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OPTIMISATION ON BIODEGRADATION OF GLYPHOSATE BY LOCALLY ISOLATED BACTERIA

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to my parents, farmers, pioneers and fellow researchers.

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

OPTIMISATION ON BIODEGRADATION OF GLYPHOSATE BY LOCALLY-ISOLATED BACTERIA

By

MOTHARASAN MANOGARAN

November 2017

Chairman: Siti Aqlima Binti Ahmad, PhD
Faculty : Biotechnology and Biomolecular Sciences

N-Phosphonomethylglycine (glyphosate) is often used to control weeds in agriculture land. In Malaysia alone, thousands of tonnes of glyphosate formulation is used annually. Although this low-cost herbicide is able to effectively in killing weeds, its extensive use has been negatively linked to the human health due to its toxicity. Moreover, many past studies have reported its negative effects on aquatic animals and vertebrates. Thus, there is a need for an eco-friendly method to manage this environmental contaminant. Furthermore, it is crucial to find glyphosate-degrading microorganisms in the soil of interest for local applications. The objective of this study is to isolate local glyphosate-degrading bacteria and optimisation of culture medium condition to improve the degradation rate. Two bacteria isolated from agriculture site located in Kedah, Malaysia were identified by physical, biochemical and 16S rRNA sequencing techniques as *Burkholderia vietnamiensis* strain AQ5-12 and *Burkholderia* sp. strain AQ5-13. These strains were found with the ability to tolerate up to 12 mL/L Roundup concentration and were successfully used to degrade glyphosate. Factors affecting glyphosate biodegradation such as carbon and nitrogen sources, pH of the medium, glyphosate concentration and temperature were optimised using one factor at time (OFAT) and response surface method (RSM) using free cells. Initial free cells of strain AQ5-12 and AQ5-13 were able to degrade 79.7% and 40.67% of 50 ppm glyphosate, respectively, within 24 h incubation. Using these optimisation processes, free cells of AQ5-12 were able to degrade 94% of 100 ppm glyphosate, whereas strain AQ5-13 degraded 94% of 50 ppm glyphosate under optimal condition. The results illustrated fructose at 8.62 g/L, ammonium sulphate at 0.5 g/L, pH 5.41, 100 ppm of glyphosate concentration and 32ºC as the optimum biodegradation conditions required by *Burkholderia vietnamiensis* strain AQ5-12, whereas the optimum biodegradation conditions for *Burkholderia* sp. strain AQ5-13 were sucrose at 8.0 g/L, ammonium sulphate at 0.5 g/L, pH 6.0, 50 ppm of glyphosate concentration and temperature at 32ºC. The optimised condition for free cells resulted in significant improvement in degradation rate. The bacteria were immobilised in gellan gum gelling agent with its conditions optimised. The results presented a degradation rate of 87.2% 100 ppm glyphosate with gellan gum concentration of 0.55 g, 285 number of beads and bead size of 0.48 cm for immobilised cells of AQ5-12.

Meanwhile, immobilised cells of AQ5-13 illustrated a degradation rate of 96.68% 50 ppm glyphosate with gellan gum concentration of 0.55 g, 280 number of beads and bead size of 0.45 cm. It was seen that immobilised form of bacteria showed better biodegradation in terms of duration as it degrades the glyphosate within 12 h compared to free cells that require 24 h degradation process in optimised media. In conclusion, these strains possess the potential of being used in the management of glyphosate contamination. Furthermore, the success of isolating bacteria from local soils in Malaysia has shown prominent ability in glyphosate degradation rate, which can be applied for glyphosate treatment in agricultural land.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

