UNIVERSITI PUTRA MALAYSIA

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF PHOSPHATE-SOLUBILIZING PSEUDOMONAS SP. ISOLATED FROM OIL PALM SOIL

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By

MOLOUD KOOSHAN

Thesis Submitted to the School of Graduate Studies Universiti Putra Malaysia in Fulfillment of the Requirements for the Degree of Master of Science

June 2015

To my lovely parents for their consistent love, help and support throughout all levels of my life and education,

To my beloved husband, Reza, who encourage me always, thank you Reza for your love, wisdom, and support, and last but not least, to my brothers, Mostafa and Morteza for their lovely support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman: Associate Professor Halimi Mohd Saud, PhD
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Phosphorous (P) is one of the most essential macronutrients required for plant growth and development. The objectives of the present study were to select *Pseudomonas* from soils of oil palm capable of solubilizing phosphate under tropical environment by various biochemical and genotypical method have been used. The study carried out to determine the population distribution of phosphate solubilizing *Pseudomonas* in oil palm soil by Standard Plate Count. The results showed that the distribution of phosphate solubilizing bacteria and phosphate solubilizing *Pseudomonas* population in UPM were higher than the rest. Twenty five isolates were able to grow and form clearing zone in NBRIP (National Botanical Research Institute's Phosphate) medium, PVK (Pikovskayas) and other different carbon and nitrogen sources. The best isolates were 43Dengkil, 9Dengkil, 10Dengkil and 15Dengkil which solubilized phosphate on NBRIP better than other isolates. Meanwhile, the best isolate on PVK was 15Dengkil. The result of culturing the isolates in NBRIP indicated that among all carbon sources glucose was the best carbon sources for phosphate solubilization and KNO_3 was less effective compared to $(NH_4)NO_3$ when it was used as a source of nitrogen. All the isolates were acid producers and among them, 633UPM and 144UPM, after 5 days of culture, 555UPM and 23Dengkil after 8 days of culture respectively, showed the lowest pH. The effective phosphate solubilizer isolates after 5 days were 48Semenyih and 45Dengkil, 5Semenyih, and after 8 days were 45Dengkil, 5Semenyih, followed by 48Semenyih respectively. Initial confirmation of their genus level identity as *Pseudomonas* was arrived by amplification of 16S rDNA sequence. The result of a BLAST search of 16S rDNA sequences of *Pseudomonas* compared with the available 16S rDNA sequences in the GenBnak database indicated that all of the *Pseudomonas* isolates belonged to gamma proteobacteria subdivision. Isolates 59Semenyih, 62Dengkil, 555UPM, 43Dengkil, 69Semenyih and 103Semenyih had more than 80% similarity to *Pseudomonas* species. The diversity among isolates was determined by REP-PCR (Repetitive Extragentic Palindromic Elements). Based on REP-PCR pattern isolates were classified into seven groups (A, B, C, D, E, F, and G). The cluster showed that in group A, isolates 9Dengkil, 10Dengkil, 15Dengkil, 23Dengkil and 45Dengkil were totally similar to each other. Cluster B included 43Dengkil, 62Dengkil isolates. Clusters C and D included isolates 103UPM and 59Semenyih respectively. Four isolates fell into cluster E and these isolates were totally similar to each other. Cluster F

contained isolates 144UPM and 625UPM. Cluster G consisted of 4 isolates and also cluster H included 5 isolates. Also, this study concluded that the cluster analysis of *Pseudomonas* based on REP-PCR identified clusters A, B, C, D, E and F approximately with genetic distance=0.34. The results presented here clearly establish that REP sequences (elements) are present in genome of phosphate solubilizing *Pseudomonas* isolated from oil palm soils.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGELASAN MOLEKUL DAN BIOKIMIA FOSFAT TERMELARUT PSEUDOMONAS SP. DIASINGKAN DARIPADA TANAH KELAPA SAWIT

