

UNIVERSITI TEKNOLOGI MALAYSIA

DECLARATION OF THESIS / UNDERGRADUATE PROJECT PAPER AND COPYRIGHT

Author's full name : WAN HASLINDA BINTI WAN AHMADDate of birth : 11 NOVEMBER 1978Title : HEXAVALENT CHROMIUM REDUCTION BY *Acinetobacter**haemolyticus* USING AGRICULTURAL WASTEAcademic Session : 2012/2013

I declare that this thesis is classified as :

CONFIDENTIAL

(Contains confidential information under the Official Secret Act 1972)*

RESTRICTED

(Contains restricted information as specified by the organization where research was done)*

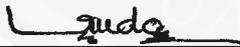
OPEN ACCESS

I agree that my thesis to be published as online open access (full text)

I acknowledged that Universiti Teknologi Malaysia reserves the right as follows:

1. The thesis is the property of Universiti Teknologi Malaysia.
2. The Library of Universiti Teknologi Malaysia has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified by :

**SIGNATURE**781111-11-5412**(NEW IC NO. /PASSPORT NO.)****SIGNATURE OF SUPERVISOR**PROF. DR. WAN AZLINA BINTI AHMAD**NAME OF SUPERVISOR**

Date : 26 December 2013

Date : 26 December 2013

NOTES :

* If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization with period and reasons for confidentiality or restriction.

**HEXAVALENT CHROMIUM REDUCTION BY *Acinetobacter haemolyticus*
USING AGRICULTURAL WASTE**

WAN HASLINDA BINTI WAN AHMAD

A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Chemistry)

Faculty of Science
Universiti Teknologi Malaysia

DECEMBER 2013

ABSTRACT

The high cost of culture growth medium is one of the problems faced in the scaling-up of biological processes involved in wastewater treatment. This makes it imperative to find a useful, cheap and easily available alternative source for culture growth medium. The possibility of using sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM) as alternative sources for culture medium is preferable as these agricultural wastes are easily available, cheap and abundantly grown. The present work highlights the use of SCB, SPW and SBM to sustain the bacterial population for the Cr(VI) reduction process. Growth of *A. haemolyticus* in agricultural wastes was measured by optical density (OD₆₀₀) followed by viable cell counts. Reduction of Cr(VI) was determined using diphenylcarbazide method. For all Cr(VI) concentrations tested (10–100 mg/L), SCB-adapted *A. haemolyticus* showed the highest reduction ranging from 92–99% followed by SPW and SBM with 40–94% and 21–85% reduction respectively. From the FESEM–EDX analysis, toxicity effect can be seen clearly from the shape of bacteria in the presence of 100 mg/L Cr(VI). The FT–IR analysis showed shifting of the C–O band absorption peak from 1252–1261 cm⁻¹ and 1048–1037 cm⁻¹ after Cr-loaded which was due to the binding of Cr(VI) to this functional group. In this study, down-ward biofilm packed-bed reactor was used. A minimum of 4 h was required for complete reduction of Cr(VI) to Cr(III) at the flow rate of 3.0 mL/min using 25 mg/L initial Cr(VI) concentration. Cr(VI) reduction mechanism study using XPS and ESR implies that the Cr bound to the SCB and SCB-adapted *A. haemolyticus* were mostly in trivalent form. SCB can serve as an alternative and cost-effective growth medium for cultivation of *A. haemolyticus* with high percent reduction of Cr(VI). Phylogenetic analysis revealed that the microbial community was dominated by *Chitinophaga terrae*, *Laribacter hongkongensis*, *Ottowia thiooxydans*, *Rhizobium cellulosilyticum*, *Candidate division OP10*, *Pedobacter sp.* and uncultured bacterium.

