UNIVERSITI PUTRA MALAYSIA

BIOREDUCTION OF HEXAVALENT MOLYBDENUM TO MOLYBDENUM BLUE USING Serratia sp. MIE2 AND PURIFICATION OF MOLYBDENUM-REDUCING ENZYME

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By

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

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Chairman: Mohd Yunus Abd Shukor, PhD
Faculty: Biotechnology and Biomolecular Sciences

Molybdenum reduction is an old phenomenon that has received very low attention compare to other well-known and extensively studied metals such as chromium, mercury and lead. Molybdenum has long been known to be toxic to ruminants and not toxic to other organisms. However, more recently it has been increasingly reported that molybdenum shows toxic effects to reproductive organs of fish, mouse and even humans at concentrations between 1 and 100 ppm. Hence its removal from the environment is highly sought after. The isolation of molybdenum reducing bacteria and the elucidation of the reducing mechanism will lead to an efficient bioremediation system. To fulfil this, a new Mo-reducing bacterium was isolated from an agriculture soil plot from Universiti Putra Malaysia. The isolate was tentatively identified as *Serratia* sp. MIE2 based on 16s rDNA molecular phylogeny. *Serratia* sp. MIE2 is a gram negative, oxidase and catalase positive bacterium. The molybdenum blue produced by *Serratia* sp. MIE2 exhibited a unique absorption spectrum with maximum peak at 865 nm and a shoulder at 700 nm. Dialysis tubing experiment showed that molybdate reduction by *Serratia* sp. MIE2 was an enzymatic process and not chemically mediated.

Characterization and optimization of molybdenum blue production by *Serratia* sp. MIE2 was carried out using one factor at a time (OFAT) and Response Surface Methodology (RSM). One factor at a time (OFAT) showed the optimum conditions supporting molybdate reduction occurred at pH 6.0, from 27 to 35 ^{o}C and 30-40 g/L sucrose as the carbon source or electron donor. The best nitrogen source was ammonium sulphate with an optimum concentration at 10 g/L. Moreover, the optimum concentrations of phosphate and molybdate were 2 and 10 mM, respectively. Molybdate reduction was maximized and optimized using response surface methodology (RSM) with optimum conditions occuring at 20 mM of molybdate, 25 g/L of sucrose, pH 6.25 and 3.95 mM of phosphate with molybdenum blue production increasing from an OFAT absorbance yield of 10.0 to higher than 20.0 as measured at 865 nm.

Modelling kinetic studies of *Serratia* sp. MIE2 using the optimum conditions obtained from the classical method (OFAT) show that the best model was Teissier followed by Luong, Aiba, Yano and Haldane with correlation coefficient, R^2 values of 0.994, 0.993, 0.992, 0.990 and 0.982, respectively. The calculated values of P_{max}, K_s and K_i of the best model were 0.89 µmole Molybdenum blue per hour, 5.84 mM and 32.23 mM respectively. Otherwise, modelling kinetics using the optimum condition obtained from RSM showed that the Luong model was the best model followed by Teissier, Aiba, Yano and Haldane with correlation coefficient, R^2 values of 0.999, 0.994, 0.993, 0.992 and 0.965, respectively. However, since Luong exhibited 4 kinetic constants while Teissier has only 3 constants, by default, Teissier model was chosen due to its mathematical simplicity. The calculated values of R^2, P_{max}, K_s and K_i of the best model, Teissier were 1.97 µmole Mo-blue per hour, 5.79 mM and 31.48 mM, respectively. Modelling kinetics showed the value of *P*max was increasing from 0.89 µmole Molybdenum blue per hour to 1.97 µmole molybdenum blue per hour indicating that molybdate reduction yield increase several fold after optimization using RSM.