BIOPENURUNAN DAN PENGOPTIMUMAN GLIFOSAT OLEH PEMENCILAN BAKTERIA TEMPATAN

Oleh

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N-Phosphonomethylglycine (glyphosate) sering digunakan untuk mengawal rumpai di tanah pertanian. Di Malaysia sahaja, beribu-ribu tan formulasi glifosat digunakan setiap tahun. Walaupun racun herba murah ini berkeupayaan berkesan dalam membunuh rumpai, penggunaannya yang luas telah dikaitkan secara negatif dengan kesihatan manusia akibat keracunannya. Selain itu, banyak kajian lepas telah melaporkan kesan negatif ke atas haiwan akuatik dan vertebrata. Oleh itu, terdapat keperluan untuk kaedah yang mesra alam untuk menguruskan pencemaran alam sekitar ini. Tambahan pula, adalah penting untuk mencari mikroorganisma yang menurunkan glifosat di tanah yang menarik untuk aplikasi tempatan. Oleh demikian, objektif kajian ini adalah untuk mendapatkan bakteria tempatan yang boleg mendegradasikan glifosat dan pengoptimuman media untuk meningkatkan kadar degradasi. Dua bakteria yang dipencilkan dari tapak pertanian yang terletak di Kedah, Malaysia telah dikenal pasti dengan teknik penjujukan fizikal, biokimia dan 16S rRNA sebagai *Burkholderia vietnamiensis* strain AQ5-12 dan *Burkholderia* sp. strain AQ5-13. Strain ini didapati dengan keupayaan untuk bertolak ansur sehingga kepekatan 12 mL/L Roundup dan berjaya digunakan untuk merendahkan glifosat. Faktor-faktor yang mempengaruhi biopenurunan glifosat seperti sumber karbon dan nitrogen, pH medium, kepekatan glifosat dan suhu dioptimalkan dengan menggunakan satu faktor pada waktu (OFAT) dan kaedah permukaan tanggapan (RSM) menggunakan sel bebas. Sel-sel bebas awal strain AQ5-12 dan AQ5-13 mampu menurunkan 79.7% dan 40.67% glifosat 50 ppm, masing-masing, dalam pengeraman 24 jam. Dengan menggunakan proses pengoptimuman ini, sel-sel bebas AQ5-12 mampu menurunkan 94% daripada 100 ppm glifosat, manakala strain AQ5-13 menurunkan 94% daripada 50 ppm glifosat dalam keadaan optimum. Keputusan menggambarkan fruktosa pada 8.62 g/L, ammonium sulfat pada 0.5 g/L, pH 5.41, 100 ppm kepekatan glyphosate dan 32ºC sebagai syarat biopenurunan optimum yang diperlukan oleh *Burkholderia vietnamiensis* strain AQ5-12, manakala keadaan biodegradasi optimum untuk Burkholderia sp. strain AQ5-13 adalah sukrosa pada 8.0 g/L, ammonium sulfat pada 0.5 g/L, pH 6.0, 50 ppm kepekatan glikosat dan suhu pada 32ºC. Keadaan yang dioptimumkan untuk sel-sel bebas menghasilkan peningkatan yang ketara dalam kadar penurunan. Bakteria telah diabadikan dalam agen gel gellan gum dengan keadaannya dioptimumkan. Hasilnya

memperlihatkan kadar penurunan 87.2% 100 ppm glifosat dengan kepekatan gellan gum 0.55 g, 285 bilangan manik dan saiz manik 0.48 cm untuk sel sekat gerak AQ5-12. Sementara itu, sel sekat gerak AQ5-13 menggambarkan kadar kemerosotan 96.68% 50 ppm glyphosate dengan kepekatan gellan gum 0.55 g, 280 bilangan manik dan saiz manik 0.45 cm. Ia telah memperlihatkan bahawa bentuk sekat gerak bakteria menunjukkan biopenurunan yang lebih baik dari segi tempoh kerana ia menurunkan glifosat dalam masa 12 jam berbanding dengan sel-sel bebas yang memerlukan 24 jam proses penurunan dalam media yang dioptimumkan. Kesimpulannya, strain ini mempunyai potensi untuk digunakan dalam pengurusan pencemaran glifosat. Tambahan pula, kejayaan memencilkan bakteria dari tanah tempatan di Malaysia telah menunjukkan keupayaan menonjol dalam kadar penurunan glifosat, yang boleh digunakan untuk rawatan glifosat di tanah pertanian.

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"It's the job that's never started as takes longest to finish."

Motharasan Manogaran, 2017

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

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LIST OF ABBREVIATIONS

%	Percent
<	Less than
>	Greater than
Abs	Absorbance
AMPA	Aminomethylphosphonic acid
ANOVA	The analysis of variance
CCD	Central composite design
cm	Centimetre
Co	Cobalt
Cr	Chromium
Cu	Copper
Da	Dalton
dH_2O	Distilled water
EDTA	Ethylene diamine tetra acetic acid
et al	and friends
EC	Half maximal effective concentration
EPSPS	5-enolpyruvyl-shikimate-3-phosphate-synthase
Fe	Iron
FFD	Fractional factorial design
G	Gram
h	Hour
IPA	Isopropylamine
Hg	Mercury
kb	Kilo base
kDa	Kilo dalton
kg	Kilogram
L	Liter
LC	Lethal concentration
M	Molar
min	Minute
mL	Mililitre
MSM	Minimal salt medium
MW	Molecular weight
NA	Nutrient agar
^oC	Degree celcius
OD	Optical density
OFAT	One-factor-at-a-time
POEA	Polyethoxylated tallow amine
RPM	Rotation per minute
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
µL	Microlitre
µm	Micrometre
µM	Micromolar

CHAPTER 1

INTRODUCTION

Herbicides are used as a means of controlling a variety of weed species. Newly formulated herbicides are fast acting and possess unique actions against different environmental conditions. Herbicides can help to increase crop yield by reducing crop loss due to uncontrolled weed growth. Meanwhile, glyphosate is a well-known herbicide in the agricultural field. The rise of glyphosate is mainly due to its fast action in controlling weed number. It is the only herbicide that targets 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (Yu et al., 2015). Hence, there are no rival herbicide analogues or classes for this herbicide (Duke and Powles, 2008). By inhibiting this crucial enzyme, plants will wither and die. Glyphosate herbicides were first introduced as Roundup brand by Monsanto Company. Since then, more than 172 glyphosate-based products can be found in Malaysia alone.

