UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING PHOSPHOMOLYBDO-REDUCTASE PRODUCE BY *Bacillus* sp. isolate A.rzi

AHMAD RAZI OTHMAN

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By

AHMAD RAZI OTHMAN

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

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DEDICATED TO MY FAMILY, LECTURES AND FRIENDS

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements of the degree of Master of Science

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Chairman: Assoc. Prof. Mohd Yunus Abdul Shukor, PhD

Faculty: Biotechnology and Biomolecular Sciences

The department of Environmental Malaysia in 2006 recorded that heavy metals were recorded at levels above the Maximum Permissible Limit at various places in Malaysia. Bioassays using enzyme and microorganism were currently in placed as these methods gave faster results and are cheaper compare to instrumental technology. This research is based on an inhibitive enzyme-based bioassay to detect heavy metals in soil and water sample using heavy metals sensitive bacteria. About 106 single colonies were isolated and assayed for molybdenum blue quantification using Low Phosphate Media agar (LPM). A colony isolated from Pulau Pangkor labeled as P2 (2) exhibited the lowest Mo-reducing activity in the presence of heavy metals and was selected for further studies. This bacterium is identified as *Bacillus* sp. isolate A.rzi based on the carbon utilization profiles using Biolog GP plate and partial 16S rDNA molecular phylogeny. The sequence of the 16s rRNA of the bacterium has been submitted to genbank with the accession no EU835195. Optimizations studies carried out for this bacterium showed that maximum temperature and pH supporting molybdate reduction were room temperature and pH 6.0, respectively. The absorption spectrum of the molybdenum blue product showed a maximum

peak at 865 nm and a shoulder at 700 nm indicating the involvement of a phosphomolybdate intermediate. Different electron donors such as glucose, sucrose, maltose, mannose, mannitol, lactose, starch, glycerol, tartarate, formate and acetate supported molybdate reduction except citrate. Molybdate reduction to Mo-blue was found to increase as molybdate concentration was raised from 0 to 50 mM and is inhibited at higher molybdate concentrations. The optimum phosphate concentration for molybdate reduction for this bacterium when molybdate concentration was fixed at 50 mM was 3 mM. Partial purification using MonoQ anion exchange chromatography showed that enzyme activity was eluted at tubes numbered between 20 and 23. Almost two fold purification was achieved after chromatography. A plot of initial rates against NADH concentrations at 10 mM LPPM registered an apparent V_{max} for NADH at 79.72 nmole Mo blue/min/mg protein and a K_m of 19.3 mM while the plot of initial rates against LPPM concentrations at 30 mM NADH (saturating) registered a V_{max} for LPPMH at 89.1 nmole Mo blue/min/mg protein and a K_m of 6.26 mM. Screening of inhibition by metals was also carried out, and the results showed that reduction was inhibited by copper, mercury and lead. The calculated IC_{50} value for copper, mercury and lead were 0.2476 mg/L, 0.3543 mg/L and 0.4875 mg/L, respectively. Field trial works using the assay showed that the most polluted samples came from Sg. Derhaka Juru, Juru, Penang, and Bukit Juntong, Bentong, Pahang which exhibited nearly 100% inhibition of enzyme activity and was corroborated by ICP analysis. Another sample from Kg. Ladang showed small amount of copper but not enough to show inhibition of the enzyme activity. Waters from clean areas and tap water all showed no inhibition to the enzyme activity and data were corroborated with ICP analysis. In general, all of the objectives have been met. This study hopefully can contribute to this country in monitoring pollution since this newly developed bioassay is low in cost, easy to handle compared to conventional methods and enable real time results.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

