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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

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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 (OD_{600}) 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.

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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*, *Candidate division OP10*, *Pedobacter sp.* dan bakteria yang tidak dikultur.

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LIST OF ABBREVIATIONS

A. haemolyticus	-	*Acinetobacter haemolyticus*
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–diphenylcarbazide
E. coli	-	*Escherichia coli*
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

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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 Spectroscope
XPS	-	X–Ray Photoelectron Spectroscope

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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 'ChromeBac^{TM} 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

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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^{TM}. This system was developed at the laboratory and pilot–scale in Universiti Teknologi Malaysia (UTM), Skudai since 2005. ChromeBac^{TM} 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^{TM} 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^{TM} 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

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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.

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1.4 Scopes of the study

The work will be carried out via the following:

1. Characterization and screening of sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM) and assessment of bacterial survival ability using the AW with the following profile: OD_{600}, CFU, total carbohydrate and pH.
2. Determination of Cr(VI) reduction by AW in the presence and absence of bacteria using DPC method and AAS.
3. Preparation and analysis of the performance of Cr(VI) reduction using the reactor system with the following profile: OD_{600}, CFU, total carbohydrate, pH, COD and Cr(VI) concentration. Optimization of important experimental parameters such as effect of influent flow rate and effect of influent Cr(VI) concentration will be carried out.
4. Identification of microbial diversity presents in biofilm of Cr(VI) reducing environment using PCR and basic molecular techniques.
5. Isolation and study the performance of Cr(VI) reduction–resistance bacteria from the biofilm formed and evaluation on the role of aerobic, semi–anaerobic and anaerobic conditions towards the Cr(VI) reduction.
6. Study the interaction between *A. haemolyticus* and Cr during Cr(VI) reduction using instrumental analysis such as XPS, ESR, FT–IR, SEM and FESEM–EDX.

1.5 Significance of the study

At the end of the study, this research shall contribute to the advancement of knowledge via suggesting on the feasibility of using other types of AW as growth medium in a biological based Cr(VI) reduction process. Results obtained from the batch and continuous modes can serve as a benchmark for the bioremediation of Cr(VI) especially for industries such as electroplating industry, metal–related industries, oil–refineries, wood preserving and also agencies involved in the remediation and preservation of environmental quality.

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CHAPTER 2

LITERATURE REVIEW

2.1 Water resources pollution

Environmental pollution has become a key focus of concern for all the nations worldwide, as not only the developing countries but developed nations as well are affected by and suffer from it. Accordingly, the main types of pollution are water, air, soil, thermal, radioactive, noise and light pollution. These pollutions contribute to health problems and lower quality of life.

Among all the environmental pollutions, pollution of water resources is a matter of great concern. Poor and developing countries are at high risk due to lack of wastewater treatment technologies. Increasing contamination of aquatic sources with large number of pollutants is not only endangering the aquatic biota but creating a worldwide shortage of recreational waters.

The sources of water resources pollution involved domestic activities, mining activities, municipal wastes, modern agricultural practices, radioactive wastes, oil spillage and industries. But the major factors causing the pollution of water resources are different industrial units. Unsystematic discharges of toxic chemicals through effluents from a wide range of industries such as textile, pharmaceuticals, ceramics, petrochemicals, food industries, steel, oil mills, sugar industries, fertilizer factories and leather tanning into water bodies pollutes these resources and causes hazardous effects on ecosystem (Azizullah *et al.*, 2011).

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Among all the pollutants, heavy metals are most dangerous one as these are non–biodegradable, recalcitrant and persistent in the environment, besides known to be toxic or carcinogenic (Fu and Wang, 2011). These pollutants enter into the water resources through both natural and anthropogenic sources. The term heavy metal refers to any metallic chemical element that has a relatively high density. The density of heavy metals is usually more than 5.0 g/cm^3. Heavy metals are known to cause deleterious effects on human health. Excessive exposure to heavy metals has been shown to cause various diseases (Bo *et al.*, 2009). In human, these metals accumulate in living tissues through the food chain and thus multiplying the danger. Some common harmful effects and health risks of some heavy metals to human beings are given in Table 2.1.

Table 2.1: Common health problems exerted on humans by heavy metals (Singh *et al.*, 2011; Barakat, 2011)

Metals	Health risks
Arsenic	Bronchitis, dermatitis, poisoning, skin manifestations, visceral cancers and vascular disease
Cadmium	Renal dysfunction, lung diseases, lung cancer, bone defects (osteomalacia, osteoporosis), increased blood pressure, kidney damage, bronchitis, gastrointestinal disorder, bone marrow and cancer
Lead	Mental retardation in children, developmental delay, fatal infant encephalopathy, congenital paralysis, epilepticus, liver, kidney, gastrointestinal damage, sensor neural deafness and acute or chronic damage to the nervous system
Manganese	Inhalation or contact causes damage to central nervous system
Mercury	Tremors, gingivitis, minor psychological changes, acrodynia characterized by pink hands and feet, spontaneous abortion, rheumatoid arthritis, diseases of the kidneys, circulatory system damage to the nervous system and protoplasm poisoning
Zinc	Zinc fumes have a corrosive effect on the skin, cause damage to nervous membrane, depression, lethargy, neurological signs and increased thirst
Chromium	Damage to the nervous system, fatigue, irritability, headache, diarrhea, nausea, vomiting and carcinogenic
Nickel	Dermatitis, nausea, chronic asthma, coughing and human carcinogen
Copper	Anemia, liver and kidney damage, intestinal irritation and insomnia

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The health risks of heavy metal ingestion thus are of wide range. Some metals cause physical discomfort while others may cause life–threatening illness,

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damage to the vital body system or cause other damages. Thus, it is very necessary to control emissions of heavy metals into the environment.

Amongst heavy metals, chromium (Cr) is one of the major contaminants in the effluents and demands special attention as it may present several oxidation states. Among the different oxidation states, Cr(VI) is of special concern due to its toxicity and potential carcinogenicity. Hence, Cr was chosen in this study.

2.2 Chromium

The name 'chromium' is derived from the Greek word for colour, 'chroma', because this metal forms a wide variety of compounds with beautiful colours. Cr compounds have been used for many years as pigments in paints and other coatings – $Na_2Cr_2O_7$ is bright orange, Cr_2O_3 is green and the Zn and Pb salts of CrO_4^{2-} are bright yellow. However, in recent years, the use of Cr pigments has diminished because Cr in the +6 oxidation state is highly toxic. Cr is also important in converting animal hides into leather. In the tanning process, the hides are treated with basic solutions of Cr(III) salts, which cause cross–linking of collagen proteins. The hides toughen and become pliable and resistant to biological decay (Olmsted and Williams, 2006).

2.2.1 Chemical and physical properties

Cr with the atomic number of 24 and atomic mass of 51.996 g/mol is found in the centre of the periodic table. Elements in Groups 6 through 18 are known as the transition metals. Cr is a hard, steel–gray, shiny metal that breaks easily. It has a melting point of 1857 °C and a boiling point of 2672 °C. The density is 7.19 g/cm^3. Cr is remarkable for its magnetic properties – it is the only elemental solid which shows antiferromagnetic ordering at room temperature (and below). Above 38 °C, it transforms into a paramagnetic state. Cr has an electronic configuration of

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$[Ar]3d^5 4s^1$, owing to the lower energy of the high spin configuration. One important property is that Cr can be polished to a high shine. Cr is a fairly active metal. It does not react with water, but reacts with most acids. It combines with oxygen at room temperature to form chromium oxide (Cr_2O_3). Cr_2O_3 forms a thin layer on the surface of the metal, protecting it from further corrosion (rusting).

Cr is an essential micronutrient required for the growth of many organisms and a component of enzyme involved in sugar metabolism (Cheung and Gu, 2007). However, at high concentrations, Cr is toxic, mutagenic, carcinogenic and teratogenic. Cr exists in several oxidation states from Cr(II) to Cr(VI). In nature, Cr can be found either as Cr(III) or Cr(VI) (Dhal *et al.*, 2013).

2.2.2 Trivalent and hexavalent chromium

Cr(III) is rather benign and easily adsorbed in soil and water, whereas Cr(VI) is the toxic form, is not readily adsorbed and is soluble (Kotas and Stasicka, 2000). Industrial wastewater contains both Cr and salt ions which have toxic effects on the microbial consortia of wastewater treatment systems (Stasinakis *et al.*, 2003). Removal of Cr(VI) either by reduction (Kamaludeen *et al.*, 2003) or biosorption (Hou *et al.*, 2012) can significantly reduce the risks to human health.

Chemically, Cr(III) behaves like Fe(III) in that it is readily hydrolyzed to an insoluble hydroxide. In this form, it is comparatively harmless as long as it remains immobilized. Because of its electronic configuration, compounds of Cr(III) tend to be chemically inert – that is, reaction such as exchange of ligands tends to be quite slow.

Cr(VI) compounds are encountered as oxo species, which are good oxidizing agents. One such application is treating steel to resist corrosion. The solution of the chromate ion, CrO_4^{2-}, is effective in 'passivating' steel, presumably by reacting to form a protective oxide layer on the surface. Wastes from such operations are a

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prime source of Cr pollution. In acid, chromate is in equilibrium with dichromate (Equation 2.1):

$$2CrO_4^{2-} + 2H_3O^+ \rightleftharpoons Cr_2O_7^{2-} + 3H_2O \quad (2.1)$$

Although organic materials may reduce Cr(VI) to the less toxic Cr(III), redox potentials predict that Cr(III) may be oxidized to Cr(VI) by oxygen in well–aerated systems. This and other possible reactions make soil contaminated by Cr(III) quite hazardous. Stabilization of Cr(III), for example by converting it to the very insoluble and unreactive sulphide, can reduce this hazard.

Figure 2.1 shows the predominant zone of Cr(VI) and Cr(III) existence in nature at different redox potential (Eh) and pH levels. The change in Cr valence correlates with the variation of pH and redox potential characteristics of the surrounding environment. Cr(III) is the most thermodynamically stable oxidation states under reducing conditions. Cr(VI) can remain stable for significant periods of time. Cr(III) predominates at pH<3.0. At pH>3.5, hydrolysis of aqueous Cr(III) yields trivalent chromium hydroxyl species ($CrOH^{2+}$, $Cr(OH)^{2+}$, $Cr(OH)_3^0$ and $Cr(OH)_4^-$). $Cr(OH)_3^0$ is the only solid species, existing as an amorphous precipitate. Hexavalent Cr exists primarily as salts of chromic acid (H_2CrO_4), hydrogen chromate ion ($HCrO_4^-$) and chromate ion (CrO_4^{2-}), depending on the pH. H_2CrO_4 predominates at pHs less than about 1.0, $HCrO_4^-$ at pHs between 1.0 and 6.0 and CrO_4^{2-} at pHs above about 6.0. The dichromate ion ($Cr_2O_7^{2-}$), a dimer of $HCrO_4^-$, minus a water molecule, forms when the concentration of Cr exceeds approximately 1g/L (Mohan and Pittman, 2006).

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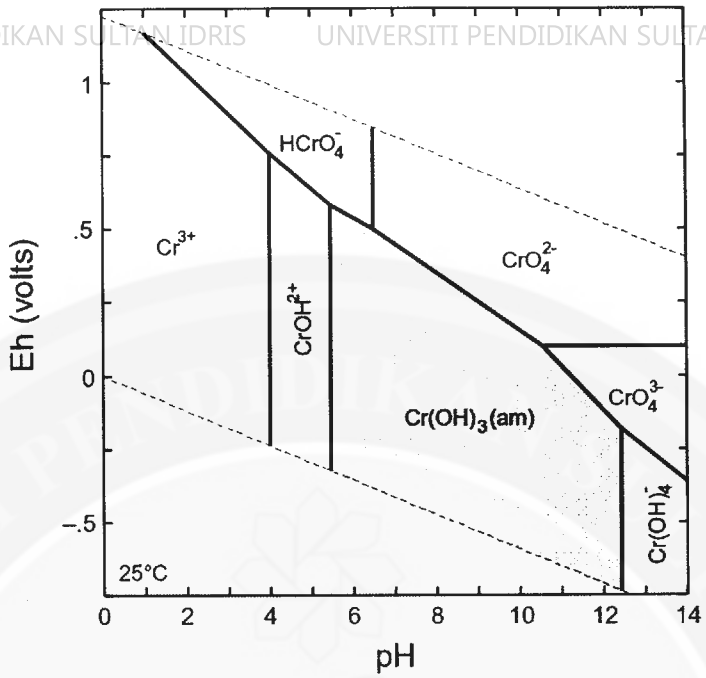


Figure 2.1: Eh–pH predominance diagram for aqueous Cr at 25 °C (Reeder *et al.*, 2006)

2.2.3 Toxicity of chromium

Of the two stable Cr oxidation states, Cr(VI), apart from being the predominant species involved in mutagenicity, carcinogenicity and teratogenicity, is approximately 100 times, while other researcher reported 500 times more toxic than Cr(III), a form considered relatively innocuous (Neagu and Mikhalovsky, 2010; Gupta *et al.*, 2010). Human toxicity includes lung cancer, as well as kidney, liver and gastric damage (Acar and Malkoc, 2004). Moreover, while Cr(VI) is highly soluble in water and forms divalent oxyanions (chromate, CrO_4^{2-} and dichromate, $Cr_2O_7^{2-}$), Cr(III) readily forms less soluble chromium hydroxides at neutral pH. The biological effects of chromate appear to be related to its uptake, since Cr(VI) passes easily across cellular membranes, the trivalent form is incapable of crossing cellular membranes (Vaiopoulou and Gikas, 2012).

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2.3 Treatment of chromium–contaminated wastewater

Cr is widely used in a number of industries. Cr contaminated wastewater can originate from a multitude of sources. Cr is widely used in the industries of electroplating, metal finishing, magnetic tapes, pigments, leather tanning, wood protection, chemical manufacturing, brass, electrical and electronic equipment and catalysis (Liu *et al.*, 2011a). It is also employed in manufacturing pulp and paper, paint and petroleum refining processes (Gupta *et al.*, 2010; Sharma and Adholeya, 2011). Consequently, effluents may contain a wide range of concentrations of either hexavalent or trivalent Cr or both. Tanning process for example, is one of the major sources of Cr pollution on a global scale. In the Cr tanning process, the leather takes up only 60–80% of applied Cr and the rest usually discharges into the wastewater causing serious environmental impact. Cr ion in liquid tanning wastes occurs mainly in trivalent form, which gets further oxidized to hexavalent form (Garg *et al.*, 2007).

A wide array of technologies is available for the removal of Cr from wastewater. Some of these are well established methods that have been in practice for decades, others are more recent innovations (Ahluwalia and Goyal, 2007; Zahoor and Rehman, 2009). According to the Environmental Quality Regulation (EQR)(Industrial Effluent) 2009, amendment on Environmental Quality Act (EQA) 1974, the permissible discharge limit for Cr(VI) is 0.05 mg/L for both Standard A and Standard B (Department of Environment, 2009), 0.20 mg/L for Standard A and 1.0 mg/L for Standard B for Cr(III) (Table 2.2). In practical applications, the choice of treatment depends on effluent characteristics such as concentration of Cr(VI), pH, temperature, flow volume, biological oxygen demand (BOD), the economics involved and the social factor like standard set by government agencies (Acharya *et al.*, 2009).

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Table 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

Parameter	Unit	Standard	
		A	B
Temperature	°C	40	40
pH value	–	6.0 – 9.0	5.5 – 9.0
BOD_5 at 20 °C	mg/L	20	50
Suspended Solids (SS)	mg/L	50	100
Mercury (Hg)	mg/L	0.005	0.05
Cadmium (Cd)	mg/L	0.01	0.02
Chromium, Hexavalent (Cr(VI))	mg/L	0.05	0.05
Chromium, Trivalent (Cr(III))	mg/L	0.20	1.0
Arsenic (As)	mg/L	0.05	0.10
Cyanide(CN)	mg/L	0.05	0.10
Lead (Pb)	mg/L	0.10	0.5
Copper (Cu)	mg/L	0.20	1.0
Manganese (Mn)	mg/L	0.20	1.0
Nickel (Ni)	mg/L	0.20	1.0
Tin (Sn)	mg/L	0.20	1.0
Zinc (Zn)	mg/L	2.0	2.0
Boron (B)	mg/L	1.0	4.0
Iron (Fe)	mg/L	1.0	5.0
Silver (Ag)	mg/L	0.1	1.0
Aluminium (Al)	mg/L	10	15
Selenium (Se)	mg/L	0.02	0.5
Barium (Ba)	mg/L	1.0	2.0
Fluoride	mg/L	2.0	5.0
Formaldehyde	mg/L	1.0	2.0
Phenol	mg/L	0.001	1.0
Free Chlorine	mg/L	1.0	2.0
Sulphide	mg/L	0.50	0.50
Oil and Grease	mg/L	1.0	10
Ammoniacal Nitrogen	mg/L	10	20
Colour ADMI*		100	200

*ADMI – American Dye Manufacturers Institute

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2.3.1 Conventional treatment methods

A wide range of physical and chemical processes are available for the removal of Cr from industrial wastewater, such as ultrafiltration (Ghosh and Bhattacharya, 2006), chemical and electrochemical processes (Almaguer–Busso *et al.*, 2009), ion exchange resins (Rengaraj *et al.*, 2003), membrane technologies (Hsu *et al.*, 2011), electrocoagulation (Akbal and Camci, 2011) and solvent extraction (Agrawal *et al.*, 2008). However, these conventional methods have severe constraints, such as ineffective metal removal at low concentrations, high reagent requirements, high operating and maintenance costs and secondary environmental problems from waste disposal (Hou *et al.*, 2012). Furthermore, such processes may be ineffective or extremely expensive when the initial heavy metal concentrations are in the range of 10–100 mg/L (Ramrakhiani *et al.*, 2011). Ion exchange is considered a better alternative technique for such a purpose. However, it is not economically appealing because of high operational cost. Such processes have its disadvantages whereby large volumes of sludge are generated and also release of obnoxious gases such as H_2S (Ahmad *et al.*, 2010a). Therefore, it is important to develop an innovative, low cost and eco–friendly method for Cr removal from the wastewater (Zahoor and Rehman, 2009).

