UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION, MODELLING AND PURIFICATION OF A MOLYBDENUM-REDUCING ENZYME FROM A SEAWATER-TOLERANT BACTERIUM

MOHD FADHIL BIN ABDUL RAHMAN

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

December 2018

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DEDICATION

This thesis is dedicated to my parents, farmers, pioneers and fellow researchers. I dedicate this thesis to my beloved family, especially to my late father and friends whose moral support, prayers and encouragement have led me to finish this journey I started a long time ago that was filled with numerous setbacks and challenges.

Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairman: Assoc. Prof. Mohd Yunus Bin Abd. Shukor, PhD
Faculty : Biotechnology and Biomolecular Sciences

Molybdenum, which is an emerging pollutant is toxic to spermatogenesis in several animal model and to ruminants. In Malaysia, the source of molybdenum pollution is from the dumping sites of waste oil lubricant. Molybdenum can be reduced to molybdenum blue by bacteria and form the basis for bioremediation of molybdenum. A novel seawater-tolerant Mo-reducing bacterium has been isolated from Pantai Merchang, Terengganu 1. The bacterium was identified by molecular phylogenetic analysis as *Kluyvera* sp. strain UPM-FR1. The optimum conditions for Mo reduction by using One-Factor-at-a-Time (OFAT) method showed that sucrose at between 30 and 50 g/L was the best carbon source. Ammonium sulphate at 10 g/L was the best nitrogen source. The optimal initial pH and temperature were pH 6.0 and from 27 to 35°C, respectively. The optimum phosphate concentration was 2 mM and the optimum molybdate concentration was 10 mM. The Mo-reducing capacity was strongly inhibited by the heavy metal mercury, followed by copper, silver and chromium. Optimization of Mo-reduction through Response Surface Method (RSM) begins with a pre-screening Plackett–Burman method which showed that molybdate (A), phosphate (B), pH (C), incubation time (G), and pH and phosphate (BC) were the significant ($P<0.05$) parameters influencing Mo-blue production. Surface plots show the optimum concentration of molybdate and phosphate occurred at 30 mM and 3.95 mM, respectively, while the optimum concentrations of molybdate concentration and pH occurred at 30 mM and 6.8, respectively. Surface plots also indicated that the optimum concentrations of molybdate concentration and time occurred at 30 mM and 36 h, respectively while the optimum concentrations of phosphate concentration and pH occurred at 3.95 mM and 6.25, respectively. The best solution satisfying the optimality goal was molybdenum at 34.89 mM, phosphate 4.04 mM, pH 6.81 and incubation time of 34.92 h, with the overall Mo-blue production of 12.6 compared to OFAT at 6.7 absorbance value. Modelling studies showed that the best primary model was modified Logistics model, while secondary modelling showed that the Luong model was the best with the calculated value for the Luong's constants, which were q_{max}, K_s, S_m, and n that

were 2.22±5 μmole Mo-blue hr^{-1}, 23.14±7.45 mM, 70.34±1.67 mM and 1.34±2.21, respectively. On the other hand, the best kinetic model for the effect of molybdate on molybdenum bioremoval rate using the dialysis tubing method was the Yano model. The calculated value for the Yano's constants, which are q_{max}, K_s, K_i, and n were 2.454 ±2.12 μmole Mo-blue hr^{-1}, 24.18±4.23 mM, 87.82±8.19 mM and 0.258±0.121, respectively. Prior to the purification of the Mo-reducing enzyme, initial experiments shows that the Mo-reducing enzyme was stable in between pH 7 and 7.5. Both DTT and β-mercaptoethanol are capable to restore the Mo-reducing enzyme activity. The results also show that none of the metabolic inhibitors inhibited the Mo-reducing enzyme from this bacterium suggesting that the electron transport system is not the site of Mo reduction to Mo-blue. The crude enzyme extract was fractionated with ammonium sulphate (40-50% fraction) and gel filtration on GF-250 yielding a 19.8 fold purification. The Mo-reducing enzyme is estimated to be at 100 kDa. The purified enzyme shows a maximum activity at pH 5.5 and a maximum activity in between 30 and 35°C. The V_{max} for NADH and NADPH were at 8.6±0.25 (± standard error value) and 8.01±0.51 nmole Mo blue/min/mg protein, respectively. The apparent K_m for NADH and NADPH were 1.55±0.16 mM and 4.15±0.64, respectively. The V_{max} and K_m for LPPM were 8.755±0.26 nmole Mo blue/min/mg protein and 3.37±0.35 mM, respectively. To conclude, all of the objectives in this thesis have been accomplished and a novel Mo-reducing bacterium with a novel seawater tolerant capacity had been isolated and characterized. This bacterium will be very useful in remediating molybdenum pollution in the coastal areas or in seawater using the dialysis tubing as an entrapment tool.

