

UNIVERSITI PUTRA MALAYSIA

MICROBIAL MOLYBDATE REDUCTION TO MO-BLUE BY A CYANIDE-DEGRADING BACTERIUM

HAFEEZ MUHAMMAD YAKASAI

FBSB 2018 2



MICROBIAL MOLYBDATE REDUCTION TO MO-BLUE BY A CYANIDE-DEGRADING BACTERIUM



HAFEEZ MUHAMMAD YAKASAI

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

December 2017

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs, and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



DEDICATION

This thesis is dedicated to my beloved family whose moral support, prayers and encouragement are undoubtedly the driving force to my achievements.



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

MICROBIAL MOLYBDATE REDUCTION TO MO-BLUE BY A CYANIDE-DEGRADING BACTERIUM

By

HAFEEZ MUHAMMAD YAKASAI

December 2017

Chairman: Associate Professor Mohd Yunus Shukor, PhDFaculty: Biotechnology and Biomolecular Sciences

Molybdenum, an emerging pollutant, has recently being demonstrated to be toxic to spermatogenesis in several animal models. It is also very toxic to ruminants causing death at very low level (parts per million). Molybdenum is mined as a small byproduct of copper and gold mining in Malaysia. Molybdenum pollution is also found at dumping sites of waste oil lubricant containing molybdenum disulfide, with levels of up to hundreds of parts per million found. Molybdenum, in the soluble form, molybdate can be reduced to molybdenum blue, a colloidal and relatively insoluble product and this phenomenon forms the basis for bioremediation of molybdenum. This research is therefore, aimed at screening, phylogenetic identification, characterization and optimization of molybdenum-reducing activity of the best isolate via one-factorat-a-time (OFAT) and response surface method (RSM); modelling the kinetics of molybdenum reduction through primary and secondary models; finally purify and characterize the molybdenum-reducing enzyme activity. One of the ten previously isolated cyanide-degrading bacteria from gold mine soils in Malaysia exhibited a novel molybdenum reduction to molybdenum-blue, with this best molybdate-reducer further studied on a molybdate low phosphate minimal salts media supplemented with glucose and ammonium sulfate as the carbon and nitrogen source, respectively. Strain HMY3 via phylogenetic analyses reveals that the isolate belongs to Serratia genus. Sucrose was the best carbon source supporting molybdate reduction in this strain and was optimum at 20 g/L. Ammonium sulfate was the best source of nitrogen for strain HMY3 and was optimal at 10 g/L. Strain HMY3 grew best at 35 °C and at pH 6.5. Response Surface Method shows the best conditions for molybdenum reduction were molybdate concentration between 55 and 57.5 mM, phosphate concentration of 3.95 mM, pH 7, sucrose concentration between 15 and 17.5 g/L and incubation time between 48 and 60 h. Molybdenum reduction was inhibited by the heavy metals such as copper, mercury, chromium and arsenic at concentrations higher than 1 ppm. However, prolonged incubation succeeded in overcoming this inhibition. Analysis of the reduction kinetics showed that molybdenum reduction over time can best be



modelled using the modified Gompertz model while the reduction kinetics was best modelled using the Luong model. Statistical analysis of these models has been carried out and they exhibit low values for RMSE and AICc, highest adjusted R² values, Ftest and with Bias Factor and Accuracy Factor nearest to unity (1.0) over other models. The calculated value for the Luong's constants which are maximal reduction rate, half saturation constant for maximal reduction, maximal concentration of substrate tolerated and curve parameter that defines the steepness of the growth rate decline from the maximum rate symbolized by q_{max} , K_s , S_m , and n were 0.06±0.1 hr⁻¹, 47.95±10.12 mM, 69.63±0.8 mM and 0.69±0.11, respectively. The Luong model clearly shows strong substrate inhibition to rate of reduction at high substrate (Mo) concentration. The Mo-reducing enzyme has been purified using an ammonium sulfate precipitation followed by gel-filtration chromatography. The results showed that the best ammonium sulfate fraction giving the highest enzyme activity was between 50 and 60% ammonium sulfate concentration. The molybdenum-reducing enzyme was monomeric with an estimated mwt of 105 kDa. The enzyme was further characterized, and the results show that the enzyme is most stable in Tris-buffer pH 7 containing 0.1 mM DTT, temperature 4 °C and not affected by metabolic inhibitors and heavy metals (1 ppm). The enzyme attains optimum catalysis at pH 5.5, temperature between 25 and 35 °C, substrate concentration (LPPM) 12 mM and electron donor (NADH) concentration 5 mM. In conclusion, a novel cyanidedegrading bacterium with a molybdenum reduction capacity has been isolated, and statistical method using RSM has succeeded in optimizing reduction. Also, the enzyme has been purified to homogeneity. The characteristic of this bacterium makes it suitable for future bioremediation works in polluted soil containing cyanide and molybdenum metal.

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

PENURUNAN MOLIBDAT BERASASKAN MIKROB KEPADA MOLIBDENUM BIRU OLEH BAKTERIA-PENGURAI SIANIDA

Oleh

HAFEEZ MUHAMMAD YAKASAI

Disember 2017

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

Molibdenum, bahan pencemar yang baru muncul pada waktu kebelakangan ini adalah toksik kepada proses spermatogenesis dalam beberapa model haiwan. Ia juga sangat toksik kepada ruminan menyebabkan kematian pada kepekatan yang sangat rendah (bahagian per juta). Molibdenum dilombong sebagai bahan produk sampingan kecil dari perlombongan kuprum dan emas di Malaysia. Pencemaran molibdenum juga dijumpai di tapak pelupusan pelincir minyak sisa yang mengandungi molibdenum disulfida di beberapa tempat di Malaysia pada kepekatan sehingga beratus-ratus bahagian per juta. Molibdenum, dalam bentuk terlarutnya adalah molibdat, dan boleh diturunkan kepada molibdenum biru, satu produk koloidal dan tidak larut, dan fenomena ini menjadi asas bagi bioremediasi molibdenum. Oleh itu, objektif penyelidikan ini adalah bertujuan untuk menyaring, mengenalpasti secara filogenetik, membuat pencirian dan memoptimumkan aktiviti penurunan molibdenum melalui kaedah satu-faktor-pada-satu-masa (OFAT) dan kaedah permukaan tindak balas (RSM); memodelkan kinetik penurunann molibdenum melalui model-model primer dan sekunder; dan terakhir sekali menulenkan dan mencirikan aktiviti enzim penurunan molibdenum. Salah satu daripada sepuluh bakteria pengurai sianida yang sebelumnya dipencilkan dari tanah perlombongan emas di Malaysia mempamerkan keupayaan novel penurunan molibdenum kepada molibdenum-biru, dengan bakterium penurunan molibdat ini dikaji selanjutnya menggunakan media garam minimal molibdat dengan fosfat rendah ditambah dengan glukosa dan ammonium sulfat sebagai sumber karbon dan nitrogen, masing-masing. Melalui analisa filogenetik strain HMY3 ini didapati tergolong dalam genus Serratia. Sukrosa adalah sumber karbon terbaik yang menyokong penurunan molibdat dalam strain ini dan adalah optimum pada 20 g/L. Ammonium sulfate adalah sumber terbaik nitrogen untuk strain HMY3 dan adalah optimum pada 10 g/L. Strain HMY3 tumbuh paling baik pada suhu 35 °C dan pada pH 6.5. Melalui kaedah permukaan tindak balas, keadaan terbaik untuk penurunan molibdenum adalah pada kepekatan molibdat di antara 55 dan 57.5 mM,



kepekatan fosfat pada 3.95 mM, pH 7, kepekatan sukrosa di antara 15 dan 17.5 g/L dan masa inkubasi di antara 48 dan 60 jam. Penurunan molibdenum direncat oleh logam berat seperti kuprum, merkuri, kromium dan arsenik pada kepekatan yang lebih tinggi daripada 1 bahagian per juta. Walau bagaimanapun, waktu pengeraman yang berpanjangan telah berjaya mengatasi perencatan ini. Analisis kinetik menunjukkan bahawa penurunan molibdenum melawan masa dapat dimodelkan dengan baik sekali menggunakan model Gompertz terubahsuai manakala kinetik penurunan adalah dapat dimodelkan dengan baik sekali menggunakan model Luong. Analisis statistik modelmodel ini mempamerkan nilai-nilai yang rendah untuk RMSE dan AICc, nilai tertinggi untuk R^2 yang diselaraskan, ujian F dan Faktor Bias dan Faktor Ketepatan yang paling dekat dengan satu (1.0) berbanding model-model lain. Nilai-nilai yang dikira untuk pemalar Luong seperti kadar penurunan maksima, pemalar tepu separuh untuk penurunan maksima, kepekatan maksima substrat yang diguna dan parameter lengkung yang menentukan kecuraman penurunan kadar pertumbuhan dari kadar maksimum yang diwakilkan oleh q_{max} , K_s , S_m , dan n adalah 0.06 \pm 0.1 jam⁻¹, 47.95 \pm 10.12 mM, 69.63 ± 0.8 mM dan 0.69 ± 0.11 . Model Luong jelas menunjukkan kesan perencatan substrat yang kuat pada kadar penurunan pada kepekatan substrat (Mo) yang tinggi. Enzim penurunan Mo telah ditulenkan menggunakan pemendakan ammonium sulfat diikuti oleh kromatografi gel-penapisan. Hasilnya menunjukkan bahawa peratusan ammonium sulfat yang memberikan aktiviti enzim tertinggi adalah di antara 50 hingga 60%. Enzim penurunan molibdenum adalah monomerik dengan anggaran berat molekul 105 kDa. Pencirian enzim ini menunjukkan enzim adalah paling stabil pada pH 7 menggunakan penimbal Tris yang mengandungi 0.1 mM DTT, pada suhu 4 °C dan tidak terjejas oleh perencat metabolik dan logam berat pada 1 bahagian per juta. Enzim ini mencapai pemangkinan optimum pada pH 5.5, suhu di antara 25 dan 35 °C, kepekatan substrat substrat (LPPM) pada 12 mM dan kepekatan penderma elektron (NADH) pada 5 mM. Sebagai kesimpulannya, satu bakteria pengurai sianida novel dengan kapasiti penurun molibdenum telah diasingkan, dan kaedah statistik menggunakan RSM telah berjaya mengoptimumkan penurunan. Juga, enzim telah dapat ditulenkan sehingga homogen. Ciri-ciri bakteria ini menjadikannya sesuai untuk kerja bioremediasi pada masa hadapan di tanah tercemar yang mengandungi logam sianida dan molibdenum.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, Most Merciful

My profound gratitude goes to my able supervisor Assoc. Prof. Dr. Mohd Yunus Shukor and his team Dr. Siti Aqlima Ahmad, Dr. Nur Adeela Yasid and Dr. Mohd Izuan Effendi Halmi for their tireless effort, constructive, logical and unreserved suggestions.

