UNIVERSITI PUTRA MALAYSIA

NUTRIENT REQUIREMENT AND KINETICS OF PHENOL DEGRADATION BY RHODOCOCCUS SP. UKMP-5M IN BATCH AND CONTINUOUS CULTURE USING STIRRED TANK BIOREACTOR

NOR SUHAILA YAACOB

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DOCTOR OF PHILOSOPHY
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By

NOR SUHAILA YAACOB

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

July 2013

This Thesis is dedicated to My Precious Beloved Husband,
Shukrie Sarjeng
Sons,
Adam & Afie
Parents,
Yaacob Othman & DZaharah Faridah
and
My Beloved Family

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

NUTRIENT REQUIREMENT AND KINETICS OF PHENOL DEGRADATION BY *RHODOCOCCUS* SP. UKMP-5M IN BATCH AND CONTINUOUS CULTURE USING STIRRED TANK BIOREACTOR

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Chairman: Arbakariya B. Ariff, PhD

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Rhodococcus UKMP-5M is a gram positive locally isolated strain that capable of degrading an impressive range of xenobiotic and hazardous compounds. This strain arises normally from contaminated soil and aquatic sediments which is highly enriched with the source of contamination. Phenol and its derivatives are known as one of the example of xenobiotic compound that always need to be removed from the environment. Biodegradation by microbial activity may be used as an effective method for phenol removal from the contaminated sites. The feasibility of using *Rhodococcus* UKMP-5M in phenol biodegradation is the main focus of this study. Nutrients requirement for the enhancement of growth of *Rhodococcus* UKMP-5M and the ability to degrade phenol was first studied in 250 mL shake-flask culture. The various parameters applied during the cultivation that influenced phenol biodegradation by *Rhodococcus* UKMP-5M were also optimized using response surface methodology (RSM) aimed at improving the biodegradation performance in

terms of percentage of phenol degraded and degradation time. The performance of using cells suspended in medium containing phenol, termed as biotransformation, in biodegradation of phenol was also studied. The effect of mode of bioreactor operation (batch and continuous culture) on phenol biodegradation by *Rhodococcus* UKMP-5M was studied using 2 L stirred tank bioreactor. The activity of phenol hydroxylase, the enzyme responsible in phenol degradation, was evaluated in various phenol biodegradation experiments. Finally, phenol hydroxylase of *Rhodococcus* UKMP-5M was purified and its characteristics in phenol degradation were identified.

From the initial screening of medium composition and cultivation condition, it was found that basal medium M1, temperature of 37^{o}C, pH of 7.5, buffer concentration of 50-150 mM, ammonium sulphate concentration of 0.4 g/L and natrium chloride of 0.1 g/L gave the highest growth of *Rhodococcus* UKMP-5M and degradation of phenol. *Rhodococcus* UKMP-5M was capable to tolerate up to 900 mg/L phenol. Phenol degradation by the growing cells of *Rhodococcus* UKMP-5M was further improved by optimization using RSM, where the degradation period for 1 g/L phenol was successfully reduced from 48 h to 27 h with phenol concentration, ammonium sulphate and temperature were the most significant variables that influenced phenol biodegradation. Although the biotransformation using whole cells of *Rhodococcus* UKMP-5M in minimal salt medium (MSM) containing phenol was successfully developed for the biodegradation of phenol, but the degradation efficiency was lower than those obtained in the growing cell system.

In the optimal conditions (agitation speed of 160 rpm, air flow rate of 1.5 vvm and controlled dissolved oxygen tension at 80% air saturation) for biodegradation of

phenol by *Rhodococcus* UKMP-5M using 2 L stirred tank bioreactor, 0.5 g/L of phenol was sucessfuly degraded in 12 h of cultivation. The continuous mode of bioreactor operation was also successfully used for phenol biodegradation by *Rhodococcus* UKMP-5M, where the phenol degradation rate of 0.18 h^{-1} obtained in the continuous culture was about 70% higher than that obtained in batch mode of bioreactor operation. In all cases, high biodegradation of phenol was corresponded well with high activity of phenol hydroxylase, suggesting that this enzyme was responsible in phenol biodegradation.