One example of current industrial application for Cr removal is the use of sulphur compounds. For the treatment of chromate–containing wastewater, the typical procedure is reduction of hexavalent to the trivalent Cr state, followed by its precipitation as chromium hydroxide, $Cr(OH)_3$. The most common industrial reducing agents are sulphur dioxide gas or sodium bisulphite in an acid solution. Both of these reactants form the same active reducing agent, sulphurous acid as shown in Equations 2.2 and 2.3.

$$SO_2 + H_2O \rightarrow H_2SO_3 \quad (2.2)$$

$$NaHSO_3 + H^+ \rightarrow H_2SO_3 + Na^+ \quad (2.3)$$

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The theoretical requirement is 3 kg of sodium bisulphite plus 2–3 kg of sulphuric acid, to reduce 1 kg of hexavalent Cr. Equation 2.4 takes place almost instantaneously at a pH of 2.5.

$$4CrO_4^{2-} + 6NaHSO_3 + 3H_2SO_4 + 8H^+ \rightarrow 2Cr_2(SO_4)_3 + 3Na_2SO_4 + 10H_2O \quad (2.4)$$

Subsequent treatment with sodium hydroxide (NaOH) precipitates the Cr(III) as indicated in Equation 2.5.

$$Cr_2(SO_4)_3 + 6NaOH \rightarrow 2Cr(OH)_3 + 3Na_2SO_4 \quad (2.5)$$

Since $Cr(OH)_3$ is precipitated, the resulting effluent will contain little or no residual Cr. The sulphur dioxide (SO_2) reacts as follows (Equation 2.6):

$$2CrO_4^{2-} + 3SO_2 + 4H^+ \rightarrow Cr_2(SO_4)_3 + 2H_2O \quad (2.6)$$

Following reduction, the effluent is treated with NaOH solution or calcium hydroxide $Ca(OH)_3$ slurry to neutralize the acidity and precipitate the Cr. Where sludge formation must be reduced to an absolute minimum, the use of NaOH solution is advised. One of the main problems using this technique is that large amounts of residual sludge are generated. The sludge presents difficulties in managing, transporting and final disposal issues as well as the associated cost. Thus, new technologies are being developed to address these problems (Barrera–Diaz *et al.*, 2012).

Another currently practiced methods is the use of ViroChrome^{TM} reagent. A pilot scale treatment of tannery sludge has been employed by the Virotec Global Solution Company, known as Viroflow^{TM} technology using ViroChrome^{TM} reagent where the Cr concentration rapidly lowered below the regulatory limit (5 mg/L) in 5 days mixing period. The optimised ViroFlow^{TM} Technology treatment involved the

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addition of appropriate quantities of ViroChrome^{TM} reagent and thorough mixing of the reagent with the contaminated sludge. ViroChrome^{TM} reagent is Virotec's proprietary reagent for the treatment and immobilization of Cr in contaminated solid waste. For the full–scale treatment, the ViroChrome^{TM} reagent was mixed with the sludge using an excavator. The Virotec Total Solution includes the bench–scale testing and pilot–scale testing before application and ensures that each solids treatment application provides a custom–designed treatment solution that will meet all regulatory limits (Virotec Global Solutions, 2011). Generally these methods have good extraction yield, however application of chemical extraction methods is limited and expensive due to operational difficulties, high chemical consumption and the requirement of long processing time (Dhal *et al.*, 2013).

2.3.2 Biological treatment methods

Biological removal of Cr(VI) aroused great interest because of its lower impact on the environment. Biological systems, such as biosorption, bioreduction or bioaccumulation using living cells have been examined for their Cr removal abilities (Sharma and Forster, 1993; Fude *et al.*, 1994; Dermou *et al.*, 2007), where these systems offer potential alternatives to existing technologies to remove Cr(VI) from wastewater (Chen and Gu, 2005).

A wide variety of microorganisms such as bacteria, yeast, algae, protozoa and fungi are found in waters receiving industrial effluents. These microorganisms have developed the capabilities to protect themselves from heavy metal toxicity by various mechanisms such as adsorption, uptake, methylation, oxidation and reduction (Zahoor and Rehman, 2009).

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2.3.2.1 Biosorbent and biosorption

Biosorbents are found to be more suitable for metal removal at relatively lower concentrations (10–100 mg/L), where other methods usually fail (Mona *et al.*, 2011). A large variety of biological based materials like wood bark, sawdust, bagasse and coir pith have been used for the sorption of a large quantity of toxic metals from the solutions (Saha and Orvig, 2010). Use of microbial biomass of algae, cyanobacteria, fungi and bacteria has proven to be capable of passively sequestering metal by the process of biosorption even from dilute solutions (Paul *et al.*, 2012). The removal of Cr(VI) from the wastewater using biological materials has long been used. Several researchers have studied the adsorption of Cr(VI) from aqueous solution using cellulosic, lignocellulosic and agricultural waste materials (Mohan and Singh, 2002; Khan *et al.*, 2004; Ahmad *et al.*, 2010b). Examples are summarized in Tables 2.3.

Table 2.3: Bacterial biomass and agricultural products as biosorbents (Saha and Orvig, 2010)

Biosorbents	Sorption capacity (mg/g)[a], (mmol/g)[b] or removal efficiency (%)[c]
Bacterial biomass	
Biofilm of *E. coli* supported on carbon	97.70 (a)
Corynebacterium glutamicum	95 (c)
Nostoc muscorum	22.92 (a)
Phormidium sp.	24.3 (a)
Pseudomonas aeruginosa	1.44 (a)
Agricultural products	
Activated tamarind seeds	29.7 (a)
Banana skin	25.5 (c)
Rice straw	26.3 (c)
Sawdust	20.70 (a)
Soya cake	0.28 (a)
Sugarcane bagasse	1.04 (b)

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Among many researchers in this research field, Park *et al.* (2008) have studied Cr(VI) biosorption by natural biosorbents. Adsorption process has proved to be the most effective for the removal of Cr from wastewater. The biggest barrier in the application of this process by the industries is the high cost of adsorbents presently available for commercial use. The cost of adsorption technology application can be reduced if the adsorbent is inexpensive. So, there is a need to develop low cost and easily available adsorbent for the removal of Cr ions from the aqueous environment (Garg *et al.*, 2007) such as from agricultural waste.

2.3.2.2 Bioreduction

Since several microorganisms possess the capability to reduce Cr(VI) to relatively less toxic Cr(III), bioremediation gives immense opportunities for the development of technologies to detoxify Cr(VI)–contaminated wastewater as an alternative to the existing physico–chemical technologies (Dhal *et al.*, 2013). A wide variety of bacteria have been reported in reducing Cr(VI) to Cr(III) (Camargo *et al.*, 2004; He *et al.*, 2011). Other microbial populations are summarized in Table 2.4. Application of Cr(VI)–resistant bacteria for detoxification of Cr(VI) has been considered as an economical, effective and safe procedure over conventional physical and chemical methods (Cheung and Gu, 2007; Polti *et al.*, 2009; Pinon–Castillo *et al.*, 2010). Enzymatic reduction of chromate by Cr(VI)–resistant bacteria proceeds aerobically, anaerobically or both (Thacker *et al.*, 2006). The physiological mechanisms involved in Cr(VI) reduction vary widely among species. In some cases, the enzyme–catalyzed reaction is membrane associated, whereas in others the enzyme is in the soluble fraction (Park *et al.*, 2000; Hsiao Pei *et al.*, 2009). However, the availability of effective Cr(VI) reducing organisms is an essential prerequisite for the bioreduction–based remediation of Cr contaminated wastewater or soil (Pattanapipitpaisal *et al.*, 2001; Mishra *et al.*, 2009).

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Table 2.4: Microbial populations that transform Cr(VI) to Cr(III)

Organisms	Substrate/Redox condition	References
Acinetobacter haemolyticus	Nutrient media, Luria–Bertani media, glucose/Aerobic	Zakaria *et al.* (2007b); Hsiao Pei *et al.* (2009)
Ochrobactrum sp.	Luria–Bertani media/Aerobic	Thacker and Madamwar (2005)
Arthrobacter oxydans	-/Aerobic	Asatiani *et al.* (2004)
Pseudomonas fluorescens LB300	Specific media, citrate/Aerobic	DeLeo and Ehrlich (1994)
Bacillus sp.	Luria–Bertani media, glucose/Aerobic	Masood and Malik (2011)
Providencia sp.	Luria–Bertani media/Aerobic	Thacker *et al.* (2006)
Cellulosimicrobium cellulans	PYG media/Aerobic	Chatterjee *et al.* (2009)
Brucella sp.	Luria–Bertani media/Aerobic	Thacker *et al.* (2007)
Agrobacterium radiobacter	Glucose, fructose, maltose, lactose, mannitol, glycerol/Aerobic	Llovera *et al.* (1993)
Desulfovibrio vulgaris	Hydrogen/Anaerobic	Lovley and Phillips (1994)
Enterobacter cloacae	Acetate, glycerol, glucose/Anaerobic	Ohtake *et al.* (1990)
Escherichia coli ATCC33456	Glucose, acetate, propionate/Aerobic and anaerobic	Shen and Wang (1993)
Streptomyces griseus	Broth–II media/Aerobic	Laxman and More (2002)

ChromeBac™ system is another cost–effective, simple and economical solution for the removal of Cr. It utilizes *A. haemolyticus* (Figure 2.2), an aerobic Gram–negative bacterium deposited into National Centre for Biotechnology Information (NCBI) with Accession No. EF369508. It was isolated from Cr(VI)–containing effluent (batek textile industry) in Kota Bharu, Kelantan. This bacterium was previously isolated and identified by Zakaria *et al.* (2005; 2007b), capable of reducing Cr(VI) to Cr(III) (Zakaria *et al.*, 2007b) and possessed the ability to resist up to 70 mg/L of Cr(VI) (Ahmad *et al.*, 2009b). At stationary phase, the cells appear as cocci, however, at log phase they are rod shaped (Brenner *et. al.*, 2005). The colonies formed on agar plate were circular, convex, smooth and translucent (Nemec *et al.*, 2003).

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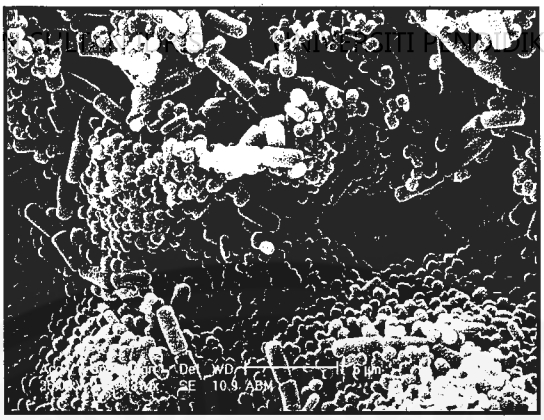


Figure 2.2: Bacterium of *A. haemolyticus* (Zakaria *et al.*, 2006)

This system combines the use of an immobilized bacterial system to reduce Cr(VI) to Cr(III) as a primary treatment followed by precipitation as a secondary treatment and filtration as a tertiary treatment (Ahmad *et al.*, 2010b). ChromeBac™ is a novel, environmentally–friendly system to treat Cr(VI)–bearing water (surface water, industrial effluent, groundwater) consisting of bioreactor packed with immobilized Cr(VI) resistant–reducing bacteria. Cr(VI)–bearing water, supplemented with a minimal amount of carbon source, was introduced into the bioreactor where it will be biologically reduced to Cr(III), which can then be removed via precipitation (Ahmad *et al.*, 2010a; 2010b). The schematic diagram for the 0.2 m^3 pilot plant of the ChromeBac™ system to remove Cr from industrial electroplating wastewater is shown in Figure 2.3.

ChromeBac™ is primarily targeted to treat Cr(VI) from the surface water of a contaminated area. It is also very useful to treat Cr(VI) from industrial wastewater (containing less than 300 mg/L Cr(VI)) or soil area exposed to Cr(VI). Typical characteristics of effluent after treatment by ChromeBac™ are as follows; oxidation–reduction potential (ORP): 96–232 mV, dissolved oxygen (DO): 5.4–6.0 mg/L, pH: 6.1–6.9, Cr(VI): not detectable. The system is expected to be incorporated into the Cr treatment system by replacing the current chemical–based Cr(VI) reduction system. By using ChromeBac™, consumption of chemicals can be reduced up to 60% compared to the conventional method. Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) concentrations were also treated to permissible discharge levels.

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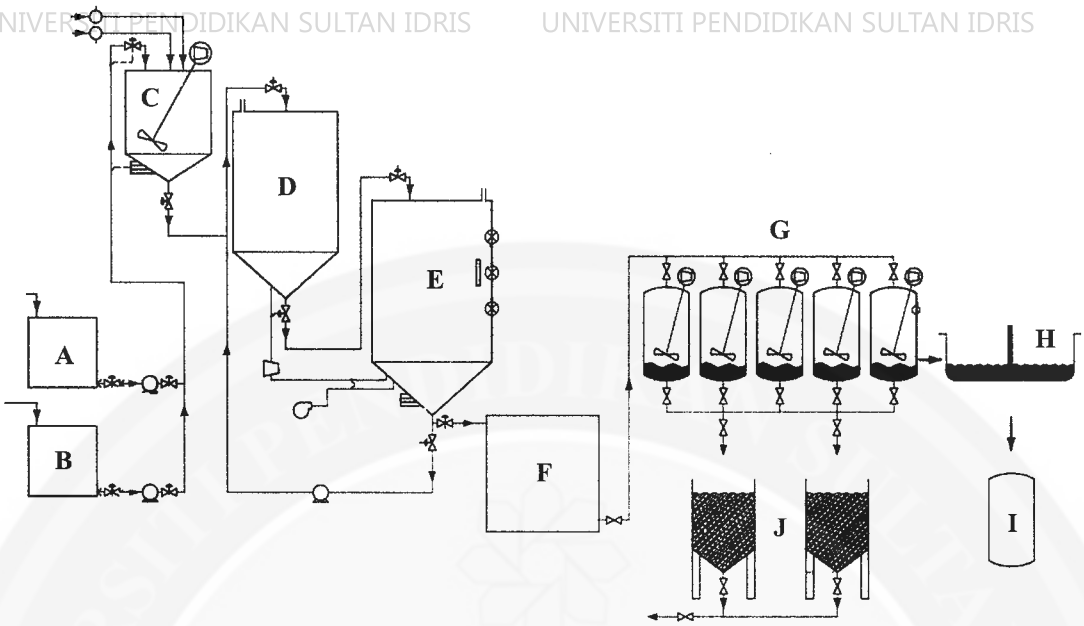


Figure 2.3: Schematic representation of the experimental set up for the Cr(VI) reduction system using a 0.2 m^3 bioreactor; A – nutrient tank, B – raw Cr(VI) wastewater tank, C – mixing tank, D – holding tank, E – bioreactor, F – receiving tank, G – flocculation and coagulation section, H – sludge drying bed, I – sludge collecting tank, J – powdered activated carbon column (Ahmad *et al.*, 2010a)

2.3.2.3 Bioaccumulation

Bioaccumulation is a metabolic dependent process, whereby the pollutant or cation is accumulated intracellularly or across the cell membrane or removed through the cell metabolic cycle (Malik, 2004). Many microorganisms are able to remove Cr(VI) from wastewater. These microorganisms have developed a variety of mechanisms to remove Cr(VI), such as adsorption to cell surfaces, transport into the cell, intracellular accumulation or reduction to non–toxic Cr(III) (Jianlong *et al.*, 2004; Zouboulis *et al.*, 2004; Dursun *et al.*, 2003). Cr(VI) bioaccumulation process might be effectively useful in wastewater treatment systems for detoxification and removal of soluble Cr(VI). However, high concentrations of Cr(VI) strongly reduce microbial activity of activated sludge (Stasinakis *et al.*, 2003).

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Therefore, the use of alternative treatment techniques such as bioremediation offers an interesting solution. One example approach is the used of membrane

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bioreactor. Chromate reduction was studied in a membrane bioreactor under action of *Pseudomonas* bacteria immobilized in agar films on the surface of synthetic membrane. Six strains of denitrifying bacteria of the genus *Pseudomonas* were studied in relation to the chromate resistance and the ability of Cr reduction. Two studied strains are low active or inactive in Cr reduction. Active strains in free form are able to reduce chromate at concentrations up to 30 mg/mL after notable induction period. For effective reduction, chromate concentration should be restricted to avoid chromate toxicity.

Bacterial cells immobilized in agar films on the surface of synthetic membranes are protected from the action of chromate at high levels. That allows a biological process for remediation of Cr(VI)–containing solutions in membrane bioreactor of dialyze type. Chromate reduction passes with high rate at concentrations up to 20 mg/L practically without induction period. Effective reactor operation occurs at condition of an equality of the diffusional transport and reduction rates or prevalence of reduction rate. Such regime excludes or minimizes a chromate penetration in receiving phase. Rates of diffusional transport are controlled by variation of chromate concentration. This factor influences also on the cell activity because of chromate toxicity. A stepwise introducing of chromate in the feed chamber of membrane bioreactor allows maintenance of optimal chromate concentration. At starting chromate concentrations of 20 mg/L, immobilized bacteria *P. mendocina* effectively reduces Cr(VI) concentration to trace levels. Reduction is suppressed on the sixth step of chromate adding as a result of reaching of higher metabolite concentrations. The cell's ability to reduce chromate is restored after changing of feed and receiving solutions. Membrane bioreactor operation during the subsequent stages after the solution change is similar to starting cycles. That allows a fulfilment of chromate reduction in membrane bioreactor with immobilized bacteria in a semi–batch mode of operation (Konovalova *et al.*, 2003).

