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BIODEGRADATION OF PHENOL BY LOCALLY ISOLATED *Acinetobacter* sp. STRAIN AQ5NOL 1 AND PURIFICATION OF PHENOL HYDROXYLASE

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

2012

This thesis is dedicated to late Che Ramlah Dollah.
My mother, my mentor, my teacher, my coach, my idol, my heroin,
my best friend and by far the coolest women I will have ever known.
I will miss you everyday and always try to make you proud.

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

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Chairman: Professor Nor Aripin Shamaan, PhD

Faculty : Biotechnology and Biomolecular Sciences

Phenol and its derivatives are categorized as one of the most toxic compounds. Phenolic compounds are ubiquitous pollutants which mainly originate from industrial processes such as resin manufacturing, pharmaceutical, petrochemical, oil refinery, plastic and textile industries. These pollutants are persistent in nature and are considered highly hazardous due to their toxicity, mutagenicity and carcinogenicity towards most biological processes even at low concentrations. At present, the bioremediation technique involving microorganisms such as bacteria, fungi and yeast has been the target of phenol remediation technologies. However, the growth of these microorganisms is inhibited at high concentrations of phenol, thus limiting the efficiency of the biodegradation. The efficiency of phenol biodegradation can be enhanced by a process of cell immobilisation. Accordingly, the aim of this study was to seek potential phenol-degrading bacteria from local bacterial strains. The study encompasses the process of screening, isolating, identifying and characterizing phenol-degrading bacteria. The effects of phenol concentration, heavy metals,

pesticides and respiratory inhibitors on the efficiency of phenol biodegradation by freely suspended and immobilised cells are also compared. This is then followed by purification and characterization of the phenol-degrading enzyme. From the 115 samples collected from different locations, 37 pure phenol-degrading bacteria were isolated of which 6 were able to degrade 100% 500 mg/L phenol. From the 6 isolates, bacterial Isolate number SA28s(i) isolated from Hutan Simpan Gunung Arong, Mersing, Johor, has the best capability to degrade phenol in a mineral salt medium, pH 7.5 at 30°C, after 4 days of incubation compared with the other isolates. Isolate SA28a(i) was identified as Gram-negative cocci. 16s rRNA analysis placed this isolate in the *Acinetobacter* genera but did not match any known species in the NCBI database. Thus, it is tentatively named it as *Acinetobacter* sp. strain AQ5NOL 1 using molecular phylogenetics analysis of the sequenced 16s rRNA gene. Studies were carried out to optimise the degradation of phenol and bacterial growth by free and immobilised cells in gellan gum. The combination of 0.04% (w/v) ammonium sulphate and 0.01% (w/v) of NaCl at pH 7 (phosphate buffer) gave optimum degradation of phenol and bacterial growth by the free cells. The combination of 0.75% (v/w) gellan gum, 300 beads, and bead size of 3 mm gave optimum phenol degradation by the immobilised cells. *Acinetobacter* sp. strain AQ5NOL 1 immobilised in gellan gum beads showed enhanced degradation of elevated concentrations of phenol (1900 mg/L) compared to the free cells (1100 mg/L) and could be reused for at least 45 cycles. Heavy metals, pesticides and respiratory inhibitors showed less inhibition of phenol degradation in immobilised cells than the free cells. Purification and characterisation of the phenol degrading enzyme known as phenol hydroxylase was conducted with anion exchange chromatography using DEAE-Sepharose®, DEAE–Sephadex®, Q-Sepharose® and gel filtration

chromatography using gel filtration of Agilent Zorbax^{TM} (GF-250). One band was visualised on the gel filtration fraction at 50 kDa using the SDS polyacrylamide-gel electrophoresis (SDS-PAGE) and native polyacrylamide-gel electrophoresis (Native-PAGE), suggesting that the enzyme preparation is homogenous. In the enzyme kinetic studies, the K_m and V_{max} obtained for phenol were 13.4 µM and 2.5 µmole/min/mg protein (Michaelis-Menten); 17.57 µM and 2.89 µmole/min/mg protein (Lineweaver-Burk) respectively. The K_m and V_{max} obtained for NADH were 84 µM and 2.31 µmole/min/mg protein (Michaelis-Menten); 50.7948 µM and 1.724 µmole/min/mg protein (Lineweaver-Burk) respectively. Phenol degradation by phenol hydroxylase was optimum at pH between 6.5-7.5 by phosphate buffer and 7-7.5 by Tris-HCl buffer at 15-25°C. The enzyme was stable at –20°C for 40 days in phosphate buffer at pH 7.5. The outcome of this study on phenol degrading bacteria and enzyme will contribute to additional knowledge on a new source of more efficient microbe in phenol degrading process, hence contributing to environmental sustainability especially for a developing country like Malaysia.

