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PARTIAL PURIFICATION AND CHARACTERIZATION OF MOLYBDENUM REDUCING ENZYME FROM Escherichia coli STRAIN K12

MOHD HARIS BIN SULAIMAN

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By

MOHD HARIS BIN SULAIMAN

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

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Chairman: Associate Prof. Mohd Yunus Abdul Shukor, PhD
Faculty: Biotechnology and Biomolecular Sciences

Molybdenum is becoming a threat to environment due to its toxic effect in high concentration due to various industrial use. Therefore improper management of this heavy metal waste will cause it to remain in the ecosystem. To date, the best way to treat heavy metals is via bioremediation. *Escherichia coli* (*E. coli*) have been reported to have the capability to reduce the heavy metal molybdenum (molybdate) to molybdenum blue. Thus, this study was conducted to determine the optimum environmental and nutrient conditions of *Escherichia coli* strain K12; to partially purify molybdenum reducing enzyme; and to characterize the molybdenum reducing enzyme. *E. coli* are Gram-negative bacteria that belong to the g-proteobacteria. They exist in a straight rod shaped cells and about 2 µm long and 0.5 µm wide, which can grow and divide rapidly by binary fission. In this experiment, *E. coli* was used to reduce molybdate Mo^{6+} forming a Mo-blue with the aid of Molybdenum reductase (Mo-reducing enzyme) which give non-toxic effect to the environment. Bacteria were cultured in a low phosphate media, pH 7.5 at temperature 35°C and incubated for 2 days. The optimization studies were carried out to optimize the production of molybdenum blue. The combination of 1% (w/v) glucose, 0.4% (w/v) ammonium chloride, 0.2% (w/v) yeast extract, and in ratio of 5mM phosphate and 80mM molybdate at pH 7.5 gave the optimum production of Molybdenum blue. Based on the maximum absorption peak at 865nm, this wavelength was used for the measurement of molybdenum blue produced in subsequent experiments. The effect of heavy metals on molybdenum blue production were studied. Thirteen metal ions and heavy metals were screened on *E.coli.* Mercury (Hg), Argentum (Ag), Copper (Cu) and Chromium (Cr) totally inhibited molybdate reductase enzyme while Zink (Zn), Nickel (Ni), Cobalt (Co), Arsenic (As), Lead (Pb) Aluminium (Al), Cadmium (Cd), and Magnesium (Mg) decreased the molybdenum reduction activity. Partial purification and characterization were conducted on molybdenum reducing enzyme with anion exchange chromatography using GE-Healthcare Mono-Q™ column and gel filtration chromatography using Agilent Zorbax™ (GF-250) column. Two bands were visualized on the gel filtration fraction at 95.64 and 84.42 kDa using SDS polyacrylamide-gel electrophoresis (SDS-PAGE). In enzyme kinetic studies, characterization of enzyme and stability of enzyme is being studied. NADH serves as the substrate for electron donor and 12-Molybdophosphate act as the substrate. The K_m and V_{max} for NADH were 2.156 mM and 15.015 units/ mg enzyme respectively. While the values for 12-MP were 3.549 mM and 54.348 units/ mg enzyme respectively. The characterization of Mo-reducing

enzyme studies were carried out at optimum pH of 6.0 using phosphate buffer at 35 ºC. For enzyme temperature stability, enzyme are stable at range of 5ºC - 40 ºC for a period of 24 hours. Based on the result obtained, *Escherichia coli* strain K12 was proven to be effective for reduction of molybdate forming molybdenum blue.

