UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF PHOSPOMOLYBDATE REDUCTASE PRODUCED BY LOCALLY ISOLATED *SERRATIA MARCESCENS* STRAIN DR.Y5

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By

MOHD FADHIL BIN ABDUL RAHMAN

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October 2007

UPM

Dedicated to my parents, family and friends.

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

PURIFICATION AND CHARACTERIZATION OF PHOSPOMOLYBDATE REDUCTASE PRODUCED BY LOCALLY ISOLATED *SERRATIA MARCESCENS* STRAIN DR.Y5

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October 2007

Chairman: Mohd Yunus Abdul Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

The threat of heavy metal pollution to public health and wildlife has led to an increased in developing systems that can remove or neutralise its toxic effect in soil, sediments and wastewater. In this work, a local molybdenum reducing bacterium was isolated. This bacterium is Gram negative and identified as *Serratia marcescens* Strain Dr.Y5 based on Biolog ID system and 16s rRNA molecular phylogenetics studies matched 99.96% to *Serratia marcescens.*The isolate was originally isolated from the grounds of King Edward VII (2) primary school in Taiping, Perak.. The optimum carbon source for Mo-blue production was sucrose at 1.0% concentration and optimally grown at 40 °C. While the optimum concentration for nitrogen source was 0.2(w/v) % and optimum yeast concentration was 0.05(w/v) %. The Mo-blue production were optimum at pH 6.0 with the best ratio of phosphate to molybdate giving optimum reduction was 2.9 mM to 20 mM, respectively. Molybdenum reducing activity of the enzyme extract was assayed at 865 nm using 20 mM 10:4 molybdophosphoric acid and 2 mM NADH at room temperature. Purification of phosphomolybdate reductase was done by using anion exchange on Macro Prep High Q and gel filration on Zorbax GFX-250. The enzyme was assayed using NADH or

NADPH as the electron donor and phosphomolybdate as the electron acceptor. The assay was completed in less than 5 minutes and produced an intense blue color with a wavelength maximum at 865 nm. The best electron donor for the enzyme is NADH (12-MP as electon acceptor) with a maximum initial velocity, V_{max} of 25.07 nmole molybdenum blue produced/min/mg/protein and a Michaelis constant, K_m at 0.44 mM. The best electron acceptor substrate is 10:4 molybdophosphate, with a K_m of 3.87 mM and a V_{max} of 24.18 nmole molybdenum blue/min (NADH as electron donor at saturated concentrations). The phosphomolybdate reductase activity has an optimum temperature at 30 °C. At 40 °C of incubation for a period of one hour, the residual phosphomolybdate reductase activity remains 80% of the control, indicating that the enzyme is stable below 40 °C. The enzyme was inactivated rapidly at temperatures higher than 54 °C and was inactivated totally at 70 °C within 30 minutes of incubation.

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

PENULENAN DAN PENCIRIAN PHOSPOMOLYBDATE REDUCTASE DARIPADA PEMENCILAN TEMPATAN *SERRATIA MARCESCENS* STRAIN DR.Y5

Oleh

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Ancaman pencemaran logam berat kepada kesihatan awam dan hidupan liar telah meningkatkan pembinaan system yang dapat menghapuskan atau meneutralkan kesan toksik logam berat dalam tanah, mendakan dan air buangan. Dalam kajian ini, bakteria penurun molibdenum telah dipencilkan. Bakteria ini adalah bakteria Gram-negatif dan dikenali sebagai *Serratia marcesces* strain Dr.Y5 berdasarkan penggunaan Biolog ID dan analisis filogenetik molekul 16S rRNA yang memberikan kebarangkalian 99.96% untuk *Serratia marcescens.* Bakteria ini telah dipencilkan daripada tanah yang diambil daripada kawasan sekitar Sekolah Kebangsaan King Edward VII, Taiping, Perak. Sumber karbon yang optima untuk penghasilan molibdenum biru adalah sukrosa pada kepekatan 1.0% (w/v) dan suhu untuk pertambahan optima adalah 40°C. Kepekatan optima sumber nitrogen adalah 0.2% (w/v) dan kepekatan yeast optima adalah 0.05% (w/v). Penghasilan molibdenum biru adalah terbaik pada pH 6.0 dengan nisbah fosfat kepada molibdat optimum untuk penurunan molibdat adalah 2.9 mM kepada 20 mM. Aktiviti penurunan molibdenum

