**UNIVERSITI PUTRA MALAYSIA**

***BIODEGRADATION OF PHENOL BY A MALAYSIAN ISOLATE Rhodococcus sp. NAM 81***

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**BIODEGRADATION OF PHENOL BY A MALAYSIAN ISOLATE *Rhodococcus* sp. NAM 81**

By

**NORAZAH MOHAMMAD NAWAWI**

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

**November 2016**

**DEDICATION**

This Thesis is dedicated to my beloved Husband,

*Mr Nor Suhaime Sukirman*

Daughters,

*Miza Nur Wajihah & Miza Nur Wafiyah*

and

My supportive daddy,

*Tuan Haji Mohammad Nawawi Abdul Rahman*

sister and brother

as well as my late mother,

*Jamilah Abdul Rahman*,

my thoughts and prayers are always with you Mak

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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**November 2016**

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

Phenol is a major threat to the environment due its toxicity effect and extensive use in various industries. Phenol can harm human and other organisms at a low dose. Phenols and phenolic compounds are organic pollutants generated from various industrial activities. Biodegradation is a technology that is currently applied for decontaminating pollutants including phenols. Biodegradation of phenols using microorganism is an eco-friendly and cost-effective approach. Bacteria are widely used in bioremediation processing. Thus, the isolation and selection of good microorganism with tolerance towards other toxicants is important to improve the performance of bioremediation. This study evaluates the potential of a local *Rhodococcus* bacteria to decompose phenol, an oxygenated hydrocarbon, one of the harmful pollutants, because of its toxic and carcinogenic effects. Ten strains of *Rhodococcus* spp. isolated from Peninsular Malaysia survived and grew in a medium that was supplemented with 0.5 $gL^{-1}$ of phenol. The 1500 bp phenol hydroxylase gene was amplified from the eight strains as a confirmation of phenol-degradation pathway and for future molecular marker for the presence of phenol-degrading bacteria. One strain, *Rhodococcus* sp. NAM 81 was selected as the most potent strains with the ability to decompose 0.6 $gL^{-1}$ phenols within 24 hours. The phenol biodegradation ability of *Rhodococcus* sp. NAM 81 was greatly affected by the presence of trace element in the medium. The parameters that supported the degradation and growth of *Rhodococcus* sp. NAM 81 were optimized initially by one-factor at a time approach. The strain exhibited highest cell growth and phenol degradation at the optimal incubation conditions of 30 °C and pH 7.5. Ammonium sulphate 0.4 $gL^{-1}$, glycine 0.3 $gL^{-1}$, and 0.1 $gL^{-1}$ NaCl were needed to enhance cell growth. Apart from phenol, the strain was able to utilizes 1 mg $L^{-1}$ of 2,4-dinitrophenol, toluene, naphthalene, diesel, acetonitrile, glycerol and waste cooking oil as the sources of carbon for growth, but the phenol degradation ability was inhibited by 1 mg $L^{-1}$ of $Ag^{+}$, $Cu^{2+}$, $Cr^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Hg^{2+}$. Statistical approach by fractional factorial design (FFD) and response surface method (RSM) improved the biodegradation of phenol with pH, phenol and NaCl concentrations were found to be important parameters. The results showed good verification between theoretical and experimental data. RSM method improves the process by 1.2 fold for phenol degradation and 1.3 fold for cell growth. Investigation on the application of immobilized cells in phenol biodegradation started with an evaluation of suitable matrices such as gellan gum, calcium alginate, agarose, agar-agar and polyacrylamide for phenol degradation using an immobilization approach. Gellan gum was found to be the most effective and suitable matrix for high phenol degradation compared to other matrices studied. Maximum phenol degradation was achieved at the gellan gum concentration of 0.75% (w/v), bead size of 3 mm diameter and bead number of 300 per 100 mL medium. Both free and immobilized bacteria exhibited similar rates of phenol degradation at the phenol concentration of 100 $mgL^{-1}$, but at higher phenol concentrations, immobilized bacteria exhibited a higher degradation rate of phenol. The immobilized cells completely degrade phenol within 108, 216, and 240 h at 1100, 1500 and 1900 $mgL^{-1}$ phenol, respectively, whereas free cells took 240 h to completely degrade phenol at 1100 $mgL^{-1}$. In overall, the rates of phenol degradation by both immobilized and free bacteria decreased gradually as the phenol concentrations were increased. It also proved that inhibition of heavy metal and respiratory inhibitors was prevented by gellan gum encapsulated cells. The immobilized cells showed no loss in phenol degrading activity after being used repeatedly for 50 repetitions of 18 h cycle and was stable after storing at 4 ºC for 28 days. Study on the best disruption methods for efficient enzyme, protein and genomic DNA isolation from *Rhodococcus* cells showed that grinding for 20 minutes under liquid nitrogen was the best approach. The ortho-cleavage pathway as applied by *Rhodococcus* sp. NAM 81 for phenol degradation was discovered by biochemical and polymerase chain reaction. The gene for phenol hydroxylase; the enzyme that catalyzes the conversion of phenol to catechol, has been cloned, expressed and purified from *Rhodococcus* sp. NAM 81. SDS-PAGE produced a single band with a molecular weight of ~ 60 kDa. The potential of resting cells of *Rhodococcus* sp. NAM 81 as an alternative to the use of free and immobilized cells for phenol biodegradation process in liquid waste was also tested. The results from this study showed that *Rhodococcus* sp. NAM 81 has an excellent potential that can be applied in bioremediation of phenol-containing wastes especially using immobilized cells.

