UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF PHENOL BY FREE AND IMMOBILISED CELLS OF LOCALLY-ISOLATED BACTERIA

ABUBAKAR AISAMI

FBSB 2018 1

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By

ABUBAKAR AISAMI

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

May 2017

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DEDICATION

This research work is dedicated to my Late father Malam Aisami Garba and our Malama Hadiza Aliyu.

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

BIODEGRADATION OF PHENOL BY FREE AND IMMOBILISED CELLS OF LOCALLY-ISOLATED BACTERIA

By

ABUBAKAR AISAMI

May 2017

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

Phenol is mainly used by the industries to produce a variety of chemical products such as resins, textiles, pesticides, plastics and explosive. Due to the wider use of phenol and other phenolic compounds by industries, this has resulted in an increased presence of these toxic compounds in the environment. In Malaysia, phenol and phenolic compounds rank among the top three scheduled wastes with thousands of tonnes being produced yearly for disposal. In Malaysia about 37.7 metric tonnes of phenol and phenol-containing wastes are produced in 2014, there is also an incident of tanker accidents Straits of Malacca in 2003 where tonnes of phenol spilt into the river and the Kapar power plant in Klang, Selangor uses coal thereby producing phenol as a by-product.These make phenol one of the environmental problem in Malaysia. Bio-removal of phenol by microorganisms especially bacteria has been demonstrated to be the most effective and economical approach compared to physio-chemical methods. The search for efficient phenol-degraders especially local sources to remediate local phenol pollution is important as indigenous bacteria usually have better survival and resilient to local geographical conditions. In this study, phenol-degrading microorganisms were isolated from local soils and water bodies. Identification was carried out using 16s rRNA gene sequencing and molecular phylogeny analysis using the Phylip software. The isolates were inoculated in mineral salt media with 0.5 g/L phenol as the sole source of carbon. Phenol degradation was determined using 4-amino antipyrine method. Physical and cultural conditions influencing phenol degradation such as pH, temperature, nature of bacteria, salinity, and nitrogen source were optimised via one-factor-at-a-time and response surface methodology (RSM). The robust and hardy Gellan gum was used for the immobilisation of bacterial cells and also the ortho and meta-pathways for phenol degradation were elucidated. The highest degradation was achieved at pH 7.5 (phosphate buffer) for all of the three isolates, with an optimum temperature of 30°C for *Pseudomonas* sp. AQ5-04 and *Alcaligenes* sp. AQ5-02 and 32.5°C for *Serratia* sp. AQ5-03. Ammonium sulphate was established