yang dimiliki oleh enzim ekstrak telah diasai pada jarakgelombang 865 nm dengan menggunakan 20 mM 10:4 asid molibdofosforik sebagai penerima elektron dan 2 mM NADH sebagai penyumbang elektron pada suhu bilik. Penulenan phosphomolibdat reduktase telah dilakukan dengan menggunakan kromatografi penukaran anion kolum Macro-Prep High-Q™ dan kromatografi penurasan gel kolum Agilent Zorbax™ GF-250. Asai tersebut dapat disempurnakan dalam masa kurang daripada 5 minit dan dapat menghasilkan warna biru pekat dengan gelombang maksima pada 865 nm. Penderma elektron terbaik untuk enzim ini adalah NADH (12-MP sebagai penerima) dengan halaju permulaan maksima, $V_{max,}$ adalah 25.07 nmol molibdenum biru/min/mg/protein dan pemalar Michaelis, K_m pada 0.44 mM. Penerima elektron terbaik adalah fosofomolibdat pada nisbah 10:4, dengan nilai K_m pada 3.87 mM dan nilai V_{max} pada 24.18 nmol molibdenum biru/min (NADH sebagai penderma elektron pada tahap kepekatan yang tepu). Aktiviti phosphomolibdat reduktase adalah tertinggi pada suhu 30 °C. Pada pengeraman selama satu jam pada suhu 40 °C, aktiviti residu phosphomolibdat reduktase kekal pada 80% berbanding kawalan menunjukan enzim ini adalah stabil pada suhu kurang dari 40 °C. Enzim ini didapati tidak aktif pada suhu lebih tinggi daripada 50 °C dan hilang aktiviti sepenuhnya pada suhu 70 °C dengan 30 minit masa pengeraman.

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I certify that an Examination Committee met on 4 October 2007 to conduct the final examination of Mohd Fadhil bin Abdul Rahman on his Master of Science thesis entitled "Purification and Characterization of Phosphomolydate Reductase produced by Locally Isolated *Serattia marcescens* Strain Dr.Y5" 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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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations 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.

MOHD FADHIL BIN ABDUL RAHMAN

Date: 3 January 2008

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL		viii
DECLARATION		x
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xix
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
	2.1 The Chemistry of Molybdenum	5
	2.2 Pollution of Heavy Metals	8
	2.3 Cases of Heavy Metals Pollution	9
	2.4 Heavy Metals Pollution in Malaysia	10
	2.5 Sources of Heavy Metal Pollution	12
	2.6 Toxicity of Heavy Metals	14
	2.7 Biochemistry of Molydenum	
	2.7.1 Molybdenum in Plant and Soil Microbial Symbiotic Interaction	16
	2.8. Molydenum in Mammals	17
	2.9 Molybdenum Toxicity	18
	2.10 Molybdenum as a Pollutant	19
	2.11 Enzymatic and Microbial Action on Molybdenum	20
	2.12 *Serratia marcescens*	23
	2.13 Bioremediation as a Alternative Technologies for Pollution Treatment	24
	2.13.1 Bioremediation of Molybdenum	26
3	**MATERIALS AND METHODS**	
	3.1 Chemicals and Equipments	27
	3.2 Isolation of Molybdate-reducing Bacterium, Maintenance and Phenotype Identification	28
	3.3 16s rDNA Gene Sequencing	30
	3.4 Phylogenetic Analysis	31
	3.5 Preparation of Crude Enzyme	31
	3.6 Molybdenum-reducing Enzyme Assay	32
	3.7 Quantification of Molybdenum Blue	33

3.8 Effect of Various Parameters on Mo-reducing Activity from *Serratia marcescens* Strain Dr.Y5 33
3.9 Effect of Nutritional of Bacteria Growth Condition
3.9.1 Effects of Various Carbon Sources 34
3.9.2 Effects of Various Nitrogen Sources 35
3.9.3 Effects of Various pH 35
3.9.4 Effects of Various Temperatures 36
3.9.5 Effect of Various Molybdate Concentrations 36
3.9.6 Effects of Yeast Extract Concentrations 36
3.10 Partial Purification of Mo-reducing Enzyme
3.10.1 Preparation of Crude Mo-reducing Enzyme Fractions 37
3.10.2 Ion-exchange Chromatography using Mono-Q™ Strong-Anion Exchanger 38
3.10.3 Gel Filtration on Zorbax GF-250 40
3.10.4 Gel Electrophoresis, Non-denaturing and Denaturing SDS-PAGE 41
3.10.5 Gel Staining and Destaining 44
3.11 Optimization of pH for Enzyme Activity 44
3.12 Optimization of Temperature for Enzyme Activity 45
3.13 Determination of Enzyme Kinetic Constants 46
3.13.1 Electron Donors (NADH and NADPH) 47
3.13.2 Electron Acceptor (molybdophosphate) 48
3.14 Protein Assay 49