The cells of *Rhodococcus* UKMP-5M were successfully disrupted using glass bead technique for the extraction of phenol hydroxylase. The optimal cell disruption was obtained at this condition: 50 mL falcon bottle, glass bead with the diameter of 425-600 μm, cell concentration of 10%, and disruption time of 30 min. Phenol hydroxylase was purified using anion exchange chromatography by DEAE-sepharose fast flow column, which gave the purification fold and yield of 10.18 and 14.28%, respectively. The molecular weight of phenol hydroxylase was 53 kDa, while the K_m and V_{max} values of NADH using Lineweaver-burk plot were 16.98 μM and 28.57 U/mg protein, respectively. The optimal temperature and pH for the maximum activity of phenol hydroxylase from *Rhodococcus* UKMP-5M was obtained at 25^{o}C and pH 7.5, respectively. Results of this study have demonstrated that *Rhodococcus* UKMP-5M is a versatile bacterium which has a great potential to be used industrially in the removal of xenobiotic compounds especially phenol.

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

KEPERLUAN NUTRISI DAN KINETIK FENOL DEGRADASI OLEH *RHODOCOCCUS* SP. UKMP-5M DI DALAM KULTUR SESEKELOMPOK DAN SELANJAR MENGGUNAKAN BIOREAKTOR BERPENGADUK

Oleh

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Rhodococcus UKMP-5M adalah bakteria gram positif yang dipencilkan daripada sumber tempatan yang berupaya untuk menguraikan sekelompok sebatian xenobiotik dan berbahaya. Pada kebiasaannya strain ini wujud daripada tanah dan mendapan akuatik yang tercemar yang sangat kaya dengan sumber pencemaran. Fenol dan terbitannya dikenali sebagai salah satu contoh sebatian xenobiotik yang perlu dihapuskan daripada persekitaran. Biodegradasi dari akiviti mikrob boleh digunakan sebagai satu cara untuk menghapuskan fenol dari kawasan yang tercemar. Keupayaan menggunakan *Rhodococcus* UKMP-5M di dalam proses biodegradasi fenol merupakan fokus utama kajian ini. Keperluan nutrisi untuk meningkatkan pertumbuhan *Rhodococcus* UKMP-5M dan keupayaan mendegradasikan phenol pada awalnya dikaji di dalam kelalang kultur bergoncang 200 mL. Pelbagai parameter yang digunakan yang mempengaruhi proses biodegradasi fenol oleh *Rhodococcus* UKMP-5M juga dioptimumkan menggunakan pengkaedahan tindalbalas permukaan (RSM) bertujuan untuk memperbaiki keupayaan biodegradasi

dari segi peratusan fenol yang terurai dan jangkamasa peruraian. Keupayaan untuk menggunakan sel-sel yang dipegunkan didalam media yang mengandungi fenol, dikenali sebagai biotransformasi di dalam proses biodegradasi phenol juga dikaji. Kesan mod operasi bioreaktor (kultur sesekelompok dan selanjar) untuk degradasi fenol oleh *Rhodococcus* UKMP-5M dikaji menggunakan 2 L bioreaktor berpengaduk. Aktiviti fenol hidroksilase, enzim yang bertanggungjawab di dalam degradasi fenol telah di tentukan di dalam pelbagai ujikaji biodegradasi fenol. Akhirnya, fenol hidroksilase daripada *Rhodococcus* UKMP-5M di tulenkan dan ciri-cirinya di dalam proses degradasi fenol telah dikenalpasti.