Oleh

MOLOUD KOOSHAN

Jun 2015

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Fosforus (P) adalah salah satu makronutrien terpenting yang diperlukan untuk pertumbuhan dan tumbesaran tumbuhan. Tujuan kajian ini adalah untuk memilih Pseudomonas dari tanah kelapa sawit yang mampu melarutkan fosfat dalam persekitaran tropika dan menentukan ciri-ciri yang bermanfaat dan peranan ekologi mereka dalam meningkatkan produktiviti pertanian. Kajian ini dijalankan untuk menentukan taburan populasi bakteria Pseudomonas pelarut fosfat dalam tanah kelapa sawit menggunakan kaedah 'Standard Plate Count.' Hasil kajian menunjukkan bahawa taburan Pseudomonas pelarut fosfat di UPM adalah lebih tinggi daripada yang lain. Dua puluh lima pencilan bleh membiak dan membentuk zon 'halo' dalam media NBRIP (National Botanical Research Institute in Phosphate), PVK (Pikovskayas) dan lain-lain sumber karbon dan nitrogen. Isolat terbaik adalah 43Dengkil, 9Dengkil, 10Dengkil dan 15Dengkil yang melarut fosfat lebih baik daripada pencilan lain. Sementara itu, isolat terbaik PVK adalah 15Dengkil. Sumber karbon glukosa adalah sumber karbon terbaik untuk fosfat dan KNO_3 kurang berkesan berbanding dengan (NH4) NO3 apabila ianya digunakan sebagai sumber nitrogen. Semua pencilan adalah pengeluar asid dan didapati bahawa 633UPM dan 144UPM selepas lima hari pengkulturan, dan 555UPM dan 23Dengkil selepas lapan hari pengkulturan mempunyai pH yang terendah. Pelarut fosfat yang paling berkesan selepas lima hari adalah 48Semenyih, 45Dengkil, dan 5Semenyih, manakala selepas lapan hari ianya adalah 45Dengkil, 5Semenyih diikuti oleh 48Semenyih. Pengesahan awal identiti genus mereka sebagai Pseudomonas telah diperolehi dengan amplifikasi jujukan 16S rDNA. Hasil carian BLAST daripada urutan 16S rDNA Pseudomonas berbanding urutan 16S rDNA dalam pangkalan data GenBank menunjukkan bahawa semua Pseudomonas yang diasingkan adalah terdiri daripada pecahan gamma proteobacteria . Pencilan 59Semenyih, 62Dengkil, 555UPM, 43Dengkil, 69Semenyih dan 103Semenyih mempunyai lebih daripada 80 % persamaan dengan spesies Pseudomonas. Berdasarkan corak REP -PCR pencilan diletakkan dalam tujuh kumpulan (A, B , C , D , E, F dan G). Kelompok A menunjukkan bahawa pencilan 9Dengkil, 10Dengkil, 15Dengkil, 23Dengkil dan 45Dengkil, adalah sama dengan satu sama lain. Kelompok B termasuk pencilan 43Dengkil, 62Dengkil. Kluster C dan D hanya ada satu pencilan, setiap satu ialah 103UPM dan 59Semenyih. Empat pencilan jatuh ke dalam kelompok E dan pencilan ini adalah sama antara satu sama lain. Kluster

F mengandungi pencilan 144UPM dan 625UPM. Kluster G terdiri daripada empat pencilan dan juga kelompok H terbentuk oleh lima pencilan. Selain itu, kajian ini membuat kesimpulan bahawa analisis kelompok Pseudomonas berdasarkan REP -PCR mengenalpasti kelompok A, B, C, D, E dan F yang mempunyai jarak genetik F = 0.34. Keputusan yang dibentangkan di sini jelas membuktikan bahawa jujukan REP (elemen) hadir dalam genom Pseudomonas pelarut fosfat yang diasingkan dari tanah kelapa sawit.

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I certify that an Examination Committee has met on 22nd June 2015 to conduct the final examination of Moloud Kooshan on her thesis entitled "Molecular and Biochemical Characterization of Phosphate-Solubilizing *Pseudomonas Sp*. Isolated

from Oil Palm Soils" in accordance with 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 Master of Science

Members of the Thesis Examination Committee were as follows:

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

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DECLARATION

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LIST OF ABBREVIATIONS

C.F.U	Colony Forming Unite
DNA	Deoxyribonucleic Acid
Dnase	Dexoyribonuclease
LB	Luria Bertoni
LMW	Low Molecular Weight
NBRIP	National Botanical Institue
PCR	Ploymerase chain Reaction
PSB	Phosphate solubilizing Bacteria
PVK	Pikovskaya
PGPR	Plant Growth Promoting Rhizobacteria
REP	Repetitive Exteragentic Palindromic
REP-PCR	Repetitive Exteragentic Palindromic-PCR
RNA	Ribonucleic Acid
Rnase	Ribonuclease
16S rDNA	16s Ribosomal Deoxyribo Nucleic Acid
ºC	Degree Celsius
Blast	Basic Local Alignment Search Tool
bp	Base Pair
OD	Optimal Density
rpm	Revolution Per Minute
VU	Ultraviolet
W/V	Wieght/Volume
Pi	Soluble Phosphorus / Orthophosphate

CHAPTER 1

INTRODUCTION

Soil is considered as a complex habitat with a large number of different microorganisms including bacteria, fungi, protozoa and algae. The rhizosphere is often separated into the endorhizosphere, the rhizoplane, and the ecotorhizosphere (Lynch, 1990). These separated parts consist of the root tissue, the top and related soil. Soil that is a distance from the rhizosphere is usually referred to as 'bulk soil'. It has been demonstrated that the immediate area of the roots or rhizosphere contains a much higher concentration of bacteria compared to the bulk soil, and is able to support higher rates of microbial development and activities than the bulk soil (Söderberg and Bååth, 1998). Phosphorous is one of the most limiting nutrients for growth of crops in tropical and subtropical regions (Ae *et al.*, 1990).