4 RESULTS AND DISCUSSIONS
4.1 Screening test 50
4.2 Molybdenum blue production and molybdate reductase activity of selected bacteria 51
4.2.1 Morphological and Characteristic of *Serratia* Strain Dr.Y5 52
4.3 Biochemical Test of *Serratia* Strain Dr.Y5 54
4.4 16S rRNA Analysis 55
4.5 Polymerase Chain Reaction (PCR) 55
4.6 Identification of the Isolate 56
4.7 Optimization of Cellular Mo-reduction Capabilities 59
4.8 Molybdate reduction and characterization studies
4.8.1 Carbon Source for Molybdate reduction 59
4.8.2 Nitrogen Source for Molybdate reduction 61
4.8.3 Optimization of pH 64
4.8.4 The Effect of Temperature on Molybdate Reductase Activity 66
4.8.5 Effect of Molybdate and Phosphate Concentrations on Molybdenum 68
4.8.6 The Effect of Yeast Extract on Enzyme Activities 70
4.9 Absorption Spectra of Molybdenum Blue Produced by *Serratia* Strain Dr.Y5 Bacterium 72
4.10 Molybdenum Blue Production and pH Profile during Molybdate Reduction 73
4.11 Purification of Molybdenum-Reducing Enzyme 74
4.11.1 : Purification Analysis of Phosphomolybdate

Reductase 79
4.11.2 Gel Electrophoresis 80
4.12 Phosphomolybdate reductase enzyme characterization. 82
4.12.1 Optimum pH 82
4.12.2 Optimum Temperature 83
4.12.3 Kinetic Constants of the Phosphomolybdate Reductase 86

5 CONCLUSIONS 94

REFERENCES 95
APPENDICES 104
BIODATA OF THE AUTHOR 113

LIST OF TABLES

Table		Page
1	Mo-reducing Capacity of Bacterial Isolates in Soils	51
2	Amount of Molybdenum Blue Produced from 24-hours Bacteria Cultures.	52
3	Partial Purification Scheme of Mo-reducing Enzyme from *Serratia* marcescens Strain Dr.Y5.	80
4	Summarization of Results Between Michaelis-Menten and Lineweaver-Burk	87

LIST OF FIGURES

Figure		Page
1	Future potential sites for molybdenum mining in Peninsular Malaysia (Mining industries statistics,-1999).	7
2	Structure of molybdenum-blue (Mo-blue). It is formed by 12 tetrahedral MoO_4^{2-} and one phosphate (PO_4^{3-}) ion.	8
3	A schematic presentation of the mechanism of molybdate reduction to Mo- blue by EC 48 (modified from Ghani *et al.,* 1993; Ariff *et al*., *1997*.	22
4	A schematic presentation of the mechanism of molybdate reduction to molybdenum blue (Shukor *et al*., 2000)	22
5	Mono Q attached to an Agilent 1100 series HPLC with fraction collector filled with ice.	40
6	Colonies of *Serratia* sp. strain Dr.Y5 on nutrient agar (A) and low phosphate media (B). Strain Dr.Y5 grew with white colonies on nutrient agar and appeared as blue colonies on low phosphate media.	53
7	Strain Dr.Y5 stained with gram staining.	54
8	Agarose gel electrophopresis. Lane M: GeneRuler 1 kb DNA Ladder markers in bp; Lane PCR product: PCR product (1500 bp, indicated by arrow).	56
9	Neighbour-joining method cladogram showing phylogenetic relationship between Strain Dr.Y5 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rDNA sequences. The numbers at branching points refer to bootstrap values, based on 1000 re-samplings. The accession number for each bacterium is indicated in brackets. The branch lengths in the cladogram are not to scale.	58
10	Cell growth (□) and molybdate reduction (■) using various carbon sources at the final concentration of 0.2% (w/v). Error bars represent the standard error of the mean between three determination.	60
11	The effect of sucrose concentrations on molybdate reduction. Error bars represent the standard error of the mean between three determinations.	61