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

PENULENAN DAN PENCIRIAN ENZIM PENURUN MOLIBDENUM DARIPADA BAKTERIA YANG RINTANG DENGAN AIR LAUT DARI KAWASAN YANG TERCEMAR DENGAN MOLIBDENUM

Oleh

MOHD FADHIL BIN ABDUL RAHMAN

Disember 2018

Pengerusi : Prof Madya Mohd Yunus Bin Abd. Shukor, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Molybdenum yang merupakan bahan pencemar yang baru muncul adalah toksik kepada spermatogenesis kepada sesetengah haiwan dan ruminan. Di Malaysia pencemaran molybdenum adalah dari sumber kawasan pembuangan minyak enjin yang telah digunakan. Molybdenum boleh diturunkan kepada molybdenum biru oleh bakteria dan menjadi asas kepada bioremidasi molybdenum. Bakteria penurun molybdenum yang toleransi dengan air laut terbaru telah berjaya dipencilkan dari pantai merchang Terengganu 1. Bakteria ini telah dikenalpasti sebagai Kluyvera sp. strain UPM-FR1 melalui analisis molecular phylogenetik Keadaan optimum untuk penurun Mo dengan menggunakan teknik satu faktor pada satu masa menunjukkan sumber karbon terbaik adalah di antara 30-50 g/L. Ammonium Sulphate pada 10 g/L adalah sumber nitrogen terbaik. Optimum pH dan suhu adalah pada pH 6.0 dan di antara 27°C ke 35°C. Kepekatan optimum phosphate adalah 2mM dan kepekatan optimum molybdate adalah 10mM. Kapasiti penurun Mo adalah direncat kuat oleh logam berat merkuri diikuti perak dan kromium. Pengoptimum penurunan Mo melalui Kaedah Tindakbalas Permukaan (RSM) dimulai dari pemeriksaan kaedah Plackett-Burnmen yang menunjukkan molybdenum(A), phosphate (B), pH (C), Masa inkubasi (G), dan pH dan phosphate (BC) adalah signifikan (<0.05). Plot permukaan menunjukkan kepekatan optimum bagi molybdate dan phosphate berlaku pada 30mM dan 3,95mM , manakala kepekatan optimum bagi kepekatan molybdate dan pH berlaku pada 30mM dan 6.8. Plot permukaan juga menunjukkan kepekatan optimum bagi kepekatan molybdate dan masa berlaku pada 30mM dan 36 J., manakala kepekatan optimum bagi kepekatan phosphate dan pH berlaku pada 3.95mM dan 6.25. Penyelesaian terbaik adalah pada tahap optimum bagi molybdenum pada 34.89mM, phosphate 4.04mM, pH 6.81 dan masa inkubasi adalah 34.92J, dengan secara keseluruhan penghasilan Mo-biru pada 12.6 berbanding dengan OFAT 6.7. Pembelajaran pembentukan model menunjukkan model utama adalah Logistik terubahsuai, manakala model kedua menunjukkan model Luong adalah terbaik berdasarkan nilai kiraan Luong yang tetap iatu qmax, Ks, Sm, dan n iaitu 2.22±5 μmole Mo-blue jam-1, 23.14±7.45 mM, 70.34±1.67 mM dan 1.34±2.21. Pada masa yang sama model kinetik terbaik bagi kesan molybdate pada nilai penyingkiran molybdate