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to be the best nitrogen source at the concentration of 0.4 g/L for all three isolates and a sodium chloride concentration of 0.1 g/L for *Alcaligenes* sp. AQ5-02 and 0.15 g/L for *Serratia* sp. AQ5-03. However, *Pseudomonas* sp. AQ5-04 could tolerate up to 0.2 g/L of sodium chloride. This indicates that these isolates are not suitable for remediation of phenol in the marine environment. Immobilisation has reduced the incubation period from 48 h to 24 h for all three isolates, with *Pseudomonas* sp. AQ5-04 showing the best reusability of 22 cycles compared to 16 and 14 cycles for *Alcaligenes* sp. AQ5-02 and *Serratia* sp. AQ5-03, respectively. The immobilised cell of *Alcaligenes* sp. AQ5-02, *Serratia* sp. AQ5-03 and *Pseudomonas* sp. AQ5-04 can degrade up to 1900 mg/L. All the three isolates have the ability to degrade phenol both in free and immobilised cells. Immobilisation has significantly enhanced their biodegradation ability. *Pseudomonas* sp. AQ5-04 has the highest reusability as well as tolerating slightly high salinity. The meta pathway for phenol degradation was detected for *Alcaligenes* sp. AQ5-02 *Pseudomonas* sp. AQ5-04 while the ortho pathway was detected for, *Serratia* sp. AQ5-03. The accuracy and statistical analysis of the kinetic models used show that the best model was Luong for all bacterial growth curves with the lowest values for root mean square error or RMSE and adjusted Akaike Information criteria AICc, highest adjusted R^2 values, and with Bias Factor and Accuracy Factor nearest to unity (1.0) for *Pseudomonas* sp. AQ05-04 and *Serratia* sp. AQ05-03, with the exception of *Alcaligenes* sp. AQ05-02 where the AICc value was not the lowest but the rest of the statistical analysis values still overwhelmingly pinpointing the Luong model as the best model for *Alcaligenes* sp. AQ05-02. The calculated value for the Luong's constants maximal growth rate, half saturation constant for maximal growth, maximal concentration of substrate tolerated and curve parameter that defines the steepness of the growth rate decline from the maximum rate, symbolized by u_{max}, K_s, S_m, and n were 0.10±0.02 hr^{-1}, 0.02±0.01 g/L, 2.05±0.06 g/L and 0.80±0.20 (± 95% confidence interval) for *Pseudomonas* sp. AQ05-04, 0.07±0.02 hr^{-1}, 0.02±0.01 g/L, 1.18±0.03 g/L and 1.16±0.23 for *Serratia* sp.AQ05-03, and 0.07±0.01 hr^{-1}, 0.18±0.03 g/L, 1.27±0.24 g/L and 6.60±0.94 for *Alcaligenes* sp. AQ05-02, respectively. It appears that the highest maximum growth rate on phenol was exhibited by *Pseudomonas* sp. AQ05-04, while both *Serratia* sp.AQ05-03 and *Alcaligenes* sp. AQ05-02 had similar lower growth rates indicating that *Pseudomonas* sp. AQ05-04 showed a higher efficient growth rate on phenol.

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

BIODEGRADASI FENOL OLEH SEL SEKAT GERAK DAN BEBAS DARI BAKTERIA ISOLASI TEMPATAN

Oleh

ABUBAKAR AISAMI

Mei 2017

Pengerusi : Profesor Madya Mohd. Yunus Bn Abd.Shukor, PhD
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Fenol digunakan terutamanya oleh industri untuk menghasilkan pelbagai bahan kimia seperti resin, tekstil, racun perosak, plastik dan bahan letupan. Oleh kerana penggunaan yang meluas oleh industri, kehadiran fenol dan sebatian fenolik lain dalam alam sekitar semakin meningkat. Di Malaysia, fenol dan sebatian fenolik adalah di antara tiga bahan buangan terjadual teratas dengan beribu-ribu tan dihasilkan setiap tahun untuk dilupuskan. Di Malaysia, lebih kurang 37.7 tan metrik bahan buangan yang mengandungi phenol dan sebatian fenolik dihasilkan pada 2014, dan ini diburukkan lagi oleh kejadian tumpahan fenol di Melaka yang berlaku pada tahun 2003. Kejadian yang sama turut berlaku di pusat penjana kuasa di Kapar , Klang Selangor di mana bahan sampingan pemprosesan arang batu dilepaskan di alam sekitar. Ini menjadikan fenol sebagai salah satu bahan pencemar alam sekitar yang penting untuk dirawat di Malaysia. Bio-penyingkiran fenol oleh mikroorganisma terutamanya bakteria merupakan satu pendekatan yang paling berkesan dan bersifat ekonomi berbanding dengan kaedah fisiko-kimia. Pencarian isolat pengurai fenol terutamanya dari sumber tempatan untuk mengatasi pencemaran fenol adalah penting kerana bakteria asli biasanya mempunyai rintangan hidup yang lebih baik dan kuat terhadap keadaan geografi tempatan. Dalam kajian ini, mikroorganisma pengurai fenol telah diisolatkan daripada tanah dan air. Identifikasi telah dijalankan dengan menggunakan penjujukan gen 16s rRNA dan analisis filogeni molekul menggunakan perisian Phylip. Isolat-isolat ini diinokulumkan dalam media garam mineral dengan 0.5 g /L fenol sebagai satu-satunya sumber karbon. Degradasi fenol ditentukan dengan menggunakan kaedah 4-amino antipirina. Ciri-ciri fizikal dan kultur yang mempengaruhi degradasi fenol seperti pH, suhu, jenis bakteria, darjah kemasinan, dan sumber nitrogen telah dioptimumkan melalui kaedah satu faktor-per-masa dan kaedah <u>gerak balas permukaan</u> (RSM). Gam Gellan yang tahan lasak telah digunakan untuk menyekatgerak sel-sel bakteria dan juga kajian tapakjalan laluan orto dan meta untuk degradasi fenol telah dikaji. Degradasi tertinggi dicapai pada pH 7.5 (penimbal fosfat)