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

PENULENAN SEPARA DAN PENCIRIAN ENZIM PENURUNAN MOLIBDENUM OLEH BAKTERIA *Escherichia coli* STRAIN K12

Oleh

MOHD HARIS BIN SULAIMAN

Mac 2015

Pengerusi: Profesor Madya Mohd Yunus Abdul Shukor, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Molibdenum di dalam kepekatan yang tinggi telah menjadi satu ancaman kepada persekitaran disebabkan oleh kesan toksiknya serta penggunaan meluas di dalam industri. Oleh itu, salah pengurusan bagi sisa logam berat akan menyebabkannya kekal di dalam ekosistem persekitaran. Setakat ini cara yang terbaik bagi merawat sisa logam berat adalah melalui bioremediasi. *Escherichia coli (E.coli)* telah dilaporkan mempunyai keupayaan untuk menurunkan logam berat molibdenum (molibdat) kepada molibdenum biru. *E.coli* merupakan bakteria gram-negatif dan tergolong dalam kumpulan g– protobakteria yang wujud dalam sel-sel berbentuk rod kira-kira 2 µm panjang dan 0.5 µm lebar yang mempunyai pertumbuhan yang cepat dan menjalani belahan dedua. Dalam eksperimen ini, *E.coli* digunakan bagi mengkaji penurunan molibdat Mo^{6+} membentuk molibdenum biru melalui enzim penurun Molibdenum. Pengoptimaan telah dikaji untuk menentukan kadar optimum penghasilan molibdenum biru. Bakteria dikultur dalam media fosfat berkepekatan rendah pada pH 7.5 dan suhu 35 °C selama 2 hari. Kombinasi 1% (w/v) kepekatan glukosa, 0.4% (w/v) ammonium klorida, 0.2% (w/v) kepekatan yis dan nisbah 5mM fosfat dan 80mM molibdat pada pH 7.5 memberikan hasil optimum molibdenum biru. Molibdenum biru diukur berpandukan gelombang pada 865nm dalam keseluruhan eksperimen. Tiga belas ion logam dan logam berat telah diuji kepada *E.coli.* Merkuri (Hg), Argentum (Ag), Kuprum (Cu), dan Kromium (Cr) merencat sepenuhnya enzim penurun molibdat manakala Zink (Zn), Nikel (Ni), Kobalt (Co), Arsenik (As), Plumbum (Pb), Aluminium (Al), Kadmium (Cd), dan Magnesium (Mg) merendahkan aktiviti enzim penurun molibdenum. Penulenan separa dan pencirian telah dijalankan kepada enzim penurun molibdenum dengan kaedah kromatografi penukaran anion menggunakan kolum GE-Healthcare Mono Q™ dan kromatografi penurasan gel fraksi menggunakan kolum Agilent Zorbax™ (GF-250). Dua jalur dapat dilihat pada penurasan gel pada fraksi 95.64 dan 84.42 kDa menggunakan SDS elektroforesis-gel poliakrilamida. Dalam kajian kinetik enzim, NADH berfungsi sebagai substrat untuk menderma elektron dan 12-MP bertindak sebagai substrat untuk menerima elektron dimana K_m dan V_{max} untuk NADH adalah 2.156mM dan 15.015 mM unit / mg enzim masing-masing. Bagi 12-MP pula nilai K_m dan V_{max} adalah 3.549 mM dan 54.348 mM unit / mg enzim masing-masing. Pencirian bagi enzim penurun molibdenum telah dijalankan dan pH optimum adalah pada pH 6.0 didalam larutan penimbal fosfat pada suhu 35 º C. Bagi ujian kestabilan suhu, enzim

adalah stabil pada julat suhu 5 º C - 40 º C selama tempoh 24 jam. Berdasarkan keputusan yang diperolehi, *Eschiricia coli* strain K12 telah terbukti berkesan untuk penurunan molibdat membentuk molybdenum biru.

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Mohd Haris Bin Sulaiman

I certify that a Thesis Examination Committee has met on 16 March 2015 to conduct the final examination of Mohd Haris Bin Sulaiman on his thesis entitled "Partial Purification and Characterization of Molybdenum-reducing Enzyme from *Escherichia coli* Strain K12" 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 Masters of Science.

Members of the Examination Committee are as follows:

Syahida Binti Ahmad, PhD
Lecturer
Faculty of Biotechnology & Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Son Radu, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal examiner)

Phang Lai Yee, PhD
Lecturer
Faculty of Biotechnology & Biomolecular Sciences
Universiti Putra Malaysia
(Internal examiner)

Mohd Azmuddin Abdullah, PhD
Associate Professor
Universiti Teknologi PETRONAS
Malaysia
(External Examiner)

_____
ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date : 5 November 2015

This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Mohd Yunus Bin Abd Shukor, PhD
Associates Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

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

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Signature:_____	Signature:_____
Name of	Name of
Chairman of	Member of
of Supervisory	Supervisory
Committee: Mohd Yunus Bin Abd Shukor	Committee: Mohd Arif Bin Syed

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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	Arsenic
ATP	Adenosine triphosphate
Cd	Cadmium
cm	Centimeter
Co	Cobalt
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	Hydrochloric acid
Hg	Mecury
HPLC	High performance liquid chromatography
HPM	High phosphate media
hr	Hour
K	Kelvin
kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
K_m	Michealis-Menten constant
L	Litre
LPM	Low phosphate media
m	Meter
M	Molar
mA	Miliampere
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-hidrogen phosphate
$Na_2MoO_4.2H_2O$	DiSodium molybdate
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
Ni	Nickel
nm	Nanometer
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
Pb	Plumbum
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
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 resources 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 it is salt water. We have very little reserve of fresh water yet great portion of our reserved water have been badly polluted or contaminated.