menggunakan kaedah tiub dialysis adalah model Yano. Nilai kiraan bagi ketetapan Yano adalah qmax, Ks, Ki, dan n were 2.454 ±2.12 μmole Mo-blue jam-1, 24.18±4.23 mM, 87.82±8.19 mM dan 0.258±0.121 Bagi penulenan enzum penurun Mo, hasil ujikaji menunjukkan enzim penurun Mo stabil diantara pH 7 dan 7.5. Kedua-dua DTT dan β-mercaptoethanol adalah mampu mengekallkan aktiviti enzim penurun Mo. Hasil ujikaji juga menunjukkan tiada perencat metabolik merencat enzim penurun Mo dari bakteria ini menunjukkan bahawa sistem pengangkutan elektron bukan tapak penurunan mo-biru. Ekstrak enzim mentah difraksinasikan dengan ammonium sulfat (pecahan 40-50%) dan penurasan gel pada GF 250 menulenkan 19.8 kali ganda penulenan. Enzim penurun Mo dianggarkan pada 100kDa. Enzim yang ditulenkan menunjukkan aktiviti maksimum diantara 30 dan 35°C. Vmax untuk NADH dan NADPH pada 8.6 ± 0.25 dan 8.01 ± 0.51 nmol Mo/min/mg mg protein, Km ketara untuk NADH dan NADPH adalah masing-masing pada 1.55 ± 0.16 mM dan 4.15 ± 0.64. Vmax dan Km untuk LPPM adalah 8.755 ± 0.26 nmol protein Mo/min/mg protein dan 3.37 ± 0.35 mM masing-masing. Sebagai kesimpulan, semua objektif dalam tesis ini telah dicapai dan bakteria Mo-penurun novel dengan kapasiti yang rintang pada air laut telah berjaya dipencilkan dan dicirikan. Bakterium ini akan sangat berguna dalam memulihkan pencemaran molibdenum di kawasan pesisiran pantai atau di dalam air laut dengan menggunakan tiub dialisis sebagai alat penyekatgerak.

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Mohd Fadhil Abdul Rahman, 2018

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Mohd Yunus Abd. Shukor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairperson)

Siti Aqlima Ahmad, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Nur Adeela Yasid, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mohd Izuan Effendi Halmi, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Ahmad Razi Othman, PhD
Senior Lecturer
Faculty of Engineering and Built Environment
Universiti Kebangsaan Malaysia
(Member)

_____
ROBIAH BINTI YUNUS, PhD
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Name and Matric No.: Mohd Fadhil Bin Abdul Rahman, GS47947

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Name of Chairman
of Supervisory
Committee: Associate Professor
Dr. Mohd Yunus Shukor

Signature: _____
Name of Member
of Supervisory
Committee: Associate Professor
Dr. Siti Aqlima Ahmad

Signature: _____
Name of Member
of Supervisory
Committee: Dr. Nur Adeela Yasid

Signature: _____
Name of Member
of Supervisory
Committee: Dr. Mohd Izuan Effendi Halmi

Signature: _____
Name of Member
of Supervisory
Committee: Dr. Ahmad Razi Othman

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LIST OF ABBREVIATIONS

%	Percent
<	Less than
>	Greater than
ɤ	Gamma
α	Alpha
β	Beta
CCD	Central composite design
cm	Centimetre
Co	Cobalt
Cr	Chromium
Cu	Copper
Da	Dalton
DEAE	Diethylaminoethylamine
DTT	Dithiothretol
EDTA	Ethylene diamine tetra acetic acid
et al	and friends
Fe	Iron
FFD	Fractional factorial design
G	Gram
h	Hour
HPLC	High Performance Liquid Chromatography
kb	Kilo base
K_m	Michaelis-Menten constant
kg	Kilogram
L	Liter
LPMAS	Low Phosphate Molybdate Artificial Seawater
M	Molar
min	Minute
mL	Mililitre
Mo	Molybdenum
MSM	Minimal salt medium
MW	Molecular weight
NADH	Nicotinamide adenine-dinucleotide reduced
nm	Nanometer
ºC	Degree celcius
OFAT	One-factor-at-a-time
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonylflouride
RPM	Rotation per minute
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
µL	Microlitre
µm	Micrometre
µM	Micromolar
V_{max}	Maximum velocity

