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BIODEGRADATION OF PHENOL BY COLD-ADAPTED BACTERIA ISOLATED FROM ANTARCTIC SOILS

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By

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BIODEGRADATION OF PHENOL BY COLD-ADAPTED BACTERIA ISOLATED FROM ANTARCTIC SOILS

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July 2018

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

Phenol is an important pollutant widely discharged as a component of hydrocarbon fuels, but its degradation in cold regions is a great challenge due to the harsh environmental conditions. To date, there is little information available concerning the biodegradation of phenol by indigenous Antarctic bacteria. This study addresses the isolation of three phenol-degrading bacterial strains from King George Island, Antarctica. Based on preliminary screening, three isolates (AQ5-05, AQ5-06, and AQ5-07) capable of completely degrading 0.5 g/L phenol within 120 h at 10°C were selected for detailed study. Two were identified as *Arthrobacter* spp., and one as *Rhodococcus* sp., based on 16S rRNA sequences. All strains were non-motile, Gram-positive, oxidase-negative and catalase-positive. A study on the effects of parameters including temperature, pH, salinity and nitrogen source was conducted to optimise the conditions for phenol degradation using one-factor-at-a-time (OFAT) and response surface methodology (RSM). Based on the results from OFAT, AQ5-05 showed highest phenol degradation at 15°C, pH 7.5, 0.1 g/L NaCl and 0.4 g/L $(NH_4)_2SO_4$, AQ5-06 achieved maximum phenol degradation at 10°C, pH 7.5, 0.1 g/L NaCl and 0.3 g/L $(NH_4)_2SO_4$ while optimum phenol degradation for AQ5-07 was observed at 10°C, pH 7.0, 0.15 g/L NaCl and 0.3 g/L $(NH_4)_2SO_4$. Statistical analysis of the results obtained from RSM showed improvement in phenol degradation for all strains compared to the conventional OFAT approach. All strains showed optimum pH of 7.0 as optimised using RSM, with maximum phenol degradation observed under slightly different combinations of conditions for each: 17.5°C for AQ5-05, 10°C and 0.1 g/L of NaCl for AQ5-06, and 12.5°C and 0.4 g/L of $(NH_4)_2SO_4$ for AQ5-07. In addition, enzyme activities, and genes encoding phenol degradative enzymes identified using whole genome sequencing (WGS), were investigated to determine the pathways of phenol degradation. Complete phenol degradative genes involved in only ortho-cleavage were detected in all three strains and the results were revalidated using enzyme assays of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase. The data obtained indicated activity of only catechol 1,2-dioxygenase in all three strains, in agreement with the results from WGS. Graphical and statistical analyses on the growth kinetic models used indicated that the best models were Luong models for strains AQ5-05 and AQ5-07, while the best model for strain AQ5-06

was the Han-Levenspiel model. Meanwhile, the calculated values for maximum growth rate (μ_{max}), half saturation constant for maximum growth (K_s), maximum concentration of substrate tolerated (S_m) and empirical constant (n) were 0.180 h^{-1}, 0.390g/L, 1.290g/L and 0.240 for strain AQ5-05, and 0.175 h^{-1}, 0.388 g/L, 1.287 g/L and 0.236 for strain AQ5-07, respectively, according to the Luong equation. For strain AQ5-06, the Han-Levenspiel equation gave the values of μ_{max}, K_s, S_m with empirical constants m and n of 0.077 h^{-1}, 0.364 g/L, 1.273 g/L, 1.879 and 2341.00, respectively. All isolated strains were psychrotolerant with the optimum temperature for phenol degradation between 10 and 15°C. This study suggests the potential use of cold-adapted bacteria in the bioremediation of phenol over a wide range of low temperatures.

