UNIVERSITI PUTRA MALAYSIA

ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF MOLYBDENUM-REDUCING ENZYMES FROM AN ANTARTICA BACTERIUM (gamma-Proteobacterium STRAIN DR.Y1)

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By

SITI AQLIMA BINTI AHMAD

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Dedicated to my parents, family and friends.

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

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Chairman: Professor Nor Aripin Shamaan, PhD

Faculty : Biotechnology and Biomolecular Sciences

Bacterial Isolate no. J7A was isolated from Jubany Station, Antarctica and it has the capability to reduce the heavy metal molybdenum (molybdate) to molybdenum blue in a solid medium agar, pH 7 at 10˚C, after for 4 days of incubation. Isolate J7A was identified as Gram-negative and gamma-Proteobacterium Strain Dr.Y1 through moleculare phylogenetics analysis of the sequenced 16s rRNA gene. The optimization studies were carried out to optimize the production of molybdenum blue. The combination of 1% (w/v) glucose, 0.3% (w/v) ammonium sulphate, 0.1% (w/v) of yeast extract, 30mM molybdate, and low phosphate medium at pH 7 give the optimum production of Molybdenum blue. Partial purification and characterization were conducted on molybdenum reducing enzyme with anion exchange chromatography using Macro-Prep High-Q^{TM} column and gel filtration chromatography using Agilent Zorbax^{TM} (GF-250) column. Three bands were visualized on the gel filtration fraction at 39, 36 and 33 kDa using the SDS polyacrylamide-gel electrophoresis (SDS-PAGE) suggesting that purification was

not achieved. In enzyme kinetic studies, NADH serves as the substrate for electron donor and 12-MP act as the substrate for electron acceptor. The K_m and V_{max} for NADH were 0.4838 mM and 21.51 units/mg enzyme respectively. While the values for 12-MP were 5.347 mM and 64.04 units/mg enzyme respectively. The characterization of Mo-reducing enzyme studies were carried out at the optimum pH of 7.5 using 50mM Tris-HCl at 15˚C. The enzyme is stable at -20°C for six days in Tris-HCL buffer at pH 7.5.

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

PEMENCILAN, PENULENAN SEPARA DAN PENCIRIAN ENZIM PENURUNAN MOLYBDENUM OLEH BAKTERIA ANTARCTICA (gamma-Proteobacterium STRAIN DR.Y1)

Oleh

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Bakteria nombor J7A telah dipencilkan daripada Jubany Station, Antarctica dan mempunyai kebolehan untuk menurunkan logam berat molybdenum (molybdate) kepada molybdenum biru di dalam medium rendah fosfat, pH 7 dalam keadaan anaerobic pada 10˚C selama empat hari. Pemencilan J7A telah diidentifikasi sebagai Gram-negatif dan strain baru untuk jujukan DNA yang dikenali sebagai gamma-Proteobacterium strain DR.Y1 menggunakan analisis filogenetik molekul 16S rRNA. Pengoptimaan telah dikaji untuk menentukan kadar optimum penghasilan molybdenum biru. Kombinasi 1% (w/v) kepekatan glukos sebagai sumber karbon, 0.3% (w/v) kepekatan ammonium sulfat sebagai sumber nitrogen, 0.1% (w/v) kepekatan yis, 30mM kepekatan molybdate, dan medium rendah fosfat pada pH 7 memberikan penghasilan optimum molybdenum biru. Penulenan separa dan pencirian telah dikonduksikan oleh enzim penurunan-molybdenum dengan kromatografi penukaran anion menggunakan kolum Macro-Prep High-Q^{TM} dan kromatografi penurasan gel menggunakan kolum Agilent Zorbax^{TM} (GF-250). Tiga ikatan telah visualisasikan pada fraksi gel filtrasi pada 39,36 dan 33 kDa

menggunakan SDS elektroforesis-gel poliakrilamid yang menunjukkan penulenan tidak tercapai.. Dalam kajian enzim, NADH bertindak sebagai substrat untuk penderma electron dan 12-MP bertindak sebagai substrat untuk penerima electron. K_m dan V_{max} untuk NADH ialah 0.4838 mM dan 21.51 unit/mg enzim. Manakala nilai untuk 12-MP ialah 5.347 mM dan 64.04 units/mg enzim. Pencirian enzim penurun-Mo telah didapati optimum pada pH 7.5 menggunakan 50mM Tris-HCl pada 15˚C. Enzim ini stabil pada -20°C selama enam hari di dalam buffer Tris-HCl pada pH 7.5.

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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.

Siti Aqlima Ahmad, 2006.