Another currently practiced method is the used of native microbial species to bio remediate the Cr contaminated soil. Kathiravan *et al.* (2011) studied the bioremediation processes using *Bacillus* sp. in batch and continuous operations. *Bacillus* sp. was isolated from tannery effluent contaminated soil and the optimum

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pH and temperature were found to be 7 and 35 °C respectively where glucose was used as an electron donor. Concentration of Cr(VI) was found to be 2.5 mg/g of soil. In packed–bed column experiments, time required for complete Cr(VI) reduction was found to be 120, 112, 96 and 100 h for 2, 3, 4 and 5 mL/min flow rate respectively at an average viable cells in the range of 3.2 X 10^8 to 3.9 X10^8 CFU/mL.

2.4 Agricultural waste as support and growth medium for treatment of chromium–contaminated wastewater

Agricultural by–products are mostly composed of lignin and cellulose, as well as other polar compounds such as alcohols, aldehydes, ketone, carboxylates, phenols and ethers. These groups are able to bind heavy metals through replacement of hydrogen ions with metal ions in solution or by donation of an electron pair from these groups to form complexes with metal ions in solution (Ofomaja and Ho, 2007). These agricultural waste materials have been used in their natural form or after some physical or chemical modification.

The possibility of using sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM) as alternative sources for culture medium in Malaysia and other tropical countries is helped by the fact that all agricultural wastes chosen is easily available, cheap and abundantly grown especially in the southern tip of peninsular Malaysia in particular, the state of Johor, Negeri Sembilan and Selangor. According to the Food and Agricultural Organization (FAO) online database (2013), Malaysia produced about 800, 000 metric tons of sugarcane and 332, 736 metric tons of pineapples in 2011. Even though Malaysia does not have soybean plantation, the existing local foodstuff industries such as Jalen Sdn. Bhd. and Zara Foodstuff Industries Sdn. Bhd. at Johor industrial area used imported soybean for their production, creating the possibility of using soybean waste for wastewater treatment.

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2.4.1 Sugarcane bagasse

Sugarcane (*Saccharum officinarum*) is a grass that is harvested for its sucrose content. After sucrose extraction, the residual plant material is called bagasse. Sugarcane bagasse (SCB) is composed approximately of 35–50% cellulose, 24–35% hemicellulose and 15–35% lignin on dry weight basis (Lee *et al.*, 2009; Mohamed *et al.*, 2010). SCB is a source of lignocellulosic biomass. It is a potential renewable energy source for ethanol production besides naturally cheap, plentiful and has high cellulose content. Lignocellulosic degradation is relatively difficult to deal with. The components exist in compact structures tied together with strong intermolecular bonds. Lignocellulosic materials do not contain readily accessible monosaccharide and chemicals but rather a polymer which needs to be hydrolyzed to release the desired compound (Walford, 2008).

In plant cells, including sugarcane plants, a secondary wall, consisting of three layers (S1, S2 and S3) is surrounded by a thin primary wall. The secondary wall is surrounded by lignin. The S1 and S3 layers contain mainly amorphous cellulose and hemicellulose. The S2 layer contains crystalline cellulose. Crystalline regions are shaped by linear forms of hydrogen bonded in a highly ordered manner. However, amorphous regions also exist in the cellulose. Amorphous cellulose, hemicellulose and lignin are present between the layers (S1, S2 and S3) (Lee, 2005). An illustration of the structure of the cell wall with its component organization is shown in Figure 2.4.

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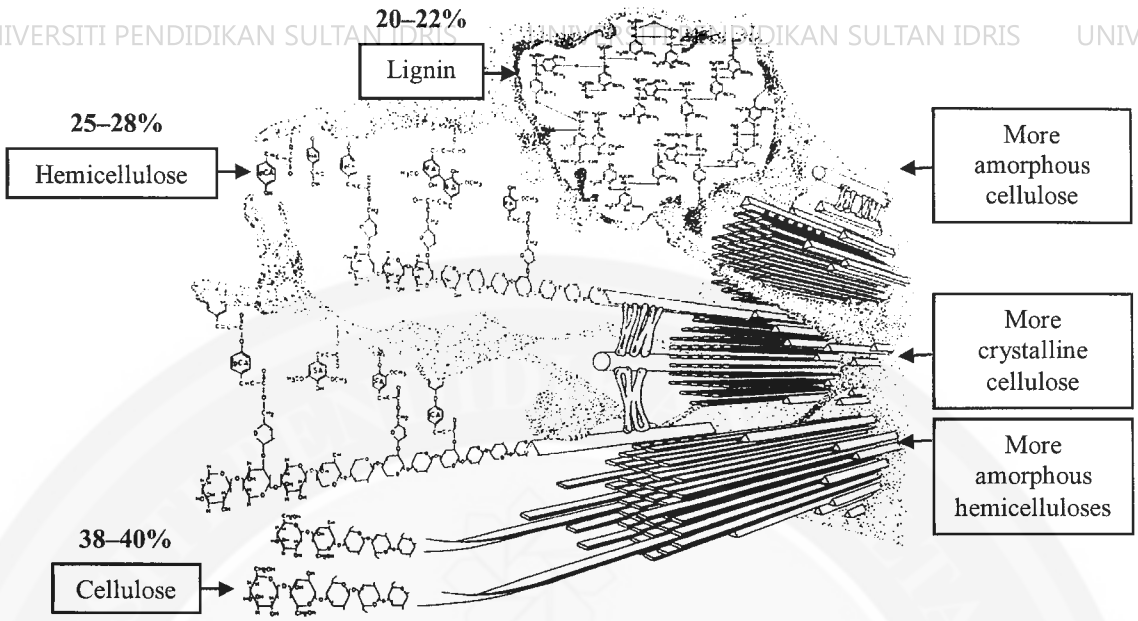


Figure 2.4: Secondary cell wall (CW) structure of cellulose, hemicelluloses and lignin in lignocellulosic materials. For SCB, the basic composition is 40% cellulose, 28% hemicelluloses and 22% lignin (Lee, 2005)

The composition of SCB as reported by Aiello *et al.* (1996) and Rodrigues *et al.* (2010) is given in Table 2.5.

Table 2.5: Basic composition of SCB

Composition	Unit	Values	
		Aiello *et al.* (1996)	Rodrigues *et al.* (2010)
Moisture content	%	9.38	–
Ash	%	4.25	2.3 ± 0.1
Cellulose	%	33.28	45.4 ± 0.8
Hemicellulose	%	22.58	24.1 ± 0.6
Klason Lignin	%	6.15	23.4 ± 0.2
Sugar content	%	31.69	–

Garg *et al.* (2009) investigated the effect of adsorbent dose, pH and agitation speed on the removal of Cr from aqueous medium using SCB–treated succinic acid. Batch mode experiments were carried out to assess the adsorption equilibrium. The influence of three parameters on the removal of Cr was examined using a response surface methodology (RSM) approach. Results showed the optimum conditions for

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maximum removal of 50 mg/L Cr(VI) from an aqueous solution were using 20 g/L of adsorbent dose, at pH 2.0 with 250 rpm stirring speed. While studies conducted by Cronje *et al.* (2011) used an adsorbent prepared from SCB with chemical activation by zinc chloride ($ZnCl_2$) to study its sorption potential on removing Cr(VI). In this work, the application of RSM is presented for optimization of Cr(VI) removal from aqueous solutions to improve adsorption conditions in batch process. The optimum adsorbent dose, temperature, initial concentration of Cr(VI) and initial pH of the Cr(VI) solution were found to be 6.85 g/L, 40 °C, 77.5 mg/L and 8.58 respectively. Under the optimal value of process parameters, high removal (>87%) was obtained for Cr(VI).

Most of these researchers reported the ability of chemically treated SCB to remove Cr(VI). SCB has been used for the production of various industrially important enzymes. Many microorganisms, including filamentous fungi, yeasts and bacteria, have been cultivated on this material in fermentation processes. Table 2.6 shows microorganisms cultivated on SCB and the products. It clearly shows that no research has been published related to Cr(VI) reduction study using SCB–cultivated bacteria, hence the novelty of this study.

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Table 2.6: Microorganism cultivated on SCB and the products (Parameswaran, 2009)

Products	Organisms
Ethanol	*Pachysolen tannophilus*; *Escherichia coli* KO11; Recombinant bacteria
Xylitol	*Candida guilliermondii*; Yeast
SCP/protein enriched feed	*Cellulomonas flavigena*; *Xanthomonas* sp.; White–rot fungus; *Candida tropicalis*; *Trichoderma reesei*
Mycoprotein	*Neurospora sitophila*
Aroma	*Ceratocystis fimbriata*
Cellulases	*Trichoderma reesei*; White–rot fungi; *Aspergillus niger*; *Aspergillus ellipticus*; *Aspergillus fumigates*
Xylanases	*Cellulomonas flavigena*; *Melanocarpus albomyces*
Laccase	*Flammulina velutipes*; *Trametes versicolor*
Gubberlic acid	*Gibberella fujikuroi*
Pigments	*Monascus purpurea*
Glutamic acid	*Brevibacterium* sp.
Ergot alkaloids	*Claviceps purpurea*
Lactic acid	*Rhizopus oryzae*; *Lactobacillus delbrueckii*
Citric acid	*Aspergillus niger*
Pectinases	*Aspergillus niger*
Bioplastics	*Ralstonia eutropha*

2.4.2 Pineapple waste

Pineapple (*Ananas comosus*) canning industry in Malaysia which contributed 1% of world pineapple production as in 2007 produces large quantities of solid and liquid waste. The solid pineapple waste (SPW) from the pineapple cannery industries is sold to the local farmers where it will be used as the feedstock sources for farm livestock. In most cases, the liquid pineapple waste (LPW) is discharged as an effluent into the nearest stream untreated; contributing to pH decrease, BOD increase, and bad odours rendering the stream unsuitable for aquatic life (Mohd Zain *et al.*, 2010). Alternatively, if the liquid waste could be transformed into value added products such as sugars and organic acid, this would certainly be advantageous to provide sustainable resources for human needs. Pineapple peel is a rich source of

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cellulose, hemicellulose and other carbohydrates. The composition of SPW is given in Table 2.7. It has been used to produce paper, banknotes and cloth (Bartholomew *et al.*, 2003). While the composition of LPW as reported by Zakaria *et al.* (2007a) and Mohd Zain *et al.* (2010) is given in Table 2.8.

Table 2.7: Composition of SPW (Abdullah and Mat, 2008)

	Unit	Values
Moisture	%	87.50
pH	-	4.00
Total solid	%	12.50
Ash	%	4.05
Reducing sugars	%	20.93
Glucose	%	8.24
Fructose	%	12.17
Sucrose	%	0.00
Crude fibre	%	10.57
Total nitrogen	%	0.83
Crude protein	%	5.18

Table 2.8: Composition of LPW

Parameters	Unit	Values	
		Zakaria *et al.* (2007a)	Mohd Zain *et al.* (2010)
pH	-	3.19 – 4.17 ± 0.28	4.0
Colour	-	Yellowish green	–
Temperature	°C	33.9 ± 1.61	–
Microbiological count	-	4 colonies isolated	–
Sulphate (SO_4^{2-})	mg/L	58.2 ± 1.41	< 0.05
Glucose	g/L	4.95	8.32
Fructose	g/L	4.49	5.8
Sucrose	g/L	Not readily detected	58.2
Arsenic (As)	mg/L	4.04 ± 1.41	–
Copper (Cu)	mg/L	9.06 ± 10.3	0.14
Iron (Fe)	mg/L	22.21 ± 2.62	2.03
Cadmium (Cd)	mg/L	0.05 ± 0.00	< 0.004
Chromium (Cr)	mg/L	1.28 ± 0.08	–

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The core waste could be used for the production of frozen pineapple juice concentrates or extracted juice for alcoholic beverages or for vinegar (Couto and Sanroman, 2006). In addition, the waste from pineapple has been used for cellulase production (Omojasola *et al.*, 2008), removal of methylene blue (Bhatnagar and Sillanpaa, 2010; Lutpi *et al.*, 2011) and for the remediation of selected trace metals (Cd, Pb and Mn) and oxyhalides (BrO_3^-, ClO_3^- and IO_3^-) in aqueous system (Abdulkadir, 2009). Moreover, the pineapple wastes have also been used as substrates for the production of methane, ethanol, citric acid and antioxidant compounds (Imandi *et al.*, 2008; Paepatung *et al.*, 2009). To date, no attempt on Cr(VI) removal study using SPW to cultivate bacteria for the reduction process has been reported.

2.4.3 Soybean meal

Soybean meal (SBM) is a co–product obtained in the processing of soybean (*Glycine max*) to produce dietary oil. After extracting most of the oil from whole soybeans the remaining product is utilized as SBM. Banaszkiewicz (2011) stated that SBM contains the following (%); crude protein: 44.40, crude ash: 6.65, crude fat: 2.18, crude fibre: 6.75, neutral detergent fibre (NDF): 15.51, acid detergent fibre (ADF): 9.5, N–free–extractive: 31.82 and starch: 6.3. A study conducted by Witek–Krowiak and Harikishore Kumar Reddy (2013) has revealed novel application of SBM for successful removal of Cr(III) and Cu(II) and gave new insights into the binding mechanism during the biosorption process. Biosorption kinetics was very fast and the kinetics data were successfully modelled using nonlinear pseudo–second–order model. A series of isotherm experiments revealed that pH 5 favoured Cr(III) and Cu(II) biosorption and the affinity order of SBM was Cu(II)>Cr(III). Biosorption mechanism was confirmed by the functional group blocking, FT–IR and SEM–EDX results. The biosorption mechanism was due to ion–exchange, chelation by carboxyl and hydroxyl groups present on the SBM surface and further precipitation of metal ions on the surface of biomass. No research based on the adaptation of SBM with bacteria for heavy metal removal has been reported.

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2.5 Microbial technology for Cr(VI) bioremediation

A number of viable process arrangements have been reported for the application of microbial technology to reduce Cr(VI) to Cr(III) including aerobic suspended growth system, aerobic attached growth system and anoxic attached growth system (Elangovan and Philip, 2009). From an industrial point of view, the continuous–flow and fixed–film bioreactors offer the most reliable mode of application due to its easiness of handling and simple operation. Hence, most of the studies are now directed towards the evaluation on the feasibility of the process at the pilot–scale level (Ahmad *et al.*, 2010b). Some of the reported studies are shown in Table 2.9.

Table 2.9: Cr(VI) reduction using the continuous–flow and fixed–film bioreactors at pilot–scale level (Ahmad *et al.*, 2010b)

Bioreactor	Microbes used	Time needed (h)	Initial Cr(VI) (mg/L)	Cr(VI) reduction (%)
Anaerobic	*Desulfomicrobium norvegicum*	7	20	~ 100
Anaerobic trickling filter	Mixed cultures	11	10 – 20	~ 100
Aerobic packed–bed	Mixed cultures	3.3	5.5	100
Aerobic trickling filter	*Acinetobacter* sp.	19	30	100
Aerobic trickling filter	Mixed cultures	0.3 – 6	5 – 100	100
Aerobic packed–bed	*Acinetobacter haemolyticus*	3 – 9	17 – 81	100

Biological Cr(VI) removal using immobilized cells in column requires additional nutrient supplementation to maintain the number of growing cells in column during Cr(VI) reduction. The used of LPW as bacterial growth medium in a laboratory–scale Cr(VI) reduction system was discussed previously by Ahmad *et al.* (2009a). In this study, choice of LPW as nutrient for the microbial community inside the biofilm was made due to its abundance and cheap pricing, even though, other types of nutrients sources such as brown sugar and molasses also showed excellent role to support bacterial growth in a biofilm system (Ahmad *et al.*, 2009b). However, the dark coloration and high COD contents in the final effluent off–set its potential application (Ahmad *et al.*, 2010b).

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In this study, immobilization cell technique was applied for increasing cell mass concentration inside the column. Cells can be immobilized by three different techniques, namely adsorption, entrapment and covalent bond formation. Entrapment and covalent bond formation require use of chemicals that add to the cost of production and perhaps restrict further propagation or increase in cell concentration inside the column. The adsorption technique is of natural origin as cells 'adsorb/adhere' to the support naturally and firmly. This has been used extensively in the literature to adsorb microbial cells (Qureshi *et al.*, 2005).

2.5.1 Biofilm reactors in Cr(VI) wastewater treatment

The application of biofilm technology in wastewater treatment originated from the industrial operation of trickling filters in the early 1880s in Wales, Great Britain (Lazaravo and Manem, 2000). Biofilm processes in wastewater treatment can be divided into two categories, namely the fixed–medium systems where the biofilm media are static in the reactors and the biological reactions take place in the biofilm developed on the static media and the moving–medium systems where the biofilm media are kept continually moving by means of mechanical, hydraulic or air forces (Rodgers and Zhan, 2003). The moving–medium systems include rotating biological contactors, moving–bed biofilm reactors, vertically moving biofilm reactors and fluidized bed biofilm reactors, while the fixed–medium systems include trickling filters and biological aerated filters (Rodgers and Zhan, 2003). Rotating biological contactors (RBC) have been widely used in biological treatment of wastewater for reducing COD/BOD and nitrification/denitrification purposes (Kargi and Eker, 2001). RBC treats wastewater streams using a thin biofilm of aerobic microorganisms on rotating cylinders or biodiscs. The rate of rotation is selected to provide optimum contact of the waste stream with the biofilm for efficient oxygen transfer and bioactivity.