ABSTRAK

Kos yang tinggi dalam penyediaan media untuk pertumbuhan bakteria adalah salah satu masalah yang timbul dalam proses rawatan air sisa menggunakan kaedah biologi pada skala besar. Maka adalah penting untuk mencari sumber alternatif yang berguna, murah dan mudah didapati untuk pertumbuhan bakteria tersebut. Penggunaan hampas tebu (SCB), sisa pepejal nenas (SPW) dan sisa kacang soya (SBM) sebagai sumber alternatif adalah disarankan kerana ianya murah, mudah dan banyak didapati. Kajian ini menekankan penggunaan SCB, SPW dan SBM untuk mengekalkan populasi bakteria bagi proses penurunan Cr(VI). Pertumbuhan *A. haemolyticus* dalam media sisa pertanian telah ditentukan berdasarkan nilai kekeruhan bakteria (OD_{600}) diikuti oleh pengiraan sel hidup. Penurunan kepekatan Cr(VI) telah ditentukan menggunakan kaedah difenilkarbazida. Bagi semua kepekatan logam kromium yang diuji (10–100 mg/L), bakteria *A. haemolyticus* yang telah menjalani penyesuaian di dalam SCB menunjukkan tahap penurunan yang tertinggi (92–99%) diikuti dengan penyesuaian di dalam SPW (40–94%) dan SBM (21–85%). Daripada analisis FESEM–EDX, kesan toksik dapat dilihat dengan jelas melalui bentuk bakteria dengan kehadiran Cr(VI) berkepekatan 100 mg/L. Analisis FT–IR pula telah menunjukkan anjakan jalur penyerapan C–O daripada 1252–1261 cm^{-1} dan 1048–1037 cm^{-1} yang disebabkan oleh pembentukan ikatan antara Cr(VI) dengan kumpulan berfungsi pada SCB. Dalam kajian ini, turus dengan aliran ke bawah telah digunakan. Masa paling minimum diperlukan bagi melengkapkan penurunan Cr(VI) kepada Cr(III) ialah 4 jam, pada kadar alir 3.0 mL/min dengan kepekatan awal Cr(VI) sebanyak 25 mg/L. Mekanisma penurunan Cr(VI) menggunakan XPS dan ESR membuktikan bahawa Cr terikat kepada SCB dan SCB yang diadaptasi dengan *A. haemolyticus* kebanyakannya dalam bentuk trivalen. SCB boleh dijadikan sebagai media pertumbuhan alternatif dengan kos yang efektif kepada pertumbuhan bakteria *A. haemolyticus* dengan peratus penurunan Cr(VI) yang tinggi. Analisis filogenetik menunjukkan bahawa komuniti mikrob telah didominasi oleh *Chitinophaga terrae*, *Laribacter hongkongensis*, *Ottowia thiooxydans*, *Rhizobium cellulosilyticum*, *Candidata division OP10*, *Pedobacter sp.* dan bakteria yang tidak dikultur.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xiv
	LIST OF FIGURES	xvii
	LIST OF ABBREVIATIONS	xxi
	LIST OF APPENDICES	xxiii
1	INTRODUCTION	
	1.1 Background of the problem	1
	1.2 Statement of the problem	2
	1.3 Objectives of the study	3
	1.4 Scopes of the study	4
	1.5 Significance of the study	4
2	LITERATURE REVIEW	
	2.1 Water resources pollution	5
	2.2 Chromium	7
	2.2.1 Chemical and physical properties	7
	2.2.2 Trivalent and hexavalent chromium	8
	2.2.3 Toxicity of chromium	10

UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI PENDID
N IDRIS	UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI F
2.3	Treatment of chromium-contaminated wastewater	11
2.3.1	Conventional treatment methods	13
2.3.2	Biological treatment methods	15
2.3.2.1	Biosorbent and biosorption	16
2.3.2.2	Bioreduction	17
2.3.2.3	Bioaccumulation	20
2.4	Agricultural waste as support and growth medium for treatment of chromium-contaminated wastewater	22
2.4.1	Sugarcane bagasse	23
2.4.2	Pineapple waste	26
2.4.3	Soybean meal	28
2.5	Microbial technology for Cr(VI) bioremediation	29
2.5.1	Biofilm reactors in Cr(VI) wastewater treatment	30
2.6	Molecular analysis of microbial communities	31
2.6.1	Microbial diversity applications	34
2.7	Cr(VI) reduction mechanism study	36
2.7.1	Microbial reduction of hexavalent chromium	36
2.7.1.1	Aerobic Cr(VI) reduction	38
2.7.1.2	Anaerobic Cr(VI) reduction	40
2.7.2	Determination of the oxidation state of chromium bound to the lignocellulosic residue using instrumental analysis	42
3	MATERIALS AND METHODS	
3.1	Bacterial strain	45
3.1.1	Growth medium	45
3.1.1.1	Nutrient broth	45
3.1.1.2	Nutrient agar	45
3.1.1.3	Luria-Bertani glycerol	46
3.1.2	Bacterial stock culture	46
UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI PENDIDIKA
DRIS	UNIVERSITI PENDIDIKAN SULTAN IDRIS	UNIVERSITI PEN