Before purification process, preliminary studies such as effect of storage and chromatographic stabilities, effects of restorative and inhibitive agents were carried out to minimise denaturation and to maximise yield of purified enzyme. The buffer use during storage and purification process was Tris-HCl at pH 7.0. Mo-reducing enzyme was stable when stored at -80^{o}C for both 24 hours and one month followed by storage on ice (0^{o}C). Temperature stability study showed that the enzyme was most stable at 25^{o}C followed by 40^{o}C with complete lost of activity at 60 and 40 minutes of incubation at 54 and 70^{o}C. EGTA or (ethylene glycol tetraacetic acid), EDTA, Triton X-100, DBS and SDS decrease 50% activity of enzyme at concentration 0.1mM, 0.1mM, 0.1%, 0.1%, and 0.1%, respectively. DTT could restore the Mo-reducing enzyme activity of up to 100% at the maximum concentration of 5 mM for DTT and 0.5 mM for β-mercaptoethanol. Effects of cofactor suggest that nickel might be an important cofactor for the enzyme. Heavy metals such as mercury and zinc effect strongly inhibited the Mo-reducing enzyme. The coenzyme such as FMN and FAD were able to restore Mo-reducing enzyme activity. Mo-reducing enzyme was not inhibited by respiratory inhibitors, therefore, the electron transport chain of this bacterium is not the site of molybdate reduction.

Purification of the Mo-reducing enzyme was done using ammonium sulphate precipitation, gel filtration on Zorbax GF-250 and Zorbax GF-450 with a 20.8 purification fold. The molecular mass was estimated to be 100 kDa by SDS-polyacrylamide gel electrophoresis and the enzyme was monomeric. Mo-reducing enzyme showed maximum activity at 35^{o}C and pH 5. The enzyme was assayed using NADH as the electron donor with the maximum initial velocity, V_{max} of 16.18 nmole molybdenum blue/min/mg protein and a Michaelis constant, K_m at 0.89 mM. The optimum concentration of phosphomolybdate (electron acceptor substrate) was 10 mM, with a V_{max} of 6.89 nmole molybdenum blue/min/mg protein (NADH as electron donor at saturated concentrations) and K_m of 6.02 mM. Identification of pure enzyme using MALDI-TOF showed only peptide DNAATRSEAMSLIHGR shows similarity to 35% to nitrile oxidoreductase and GTP cyclohydrolase I. The low similarity value prohibited further analysis to be carried out. Thus, the enzyme is assigned as hypothetical protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENURUNAN HEKSAVALEN MOLIBDENUM KEPADA MOLIBDENUM BIRU OLEH *Serratia* sp. MIE2 DAN PENULENAN ENZIM PENURUN MOLIBDENUM

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Penurunan molibdenum adalah suatu fenomena yang kurang mendapat perhatian berbanding dengan logam lain-lain yang terkenal dan dikaji secara meluas seperti kromium, merkuri dan plumbum. Molibdenum telah lama diketahui adalah toksik kepada ruminan dan tidak toksik kepada organisma lain. Walau bagaimanapun, baru-baru ini terdapat laporan yang menujukan bahawa molibdenum menunjukkan kesan toksik kepada organ-organ pembiakan ikan, tikus dan juga manusia pada kepekatan antara 1 dan 100 ppm. Oleh itu penyingkirannya daripada alam sekitar mendapat perhatian yang tinggi. Pengasingan bakteria penurun molibdenum dan pengetahuan mengenai mekanisme penurunan akan membawa kepada sistem biopemulihan yang cekap. Satu bakteria Mo-penurun telah diasingkan daripada plot tanah pertanian dari Universiti Putra Malaysia. Isolat ini secara sementara ini dikenalpasti sebagai *Serratia* sp. MIE2 berdasarkan analisa filogenetik molekul 16s rDNA. *Serratia* sp. MIE2 adalah gram negatif, oksidase dan katalase positif. Molibdenum biru dihasilkan oleh *Serratia* sp. MIE2 mempamerkan spektrum penyerapan unik dengan puncak maksimum pada 865 nm dan bahu di 700nm. Eksperimen tiub dialisis menunjukkan bahawa penurunan molibdenum oleh *Serratia* sp. MIE2 adalah suatu proses melibatkan enzim dan bukan secara kimia.