Daripada saringan awal, komposisi media dan keadaan pengkulturan, telah dikenalpasti bahawa medium asas M1, suhu 37^{o}C, pH 7.5, kepekatan penimbal 50-150 mM, kepekatan ammonium sulfate 0.4 g/L dan 0.1 g/L natrium klorida memberikan pertumbuhan yang paling tinggi bagi *Rhodococcus* UKMP-5M dan degradasi fenol. *Rhodococcus* UKMP-5M berupaya untuk hidup di dalam kepekatan fenol sehingga 900 mg/L. Proses biodegradasi fenol dengan menghidupkan sel-sel *Rhodococcus* UKMP-5M di pertingkatkan lagi dengan mengoptimumkannya menggunakan RSM, dimana tempoh degradasi bagi 1 g/L fenol telah berjaya dikurangkan daripada 48 jam ke 27 jam dengan kepekatan fenol, ammonium sulfate dan suhu adalah pembolehubah yang paling signifikan yang mempengaruhi proses biodegradasi fenol. Walaupun proses biotransformasi menggunakan keseluruhan sel-sel *Rhodococcus* UKMP-5M di dalam medium MSM yang mengandungi fenol telah berjaya dihasilkan untuk proses biodegradasi fenol, kecekapan proses degradasi adalah lebih rendah daripada yang diperolehi melalui sistem sel yang ditumbuhkan.

Di dalam keadaan optima (kelajuan pergerakan 160 rpm, kadar alir udara 1.5 vvm dan kepekatan oksigen terlarut pada kepekatan 80%) untuk biodegradasi fenol oleh *Rhodococcus* UKMP-5M menggunakan 2L bioreaktor berpengaduk, 0.5 g/L fenol telah berjaya didegradasikan di dalam masa 12 jam pengkulturan. Mod operasi bioreaktor selanjar juga berjaya digunakan bagi biodegradasi fenol oleh *Rhodococcus* UKMP-5M dimana kadar degradasi fenol adalah 0.18 j^{-1} yang diperolehi di dalam operasi selanjar adalah lebih kurang 70% lebih tinggi berbanding yang diperolehi di dalam mod operasi bioreaktor sesekelompok. Di dalam kesemua kes, biodegradasi fenol yang tinggi adalah sangat berkait rapat dengan akitiviti fenol hidroksilase yang tinggi, dicadangkan enzim ini adalah bertanggungjawab di dalam degradasi fenol.

Sel *Rhodococcus* UKMP-5M telah berjaya dipecahkan menggunakan manik-manik kaca bagi pengestrakan fenol hidroksilase. Pemecahan sel yang optimum adalah diperolehi di dalam keadaan berikut: botol falcon 50 mL, diameter manik kaca 425-600 μM, kepekatan sel 10% dan masa pemecahan 30 minit. Fenol hidroksilase ditulenkan dengan kromatografi penukaran anion menggunakan kolum aliran cepat DEAE-sepharose, yang memberikan jumlah penulenan dan hasil sebanyak 10.18 dan 14.28% masing-masing. Berat molekul fenol hidroksilase adalah 53 kDa, manakala nilai K_m dan V_{max} NADH menggunakan plot Lineweaver-burk adalah 16.98 μm dan 28.57 U/mg protein, masing-masing. Suhu dan pH optima bagi aktiviti fenol hidroksilase daripada *Rhodococcus* UKMP-5M adalah diperolehi pada suhu 25^{o}C dan pada pH 7.5, masing-masing. Hasil daripada kajian ini menunjukkan *Rhodococcus* UKMP-5M adalah bakteria serba boleh yang mempunyai potensi yang sangat baik untuk digunakan bagi menghapuskan sebatian xenobiotik terutamanya fenol.

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I certify that a Thesis Examination Committee has met on 24th July 2013 to conduct the final examination of Nor Suhaila binti Yaacob on her thesis entitled "Nutrient Requirement and Kinetics of Phenol Degradation by *Rhodococcus* sp. UKMP-5M in Batch and Continuous Culture using Stirred Tank Bioreactor" 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions

NOR SUHAILA YAACOB

Date: 24 July 2013

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mL	millilitre
L	litre
g	gram
g/L	gram per litre
Mg/L	milligram per litre
mM	millimolar
µg/L	microgram per litre
mg/L	milligram per litre
µM	micromolar
µL	microlitre
M	Molar
µm	micrometer
RSM	Response Surface Methodology
^{o}C	degrees Celsius
h^{-1}	per hour
Abs	absorbance
%	percent
OD	optical density
t	time (h)
min	minute
h	hour
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

