic bacteria help the associated legume by increasing nutrient availability, promoting plant growth hormones, and enhancing the bioavailability of hydrocarbons (Alaru *et al.*, 2014; Jerez Ch and Romero, 2016; Remigi *et al.*, 2016; Saadani *et al*., 2016). *Cajanus cajan* (pigeon pea) (kacang dhal) in Malay is a common legume crop which is commonly cultivated in tropical countries, and it serves as a very important source of protein in human diets, it has a long root system which withstands

different soil condition and properties (Orwa et al. 2009). It tolerates pH over a wide-ranging range, and temperatures from 10 to 35 ºC (Singh *et al*., 2012).

1.2 Problem statement

Soil for a long time, has been a reservoir of various natural and anthropogenic discharges (Maqbool *et al*., 2012). With the emergence of petroleum industry, soil contamination due to petroleum and its derived products has been a problem (Xu *et al*., 2011). However, the global concern on soil health and sustainable food security is making scientists to consider restoration of polluted lands, especially where oily sludge contamination limits the use of such soils, considering the length at with the contaminated soil can be regenerated into pristine conditions (Prakash *et al*., 2015; Wang *et al*., 2016). During the past years, a variety of oily sludge treatment methods comprising of physical and chemical methods are used (Islam, 2015). By employing these technologies, the content of hazardous constituents can be reduced or eliminated, and its deleterious environmental and health impacts can thus be mitigated (Hu *et al*., 2013; Prakash *et al.*, 2015).

However, due to the recalcitrant persistent nature of oily sludge and especially the high heavy metal content of the oil (Ubani *et al*., 2013), the search for treatment method with a compromised balance of satisfying strict environmental regulations and reducing treatment costs (Islam, 2015). The complexity of oily sludge composition of hydrocarbon, heavy metals and others made it highly resistant and made it less bioavailable for microbial degradation (Hu *et al*., 2013). Legume plants especially *Cajanus cajan* has never been explored as a remediation agent for oily petroleum sludge, but it has its advantageous based on its ability to fix nitrogen and harboring numerous xenobiotic-degrading microorganisms in its rhizome.

1.3 Justification of the study

To encourage the effectiveness of microbial petroleum oily sludge degradation, an effective legume plant tolerant to petroleum oily sludge is essential as non-legumes used in the phytoremediation studies were not successful as external augmentation required (Agnello *et al*., 2015; Nanekar *et al*., 2015; de Oliveira *et al*., 2012). Legume plants are known to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen which is strategic to establish nutrient rich rhizosphere as environments contaminated with oily sludge had a deficit in C: N ratio (Vázquez-Luna, 2015).

Identification of rhizosphere population that would enhance microbial growth and subsequent degradation of hydrocarbons would to greater extent help in reducing the hydrocarbon contaminants. *Cajanus cajan* used in this study have been reported to bioremediate spent engine oil contaminated soil (Ismail *et al*., 2014) and light crude petroleum oil in soil (Ibrahim *et al*., 2013) no study has reported its application for

remediation of petroleum oily sludge in soil. Therefore, to determine the effectiveness of *Cajanus cajan* and its rhizosphere microbial associate in phytoremediation of petroleum oily sludge-contaminated soil, it is essential as it is among important leguminous plants in tropical countries.

1.4 Research objectives

The study aimed to remediate petroleum oily sludge spiked soil using *Cajanus cajan* association with microbial communities in its rhizosphere.

The objectives of this study are;

1. To examine growth parameters and responses of *Cajanus cajan* to oily petroleum sludge
2. To isolate, enumerate, characterize and identify bacteria associated with the rhizosphere of *Cajanus cajan*
3. To examine the microbial community in *Cajanus cajan* rhizosphere
4. To measure the rate and extent of petroleum oily sludge biodegradation in the soil
5. To determine the rate of accumulation of heavy metals by *Cajanus cajan*

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