It should be noted that before a biofilm–based treatment system is to be considered for the treatment of wastewater, it is necessary to determine whether the naturally occurring microorganisms are able to produce biofilms, while

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simultaneously reducing the COD of the wastewater, or if there is a need to inoculate the reactor with external bacterial strains. The most commonly used RBC is the rotating biodisc and its various modifications (Kargi and Eker, 2001). For the treatment of high strength wastewater, gentle aeration of the liquid phase has been shown to improve the COD reduction of the system by about 40%. In a related study, Kargi and Eker (2001) have shown that a rotating–perforated–tube biofilm reactor is effective in COD removal from pure Cr(VI) solution composed of diluted molasses, urea, KH_2PO_4 and $MgSO_4$. The liquid phase in the tank was not aerated (the total biofilm surface area, A was 1.34 m^2) and the rotation speed of the tubes was 5 rpm. Successful application of a bioremediation process relies upon an understanding of interactions among microorganisms, organic contaminants and soil or aquifer materials.

2.6 Molecular analysis of microbial communities

The application of molecular techniques for the identification and/or enumeration of different microbial groups have increased enormously during the past decade. Especially the 16S rRNA–based detection and enumeration techniques have advanced greatly (Table 2.10). Molecular techniques enable faster detection and enumeration of specific bacteria than conventional culture–based methods. However, culture–based methods are still the only validated methods able to quantitate viable bacteria from industrial environments (Maukonen and Saarela, 2009).

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Table 2.10: Current techniques that can be used to study wastewater microorganisms (Gilbride *et al.*, 2006)

Technique	Benefits	Limitations	Examples of usage
Microscopy	Fast; direct observation of microbial cell	Majority of bacteria population cannot be identified	Association of filamentous bacteria with sludge bulking
Media–based methods	Easy to perform; identification of individual microorganisms	Majority of bacteria cannot be easily cultivated on general purpose media	Domination of aerobic and facultative anaerobic heterotrophs in kraft pulp treatment systems; common bacterial isolates from kraft bleached pulp mill
Indicator microorganism–based pathogen estimation	Easy to perform; current standard for coliforms	Labour intensive and time consuming; indirect estimation of pathogens rather than direct detection	F+ RNA and DNA coliphage densities as an indicator of water quality
Amplified ribosomal DNA Restriction Analysis (ARDRA)	Culture–independent; suitable for analysis of a wide range of microorganisms	DNA extraction and PCR biases; not quantitative	Microbial diversity of activated sludge
ribosomal RNA Intergenic Spacer Analysis (RISA)	Culture–independent; suitable for analysis of a wide range of microorganisms; significant heterogeneity in length and sequence among bacteria	DNA extraction and PCR biases; not quantitative; significant heterogeneity in length and sequence among bacteria	Bacterial diversity and community structure in pulp and paper wastewater; bacterial community analysis from different pulp and paper wastewater treatment systems

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*Table 2.10 continued

Technique	Benefits	Limitations	Examples of usage
Denaturing Gradient Gel Electrophoresis (DGGE)	Cuture–independent; suitable for analysis of a wide range of microorganisms; use of rRNA gene sequence heterogeneity	DNA extraction and PCR biases; not quantitative; specificity can be an issue because of short target sequences	Microbial community composition; population shift; succession of bacterial population
Terminal–Restriction Fragment Length Polymorphism (t–RFLP)	Culture–independent; suitable for analysis of a wide range of microorganisms; fast and semi–quantitative	DNA extraction and PCR biases	Composition of pulp mill microbial community; bacterial community composition from sewage treatment plants
Fluorescent *In Situ* Hybridization (FISH)	Quantitative; direct visual resolution of microbial cells including non–culturables	Inactive cells may not be detected	*In situ* analysis of microbial community structure in activated sludge; observation of sludge floc forming microorganisms
FISH and Confocal Scanning Laser Microscopy (CSLM)	Quantitative; direct visual resolution of microbial cells including slow growing and non–culturables	Expensive instrumentation	Visualization of nitrifying bacteria
Multiplex PCR	Rapid and simultaneous detection of several target microorganisms	Combinations of primer pairs must function in a single PCR reaction	Detection of bacterial pathogens in wastewater and viruses
Nucleic acid microarrays	High throughput design; various applications	Low sensitivity for environmental samples; sample processing complexities	Pathogen detection in water, foods and wastewater
On–chip technology	Combination of PCR with nucleic acid hybridization on a single chip; less interference between parallel reactions	Integration and packaging	Detection of bacterial pathogens

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Because of the inherent limitations of culture–based methods, soil microbial ecologists are turning increasingly to culture–independent methods of community analysis. Using culture–independent methods, the composition of communities can be inferred based on the extraction, quantification and identification of molecules from soil that are specific to certain microorganisms or microbial groups, or advanced fluorescence microscopic techniques. Useful molecules for such studies include phospholipid fatty acids and nucleic acids, whereas the microscopic techniques involve either the hybridization of fluorescent–labelled nucleic acid probes with total RNA extracted from soils or hybridizations with cells *in situ* (Hill *et al.*, 2000).

Molecular methods based on the sequence of PCR–amplified genes, especially 16S rRNA genes, have become very important for revealing the genetic and functional diversity of microbial communities. These methods include PCR fragment cloning, followed by restriction and sequence analysis and finally genetic fingerprinting techniques such as Restriction Fragment Length Polymorphism (RFLP) (Geets *et al.*, 2008).

2.6.1 Microbial diversity applications

For centuries, the conventional identification and characterization of microbial communities were limited to those that are culturable under laboratory conditions. However, these culturable communities only represent less than 1% of the total diversity (Amann *et al.*, 1995; Davies *et al.*, 2005). With the advancements in molecular biology technique, the application of culture–independent approaches (without the need for culturing the microbe) to study microbial populations have led to the discovery of unique and previously unrecognized microorganisms. These approaches not only reveal the actual diversity of microbes in particular environments or system but also disclosed some biological information for the benefit of other potential applications such as bioremediation of heavy metals. The culture–independent approach based on 16S rDNA sequence analysis has been proven extremely useful in assessing the changes in microbial community structures.

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This method has been used to access the microbial communities in compost (Liew *et al.*, 2009), sludge (Jin *et al.*, 2011), thermophilic microbial fuel cells (Jong *et al.*, 2006), soil microbial ecology (Hirsch *et al.*, 2010), effluent treatment plants (Rani *et al.*, 2008) and metal bioremediation (Vargas–Garcia *et al.*, 2012).

Mendes *et al.* (2007) whose study focused on the diversity of cultivated endophytic bacteria from SCB reported that 16S rRNA gene sequence analysis from selected isolates of the endophytic bacterial community of sugarcane belong to the genera of *Burkholderia*, *Pantoea*, *Pseudomonas* and *Microbacterium*. Bacterial isolates belonging to the genus *Burkholderia* were the most predominant among the endophytic bacteria. Many of the *Burkholderia* isolates produced the antifungal metabolite pyrrolnitrin and all were able to grow at 37 °C. Phylogenetic analyses of the 16S rRNA gene and *recA* gene sequences indicated that the endophytic *Burkholderia* isolates from sugarcane are closely related to clinical isolates of the *Burkholderia cepacia* complex and clustered with *B. cenocepacia* (gv. III) isolates from cystic fibrosis patients.

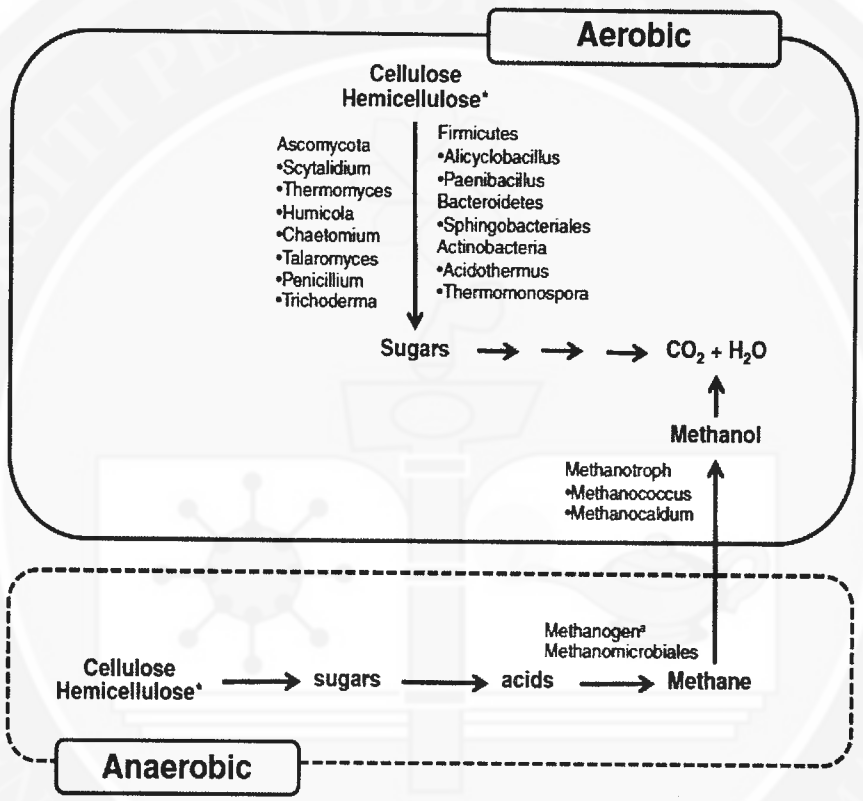
Silva *et al.* (2009) described the isolation and identification of the microbiota present in SCB and coast–cross straw compost prepared for the production of *Agaricus brasilienses*. Bacteria mainly *Bacillus* and *Paenibacillus* spp. and members of the *Enterobacteriaceae* were the predominant microorganisms present throughout the composting process with an average population density of 3 X 10^8 CFU/g. *Actinomycetes* and especially members of the genus *Streptomyces*, were well represented with a population density of 2–3 X 10^8 CFU/g. The filamentous fungi however exhibited much lower population densities and were less diverse than the other microorganisms, although *Aspergillus fumigatus* was present during the whole composting process and after pasteurisation.

Another study by Rattanachomsri *et al.* (2011) investigated the microbial community in industrial SCB feedstock piles. Molecular biodiversity analysis of 16S rDNA sequences revealed the presence of a complex bacterial community. A diverse group of mainly aerobic and facultative anaerobic bacteria was identified reflecting the aerobic and high temperature microenvironmental conditions under the pile

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surface. The major bacterial taxa present were identified as *Firmicutes*, *Alpha–* and *Gammaproteobacteria*, *Acidobacteria*, *Bacteroidetes* and *Actinobacteria*. A microbial interaction model was proposed focusing on lignocellulose degradation and methane metabolism (Figure 2.5). The insights into the microbial community from the study provide a basis for efficient utilization of bagasse in lignocellulosic biomass–based industries.



[a] Rattanachomsri *et al.*, unpublished

Figure 2.5: Biochemical processes in industrial bagasse feedstock piles based on microbial biodiversity (Rattanachomsri *et al.*, 2011)

2.7 Cr(VI) reduction mechanism study

2.7.1 Microbial reduction of hexavalent chromium

The use of microorganisms received much more attention in recent years since they carry a wide range of binding sites for heavy metal ions (Goyal *et al.*,

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2003). Advances in the field of environmental microbiology and biotechnology indicate that bacteria, fungi, yeast and algae either as pure or mixed cultures, can remove Cr(VI) from aqueous solutions under aerobic and/or anaerobic conditions (Ramirez–Ramirez *et al.*, 2004; Morales–Barrera and Cristiani–Urbina, 2006; Tekerlekopoulou *et al.*, 2010). The bioreduction rate of Cr(VI) is more rapid in aerobic conditions than in anaerobic (Jeyasingh and Philip, 2005). Additionally, aerobic cultures grew well in the presence of high initial Cr(VI) concentrations, in contrast to anaerobic conditions where growth was completely inhibited by exposure to 500 mM Cr(VI) (Pattanapipitpaisal *et al.*, 2001). There are many studies concerning the removal of Cr(VI) using pure or mixed cultures of different microorganisms. However, the main disadvantage of using pure cultures to remove Cr compounds is related to the use of sterile conditions to prevent external microbial contamination, thus increasing the treatment costs and hindering their wide applicability. For this reason, results obtained in the study of Cr(VI) removal by mixed cultures are more relevant to optimize the design and operation of actual biological Cr(VI) reduction systems.

The mechanisms that microorganisms use to reduce Cr(VI) to Cr(III) are variable and species dependent. The processes by which microorganisms interact with toxic metals enabling their removal/and recovery are enzymatic reduction, bioaccumulation and biosorption (Sharma and Forster, 1993; Shen and Wang, 1993; Fude *et al.*, 1994). Many factors affect the rate and the extent of microbial Cr(VI) reduction such as the initial biomass concentration, initial Cr(VI) concentration, pH, temperature, oxidation–reduction potential and metal cations (Wu *et al.*, 2010; Ye *et al.*, 2010). Also the presence of suitable carbon source is necessary to enhance the Cr(VI) reduction capacity (Tekerlekopoulou *et al.*, 2010). Similar observations were also referred in previous studies by Ferro Orozco *et al.* (2010) and Contreras *et al.* (2011). Changes in these parameters can vary greatly in the environment, affecting the ability of microorganisms to reduce pollutants (Tekerlekopoulou *et al.*, 2013).

Bacteria can enzymatically reduce Cr(VI) by both aerobic and anaerobic pathways. However, other nonbiological Cr reduction pathways compete with the biological pathways. Under anaerobic conditions, biological reduction is slow so

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abiotic reduction by Fe(II) or hydrogen sulphide is expected to dominate. Microbial reduction only becomes kinetically important in aerobic environments (Fendorf *et al.*, 2001). Oxygen concentrations in the system are the primary factor influencing the reduction rate, followed by pH and geochemical conditions.

2.7.1.1 Aerobic Cr(VI) reduction

In the presence of oxygen, bacterial Cr(VI) reduction occurs as a two or three step process with Cr(VI) initially reduced to the short–lived intermediates Cr(V) and/or Cr(IV) before further reduced to the thermodynamically stable end product, Cr(III). Cr(V) undergoes a one electron redox cycle to regenerate Cr(VI) by transferring the electron to oxygen. The process produces a reactive oxygen species (ROS) that easily combines with DNA–protein complexes. Nevertheless, it is presently unclear whether the reduction of Cr(V) to Cr(IV) and Cr(IV) to Cr(III) is spontaneous or enzyme mediated (Cheung and Ji–Dong, 2007). Chromate reductase (ChrR) assay from an alkaliphilic Gram–positive *Bacillus subtilis* indicated that the Cr(VI) reduction was mediated by constitutive membrane bound enzymes and a decrease in pH with growth of the bacterium signified the role played by metabolites (organic acids) in Cr resistance and reduction mechanism (Mary Mangaiyarkarasi *et al.*, 2011). Nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH) and electrons from the endogenous reserve are implicated as electron donors in the Cr(VI) reduction process (Cheung and Ji–Dong, 2007). Reductases as ChrR, YieF and *Bacillus megaterium* TKW3 reduce Cr(VI) species by electron shuttle to form Cr(III). Strain *Lysinibacillus fusiformis* ZC1 was found to contain large numbers of metal resistance genes. Specifically, a chrA gene encoding a putative chromate transporter conferring chromate resistance was identified. Furthermore, a yieF gene and several genes encoding reductase that were possibly involved in chromate reduction were also found. Expression of adjacent putative chromate reduction related genes, nitR and yieF, were found to be constitutive. The large numbers of NADH dependent chromate reductase genes may be responsible for the rapid chromate reduction in order to detoxify Cr(VI) and

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survive in the harsh wastewater environment (He *et al.*, 2011; Barrera–Diaz *et al.*, 2012).

Also, isolated bacteria putatively identified by 16S ribosomal RNA (rRNA) gene sequencing as *Arthrobacter aurescens* strain MM10, *Bacillus atrophaeus* strain MM20 and *Rhodococcus erythropolis* strain MM30 that present chromate reductase gene sequences found in Gram–negative bacteria (*Escherichia coli* and *Shigella* spp.) have the ability to rapidly reduce highly toxic concentrations of Cr(VI) to Cr(III) when grown in minimal medium supplemented with glucose as the sole carbon source (Patra *et al.*, 2010). Two reaction steps have been suggested to be involved in the reduction reactions, first Cr(VI) accepts one molecule of NADH and generates Cr(V) as an intermediate (Equation 2.7) and then Cr(V) accepts two electrons to form Cr(III) (Equation 2.8) (Singh *et al.*, 2011):

$$Cr^{6+} + e^{-} \rightarrow Cr^{5+} \quad (2.7)$$

$$Cr^{5+} + 2e^{-} \rightarrow Cr^{3+} \quad (2.8)$$

Two processes are responsible for the reduction of Cr(VI) when aerobic heterotrophic cells, non–growing cells, growing cells with chromate reductase activity and growing cells that have lost the chromate reductase activity were used. The first one is the reduction of Cr(VI) coupled with growth and the second process is coupled with the endogenous decay of the biomass (Contreras *et al.*, 2011).

The presence and influence of proteins (Zahoor and Rehman, 2009) and electron donors such as glucose, fructose, sucrose and bagasse extract were found to offer great Cr(VI) reduction rates when *Bacillus* sp. and *Staphylococcus capitis* are used. Among the different electron donors, glucose provided the highest Cr(VI) reduction compared to other electron donors (Kathiravan *et al.*, 2011). A new mechanism has been proposed for chromate reduction by *Brevibacterium casei*, which can reduce Cr(VI) with an azo dye Acid Orange 7 (AO7). Under nutrient–limiting conditions, AO7 was used as an electron donor by the reduction enzyme(s)

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of *B. casei* for the reduction of Cr(VI). The resulted Cr(III) then complexes with the oxidized AO7 to form a purple–coloured intermediate (Ng *et al.*, 2010). A diagram illustrating the mechanism of enzymatic Cr(VI) reduction under aerobic conditions is shown in Figure 2.6 (upper).