3.3.5	Growth and Cr(VI) reduction by selected agricultural waste–adapted <i>A. haemolyticus</i>	58
3.3.5.1	Denatured and absolute ethanol as disinfecting agents	58
3.3.5.2	Effect of different concentrations of denatured ethanol on growth of <i>A. haemolyticus</i>	59
3.3.5.3	Growth profile of selected agricultural waste–adapted <i>A. haemolyticus</i>	59
3.3.5.4	Cr(VI) reduction study of selected agricultural waste–adapted <i>A. haemolyticus</i>	60
3.3.6	Chromium desorption study	61
3.4	Continuous Cr(VI) reduction process in packed–bed reactor	61
3.4.1	Laboratory packed–bed reactor set up	61
3.4.2	Immobilization of adapted <i>A. haemolyticus</i> onto SCB	63
3.4.3	Cr(VI) reduction using Cr(VI) stock solution	64
3.4.3.1	Effect of influent flow rate	64
3.4.3.2	Effect of influent Cr(VI) concentration	64
3.4.3.3	Packed–bed reactor performance study	65
3.4.3.4	Evaluation of bacterial growth using dislodging methods	66
3.4.3.5	Assessment of bio film morphology using electron microscopy	66

4 RESULTS AND DISCUSSION

4.1	Characterization of agricultural wastes	80
4.2	Growth and Cr(VI) reduction of <i>A. haemolyticus</i> in agricultural wastes in a batch system	84
4.2.1	Screening of agricultural wastes as an alternative growth medium for <i>A. haemolyticus</i>	84
4.2.2	Adaptation of <i>A. haemolyticus</i> in agricultural wastes	87
4.2.3	Cr(VI) reduction study of agricultural wastes adapted and non-adapted <i>A. haemolyticus</i>	88
4.2.4	Growth and Cr(VI) reduction by SCB-adapted <i>A. haemolyticus</i>	91
4.2.4.1	Denatured and absolute ethanol as disinfecting agents	91
4.2.4.2	Effect of different concentrations of denatured ethanol on growth of <i>A. haemolyticus</i>	92
4.2.4.3	Growth profile of SCB-adapted <i>A. haemolyticus</i>	93
4.2.4.4	Cr(VI) reduction study of SCB-adapted <i>A. haemolyticus</i>	96
4.2.5	Chromium desorption study	101
4.3	Continuous Cr(VI) reduction process in packed-bed reactor	102
4.3.1	Immobilization of adapted <i>A. haemolyticus</i> onto SCB	102
4.3.2	Cr(VI) reduction using Cr(VI) stock solution	104
4.3.2.1	Effect of influent flow rate	104

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Common health problems exerted on humans by heavy metals (Singh <i>et al.</i> , 2011; Barakat, 2011)	6
2.2	Environmental Quality Regulation (Industrial Effluent) 2009, amendment on Environmental Quality Act 1974 (Department of Environment, 2009). Standard A – industrial wastewater within the catchment area, Standard B – industrial wastewater outside the catchment area	12
2.3	Bacterial biomass and agricultural products as biosorbents (Saha and Orvig, 2010)	16
2.4	Microbial populations that transform Cr(VI) to Cr(III)	18
2.5	Basic composition of SCB	24
2.6	Microorganism cultivated on SCB and the products (Parameswaran, 2009)	26
2.7	Composition of SPW (Abdullah and Mat, 2008)	27
2.8	Composition of LPW	27
2.9	Cr(VI) reduction using the continuous-flow and fixed-film bioreactors at pilot-scale level (Ahmad <i>et al.</i> , 2010b)	29
2.10	Current techniques that can be used to study wastewater microorganisms (Gilbride <i>et al.</i> , 2006)	32
3.1	The sources of AW and the fibre preparation for characterization	47
3.2	Operating parameters for anion separation by suppressed IC with an anion-exchange column under isocratic conditions	54