Sincere appreciation to my beloved parents Dr. Muhammad Dauda Yakasai and Hajiya Amina Datti Yola; to my loving wife Yusra and daughter Mariya (Mufeeda) and to my brothers; Zahraddeen, Abdulwahab and sisters Aisha, Hafsat, Asma'u and Zahra'u for your support, prayers and encouragement.

To my Teachers, families, friends and colleagues particularly my lab mates in Bioremediation and Biomonitoring laboratory for being of help at all time through this great journey in UPM.

To my employer, Bayero University Kano and sponsors, TETFund for awarding me with Ph.D. scholarship; not forgetting the mentorship of his Excellency Professor Hafiz Abubakar. Together you made my dream a reality.

Alhamdulillah.

Hafeez Muhammad Yakasai, 2017.

This thesis was submitted to the Senate of the 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:

Mohd Yunus Shukor, PhD

Associate Professor Faculty of Biotechnology and Biomolecular Sciences Universiti Putra Malaysia (Chairman)

Siti Aqlima Ahmad, PhD

Senior Lecturer Faculty Biotechnology and Biomolecular Sciences Universiti Putra Malaysia (Member)

Nur Adeela Yasid, PhD

Senior Lecturer Faculty of Biotechnology Universiti Putra Malaysia (Member)

Mohd Izuan Effendi Halmi, PhD

Senior Lecturer Faculty of Agriculture Universiti Putra Malaysia (Member)

ROBIAH BINTI YUNUS, PhD Professor and Dean

School of Graduate Studies Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature:	Date:

Name and Matric No: Hafeez Muhammad Yakasai, GS43294

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.

Signature: Name of Ch	airman		
of Superviso	rv Associate Pro	ofessor	
Committee:	Dr Mohd Yu	nus Shukor	
Signature:			
Name of Me	ember		The second se
of Superviso	rv		
Committee:	Dr. Siti Aalir	na Ahmad	
		\bigcirc	
Signature:			
Name of Me	ember		
of Superviso	ry		
Committee:	Dr. Nur Adee	ela Yasid	
Signature:			
Name of Me	ember		
of Superviso	ry		
Committee:	Dr. Mohd Izu	an Effendi Halmi	

TABLE OF CONTENTS

Page

ABS ABS ACK APP DEC LIST LIST LIST	TRACT TRAK TRAK NOWLEDGEMENTS ROVAL LARATION COF TABLES COF FIGURES COF ABBREVIATIONS	i iii v vi viii xiv xvi xx
СНА	PTER	
1	GENERAL INTRODUCTION	1
	1.1 Research background	1
	1.2 Problem statements	2
	1.3 Hypothesis	3
	1.4 Objectives	3
2		4
2	2.1 Molyhdenum (Mo)	4
	2.1 Molybdenum (Mo) 2.2 Molybdenum entry routes in animals	4 4
	2.2 Molybdenum toxicity	- 5
	2.3.1 Toxicity to spermatogenesis and oogenesis	6
	2.3.2 Mechanism of molybdenum toxicity in ruminants	6
	2.4 Bioremediation	8
	2.4.1 Molybdenum pollution	8
	2.4.2 Molybdenum bioremediation	9
	2.4.3 Mechanism of molybdenum reduction to Mo-blue	10
	2.5 Characteristics of previously isolated molybdenum-reducing	
	bacteria	13
	2.6 Mathematical modelling of molybdenum reduction profile and	10
	kinetics	19
	2.7 Characteristic of partially purified molybdenum-reducing enzyme	24
	1 rom other bacterial sources	24
	2.8 Electron transport chain inhibitors	26
3	MATERIALS AND METHODS	27
	3.1 Experimental design	27
	3.2 Chemicals and equipment	27
	3.3 Culture media preparation	28
	3.3.1 Low phosphate-molybdate (LPM) agar	28
	3.3.2 Low phosphate-molybdate medium (LPM)	28
	3.3.3 High phosphate-molybdate media (HPM)	28
	3.4 Screening of Mo-reducing bacteria	29

Х

3.5	Spectra	al scanning	g of molybdenum-blue	29
3.6	Identif	ication of 1	Mo-reducing bacteria	29
	3.6.1	Gram stai	n	29
	3.6.2	Catalase t	est	30
	3.6.3	Oxidase t	est	30
	3.6.4	16s rRNA	Gene Sequencing	30
	3.6.5	Phylogen	etic analysis	30
3.7	Optimi	ization of p	barameter affecting molybdate reduction in strain	
	HMY3	using One	e Factor at a Time (OFAT)	31
	3.7.1	Effect of	different electron donor sources on molybdate	
		reduction	5	31
	3.7.2	Effect of	electron donor concentrations	32
	3.7.3	Screening	of nitrogen sources	32
	3.7.4	Effect of	ammonium sulfate concentrations	32
	375	Effect of i	initial pH of LPM	33
	376	Effect of t	temperature	33
	377	Effect of	phosphate and molybdate concentrations	33
38	Ontimi	ization of r	nolybdate reduction using Response Surface	55
5.0	Metho	d (RSM)	norybuate reduction using Response Surface	34
	3.8.1	Screening	of significant parameters using Placket-Burman	34
	3.8.2	Ontimizat	ion of significant parameters using central	54
	5.0.2	composite	design	35
3.0	Model	ling the ki	etics of molybdenum reduction	35
5.7	3 0 1	Fitting of	the data	35
	3.9.1	Statistical	analysis	36
3 10	Durific	ation of th	a Mo Reducing Enzyme	38
5.10	3 10 1	Crude en	ume preparation	38
	3.10.1	Drotoin of		20
	3.10.2	Determine	say	30
	3.10.3	Mo roduo	ing angume stability study	39 40
	5.10.4	2 10 4 1	Effect of storage pH	40
		5.10.4.1	Effect of storage pri	41
		3.10.4.2	PEG at a f at an a standard terrar	41
		3.10.4.3	Effect of storage temperature	41
		5.10.4.4 2.10.4.5	Effect of choloting agent, determent and ergenic	41
		5.10.4.5	Effect of chefating agent, detergent and organic	40
		2 10 4 6	Solvents	42
		5.10.4.0 2.10.4.7	Effect of suffrydryf agents	42
		3.10.4.7	Effect of metal collectors	42
		3.10.4.8	Effect of neavy metals	43
	2 10 5	5.10.4.9	Effect of metabolic inhibitors	43
	3.10.5	Purification	on of enzyme by ammonium suifate fractionation	43
	3.10.6	Purification	on on Agilent Zorbax ^{1M} (GF-250) gel filtration	44
	3.10.7	Preparatio	on of SDS-polyacrylamide gel	45
		3.10.7.1	Sample and running gel preparation	46
	0.10.0	3.10.7.2	Gel staining	46
	3.10.8	Character	ization of Mo-reducing enzyme	47
		3.10.8.1	Effect of pH on molybdenum-reducing enzyme	
			activity	47

		3.10.8.2	Effect of temperature on molybdenum-reducing	
			enzyme activity	47
	3.10.9	Kinetics of	of Mo-reducing enzyme	47
		3.10.9.1	Determination of Km and Vmax NADH/	
			NADPH as electron donor substrates	48
		3.10.9.2	Determination of K_m and V_{max} LPPM as electron	
			acceptor substrate	48
4 RESU	LTS A	ND DISCU	USSION	49
4.1	Screen	ing for mo	lybdenum-reducing bacteria	49
4.2	Spectra	al scanning	of molybdenum-blue produced by strain HMY3	49
4.3	Identif	ication of l	bacterial strain	50
4.4	Charac	terization	and optimization of molybdate reduction using	
	one-fa	ctor-at-a-ti	me approach (OFAT)	52
	4.4.1	Screening	of electron donor sources	52
	442	Effect of	sucrose concentration	53
	443	Screening	of nitrogen sources	54
		Effect of	various concentrations of ammonium sulfate	55
	1 1 5	Effect of	initial pH	56
	446	Effect of t	amperature	50
	4.4.7	Effect of	phosphate concentration	5) 60
	4.4.7	Effect of	molyhdata concentration	61
1.5	4.4.0	Effect of a	nolybdate concentration	01
4.5	Matha	dology (D	norybuate reduction using Response Surface	62
		Concerning	SIVI)	03
	4.3.1	Dumman	, for significant parameters using Plackett-	$\mathcal{C}\mathcal{D}$
	450	Burman d	esign	03
	4.5.2	Optimizat	ion of molybdate reduction using RSM	60
	4.5.3	Determina	ation and validation of optimum conditions	69 71
	4.5.4	Effect of	process variables using response surface plot	/1
		4.5.4.1	Molybdate concentration vs. phosphate	
			concentration	71
		4.5.4.2	Molybdate concentration vs. pH	72
		4.5.4.3	Molybdate concentration vs. sucrose	
			concentration	73
		4.5.4.4	Molybdate concentration vs. time	74
		4.5.4.5	Phosphate concentration vs. pH	75
		4.5.4.6	Phosphate concentration vs. sucrose	
			concentration	76
		4.5.4.7	Phosphate concentration vs. time	77
		4.5.4.8	pH vs. sucrose concentration	78
		4.5.4.9	pH vs. time	79
		4.5.4.10	Sucrose concentration vs. time	80
	4.5.5	Modelling	g of molybdenum reduction kinetics after RSM	81
4.6	Effect	of heavy n	netals on bacterial molybdate reduction	91
	4.6.1	Effect of	copper	91
	4.6.2	Effect of	mercury	92
	4.6.3	Effect of a	silver	93
	4.6.4	Effect of I	lead	94