S	Substrate concentration (g/L)
K_s	Substrate concentration at half the value of μ_{max} (g/L)
K_m	Michaelis constant (g/L)
V_{max}	Maximum velocity of an enzyme catalyzed Reaction
X_0 or X	Concentration of cell (g/L)
D	Dilution rate
MW	Molecular weight
kDa	Kilodalton
kb	Kilobase
NA	Nutrient agar
Kg	Kilogram
OD	optical density
t	time (h)
X_m	Maximum biomass concentration (g/L)
P_m	Maximum product concentration (g/L)
$Y_{x/s}$	Cellular yield coefficient (g/g)
$Y_{p/s}$	Product yield coefficient (g/g)
S_0	Outlet concentration of limiting nutrient
m	Maintenance requirement
q	Specific rate of product formation (g/g/hr)
V	Volume (L)
F	Medium flow rate (L/h)
α	Specific death cell (h^{-1})

r_x	Rate of cell growth
r_p	Rate of product formation
r_s	Substrate consumption rate
K_{La}	Oxygen transfer rate (h^{-1})
qO_2	Specific oxygen uptake rate (mg O^2/g $cell^{-1}h^{-1}$)
DEAE	Diethylaminoethylamine
dH_2O	Distilled water
MSM	Minimal salt medium
PAGE	Polyacrylamide gel electrophoresis
RPM	Rotation per minute
SDS	Sodium dodecyl sulphate

CHAPTER 1

INTRODUCTION

Many industries now utilize phenol in their process without proper disposal methods which leads to environmental pollution. Chemical treatment normally used for phenol remediation may cause serious effect and high cost. Therefore, the biological treatment using microbial is an alternative to overcome this problem. Chlorinated aromatic compounds pose one of the most serious contemporary environmental problems worldwide because they have been used in large quantities as herbicides, pesticides and solvents (Ogawa and Miyashita, 1995). In the 1980s, rapidly increasing environmental contamination raised concerns about health of ecosystems and human lead the interest into biological methods of pollution cleanup (bioremediation) as they are cost effective and environmental friendly (Martinkova *et al*., 2009; Ali *et al*., 2009).

Phenol is a characteristic pollutant in wastewaters of coal conversion processes, effluents from crude oil and treatment plants (Alamzadeh *et al.,* 2002; Kavitha and Palanively, 2004; Sung *et al*., 2000; El Sayed *et al*., 2003; Yan *et al*., 2008) It is either toxic or lethal to fish, and most types of microorganisms at relatively low concentrations (Hill and Robinson, 1975). Phenols are hydroxy compounds of aromatic hydrocarbons and the derivatives are widely used in many petro-chemical industries and manufacturing including the production of textile, plastic, resin and oil refineries, pesticide, steel and pharmaceutical products (Bajaj *et al*., 2009; Schie and Young, 2000; Edalatmanesh *et al.*, 2008). The annual production of phenol is around

1.25 x 10^9 kg (Boopathy, 1997). These compounds are toxic either by ingestion, skin contact or inhalation. Acute exposure to phenol causes central nervous system disorders leading to collapse and coma. Muscular convulsions with significant reduction in body temperature are also noted due to phenol toxicity. Renal damage and salivation may be induced by continuous exposure to phenol (Nair *et al*., 2008). Therefore, biodegradation of phenol at high concentration has been an interesting topics of research for many years (Bajaj *et al*., 2009) and phenol biodegradation has been chosen as a method to remediate environments contaminated by phenol (Bastos *et al.*, 2000; Zhao *et al*., 2009; Veenagayathri and Vasudevan, 2010 and Liu *et al*., 2010).