3.3	Adaptation of <i>A. haemolyticus</i> in AW for growth profile monitoring	55
3.4	Cr(VI) and active culture used for the Cr(VI) reduction	58
3.5	Different concentrations of ethanol for growth profile monitoring	59
3.6	Cr(VI) and SCB-adapted <i>A. haemolyticus</i> culture used for time-dependent study	61
4.1	Composition of SCB, SPW and SBM. Data shown are the mean value of three different batches	81
4.2	Turbidity and cell concentration profiles for 10% (w/v) AW-adapted <i>A. haemolyticus</i> , 85% (v/v) deionised water, 5% (v/v) denatured ethanol and 10% (v/v) active culture	88
4.3	Percentage reduction of 10–100 mg/L Cr(VI) by AW-adapted and non-adapted <i>A. haemolyticus</i> after 48 h contact time	89
4.4	Effect of ethanol pre-treatment on SCB	92
4.5	Turbidity and cell concentration profiles for <i>A. haemolyticus</i> grown in pre-treated SCB	93
4.6	Turbidity and cell concentration profiles for <i>A. haemolyticus</i> grown overnight in increasing volumes of deionised water in the presence of 10% (w/v) SCB	94
4.7	Percentage of Cr desorption	102
4.8	The plasmid DNA concentration and purity of each selected clone for (a) control (C5) and (b) test (T5) samples	120
4.9	Taxonomic affiliations and abundance of 16S rRNA sequence types as defined by RFLP analysis	124
4.10	Elemental composition of raw and Cr-loaded SCB as determined using XPS	128
4.11	Components of Cr2p high-resolution spectra for Cr(VI)-loaded SCB samples as obtained using XPS. Initial Cr(VI) concentration (100 mg/L), biomass dosage (10% w/v), exposure time (48 h) and pH (3.29–4.36)	131

4.12

FT-IR analysis of *A. haemolyticus* grown onto SCB with and without Cr(VI) after 48 h contact time



LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Eh–pH predominance diagram for aqueous Cr at 25 °C (Reeder <i>et al.</i> , 2006)	10
2.2	Bacterium of <i>A. haemolyticus</i> (Zakaria <i>et al.</i> , 2006)	19
2.3	Schematic representation of the experimental set up for the Cr(VI) reduction system using a 0.2 m ³ bioreactor; A – nutrient tank, B – raw Cr(VI) wastewater tank, C – mixing tank, D – holding tank, E – bioreactor, F – receiving tank, G – flocculation and coagulation section, H – sludge drying bed, I – sludge collecting tank, J – powdered activated carbon column (Ahmad <i>et al.</i> , 2010a)	20
2.4	Secondary cell wall (CW) structure of cellulose, hemicelluloses and lignin in lignocellulosic materials. For SCB, the basic composition is 40% cellulose, 28% hemicelluloses and 22% lignin (Lee, 2005)	24
2.5	Biochemical processes in industrial bagasse feedstock piles based on microbial biodiversity (Rattanachomsri <i>et al.</i> , 2011)	36
2.6	Biochemical processes in industrial bagasse feedstock piles based on microbial biodiversity (Rattanachomsri <i>et al.</i> , 2011)	40
3.1	Schematic representation of laboratory packed–bed reactor of the Cr(VI) reduction system	62
3.2	Sampling site for pineapple liquid effluent at the waste treatment plant of a pineapple–processing facility in Tampoi, Johor Bahru	63
3.3	Schematic representations for purification of PCR products	70