		4.6.5	Effect of	chromium	95
		4.6.6	Effect of	arsenic	96
		4.6.7	Effect of	cadmium	97
		4.6.8	Effect of	nickel	98
	4.7	4.7 Pu	rification	of Mo-reducing enzyme from strain HMY3	99
		4.7.1	4.7.1 Mc	p-reducing enzyme stability studies	99
			4.7.1.1	Effect of storage pH	100
			4.7.1.2	pH stability study	101
			4.7.1.3	Effect of storage temperatures	102
			4.7.1.4	Temperature stability study	103
			4.7.1.5	Effect of chelating agents, detergents and	
				organic solvents	103
			4.7.1.6	Effect of sulfhydryl reagents	104
			4.7.1.7	Effect of cofactors	105
			4.7.1.8	Effect of heavy metals on Mo-reducing enzyr	ne 106
			4.7.1.9	Effect of inhibitors	107
		4.7.2	Purificat	ion of Mo-reducing enzyme	108
			4.7.2.1	SDS polyacrylamide gel electrophoresis	110
		4.7.3	Characte	rization and kinetics studies	111
			4.7.3.1	Optimum pH	111
			4.7.3.2	Optimum temperature	112
			4.7.3.3	Determination of Kinetic Parameters	113
5	SUM	MARY.	CONCL	USION AND RECOMMENDATIONS FOR	
U	FUTU	RE RE	SEARCH		116
	5.1	Summ	arv		116
	5.2	Conclu	ision		116
	53	Recom	mendatio	ns for future reserch	117
	2.2	neeon	mondullo		
REFE	RENC	ES			118
APPE	NDICH	ES			134
BIOD	ATA O	FSTU	DENT		142
LIST (OF PU	BLICA	TIONS		143

LIST OF PUBLICATIONS

G

LIST OF TABLES

	Table		Page
	2.1	Molybdenum levels at different waste oil lubricant pollution sites in Selangor Malaysia	9
	2.2	Characteristics of previously isolated Mo-reducing bacteria	16
	2.3	Mo-blue production models used in this study	21
	2.4	Various mathematical models developed for degradation kinetics involving substrate inhibition	23
	3.1	Plackett-Burman design for tested variables	34
	3.2	Central composite design for tested variables	35
	3.3	Preparation of resolving gels (12%) for Tris-glycine SDS- polyacrylamide gel electrophoresis	46
	3.4	Preparation of stacking gels (5%) for Tris-glycine SDS- polyacrylamide gel electrophoresis	46
	4.1	Molybdate reduction to Mo-blue by cyanide-degrading isolates	49
	4.2	Plackett–Burman design to evaluate significant parameters influencing molybdenum reduction by strain HMY3	64
	4.3	Plackett-Burman factorial model and analysis of variance (ANOVA) for molybdenum reduction	65
	4.4	Central composite matrix for experimental design and predicted response using RSM	67
	4.5	ANOVA for fitted quadratic polynomial model to optimize molybate reduction in strain HMY3	69
	4.6	Predicted and experimental value for the responses at optimum condition using RSM	70
	4.7	Summary of optimum conditions for strain HMY3 using One Factor at a Time (OFAT)	71
	4.8	Statistical analysis of the various fitted models	83

4.9	Mo-blue production coefficients at various molybdate concentrations as modelled using the modified Gompertz model	84
4.10	Statistical analysis of kinetic models	90
4.11	Effect of storage temperature on Mo-reducing enzyme storage Stability	102
4.12	Effect of some chemical agents on Mo-reducing enzyme storage stability	104
4.13	Fractionation by ammonium sulfate	109
4.14	Purification scheme for the Mo-reducing enzyme from strain HMY3	110

 \bigcirc

LIST OF FIGURES

]	Figure		Page
	2.1	Schematic presentation of the mechanism of molybdate reduction to Mo-blue	10
	2.2	12-phosphomolybdate structure	11
	2.3	Spectral scanning of Mo-blue from 3 different isolates	12
	2.4	Spectral scanning of Mo-blue from silicomolybdate, sulphomolybdate and phosphomolybdate	13
	3.1	Experimental design for molybdate reduction to Mo-blue by cyanide-degrading bacteria	27
	4.1	Spectral scanning of Mo-blue produced by strain HMY3 following 24 hours incubation	50
	4.2	Phylogram (xvi eighbor-joining method) showing the genetic relationship between <i>Serratia</i> sp. strain HMY3 and other related references microorganisms based on the 16S rRNA gene sequence analysis from the GenBank database	51
	4.3	Effect of various electron donor sources on optimum Mo-blue production by strain HMY3	53
	4.4	Effect of various sucrose concentrations on optimum Mo-blue production by strain HMY3	54
	4.5	Effect of various nitrogen sources on Mo-blue production by strain HMY3	55
	4.6	Effect of various ammonium sulfate concentrations on optimum Mo-blue production by strain HMY3	56
	4.7a	Effect of initial pH on optimum Mo-blue production by strain HMY3 HMY3	58
	4.7b	Effect of different initial pH on Mo-blue production rate by strain HMY3	58
	4.8a	Effect of temperature on optimum Mo-blue production by strain HMY3	59

4.8b	Effect of different temperature on Mo-blue production rate by strain HMY3	60
4.9	Effect of phosphate concentration on optimum Mo-blue production by strain HMY3	61
4.10	Effect of molybdate concentration on Mo-blue production by strain HMY3	62
4.11	Effect of different molybdate concentrations on Mo-blue production rate measured periodically for 72 h	62
4.12	3D and contour plots showing effect of molybdate concentration and phosphate on Mo-blue production by strain HMY3	72
4.13	3D and contour plots showing effect of molybdate concentration and pH on Mo-blue production by strain HMY3	73
4.14	3D and contour plots showing effect of molybdate and sucrose concentrations on Mo-blue production by strain HMY3	74
4.15	3D and contour plots showing effect of molybdate concentration and time on Mo-blue production by strain HMY3	75
4.16	3D and contour plots showing effect of phosphate concentration and pH on Mo-blue production by strain HMY3	76
4.17	3D and contour plots showing effect of phosphate and sucrose concentrations on Mo-blue production by strain HMY3	77
4.18	3D and contour plots showing effect of phosphate concentration and time on Mo-blue production by strain HMY3	78
4.19	3D plot and contour plots showing effect of pH and sucrose concentration on Mo-blue production by strain HMY3	79
4.20	3D plot and contour plots showing effect of pH and time on Mo- blue production by strain HMY3	80
4.21	3D plot and contour plots showing effect of sucrose and time on Mo-blue production by strain HMY3	81
4.22	The Mo-blue production curves of <i>Serratia</i> sp. strain HMY3 at various concentrations of sodium molybdate over time after RSM optimization	82

4.23	The Mo-blue production curves of <i>Serratia</i> sp. strain HMY3 on various concentrations of sodium molybdate fitted using the Gompertz model	83
4.24	Fitting experimental data with the Luong model	86
4.25	Fitting experimental data with the Yano model	86
4.26	Fitting experimental data with the Teissier model	87
4.27	Fitting experimental data with the Aiba model	87
4.28	Fitting experimental data with the Haldane model	88
4.29	Fitting experimental data with the Monod model	88
4.30	Fitting experimental data with the Han-Levenspiel model	89
4.31	Effect of various copper concentrations on Mo-blue production rate by strain HMY3	92
4.32	Effect of various mercury concentrations on Mo-blue production rate by strain HMY3	93
4.33	Effect of various silver concentrations on Mo-blue production rate by strain HMY3	94
4.34	Effect of various lead concentrations on Mo-blue production rate by strain HMY3	95
4.35	Effect of various chromium concentrations on Mo-blue production rate by strain HMY3	96
4.36	Effect of various arsenic concentrations on Mo-blue production rate by strain HMY3	97
4.37	Effect of various cadmium concentrations on Mo-blue production rate by strain HMY3	98
4.38	Effect of various nickel concentrations on Mo-blue production rate by strain HMY3	99
4.39	Effect of pH buffers on Mo-reducing enzyme storage stability of Mo-reducing enzyme	100
4.40	Effect of preincubation pH on Mo-reducing stability	101

4.41	Effect of preincubation temperatures on Mo-reducing enzyme stability	103
4.42	Effect of the sulfhydryl reagents DTT and β -mercaptoethanol on Mo-reducing enzyme storage stability	105
4.43	Effect of potential cofactors to the storage stability of the Mo-reducing enzyme	106
4.44	Effect of heavy metals on Mo-reducing enzyme storage stability	106
4.45	Effect of metabolic inhibitors to the Mo-reducing enzyme	108
4.46	Gel filtration on Zorbax GFC-250 indicating Mo-reducing enzyme activity and protein content at 280 nm	110
4.47	SDS-PAGE electrophoretogram of purified Mo-reducing enzyme	111
4.48	Optimum pH for enzyme activity using a 25 mM citrate- phosphate buffer	112
4.49	Optimum temperature for enzyme activity	113
4.50	Michaelis-Menten plot for the amount of Mo-blue formed (nmol/min/mg protein) versus the electron donor substrate (NADH)	114
4.51	Michaelis-Menten plot for the amount of Mo-blue formed (nmol/min/mg protein) versus the electron donor substrate (NADPH)	115
4.52	Michaelis-Menten plot of amount of Molybdenum-blue formed (nmole/min/mg protein) versus the electron acceptor substrate	115

(LPPM)

LIST OF ABBREVIATIONS

	%	Percent
	<	Less than
	>	Greater than
	Ŷ	Gamma
	β	Beta
	α	Alpha
	Abs	Absorbance
	CCD	Central composite design
	cm	Centimetre
	Мо	Molybdenum
	As	Arsenic
	Ag	Silver
	Cd	Cadmium
	Ni	Nickel
	Со	Cobalt
	Cr	Chromium
	Cu	Copper
	cm	Centimetre
	Da	Dalton
	dH ₂ O	Distilled water
	EDTA	Ethylene diamine tetra acetic acid
	et al	and friends
	Fe	Iron
	FFD	Fractional factorial design
	G	Gram
	h	Hour