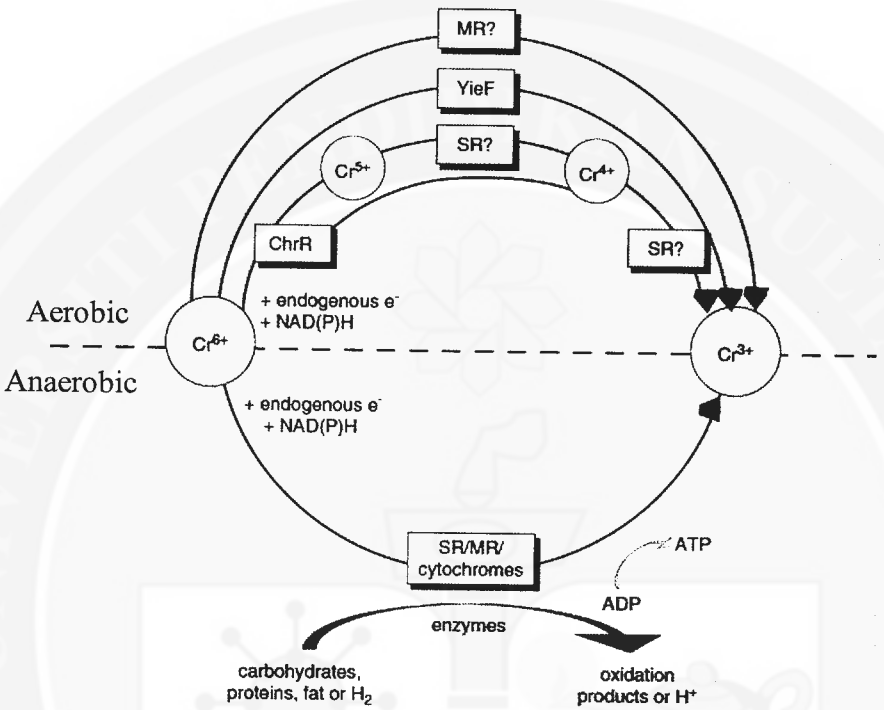


Figure 2.6: Plausible mechanisms of enzymatic Cr(VI) reduction under aerobic (upper) and anaerobic (lower) conditions (Cheung and Gu, 2007)

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2.7.1.2 Anaerobic Cr(VI) reduction

In the absence of oxygen, Cr(VI) can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors, including carbohydrates, proteins, fats, hydrogen, NADPH and endogenous electron reserves. Both soluble and membrane–associated enzymes have been found to mediate the process of Cr(VI) reduction under anaerobic conditions. Unlike the Cr(VI)–reductases isolated from aerobes, the Cr(VI)–reducing activities of anaerobes are associated with their electron transfer systems ubiquitously catalyzing the electron shuttle along the respiratory chain (Cheung and Ji–Dong, 2007). The overall bioreduction of Cr(VI) and precipitation of Cr(III) is illustrated in Equations 2.9 and 2.10. Under anaerobic

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conditions with glucose as an electron donor, the Cr(VI) microbial reduction is related to Equation 2.11 (Singh *et al.*, 2011).

$$CrO_4^{2-} + 8H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O \quad (2.9)$$

$$Cr^{3+} + 3H_2O \rightarrow Cr(OH)_3 + 3H^+ \quad (2.10)$$

$$C_6H_{12}O_6 + 8CrO_4^{2-} + 14H_2O \rightarrow 8Cr(OH)_3 + 10OH^- + 6HCO^- \quad (2.11)$$

Pannonibacter phragmitetus has strong aerobic and anaerobic Cr(VI)–reduction potential under alkaline conditions but its anaerobic reduction potential is higher than aerobic, showing a potential application for Cr(VI) detoxification (Xu *et al.*, 2011b). Compared to free cells, *P. phragmitetus* cells coated with polyethylenimine–functionalized magnetic nanoparticles, not only had the same Cr(VI)–reduction activity but could also be easily separated from reaction mixtures by magnetic force. In addition, the magnetically immobilized cells retained high specific Cr(VI)–reduction activity over six batch cycles. The results suggest that the magnetic cell separation technology has potential application for Cr(VI) detoxification in alkaline wastewater (Barrera–Diaz *et al.*, 2012).

An efficient mediated microbial reduction of Cr(VI) by *E. coli* is promising for rapid anaerobic removal and the reduction of chromate by resting *E. coli* cells were significantly enhanced by the quinone redox mediators lawsone, menadione, anthraquinone–2–sulfonate, anthraquinone–2,6–disulfonate and also by the addition of glucose as an electron donor (Liu *et al.*, 2011). Furthermore, *E. coli* biofilm on chitosan beads (EBCB) bio regenerated the chitosan beads after *E. coli* biofilm has grown significantly allowing Cr(VI) adsorption by chitosan beads and Cr(VI) bioreduction by *E. coli* cells (Lin *et al.*, 2011). Also *E. coli* cells in the presence of quinone redox mediators have enhanced chromate reduction (Liu *et al.*, 2010).

Sulphate and iron reducing bacteria (SRB and IRB) are important members of anaerobic microbial communities with economic, environmental and

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biotechnological interest. Cr(VI) reduction by biogenic Fe(II) and sulphides generated by IRB and SRB is 100 times faster than Cr reducing bacteria (CRB) alone. SRB produces H_2S, which serves as a Cr(VI) reductant and involves three stages (Somasundaram *et al.*, 2011); (a) reduction of sulphates (Equation 2.12), (b) reduction of chromate by sulphides (Equation 2.13) and (c) precipitation of Cr(VI) by sulphide (Equation 2.14).

$$SO_4^{2-} + 2CH_2O + H^+ \rightarrow HS^- + 2H_2O + 2CO_2 \quad (2.12)$$

$$8CrO_4^{2-} + 3HS^- + 17H_2O \rightarrow 8Cr(OH)_3 + 3SO_4^{2-} + 13OH^- \quad (2.13)$$

$$Cr^{6+} + 3HS^- \rightarrow CrS_3 + 3H^+ \quad (2.14)$$

The reduction of Cr(VI) by Fe(II) occurs when IRB reduces Fe(III) to Fe(II) which in turn reduces Cr(VI) to Cr(III) (Equations 2.15–2.17) (Somasundaram *et al.*, 2011).

$$C_6H_{12}O_6 + 24Fe^{3+} + 12H_2O \rightarrow 6HCO_3^- + 24Fe^{2+} + 30H^+ \quad (2.15)$$

$$3C_3H_5O_3^- + 12Fe(OH)_3 \rightarrow 3C_2H_3O_2^- + 12Fe^{2+} + 3HCO_3^- + 8H_2O + OH^- \quad (2.16)$$

$$3Fe^{2+} + HCrO_4^- + 8H_2O \rightarrow 3Fe(OH)_3 + Cr(OH)_3 + 5H^+ \quad (2.17)$$

The typical anaerobic Cr^{6+} reduction is shown in Figure 2.6 (lower).

2.7.2 Determination of the oxidation state of chromium bound to the lignocellulosic residue using instrumental analysis

Various biomaterials can retain relatively high quantities of metal ions by passive sorption and/or complexation i.e. this is commonly known as biosorption

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(Bailey *et al.*, 1999). Since a report on the use of sawdust by Srivastava *et al.* (1986), many researchers have tested various biomaterials such as non–living bacteria, microalgae, fungi, seaweed, agricultural by–product and industrial biowaste as an adsorbent for Cr(VI) removal (Mohan and Pittman, 2006).

To verify the oxidation state of the Cr bound to biomaterials, X–Ray Absorption Spectroscope (XAS) or X–Ray Photoelectron Spectroscope (XPS) (Park *et al.*, 2006) can be used. Regardless of whether or not researchers have recognized and/or confirmed the occurrence of Cr(VI) reduction to Cr(III) by the biomaterial tested, most of them have accepted 'adsorption–coupled reduction' as the mechanism of Cr(VI) biosorption (Verma *et al.*, 2006; Garg *et al.*, 2007). However, a number of researchers still believe that 'anionic adsorption' is the removal mechanism of Cr(VI) in their systems (Agarwal *et al.*, 2006; Kiran *et al.*, 2007).

Previous research elucidated the mechanisms of Cr(VI) resistance and removal by *A. haemolyticus* via instrumental analysis. Hsiao Pei *et al.* (2009) reported the tolerance to 90 mg/L Cr(VI) in LB broth compared to only 30 mg/L Cr(VI) in LB agar. From the FT–IR analysis, the Cr(III) species formed was also most likely to form complexes with carboxyl, hydroxyl and amide groups from the bacteria. A TEM study showed the absence of precipitates on the cell wall region of the bacteria. Instead, microprecipitates were observed in the cytoplasmic region of the cells, suggesting the transportation of Cr(VI) into the cells. Intracellular reduction of Cr(VI) was supported by a reductase test using soluble crude cell–free extracts. The specific reductase activity obtained was 0.52 μg Cr(VI) reduced per mg of protein an hour at pH 7.2 and 37 °C.

Besides exploring the metal–microbe interaction in the presence of agricultural waste as a culture medium, other researchers also reported the use of natural and modified lignocellulosic residues for Cr adsorption. The Cr maximum adsorption capacity and the adsorption mechanisms under different experimental conditions are also reported using instrumental analysis such as X–Ray Photoelectron Spectroscopy, XPS (Park *et al.*, 2007), X–Ray Absorption Near–Edge Structure, XANES and Extended X–Ray Absorption Fine Structure, EXAFS

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(Gardea–Torresdey *et al.*, 2000), Electronic Paramagnetic Resonance, EPR or Electronic Spin Resonance, ESR (Flogeac *et al.*, 2003) for determination of the oxidation state of Cr bound to the lignocellulosic residue. Determination of functional groups present in the biomass was usually performed by Fourier Transform Infra–Red, FT–IR (Garg *et al.*, 2007; Qaiser *et al.*, 2007; Krishnani *et al.*, 2008).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strain

A Cr(VI) resistant–reducing bacteria, *A. haemolyticus* described previously in section 2.3.2.2 was used throughout this study.

3.1.1 Growth medium

3.1.1.1 Nutrient broth

Nutrient broth (NB) is an enriched medium composed of peptone from meat extract. It is commonly used as a growth medium for the bacteria. For the preparation of NB, 8 g of NB powder (Merck) was dissolved in 1 L of deionised water followed by autoclaving at 121 °C, 1.03 kPa for 15 min (Hirayama Hiclave HVE–50).

3.1.1.2 Nutrient agar

Nutrient agar (NA) plates were prepared by dissolving 20 g NA powder (Merck) in 1 L deionised water and autoclaved at 121 °C, 1.03 kPa for 15 min. Once cooled to ±55 °C, the NA was dispensed aseptically into sterile petri dishes and

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allowed to solidify. The plates were left to dry overnight at 30 °C in the incubator (Memmert) and stored in a refrigerator (4 °C) prior to use.

3.1.1.3 Luria–Bertani glycerol

For long–term storage of bacterial strain, a rich medium containing Luria–Bertani (LB) with 15% (v/v) glycerol was used. The composition of LB glycerol was 1.0 g tryptone (Oxoid), 0.5 g yeast extract (Oxoid), 0.05 g NaCl (Merck) and 15 g of glycerol (J. T. Baker). All these components were dissolved in 100 mL of deionised water and autoclaved at 121 °C, 1.03 kPa for 15 min.

3.1.2 Bacterial stock culture

Short–term bacterial stock culture was prepared first by streaking a loopful of *A. haemolyticus* onto NA plate. The plate was incubated for 24 h at 30 °C and can be used immediately or stored in the refrigerator (4 °C) for further use. For long–term bacterial stock culture preparation, a single colony of *A. haemolyticus* was streaked onto two different NA plates and incubated at 30 °C for 24 h. Sterile LB glycerol solution (3 mL) was then pipetted onto the first freshly grown culture plate. A sterile glass spreader was used to resuspend the bacterial cells. The cell suspension was later transferred onto a second NA plate and the resuspension process was repeated. The suspension was then transferred into sterile Bijou bottles and kept in the freezer at –20 °C.

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3.2 Agricultural wastes

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3.2.1 Sampling of agricultural wastes

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Three types of agricultural wastes (AW) were used in this study; sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM). All AW samples (5 kg each) were selected using coning and quartering method (Harvey, 2008). The AW was transferred into a plastic container and stored in the refrigerator at 4 °C prior to use. All three AW was used without any chemical treatment. The sources of AW, physical properties and the pre–treatment are summarized in Table 3.1.

Table 3.1: The sources of AW and the fibre preparation for characterization

AW	Sources	Physical Properties	Pre–treatment	Photographs
SCB	Local sugarcane stall, Skudai, Johor	long fibre, 50–100 cm, pale yellow	Fibres were cut into segments ranging between 1–2 cm	
SPW	Lee Pineapple Sdn. Bhd., Tampoi, Johor	short fibre, 1–2 cm, yellow to green	None	
SBM	Zara Foodstuff Industries Sdn. Bhd., Larkin, Johor	mixes slurry, 1 cm, brown	None	

3.2.2 Characterization of agricultural wastes

The AW were air–dried at 30 °C, ground and sieved (18 mesh) to yield fibres of less than 1 mm and stored in a tight container until further use. The dried AW samples were characterized for the following properties; ash content, total solids, α–cellulose and lignin. However, the moisture content and pH of the samples were determined using fresh AW samples. The α–cellulose and lignin contents were determined from SCB only as it was the most feasible growth substrates for the

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Cr(VI) reduction study. Characterization for each parameter was carried out in triplicates to ensure reproducibility of results.

3.2.2.1 Moisture, ash content and total solids determination

Moisture content determination was carried out according to the American Society for Testing and Materials (ASTM E1756–08, 2008). Fresh AW samples at 2.5 g were transferred into a pre–weighed dried crucible and placed in a drying oven (Memmert) at 105±2 °C for 4 h. The samples were then cooled to room temperature in a desiccator and weighed. This procedure was repeated until a constant weight was obtained. The moisture content was calculated according to Equation 3.1.

$$Moisture\ content\ (\%) = \left[\frac{(A-B)}{A}\right] \times 100 \quad (3.1)$$

where, A is the mass of the sample (g) and B is the mass of oven–dried sample (g).

Similar samples were determined for ash content according to American Society for Testing and Materials (ASTM E1755–01, Reapproved 2007). Each sample (2.5 g) was initially heated to 250 °C at a rate of 10 K/min for 30 min in a muffle furnace (Carbolite) followed by ignition at 575±25 °C for a minimum of 3 h. The samples were then cooled in a desiccator and the remaining solids (ash) were weighed. The ash content was calculated according to Equation 3.2.

$$Ash\ content\ (\%) = \left(\frac{A}{B}\right) \times 100 \quad (3.2)$$

where, A is the mass of ash (g) and B is the mass of oven–dried sample (g).

Data obtained from moisture content was directly used to calculate the total solids in each AW. Total solids were calculated based on Equation 3.3.

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$$Total\ solids\ (\%) = \left(\frac{A}{B}\right) \times 100 \quad (3.3)$$

where, A is the mass of oven–dried sample (g) and B is the mass of the sample (g).

3.2.2.2 pH

Fresh AW (5 g) was immersed in 200 mL of deionised water in 250 mL beaker for 1 h. The mixture was homogenized and the pH was recorded with a pH meter (Eutech Instruments).

3.2.2.3 α–cellulose content determination

α–cellulose content of SCB was determined using the modified procedure of American Society for Testing and Materials (ASTM D1103–60, Reapproved 1977). This analysis was carried out at the Pulp and Paper Testing Laboratory, Forest Research Institute Malaysia (FRIM), Selangor. In order to analyze α–cellulose content in SCB, ethanol–toluene solubility test (ASTM D1107–56, Reapproved 1972) was initially carried out followed by determination of holocellulose content (ASTM D1104–56, Reapproved 1978).

3.2.2.4 Lignin analysis

Lignin analysis from SCB was determined using the American Society for Testing and Materials (ASTM D1106–96, Reapproved 2007) procedure carried out at the Pulp and Paper Testing Laboratory, Forest Research Institute Malaysia (FRIM), Selangor. Lignin content was evaluated based on isolation of lignin after polysaccharide hydrolysis by H_2SO_4 (72% v/v).

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3.2.2.5 Heavy metal content analysis

To determine the heavy metal content in the AW, samples were digested using concentrated HNO_3. Acid digestion method was carried out to reduce interference by organic matter and to convert metals associated with particulates to a form (usually as free metal) that can be determined by Flame Atomic Absorption Spectrometry (AAS) or Inductively–Coupled Plasma Mass Spectrometry (ICP–MS). Digestion for metals was conducted according to American Public Health Association, APHA 3030–D (Eaton *et al.*, 2005).

Acid digestion of AW samples was carried out as follows; ash sample (1 g) was mixed with 5 mL HNO_3, 70% (w/w) (J. T. Baker) in 250 mL beakers, covered with a watch glass to minimize contamination. Boiling chips were added to aid boiling and minimize spattering. The samples were heated and evaporated on a hot plate until the volume obtained was less than 1 mL. Heating and HNO_3 addition were continued until all AW were completely solubilised indicated by the formation of light coloured or clear solution. The digested AW was finally recovered and transferred into 100 mL volumetric flasks, cooled and used for metal analysis. A blank, consisting of only HNO_3 was also prepared using the same procedure without the addition of samples.