Pencirian dan pengoptimuman pengeluaran molybdenum biru oleh *Serratia* sp. MIE2 telah dijalankan dengan menggunakan satu-faktor-pada-satu-masa (OFAT) dan Kaedah Metodologi Permukaan (RSM). Kaedah satu-faktor-pada-satu-masa (OFAT) menunjukkan keadaan optimum menyokong pengurangan molibdenum berlaku pada pH 6.0, 27-35^{o}C dan 30-40 g/L sukrosa sebagai sumber karbon atau elektron penderma. Sumber terbaik nitrogen adalah ammonium sulfat dengan kepekatan optimum pada 10 g/L. Selain itu, kepekatan optimum fosfat dan molibdat adalah 2 dan 10 mM, masing-masing. Pengurangan molibdenum telah dimaksimumkan dan dioptimumkan menggunakan Kaedah Metodologi Permukaan (RSM) dengan keadaan penghasilan molibdenum biru optimum berlaku pada 20 mM molibdat, 25 g/L sukrosa, pH 6.25 dan 3.95 mM fosfat dengan pengeluaran molibdenum biru meningkatkan dari serapan 10.0 menggunakan kaedah OFAT kepada serapan 20.0 pada jarak gelombang 865 nm menggunakan kaedah RSM.

Model kajian kinetik penurunan oleh *Serratia* sp. MIE2 menggunakan kondisi optimum yang diperolehi daripada kaedah klasik (OFAT) menunjukkan bahawa model yang terbaik ialah Teissier diikuti dengan Luong, Aiba , Yano dan Haldane dengan nilai R^2 0.994, 0.993, 0.992, 0.990 dan 0.982, masing-masing. Nilai P_{max}, K_s dan K_i model yang terbaik adalah 0.89 µmol Mo-biru per jam, 5.84 mM dan 32.23 mM, masing-masing. Kinetik pemodelan menggunakan keadaan optima yang diperolehi daripada kaedah RSM pula menunjukkan bahawa model Luong adalah model terbaik diikuti oleh Teissier, Aiba, Yano dan Haldane dengan, nilai R^2 0.999, 0.994, 0.993, 0.992 dan 0.965, masing-masing. Walau bagaimanapun, sejak Luong menggunakan 4 pemalar kinetik manakala Teissier hanya menggunakan 3 pemalar, maka secara tetapan model Teissier dipilih berdasarkan kesederhanaan matematik. Nilai P_{max}, K_s dan K_i model yang terbaik adalah 1.97 µmole Mo-biru sejam, 5.79 mM dan 31.48 mM, masing-masing. Kinetik pemodelan Teissier menunjukkan nilai P_{max} telah meningkat daripada 0.89 µmol molibdenum biru/jam kepada 1.97 µmole molibdenum biru per jam yang menunjukkan bahawa pengurangan molibdenum meningkatkan hasil beberapa kali ganda selepas pengoptimuman menggunakan kaedah RSM.

Sebelum proses penulenan, kajian awal seperti kesan penyimpanan dan pengstabilan semasa kromatografi, kesan agen pemulihan enzim dan agen perencat telah dijalankan untuk mengurangkan denaturasi dan memaksimumkan hasil enzim yang akan ditulenkan. pH optima untuk tujuan penyimpanan dan proses kromatografi adalah Tris-HCl pada pH 7. Mo-penurun enzim adalah stabil apabila disimpan pada -80ºC samada pada 24 jam atau satu bulan dan diikuti dengan penyimpanan di dalam ais (0ºC). Kajian kestabilan suhu menunjukkan bahawa enzim adalah yang paling stabil pada 25ºC diikuti dengan 40ºC dengan kehilangan aktiviti berlaku pada 60 dan 40 minit pengeraman pada suhu 54 dan 70ºC, masing-masing. EGTA (etilena glikol asid tetraasetik), EDTA (etilena diamina asid tetraasetik), Triton X-100, DBS (dodesil benzena sulfat) dan SDS (sodium dodesil sulfat) mengurangkan aktiviti enzim sebanyak 50% pada kepekatan 0.1mM, 0.1 mM , 0.1 % , 0.1 %, dan 0.1 % masing-masing. DTT boleh memulihkan aktiviti enzim penurun molibdenum sehingga 100 % pada kepekatan maksimum 5 mM untuk DTT dan 0.5 mM untuk 2-merkaptoetanol. Kesan kofaktor menunjukkan bahawa nikel mungkin menjadi kofaktor penting bagi enzim ini. Logam berat seperti merkuri dan zink merencat aktiviti enzim penurun molibdenum. Koenzim seperti FMN (flavin mononukleotida) dan FAD (flavin adenina dinukleotida) dapat memulihkan aktiviti Mo-penurun enzim. Aktiviti enzim penurun molibdenum tidak direncat oleh perencat respirasi, oleh itu, rantaian pengangkutan elektron daripada bakteria ini bukan merupakan tapak aktiviti penurunan molibdenum.