	Hg	Mercury
	Km	Michaelis-Menten constant
	Vmax	Maximum velocity
	kb	Kilo base
	kDa	Kilo dalton
	kg	Kilogram
	KCN	Potassium Cyanide
	L	Liter
	М	Molar
	mAu	Milli absorbance unit
	min	Minute
	mL	Mililiter
	(NH4)2SO4	Ammonium sulfate
	MgSO ₄	Magnesium sulfate
	NaCl	Sodium chloride
	MSM	Minimal salt medium
	MW	Molecular weight
	NA	Nutrient agar
	°C	Degree celcius
	w/v	Weight/volume
	OD	Optical density
	RPM	Rotation per minute
	RSM	Response surface method
	SDS	Sodium dodecyl sulfate
	pH	-log concentration of H ⁺ ion (Puissance hydrogen)
	PO4 ³⁻	Phosphate
	RNA	Ribonucleic Acid
	rRNA	Ribosomal ribonucleic acid

μL	Microlitre
nm	Nanometer
nmol	Nanomole
μm	Micrometer
μΜ	Micromolar
Mo-blue	Molybdenum-blue
NADH	Nicotinamide adenine-dinucleotide reduced
DTT	Dithiothretol
PMSF	phenylmethylsulfonylflouride
DEAE	Diethylaminoethylamine
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
PAGE	Polyacrylamide gel electrophoresis
HCI	Hydrochloric acid
HPLC	High Performance Liquid Chromatography

C

CHAPTER 1

GENERAL INTRODUCTION

1.1 Research background

Today, our environment is under constant threat from our own activities. Humans expanding population, industrialization, urbanization and intensive agriculture have caused tremendous damage to our environment. Man's ignorance of the laws of nature and over exploitation of natural resources has further aggravated the problem (Bhatia, 2006; Hamid *et al.*, 2010).

Metals and their compounds have been long recognized as toxic agents, causing a range of acute to chronic toxicity cases in occupational and environmental highexposure settings. Heavy metals are elements that occur naturally in the environment. However, their level has increased tremendously since the pre-industrial times due to anthropogenic activities (Kaplan 2013; Meyer et al., 2014; Ilyin et al., 2015). Presently, increasing population and industrialization have led to a considerable and indiscriminate release of pollutants into the environment (Soares and Soares, 2012; Dixit et al., 2015). When heavy metals levels exceeded the so-called critical loads, they exert harmful effects to human health and biota (Alloway, 1995; Meyer et al., 2014; Ilyin et al., 2015; Gupta et al., 2016). Metals such as arsenic, cadmium, chromium, cobalt, copper, lead, mercury, molybdenum, nickel, silver and zinc are known to be toxic in their elemental forms and different chemical combinations (Rosca et al., 2015). They are non-degradable (Sarubbo et al., 2015), hence, accumulate in the food chain posing a serious threat to the ecosystem (Li and Tao, 2013) due to their carcinogenic and mutagenic property (Singh and Prasad, 2011). Today, pollution by heavy metals has become a global public health concern. Thus, their removal from the environment is of great importance.

Molybdenum is an essential trace element and a micronutrient required as a cofactor by more than 50 enzymes (Zhai *et al.*, 2013; Wu *et al.*, 2014). It promotes cellular function by catalyzing a variety of hydroxylation and redox transfer reactions, thus play an important role in animal and plant physiology (Pandey and Singh, 2002). Earlier studies have shown that molybdenum is an endocrine disruptor and affect spermatogenesis in several animal models, nevertheless, it is ubiquitously found in a number food and water sources (Kargar *et al.*, 2011; Zhai *et al.*, 2013). The wide 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 has increased the risk of humans exposer to its toxicity (Pandey and Singh, 2002; Zhai *et al.*, 2013). For instance, an elevated level of molybdenum in ground water around the mining areas up to 0.2 mg/L was previously reported, which is above WHO recommended limit of 0.07 mg/L in drinking water. Thus, animals in contact with molybdenum in drinking water or while foraging for plants are likely to reflect symptoms of hypocuprosis or suffer from

 \bigcirc

molybdenosis over a long exposure period (Zhai et al., 2013).

Microbial molybdate reduction to Mo-blue is a phenomenon that has puzzled scientists for over a century. It was until recently that the phenomenon was proven to be enzymatic rather than abiotic. Before the work of Halmi *et al.* (2013), previous works on bacterial molybdate reduction are centered towards isolating molybdenum-reducer with a higher Mo-blue production capacity as a tool in bioremediation. However, since most polluted sites contain mixed contaminants of both organics and inorganics origin, makes effective remediation a complex one. During the last five years, attention has been shifted towards isolating microorganisms with multi-reduction and/or degrading potentials that could be used to remediate co-contaminated areas. To date, about eight molybdenum-reducing bacteria with the potential to degrade other organic contaminants were isolated. Therefore, the detailed understanding of the reduction mechanism and kinetics of Mo-reducing enzyme through various optimization processes, will assist in unravelling the mystery that lingers around the phenomenon of molybdate reduction to Mo-blue, thus an important step towards effective translation of laboratory findings to the field practice.

1.2 Problem statements

Mining sites in Malaysia, especially gold mining often contain multi-pollutant including cyanide and heavy metals like molybdenum. These sites can be potential molybdenum toxicity source especially if the areas are grazing areas for ruminant. Also, new information has surfaced that molybdenum at levels above the toxic levels for ruminants (2 to 200 ppm) is found at several waste oil lubricant dumping sites in Malaysia. Alarmingly, these sites are also grazing areas for free wandering cows presenting a greater threat. In addition, molybdenum recycling from industrial waste waters can also take advantage of the bacterial reduction process as both a recycling activity and a reduction of metal content from the wastewater before discharge (Blokhin et al., 2000; AhmadPanahi et al., 2014; Halmi et al., 2014a; Shukor and Shukor, 2015). Taking these issues into consideration, isolation of cyanide-degrading bacteria with high molybdenum tolerance can be used to remediate these sites or recycle molybdenum from wastewater. The conversion of soluble molybdenum to insoluble molybdenum-blue (reduction product) as was demonstrated in Tyrol Austria present a real and possible remediation technology using microorganisms (Neunhäuserer et al., 2001). Bioremediation is generally cost effective and provides an alternative to the existing physicochemical methods of heavy metals removal. Some physicochemical techniques such as evaporation, chemical precipitation, filtration, ion exchange, membrane technology, reverse osmosis and electrochemical treatment are used to treat industrial wastes. However, these methods remain ineffective at lower heavy metals concentrations, are laborious and often expensive (Rosca et al., 2015). Similarly, the solubility of heavy metal salts in wastewater made their effective removal via physical technique a complex process.

Several of the ways to improve reduction and further understand the mechanism of reduction are studied at the molecular level. For instance, the genome of the molybdenum resistant Bacillus subtilis LM 4-2 have been sequenced, and 116 redox protein-coding genes have been identified (You et al., 2015). Also, a 100 KDa protein has been shown to be a potential identity of the Mo-reducing enzyme in Serratia sp. strain DrY5 (Shukor et al., 2014). However, the protein was not sequenced, and the true identity of the enzyme remains unknown. Furthermore, modelling of Moreduction and molybdenum inhibition kinetics have revealed important constants of growth and inhibition that can be used for further modelling of molybdenum reduction (Othman et al., 2013; Halmi et al., 2016). Thus, this study focused on screening previously isolated cyanide-degrading bacterium for molybdenum-reducing capability and to optimize molybdenum reduction in this bacterium using OFAT and RSM. Other studies that need to be carried out include the kinetics of reduction, the effect of heavy metals on reduction, the possibility of molybdenum reduction in the presence of cyanide and the purification of the molybdenum-reducing enzyme from this bacterium including the enzyme characteristics. These studies are not only very important fundamentally but important for further understanding the capability and limitations of this bacterium in the future bioremediation or bioremoval of molybdenum.

1.3 Hypothesis

Cyanide-degrading isolate can efficiently reduce molybdate to molybdenum-blue via enzymatic reaction; that kinetic studies of the molybdenum reduction process can yield important constants and limitation of the system; that further optimization through RSM can increase the reduction efficiency and permit the production of adequate amount of enzyme to enable the purification of Mo-reducing enzyme.

1.4 Objectives

This research is aimed at characterizing the molybdenum-reducing property of previously isolated cyanide-degrading bacteria. With this in mind the objectives of this study are as follows;

- 1. To screen for the best molybdenum-reducer from previously isolated cyanidedegrading bacteria and identify the best isolate using molecular phylogenetic methods.
- 2. To characterize the Mo-reducing properties of the best cyanide-degrading 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.
- 4. To purify and characterize the Mo-reducing enzyme from this bacterium.