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untuk ketiga-tiga isolat, dengan suhu optimum 30 °C untuk *Pseudomonas* sp. AQ5-04 dan *Alcaligenes* sp. AQ5-02 dan 32.5 °C untuk *Serratia* sp. AQ5-03. Amonium sulfat merupakan sumber nitrogen yang terbaik pada kepekatan 0.4 g / L bagi ketiga-tiga isolat, dan natrium klorida pada kepekatan 0.1 g/L untuk *Alcaligenes* sp. AQ5-02 dan 0.15 g/L untuk *Serratia* sp. AQ5-03. Walaubagaimanapun, *Pseudomonas* sp. AQ5-04 boleh bertahan sehingga kepekatan natrium klorida pada 0.2 g/L. Ini menunjukkan bahawa isolat-isolat ini tidak sesuai untuk meremediasi fenol dalam persekitaran marin. Sekatgerak telah mengurangkan tempoh pengeraman dari 48 jam ke 24 jam untuk ketiga-tiga isolat, dengan *Pseudomonas* sp. AQ5-04 boleh digunapakai sehingga 22 kitaran berbanding 16 dan 14 kitaran untuk *Alcaligenes* sp. AQ5-02 dan *Serratia* sp. AQ5-03, masing-masing. Sel tersekatgerak daripada *Alcaligenes* sp. AQ5-02, *Serratia* sp. AQ5-03 dan *Pseudomonas* sp. AQ5-04 boleh mengurai fenol sehingga 1900 mg/L. Ketiga-tiga isolat mempunyai keupayaan untuk menguraikan fenol dalam keadaan bebas dan tersekatgerak. Proses sekatgerak telah mempertingkatkan keupayaan biodegradasi isolat-isolat ini dengan ketara. *Pseudomonas* sp. AQ5-04 mempunyai kebolehgunaan serta mempunyai kerintangan paling tinggi pada garam. Tapakjalan meta untuk penguraian fenol telah dikenalpasti untuk *Alcaligenes* sp. AQ5-02 *Pseudomonas* sp. AQ5-04 manakala tapakjalan orto pula dikenalpasti untuk *Serratia* sp. AQ5-03. Ketepatan dan analisis statistikal pada model kinetik yang digunakan menunjukkan bahawa model yang paling terbaik adalah model Luong untuk semua kelok pertumbuhan bakteria dengan nilai paling rendah untuk akar min ralat kuasa dua RMSE dan kriteria maklumat Akaike terselaras AICc, nilai paling tinggi untuk R^2 diselaras, dan dengan Faktor Berat Sebelah dan Faktor Ketepatan paling hampir kepada satu (1.0) untuk *Pseudomonas* sp. AQ05-04 dan *Serratia* sp. AQ05-03, melainkan *Alcaligenes* sp. AQ05-02 di mana nilai AICcnya bukanlah yang paling rendah tetapi analisis statistik menunjukkan nilai-nilai penunjuk lain adalah lebih menjurus kepada model Luong sebagai model terbaik untuk *Alcaligenes* sp. AQ05-02. Nilai yang dikira bagi pemalar Luong ini seperti kadar pertumbuhan maksimum, pemalar ketepuan separa untuk pertumbuhan maksimum, kepekatan maksimum substrat yang dapat ditoleransi dan parameter lengkung yang mentakrifkan kecuraman penurunan kadar pertumbuhan daripada kadar maksimum yang dilambangkan oleh by u_{max}, K_s, S_m, dan n adalah 0.10±0.02 hr^{-1}, 0.02±0.01 g/L, 2.05±0.06 g/L dan 0.80±0.20 (± 95% sela keyakinan) untuk *Pseudomonas* sp. AQ05-04, 0.07±0.02 hr^{-1}, 0.02±0.01 g/L, 1.18±0.03 g/L dan 1.16±0.23 unutk *Serratia* sp.AQ05-03, dan 0.07±0.01 hr^{-1}, 0.18±0.03 g/L, 1.27±0.24 g/L dan 6.60±0.94 untuk *Alcaligenes* sp. AQ05-02, masing-masing. Kadar pertumbuhan maksimum tertinggi untuk fenol telah dipamerkan oleh *Pseudomonas* sp. AQ05-04, manakala kedua-dua *Serratia* sp.AQ05-03 dan *Alcaligenes* sp. AQ05-02 mempunyai kadar pertumbuhan rendah yang sama menunjukkan bahawa *Pseudomonas* sp. AQ05-04 mempunyai kadar pertumbuhan menggunakan fenol yang lebih efisien.