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Oleh

AHMAD RAZI OTHMAN

Oktober 2010

Pengerusi: Prof. Madya Mohd Yunus Abdul Shukor, PhD

Fakulti: Fakulti Bioteknologi dan Sains Biomolekul

Jabatan Alam Sekitar telah merekodkan bahawa pada tahun 2006 pelbagai tempat di Malaysia mengandungi kandungan logam berat yang melebihi kadar maksimum yang dibenarkan. Bioasai menggunakan enzim dan mikroorganisme pada masa ini banyak digunakan kerana kaedah-kaedah ini adalah lebih pantas dan murah berbanding menggunakan teknologi berasaskan instrumen. Penyelidikan ini adalah berdasarkan bioasai perencatan-enzim untuk mengukur kehadiran logam berat di dalam tanah dan air menggunakan bakteria sensitif kepada logam berat. Sebanyak 106 koloni tunggal telah diasingkan dan di assai untuk menentukan kuantifikasi molybdenum biru dengan menggunakan media agar rendah fosfat (LPM). Koloni yang di ambil dari Pulau Pangkor yang dilabel P2 (2) telah menunjukan aktiviti penurunan molibdenum paling rendah dengan kehadiran logam berat dan telah dipilih untuk kajian selanjutnya. Bakteria ini telah dikenalpasti sebagai *Bacillus* sp. isolate A.rzi berdasarkan profil kebolehan menggunakan unsur karbon menggunakan plat Biolog GP dan analisis molekul filogenetik separa untuk gen16s rDNA. Jujukan 16s rDNA telah dihantar ke bank gen dengan nombor aksesi EU835195.

Pengoptimuman bakterium ini menunjukan suhu dan pH maksimum pada suhu bilik dan pH 6.0. Spektrum penjerapan bagi produk molybdenum biru menunjukan puncak maksimum pada 865 nm dengan peningkatan bermula pada 700 nm menunjukan penglibatan perantara fosfomolibdat. Penderma electron yang berbeza seperti glukosa, sukrosa, maltosa, mannosa, manitol, laktosa, kanji, gliserol, tartarat, format dan asetat membantu dalam penurunan molibdat kecuali sitrat. Penurunan molibdat ke molybdenum biru di dapati meningkat dengan peningkatan kepekatan molibdat dari 0 mM ke 50 mM dan direncat pada kepekatan yang lebih tinggi. Kepekatan optimum bagi fosfat dalam proses penurunan molibdat apabila kepekatan molibdat ditetapkan pada 50 mM adalah 3 mM. Penulenan separa menggunakan penukaran anion MonoQ kromatografi menunjukan kehadiran aktiviti enzim antara tiub 20 dan 23. Hampir dua kali penulenan dicapai selepas kromatografi. Plot kadar awal melawan NADH pada kepekatan LPPM pada 10 mM memberikan V_{max} untuk NADH pada 79.72 nmole Mo blue/min/mg protin dan K_m 19.3 mM manakala plot kadar awal melawan kepekatan LPPM pada kepekatan NADH 30 mM (tepu) memberikan V_{max} untuk LPPM at 89.1 nmole Mo blue/min/mg protin dan K_m sebanyak 6.26 mM. Pencerakinan kesan terhadap logam berat telah dijalankan dan keputusan menunjukan penurunan molibdenum kepada molibdenum biru direncat oleh kuprum, merkuri dan plumbum. Nilai IC_{50} yang telah dikira untuk kuprum, merkuri dan plumbum adalah 0.2476 mg/L, 0.3543 mg/L dan 0.4875 mg/L, masing-masing. Ujian lapangan menggunakan asai ini menunjukan sampel yang paling tercemar adalah dari Sg. Derhaka Juru, Juru, Penang, dan Bukit Juntong, Bentong, Pahang di mana sampel menunjukan hanpir 100% perencatan terhadap aktiviti enzim dan telah disahkan dengan analisis ICP. Sampel dari Kg. Ladang menunjukan kehadiran sejumlah kecil kuprum tetapi tidak cukup untuk menunjukan kesan rencatan terhadap aktiviti enzim. Sampel air bersih dan air paip menunjukan tiada kesan rencatan terhadap aktiviti enzim dan data disahkan oleh analisis ICP. Secara amnya, kesemua objektif telah dicapai. Kajian ini

diharap dapat menyumbang sesuatu yang berfaedah kepada negara ini dalam mengawasi pencemaran memandangkan penghasilan sistem bioasai baru ini bermodal rendah, senang dikendalikan berbanding kaedah konvensional dan memberikan keputusan dalam masa yang singkat.

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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 Master of Science. The members of the Supervisory Committee were as follows:

Mohd Yunus Abdul Shukor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
University Putra Malaysia
(Chairman)

Mohd Arif syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
University Putra Malaysia
(Member)

_____
BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
University Putra Malaysia

Date:

DECLARATION

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

AHMAD RAZI OTHMAN

Date: 05 October 2010

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