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

**BIODEGRADASI FENOL OLEH *Rhodococcus* sp. NAM 81 PENCILAN MALAYSIA**

Oleh

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Fenol merupakan satu bahan yang mengancam alam sekitar kerana kesan ketoksikan dan penggunaan yang meluas dalam pelbagai industri. Fenol membahayakan manusia dan organisma lain walaupun dalam dos yang minimum. Fenol dan sebatian fenolik merupakan pencemar organik yang dihasilkan dari pelbagai aktiviti industri. Biodegradasi adalah teknologi yang boleh digunakan untuk menguraikan bahan pencemar termasuk fenol. Biodegradasi fenol menggunakan mikroorganisma adalah satu pendekatan yang mesra alam dan ekonomi. Bakteria digunakan secara meluas dalam proses pemulihan alam sekitar. Maka, pemencilan dan pemilihan mikroorganisma yang bagus dengan toleransi terhadap toksik lain adalah penting untuk meningkatkan prestasi pemulihan persekitaran. Kajian ini menilai potensi bakteria *Rhodococcus* tempatan untuk mengurai fenol, iaitu hidrokarbon oksigen, yang merupakan salah satu daripada bahan cemar berbahaya kerana kesan toksik dan karsinogeniknya. Sepuluh strain *Rhodococcus* spp. dipencilkan dari Semenanjung Malaysia mampu tumbuh dalam medium yang mengandungi 0.5 $gL^{-1}$ fenol. 1500 bp gen fenol hydroxylase gen telah diamplifikasi dari lapan strain sebagai penanda molekul. Satu strain, *Rhodococcus* sp. NAM 81 yang telah dipilih sebagai strain yang paling efektif menguraikan 0.5 $gL^{-1}$ fenol dalam masa 24 jam. Keupayaan biodegradasi *Rhodococcus* sp. NAM 81 terjejas dengan kehadiran unsur surih dalam medium. Sebagai permulaan, parameter yang membantu biodegradasi dan pertumbuhan *Rhodococcus* sp. NAM 81 telah dioptimumkan menggunakan satu faktor pada satu masa. Pertumbuhan sel tertinggi dan degradasi fenol adalah optimum pada (30 °C, pH 7.5). Ammonium sulfat 0.4 $gL^{-1}$, glycine 0.3 $gL^{-1}$ dan 0.1 $gL^{-1}$ NaCl diperlukan untuk meningkatkan pertumbuhan sel. Selain daripada fenol, strain ini juga mampu menggunakan 2,4-dinitrophenol, toluena, naftalena, diesel, asetonitril, gliserol dan sisa minyak memasak sebagai sumber karbon. Namun, kebolehan *Rhodococcus* sp. NAM 81 direncat dengan kehadiran logam berat di dalam medium yang mengandungi 1 $mgL^{-1}$ $Ag^{+}$, $Cu^{2+}$, $Cr^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Hg^{2+}$. Kaedah statistik melalui rekabentuk faktor fraksi (FFD) dan pengkaedahan tindakbalas permukaan (RSM) meningkatkan keupayaan biodegradasi fenol dengan pH, kepekatan fenol dan NaCl sebagai parameter penting yang disahkan melalui teori dan data eksperimen. RSM telah memperbaiki proses dengan 1.2 kali ganda bagi degradasi fenol dan 1.3 kali ganda bagi pertumbuhan sel. Kajian tentang penggunaan sel sekat gerak dalam biodegradasi fenol bermula dengan penilaian matriks yang sesuai merangkumi gam gellan, kalsium alginat, agarose, agar-agar dan polyacrylamide. Gam gellan didapati matriks yang sesuai berbanding dengan matriks-matriks lain. Maksimum degradasi fenol dicapai pada kepekatan gam gellan sebanyak 0.75 %, saiz manik 3 mm dengan bilangan 300 biji dalam 100 ml media. Kedua-dua sel bebas dan sel sekat gerak mengurai pada kadar yang sama pada kepekatan fenol 100 $mgL^{-1}$, namun pada kepekatan yang tinggi, sel sekat gerak sahaja mengurai pada kadar yang tinggi. Fenol terurai sepenuhnya dalam masa 108, 216, dan 240 jam dalam kepekataan fenol masing-masing 1100, 1500 dan 1900 $mgL^{-1}$, manakala sel bebas mengambil masa 240 jam untuk mengurai sepenuhnya 1100 $mgL^{-1}$ fenol dan tidak berupaya mengurai sepenuhnya fenol pada kepekatan yang tinggi. Secara keseluruhan, kadar degradasi fenol oleh sel sekat gerak dan sel bebas menurun selari dengan peningkatan kepekatan fenol. Logam berat dan perencat respirasi memberikan kesan yang rendah pada penguraian fenol oleh sel tersekat-gerak berbanding sel bebas. Sel tersekat-gerak boleh digunakan berulang kali selama 50 kitaran 18 jam dan stabil dalam penyimpan pada 4 °C selama 28 hari. Kajian tentang teknik untuk memecahkan sel bagi mengeluarkan enzim, protein dan DNA daripada sel *Rhodococcus* menunjukkan pemecahan menggunakan nitrogen cecair dalam masa 20 minit adalah kaedah terbaik. Bakteria ini menggunakan tapak jalan ortho untuk menguraikan fenol yang telah ditentukan menggunakan pendekatan biokimia dan tindak balas berantai polimerase. Gen untuk fenol hydroxylase, iaitu enzim yang memangkinkan penukaran fenol kepada catechol telah diklon, dirembes serta ditulenkan daripada *Rhodococcus* sp. NAM 81. Gel SDS-PAGE menghasilkan jalur tunggal dengan berat molekul ~ 60 kDa. Potensi sel rehat bagi *Rhodococcus* sp. NAM 81 sebagai alternatif kepada penggunaan sel bebas dan tersekat gerak untuk bidegradasi fenol dalam pencemar cecair juga telah diuji. Keputusan daripada kajian ini menunjukkan bakteria *Rhodococcus* sp. NAM 81 mempunyai potensi yang baik yang boleh diaplikasikan dalam proses biopemulihan sisa mengandungi fenol terutamanya menggunakan sel tersekat-gerak.