The heavy metal analysis was carried out using Inductively Coupled Plasma–Mass Spectrometer, ICP–MS (Perkin Elmer ELAN 6100) at Unit Perkhidmatan Analisis (UPA), Department of Chemistry, Faculty of Science, UTM, Johor. For complete mineralization of solid samples, results were reported based on dry–weight basis. The metal concentration was calculated according to Equation 3.4.

$$Metal\ concentration\ \left(\frac{mg}{kg}\right) = \left[\frac{(A \times B)}{C}\right] \times \left(\frac{100}{D}\right) \qquad (3.4)$$

where, A is the concentration of metal in digested solution detected by ICP–MS analysis (mg/L), B is the final volume of digested solution (mL), C is the mass of the sample (g) and D is the percentage of total solid (%).

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3.2.2.6 Monosaccharide determination

Carbohydrate analyses from AW were carried out using the procedure of the American Society for Testing and Materials (ASTM E1758–01, Reapproved 2007). Monosaccharide content was quantified by ion–moderated partition High Performance Liquid Chromatography (HPLC). Sugar recovery standard (SRS) was measured and the calculated recovery of the SRS was used to correct for losses caused by the destruction of sugars during the hydrolysis process.

AW samples (0.3 g) were digested in 3 mL concentrated H_2SO_4 (72% w/w) in glass test tubes. The samples were hydrolyzed in the acid solution for 1 h at 30 °C where the mixing was performed by vortexing every 15 min interval. Each hydrolyzate was transferred to a glass bottle and diluted with 84 mL of deionised water in order to obtain a final concentration of 4% (w/w) prior to autoclaving for 1 h at 121 °C and 1.03 kPa. The samples were then allowed to cool at room temperature. Aliquot of each hydrolyzate (20 mL) was transferred and the pH was adjusted with calcium carbonate, $CaCO_3$ (Emory) to approximately pH 5–6. The mixture was then centrifuged (Kubota, Japan) at 3000 rpm for 3 min. The supernatant was carefully and slowly drawn out and pH was measured prior to filtration through a 0.2 µm syringe filter into HPLC vials (Waters).

A similar procedure was used for SRS by replacing samples with high purity sugar standards i.e. xylose, arabinose, fructose, glucose, galactose and sucrose. The amount of sugar recovered from each sugar recovery standard was calculated according to Equation 3.5.

$$Sugar\ recovery\ (\%) = \left(\frac{A}{B}\right) \times 100 \qquad (3.5)$$

where, A is the concentration of SRS detected by HPLC after hydrolysis (mg/mL) and B is the known concentration of SRS before hydrolysis (mg/mL).

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Sugar concentration values obtained from HPLC for each hydrolyzed sample was corrected by using the percentage of sugar recovery from each SRS and was calculated according to Equation 3.6.

$$Corrected\ sugar\ concentration\ \left(\frac{mg}{mL}\right) = \left[\frac{A}{(100 \times B)}\right] \tag{3.6}$$

where, A is the concentration of sugar in hydrolyzed sample detected by HPLC (mg/mL) and B is the percentage of sugar recovery (%).

Calculation of each sugar present in the AW was calculated according to Equation 3.7.

$$Sugar\ (\%) = \left[\frac{(A \times B)}{C \times \left(\frac{D}{100}\right)}\right] \times 100 \tag{3.7}$$

where, A is the corrected sugar concentration (mg/mL), B is the volume of filtrate (mL), C is the initial mass of sample (mg) and D is the percentage of total solids of oven–dried sample (%).

Sugar standards with a concentration range of 1–4 mg/mL were prepared in deionised water. Calibration sugar standards, the hydrolyzed sugar recovery standards (SRS) and the hydrolyzed samples were analyzed using HPLC (Waters 717 plus Autosampler, USA and Waters 1525 Binary HPLC Pump). The analysis was carried out at the Food Science Laboratory, School of Chemical Sciences and Food Technology, Faculty of Science and Technology, UKM, Selangor. Separation was accomplished using a Chromolith® Performance NH_2 100–4.6 mm HPLC column sorbent (Merck, Germany) with Chromolith® NH_2 5–4.6 mm guard column kit (Merck, Germany) and quantified with a polymer laboratories PL–ELS 1000 detector.

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3.2.2.7 Total carbohydrate analysis

The total carbohydrates of AW were analyzed using the phenol–sulphuric acid method as suggested by Dubois *et al.* (1956). The method is based on the formation of highly coloured materials from the reaction between acid degradation products and polyaromatic compounds. Sugars have a strong characteristic dehydration reaction in concentrated sulphuric acid where pentose sugar yields furfural while hexose sugars give 5–hydroxymethylfurfural. The aldehyde–based products formed will undergo condensation with various types of phenols to yield characteristically coloured products (Grasshoff *et al.*, 1983).

In this analysis, 2.0 mL aqueous phenol solution (5% w/w) (Scharlau, crystallized reagent grade, ACS) and 10.0 mL of concentrated H_2SO_4 95–97% (QRec) were added to 2.0 mL of digested AW samples in a series of glass tubes. The tubes were then vortexed (Maxi Max II Thermolyne) and allowed to stand for about 10–20 min in a water bath at 25–30 °C before measurement. The glucose standard solutions were prepared by diluting glucose stock solution (1000 mg/L) in the range of 25–200 mg/L. The absorptions were measured in a cuvette at 485 nm using spectrophotometer (Genesys 20 Thermospectronic). A blank solution containing 2.0 mL of deionised water, 2.0 mL of 5% (w/w) aqueous phenol solution and 10.0 mL of concentrated H_2SO_4 was used as reference.

3.2.2.8 Anion content

The AW was analyzed for chloride (Cl^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}). The anion standard solutions were prepared by diluting anion stock solution (1000 mg/L) in the range of 1–5 mg/L. The digested AW samples (20 µL) as prepared during monosaccharide analysis were filtered through 0.45 µm syringe filter. Analysis was carried out using Ion Chromatography (IC) (Metrohm, Switzerland) at Unit Perkhidmatan Analisis (UPA), Department of Chemistry, Faculty of Science, UTM, Johor. Separation was accomplished using a Metrosep A Supp 5 column (Metrohm, Switzerland). The IC Net program was used for the data

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acquisition and as control software for IC system. Details of the operating conditions of the IC system are given in Table 3.2.

Table 3.2: Operating parameters for anion separation by suppressed IC with an anion–exchange column under isocratic conditions

	Parameters
Column	Metrosep A Supp 5 (4 X 150 mm), particle size 5 μm
	Metrosep A Supp 4/5 guard
Eluents	3.2 mmol/L Na_2CO_3 and 1.0 mmol/L $NaHCO_3$
Eluent flow rate	0.70 mL/min
Injection volume	20.0 μL
Detection	Suppressed conductivity

3.3 Growth and Cr(VI) reduction of *A. haemolyticus* in agricultural wastes in a batch system

The growth of *A. haemolyticus* was monitored in AW via cell turbidity (OD_{600}) and viable cell count (CFU/mL) using the spread plate method. The effect of Cr(VI) on bacterial growth was determined based on percentage Cr(VI) reduction using pure Cr(VI) solution.

3.3.1 Screening of agricultural wastes as an alternative growth medium for *A. haemolyticus*

To evaluate the suitability of AW to be used as a bacterial growth medium, growth of *A. haemolyticus* in AW study was conducted. The bacterial growth was carried out as follows; AW (2–10% w/v) were transferred into a series of 1 L Erlenmeyer flasks containing 85 mL of deionised water and added with 5% (v/v) denatured ethanol (Nordin and Ahmad, 2008; Ahmad *et al.*, 2009a). The pH of the mixture was adjusted to 7.0 using either 1.0 M HCl (QRec) or 1.0 M NaOH (QRec). To the mixture was then added 10% (v/v) of overnight grown *A. haemolyticus*

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followed by shaking at 200 rpm, 30 °C for 48 h. A similar set up without bacterial cells served as control. Aliquots (4 mL) were aseptically pipetted at 24 h and 48 h, and bacterial growth was measured for turbidity (OD_{600}), cell concentration (CFU/mL), total carbohydrate and pH.

3.3.2 Adaptation of *A. haemolyticus* in agricultural wastes

A. haemolyticus culture was prepared by aseptically transferring a loopful of *A. haemolyticus* cells from the NA stock culture and inoculated into 250 mL Erlenmeyer flask containing 25 mL of NB medium. The flask was then shaken at 30 °C, 200 rpm overnight in an orbital shaker (Certomat–R, B. Braun). The active culture of *A. haemolyticus* (10% v/v) was then inoculated into 1 L flask containing 10% (w/v) AW, 85% (v/v) NB, 5% (v/v) denatured ethanol and incubated at 30 °C with continuous agitation at 200 rpm for 24 h. After that, the turbidity of cells (OD_{600}) and bacterial count (CFU/mL) were recorded for the determination of bacterial growth. The adaptation process was repeated with increasing percentage of deionised water and decreasing percentage of NB (at ratios of 0:100, 20:80, 40:60, 80:20 and finally 100:0). The final system consists of 10% (w/v) AW, 85% (v/v) deionised water, 5% (v/v) denatured ethanol and 10% (v/v) active culture. The composition of the mixture are shown in Table 3.3. The AW–adapted *A. haemolyticus* was maintained in LB glycerol solution and stored in the freezer at –20 °C for long–term bacterial stock culture.

Table 3.3: Adaptation of *A. haemolyticus* in AW for growth profile monitoring

	Number of flasks				
	1	**2**	**3**	**4**	**5**
AW (10% w/v)	10	10	10	10	10
Deionised water (mL)	0	17	51	68	85
Nutrient broth (mL)	85	68	34	17	0
Denatured ethanol (5% v/v)	5	5	5	5	5
Active culture (10% v/v)	10	10	10	10	10

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3.3.3 Chromium analysis

3.3.3.1 Preparation of Cr(VI) stock solution

For the preparation of pure Cr(VI) solution, all chemicals used were of analytical reagent grade. Cr(VI) stock solution (1000 mg/L) was prepared by dissolving 2.8287 g of 99.9% potassium dichromate, $K_2Cr_2O_7$ (294.18 g/mol, Fluka) in 1 L of deionised water (UHQII, Elgastat). The pH of the Cr(VI) solution was adjusted to 7.0 using 1.0 M NaOH or 1.0 M HCl before sterilizing using 0.45 μm cellulose acetate filter paper (Whatman).

3.3.3.2 Determination of total chromium concentration using Flame Atomic Absorption Spectrophotometer

Total Cr concentrations were determined using a Flame Atomic Absorption Spectrometer, AAS (Perkin Elmer AAnalyst 400) at λ = 425.44 nm with a detection limit range between 1.0–5.0 mg/L. All analyses were performed according to standard methods for the examination of water and wastewater, APHA (Eaton *et al.,* 2005). The Cr(III) content in solution was obtained by subtracting Cr(VI) concentration from total chromium present in the solution.

3.3.3.3 Determination of Cr(VI) concentration using diphenylcarbazide method

Reduction of Cr(VI) to Cr(III) was determined using diphenylcarbazide (DPC) method with a detection limit of 5 μg/L, APHA 3500–Cr B (Eaton *et al.,* 2005). In the presence of very acidic condition (pH ≤ 1.0), chromate or dichromate ions will oxidize DPC to diphenylcarbazone (DPCO) and Cr(VI) will be reduced to Cr(III). The resulting DPCO and Cr(III) will form soluble and strongly red–violet to purple compounds which absorbs strongly at 540 nm (Llobat–Estelles et al., 2001).

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For 10 mg/L of Cr(VI) preparation in a 10 mL volumetric flask, 1.0 mL of sample was mixed with 9.0 of 0.2 M H_2SO_4. Then 0.2 mL of freshly prepared 0.25% (w/v) DPC (Fluka) in acetone was added to the volumetric flask. The mixture was then shaken to obtain a homogenized solution and left to stand between 4–5 min for full colour development. The red–violet to purple colour formed was then measured at λ = 540 nm using a spectrophotometer (Genesys 20 Thermospectronic) and distilled water was used as reference. The standard curve for Cr(VI) concentration was prepared with Cr(VI) concentration ranging from 0.2–2.0 mg/L Cr(VI) prepared from Cr(VI) stock solution (1000 mg/L). Cr(VI) reduction was expressed as percentage of difference between initial and final Cr(VI) concentration as shown in Equation 3.8.

$$Cr(VI)\ reduction\ (\%) = \left[\frac{(A - B)}{A}\right] \times 100 \qquad (3.8)$$

where, A is the initial Cr(VI) concentration (mg/L) and B is the final Cr(VI) concentration (mg/L).

3.3.4 Cr(VI) reduction study of agricultural wastes adapted and non–adapted *A. haemolyticus*

Active culture of adapted and non–adapted *A. haemolyticus* (10% v/v) were inoculated into a series of 1 L Erlenmeyer flasks containing AW (10% w/v) and respective volumes of deionised water and denatured ethanol (5% v/v) (Table 3.4) followed by incubation at 200 rpm, 30 °C for 12 h (early stationary phase). Then, Cr(VI) concentrations ranging from 1.0–10.0 mL were added to the respective flasks to obtain a final Cr(VI) concentration of 10–100 mg/L. The mixture was further shaken for 48 h. After 24 h and 48 h, aliquots (4 mL) were withdrawn, centrifuged (Hettich Zentrifugen Universal 320) at 5000 rpm for 5 min and the supernatant analyzed for residual Cr(VI) by colorimetric analysis at 540 nm according to the diphenylcarbazide (DPC) method. Bacterial growth (OD_{600} and CFU/mL) and pH were determined at the end of the incubation period. Similar experimental set up was

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carried out in the absence of bacterial cells, which acted as control i.e. evaluation on any abiotic Cr(VI) reduction.

Table 3.4: Cr(VI) and active culture used for the Cr(VI) reduction

Cr(VI) concentration (mg/L)	Control					Test				
	10	**30**	**50**	**70**	**100**	**10**	**30**	**50**	**70**	**100**
AW (10% w/v)	10	10	10	10	10	10	10	10	10	10
Deionised water (mL)	94	92	90	88	85	84	82	80	78	75
Denatured ethanol (5% v/v)	5	5	5	5	5	5	5	5	5	5
Active culture (10% v/v)	-	-	-	-	-	10	10	10	10	10
Cr(VI) 1000 mg/L (mL)	1	3	5	7	10	1	3	5	7	10

3.3.5 Growth and Cr(VI) reduction by selected agricultural waste–adapted *A. haemolyticus*

The growth of SCB–adapted *A. haemolyticus* was assessed by cell turbidity at OD_{600} and viable cell count (CFU/mL) while the lessening effect of Cr(VI) toxicity was determined by the percentage of Cr(VI) reduction to Cr(III).

3.3.5.1 Denatured and absolute ethanol as disinfecting agents

The effect of ethanol pre–treatment on SCB was also monitored by using different types of 5% (v/v) ethanol, i.e. denatured and absolute ethanol. Sample preparation was carried out using 10% (w/v) SCB and was monitored for the bacterial growth. The mixture was incubated at 30 °C, 200 rpm for 48 h. At various time intervals, samples (4 mL) were pipetted aseptically for the CFU counting (CFU/mL) and turbidity (OD_{600}) measurement and results obtained were compared to assess the effectiveness of disinfecting agent used.

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3.3.5.2 Effect of different concentrations of denatured ethanol on growth of *A. haemolyticus*

To assess the effect of pre–treatment of SCB on bacterial growth, the SCB was treated with 1–10% (v/v) of denatured ethanol. The mixtures for the preparation of different concentrations of denatured ethanol are shown in Table 3.5. At regular time intervals, aliquots (4 mL) were withdrawn and bacterial growth (OD_{600} and CFU/mL) was determined at the end of the incubation period. The treated SCB without the inoculation of *A. haemolyticus* culture acted as control experiments.

Table 3.5: Different concentrations of ethanol for growth profile monitoring

Ethanol concentration (% v/v)	Control					Test				
	1	**3**	**5**	**7**	**10**	**1**	**3**	**5**	**7**	**10**
SCB (10% w/v)	10	10	10	10	10	10	10	10	10	10
Deionised water (mL)	99	97	95	93	90	89	87	85	83	80
Denatured ethanol (mL)	1	3	5	7	10	1	3	5	7	10
Active culture (10% v/v)	-	-	-	-	-	10	10	10	10	10

3.3.5.3 Growth profile of selected agricultural waste–adapted *A. haemolyticus*

Growth profile of SCB–adapted *A. haemolyticus* was carried out using deionised water and SCB as a medium for the bacterial growth. In 1 L volumetric flask, 170 mL of deionised water and 10 mL of denatured ethanol was added to 10% (w/v) SCB. Then, bacterial cell suspension (10% v/v) was added to the mixtures and incubated at 30 °C, 200 rpm for 48 h. Cultures (4 mL) were withdrawn at regular time intervals for optical density (OD) measured at a wavelength of 600 nm using a spectrophotometer. Growth was also monitored by viable cell counts. Viability was assessed by serial dilutions of culture samples in SCB growth medium, which were immediately plated on the NA to determine the number of viable cells (CFU/mL). A similar set up without bacterial cell served as a control.

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3.3.5.4 Cr(VI) reduction study of selected agricultural waste–adapted *A. haemolyticus*

Cr(VI) reduction study using SCB–adapted *A. haemolyticus* culture was carried out using Cr(VI) concentration of 100 mg/L. Samples (4 mL) were pipetted aseptically for the determination of Cr(VI) concentration using the DPC method and total chromium by AAS and also for the OD_{600} and bacterial counts (CFU/mL) after 24 h and 48 h incubation. The effectiveness of Cr(VI) reduction of SCB–adapted *A. haemolyticus* was also measured by decreasing the SCB dosage (from 10% w/v to 0.5% w/v) and increasing the Cr(VI) concentration (from 100 mg/L to 100–500 mg/L) and was monitored at time intervals from 0–142 days.

