UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF AN INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING MOLYBDENUM-REDUCING ENZYME FROM *Staphylococcus* sp. isolate liz1

NURLIZAH BINTI ABU BAKAR

FBSB 2010 26

DEVELOPMENT OF AN INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING MOLYBDENUM-REDUCING ENZYME FROM *Staphylococcus* sp. isolate liz1

NURLIZAH BINTI ABU BAKAR

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA
2010

© COPYRIGHT UPM

DEVELOPMENT OF AN INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING MOLYBDENUM-REDUCING ENZYME FROM *Staphylococcus* sp. isolate liz1

© COPYRIGHT UPM

By

NURLIZAH BINTI ABU BAKAR

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

October 2010

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

DEVELOPMENT OF AN INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING MOLYBDENUM-REDUCING ENZYME FROM *Staphylococcus* sp. isolate liz1

By

NURLIZAH BINTI ABU BAKAR

October 2010

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

Faculty : Biotechnology and Biomolecular Sciences

Molybdenum-reducing bacteria were isolated from soil and water samples throughout Malaysia and one isolate from workshop area in Seri Kembangan, Selangor was identified to reduce molybdenum to molybdenum blue in less than 48 hours with the most intense blue-colour colony formation. Further analysis using biochemical test, 16S rRNA ribosomal gene sequence comparison and molecular phylogenetics analysis showed that isolate Point 1 is Gram-positive coccus and grouped under genus Staphylococcus and was submitted to Genbank under accession number of EU828636 as *Staphylococcus* sp. isolate liz1. The characterization studies showed that the bacterium gave the highest Mo-reducing activity under the combination of 1% glucose, 50 mM molybdate, 5 mM phosphate, at pH 6.0 and temperature 37°C in 48 hours of incubation time. Partial purification and characterization were conducted on crude Mo-reducing enzyme with two anion exchange chromatography columns (Q-Sepharose and Mono Q). Three bands

© COPYRIGHT UPM

were obtained on the Mono-Q fraction at 25, 40 and 55 kDa using reducing SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Characterization of Mo-reducing enzyme kinetics gave the K_m and V_{max} values of 5.722 mM and 127.1 nmole Mo-blue/min/mg protein for laboratory-prepared phosphomolybdate (LPPM), and 15.58 mM and 152.8 nmole Mo-blue/min/mg protein for NADH, respectively. The optimum pH and temperature for enzyme were pH 5.0 and 37°C, respectively. The inhibition profiles of heavy metal on Mo-reducing activity were determined and IC_{50} values were calculated. The results showed that the enzyme has high sensitivity towards copper (0.2845 mg L^{-1}), silver (0.2773 mg L^{-1}), and mercury (0.4187 mg L^{-1}). Three commercialized herbs samples were tested using the developed enzyme assay and the results were compared with that of atomic absorption spectroscopy (AAS) analysis. The results showed that one of the herb sample contained high level of mercury and copper when analysed using AAS (1.016 mg L^{-1} and 0.421 mg L^{-1}, respectively). The findings were similar to the biological assay system developed for the detection of selected heavy metals, indicating the usefulness of the assay.

© COPYRIGHT UPM

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

DEVELOPMENT OF AN INHIBITIVE ENZYME ASSAY FOR HEAVY METAL DETECTION USING MOLYBDENUM-REDUCING ENZYME FROM *Staphylococcus* sp. isolate liz1