Many studies have been carried out by researchers for the improvement of phenol degradation process such as using *Acinetobacter* sp. (Hao *et al*., 2002; Ahmad *et al*., 2011a), *Alcaligenes* sp. (Bastos *et al*., 2000; Bai *et al*., 2007). *Candida* sp. (Yan *et al*., 2005; Liu *et al*., 2010); *Ewingella americana* (Khleifat, 2006), *Pseudomonas* sp. (Kumar *et al*., 2005; Kotresha and Vidyasagar, 2008; Wang *et al*. 2010), *Aureobasidium pullulans* (Santos *et al.*, 2009) and *Rhodococcus* sp. (Prieto *et al*., 2002; Pizzul *et al*., 2006; Hori *et al*., 2009; Shen *et al*., 2009). It is well known that nutrient and cultural conditions are required to improve the degradation of aromatic compound. The optimization of the growth conditions and medium for degradation of phenol is of primary importance in the development of the bioprocess. Phenol degradation by the activity of microorganism was regulated by some factors such as pH, temperature, nitrogen source, salt ions, phenol and glucose concentration (Ahmad *et al.*, 2011a; Annadurai *et al.*, 2000; Khleifat, 2006; Bai *et al.*, 2007; Basha *et al*., 2010). Response Surface Methodology (RSM) is an important technique to

improve the degradation of phenol. RSM is a useful approach in designing a sequence of experiments to get optimal response and quantify the relationships between measured responses and the variables by first and second-order polynomial equations to estimate interaction and quadratic effects (Tan *et al*., 2011). RSM reduces the number of experimental trials need to evaluate multiple parameters and their interaction therefore less time consuming and laborious required (Lee *et al.*, 2006).

Among the scope of interest that have been carried out to biodegrade the phenols is utilization of *Rhodococcus* species. *Rhodococci* may be naturally present in contaminated environment and are promising candidates for bioremediation (Konovalova *et al*., 2009; Rehfuss and Urban, 2005; Larkin *et al*., 2005 and Shumkova *et al*., 2009). *Rhodococcus* is Gram-positive non-motile aerobic bacteria, and are closely related to the other mycolic acid containing genera such as *Nocardia, Corynebacterium* and *Mycobacterium* (Goodfellow 1989; Larkin *et al*., 1998). There are currently 12 established *Rhodococcus* species, namely *R.coprophilus*, *R. equi, R. fascians, R. erythropolis, R. globerulus, R. marinonascens, R. opacus, R. percolatus, R. rhodnii, R. rhodochrous, R. ruber* and *R. zopfii* (Bell *et al*., 1998). Recent studies on their metabolic activities have shown *rhodococci* to be of important use in industrial, pharmaceutical and environmental biotechnology. Beside, it is also proving useful in pharmaceuticals as antibiotic, anti-fungal and anti-tumor. This genus attracted attention because of their extensive catabolic activities toward a wide variety of organic compounds, including polychlorinated biphenyls (PCBs), aliphatic and aromatic hydrocarbons, and also crude oil, thus suggesting their use as potential agents for bioremediation of contaminated environments (Perry, 2007).

Although there are suitable bacteria groups capable to degrade phenol, a proper kind of reactor to promote treatment is also necessary. Generally, a stirred tank reactor is used in phenol degradation process. The advantage of using this reactor is that it's operation and retention time adjustment is simple (Hwa Kim *et al*., 2002). Beside stirred tank bioreactor, airlift bioreactor (Jia *et al.*, 2006); immersed membrane bioreactor (Marrot et al., 2006) and packed bed reactor (Paca Jr *et al*., 2005; Tziotzios *et al*., 2007) were also used for phenol degradation study.

This study explore into the possibility of biodegradation of phenol using locally isolated *Rhodococcus* UKMP-5M. Therefore, the objectives of this study are;

1. To evaluate the effect of phenol degradation by *Rhodococcus* UKMP-5M in different media and culture conditions in shake-flasks.
2. To optimize the degradation of phenol by *Rhodococcus* UKMP-5M using response surface methodology (RSM).
3. To purify and determine the mechanism of phenol degradation by characterization of phenol hydroxylase enzymes.
4. To determine the efficiency of phenol degradation using biotransformation technique.
5. To investigate the performance of phenol degradation using different modes of bioreactor operation (batch and continuous) in stirred tank reactor.

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