The total phosphate (P) in the soil is estimated to be around 0.04 % to 0.1 % (Son *et al.*, 2006; Chen *et al.*, 2008). Though abundant phosphate is found in soil, the concentration of orthophosphate is extremely low (Yi *et al.*, 2008). Lack of soluble phosphate could hinder plant growth and the development of crops and this had led to the intensive use of synthetic fertilizers. However, the application of chemical phosphate fertilizers may supply orthophosphate for only a short period and consequently changes the soluble phosphate to insoluble form via precipitation or adsorption (Lin *et al.*, 2006). Over-application of synthetic fertilizer for long periods leads to the accumulation of a large amount of fixed phosphate in arable soils and one of the long-term effects of over application of synthetic phosphate fertilizer is eutrophication (Son *et al.*, 2006), an excess of nutrients in water. It has also led to adverse impact on the water reservoirs and environment as well as the disruption of the food chain of aquatic organisms.

Besides that, the production of synthetic phosphate fertilizers involves huge costs. Thus, a substantial import bill for the purchase of synthetic phosphate fertilizers from other countries may cause economic constraints for a developing country like Malaysia. Depletion of the natural phosphate sources and lack of available phosphate in arable soil is of great concern as it will adversely affect the development of sustainable agriculture systems. Therefore it is very crucial to find suitable alternatives for natural phosphate, synthetic phosphate and unavailable phosphate in soils. In this respect, the role of bacteria is of great significance in providing phosphate for plants by their metabolic activity. Bacteria are the most important and more dominant microorganisms which have better ability to solubilize mineral phosphate compared to fungi or actinomyces (Kucey, 1983; Yin, 1988). There are several advantages in using bacteria as these microorganisms are known to have the ability to solubilize phosphate in

different environments through adsorption, metabolism and/or transport. Organic acids are naturally and inexpensively produced by bacteria, their rapid production rates make it possible to process large volumes of different organic acids in a timely manner, and the high selectivity organisms are able to solubilize different kinds of phosphate in different specific environments (McGrath *et al.*, 1998). Knowledge of mineral phosphate solubilization is vital for the production of biofertilizers or bioinoculants. Biofertilizers play an important role in solubilizing insoluble soil phosphate; increasing the amount of phosphate uptake by crops and eventually boosting the growth and yield of the crops (Malboobi *et al.*, 2009; Vyas and Gulati, 2009).

Generally, useful or pathogenic bacteria and fungi have a tendency to converge around plant roots and this is what provides a significant impact on plant growth as well as development and enhanced productivity. In such a situation, the colonies of bacteria, and fungi interact with the roots resulting in positive, negative or neutral outcomes due to the symbiotic interaction and soil conditions (Smith and Read, 1997). As a result of bacterial inoculation, plants are able to receive a balanced nutrition that includes nitrogen and phosphorus via the roots, which is the main interactive conduit between plants and bacteria (Belimov *et al.*, 1995). *Pseudomonas sp* is introduced in the soil of agricultural lands and it is the behavior of the bacteria that makes them suitable as PGPR (Saharan and Nehra, 2011).

Observations showed that some *Pseudomonas* species enhance the absorption of nutrients, such as N, P and K, besides their function as agents of biocontrol of phytopathogenic fungi and promoting plant growth by production of phytohormones in the rhizosphere (O'sullivan and O'Gara, 1992). To characterize phosphate solubilizing *Pseudomonas* isolates, various biochemical and genotypical methods have been developed and used so far. Several researchers have used polymerase chain reaction (PCR) technology to detect and study the variability of the *Pseudomonas* species (Yamamoto and Harayama, 1995). The 16S ribosomal RNA sequence (16S RNA) has been used for deducing phylogenic and evolutionary relationships among bacteria and other prokaryote species (Anzai *et al.*, 2000). Another PCR-based technique, known as REP-PCR has also been used in the identification and classification of bacteria (Versalovic *et al.*, 1991). It is a repeating sequence-based genomic fingerprinting that employs primers corresponding to the endogenous interspersed repetitive sequence of the bacteria. These scattered sequences are highly maintained features in the prokaryotic genomes. These elemental sequences have been widely considered in many prokaryotic microorganisms (Versalovic *et al.*, 1991). This research focused on the phenotypic and genotypic characterization of phosphate solubilizing *Pseudomonas* from different oil palm soils by the application of biochemical and molecular techniques. The main objectives of this research were:

i. To biochemically characterize the phosphate solubilizing Pseudomonads isolated from oil palm soils.

ii. To identify the phosphate solubilizing *Pseudomonas sp*. isolates from oil palm fields using 16srRNA.
iii. To study the genetic variation of the phosphate solubilizing *Pseudomonas sp.* from oil palm soils by using REP-PCR.

The results will give a better insight on the molecular diversity of *Pseudomonas* isolates from soils planted with oil palms.

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