Cr(VI) reduction behaviour of SCB–adapted *A. haemolyticus* was examined by measuring the time–dependent concentrations of Cr(VI) and total Cr in batch systems. A series of Cr(VI) concentration (100, 150, 300 and 500 mg/L) were prepared by dissolving 25–125 mL of 1000 mg/L stock Cr(VI) solution into 1 L Erlenmeyer flasks of 250 mL total volume containing 0.5% (w/v) SCB, deionised water and 5% (v/v) denatured ethanol. The pH was adjusted to 7.0 using either 1.0 M HCl or 1.0 M NaOH. The medium was then inoculated with 10% (v/v) of the SCB–adapted *A. haemolyticus* culture and the flasks were shaken at 200 rpm and 30 °C. Flasks for the Cr(VI) reduction study is shown in Table 3.6. The samples (5 mL) were intermittently sampled and centrifuged (Hettich Zentrifugen Universal 320) at 5000 rpm for 5 min, followed by analyses of Cr(VI) concentration (DPC method), total chromium concentration (using AAS), turbidity (OD_{600}) and pH of the supernatant. The mixture of 0.5% (w/v) SCB, deionised water, 5% (v/v) denatured ethanol and Cr(VI) 100–500 mg/L without the bacterial cells acted as control.

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Table 3.6: Cr(VI) and SCB–adapted *A. haemolyticus* culture used for time–dependent study

Cr(VI) concentration (mg/L)	Control				Test			
	100	150	300	500	100	150	300	500
AW (0.5% w/v)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Deionised water (mL)	212.5	200.0	162.5	112.5	187.5	175.0	137.5	87.5
Denatured ethanol (5% v/v)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Active culture (10% v/v)	-	-	-	-	25	25	25	25
Cr(VI) 1000 mg/L (mL)	25.0	37.5	75.0	125.0	25.0	37.5	75.0	125.0

3.3.6 Chromium desorption study

The Cr desorption study was carried out using methods suggested by Selvaraj *et al.* (2003). The preparation is as follows; Cr–loaded SCB from Cr(VI) concentrations of 10–100 mg/L was removed by gentle washing with 50 mL distilled water to remove unadsorbed Cr(VI) followed by agitating the metal loaded adsorbent with 50 mL of 0.015 M NaOH (QRec) at 100 rpm, room temperature (30 °C) for 2 h. Samples (4 mL) were collected at each stage of chromium desorption for the determination of total chromium concentration using AAS and Cr(VI) concentration using DPC method. The experiments were carried out in duplicates.

3.4 Continuous Cr(VI) reduction process in packed–bed reactor

3.4.1 Laboratory packed–bed reactor set up

SCB was used as the packing material inside the reactor for this study. Fresh SCB with a total weight around 80–90 g was cut to irregular lengths between 1–2 cm and used without any chemical treatments. SCB packed–bed reactor was then rinsed with deionised water to remove any impurities before immobilization with *A. haemolyticus*. An acrylic reactor with 50 cm height, 4.6 cm inner diameter (id) and 4.9 cm outer diameter (od) was used. Inlet and outlet points were set at 3 cm from the

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top and 2 cm from the bottom of the reactor respectively. Teflon tubing was fitted to the inlet and outlet points. The teflon tubes were sterilized by soaking in 100% ethanol before use. Inert stones were packed to 50 cm^3 at the bottom of the reactor to ensure good flow distribution inside the reactor and to retain the reactor content. Following this, SCB was packed into the reactor to a volume of 665 cm^3. Inert stone was then packed on the top of the working volume of 33 cm^3. A headspace of around 83 cm^3 was allowed in the reactor. The total volume of the reactor was therefore 831 cm^3. Schematic representation of the reactor used in Cr(VI) reduction system in this study is shown in Figure 3.1. Inert stone used was coloured, uniform shape and sized between 1–2 cm. Before use, the stone was soaked in water for 24 h, rinsed with water and dried at room temperature (30 °C).

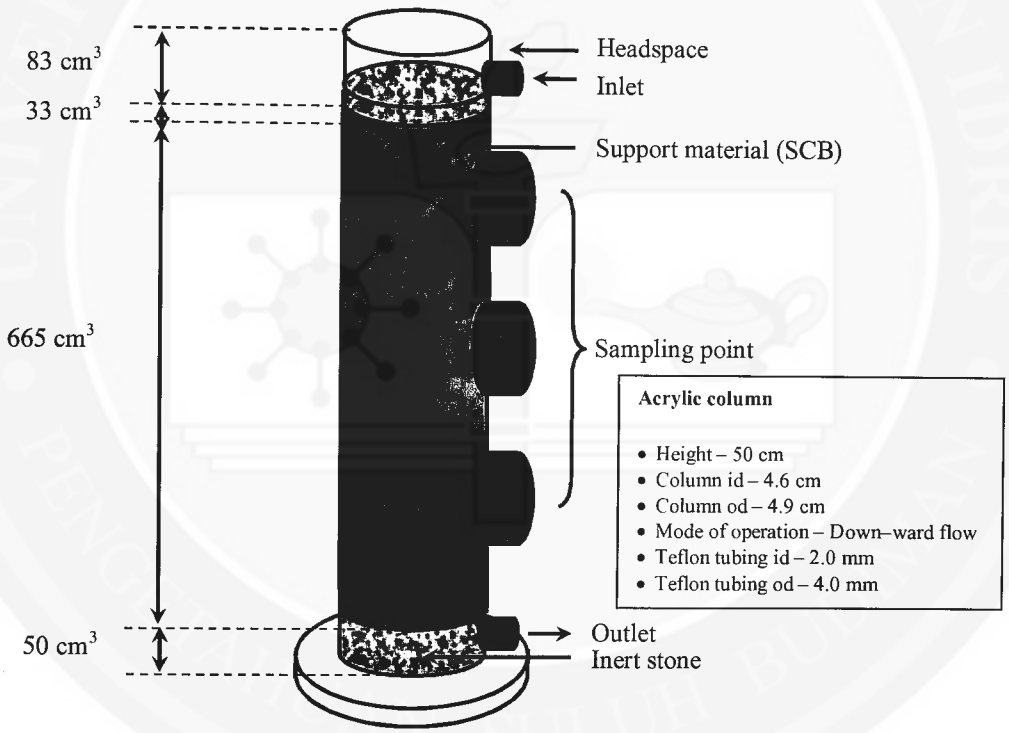


Figure 3.1: Schematic representation of laboratory packed–bed reactor of the Cr(VI) reduction system

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3.4.2 Immobilization of adapted *A. haemolyticus* onto SCB

Adapted *A. haemolyticus* was immobilized onto SCB via the following procedures; by using a peristaltic pump (Eyela MP–1000 D), the reactor was first rinsed with deionised water at 3 mL/min flow rate to prevent clogging by large particulate substances on the support material and to allow the SCB surface material to acquire a necessary charge for bacterial attachment. Then, the reactor was inoculated with 1 L adapted *A. haemolyticus* culture with continuous circulation for 3 days using the same flow rate as a rinsing step to allow initial bacterial attachment. Immobilization steps were carried out using modified methods from previous researchers (Mohammed *et al.*, 2011; Ahmad *et al.*, 2010b).The reactor was then supplemented with 1 L of NB for 2 days to ensure initial bacterial attachment. Following this, a 1 L of LPW 10% (v/v) was pumped continuously for 24 h to allow initial biofilm formation by the attached bacteria. A similar reactor was setup without *A. haemolyticus* acted as the control.

Liquid pineapple waste (LPW) used as nutrient supplementation for the attached bacteria was obtained from Lee Pineapple Sdn. Bhd. located in Tampoi, Johor Bahru (Figure 3.2). The LPW samples collected were stored in the refrigerator at 4 ^{o}C prior to use. The LPW was filtered twice through muslin cloth to remove suspended materials and diluted to 10% (v/v). The pH of the solution was adjusted to 7.0 using either 1.0 M HCl or 1.0 M NaOH before passing through the reactor.



Figure 3.2: Sampling site for pineapple liquid effluent at the waste treatment plant of a pineapple–processing facility in Tampoi, Johor Bahru

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3.4.3 Cr(VI) reduction using Cr(VI) stock solution

3.4.3.1 Effect of influent flow rate

Cr(VI) solution (50 mg/L) was prepared by adding 45 mL of stock Cr(VI) solution into 1 L Erlenmeyer flask containing 100 mL of LPW 10% (v/v) at a ratio of 9:1. The pH of the mixture was adjusted to 7.0 using either 1.0 M HCl or 1.0 M NaOH before passing through adapted *A. haemolyticus* immobilized–SCB packed into the reactor in a downward flow mode. Throughout the experiment, profile of Cr(VI) reduction was determined using the DPC method for every 2 h until 100% reduction was achieved and was observed at a different influent flow rate of 2.0, 3.0 and 5.0 mL/min. At the end of the reactor operation (observed when 100% reduction was achieved), 5 mL effluent was collected from the outlet point and centrifuged at 5000 rpm for 5 min followed by analyses of Cr(VI) concentration (DPC method). Reactor retention time, R_t is directly related to flow rate as described in Equation 3.9.

$$Retention\ time\ (\text{min}) = \left(\frac{A}{B}\right) \tag{3.9}$$

where, A is the volume of the reactor (cm^3) and B is the flow rate (cm^3/min).

3.4.3.2 Effect of influent Cr(VI) concentration

Cr(VI) solution (25 mg/L) was prepared by adding 25 mL of 1000 mg/L Cr(VI) stock solution and was mixed with LPW 10% (v/v) to give a total volume of 1 L. The pH of the mixture was adjusted to 7.0 using either 1.0 M HCl or 1.0 M NaOH. The solution was then pumped into the adapted *A. haemolyticus* immobilized–SCB packed into the reactor at 3.0 mL/min. Cr(VI) concentration was measured using the DPC method and was recorded for every 2 h until 100% reduction was achieved. The same procedure was also applied to the control reactor. The reactor was then continuously pumped with Cr(VI) solutions of 50 mg/L and 100 mg/L respectively. At the end of reactor operation, 5 mL effluent was withdrawn

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from the outlet point and centrifuged at 5000 rpm for 5 min. Analyses for the Cr(VI) concentration (DPC method) was carried out.

3.4.3.3 Packed–bed reactor performance study

Optimum flow rate and Cr(VI) concentration obtained was then used in the following 32 days of reactor operation. The following parameters were periodically measured; total carbohydrate using Dubois method, COD, turbidity (OD_{600}), pH, Cr(VI) concentration using DPC method and total Cr concentration (mg/L) using AAS. For the microbiological count (CFU/mL), samples were collected from the effluent and the SCB (packing material) at the top, middle and bottom section of the reactor.

For the COD analysis, the method is based on the measurement of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant, potassium dichromate (Eaton *et al.,* 2005). The COD test measured the amount of dichromate (oxidant) consumed in the breakdown of organic matter where more oxidant consumed showed high levels of organics and less oxidant consumed showed low levels of organics. Organic compounds are oxidized by potassium dichromate ($K_2Cr_2O_7$) and sulphuric acid (H_2SO_4). Oxidation of organic results in the reduction of Cr(VI) to Cr(III). A reduction which means consumption of oxidant can be monitored colorimetrically. As organics are oxidized and Cr(VI) is reduced to Cr(III), the colour of the solution changes from yellow to green. The amount of green colour formed is proportional to the COD of the sample (Pust, 2004).

Using a COD reagent vial (HACH), 2.0 mL of diluted samples was transferred into the vial which consists of $K_2Cr_2O_7$, 50% of H_2SO_4, silver salt (function as catalysts) and mercuric salt (to inhibit chloride interference). Samples were then heated at 150 °C for 2 h (DRB 200 COD reactor, HACH). The vial was allowed to cool for about 20 min before the readings were taken using the HACH

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DR5000 Spectrophotometer. The absorption was measured at 620 nm against a spectrophotometric blank, consisting of a COD reagent vial without sample.

3.4.3.4 Evaluation of bacterial growth using dislodging methods

In order to perform a viable cell or a total cell enumeration on SCB, the biofilm must be removed from the porous media and further disaggregated into a homogeneous cell suspension (Hamilton *et al.*, 2003). Based on the dislodging methods described by Lindsay and Von Holy (1997) for the removal and quantitative analysis of adherent microorganisms, the samples were prepared as follows; SCB–attached bacteria (1 g) were placed in Bijou bottle containing sterilized deionised water (10 mL) and vortexed at high speed. For quantification of the bacteria, an aliquot (1 mL) of the suspension was diluted and spread on the NA plates. The NA plates were then incubated at 30 °C for 24 h in the incubator before the CFU was obtained.

3.4.3.5 Assessment of biofilm morphology using electron microscopy

In order to examine the biofilm development, Field Emission Scanning Electron Microscope coupled with Energy Dispersive X–Ray (FESEM–EDX) was used. Samples (SCB–adapted *A. haemolyticus* and SCB–adapted *A. haemolyticus* in the presence of Cr(VI)) were withdrawn and prepared for FESEM–EDX analysis. The dried samples were then mounted on a sample holder with carbon–conductive adhesive tapes and coated with a thin layer of platinum under vacuum (Auto fine coater JFC–1600, JEOL) prior to viewing under the electron microscope (FESEM JSN–670 1F, JEOL) at the Ibnu Sina Institute for Fundamental Science Studies (IIS), UTM, Johor. Samples of SCB without attached bacteria served as a control.

Scanning Electron Microscopy (SEM) was employed to assess the morphology of the biofilm after reactor performance study operation. After 32 days,

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the biofilm–containing SCB was collected from the middle section of the reactor as described in section 3.4.3.3. SEM (Philips XL–30, England) analysis was conducted at the Electron Microscopy Unit, Institute for Medical Research (IMR), Kuala Lumpur. Microbial population present in the biofilm of Cr(VI) reducing environment was also identified using PCR and basic molecular techniques.

3.5 Biofilm microbial diversity study

Microbial population present in SCB was identified by 16S rRNA gene library analysis. The objective of this study is to determine the total bacteria communities (including culturable and unculturable bacteria) present in the biofilm of Cr(VI) reducing environment. The molecular experimental procedures for microbial population observations inside the reactor were carried out based on the following steps, i.e. extraction of total genomic DNA from the treated SCB, amplification of 16S rRNA gene using polymerase chain reaction (PCR) approach, purification of PCR products, cloning of PCR products, transformation of recombinant plasmids, colony PCR and Restriction Fragment Length Polymorphism (RFLP). The microbial diversity study was carried out at the Agrotechnology and Bioscience Division, Malaysian Nuclear Agency (Nuclear Malaysia), Selangor.

3.5.1 Ampicillin stock solution

A stock solution of ampicillin (100 mg/mL) was prepared by dissolving 1 g of ampicillin in 10 mL of distilled water. The solution was then filter–sterilized using 0.2 μm pore size sterile cellulose acetate filter (Whatman, England) and stored in aliquots at –20 °C in a sterile falcon centrifuge tube.

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3.5.2 Luria–Bertani agar incorporated with ampicillin

Luria–Bertani (LB) LB agar was prepared by dissolving 37 g/L of LB agar (Merck, Germany) in 500 mL distilled water. The pH of the solution was adjusted to pH 7.5 with 0.1 M NaOH and was sterilized by autoclaving at 121 °C, 115 kPa for 15 min. The sterilized LB agar was allowed to cool to 45–55 °C before adding ampicillin to give a final concentration of 80 µg/mL. The molten agar was then poured into sterile disposable petri dishes and allowed to solidify. The plates were stored at 4 °C prior to use.

3.5.3 Extraction of total genomic DNA from treated SCB

Total genomic DNA of treated SCB was extracted from the middle section of reactors for both control and test. To extract the total genomic DNA, 10 g samples were aseptically collected into sterile Schott bottles and were minced with a sterile surgical blade in a sterile disposable petri dish. The DNA extraction was carried out using the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA).

The procedures of extraction were as follows; PowerBead Solution (15 mL) was added to PowerMax® Bead Tube followed by 10 g of each sample. Mixtures were vortexed vigorously for 15 min. Solution C1 (1.2 mL) was added and samples were vortexed for 10 min. This was followed by vertically vortexing the mixtures using the highest speed for another 30 min and centrifuged at 6000 rpm for 3 min at room temperature. The supernatant was transferred to a new collection tube and mixed by inversion with 5.0 mL Solution C2. The samples were inverted twice and incubated at 4 °C for 10 min. Centrifugation, transfer of supernatant and incubation were repeated using 4.0 mL Solution C3 and 30 mL Solution C4. Supernatants from the last step were centrifuged three times at 6000 rpm for 2 min using a spin filter. At each centrifugation step, the flow through was discarded. This was followed by addition of 10 mL of Solution C5 and centrifuged at 6000 rpm for 3 min. The centrifugation was repeated using only spin filter for further 5 min. The spin filter

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was placed in a new collection tube and 4.0 mL of Solution C6 was added to the centre of spin filter membrane and centrifuged at 6000 for 3 min at room temperature. The DNA in the tubes was pooled and stored at –20 °C.

3.5.4 Amplification of 16S rRNA gene fragment

Polymerase chain reaction (PCR) is a technique for amplification of selective DNA fragment *in vitro*. PCR allows the generation of a large quantity of DNA product (up to several mg) from only a few starting copies. A typical PCR step involves initial denaturation, denaturation, annealing, extension and final extension.