Heavy metal is one of the major concerns that contributed to pollution of our water ways and has become a growing threat to the environment and humanity. Heavy metals such as mercury, lead and arsenic are widely recognized as highly toxic and dangerous to organisms. Major sources of heavy metal pollution today come from the combustion of leaded gasoline, mining and processing, steel, iron, cement and fertilizer production, nuclear and other industrial effluents and sludge, dumping and land filling of industries wastes, biocide and preservatives including organometallic compounds.

Molybdenum has been widely used in industrial applications which cause vast contamination of molybdenum in the environment. Many reports on molybdenum pollution due to molybdenum mining activity such as at Tokyo Bay and Black Sea in 1991, Red Sea in1996 and Tyrol in 2000 (Davis, 1991; Slifer 1996; Neuhauserer *et al.*, 2000). Exposure to higher concentration of molybdenum affected the reproduction and caused mortality in humans and animals.

Heavy metal is different from organic pollutants because it cannot be detoxified by degradation and remains in the ecosystem (Shukor *et al*., 2000). The best way to remove heavy metals is via bioremediation. Bioremediation is a process which involves the transformation/detoxification of pollutants using microorganisms and plants. Bioremediation cleans up the environment effectively and cheaper than any other methods.

In the early 90's, Ghani and his team (Ghani *et al.*, 1993) isolated a heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48) from Chengkau, Malaysia which was able to reduce molybdate (molybdenum 6+) to molybdenum blue (molybdenum 5+) with NADH as an electron donor. This study brought the Molybdenum-blue research to University Putra Malaysia. There were many other studies which have indicated that molybdenum can be reduced to molybdenum blue using several bacteria, such as *Serratia Marcescens* strain DRY6 (Shukor *et al.*, 2008a), *Serratia sp*. strain Dr.Y8 (Shukor *et al.*, 2009a), *Pseudomonas sp* strain DRY2 (Shukor *et al.*, 2009b), *Enterobacter sp*. strain Dr.Y13 (Shukor *et al.*, 2009c), *Serratia marcescens* strain Dr.Y9 (Shukor *et al.*, 2009d), *Serratia sp*. strain DRY5 (Shukor *et al.*, 2009e) and *Acinetobacter calcoaceticus* strain Dr.Y12 (Shukor *et al.*, 2010).

The earliest work on the Mo-reducing bacterium was on *E. coli* (Capaldi and Proskauer, 1896) followed by the work of Campbell *et al.*, (1985).
In order to present a better comparison on the efficiency and characteristics of *E. coli* more specifically *E. coli* strain K12, characterisation of this bacterium on low phosphate media as used by all of the more recent isolates is warranted.

E.coli strain K12 was chosen in this experiment as it is easy to handle, complete gene sequence, has the ability to grow under both aerobic and anaerobic condition and the outcome for post-bioremediation are less harmful as compared to bacteria studied previously.

The importance of purifying and characterizing the molybdenum-reducing enzyme allows the elucidation of the mechanism of molybdenum-reducing activity. Purification of molybdenum-reducing enzyme could identify the protein take role in molybdenum-reducing activity and determine the protein structure thus contribute in protein genetic engineering process. Comparison can be made with other purified molybdenum reducing enzymes from different sources in terms of homologous sequences and also for the best enzyme characteristics such as broad substrates capacity for the source of reducing power, strong affinity for heteropolymolybdates and heat stability for the purpose of bioremediation. This information contributes in future molybdate reduction and bioremediation studies.

Thus the objectives of this study are:

- To determine the optimum environmental and nutrient conditions of *Escherichia coli* strain K12 on Low Phosphate Media (LPM),
- To partially purify Mo-reducing enzyme, and
- To characterize the Molybdenum reducing enzyme.

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