3.4	Blue/white screening on the LB agar plate incorporated with 80 µg/mL ampicillin	72
3.5	Plate of colony PCR	74
4.1	Effect of different concentration of SCB, SPW and SBM, sterilized with 5% (v/v) denatured ethanol towards bacterial counts	85
4.2	Profile for total carbohydrate for 10% (w/v) SCB, SPW and SBM during growth of <i>A. haemolyticus</i>	86
4.3	pH profiles for 10% (w/v) SCB, SPW and SBM during growth of <i>A. haemolyticus</i>	87
4.4	CFU/mL of <i>A. haemolyticus</i> grown in 10% (w/v) SCB in deionised water	95
4.5	Percentage reduction of 100 mg/L Cr(VI) by SCB control and SCB-adapted <i>A. haemolyticus</i>	96
4.6	Cr(VI) reduction profile of SCB control, SCB-adapted and non-adapted <i>A. haemolyticus</i> of (a) total Cr, (b) Cr(VI) and (c) Cr(III)	97
4.7	Time-dependent concentrations profile of SCB-adapted <i>A. haemolyticus</i> based on percentage of reduction achieved	99
4.8	Distribution of <i>A. haemolyticus</i> cells in (a) effluent and (b) SCB. Ah culture = adapted <i>A. haemolyticus</i> culture, Ah/DI3 = adapted <i>A. haemolyticus</i> culture with continuous circulation for 3 days, NB2 = supplementation with NB for 2 days and LPW1 = supplementation with LPW for 1 day	104
4.9	Time to achieved complete reduction towards different of influent flow rate	105
4.10	Time to achieved complete reduction towards different of influent Cr(VI) concentration	107
4.11	Profile of chromium concentration during Cr(VI) reduction using seventh batches of Cr(VI) (B1–B7) at a flow rate of 3 mL/min and 25 mg/L Cr(VI) concentration.	108
4.12	Profiles of abiotic reduction of LPW for all batches studied (B1–B7). 0 = Cr(VI), (0)* = Cr(VI):LPW, (0)** = Cr(VI):LPW pH 7	109

- 4.13 Profile of total carbohydrate, COD, OD₆₀₀ and pH during Cr(VI) reduction of test reactor. DI = effluent after rinsed with deionised water, Ah3 = adapted *A. haemolyticus* culture with continuous circulation for 3 days, NB2 = supplementation with NB for 2 days, LPWif = initial reading for filtered LPW, LPWff = final reading for filtered LPW and B1–B7 = Cr(VI) of 25 mg/L for first batch to seventh batches 111
- 4.14 Formation of biofilm inside the reactor (a) before rinse with sterile deionised water, (b) after immobilization with pure *A. haemolyticus* culture (4 days) and (c) after introduction of seventh batches of Cr(VI) at 25 mg/L (32 days) 113
- 4.15 FESEM micrographs and EDX spectra of (a) control reactor and (b) test reactor after supplementation with LPW (7 days reactor operation), while (c) control reactor and (d) test reactor after first batch treatment of Cr(VI) (8 days reactor operation) at magnification of 10, 000X. Pt peak is associated with platinum sputter coating 114
- 4.16 SEM micrographs of (a) control reactor and (b) test reactor after 61 days of reactor operation at magnification of 6400X 115
- 4.17 Agarose gel electrophoresis of PCR–amplified product of 16S rDNA. M: 100 bp plus marker; C: Control sample; T: Test sample, 2 and 5: Volume of DNA used (μL) 116
- 4.18 Agarose gel electrophoresis of positive and negative colony PCR band. Numbers in red circle show the negative colony. M: 100 bp plus marker 117
- 4.19 Agarose gel electrophoresis of purified PCR products. M: 100 bp plus marker; (a): Control sample, C5; (b): Test sample, T5 118
- 4.20 Agarose gel electrophoresis of RFLP bands of purified PCR products. M: 100 bp plus marker; (a): Control sample, C5 (5 clones with different RFLP patterns); (b): Test sample, T5 (8 clones with different RFLP patterns) 119
- 4.21 Phylogram shows phylogenetic relationships of selected 13 dominant patterns 125