I certify that an Examination Committee met on 20th December 2006 to conduct the final examination of Siti Aqlima binti Ahmad on her Master of Science thesis entitled "Isolation, Partial Purification and Characterization of Molybdenum-reducing Enzyme from An Antarctica Bacterium (The gamma-Proteobacterium strain DR.Y1)" 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 fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

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

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SITI AQLIMA BINTI AHMAD

Date:

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LIST OF ABBREVIATIONS

%	Percent
$(NH_4)_2SO_4$	Ammonium sulphate
<	Less than
>	More than
°C	Degree celsius
µl	Microlitre
µM	Micromolar
12-MP	Twelve-molybdophosphate
Ag	Argentum
As	Asenic
ATP	Adenosine triphosphate
Cd	Cadmium
cm	Centimeter
Co	Cuprum
Cr	Chromium
Cu	Copper
DEAE	Diethylaminoethylamine
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
et al	And friends

Fe	Ferum
Glc	Glucose
g	Gravity (relative centrifugal force)
HCl	Hydrogen chloride
Hg	Mercury
HPLC	High performance liquid chromatography
HPM	High phosphate medium
hr	Hour
K	Kelvin
kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
K_m	Michaelis-Menten constant
L	Litre
LPM	Low phosphate medium
m	Meter
M	Molar
mA	Milliampere
mAu	Mili absorbance unit
mg	Miligram
$MgSO_4$	Magnesium sulphate
min	Minutes
mM	Milimolar
Mo	Molybdenum
Mo-blue	Molybdenum blue

Mo-reducing bacteria	Molybdenum reducing bacteria
Mo-reducing enzyme	Molybdenum reducing enzyme
MT	Milestones
MW	Molecular weight
$Na_2HPO_4.2H_2O$	DiSodium-hidrophosphate
$Na_2MoO_4.2H_2O$	DiSodium molybdate
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
Ni	Nikel
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
Pb	Plumbum
PCR	Polymerase chain reaction
pH	-Log concentration of H^+ ion (*Puissance hydrogene)*
PMSF	Phenylmethylsulfonylfluoride
PO_4^{3-}	Phosphate
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
Sn	Stanum
T50-7.5-buffer	50 mM Tris-HCl at pH 7.5
TEMED	N,N,N’,N’-tetramethyl-ethylenediamine
UV	Ultraviolet

v/v	Volume/ volume
V_{max}	Maximum velocity
w/v	Weight/ volume
XOD	Xanthine oxidase
Zn	Zink

CHAPTER 1

INTRODUCTION

Water pollution due to heavy metals is a very important issue as it reduces the viable water resource by creating a negative feedback loop involving increasing economic pressure and decreasing quality of supply. Water covers nearly 70% of our planet, yet the majority of this water is salt water. We have very little reserves of fresh water for use. However, we have polluted or contaminated a great majority of our water sources with little thought about our future needs.

Heavy metals in water, air and soil are global problems that have become a growing threat to the environment and humanity. Heavy metal such as mercury, lead and arsenic are widely recognized as highly toxic and dangerous to organism (He *et al.*, 2005; Patra *et al.*, 2004). As a result of widespread application in numerous industrial processes, heavy metal has become a contaminant of many environmental systems (Bird *et al.*, 2005; Hasselriis and Licata, 1996). Major sources of heavy metal pollution today come from the combustion of leaded gasoline, mining and processing, steel, iron, cement and fertilizers production, nuclear and other industrial effluents and sludges, dumping and land filling of industry wastes, biocides and preservatives including organometalic compounds.

From industrial applications, molybdenum has been found in discharged effluents, which results in the widespread contamination of molybdenum to the environment (Davis, 1991). There were many reports on molybdenum pollution due to

molybdenum mining activity such as at Tokyo Bay and Black Sea in 1991, Red Sea in 1996 and Tyrol in 2000 (Davis, 1991; Slifer, 1996; Neuhauserer *et al.*, 2000). Exposure to high concentration of molybdenum affected the reproduction and caused mortality in humans and animals.

Heavy metal is different from to organic pollutants because it cannot be detoxified by degradation and remains in the ecosystem (Shukor *et al.*, 2000). So, the best strategy is to remove the heavy metals by bioremediation. Bioremediation is a process which involves the transformation/detoxification of pollutants using microorganisms and plants. Bioremediation cleans up the environment effectively and is cheaper than any other methods (King *et al.*, 1992; Vidali, 2001). This research emphasizes the biotransformation of molybdenum using bacterium isolated from Antarctica. The first psychrophiles Mo-reducing bacterium and Mo-reducing enzyme that have high potential for bioremediation will be studies.

The objectives of this study are:

- to isolate and screen psychrophilic Mo-reducing bacteria.
- to determine the optimum environmental and nutrient conditions of a screened bacterium.
- to identify the selected Mo-reducing bacterium to species level.
- to partially purify and characterize the Mo-reducing enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Molybdenum

2.1.1 History on Molybdenum

A 14th century Japanese sword has been found to contain molybdenum. However, molybdenum was only discovered during the latter part of the 18th century and did not occur in metallic form in nature. Molybdenum has been discovered by the Swedish scientist, Carl Wilhelm Scheele, in 1778. He was able to positively identify molybdenum. He decomposed molybdenite, which is molybdenum predominant metal, by heating it in air to obtain a white oxide powder. Four years later, in 1782, Peter Jacob Hjelm reduced the oxide with carbon to obtain a dark metallic powder, which he named “Molybdenum”. Molybdenum came from the Greek word “molybdos”, which means lead-like.

Molybdenum was first used in 1891 by the French company, Scneider & Co. as an alloying element in the production of armour plates. Molybdenum was found to be an effective replacement for tungsten in numerous steel alloying applications. In World War 1, molybdenum has been extensively used as a substitute for tungsten in many hard and impact-resistant steels, and the increased demand has initiated an intensive search for new sources of molybdenum supply. There were marked developments of the massive Climax deposit in Colorado, USA in 1918. After 12 years, in 1930, the proper temperature range for the forging and heat treatment of