Oleh

NURLIZAH BINTI ABU BAKAR

Oktober 2010

Pengerusi: Prof. Madya Mohd Yunus Abdul Shukor, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Bakteria penurun molibdenum telah berjaya dipencilkan dari sampel tanah dan air dari beberapa tempat seluruh Malaysia dan satu bakteria dari kawasan bengkel di Seri Kembangan, Selangor telah dikenalpasti mempunyai kemampuan penurun molibdenum dalam tempoh 48 jam dan pembentukan koloni berwarna biru dengan kepekatan yang tertinggi. Kajian selanjutnya yang dijalankan ke atas bakteria menggunakan teknik analisis biokimia, jujukan gen ribosom 16S rRNA dan filogenetik molekul menunjukkan bahawa bakteria 'Point 1' adalah Gram positif kokus, diklasifikasikan di bawah genus Staphylococcus dan didaftarkan di Genbank dengan nombor akses EU828636 sebagai *Staphylococcus* sp. isolate liz1. Pencirian ke atas bakteria memberikan aktiviti penurun molibdenum tertinggi dalam kombinasi 1% glukos, 50 mM molybdat, 5 mM fosfat, pada pH 6.0 dan suhu 37°C untuk tempoh 48 jam. Penulenan separa dan pencirian telah dijalankan ke atas enzim mentah menggunakan kolum kromatografi penukaran anion (Q-Sepharose and Mono Q). Tiga ikatan telah ditunjukkan dari fraksi Mono-Q pada 25, 40

© COPYRIGHT UPM

and 55 kDa menggunakan SDS elektroforesis-gel poliakrilamid terturun (SDS-PAGE). Pencirian kinetik enzim penurun-molibdenum memberikan nilai K_m dan V_{max} 5.722 mM; 127.1 nmole Mo-blue/min/mg protein untuk phosphomolibdat penyediaan dari makmal (LPPM), dan 15.58 mM; 152.8 nmole Mo-blue/min/mg protein untuk NADH. Nilai pH dan suhu optimum untuk aktiviti enzim adalah pH 5.0 dan 37°C. Profil perencatan logam berat ke atas aktiviti enzim penurun-molibdenum dan nilai IC_{50} ditentukan. Keputusan menunjukkan enzim mempunyai ciri sensitiviti yang tinggi terhadap kuprum (0.2845 mg L^{-1}), argentum (0.2773 mg L^{-1}), dan merkuri (0.4187 mg L^{-1}). Tiga sampel herba komersial telah diuji menggunakan sistem asai enzim yang dihasilkan dan keputusan dibandingkan dengan menggunakan analisis spektroskopi penyerapan atom (AAS). Keputusan yang diperolehi menunjukkan salah satu dari sampel mengandungi nilai logam berat merkuri dan kuprum yang tinggi iaitu masing-masing 1.016 mg L^{-1} dan 0.421 mg L^{-1}. Keputusan ini adalah bersamaan dengan keputusan apabila menggunakan sistem asai biologi yang dihasilkan untuk mengesan kehadiran logam berat, membuktikan kepentingan dan kegunaan sistem yang dihasilkan.

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, the Most Merciful.

I would like to thank my supervisors, Assoc. Prof. Dr. Mohd. Yunus Shukor and Prof. Dr. Mohd. Arif Syed, for their guidance and help throughout my studies. I also would like to dedicate my dissertation to my family, especially to my parents. Only with their blessings did I managed to complete this thesis. I am sorry to them for all burdens and stressful moments caused by my unintelligent mind and lack of self-control.

To all of my dearest friends in Department of Biochemistry and Microbiology, my lab members, especially to Lubna and Keem, their help and support are most appreciated.

I also would like to thank my best friend, Muhammad Hazwan Shaharin Azhar, for his time, patience, love and moral support in the hardest moment of my life. Without his words, his advices, and his sincere helps, I would really unable to complete this thesis.

Lastly, to my husband, I love only you and for sure it is more than myself, may God bless us. And thank you.