REFERENCES

- AbdEl-Mongy, M.A., Shukor, M.S., Hussein, S., Ling, A.P.K., Shamaan, N.A. and Shukor, M.Y. (2015). Isolation and characterization of a molybdenumreducing, phenol- and catechol-degrading *Pseudomonas putida* strain amr-12 in soils from Egypt. *Scientific Study & Research Chemistry & Chemical Engineering, Biotechnology, Food Industry* 16, 353–369.
- Abo-Shakeer, L.K.A., Ahmad, S.A, Shukor, M.Y. and Syed, M. (2013). Isolation and characterization of a molybdenum-reducing *Bacillus pumilus*strain lbna. *Journal of Environmental Microbiology and Toxicology* 1, 9–14.
- Ahmad, S.A., Shamaan, N.A., Arif, N.M., Koon, G.B., Shukor, M.Y.A. and Syed, M.A. (2012). Enhanced phenol degradation by immobilized *Acinetobacter* sp. strain AQ5NOL 1. World Journal of Microbiology and Biotechnology 28, 347–352.
- Ahmad, S.A., Shukor, M.Y., Shamaan, N.A., Mac Cormack, W.P. and Syed, M.A. (2013). Molybdate reduction to molybdenum blue by an antarctic bacterium. *BioMed Research International* 1–10.
- Ahmad, W.A., Zakaria, Z.A., Zakaria, Z. and Surif, S. (2009). Hexavalent chromium reduction at different growth phases of *Acinetobacter haemolyticus*. *Environmental Engineering Science* 26, 1275–1278.
- AhmadPanahi. H., Hosseinzadeh, M., Adinehlo, H., Moniri, E. and Manoochehri, M. (2014). Removal of molybdenum from environmental sample by adsorption using modified aniline-formaldehyde with salicylic acid. World Applied Sciences Journal 30, 1892–1898.
- Aiba, S., Shoda, M. and Nagatani, M. (1968). Kinetics of product inhibition in alcohol fermentation. *Biotechnology and Bioengineering* 10(6), 845–864.
- Akaike, H. (1974). New look at the statistical model identification. *IEEE Transactions* on Automatic Control 19, 716–723.
- Alloway, B.J. (1995) 'Heavy metals in soils trace metals and metalloids in soils and their bioavailability.' (BJ Alloway, Ed.). (Springer Dordrecht Heidelberg New York London) doi:10.1007/9789400744707.
- Amoozegar, M.A., Ghasemi, A., Razavi, M.R. and Naddaf, S. (2007). Evaluation of hexavalent chromium reduction by chromate-resistant moderately halophile, *Nesterenkonia* sp. strain MF2. *Process Biochemistry* 42, 1475–1479.
- Amor, L., Kennes, C. and Veiga, M.C. (2001). Kinetics of inhibition in the biodegradation of monoaromatic hydrocarbons in presence of heavy metals. *Bioresource Technology* 78, 181–185.

- Angelo, A.J.S. and Kuck, J.C. (1977). Effects of cyanide on peanut lipoxygenase. *Lipids* 12, 682–683.
- Annadurai, G., Ling, L.Y. and Lee, J. (2008). Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*. *Journal of Hazardous Materials* 151, 171–178.
- Ariff, A.B., Rosfarizan, M., Ghani, B., Sugio, T. and Karim, M.I.A. (1997). Molybdenum reductase in *Enterobacter cloacae*. World Journal of Microbiology and Biotechnology 13, 643–647.
- Babák, L., Šupinová, P. and Burdychová, R. (2012). Growth models of *Thermus* aquaticus and *Thermus scotoductus*. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 60, 19–26.
- Balusu, R., Paduru, R.R., Kuravi, S.K., Seenayya, G. and Reddy, G. (2005). Optimization of critical medium components using response surface methodology for ethanol production from cellulosic biomass by *Clostridium thermocellum* SS19. *Process Biochemistry* 40, 3025–3030.
- Baranyi, J. (1995). Mathematics of predictive food microbiology. International Journal of Food Microbiology 26, 199–218.
- Baranyi, J. and Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* 23, 277–294.
- Barceloux, D.G. (1999). Copper. *Journal of Toxicology Clinical Toxicology* 37, 217–230.
- Berssnyi, A., Berta, E., Kadar, I., Glavits, R., Szilagyi, M. and Fekete, S.G. (2008). Effects of high dietary molybdenum in rabbits. *Acta Veterinaria Hungarica* 56, 41–55.
- Bhatia, S.C. (2006). Environmental Chemistry. CBS Publishers and Distributors (1st Ed.) New Delhi (India) pp 549
- Blokhin, A.A., Kopyrin, A.A., Boev, A.A. and Kirillova, M.K. (2000). Removal of molybdenum(VI) from tungstate solutions with weakly basic anion exchangers. *Russian Journal of Applied Chemistry* 73, 404–408.
- Bolker, B.M. (2008). 'Ecological Models and Data in R.' *Princeton University Press: Princeton*, New Jersey, pp 395.
- Boon, B. and Laudelout, H. (1962). Kinetics of nitrite oxidation by Nitrobacter winogradskyi. *The Biochemical Journal*, 85, 440–447.
- Bopp, L.H. and Ehrlich, H.L. (1988). Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. Archives of Microbiology 150, 426– 431.

- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72 (1-2), 248–254
- Buchanan, R.L. (1993). Predictive food microbiology. *Trends in Food Science and Technology* 4, 6–11.
- Buchanan, R.L., Whiting, R.C. and Damert, W.C. (1997). When is simple good enough: A comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food Microbiology* 14, 313–326.
- Burnham, K.P. and Anderson, D.R. (2002). 'Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach.' *Springer Science & Business Media*, pp 488.
- Camakaris, J., Voskoboinik, I. and Mercer, J.F. (1999). Molecular mechanisms of copper homeostasis. *Biochemical and Biophysical Research Communications* 261, 225–232.
- Campbell, A.M., Del Campillo-Campbell. A. and Villaret, D.B. (1985). Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. *Proceedings of the National Academy of Sciences of the United States of America* 82, 227–231.
- Campillo-campbell, A. and Villaret, D.B. (1985). Correction: Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. *Proceedings of the National Academy of Sciences* 82, 3532–3532.
- Cappuccino, J.G. and Sherman, N. (2005). Microbiology: A Laboratory Manual 7th Edition. Pearson Education, Inc, San Francisco. 45-47.
- Cervantes, C., Ji, G., Ramírez, J.L. and Silver, S. (1994). Resistance to arsenic compounds in microorganisms. *FEMS Microbiology Reviews* 15, 355–367.
- Chai, B., Wu, Y., Liu, P., Liu, B. and Gao, M. (2011). Isolation and phosphatesolubilizing ability of a fungus, *Penicillium* sp. from soil of an alum mine. *Journal of Basic Microbiology* 51, 5–14.
- Clesceri, L.S., Greenberg, A.E. and Trussel, R.R., (1989). Standard methods for the examination of wastewater. *American Public Health Association, Port City Press*, Baltimore, Maryland. pp 4-166
- Counotte, G.H.M. and Prins, R.A. (1979). Calculation of *Km* and *Vmax* from substrate concentration versus time plot. *Applied and Environmental Microbiology* 38, 758–760.
- Davis, G.K. (1991). Molybdenum. In Merian, E. (ed) Metals and their compounds in the environment, occurrence, analysis and biological relevance. New York: VCH Weinheim. pp. 1089-1100

- Dawson, R.M.C., Elliot, D.C. and Elliot, W.H., (1969). Data for biochemical research", *Clarendon Press*, Oxford.
- Debbie Furber (2009). Is molybdenum lurking in your forages? https://www.canadiancattlemen.ca/2009/05/14/is-molybdenum-lurking-inyour-forages/
- Deeb, B.E. and Altalhi, A.D. (2009). Degradative plasmid and heavy metal resistance plasmid naturally coexist in phenol and cyanide assimilating bacteria. *American Journal of Biochemistry and Biotechnology* 5, 84–93.
- Deutscher, M.P. (2009). Setting Up a Laboratory. *Methods in Enzymology* (1st ed., Vol. 463). Elsevier Inc.
- Devereux, R. and Wilkinson, S.S. (2004). Amplification of ribosomal RNA sequences. *Molecular Microbial Ecology Manual* 3, 509–522.
- Dey, S. and Paul, A.K. (2012). Optimization of cultural conditions for growth associated chromate reduction by *Arthrobacter* sp. SUK 1201 isolated from chromite mine overburden. *Journal of Hazardous Materials* 213, 200–206.
- Dey, S. and Paul A.K. (2014). Reduction of hexavalent chromium by immobilized viable cells of *Arthrobacter* sp. SUK 1201. *Bioremediation Journal* 18, 1–11.
- Dinarvand, M., Rezaee, M., Masomian, M., Jazayeri, S.D., Zareian, M., Abbasi, S. and Ariff, A.B. (2013). Effect of C / N ratio and media optimization through response surface methodology on simultaneous productions of intra- and extracellular inulinase and invertase from *Aspergillus niger* ATCC 20611. *BioMed Research International*, 2013, 1–13.
- Dixit, R., Malaviya, D., Pandiyan, K., Singh, U.B., Sahu, A., Shukla, R., Singh, B.P., Rai, J.P., Sharma, P.K., Lade, H. and Paul, D. (2015). Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes. *Sustainability* 7, 2189–2212.
- Ebrahimpour, A., Rahman, R.N.Z.R.A., Ean, D.H., Basri, M. and Salleh, A. (2008). A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus* sp. strain AMR. *BMC Biotechnology* 8, 1–15.
- Elekwachi, C.O., Andresen, J., Hodgman, T.C. (2014). Global use of bioremediation technologies for decontamination of ecosystems. *Journal of Bioremediation & Biodegradation* 05, 1–9.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolutuion* 39 (4), 783-791.
- Fujikawa, H. (2010). Development of a new logistic model for microbial growth in foods. *Biocontrol Science* 15, 75–80.

- Gaines, J.M., Carty, N.L., Colmer-Hamood, J.A. and Hamood, A.N. (2005). Effect of static growth and different levels of environmental oxygen toxA and ptxR expression in the *Pseudomonas aeruginosa* strain PAO1. *Microbiology* 151, 2263–2275.
- Garbisu, C., Alkorta, I., Llama, J. and Serra, J.L. (1998). Aerobic chromate reduction by *Bacillus subtilis*. *Biodegradation* 9, 133–141.
- Ghani, B., Takai, M., Hisham, N.Z., Kishimoto, N., Ismail, A.K.M., Tano, T. and Sugio, T. (1993). Isolation and characterization of a Mo⁶⁺-reducing bacterium. *Applied and Environmental Microbiology* 59, 1176–1180.
- Gibson, A.M., Bratchell, N. and Roberts, T.A. (1987). The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. *Journal of Applied Bacteriology* 62, 479– 490.
- Glenn, J.L. and Crane, F.L. (1956). Studies on metalloflavoproteins v. the action of silicomolybdate in the reduction of cytochrome c by aldehyde oxidase. *Biochimica Et Biophysica Acta* 22, 111–115.
- Gluszcz, P., Petera, J. and Ledakowicz, S. (2011). Mathematical modeling of the integrated process of mercury bioremediation in the industrial bioreactor. *Bioprocess and Biosystems Engineering* 34, 275–285.
- Gompertz, B. (1825). On the nature of the function expressive of the law of human mortality, and on a new mode of determining the value of life contingencies. *Philosophical Transactions of Royal Society of London* 115, 513–585.
- Gupta, A., Joia, J., Sood, A., Sidhu, C. and Kaur, G. (2016). Microbes as potential tool for remediation of heavy metals: A Review. *Journal of Microbial & Biochemical Technology* 8, 364–372.
- Gusmanizar, N., Halmi, M., Rusnam, M., Rahman, M., Shukor, M., Azmi, N. and Shukor, M.Y. (2016). Isolation and characterization of a molybdenumreducing and azo-dye decolorizing *Serratia marcescens* strain neni-1 from Indonesian soil. *Journal of Urban and Environmental Engineering* 10, 113– 123.
- Halmi, M.I.E. (2014). A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Malaysia.
- Halmi, M.I.E., Abdullah, S.R.S., Johari, W.L.W., Ali, M.S.M., Shaharuddin, N.A., Khalid, A. and Shukor, M.Y. (2016). Modelling the kinetics of hexavalent molybdenum (Mo⁶⁺) reduction by the *Serratia* sp. strain MIE2 in batch culture. *Rendiconti Lincei* 27, 653–663.