CHAPTER 1

INTRODUCTION

Our environment is currently being endangered by our own hands. As the growth of human's population is increasing, intensive industrialization along with urbanization and agriculture have caused much damages to the environment itself. In fact, overexploitation of natural resources as well as man ignorance towards the laws of nature make the problems become much worse (Shukor et al, 2006; Halmi *et al.,* 2016a). Pollution caused by hydrocarbons and metal ions have been on the increased over the years globally (Aftab *et al.,* 2013, 2016) and this is a big problem to be solved.

A range from acute to chronic toxicity cases within occupational and environmental high-exposure settings have been identified to be caused by toxic agents from metals and their compounds. Typically, heavy metals exist naturally in the environment. Since the pre-industrial times, the level of heavy metals in present years has increased greatly due to anthropogenic activities (Kaplan 2013; Meyer *et al.,* 2014; Ilyin *et al.,* 2015). Considerable and indiscriminate released of pollutants into the environment is due to the increasing number of population and industrialization activities (Soares and Soares 2012; Dixit *et al.,* 2015). Harmful effects towards human health and biota can be exerted when heavy metals levels reach beyond the critical loads (Alloway 1995; Meyer *et al.,* 2014; Ilyin *et al.,* 2015; Gupta *et al.,* 2016). In their elemental forms and different combinations, metals such arsenic, cadmium, chromium, cobalt, copper, lead, mercury, molybdenum, nickel, silver and zinc are known to be toxic (Rosca *et al.,* 2015) and they are also non-degradable (Sarubbo *et al.,* 2015). Thus, the accumulation of the metals within the food chain can lead to a serious threat towards the ecosystem (Li and Tao 2013) with regards of their carcinogenic and mutagenic properties (Singh and Prasad 2011). Pollution caused by heavy metals has become a global public health concern nowadays hence their removal from the environment is vital.

Molybdenum can be described as one of the important trace elements and acts as a micronutrient which is required as a co-factor for more than 50 enzymes (Zhai *et al.,* 2013; Wu *et al.,* 2014). In animal and plant physiology it helps in promoting the cellular function by catalyzing a variety of hydroxylation and redox transfer reactions (Pandey and Singh 2002). Previous studies have shown that molybdenum is an endocrine disruptor and affects the spermatogenesis in several animal models (Kargar *et al.,* 2011; Zhai *et al.,* 2013). With the broad distribution of molybdenum in the industrial manufacture of ceramics, glass and contact lens solution, metallurgical processes, lubricants, pigment, catalyst, electronic products and as color additives in cosmetics, the risks towards humans exposed to its toxicity also has arises (Pandey and Singh 2002; Zhai *et al.,* 2013). It has been reported that incremental level of molybdenum is discovered in the ground water around the mining areas of up to 0.2 mg/L which is above the WHO recommended limit of 0.07 mg/L in drinking water. For animals that have been in direct contact with molybdenum taken via drinking water or while foraging for plants, they are most likely to portray symptoms of hypocuprosis or suffer from molybdenosis over a long exposure period (Zhai *et al.,* 2013).