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I certify that a Thesis Examination Committee has met on 30 May 2017 to conduct the final examination of Abubakar Aisami on his thesis entitled "Biodegradation of Phenol by Free and Immobilised Cells of Locally-Isolated Bacteria" 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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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF ABBREVIATIONS

$(NH4)_2SO_4$	Ammonium sulphate
>	Greater than
%	Percent
<	Less than
µL	Microliter
^oC	Degrees Celsius
µM	Micro molar
As	Arsenic
Ag	Argentum
ATP	Adenosine triphosphate
CFU	Colony Forming Unit
Cd	Cadmium
cm	Centimetre
Cr	Chromium
Co	Cobalt
Cu	Copper
dH_2O	Distilled water
DEAE	Diethylaminoethylamine
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
Fe	Iron
et al	and friends
G	Gram
Hg	Mercury
HCl	Hydrogen chloride
h	Hours
Kb	Kilobase
kDa	Kilodaltons
KCN	Potassium Cyanide
Kg	Kilogram
L	Litre
Km	Michaelis-Menten constant
M	Meter
mA	Milliampere
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
Min	Minutes
$MgSO_4$	Magnesium Sulphate
mM	Millimolar
MSM	Mineral Salt Medium
MW	Molecular Weight
K_2HPO_4	di-Potassium Hydrogen Phosphate
NA	Nutrient Agar
KH_2PO_4	Potassium Dihydrogen Phosphate

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NaCl	Sodium Chloride
Ni	Nickel
OD	Optical Density
PCR	Polymerase Chain Reaction
Pb	Lead
PO_4^{3-}	Phosphate
ppm	Parts Per Million
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
v/v	Volume/Volume
UV	Ultraviolet
V_{max}	Maximum Velocity
Zn	Zinc
w/v	Weight/Volume
ɤ	Gamma
β	Beta
α	Alpha

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CHAPTER 1

INTRODUCTION

Environmental pollution is one of the major concerns in the 21st century; where billions of tonnes of harmful chemicals are produced by industries such as petroleum, paints, food, rubber, and plastic. These toxicants get their ways into the environment through air, soil, and water. Combustion of fuel, burning activities and power stations are the major sources of air pollution where volatile hydrocarbons are released into the air (DOE, 2009). Air pollutions can lead to many respiratory, cardiovascular and liver diseases (Brook et al., 2004; Ko and Hui, 2010). Discharging untreated harmful compounds and heavy metals are the primary sources of water and soil pollution. Also, oil spillage from petroleum industries contributes a lot to the global incidence of soil and water pollutions (Hossain et al., 2009). Among the phenolic compounds, phenol is the most commonly used by industries and is the precursor for the synthesis of many industrial chemicals.