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

BIODEGRADASI FENOL OLEH PENCILAN TEMPATAN *Acinetobacter* sp. STRAIN AQ5NOL 1 DAN PENULENAN FENOL HIDROKSILASE

Oleh

SITI AQLIMA BINTI AHMAD

February 2012

Pengerusi: Profesor Nor Aripin Shamaan, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Fenol dan terbitannya dikategorikan sebagai salah satu sebatian yang paling toksik. Sebatian fenolik adalah bahan umum di dalam sisa kumbahan industri seperti industri farmasi, petrokimia, plastik dan tekstil. Bahan pencemar ini adalah bersifat kekal di alam semulajadi dan dianggap sangat berbahaya disebabkan ketoksikan, kemutagenan dan kekarsinogenannya ke atas kebanyakan proses biologi walaupun pada kepekatan yang rendah. Pada masa kini, teknik bioremediasi yang melibatkan mikroorganisma seperti bakteria, fungi dan yis menjadi matlamat utama dalam teknologi meremediasikan fenol. Walau bagaimanapun, pertumbuhan mikroorganisma ini direncat pada kepekatan fenol yang tinggi, sekali gus menghadkan kecekapan biodegradasi. Kecekapan biodegradasi fenol boleh dipertingkatkan melalui proses penyekat-gerak sel. Oleh itu, tujuan kajian ini adalah mencari bakteria pengurai fenol yang berpotensi daripada strain bakteria tempatan. Kajian ini merangkumi proses penyaringan, pemencilan, identifikasi dan pencirian bakteria pengurai fenol oleh sel bebas dan tersekat-gerak. Kesan kepekatan fenol, logam berat, racun serangga dan perencat respirasi pada kecekapan biodegradasi

fenol juga dibandingkan oleh sel-sel bebas dan tersekat-gerak. Kajian seterusnya diikuti dengan penulenan dan pencirian enzim pengurai fenol. Daripada 115 sampel yang dikumpul daripada pelbagai lokasi, 37 bakteria pengurai fenol tulen telah dipencilkan dimana 6 daripadanya berkebolahan menurunkan 100% 500 mg/L fenol. Daripada 6 isolat, bakteria SA28a(i) yang telah dipencilkan daripada Hutan Simpan Gunung Arong, Mersing, Johor mempunyai kebolehan untuk menguraikan fenol di dalam medium garam mineral, pH 7.5 pada 30ºC selama empat hari berbanding dengan pemencilan yang lain. Isolat SA28a(i) telah diidentifikasi sebagai Gram-negatif cocci. Analisis 16s RNA meletakkan isolat ini dalam genera *Acinetobacter* tetapi tidak sepadan dengan mana-mana spesies yang diketahui dalam pangkalan data NCBI. Oleh itu, bakteria ini dinamakan sebagai *Acinetobacter* sp. Strain AQ5NOL 1 menggunakan analisis filogenetik molekul 16S rRNA. Kajian dijalankan untuk menentukan kadar optimum penguraian fenol dan pertumbuhan bakteria oleh sel bebas dan sekat gerak dalam gam gellan. Kombinasi 0.04% (w/v) ammonium sulfat, 0.01% (w/v) of NaCl, pada pH 7 dengan penimbal fosfat menghasilkan penguraian fenol dan pertumbuhan bakteria optimum oleh sel bebas. Kombinasi kepekatan gam gellan 0.75% (v/w), bilangan biji sebanyak 300 dan saiz biji 3 mm menghasilkan penguraian fenol optimum oleh sel sekat gerak. *Acinetobacter* sp. strain AQ5NOL 1 yang tersekat di dalam butir gam gellan menunjukan penguraian yang lebih baik pada kepekatan fenol yang tinggi (1900 mg/L) berbanding sel bebas (1100 mg/L) dan boleh digunakan semula sekurang-kurangnya 45 kitaran. Logam berat, racun serangga dan perencat respirasi memberikan kesan rendah pada penguraian fenol oleh sel tersekat-gerak berbanding sel bebas. Penulenan dan pencirian telah dilakukan ke atas enzim penguraian-fenol yang dikenali sebagai fenol hidroksilase dengan kromatografi penukaran anion menggunakan kolum DEAE-Sepharose®,

DEAE–Sephadex®, Q-Sepharose® dan kromatografi penurasan gel menggunakan kolum Agilent Zorbax™ (GF-250). Satu jalur telah dihasilkan daripada fraksi gel filtrasi pada 50 kDa menggunakan elektroforesis-gel poliakrilamid SDS dan elektroforesis-gel poliakrilamid natif, mencadangkan persediaan enzim adalah homogenous. Dalam kajian kinetik enzim, K_m dan V_{max} untuk fenol ialah 13.4 µM and 2.5 µmol/min/mg protein (Michaelis-Menten); 17.57 µM and 2.89 µmol/min/mg protein (Lineweaver-Burk) masing-masing. Nilai K_m dan V_{max} untuk NADH ialah 84 µM dan 2.31 µmol/min/mg protein (Michaelis-Menten); 50.7948 µM and 1.724 µmol/min/mg protein (Lineweaver-Burk) masing-masing. Dalam kajian pencirian enzim, penguraian-fenol optimum telah didapati pada diantara pH 6.5-7.5 dengan penimbal fosfat dan 7- 7.5 dengan penimbal Tris-HCl pada 15-25˚C. Enzim ini stabil pada suhu -20°C selama 40 hari di dalam penimbal fosfat pada pH 7.5. Keputusan kajian penguraian fenol oleh bakteria dan enzim dalam kajian ini mampu meningkatkan pengetahuan mengenai sumber baru dari mikrob yang lebih cekap dalam proses menguraikan fenol; oleh itu menyumbang kepada kelestarian persekitaran terutama untuk negara-negara membangun seperti Malaysia.

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I always believe that Allah always with me, no matter how hard this journey is. And, I keep on belief that "In every difficulty, lies opportunity". Thank you God. Thanks indeed.

"Believe the dreams come true every day, because they do."

Siti Aqlima Ahmad, 2012.

I certify that an Examination Committee met on 21st December 2011 to conduct the final examination of Siti Aqlima binti Ahmad on her Doctor of Philosophy thesis entitled "Biodegradation of Phenol by Locally Isolated *Acinetobacter* sp. Strain AQ5NOL 1 and Its Purification Phenol Hydroxylase" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are 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 institution.

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SITI AQLIMA BINTI AHMAD

Date:

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