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

PENGURAIAN FENOL OLEH SEJUK BERADAPTASI BAKTERIA YANG DIPENCILKAN DARIPADA TANAH ANTARTIKA

Oleh

GILLIAN LEE LI YIN

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Fenol adalah bahan pencemar penting yang dihasilkan secara meluas sebagai salah satu komponen daripada bahan api hidrokarbon, tetapi penguraiannya di kawasan sejuk adalah satu cabaran yang besar disebabkan keadaan persekitaran yang sukar. Sehingga kini, terdapat sedikit maklumat yang sah mengenai bio-penguraian fenol oleh bakteria asli Antartika. Kajian ini membincangkan isolasi tiga bakteria pengurai fenol dari King George Island, Antartika. Berdasarkan penyaringan awal, tiga pencilan (AQ5-05, AQ5-06, dan AQ5-07) yang mampu mengurai 0.5 g/L fenol dalam masa 120 jam pada suhu 10°C telah dipilih untuk kajian terperinci berikut. Dua dikenal pasti sebagai *Arthrobacter* spp., dan satu sebagai *Rhodococcus* sp. berdasarkan penjujukan gen 16s rRNA. Semua strain adalah tidak motil, Gram-positif, oxidase-negatif dan catalase-positif. Kajian mengenai kesan parameter termasuk suhu, pH, kandungan garam dan sumber nitrogen telah dijalankan dengan menggunakan pendekatan 'satu-faktor-pada-satu-masa" (OFAT) dan pengkaedahan tindakbalas permukaan (RSM) untuk mengoptimumkan keadaan untuk penguraian fenol. Berdasarkan keputusan ujian OFAT, pencilan AQ5-05 menunjukkan penguraian fenol yang tertinggi pada suhu 15°C, pH 7.5, 0.1 g/L NaCl dan 0.4 g/L $(NH_4)_2SO_4$, AQ5-06 mencapai penguraian fenol maksimum pada suhu 10°C, pH 7.5, 0.1 g/L NaCl dan 0.3 g/L $(NH_4)_2SO4$ manakala AQ5-07 menunjukkan penguraian fenol tertinggi pada suhu 10°C, pH 7.0, 0.15 g/L NaCl dan 0.3 g/L $(NH_4)_2SO_4$. Di samping itu, aktiviti enzim dan gen untuk pengekodan enzim pengurai fenol dikenalpasti melalui penjujukan kesuluruhan genom (WGS) telah diperiksa untuk mengenalpasti tapak jalan penguraian fenol. Genom daripada ketiga-tiga strain menunjuk gen pengurai fenol lengkap yang terlibat adalah tapakjalan ortho dan keputusan berikut telah disahkan dengan pengujian aktiviti-aktiviti katekol 1,2-dioxygenas dan katekol 2,3-dioxygenas. Data yang diperolehkan menunjukkan aktiviti katekol 1,2-dioxygenas sahaja dalam ketiga-tiga strain, bersesuaian dengan keputusan daripada WGS. Analisis grafik dan statistik mengenai model kinetik pertumbuhan yang digunakan menunjukkan bahawa model terbaik ialah model Luong untuk strain AQ5-05 dan AQ5-07, manakala model terbaik untuk strain AQ5-06 adalah model Han-Levenspiel. Sementara itu, nilai-nilai yang dikira bagi pemalar Luong seperti kadar pertumbuhan maksimum (μ_{max}), pemalar ketepuan separa untuk pertumbuhan maksimum (K_s), kepekatan maksimum substrat

yang dapat ditolerasi (S_m) dan pemantauan empirikal (n) adalah 0.180 h^{-1}, 0.390 g/L, 1.290 g/L dan 0.240 untuk pencilan AQ5-05, dan 0.175 h^{-1}, 0.388 g/L, 1.287 g/L dan 0.236 untuk pencilan AQ5-07, masing-masing. Untuk pencilan AQ5-06, model Han-Levenspiel memberikan nilai-nilai μ_{max}, K_s, S_m dengan pemalar empirikal m dan n adalah 0.077 h^{-1}, 0.364 g/L, 1.273 g/L, 1.879 dan 2341.00, masing-masing. Semua strain adalah psikrotropik dengan suhu optimum untuk mengurai fenol antara 10 dan 15°C. Kajian ini mencadangkan potensi penggunaan bacteria yang sejuk beradaptasi dalam bioremediasi fenol pada suhu yang rendah.

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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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

%	Percent
% (v/v)	Percent concentration volume / volume
% (w/v)	Percent concentration weight / volume
ºC	Degree celsius
µL	Microlitre
µm	Micrometre
x g	Relative centrifugal force
ATP	Adenosine triphosphate
bp	Base pair
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
et al.,	And colleagues
g	Gram
gDNA	Genomic DNA
HCl	Hydrochloric acid
K	Kelvin
kb	Kilobase
L	Litre
M	Molar
min	Minute
Mg	Magnesium
mg	Milligram
mL	Mililitre
mM	Milimolar
mm	Millimetre
mRNA	Messenger RNA
ng	Nanogram
nM	Nanomolar
nm	Nanometer
OD	Optical density
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolution per minute
sp.	Species (singular)
spp.	Species (plural)
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
tRNA	Transfer RNA
UV	Ultraviolet
V	Voltage

CHAPTER 1

INTRODUCTION

Antarctica can no longer be considered as the last pristine continent, as pollution has locally affected terrestrial and marine coastal ecosystems (Bargagli, 2005). Due to the continent's low temperatures, ecosystems in Antarctica are very sensitive to environmental changes, even those associated with minor incidents. Development of scientific research and tourism due to the increase in interest and easier access to the continent are the main factors of increase in anthropogenic impacts in Antarctica (Prus et al., 2015; Cabrerizo et al., 2016). Hydrocarbon pollution resulting from human activities such as transportation and power generation poses serious threats to the Antarctic environment (Luz et al., 2006; Tin et al., 2009). Particularly, accidental spillage of fuel has been reported as an issue of high concern in Antarctica (Luz et al., 2006; Jesus et al., 2015b; Prus et al., 2015). Several major contaminants have been reported in Antarctica including hydrocarbons (Powell et al., 2007; Shukor et al., 2009b), phenol and phenolic compounds (Chang et al., 2016), trace elements (Amaro et al., 2015), pesticide residues and radiation contamination (Bharti & Niyogi, 2015).