4.22	Wide scan of XPS spectra for (a) SCB adapted <i>A. haemolyticus</i> , (b) SCB in the present of 100 mg/L Cr(VI), (c) SCB adapted <i>A. haemolyticus</i> in the present of 100 mg/L Cr(VI), (d) SCB, (e) standard Cr(III) and (f) standard Cr(VI)	128
4.23	Narrow-resolution spectra collected from the Cr2p core region; (a) standard Cr(VI) and Cr(III) used and (b) Cr-loaded SCB and Cr-loaded SCB adapted <i>A. haemolyticus</i>	130
4.24	Narrow range of Cr2p spectra for (a) SCB in the present of 100 mg/L Cr(VI) and (b) SCB adapted <i>A. haemolyticus</i> in the present of 100 mg/L Cr(VI)	131
4.25	ESR spectra of standard Cr(III) and Cr(VI) at wide range for (a) solid and narrow range for (b) aqueous Cr(III) and (c) aqueous Cr(VI) at concentration of 100, 150, 300 and 500 mg/L	135
4.26	ESR spectra of (a) solid and (b) liquid samples	136
4.27	ESR spectra of (a) solid and (b) aqueous samples of Cr-loaded SCB-adapted <i>A. haemolyticus</i> at Cr(VI) concentration of 150, 300 and 500 mg/L	137
4.28	Bacterial attachments (a) in the pore and (b) at the surface of SCB	141
4.29	SEM micrograph and EDX spectra of (a) raw SCB, (b) <i>A. haemolyticus</i> cells grown on SCB, (c) SCB in the presence of 100 mg/L Cr(VI) and (d) <i>A. haemolyticus</i> cells grown on SCB in the presence of 100 mg/L Cr(VI) at magnification of 3000X	143
4.30	Proposed mechanism of Cr(VI) reduction (modified from Park <i>et al.</i> , 2007)	144

LIST OF ABBREVIATIONS

<i>A. haemolyticus</i>	-	<i>Acinetobacter haemolyticus</i>
AAS	-	Atomic Absorption Spectrophotometer
ADMI	-	American Dye Manufacturers Institute
APHA	-	American Public Health Association
ARDRA	-	Amplified Ribosomal Deoxyribonucleic acid Restriction Analysis
ASTM	-	American Society for Testing and Materials
ATP	-	adenosine triphosphate
AW	-	agricultural wastes
BLASTn	-	Basic Local Alignment Search Tool
bp	-	base pairs
CFU	-	colony forming unit
COD	-	chemical oxygen demand
Cr(III)	-	Chromium (III)
Cr(VI)	-	Chromium (VI)
DGGE	-	Denaturing Gradient Gel Electrophoresis
DNA	-	deoxyribonucleic acid
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
DOE	-	Department of Environment
DPC	-	1,5-diphenylcarbazine
<i>E. coli</i>	-	<i>Escherichia coli</i>
EPR	-	Electronic Paramagnetic Resonance
ESR	-	Electron Spin Resonance
EXAFS	-	Extended X-Ray Absorption Fine Structure
FESEM-EDX	-	Field Emission Scanning Electron Microscope coupled with Energy Dispersive X-Ray

FRIM	-	Forest Research Institute Malaysia
FT-IR	-	Fourier Transform–Infra Red
ICP-MS	-	Inductively Coupled Plasma–Mass Spectrometer
id	-	inner diameter
LB	-	Luria–Bertani
LPW	-	liquid pineapple waste
NA	-	Nutrient agar
NADH	-	nicotinamide adenine dinucleotide
NB	-	Nutrient broth
NCBI	-	National Center for Biotechnology Information
OD	-	optical density
od	-	outer diameter
PCR	-	polymerase chain reaction
rDNA	-	ribosomal deoxyribonucleic acid
RFLP	-	Restriction Fragment Length Polymorphism
RNA	-	ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
SBM	-	soybean meal
SCB	-	sugarcane bagasse
SPW	-	solid pineapple waste
TAE	-	tris–acetate–EDTA
TEM	-	Transmission Electron Microscope
T-RFLP	-	Terminal Restriction Fragment Length Polymorphism
v/v	-	volume per volume
w/v	-	weight per volume
XANES	-	Absorption Near–Edge Structure
XAS	-	X–Ray Absorption Spectroscopy
XPS	-	X–Ray Photoelectron Spectroscopy

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	Blast results of dominant clones	172
B	GenBank database (Accession numbers: EF369508)	173
C	GenBank database (Accession numbers: KC295702 to KC295714)	174
D	FT-IR spectra	187
E	List of publication (journal/article) and paper presentation	191



CHAPTER 1

INTRODUCTION

1.1 Background of the problem

Chromium (Cr) contaminated wastewater can originate from a multitude of sources. Cr was employed in leather tanning, textile dyeing and wood preserving. Consequently, effluents may contain a wide range of concentrations of either Cr(VI) or Cr(III) or both. Conventional methods for removing chromates from effluents include ion exchange, electrochemical treatments and membrane technologies. Nevertheless, these methods are expensive due to their requirements for high energy or used large quantities of chemicals and may be ineffective for the lower concentrations. Therefore, a biological based system comprising of living cells and untreated agricultural wastes (AW) were used for the removal of Cr(VI) from industrial wastewater effluent. Bacterial biofilm formed during the immobilization and supplementation process was used as the agent to reduce Cr(VI) to Cr(III). A 'ChromeBacTM system' was developed and applied to solve the Cr problem in the industrial wastewater.