12	Cell growth (□) and molybdate reduction (■) using various nitrogen sources. Error bars represent the standard deviations between three determinations	63
13	The effect of ammonium sulfate concentrations on molybdate reduction. Error bars represent the standard error of the mean between three determinations	64
14	Bell-shape curve of pH profile of molybdenum blue production by *Serratia* strain Dr.Y5 bacterium. The error bars represent mean standard deviation for three replicates. .	66
15	The effect of temperature on molybdate reduction. Error bars represent the standard error of the mean between three determinations.	67
16	The effect of molybdate concentrations on molybdate reduction. Error bars represent the standard error of the mean between three determinations.	69
17	The effect of phosphate concentrations in the media on molybdate reduction by bacterium strain. The media was adjusted to pH 7.0. Error bars represent the standard error of the mean between three determinations.	70
18	The effect of yeast extract concentrations on molybdate reduction. Error bars represent the standard error of the mean between three determinations.	71
19	Scanning spectra of molybdenum blue produced by *Serratia* strain Dr.Y5 bacterium.	72
20	Changes in pH (○) of the media during the course of molybdate reduction (●) by Strain Dr.Y5. Error bars represent the standard error of the mean between three determinations.	74
21	Ion exchange of Mo-reducing enzyme on Mono Q on an Agilent 1100 series.	76
22	Second Ion exchange of Mo-reducing enzyme on Mono Q on an Agilent 1100 series.	77
23	Gel filtration of Mo-reducing enzyme on Zorbax GFC-250 on an Agilent 1100 series with a 200 µl sample loop.	78
24	Non-denaturing SDS-PAGE of purified and crude fraction of Mo-reducing enzyme.	81
25	Denaturing SDS-PAGE of purified and crude fraction of Mo-reducing enzyme.	81

26	Activity of Phosphomolybdate reductase at a range of pH from pH 3.0 to 8.5. The error bars represent mean ± standard deviation for three replicates.	83
27	Activity of Phosphomolybdate reductase at a range of temperature from 20 to 60 °C. The error bars represent mean ± standard deviation for three replicates.	85
28	Effects of temperature on stability of the phosphomolybdate reductase for different time. 25 °C (∗), 34 °C (x), 40 °C (▲), 54 °C (■), and 70 °C (♦). The error bars represent mean ± standard deviation for three replicates.	85
29	The change initial velocity rate of reaction at the different initial substrate concentration, [NADH] in Michaelis-Menten plot. The concentration of the e^- acceptor, 12-MP, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	88
30	Lineweaver-Burk plot of reciprocal initial velocity at the different reciprocal e^- donor substrate; [NADH]. The concentration of the e^- acceptor, 12-MP was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	89
31	The change initial velocity rate of reaction at the different initial substrate concentration, [NADPH] in Michaelis-Menten plot. The concentration of the e^- acceptor, 12-MP, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	90
32	Lineweaver-Burk plot of reciprocal initial velocity at the different reciprocal e^- donor substrate; NADPH. The concentration of the e^- acceptor, 12-MP was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	91
33	The change initial velocity rate of reaction at the different initial substrate concentration, substrate[12-MP] in Michaelis-Menten plot. The concentration of the e^- donor, NADH, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	92
34	Lineweaver-Burk plot of reciprocal initial velocity at the different reciprocal e^- acceptor substrates, for 12-MP. The concentration of the e^- donor, NADH, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.	93

LIST OF ABBREVIATIONS

%	percent
$(NH_4)_2SO_4$	Ammonium sulphate
°C	degree Celsius
µl	microlitre
µM	micromolar
12-MP	Twelve-Molybdophosphate
Ag	Argentum
As	Asenic
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
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	calvin
kb	kilobase
kDa	kilodalton
Kg	kilogram
K_m	Michaelis-Menten Constant
L	Liter
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
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	Zinc

CHAPTER 1

INTRODUCTION

The term heavy metals refers to all inorganic metallic elements that have characteristics luster or shine and show poisonous effect and highly toxic in small amounts of exposure. Globally, molybdenum pollution has been documented. In the Tokyo Bay and the Black Sea, molybdenum level is in the range of hundreds of ppm (parts per million) (Tsubaki & Irukayama, 1977). In Tyrol, Austria, molybdenum pollution is caused by industrial effluents and has contaminated large pasture areas, reaching as high as 200 ppm causing scouring in ruminants (Neunhäuserer *et al*., 2001). Its uses include super alloys, nickel base alloys, lubricants, chemicals, glass workings, electronics and many other applications. It is from these industries that molybdenum can be found in the discharged effluents (Shineldecker *et al*., 1992).