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*Norazah Mohamad Nawawi, 2016*

I certify that a Thesis Examination Committee has met on 10 November 2016 to conduct the final examination of Norazah binti Mohammad Nawawi on her thesis entitled "Biodegradation of Phenol by a Malaysian Isolate *Rhodococcus* sp. NAM 81" 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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| | |
|---|---|
| Abs | Absorbance |
| CCD | Central composite design |
| Cd | Cadmium |
| cm | Centimetre |
| Cr | Chromium |
| Co | Cobalt |
| Cu | Copper |
| ºC | Degree celcius |
| $dH_2O$ | Distilled water |
| Da | Dalton |
| EDTA | Ethylene diamine tetra acetic acid |
| FFD | Fractional factorial design |
| G | Gram |
| $gL^{-1}$ | Gram per litre |
| > | Greater than |
| h | Hour |
| Fe | Iron |
| kDa | Kilo dalton |
| kb | Kilo base |
| kg | Kilogram |
| < | Less than |
| L | Liter |
| Hg | Mercury |
| mL | Mililiter |

| | |
|---|---|
| $mgL^{-1}$ | Miligram per litre |
| mM | Milimolar |
| $\mu gL^{-1}$ | Microgram per litre |
| µM | Micromolar |
| µL | Microlitre |
| min | Minute |
| MW | Molecular weight |
| MSM | Minimal salt medium |
| M | Molar |
| µm | Micrometer |
| $h^{-1}$ | Per hour |
| $L^{-1}$ | Per liter |
| % | Percent |
| *et al* | and friends |
| NA | Nutrient agar |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| RPM | Rotation per minute |
| RSM | Response surface method |
| SDS | Sodium dodecyl sulphate |
| SEM | Scanning electron microscope |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Research background

Malaysia as a modern Islamic country has made a progressive living and operating environment in a stable political and consistent economic growth from various industries. Industrializations will lead to numerous environmental problems that is harmful to ecosystems and communities (Hsu *et al.*, 2014). In the Fourth Regional 3R Forum in Asia, it has been reported that about 3,281,569.21 metric tonnes of scheduled wastes have been generated in Malaysia compared to 3,087,496.84 metric tonnes (6.29%) in 2010. Besides dross, slag, clinker, ash, gypsum, mineral sludge and heavy metals sludge, about 7,904.42 metric tonnes of phenol have been also released, which contributes to about 0.49% of the total waste as the main categories of waste generated in the country (DOE, 2011).