Herbicides are mainly used to kill and destroy unwanted terrestrial weeds. However, during raining seasons, applied Roundup formulations on agricultural land are washed away and ended up in an aquatic medium. Aquatic plants and algae are consequently the most sensitive group of aquatic nontarget organisms. Moreover, they play significant role in aquatic ecosystems. Destruction of these aquatic plants reduces the stability of sediments in the lakes and running water, thus destroying the habitat where juvenile fishes and crustaceans take shelter (Gurnell et al., 2012). Besides, exposure to glyphosate on amphibians resulted in abnormalities. A high percentage of morphology alterations was observed in sharp-snouted tree frog (*Ssinax nassicus*) incubated with 3 - 7 mg/L of glyphosate, which is the exact amount used in the sub-agricultural field (Sviridov et al., 2014).

Increased awareness on the profound effects of environmental problems such as traces of glyphosate in drinking waters and accepts of herbicide usage has encouraged necessary investigations to avoid, reduce or eliminate these problems. Applications of microbial inoculants for in-situ treatment of contamination of soil include the enhancement of associative N_2 fixation and symbiotic, prevention of soil-borne plant pathogens, biological control of frost injury on the tubular plant and biodegradation of xenobiotic compounds (Elsas and Heijnen, 1990). This microorganism has been introduced into contaminated sites in the form of liquid suspensions or adsorbed into different physical carriers (Sparrow and Ham, 1983). Nevertheless, the efficiency of glyphosate biodegradations depends on the success in isolating glyphosate-degrading strains.

A number of glyphosate degrading microorganisms with varying inherent capability to utilise glyphosate as carbon or phosphorus source have been isolated and reported from different environmental sources. However, high tolerance glyphosate degrading bacteria were frequently isolated from agricultural contaminated sites. To date, *Bacillus subtilis*

Bs-15 is one of the best glyphosate degraders with the maximum concentration tolerated by this bacterium reaches as high as 40,000 mg/L isolated from glyphosate exposed soil (Fan et al., 2012). The growth of these bacteria and their ability to effectively degrade glyphosate are significantly influenced by various physical and nutritional factors. Optimisation of these factors is therefore crucial to maximise their degradation ability. One factor at a time (OFAT) optimisation technique is often deployed to achieve optimum glyphosate degradation. However, this method results in some limitations; some of them are the lack of capability to address the interactive effects between factors and time-consuming as the parameters cannot be simultaneously run (Okoroma et al., 2012). Statistical optimisation approach using response surface methodology (RSM) is used to correct the limitations of the OFAT, which has successfully optimised many biological processes (Karamba et al., 2016; Nawawi et al., 2016; Fakhfakh-Zouari et al., 2010)

Most of these bacteria are isolated using glyphosate alone without introducing any herbicide formulation in the medium. Although it has been reported that *Bacillus subtilis* Bs-15 able to degrade up to 40,000 ppm of glyphosate, the application in situ is still questionable (Fan et al., 2012). This is because these bacteria need to withstand the toxic compounds that usually come together with glyphosate formulation. Chemicals such as polyethoxylated tallow amine, acrylamide and urea, which can be found in major glyphosate formulation, can significantly reduce bacterial growth or kill them (Mesnage et al., 2014). Therefore, it is important to isolate bacteria using Roundup than using glyphosate alone as glyphosate alone isolated bacteria may not perform as expected in the application of bioremediation. Furthermore, only two glyphosate degrading bacteria were isolated from Malaysia (Nourouzi et al., 2011). Moreover, no reports or studies were found on glyphosate degradation using gellan gum for immobilisation. The advantage encapsulation of bacterial cells in gellan gum using immobilisation technique provides the ability to be used in real life application with fewer risks. Beads, made of gellan gum has the ability to be repeatedly used and the composition of the beads are made of non-toxic materials which is safer towards the environment (Karamba et al., 2016). Moreover, this beads prevents the target bacterial cells leaked out which could be pathogenic to native strains. These gaps need to be filled as data from this study could provide significant understanding on the effects of immobilisation using native strains in Malaysia.

Based on the statements above, this study aims at isolating a native bacterium from Malaysia with a potential of withstanding the toxicity of Roundup and ability to degrade glyphosate.Thus, the objectives of this study are:

1. To isolate, characterise, identify and select glyphosate-degrading bacterium from the soil.
2. To determine optimise nutritional and physical conditions of the bacterium for a maximum glyphosate degradation using OFAT approach and RSM by free cells.
3. To study the glyphosate degradation rate using immobilised bacterium, optimise the immobilisation conditions and investigate the degradation ability of the immobilised cells via OFAT approach and RSM.

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