Phenol and its derivatives infiltrate ecosystems as the consequence of drainage of the Metropolitan or industrial sewage to shallow water bodies and soil. Phenolic compounds pollutions in the aquatic environment can alter the biodiversity of this environment due to their toxicity (Lika and Papadakis, 2009; Pradeep et al., 2015). Acute exposure to phenol is recognised to cause discomfort of the gastrointestinal, headaches, and irritation of the skin in human. Phenolic compounds are readily absorbed through skin and mucosa and may be toxic to the nervous system, heart, kidneys, and the liver (Wang et al., 2011). Toxicity of phenol towards plants has been ascertained although plants are relatively resistant towards phenol. For instance, wilting and ultimately death was observed when willow tree was exposed to phenol as high as 1,000 mg/L (Ucisik and Trapp, 2008). In animals, phenol can also prevent synthesis and replication of DNA in cells. A study discovered that phenol inhibits replication of DNA in diploid human fibroblasts (Michalowicz and Duda, 2007). Exposure to phenol for less than 14 days (short-term exposure) and the long-term exposure (more than 14 days) can cause a health problem.

Plastic, coke and petroleum industries produce the highest effluents containing phenol (up to 7 g/L). These industrial waste are treated with physiochemical methods. However, the physiochemical methods alone are not efficient due to the high cost and also the generation of secondary pollutions (González et al., 2006; Suhaila et al., 2013). In Malaysia, nearly all of the monitoring station for monitoring groundwater quality showed phenol levels exceeding the National Guidelines for Drinking Water Quality 2000 (NGDWQ) indicating a serious issue with phenol pollution that needs urgent attention (DOE, 2015). About 37.7 metric tonnes of phenol and phenol-containing wastes are produced in 2014 in Malaysia (DOE, 2015). Phenol pollution is also a problem in the busy Straits of Malacca with several incidents where tonnes of phenol have been spilt during tanker accidents (Bottema and Bush, 2012; Gami et al., 2014). The long presence of phenol in the environment has allowed microbes to direct

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their metabolic machinery to utilise phenol as the lone source of carbon and energy which comprises of both aerobic and anaerobic microorganisms (Pradeep et al., 2015; Sridevi and Pradesh, 2009). The existence of these microorganisms can be used for the biodegradation of phenol and other phenolic compounds.

Bioremediation as an alternative method to physicochemical methods is a very cost effective method, and environmentally friendly way of controlling pollutions (Ali et al., 2009; Desai et al., 2010). To optimise the bioremediation ability of microorganisms to biodegrade phenol, an appropriate inoculum size, pH and temperature are crucial factors (Pradeep et al., 2015).

Although a lot of research has been carried out on the degradation of phenol by microorganisms yet there is a need for identifying more organisms that are capable of degrading phenols. To date, there are very few locally-isolated phenol-degrading microorganisms (Ahmad et al., 2011; Fereidoun et al., 2007). There is a need to increase the reservoir of a phenol-degrading microorganism to prepare for phenol remediation in the current and future scenario. Locally isolated phenol-degrading bacteria can suit the local environmental conditions much better than untested imported commercial microbes that may cause an ecological disaster. Since the current trend of growth and degradation optimisation involves the use of statistical optimisation approaches such as RSM, this will also be explored in this thesis. In addition, previous studies have shown that immobilisation especially using Gellan gum is the best matrix for improving degradation and resistant to heavy metals, and this will be studied as well.

In the view of the above, the objectives of this study are:

1. To screen and identify locally isolated phenol-degrading bacteria
2. To optimise the factors affecting phenol degradation using one-factor-at-a-time (OFAT) and response surface method (RSM)
3. To study the growth kinetics models of free cells on phenol by all three isolates
4. To immobilise all three isolates in Gellan gum and to compare their phenol degradation and heavy metals resistant to free cells
5. To determine the metabolic pathways for phenol degradation by all three isolates

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