Phenol ($C_6H_5OH$) is toxic and relatively recalcitrant to biological degradation process (Li *et al*., 2015; ATSDR, 2014). Phenol occurs naturally and can also originate from industrial effluents such as pharmaceutical, food industries, oil refineries and coal conversion processes (Yemendzhiev *et al*., 2008; Passos *et al*., 2010; Pradeep *et al*., 2014; Aida and Hanan, 2014). Phenol and phenolic compounds are also released into the wastewater from steel industries, petrochemicals, polymeric resins and dye manufacturing units. Phenol is important as raw materials and additives for industrial purposes, especially in laboratory processes, chemical industry, chemical engineering, wood and plastics processing. Phenol and phenolics are soluble in water; hence causing the carbolic odour in water. It disturbs the ecological balance since it is toxic to aquatic flora and fauna (Michalowicz and Duda, 2007; Clodio *et al*., 2009; Soudi and Kolahchi, 2011). It could also diffuse across a cell's membrane of an organism. Studies showed that constant administration of phenol by animals could cause pathological changes (ATSDR, 1998). Phenol discharge towards the water body will endangers aquatic life, even at concentration of between 500 and 2500 $mgL^{-1}$ (Yan *et al.,* 2005).

## 1.2 Statement of problem

Based on the toxicity and risks to organism and environment, phenol removal is crucial for lowering the concentration of phenol below the regulatory limit that requires appropriate treatment of waste containing phenol before being discharged to the nature. A report prepared by the United States Government Accountability Office mentioned that their agencies have spent almost US$30 billion from 1986 to 2008 for all environmental cleanup and restoration activities (GAO, 2010). Physical, chemical and biological methods have been widely applied to remove contaminants from the polluted area. Yet, among the techniques, biological removal offers the cheapest and safest method through bioremediation (Bijay *et al*., 2012). It is an ideal solution for pollution abatement nowadays being a green technology that uses biological systems for the treatment of contaminants (Desai *et al*., 2010). Biological approach is a method of choice as it is low in cost and offers complete mineralisation compared to other methods (Shweta and Dhandayuthapani, 2013; Agarry *et al.,* 2008). Even though this recent technology is using a multidisciplinary approach, its central thrust depends on microbiology, especially with the application of bacteria in phenol biodegradation (Gami *et al.*, 2014; Aysha and Mumtaj, 2014; Shah, 2014; Krastanov *et al*., 2013).

## 1.3 Significance of study

Realising the importance of using microorganisms as a potential remediation system for xenobiotic compounds in waste, the Malaysian Government, through the Malaysia Genome Institute (MGI) established a project on the isolation of such microbes for industrial and environmental applications. The study on the ecology of marine and terrestrial microbes around Peninsular Malaysia is in progress with a number of microorganisms from different ecological niches that have been isolated. A group of microorganisms that have been chosen for further research is the mycolic acid-containing actinomycetes. The broad metabolic diversity of the mycolic acid-containing actinomycetes have attracted the pharmaceutical, environmental, chemical and energy industries. The discovery of novel xenobiotics-degrading metabolic capability and bioactive compounds from this group of microbes is far from exhausted (Davies and Davies 2010; Arenskotter *et al*., 2004). The rhodococci is a group of aerobic, non-motile actinomycetes (Larkin *et al*., 2005). *Rhodococci* degrade an extensive array of recalcitrant pollutants that makes them promising candidates to be used in bioremediation. In addition, there are numerous other industrial applications where rhodococci, or their gene products, are actually being used or are in the pipeline. The economic significance of *Rhodococcus* species are being widely recognised. In Malaysia, informations available on the potential role of *Rhodococcus* spp. especially in phenol detoxification is slowly being discovered.

Environmental-friendly technologies are gaining increasing prominence as a safe remediation technique for phenol and phenolics pollution (Agrawal and Shahi*,* 2015; Dash *et al*., 2009). In this study, the evaluation of various locally isolated *Rhodococci* will broaden the reservoir of existing phenol-degrading microbial populations that are competent in phenol degradation especially in a temperate climate.

## 1.4 Research objectives and research approach

Keeping all these points in view, the overall aim of this project was to develop an approach for phenol biodegradation performed by locally-isolated microorganism. The hypothesis for this research were; (a) the locally isolated *Rhodococcus* is able to tolerate higher concentration of phenol (b) the immobilized *Rhodococcus* sp. offer better degradation rate and (c) the enzymes and gene encoded for the enzymes could be isolated and characterized. In view of this, the following objectives are outlined:

1. To screen and characterize phenol-degrading *Rhodococcus*
2. To optimize the degradation of phenol by the selected phenol-degrading *Rhodococcus* using one factor at a time (OFAT) approach and response surface methodology (RSM) by free and immobilized cells.
3. To immobilize the phenol-degrading *Rhodococcus* to improve biodegradation efficiency.
4. To determine the mechanism of phenol degradation by characterization of the phenol-degrading enzymes
5. To evaluate the potential of resting cells for phenol degradation

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