For over a century, microbial molybdate reduction to molybdenum blue (Mo-blue) is an unsolved puzzle among scientists. This phenomenon was only discovered and proved to be enzymatic in the early 2000s (Shukor et al., 2002). Previously, in bacterial molybdate reduction, the focus was centred towards isolating molybdenum reducers having higher Mo-blue production capacity need as a tool for bioremediation (Halmi *et al.,* 2013). However, as most polluted sites contained mixed contaminants from organic and inorganic origin, effective remediation ha thus become a complex endeavour. During the last five years, attention has shifted towards isolating microorganisms with multi-reduction and/or degrading potentials which could be used to remediate co-contaminated areas. Till recent years, several molybdenum-reducing bacteria with the potential to degrade other organic contaminants have been isolated (Halmi et al., 2013; Gusmanizar et al., 2016; AbdEl-Mongy et al., 2015; Othman et al., 2015; Masdor et al., 2015; Ibrahim et al., 2015a; Sabullah et al., 2016; Shukor et al., 2016; Mansur et al., 2016; Khayat et al., 2016; Halmi et al., 2016a). Therefore, further understanding of the reduction mechanism and kinetics of Mo-reducing enzyme through various optimization processes will help in solving the phenomenon of molybdate reduction to Mo-blue. Hence, it will then become an important step towards the effective translation of laboratory findings to the field practice.

1.1 Statement of the problems and significance of the study

As of Jan 2018, In the case of chromate, literature search using the phrase search "chromate reduction" and bacterium through the Scopus database (www.scopus.com) showed about 1162 hits whilst the search for "phenol-degrading" and bacterium as the search criteria shows more than 350 hits. This shows that the search for better heavy metal reducers (detoxifiers) and better degraders of xenobiotics is an ongoing process.

To date, all studies on molybdenum reduction to molybdenum blue by bacteria have concentrated on terrestrial sites and not on coastal sites where high salinity might be a huge problem to remediation using nonsalinity-tolerant bacteria. These sites can potentially be a molybdenum toxicity source especially the areas that are used for grazing the ruminants. Molybdenum higher above the toxic levels for ruminants (2 to 50 ppm) was found at several waste oil lubricant dumping sites in Malaysia recently (Yakasai *et al.,* 2017). As these sites are also being used as grazing areas for free wandering cows, hence a greater threat has been developed. The molybdenum recycled from industrial waste waters therefore can be a major help in the bacterial reduction process as it can involve within both recycling activity and reduction of metal content obtained from the waste water before discharge (Blokhin *et al.,* 2000; AhmadPanahi *et al.,* 2014; Halmi *et al.,* 2014; Shukor and Shukor 2015). From this example, the isolation of molybdenum-reducing bacteria with seawater tolerance thus can be used to remediate coastal sites. In Tyrol Austria, the conversion of soluble molybdenum to insoluble molybdenum-blue (reduction product) was demonstrated thus this presents a real and possible remediation technology with the use of microorganisms (Neunhäuserer *et al.,* 2001). In general, several physicochemical techniques for examples evaporation, chemical precipitation, filtration, membrane technology, reverse osmosis and electrochemical treatment are ineffective or costly at lower heavy metals concentrations (Rosca *et al.,* 2015) and this present a real role of bioremediation.

In addition, it has been increasingly found that the bioremediation efficacies of microbes are geographically confined at the macro and micro scale. For instance, bacterial degraders from the tropical countries might not be useful to be used in temperate countries or even in Polar Regions. Microbes isolated from specific areas such as from freshwater or nonsalty soils might not be effective for treating pollutants in the sea and vice versa. In many cases, microbes isolated from polluted soils, grown to a large scale and reintroduced into the polluted soils (autochthonous microbes) are more effective compared to using commercial strains or other degraders from other geographical location (allochthonous microbes) (Zhu *et al.*, 2001). This is the reason for the ever-increasing reporting of novel strains of microbes able to detoxify a particular heavy metal or degrade a particular xenobiotic.