Penulenan enzim penurun molibdenum telah dilakukan dengan menggunakan fraksinasi ammonium sulfat, gel penapisan menggunakan Zorbax GF-250 dan Zorbax G-450 dengan pekali penulenan sebanyak 20.8. Jisim molekul enzim penurun molibdenum dianggarkan 100 kDa menggunakan kaedah gel elektroforesis-SDS dan enzim penurun molibdenum adalah monomerik. Enzim penurun molibdenum menunjukkan aktiviti maksimum pada suhu 35ºC dan pH 5. Enzim ini telah diasai menggunakan NADH sebagai penderma elektron dengan halaju awal maksimum, V_{max} adalah 16.18 nmole molibdenum biru /min/ mg protein dan pemalar Michaelis,

K_m adalah 0.89 mM. Kepekatan optimum fosfomolibdate (elektron penerima substrat) adalah 10 mM , dengan V_{max} adalah 6.89 nmole molibdenum biru/min/mg protein (NADH sebagai penderma elektron pada kepekatan tepu) dan K_m adalah 6.02 mM. Pengenalan enzim tulen menggunakan kaedah MALDI-TOF menunjukan hanya peptide DNAATRSEAMSLIHGR mempunyai persamaan 35% kepada nitril oksidoreduktase and GTP siklohidrolase 1. Persamaan yang rendah menghalang analisis yang seterusnya. Oleh itu enzim ini dinamakan protein hipotetikal.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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%	percentage
EDTA	ethylene diamine tetraacetic acid
kDa	kiloDalton
M	molarity
min	minute
mM	milimolar
°C	degree Celcius
SDS	sodium dodecyl sulfate
U	unit
µg	microgram
UV	ultraviolet
Abs	Absorbance
et al.	and all
g	gram
HCl	hydrochloric acid
mg	Milligram
mL	milliliter
L	liter

uL	microliter
w	weight
mg/ L	milligram perliter
DNA	Deoxyribonucleic acid
g/cm^3	gram per centimeter cube
g/L	gram per volume
V/V	volume per volume
HPLC	High performance liquid chromatography
MW	molecular weight
AAS	Atomic absorption spectrometry
mg/L	milligram per litre
µg/L	microgram per litre
WHO	World Health Organization
EPA	Environmental Protection Agency
SEM	standard error of the mean
MPL	Maximum Permissible Limit
DOE	Department of Environment
OD	Optical density
n.d	not detected

N/A	not available
ppm	parts per million
OFAT	One Factor at a Time
RSM	Response Surface Methodology
A	Absorbance
Mo	Molybdenum
HP	Hypothetical Protein
AAN	Artificial neural Network

CHAPTER 1

1.0 INTRODUCTION

Heavy metals pollution is a silent threat that has affected water bodies and soils all around the world (Rajkumar et al., 2012; Shukor et al., 2009a; Zakaria et al., 2007; Fadzilah et al., 2014; Sany et al., 2013). There has been a growing concern over public health by heavy metals contamination (Chen et al., 2013; Wee et al., 2014). Molybdenum is one of the essential heavy metals that are required at trace amount and toxic at certain concentration (Othman et al., 2013; Halmi et al., 2013; Yamaguchi et al., 2007; Meeker et al., 2008). Hexavalent molybdenum (Mo^{6+}) exhibit toxic properties due to it solubility in water compared to molybdenum blue which is insoluble in water, thus exhibiting nontoxic properties and limited environmental disruption (Raab & Feldmann, 2003; Lloyd, 2003).