- Halmi, M.I.E., Ahmad, S.A., Syed, M.A., Shamaan, N.A. and Shukor, M.Y. (2014a). Mathematical modelling of the molybdenum reduction kinetics in *Bacillus pumilus* strain Lbna. *Bulletin of Environmental Science and Management* 2, 24–29.
- Halmi, M.I.E., Wasoh, H., Sukor, S., Ahmad, S.A., Yusof, M.T. and Shukor, M.Y. (2014b). Bioremoval of molybdenum from aqueous solution. *International Journal of Agriculture and Biology* 16, 848–850.
- Halmi, M.I.E., Zuhainis, S.W., Yusof, M.T., Shaharuddin, N.A., Helmi, W., Shukor, Y., Syed, M.A. and Ahmad, S.A. (2013). Hexavalent molybdenum reduction to Mo-blue by a sodium-dodecyl-sulfate- degrading *Klebsiella oxytoca* strain dry14. *BioMed Research International* 2013, 1–8. Article number 384541.
- Hamid, A.A., Usman, L.A., Elaigwu, S.E. and Zubair, M.F. (2010). Environmental and health risk of bush burning. *Advances in Environmental Biology* 4(2), 241–249.
- Hamitouche, A.E., Bendjama, Z., Amrane, A., Kaouah, F. and Hamane, D. (2012). Relevance of the Luong model to describe the biodegradation of phenol by mixed culture in a batch reactor. *Annals of Microbiology* 62(2), 581–586.
- Han, K. and Levenspiel, O. (1988). Extended monod kinetics for substrate, product, and cell inhibition. *Biotechnology and Bioengineering* 32, 430–437.
- Haywood, S., Dincer, Z., Jasani, B. and Loughran, M.J. (2004) Molybdenumassociated pituitary endocrinopathy in sheep treated with ammonium tetrathiomolybdate. *Journal of Comparative Pathology* 130, 21–31.
- Hettiarachchi, G.M., Pierzynski, G.M. and Ransom, M.D. (2000). In situ stabilization of soil lead using phosphorus and manganese oxide. *Environmental Science and Technology* 34, 4614–4619.
- Hoffman, D.R., Okon, J.L., Sandrin and T.R. (2005). Medium composition affects the degree and pattern of cadmium inhibition of naphthalene biodegradation. *Chemosphere* 59, 919–927.
- Hong, H.B., Nam, I.H., Kim, Y.M., Chang, Y.S., Schmidt, S. (2007). Effect of heavy metals on the biodegradation of dibenzofuran in liquid medium. *Journal of Hazardous Materials* 140, 145–148.
- Hora, A. and Shetty, V. (2014). Inhibitory and stimulating effect of single and multimetal ions on hexavalent chromium reduction by *Acinetobacter* sp. Cr-B2. *World Journal of Microbiology and Biotechnology* 30, 3211–3219.
- Huang, L. (2013). Optimization of a new mathematical model for bacterial growth. *Food Control* 32, 283–288.

- Ibrahim, Y., Abdel-Mongy, M., Shukor, M.S., Hussein, S., Ling, A.P.K. and Shukor, M.Y. (2015). Characterization of a molybdenum-reducing bacterium with the ability to degrade phenol, isolated in soils from Egypt. *Biotechnologia: Journal of Biotechnology, Computational Biology and Bionanotechnology* 96, 234–245.
- Ibrahim, S., Shukor, M.Y., Khalil, K.A., Halmi, M.I.E., Syed, M.A. and Ahmad, S.A. (2015). Application of response surface methodology for optimising caffeinedegrading parameters by *Leifsonia* sp. strain SIU. *Journal of Environmental Biology* 36, 1215–1221.
- Ilyin, I., Rozovskaya, O., Travnikov, M., Varygina, M. (2015). Heavy metals: Analysis of long-term trends, country-specific research and progress in mercury regional and global modelling. *EMEP Status report* 3, 1-70
- Jan, A.T., Azam, M., Ali, A., Haq, Q.M.R. (2013). Prospects for exploiting bacteria for bioremediation of metal pollution. *Critical Reviews in Environmental Science and Technology* 519–560.
- Jeter, M.A. and Davis, G.K. (1953). The effect of dietary molybdenum upon growth, hemoglobib, reproduction and lactation of rats. *The Journal of Nutrition* 217–220.
- Johnsen, A.R., Binning, P.J., Aamand, J., Badawi, N. and Rosenbom, A.E. (2013). The Gompertz function can coherently describe microbial mineralization of growth-sustaining pesticides. *Environmental Science and Technology* 47, 8508–8514.
- Jukes, T.H. and Cantor, C.R. (1969). Evolution of protein molecules. In 'Mammalian Protein Metabolism' (Ed. HN Munro.) Academic Press, New York pp. 21–132.
- Kaplan, D. (2013). Absorption and adsorption of heavy metals by microalgae. Handbook of Microalgal Culture: Applied Phycology and Biotechnology 602– 611.
- Karamba, K.I., Ahmad, S.A., Zulkharnain, A., Syed, M.A., Khalil, K.A., Shamaan, N.A., Dahalan FA, Shukor MY (2016). Optimisation of biodegradation conditions for cyanide removal by *Serratia marcescens* strain AQ07 using one-factor-at-a-time technique and response surface methodology. *Rendiconti Lincei* 27, 533–545.
- Karamba, K.I., Shukor, M.Y., Syed, M.A., Zulkharnain, A. and Adeela, N. (2015). Isolation, screening and characterisation of cyanide-degrading *Serratia marcescens* strain AQ07. *Journal of Chemical and Pharmaceutical Sciences* 8, 401–406.
- Kargar, M., Khorasani, N., Karami, M., Rafiee, G. and Naseh, R. (2011). Study of aluminum, copper and molybdenum pollution in groundwater sources surrounding (Miduk) Shahr-E-Babak copper complex tailings dam. World Academy of Science, Engineering & Technology 52, 278–282.

- Khayat, M.E., Abd Rahman, M.F., Shukor, M.S., Ahmad, S.A., Shamaan, N.A. and Shukor, M.Y. (2016). Characterization of a molybdenum-reducing *Bacillus* sp. strain khayat with the ability to grow on SDS and diesel. *Rendiconti Lincei* 27, 547–556.
- Khuri, I.A. and Mukhopadhyay, S. (2010). Response surface methodology. *Advance Review WIREs computational statistics, John Wiley & Sons Inc* 2, 128–149.
- King, R.B., Long, K. and Sheldon, J.K. (1992). 'Practical Environmental Bioremediation: The Field Guide (2nd Edition), *Lewis Publisher, Florida*, pp 208.
- Lee, J.D. (1977). 'Concise Inorganic Chemistry.' (3rd Edition), *Van Reinhold Co., New York*, pp 325.
- Lee, K. and Park, G. (2006). A global robust optimization using Kriging based approximation model. *JSME International Journal Series C* 49, 779–788.
- Levine, V.E. (1924). The reducing properties of microorganisms with special reference to selenium compounds. *Journal of Bacteriology* X, 217–262.
- Li, P. and Tao, H. (2015). Cell surface engineering of microorganisms towards adsorption of heavy metals. *Critical reviews in microbiology* 51(2), 140-149.
- Liau, S.Y., Read, D.C., Pugh, W.J., Furr, J.R. and Russell, A.D. (1997). Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions. *Letters in Applied Microbiology* 25, 279–283.
- Lim, H.K., Syed, M.A. and Shukor, M.Y. (2011). Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. *Journal of Basic Microbiology* 51, 1–10.
- Lim, H.K., Syed, M.A. and Shukor, M.Y. (2012). Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. *Journal of Basic Microbiology* 52, 296–305.
- Lloyd, J.R. (2003). Microbial reduction of metals and radionuclides. *FEMS Microbiology Reviews* 27, 411–425.
- López, S., Prieto, M., Dijkstra, J., Dhanoa, M.S. and France, J. (2004). Statistical evaluation of mathematical models for microbial growth. *International Journal of Food Microbiology* 96, 289–300.
- Luong, J. H. (1987). Generalization of monod kinetics for analysis of growth data with substrate inhibition. *Biotechnology and Bioengineering* 29(2), 242–248.
- Lyubimov, A.V., Smith, J.A., Rousselle, S.D., Mercieca, M.D., Tomaszewski, J.E., Smith, A.C. and Levine, B.S. (2004). The effects of tetrathiomolybdate (TTM, NSC-714598) and copper supplementation on fertility and early embryonic development in rats. *Reproductive Toxicology* 19, 223–233.