Phenol is an aromatic hydrocarbon found in fuel that poses severe risks to aquatic and terrestrial ecosystems, especially in the cold region of the globe (Ahmad et al., 2011a; Zangrando et al., 2016). Phenol is widely produced in pharmaceutical, plastic manufacturing and petroleum refining. Due to its anti-bacterial properties, phenol has long been used as an antiseptic, disinfectant or preservative agent. Consequently, natural removal of phenol has been described as one of the foremost challenges, especially in environments under severe climatic conditions or with limited nutrient availability (Gerginova et al., 2013a). According to Margesin et al. (2005) and Litova et al. (2014), phenol tends to persist in cold environments due to the low rate in natural biodegradation process.

In recent years, study of the potential of Antarctic microorganisms in bioremediation has been of increasing interest, due to their adaptations to harsh conditions and their metabolic potential in removing a wide variety of organic pollutants at low temperature (Domenico et al., 2004; Litova et al., 2014). The utilisation of indigenous bacteria has been proposed as the most cost effective and environmentally-friendly method in the treatment of phenol-contaminated sites in comparison with physical and chemical methods (Ahmad et al., 2011b). Most existing studies have concentrated on phenol degradation by mesophilic microorganisms. However, there is currently inadequate information available about indigenous Antarctic phenol degraders. Previous studies on phenol degradation in Antarctica have also focused more on fungi, with very limited information available on indigenous phenol-degrading bacteria (Gerginova et al., 2013a, 2013b; Litova et al., 2014; Fernández et al., 2017). Furthermore, the use of indigenous microorganisms is ideally required in bioremediation of contaminated sites in Antarctica, since the introduction of alien species is prohibited without strict permitting requirements under the Antarctic Treaty (Aislabie et al., 2000). Several authors have reported indigenous Antarctic microorganisms capable of degrading pollutants such as

hydrocarbons, polychlorinated biphenyls, diesel and phenol (Vasileva-Tonkova & Gesheva, 2004; Domenico et al., 2004; Gerginova et al., 2013a).

Environmental variables such as temperature, pH, salinity and nitrogen source are important factors affecting the efficiency of phenol degradation. Therefore, conditions of biodegradation of phenol have to be optimised to ensure successful treatment of phenol (Ahmad et al., 2011b; Nawawi et al., 2016). Besides hydrocarbons from fuels, heavy metals such as Fe, Al, Ca and Ti from the earlier waste-dumping activity persist in Antarctic environments for many years after release (Santos et al., 2005; Aronson et al., 2011). Hence, a future study is required to analyse the presence of other compounds and heavy metals and to study their effects on phenol-degrading activities of the microorganisms prior to real field application. For example, Fernández et al. (2017) evaluated the phenol removal performance of Antarctic yeasts in the presence of heavy metals and the results showed only half of them tolerate to those metal ions tested.

In addition, kinetic studies evaluate the effectiveness of the microbial system in biodegradation. It is important that knowledge of growth kinetics is used in the evaluation of bacterial tolerance of the anti-microbial properties of phenol (Basha et al., 2010). Futhermore, the efficient utilisation of phenol as a carbon and energy source also depends on the availabilities and capabilities of the key enzymes in phenol degradation (Gerginova et al., 2013a, 2013b). The development of high-throughput sequencing technologies has contributed to the characterisation of novel biochemical pathways of biogeochemical significance, and of the phylogenetic and functional diversity of microorganisms (Bihari, 2013; Choi et al., 2013; Kostka et al., 2014). Moreover, bioinformatics based on knowledge of generally known biochemical pathways and enzymes to identify enzyme function enables exploration of the enzymatic diversity of microbial biodegradative enzymes (Galvão et al., 2005). Without doubt, studies of the mechanisms of phenol degradation by indigenous Antarctic bacteria could make an important contribution to knowledge of microbial biodiversity and function in Antarctica. The central predictions of this study are that phenol-degrading isolates will be successfully isolated from Antarctic soils, and that they will be capable of significantly degrading phenol at low temperatures (0-15°C). Overall, this study will not only provide an important increase in knowledge of the microbial biodiversity of Antarctica, but also on the potential use of cold-adapted bacteria in treating phenol-contaminated sites and therefore contribute to the environmental sustainability.

The main objectives of this study are the isolation and characterisation of cold-adapted phenol-degrading isolates from Antarctic soils as a new bioremediation tool in phenol removal, as well as study of the phenol catabolic pathway of Antarctic bacterial isolates.

The objectives of this study are;

1. To isolate, screen and identify cold-adapted phenol-degrading bacteria from Antarctic soils.
2. To determine the optimum conditions for phenol degradation using one-factor-at-a-time (OFAT) and response surface methodology (RSM).

3. To identify the pathway(s) of phenol degradation using whole genome sequencing (WGS) and enzyme assays.
4. To investigate the growth kinetics of phenol-degrading bacteria selected from the first part of the study.

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