Both further understandings for the mechanism and ways to improve the reduction thus are studied at the molecular level. The examples of them can be found in the genome of the molybdenum resistant *Bacillus subtilis* LM 4–2 which have been sequenced, and 116 redox protein-coding genes have been identified (You *et al.*, 2015). In fact, a potential identity of the Mo-reducing enzyme in *Serratia* sp. strain DrY5 has been purified and exhibited a 100 kDa-sized protein (Shukor *et al.*, 2014b). As the protein was not yet sequenced, the true identity of the enzyme remains to be unknown. From the modelling of Mo-reduction and molybdenum inhibition kinetics, several important constants of growth and inhibition can be obtained thus that can further be used for modelling of molybdenum reduction (Othman *et al.*, 2013; (Halmi *et al.*, 2016a). Therefore, this study is aimed to isolate molybdenum-reducing bacterium for molybdenum-reducing capability in seawater and coastal areas along with optimizing molybdenum reduction in this bacterium using OFAT and RSM. Apart from that, the kinetics of reduction, the effect of heavy metals on reduction, the purification of the molybdenum-reducing enzyme from this bacterium including the enzyme characteristics are also needing to be carried out. Thus, these studies are not just only fundamentally vital but also important for further understanding of the capability and limitations of this bacterium for future bioremediation or bioremoval of molybdenum.

Another major concern that is highly significant is that metal reduction by microbes have been reported to be caused by chemicals in the media or excreted by the cells. This abiotic reduction could lead researcher to the wrong direction and conclusion as is reported by Yong *et al.* (1997) on Mo-blue reduction by *Acidothiobacillus ferreoxidans*. In chromate reduction, Wang *et al.* (1989) has reported that organic acids and reducing sugars can reduce chromate abiotically. Hence, the experiment using dialysis tubing to prove molybdenum reduction in this bacterium is mediated by enzyme is important. Another important study is the effect of respiratory inhibitors especially cyanide, rotenone and Antimycin A on molybdenum reduction as this has been proven respiratory system not the site of reduction (Shukor *et al.*, 2010a).

In the case of molybdenum reduction, one of the biggest unsolved issues is the identification of the Mo-reducing enzyme. Although the enzyme has been purified from *Serratia* sp. strain Dr.Y5 (Shukor *et al.*, 2009a), the yield of the enzyme is too low for sequencing process. Hence, there is the need to search for better molybdenum reducers so that the amount of optimized enzyme per given cell is very high to start

with. The purification of intracellular enzyme is a challenging process due to the high interference by other proteins. High reducers allow more purified enzyme to be obtained. In addition, higher reducers are an excellent candidate for bioremediation. The work in this thesis is aimed at isolating such a reducer.

1.2 Hypothesis

Molybdenum-reducing bacterium isolated from marine and coastal areas can exhibit seawater tolerant; that kinetic studies of the molybdenum reduction process can yield important constants and limitation of the system which further optimization through RSM can increase the reduction efficiency and permit the production of adequate amount of enzyme to enable the purification of the Mo-reducing enzyme.

1.3 Objectives

This research is aimed at characterizing the first reported molybdenum-reducing and salinity tolerant bacterium. With this in mind the objectives of this study are as follows;

1. To screen for the molybdenum-reducers that can resist seawater and identify the best molybdenum-reducing isolate using molecular phylogenetic methods.
2. To characterize the Mo-reducing properties of the molybdenum-reducing isolate using one-factor-at-a-time (OFAT) and response surface method (RSM).
3. To carry out primary and secondary modelling of the kinetics process for molybdenum reduction in the molybdenum-reducing isolate.
4. To carry out bioremoval of molybdenum using dialysis tubing in minimals salts medium supplemented with salt to mimic bioremediation under saline conditions
5. To purify and characterize the Mo-reducing enzyme from this bacterium.

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BIODATA OF STUDENT

The student, Mohd Fadhil bin Abdul Rahman was born in Kuala Lumpur, in 1982. He is the third in the family. In April 2001, he completed his pre-university studies in matriculation under ministry of Education Malaysia. In July 2001, he had enrolled in Universiti Putra Malaysia to continue his first degree. In November 2004, he obtained his first degree of Bachelor Science (Hons) Biochemistry from UPM. In February 2004, he continued his postgraduate studies in Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia and graduated in 2007. His research interests are in the fields of biochemistry and environmental toxicology.

LIST OF PUBLICATIONS

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(Signature of Chairman of Supervisory Committee)
Name:

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