- Majak, W., Steinke, D., Mcgillivray, J. and Lysyk, T. (2004). Clinical signs in cattle grazing high molybdenum forage. *Rangeland Ecology & Management* 57, 269–274.
- Mansur, R., Gusmanizar, N., Dahalan, F.A., Masdor, N.A., Ahmad, S.A., Shukor, M.S., Roslan, M.A.H. and Shukor, M.Y. (2016). Isolation and characterization of a molybdenum-reducing and amide-degrading *Burkholderia cepacia* strain Neni-11 in soils from west Sumatera, Indonesia. *Journal - Institute of Integrative Omics and Applied Biotechnology* 7, 28–40.
- Margush, T. and McMorris, F.R. (1981). Consensus n-trees. *Bulletin of Mathematical Biology* 43, 239–244.
- Masdor, N., Shukor, M.S., Khan, A., Halmi, M.I.E., Abdullah, S.R.S., Shamaan, N.A. and Shukor, M.Y. (2015) Isolation and characterization of a molybdenumreducing and SDS- degrading *Klebsiella oxytoca* strain Aft-7 and its bioremediation application in the environment. *BIODIVERSITAS* 16, 238– 246.
- Masood, F. and Malik, A. (2011). Hexavalent chromium reduction by *Bacillus* sp. strain FM1 isolated from heavy-metal contaminated soil. *Bulletting of Environmental Contamination and Toxicology* 86, 114–119.
- Meyer, M., Pesch, R., Schröder, W., Steinnes, E. and Uggerud, H.T. (2014). Spatial patterns and temporal trends of heavy metal concentrations in moss and surface soil specimens collected in Norway between 1990 and 2010. *Environmental Sciences Europe* 26, 1–18.
- Miller, J.K., Moss, B.R., Bell, M.C. and Sneed, N.N. (1972). Comparison of ⁹⁹Mo metabolism in young cattle and swine. *Journal of Animal Science* 34(5), 846–850.
- Miyazaki, K., Wintrode, P.L., Grayling, R.A., Rubingh, D.N. and Arnold, F.H. (2000). directed evolution study of temperature adaptation in a psychrophilic enzyme. *Journal of Molecular Biology* 297, 1015–1026.
- Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology* 3(1), 371–394.
- Motulsky, H.J. and Ransnas, L.A. (1987). Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1, 365–374.
- Mulchandani, A., Luong, J.H.T. and Groom, C. (1989). Substrate inhibition kinetics for microbial growth and synthesis of poly-fl-hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697:f. *Applied Microbiology and Biotechnology* 30, 11–17.

- Neunhäuserer, C., Berreck, M. and Insam, H. (2001). Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. *Water, air, and soil pollution* 128, 85–96.
- Nickzad, A., Mogharei, A., Monazzami, A., Jamshidian, H. and Vahabzadeh, F. (2012). Biodegradation of phenol by *Ralstonia eutropha* in a kissirisimmobilized cell bioreactor. *Water Environmental Research* 84(4), 626-634.
- Olaniran, A.O., Balgobind, A. and Pillay, B. (2013). Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. *International Journal of Molecular Sciences* 14(5), 10197–10228.
- Opperman, D.J., Piater, L.A., Van Heerden, E. (2008). A novel chromate reductase from *Thermus scotoductus* SA-01 related to old yellow enzyme. *Journal of Bacteriology* 190, 3076–3082.
- Osborne, F.H. and Ehrlich, H.L. (1976). Oxidation of arsenite by a soil isolate of *Alcaligenes. Journal of Applied Biotecnology* 41, 295–305.
- Othman, A.R., Abu Zeid, I.M., Rahman, M.F., Ariffin, F. and Shukor, M.Y. (2015). Isolation and characterization of a molybdenum-reducing and orange Gdecolorizing *Enterobacter* sp. strain Zeid-6 in soils from Sudan. *Bioremediation Science and Technology Research* 3, 13–19.
- Othman, A.R., Bakar, N.A., Halmi, M.I.E., Johari, W.L.W., Ahmad, S.A., Jirangon, H., Syed, M.A. and Shukor, M.Y. (2013) Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. *BioMed Research International* 2013, 1–9. Article number 371058.
- Padhir, A.R. and Modi, H.A. (2013). Optimization of lipase production by Saccharomonospora azurea ap 11 / 18 using plackett-burman design and response surface methodology. International Journal of Bio-Technology and Research (IJBTR) 3, 59–66.
- Page, R.D.M. (1996). On consensus, confidence, and 'total evidence'. *Cladistics* 12, 83–92.
- Pandey, R. and Singh, S.P. (2002). Effects of molybdenum on fertility of male rats. *BioMetals* 15, 65–72.
- Park, C.H., Keyhan, M., Wielinga, B. and Fendorf, S. (2000). Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Applied and Environmental Microbiology* 66, 1788–1795.
- Pitt, M.A. (1976). Review Molybdenum Toxicity: Interactions between copper, molybdenum and sulphate. *Agents and Actions* 6, 758–769.

- Prapulla, S.G., Jacob, Z., Chand, N., Rajalakshmi, D. and Karanth, N.G. (1992). Maximization of lipid production by *Rhodotorula gracilris* CFR- 1 using response surface methodology. *Biotechnology and Bioengineering* 40, 965– 970.
- Prive, G.G. (2007). Detergents for the stabilization and crystallization of membrane proteins. *Methods* 41, 388–397.
- Rahman, M.F.A., Shukor, M.Y., Suhaili, Z., Mustafa, S., Shamaan, N.A. and Syed, M.A. (2009). Reduction of Mo(VI) by the bacterium *Serratia* sp. strain DRY5. *Journal of Environmental Biology* 30, 65–72.
- Rajagopalan, K.V. (1988). Molybdenum: An Essential Trace Element in Human Nutrition. *Annual Review of Nutrition* 8, 401–427.
- Richards, F.J. (1959). A flexible growth function for empirical use. *Journal of Experimental Botany* 10, 290–300.
- Ricker, F.J. (1979). Growth rates and models. 'Fish Physiol.' (Eds WS Hoar, JR Brett, DJ Randall) Bioenergetics and Growth. pp. 677–743.
- Robinson, M.F., McKenzie, J.M., Tomson, C.D. and van Rij, A.L. (1973). Metabolic balance of zinc, copper, cadmium, iron, molybdenum and selenium in young New Zealand women. *The British Journal of Nutrition* 30, 195–205.
- Rosca, M., Hlihor, R.M., Cozma, P., ComAniţA, E.D., Simion, I.M. and Gavrilescu, M. (2015). Potential of biosorption and bioaccumulation processes for heavy metals removal in bioreactors. *The 5th IEEE International Conference on E-Health and Bioengineering Conference, EHB* 31–34.
- Ross, D.A. (2004). The toxicology of mercury. *The New England Journal of Medicine* 350(9), 945–947.
- Ross, T., McMeekin, T.A. (1994). Predictive microbiology. International Journal of Food Microbiology 23, 241–264.
- Runnells, D.D. (1976). Wastewaters in the vadose zone of arid regions: Geochemical interactions. *Ground Water* 14, 374–385.
- Sabullah, M.K., Rahman, M.F., Ahmad, S.A., Sulaiman, M.R., Shukor, M.S., Shamaan, N.A. and Shukor, M.Y. (2016). Isolation and characterization of a molybdenum-reducing and glyphosate-degrading *Klebsiella oxytoca* strain saw-5 in soils from sarawak. *Agrivita* 38, 1–13.
- Sahinkaya, E. and Dilek, B.D. (2007). Modeling chlorophenols degradation in sequencing batch reactors with instantaneous feed-effect of 2,4-DCP presence on 4-CP degradation kinetics. *Biodegradation* 18, 427–437.

- Said, W.A. and Lewis, D.L. (1991). Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. *Applied and Environmental Microbiology* 57, 1498–1503.
- Saitou, N., and Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4), 406–425.
- Sandrin, T.R., Chech, A.M. and Maier, R.M. (2000). A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. *Applied and Environmental Microbiology* 66, 4585–4588.
- Sandrin, T.R. and Maier, R.M. (2003). Impact of metals on the biodegradation of organic pollutants. *Environmental Health Perspectives* 111, 1093–1101.
- Sani, R.K., Peyton, B.M. and Brown, L.T. (2001). Copper-induced inhibition of growth of *Desulfovibrio desulfuricans* G20: assessment of its toxicity and correlation with those of zinc and lead. *Applied and Environmental Microbiology* 67(10), 4765–4772.
- Sarubbo, L.A., Rocha, R.B., Luna, J.M., Rufino, R.D., Santos, V.A. and Banat, I.M. (2015). Some aspects of heavy metals contamination remediation and role of biosurfactants. *Chemistry and Ecology* 7540, 1–17.
- Schroeder, H.A., Frost, D.V. and Balass, J.J. (1970). Essential metals in man: selenium. *Journal of Chronic Diseases* 23, 227–243.
- Scopes, R.K. (1994). 'Protein Purification: Principle and practice.' (CR Cantor, Ed.). (Springer Science+Business Media New York: New York)
- Sebenik, R.F., Burkin, A.R. and Dorfler, R.R. (2013). Molybdenum and molybdenum compounds. (Neitherland) doi:10.1002/14356007.a16_655.
- Shen, H. and Wang, Y. (1995). Simultaneous chromium reduction and phenol degradation in a coculture of *Escherichia coli* ATCC 33456 and *Pseudomonas putida* DMP-1. *Applied and Environmental Microbiology* 61, 2754–2758.
- Shukor, M.Y. (2014). Short Communications: Revisiting the role of the electron transport chain in molybdate reduction by Enterobacter cloacae strain 48. *Indian Journal of Biotechnology* 13, 404–407.
- Shukor, Y., Adam, H., Ithnin, K., Yunus, I., Shamaan, N.A. and Syed, A. (2007). Molybdate reduction to molybdenum blue in microbe proceeds via phosphomolybdate intermediate. *Journal of Biological Sciences* 7, 1448– 1452.
- Shukor, M.Y., Ahmad, S.A., Nadzir, M.M.M., Abdullah, M.P., Shamaan, N.A. and Syed, M.A. (2010a). Molybdate reduction by *Pseudomonas* sp. strain DRY2. *Journal of Applied Microbiology* 108, 2050–2058.