© COPYRIGHT UPM

© COPYRIGHT UPM

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements 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
Universiti Putra Malaysia
(Chairman)

Mohd. Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

© COPYRIGHT UPM

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

Date :

DECLARATION

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

© COPYRIGHT UPM

NURLIZAH BINTI ABU BAKAR

Date : 26 October 2010

TABLE OF CONTENTS

Page

ABSTRACT i
ABSTRAK iii
ACKNOWLEDGEMENTS v
APPROVAL vi
DECLARATION viii
LIST OF TABLES xi
LIST OF FIGURES xii
LIST OF ABBREVIATIONS xiv

CHAPTER
1. **INTRODUCTION** 1

2. **LITERATURE REVIEW**
   - 2.1. Environmental pollution in Malaysia 4
     - 2.1.1. Type of pollutants 4
       - 2.1.1.1. Organic pollutants 4
       - 2.1.1.2. Inorganic pollutants 5
   - 2.2. Heavy metals 6
     - 2.2.1. Toxicity of heavy metals 8
     - 2.2.2. Medicinal herbs 10
     - 2.2.3. Heavy metals pollutions in medicinal herbs 12
   - 2.3. Heavy metals detection methods 15
     - 2.3.1. Atomic Absorption Spectroscopy 15
     - 2.3.2. Microbial bioindicator 16
     - 2.3.3. Enzyme-based bioassay 17
   - 2.4. Molybdenum-reducing enzyme 19
     - 2.4.1. Uses of Molybdenum-reducing enzyme as bioassay 20

3. **MATERIALS AND METHODS**
   - 3.1. Equipments, Chemicals, Buffers and Chemical solutions 23
   - 3.2. Screening and isolation of Mo-reducing bacterium 23
   - 3.3. Identification of bacterium
     - 3.3.1. Primary identification 25
       - 3.3.1.1. Gram staining 25
       - 3.3.1.2. Oxidase test 26
       - 3.3.1.3. Catalase test 26
       - 3.3.1.4. Biolog™ 27
     - 3.3.2. Secondary identification 27
     - 3.3.3. Phylogenetic analysis 28
   - 3.4. Characterization of *Staphylococcus* sp. isolate liz1
     - 3.4.1. Characterization for carbon sources 29
     - 3.4.2. Characterization for temperature 30
     - 3.4.3. Characterization for pH 30

© COPYRIGHT UPM

3.4.4. Characterization for molybdate concentration 31
3.4.5. Characterization for phosphate concentration 31
3.5. Preparation of crude enzyme
3.5.1. Column Chromatography Preparation 32
3.5.2. SDS-polyacrylamide gel electrophoresis 33
3.5.3. Gel Staining 34
3.6. Protein determination 34
3.7. Kinetics studies 35
3.8. Molybdenum-reducing enzyme assay 36
3.9. Preparation of heavy metals solutions 37
3.10. Field trial 37
3.11. Statistical analysis 38

4. RESULTS AND DISCUSSION
4.1. Soil sampling and preliminary screening procedures 39
4.2. Identification of bacterium
4.2.1. Primary identification of isolate Point 1 42
4.2.2. Secondary identification of isolate Point 1 45
4.3. Absorption spectra of Molybdenum blue produced by strain liz1 48
4.4. Characterization of Mo-reducing activity
4.4.1. Incubation time 50
4.4.2. Carbon sources 52
4.4.3. Molybdate concentration 54
4.4.4. pH 55
4.4.5. The effects of phosphate concentration 57
4.4.6. The effects of temperature 59
4.5. Mo-reducing enzyme characterization studies
4.5.1. Partial purification of Mo-reducing enzyme 62
4.5.2. SDS-PAGE of partially purified Mo-reducing enzyme 65
4.5.3. Determination of K_m and V_{max} for LPPM 67
4.5.4. Determination of K_m and V_{max} for NADH 69
4.5.5. Effects of pH on Mo-reducing enzyme activity 71
4.5.6. Effects of temperature on Mo-reducing activity 72
4.6. Inhibitive enzyme assay for heavy metals
4.6.1. Effect of metal ions on Mo-reducing enzyme 73
4.6.2. Inhibition profile of heavy metals
4.6.2.1. Copper (Cu), Silver (Ag) and Mercury (Hg) 75
4.6.2.2. Lead (Pb), Arsenic (As) and Cadmium (Cd) 79
4.7. Commercial medicinal herbs samples test 81

5. CONCLUSIONS 85

REFERENCES 86
APPENDICES 94
BIODATA OF STUDENT 99

© COPYRIGHT UPM