In Malaysia, molybdenum in the form of molybdenite is mined as a by-product of copper mining from the early 70's until the late 90's (Kosaka & Wakita, 1978). There are several cases of pollution in the locality caused by accidental leakage of pipe-carrying metal system and also leaching of the metals from the mining site causing contamination of a paddy field and the Ranau River (Shin, 2000). Another potential and perhaps undocumented source of molybdenum pollution In Malaysia is from scheduled waste. A survey by the Malaysian Department of Environment in 2001 showed that 10.4% of the 420,000 tones of scheduled wastes in the form of sludge generated in 2001 contained heavy metals including molybdenum. Molybdate bioremediation using

indigenous microbe from contaminated site have been demonstrated and have shown positive results (Neunhäuserer *et al*., 2001). Due to this, potential local molybdenum bioremediation system for polluted sites must be prepared in advance. Hence there is a need to isolate and characterize more local molybdate-reducing bacterium for the purpose of bioremediation and fundamental studies to better understand the metal reduction aspect.

There are a total of 65 metallic elements. Common to all heavy metals is that they are considered to be toxic and some are unneeded to organisms for their nutrient uptake. This statement is also partly untrue because it excludes the nutrient metals, which are important in small amounts. The biological implication of metals in the environment takes on new dimension now that the nature of the fossil fuels being used by most civilized countries is changing. Unfortunately, very few records on the environmental concentration of "heavy metals" exist for the period when coal was the principal source of energy production. It is important to know more about the physiological effects of the heavy metals that coal may liberate into the environment (Bruins, 2000).

The effects of chemical pollutants on populations and communities are affected by physical, chemical, and biological processes in the environment. Evaluation of these effects eventually requires as assessment of the toxicity of residues to species existing in the total environment. The direct effects of chemicals in the environment are those occurring immediately subsequent to their first introduction into specific ecosystem, resulting in the exposure of living individuals to lethal toxic effects which can measure in a short-term laboratory bioassay.

The term ‘trace element “has been suggested as a substitute for heavy metals), but it’s still inaccurate definition because it excludes some metals such as Aluminium (Al) and Iron (Fe). All of these heavy metals showed their beneficial characteristics, especially for human development. Since the early age, people discovered and noticed that this compound can help them to make building and weapons for their survival. Nowadays, people are much smarter because of the new technology and discovered a lot of its other function, not of its other function, not only for the physical civilization but also for them (Alloway, 1990).

As a result, they knows that for human and any other organism’s growth, some heavy metals such as Cobalt (Co), copper (Cu), Manganese (Mn), Nickel (Ni), Selenium (Se) and Zinc (Zn) are essential at lower concentration. But unfortunately, it also can produce toxic effect if taken too much at higher level (Hare, 1992). Besides that, there are some heavy metals that showed no benefit at all for human health such as mercury (Hg) and cadmium (Cd). These metals play no role in metabolism, as no enzyme has been identified which purposely requires cadmium or mercury as a cofactor. These two metals are, however, extremely harmful to life and have been involved in historic poisoning episodes of human population and wildlife resulting from ingestion of contaminated food and prey. Mercury is considered, as the most toxic heavy metals exist ever.

The potential of molybdate-reducing microbes as a bioremediation tool was suggested by Ghani *et al.* in 1993 due to the ability of the microbe to immobilize soluble molybdate into colloidal low-solubility molybdenum blue. Since this work was

published little works have been carried out to purify the Mo-reducing enzyme activity and to screen for better reducers; the latter is an important prerequisite for efficient bioremediation. Purification of the Mo-reducing enzyme in the heterotrophs have not been successful to date and this has hampered the basic understanding of how the microbes reduces molybdate in toxic stage to molybdenum blue that is non toxic stage. This work has several scopes; the important ones are the search for better molybdate reducers than EC 48 and the purification and characterization of the Mo-reducing enzyme. In order to fulfill this scope, several objectives must be met. They are;

1. To isolate, characterize and identify Mo-reducing bacterium from local soils
2. To optimize growth conditions of the Mo-reducing bacterium prior to large scale growth for the purposes of maximizing enzyme synthesis
3. To purify and characterize the Mo-reducing enzyme from this bacterium