- Shukor, M.Y., Habib, S.H.M., Rahman, M.F.A., Jirangon, H., Abdullah, M.P.A., Shamaan, N.A. and Syed, M.A. (2008a). Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain Dr. Y6. *Applied Biochemistry and Biotechnology* 149, 33–43.
- Shukor, M.Y., Halmi, M.I.E., Rahman, M.F.A., Shamaan, N.A. and Syed, M.A. (2014). Molybdenum reduction to molybdenum blue in *Serratia* sp. strain DRY5 is catalyzed by a novel molybdenum-reducing enzyme. *BioMed Research International* 2014, 1–8. Article ID 853084,
- Shukor, M., Hamdan, M.H., Othaman, M.A., Shamaan, N.A. and Syed, M.A. (2009b). Mo (VI) reduction to molybdenum blue by *Serratia marcescens* strain Dr. Y9. *Polish Journal of Microbiology* 58, 141–147.
- Shukor, M.S., Khan, A., Masdor, N., Halmi, M.I.E., Abdullah, S.R.S. and Shukor, M.Y. (2016). Isolation of a novel molybdenum-reducing and azo dye decolorizing *Enterobacter* sp. strain Aft-3 from Pakistan. *Chiang Mai University Journal of Natural Sciences* 15, 95–114.
- Shukor, M.Y., Lee, C.H., Omar, I., Karim, M.I.A., Syed, M.A. and Shamaan, N.A. (2003). Isolation and characterization of a molybdenum-reducing enzyme in *Enterobacter cloacae* strain 48. *Pertanika Journal of Science and Technology* 11, 261–272.
- Shukor, M.Y., Rahman, M.F.A., Shamaan, N.A., Lee, C.H., Karim, M.I.A. and Syed, M.A. (2008b). An improved enzyme assay for molybdenum-reducing activity in bacteria. *Applied Biochemistry & Biotechnology* 144, 293–300.
- Shukor, M.Y., Rahman, M.F., Shamaan, N.A. and Syed, M.S. (2009c). Reduction of molybdate to molybdenum blue by *Enterobacter* sp. strain Dr.Y13. *Journal of Basic Microbiology* 49, 43–54.
- Shukor, M.Y., Rahman, M.F., Suhaili, Z., Shamaan, N.A., Syed, M.A. (2009a). Bacterial reduction of hexavalent molybdenum to molybdenum blue. *World Journal of Microbiology and Biotechnology* 25, 1225–1234.
- Shukor, M.Y., Rahman, M.F., Suhaili, Z., Shamaan, N.A., Syed, M.A. (2010b. Hexavalent molybdenum reduction to Mo-blue by *Acinetobacter* calcoaceticus. Folia Microbiologica 55, 137–143.
- Shukor, M.Y.A., Shamaan, N.A., Syed, M.A., Lee, C.H. and Karim, M.I.A. (2000). Characterization and quantification of molybdenumm blue production in *Enterobacter cloacae* strain 48 using 12-molybdophophate as reference compound. Asia Pacific Journal of Molecular Biology and Biotechnology 8, 167–172.
- Shukor, M.S. and Shukor, M.Y. (2014). Statistical diagnostic tests of the Luong model in fitting molybdenum reduction from the bacterium *Bacillus* sp. strain A.rzi. *Journal of Environmental Microbiology and Toxicology* 2, 53–57.

- Shukor, M.S. and Shukor, M.Y. (2015). Bioremoval of toxic molybdenum using dialysis tubing. *Chemical Engineering Research Bulletin* 18, 6–11.
- Shukor, M.Y. and Syed, M.A. (2010c). Microbiological reduction of hexavalent molybdenum to molybdenum blue. 'Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol.' (Ed A Mendez-Vilas) Microbiology Book Series.(Formatex Research Center: Badajoz, Spain)
- Shukor, M.Y., Syed, M.A., Lee, C.H., Karim, M.I.A. and Shamaan, N.A. (2002). A method to distinguish between chemical and enzymatic reduction of molybdenum in *Enterobacter cloacae* strain 48. *Malaysian Journal of Biochemistry* 7, 71–72.
- Silver, S. and Phung, L.T. (1996). Bacterial Heavy Metal Resistance: New Surprises. *Annual Review of Microbiology* 50, 753–789.
- Singh, A. and Prasad, S.M. (2011). Reduction of heavy metal load in food chain: technology assessment. *Review in Environmental Science & Biotechnology* 10, 199–214.
- Sinnakkannu, S., Abdullah, A.R., Tahir, N.M. and Abas, M.R. (2004). Degradation of metsulfuron methyl in selected Malaysian agricultural soils. *Fresenius Environmental Bulletin* 13, 258–261.
- Soares, E.V. and Soares, H.M.V.M. (2012). Bioremediation of industrial effluents containing heavy metals using brewing cells of *Saccharomyces cerevisiae* as a green technology: A review. *Environmental Science and Pollution Research* 19, 1066–1083.
- Soda, S.O., Yamamura, S., Zhou, H., Ike, M. and Fujita, M. (2006). Reduction kinetics of As (V) to As (III) by a dissimilatory arsenate-reducing bacterium, *Bacillus* sp. SF-1. *Biotechnology and Bioengineering* 93, 812–815.
- Soo, E.L., Salleh, A., Basri, M., AbdulRahman, R.N.Z.R. and Kamaruddin, K. (2003).
 Optimization of the enzyme-catalyzed synthesis of amino acid-based surfactants from palm oil fractions. *Journal of Biosience and Bioengineering* 95, 361–367.
- Stafford, J.M., Lambert, C.E., Zyskowski, J.A., Engfehr, C.L., Fletcher, O.J., Clark, S.L., Tiwary, A., Gulde, C.M. and Sample, B.E. (2016). Dietary toxicity of soluble and insoluble molybdenum to northern bobwhite quail (*Colinus virginianus*). *Ecotoxicology* 25, 291–301.
- Stoll, V.S. and Blanchard, J.S. (1990). Buffers: Principles and Practice. *Methods Enzymology* 182, 24–38.
- Sugio, T., Tsujita, Y., Katagiri, T., Inagaki, K. and Tano, T. (1988). Reduction of Mo⁶⁺ with elemental sulfur by *Thiobacillus ferrooxidans*. *Journal of Bacteriology* 170, 5956–5959.

- Sukumar, M. (2010). Reduction of hexavalent chromium by *Rhizopus oryzae*. African Journal of Environmental Science and Technology 4, 412–418.
- Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). Clustal W: Improving the sensitivity of progressive multiple sequence alignent through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Truex, M.J., Peyton, B.M., Valentine, N.B. and Gorby, Y.A. (1997). Kinetics of U(VI) reduction by a dissimilatory Fe(iii)-reducing bacterium under non-growth conditions. *Biotechnology and Bioengineering* 55, 490-496.
- Vimalashanmugam, K. and Viruthagiri, T. (2013). Research article medium optimization for solid state fermentative production of xylanase by *Aspergillus terreus* using central composite design. *Innovative Romanian Food Biotechnology* 13, 18–29.
- Ward, G.M. (1978). Molybdenum toxicity and hypocuprosis in ruminants : A review. *Journal of Animal Science* 46, 1078–1085.
- Wayman, M. and Tseng, M.C. (1976). Inhibition-threshold substrate concentrations. *Biotechnology and Bioengineering* 18(3), 383–387.
- Wheeler, P.A. and Kirchman, D.L. (1986). Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnology and Oceanographynology and Oceanography* 31, 998–1009.
- World Health Organization, WHO (2011). Molybdenum in Drinking-water Background document for development of WHO Guidelines for Drinkingwater Quality.
- Wu, S., Hu, C., Tan, Q., Nie, Z. and Sun, X. (2014). Effects of molybdenum on water utilization, antioxidative defense system and osmotic-adjustment ability in winter wheat (*Triticum aestivum*) under drought stress. *Plant Physiology and Biochemistry* 83, 365–374.
- Wuana, R.A. and Okieimen, F.E. (2011). Heavy metals in contaminated soils : A review of sources, chemistry, risks and best available strategies for remediation. *International Scholarly Research Network Ecology*, Volume 2011.
- Yamaguchi, S., Miura, C., Ito, A., Agusa, T., Iwata, H., Tanabe, S., Tuyen, B.C. and Miura, T. (2007). Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: Monitoring at Mekong Delta area and in vitro experiment. *Aquatic Toxicology* 83, 43–51.
- Yano, T. and Koga, S. (1969). Dynamic behavior of the chemostat subject to substrate inhibition. *Biotechnology and Bioengineering* 11(2), 139–153. http://doi.org/10.1002/bit.260110204.

- Yoshimura, K., Ishii, M. and Tarutani, T. (1986). Microdetermination of phosphate in water by gel-phase colorimetry with molybdenum blue. *Analytical Chemistry* 58, 591–594.
- Yong, N.K., Oshima, M., Blake, R.C. and Sugio, T. (1997). Isolation and some properties of an iron-oxidizing bacterium *Thiobacillus ferrooxidans* resistant to molybdenum ion. *Bioscience Biotechnology Biochemistry*, 61(9), 1523– 1526.
- You, X.Y., Wang, H., Ren, G.Y., Li, J.J., Duan, X., Zheng, H.J. and Jiang, Z.Q. (2015). Complete genome sequence of the molybdenum-resistant bacterium *Bacillus subtilis* strain LM 4–2. *Standards in Genomic Sciences* 10, 127.
- Zakaria, Z.A., Zakaria, Z., Surif, S. and Ahmad, W.A. (2007). Hexavalent chromium reduction by *Acinetobacter haemolyticus* isolated from heavy-metal contaminated wastewater. *Journal of Hazardous Materials* 146, 30–38.
- Zhai, X., Zhang, Y., Qi, Q., Bai, Y., Chen, X., Jin, L., Ma, X., Shu, R., Yang, Z. and Liu, F. (2013). Effects of molybdenum on sperm quality and testis oxidative stress. Systems Biology in Reproductive Medicine 59, 1–5.
- van Zwieten, L., Ayres, M.R. and Morris, S.G. (2003). Influence of arsenic cocontamination on DDT breakdown and microbial activity. *Environmental Pollution* 124, 331–339.
- Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology* 56, 1875–1881.