The high cost of culture growth medium is one of the problems faced in the scaling-up of biological processes involved in wastewater treatment. This makes it imperative to find a useful, cheap and easily available alternative source for culture growth medium (Ahmad *et al.*, 2009a). Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues. Agricultural waste can replace glucose and other nutrient sources in the media. Application of agro-industrial residues in bioprocesses on the one hand, provides

alternative substrates, and on the other hand helps in solving pollution problems, which their disposal may otherwise cause.

1.2 Statement of the problem

This study is an extension of the previous study completed from the Cr(VI) reduction system i.e. ChromeBac™. This system was developed at the laboratory and pilot-scale in Universiti Teknologi Malaysia (UTM), Skudai since 2005. ChromeBac™ is a novel and environmentally-friendly system to treat Cr(VI) bearing water consisting of bioreactor packed with sawdust-immobilized Cr(VI) resistant-reducing bacteria (*Acinetobacter haemolyticus*). This bacteria (*A. haemolyticus*, GenBank Accession No. EF369508) acts as the primary bacterium in the ensuing biofilm formed during the non-sterile Cr(VI) reduction process using real Cr(VI) containing industrial wastewaters. During the ChromeBac™ process, there are three important observations that need to be immediately addressed or explained.

Firstly, the issue of having liquid pineapple waste (LPW) as a nutrient. Even though, LPW acts as an excellent, cheap and abundant source of nutrients, it also contributes to the high COD content in the effluent before the post treatment step. Therefore, this study aims to look into the possibility of having other types of excellent, cheap and abundant source of nutrients (targeted from discharge of the agricultural industries).

Secondly, the effect of nutrient supplemented by LPW on the microbial community thriving on the biofilm formed. Previous research has reported on the formation of biofilm during the ChromeBac™ process, and the isolation of bacterial species present has been attempted (Zakaria *et al.*, 2007a; Ahmad *et al.*, 2009b). However, the attempts were not successful due to the morphology based isolation procedure and the possible presence of uncultivable bacteria in the biofilm. Therefore, this study plans to apply other techniques such as culture-independent

approach as suggested by other researchers (Wagner–Dobler *et al.*, 2000; Von Canstein *et al.*, 2002).

Thirdly, the Cr(VI) reduction–resistance mechanisms of the bacteria, primarily *A. haemolyticus*. Previous research demonstrated that the Cr(VI) reduction–resistance mechanisms for the bacterium occur aerobically in the soluble protein fraction (Zakaria *et al.*, 2007b; Hsiao Pei *et al.*, 2009). However, the Cr(VI) reduction also proceeds in an anaerobic/semi–anaerobic environments which could be, due to the diversity of microbial species present, in the biofilm formed. This study plans to address this issue by studying the Cr(VI) reduction–resistance mechanisms of the bacteria isolated from the biofilm during the treatment process.

The study will be carried out in both batch and continuous modes. The feasibility of other AW as nutrient will be carried out in the batch mode, analysis on microbial community present in the biofilm will be conducted using continuous mode, while Cr(VI) reduction–resistance mechanisms of selected AW will be elucidated using batch mode.

1.3 Objectives of the study

The objectives of this work are:

1. To evaluate the Cr(VI) reduction–resistance of *A. haemolyticus* in the presence of selected agricultural waste as growth medium and support material.
2. To evaluate the effect of agricultural waste on the microbial community in the biofilm formed by using PCR and basic molecular techniques.
3. To analyze the Cr(VI) reduction–resistance mechanisms of *A. haemolyticus* isolated from the biofilm in the anaerobic/semi–anaerobic environment.