Molybdenum has many important functions in various applications. Molybdenum is a valuable alloying agent that inhibits corrosion in water-base hydraulic systems and automobile engine anti-freeze (Ilevbare & Burstein, 2001). Molybdenum replaces chromium for inhibition of corrosion in mild steel over a wide range of pH (Twite &Bierwagen, 1998). Molybdenum is used due to its low toxicity and is a less aggressive oxidant towards organic additives (Philip, 1992). Another common use of molybdenum is as lubricant in the form of molybdenum disulphide (Lansdown, 1999).

The wide application of molybdenum in industry has resulted in several water pollution cases all around the world such as in the Tokyo Bay and the Black Sea, Japan (Davis, 1991) and Tyrol in Austria (Neunhäuserer et al., 2001), where molybdenum level reaches in the hundreds of ppm. Poland is the latest case where molybdenum reached as high as 10 ppm in soil in Silesian Upland (%XUHü HW DO 2013). In Malaysia, molybdenum is mined as a byproduct of copper and molybdenum mining area in Sabah and there have been episodic cases of pollution in the surrounding area (Yong, 2000).

Molybdenum is very toxic to ruminants with levels as low as several parts per million causing scouring and even deaths (Greenwood and Earnshaw, 1984; Stojek, 2013). It was discovered that molybdenum shows its toxicity by inhibiting spermatogenesis in catfish and mice at levels as low as several parts per million (Yamaguchi et al., 2007; Zhai et al., 2013; Bi et al., 2013; Zhang et al., 2013). This new findings would increase molybdenum exposure as a toxic heavy metals similar to chromium and would increase the number of works on its removal from soil and water bodies. In the past decades researchers have focused on bioremediation as an environmental friendly and low cost method to solve this problem.

Bioremediation is one of the ways to remove toxic metals from the environment (Sar et al., 2013). A variety of molybdenum reducing bacteria has been reported with all

of them required a semi-aerobic condition for maximal production of molybdenum blue (Campbell et al., 1985; Ghani et al., 1993; Shukor et al., 2008; Shukor et al., 2009c; Shukor et al., 2009d; Abo-Shakeer et al., 2013; Shukor et al., 2009a; Shukor et al., 2010a; Ahmad et al., 2013; Shukor et al., 2009e; Shukor et al., 2010b; Othman et al., 2013.). According to Levine, Molybdate reduction was first reported in 1896 by Capaldi and Proskauer (Levine, 1925; Capaldi & Proskauer, 1896). Since then, many more reducers have been isolated (Ghani et al., 1993; Shukor et al., 2008; Shukor et al., 2009a-2009d; Shukor et al., 2010a-2010b). The first successful molybdenum remediation was carried out on an agricultural soil contaminated with molybdenum in Tyrol, Autria. Cows grazing on this soil showed signs of molybdenum toxicity or molybdenosis. The toxicity is actually a Cu deficiency, since Mo decreases Cu uptake in ruminants. The use phytoremediation and microbes from sewage and from the soil itself manages to immobilize the molybdenum into nonsoluble form ultimately reducing its toxicity (Neunhausserer et al., 2001).

Despite this, all of the molybdenum-reducing bacterium isolated so far is not from agricultural soil while molybdenum is particularly very toxic to ruminants. In addition, genetic and strain improvement of the Mo-reducing activity from potent Mo-reducing bacterium using biotechnology would enhance the remediation process. Previously, the first Mo-reducing enzyme was purified from *Serratia* sp. strain DrY5 (Shukor et al., 2014). However the yield of the purified enzyme was very low and prevents identification through sequencing process. To solve this problem, a novel Mo-reducing bacterium isolated from agricultural soil and screened for high Mo-reducing activity is needed. The identification, physiological and biochemical characterization of the isolated bacterium as well as the purification of the Mo-reducing enzyme will be carried out.

1.1 Thesis Objectives

Based on the problem statement and significant of the study, the following objectives are outlined:

1. To isolate and characterize a novel Mo-reducing bacterium from agriculture soil
2. To optimize Mo-blue production through one-factor-at-a±time (OFAT) and Response Surface Methodology (RSM)
3. To determine the kinetics of Mo-blue production in the bacterium before and after RSM
4. To determine the effect of storage pH and temperature, metabolic inhibitor, coenzyme and metal ions on Mo-blue production in the bacterium
5. To purify characterize and identify the Mo-reducing enzyme from the bacterium

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