The eluted DNAs extracted from the treated SCB for both control and test samples were diluted to 30 ng/μL for PCR amplification. The 16S rRNA gene fragments were PCR–amplified using 10X bacterial–specific primer 27F (5'–AGA GTT TGA TCC TGG CTC AG–3') paired with the 10X bacterial–specific primer 1492R (5'–GGT TAC CTT GTT ACG ACT T–3') (Lane, 1991). PCR was carried out in a 100 μL reaction mixture containing 10 μL of 10X PCR reaction buffer, 10 μL of 10X 27F/1492R 2.5 μM each, 2 μL of 10 mM PCR–grade of deoxynucleoside triphosphate (dNTP), 30 ng/μL of DNA template, 62 μL of sterile Milli–Q water and 1 μL (5 U/μL) of *Taq* DNA polymerase (Yeastern Biotech Co. Ltd., Taiwan). The PCR was carried out in a Bio–Rad MyCycler thermal cycler (Bio–Rad, USA) with an initial denaturation step at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min.

3.5.5 Purification of PCR products

The PCR product was purified using High pure PCR product purification kit (Roche Applied Science, Germany). Purification steps were carried out as follows; PCR products (50 μL) was mixed together with 250 μL of binding buffer and was transferred to High Pure filter tube, followed by centrifugation at 13, 000 rpm for

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60 s. The flow through from centrifugation was discarded. The tube was added with 500 µL of washing buffer and centrifugation was repeated at the same speed for another 1 min. The flow through was then discarded again. This was followed by the addition of 200 µL of another washing buffer and the centrifugation was repeated for an additional 1 min to remove residual wash buffer. The tube was centrifuged again at 13, 000 rpm for 1 min and transferred to a new 1.5 mL tube, added with 50 µL elution buffer at the centre of the tube membrane and centrifuged at 13, 000 rpm for 2 min. The flow through was collected as purified DNA products and was used for cloning and transformation or stored at –20 °C. The diagrammatic representation of purification of the PCR products is shown in Figure 3.3.

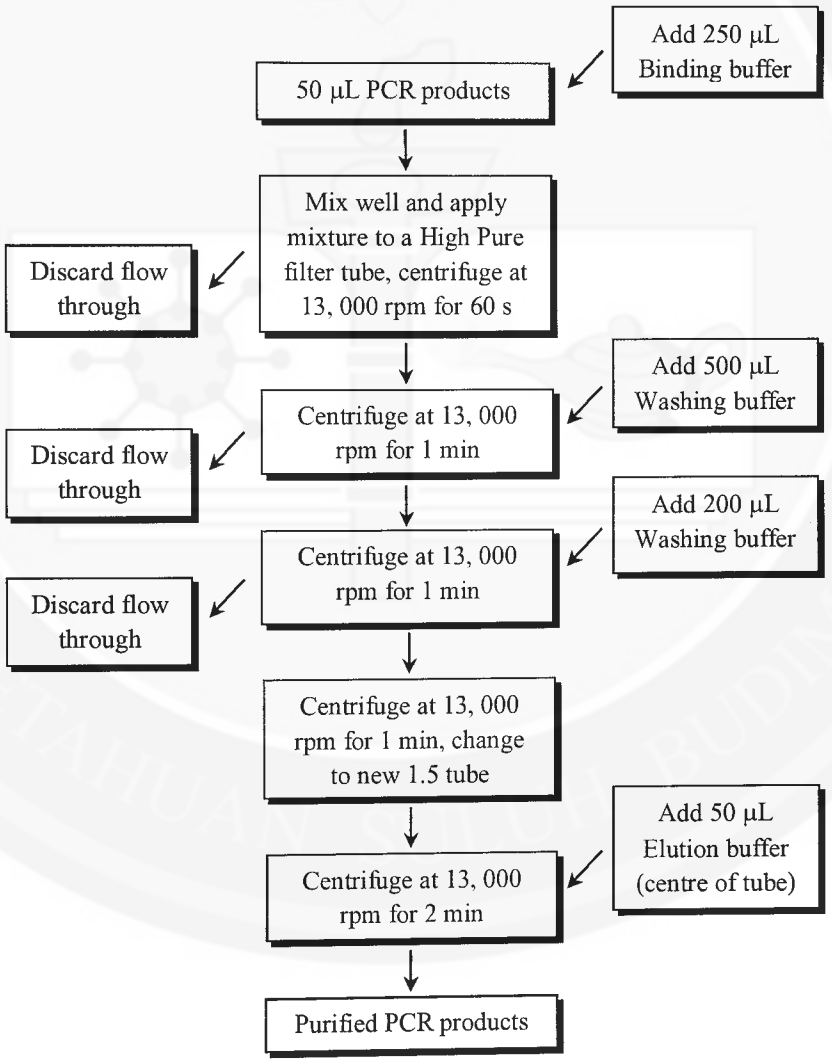


Figure 3.3: Schematic representations for purification of PCR products

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3.5.6 Cloning of PCR products

DNA ligation is a process of constructing new DNA molecules. The desired DNA molecule was first purified and then combined with linearised cloning vector. The products of the ligation reaction are then used to transform an appropriate strain of bacterial host.

The cloning vector (Yeastern Biotech Co. Ltd., Taiwan) was first centrifuged briefly and the contents were collected at the bottom of the tubes. The ligation buffer was vigorously vortexed. The reaction mixture (10 µL) was prepared containing 1 µL of ligation buffer A, 1 µL of ligation buffer B, 2 µL of cloning vector (Yeastern Biotech Co. Ltd., Taiwan), 5 µL of purified PCR products and 1 µL of T_4 DNA ligase. The mixture was vortexed and incubated for 45 min at 22 °C. The competent cells were ready to transform to *Escherichia coli* (*E. coli*).

3.5.7 Transformation of recombinant plasmids

The transformation was conducted using heat–shock technique as instructed by manufacturer (Yeastern Biotech Co. Ltd., Taiwan). Transformation is a process which involved uptake and expression of foreign DNA sequence into a bacterial host cell. DNA fragment of interest will be incorporated into a vector, forming a recombinant DNA plasmid through ligation. The recombinant plasmid is then transformed into a bacterial host, i.e. *E. coli.* Upon transformation, the foreign DNA genotype is replicated and expressed by the bacterial host.

Frozen *E. coli* DH5α (ECOS™ 101) competent cells (Yeastern Biotech Co. Ltd., Taiwan) (typically 100 µL) was defrosted under running tap water until 1/3 portion was thawed. Plasmid DNA was added immediately, mixed by brief vortex and incubated on ice (pre–chilled) for 1 min to increase the efficiency. The cells were then heat–shocked in a pre–warmed 42 °C water bath for 45 seconds. This was followed by plating the *E. coli* cells on pre–chilled LB agar plate incorporated with

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80 μg/mL ampicillin. The plates were incubated at 37 °C overnight to screen for transformants.

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Plasmids which have been transformed contain the *LacZ* operon which complementation for blue/white screening. Blue–white screening on the plates was used as a selective marker for successful ligation and transformation (Figure 3.4). After incubation, white colonies indicate insertion of foreign DNA on *LacZ* operon and blue colonies indicate the functional β–galactosidase ability to hydrolyse the *Xgal*.

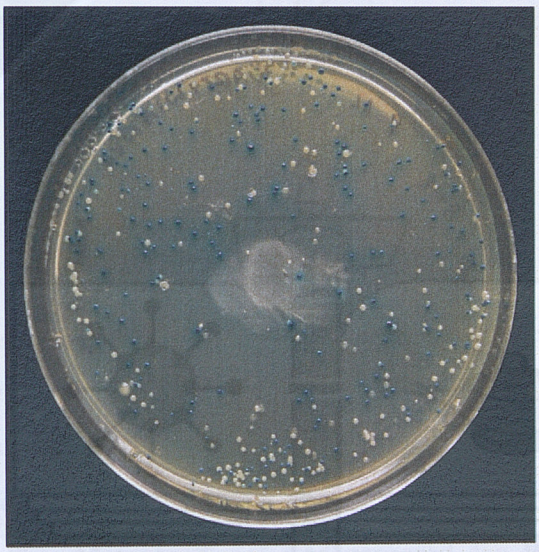


Figure 3.4: Blue/white screening on the LB agar plate incorporated with 80 μg/mL ampicillin

3.5.8 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA molecules by size. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic acids of different sizes to be separated. Shorter molecules move faster and migrate further. The buffer used for agarose gel electrophoresis is TAE (Tris–Acetate–EDTA). The gel loading dye was added to the samples before loading to increase the density of the samples to ensure the samples

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sink into the wells, to add colour to the samples through the use of dyes such as bromophenol blue and to allow tracking of the electrophoresis due to co–migration of the dye with DNA fragments of a specific size.

3.5.8.1 Preparation of 1% (w/v) agarose gel

A 1% (w/v) agarose gel was prepared by dissolving 1 g of SeaKem® LE Agarose gel (Cambrex, USA) in 100 mL of 1X TAE buffer followed by melting using microwave (Panasonic) at high power until fully dissolved. The gel solution was then cooled to 50–60 °C prior to casting (40 mL) and allowed to solidify.

3.5.8.2 Preparation of 3% (w/v) Metaphor® gel

Metaphor® gel was prepared by dissolving 1.5 g of MetaPhor® agarose gel (Cambrex Bio Science Rockland, USA) in 50 mL of 1X TAE buffer by stirring with a magnetic bar on a hot plate stirrer. The solution was then heated using microwave (Panasonic) at high power until the formation of clear solution prior to casting and allowed to solidify. This Metaphor® gel was prepared for Restriction Fragment Length Polymorphism (RFLP) analysis.

3.5.8.3 Colony PCR

Positive (white) transformants (from section 3.5.7) were picked with sterile toothpicks and spotted onto fresh LB agar plates containing 80 µg/mL ampicillin, as prepared in section 3.5.2, for incubation overnight at 37 °C. Plates were then stored at 4 °C (Figure 3.5). After spotting the colonies onto the plate, the toothpicks were subsequently deposited by scratching against the inner wall of a 0.2 mL PCR tube bottom. Then, 10 µL of PCR reaction mixture containing 1 µL of 10X PCR reaction

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buffer, 0.25 µL of 10X M13F/M13R 10 µM each, 0.2 µL of 10 mM PCR–grade of deoxynucleoside triphosphate (dNTP), 8.25 µL of sterile Milli–Q water and 0.05 µL of *Taq* DNA polymerase, 5 U/µL (Yeastern Biotech Co. Ltd., Taiwan) were added to each PCR tube. PCR amplification was performed in a Bio–Rad MyCycler thermal cycler (Bio–Rad, USA) with an initial denaturation step at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. The positive clones for each control and test samples carrying the desired insert sizes of approximately 1500 bp were collected for subsequent analysis.

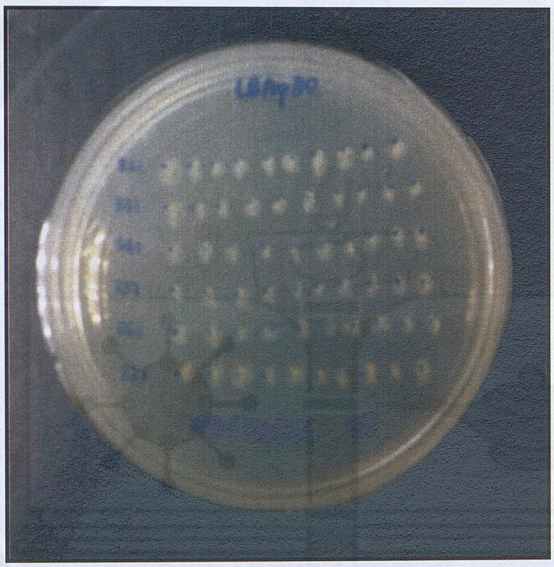


Figure 3.5: Plate of colony PCR

The PCR products (2 µL) were mixed with gel loading dye and carefully loaded into the preformed wells in a 1% (w/v) agarose gel. Each gel was prepared in 1X TAE buffer. The gel was subjected to electrophoresis for 20 min at 110 V in 1X TAE buffer. After electrophoresis, the gel was stained with 0.5 mg/mL ethidium bromide (EtBr) solution for 15 min followed by de–staining in distilled water for another 15 min. The gel was visualised under UV transilluminator (Alpha Innotech MultiImage® II coupled with Alpha Imager® HP, Southern Africa) for nucleic acid visualization and analysis. GeneRuler™ 100 bp DNA ladder plus 3 µL (Fermentas) ranging from 100–3000 bp were loaded (1.5 µL) as molecular weight DNA marker to determine fragment sizes and estimate product yields. The remaining PCR

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products were stored at 4 °C. Only products showing the correct insertion size (~1.5 kb) were used for further analyses of Restriction Fragment Length Polymorphism (RFLP).

3.5.8.4 Restriction Fragment Length Polymorphism analysis

Cloned 16S rDNA fragments were double–digested with tetrameric restriction enzymes *Hae*III and *Hinf*I (New England Biolabs, England). Restriction enzyme digestions of the PCR products were carried out according to the manufacturer's protocols using 1 µL of 10X NE buffer, 0.2 µL of *HinfI REII* and 0.2 µL of *HaeIII REI* and 3.6 µL of sterile Milli–Q water (18.2 mΩ). The mixtures were then added to each PCR product (5 µL), vortexed, followed by incubation at 37 °C overnight using a heating block (Bioer, China). After incubation, the mixtures were vortexed and spinned briefly, followed by electrophoresis.

Digested DNA (10 µL) were mixed with gel loading dye and run on 3% (w/v) MetaPhor® agarose gel (Cambrex Bio Science Rockland, USA) at 110 V for 40 min using GeneRuler™ 100 bp DNA ladder plus 3 µL (Fermentas) in 1X TAE buffer. The gel was stained with 0.5 mg/mL ethidium bromide (EtBr) solution for 15 min after electrophoresis and then de–stained using distilled water for additional 15 min. DNA was visualized via UV transilluminator (Alpha Innotech MultiImage® II coupled with Alpha Imager® HP, Southern Africa). The RFLP patterns were compared and clones with identical patterns were grouped into the same patterns. Selected clones carrying discrete restriction patterns were sub–cultured to obtain single colonies for DNA sequencing and bioinformatic analysis.

3.5.9 DNA sequencing

DNA sequencing was carried out by Medigene Sdn. Bhd., Malaysia using both M13 Reverse (M13R) and M13 Forward (M13F) primers as sequencing primers.

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Amplicons were obtained by colony PCR amplification and were verified by Restriction Fragment Length Polymorphism (RFLP) analysis. The PCR products of approximately 1500 bp in size were purified using High Pure PCR Product Purification Kit (Roche Applied Science, USA) as described in section 3.5.5. The concentration and purity of PCR products were determined using NanoDrop ND–1000 UV/Vis Spectrophotometer ND–1000 V3.5.2 (USA). Sequencing reactions of purified amplicons were then carried out for selected plasmid clones (based on the predominant patterns) that represent the different RFLP patterns.

3.5.10 Bioinformatic analysis

Results obtained for each clone was matched against the non–redundant sequence database at the NCBI using the nucleotide–nucleotide Basic Local Alignment Search Tool (BLASTn) to identify the closest relatives. The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and aligned with the library sequences with the multiple alignment tool ClustalW (Thompson *et al.*, 1994) in the MEGA 4.1 program (Kumar *et al.*, 2008). A phylogenetic tree was constructed using neighbour–joining methods (Saitou and Nei, 1987). Bootstrap analysis of 1000 data set was performed to evaluate the tree topologies.

3.5.11 Nucleotide sequence accession numbers

The 16S rRNA gene sequences from this study were submitted to the GenBank database under the accession numbers of KC295702 to KC295714.

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3.6 Cr(VI) reduction mechanism study

Samples for Cr(VI) reduction mechanism study were obtained through contact with 100 mg/L of Cr(VI) solutions at neutral pH, shaking at 30 °C and 200 rpm for 48 h. The same sample was applied to instrumental analysis for Cr(VI) reduction mechanism study, i.e. X–Ray Photoelectron Spectroscopy (XPS), Electron Spin Resonance (ESR), Fourier Transform–Infra Red (FT–IR) and Scanning Electron Microscopy coupled with Energy Dispersive X–Ray (SEM–EDX).

3.6.1 Oxidation state determination using X–Ray Photoelectron Spectroscopy

XPS analysis was carried out at Centre for Research and Instrumentation Management (CRIM), UKM, Selangor. The Cr–laden SCB was washed with deionised water several times, and then freeze–dried in a vacuum freeze dryer (Christ Alpha 1–2). $CrCl_3 \cdot 6H_2O$ (Fluka) and $K_2Cr_2O_7$ (BDH) were used as Cr(III) and Cr(VI) reference compounds respectively. XPS spectra were recorded with a Kratos Axis Ultra DLD electron spectrometer. The sample spot was analyzed using an AlKα radiation (1486.6 eV) X–ray source, at 150 W and pass energy of 20 eV. The system was operated at a base pressure of 2×10^{-9} torr. The calibration of the binding energy of the spectra was performed with the C1s peak of the aliphatic carbons, which is 284.5 eV.

3.6.2 Oxidation state determination using Electron Spin Resonance

ESR measurements were conducted with JEOL Spectrometer (JES–FA100). The freeze–dried sample obtained from section 3.6.1 were analyzed at 4 mW microwave power, 9.01 GHz microwave frequency, 320±250 mT external magnetic field, 250 mT modulation amplitude and 0.01 mT modulation width. For standard Cr(III) and Cr(VI), the reference compounds used were $CrCl_3 \cdot 6H_2O$ (Fluka) and

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$K_2Cr_2O_7$ (BDH) respectively. ESR was conducted at Ibnu Sina Institute for Fundamental Science Studies (IIS), UTM, Johor.

3.6.3 Functional group determination using Fourier Transform–Infra Red

Possible involvement of functional groups from SCB during Cr(VI) reduction was elucidated using the FT–IR analysis. The SCB after Cr(VI) reduction was dried in an oven at 60 °C for 48 h. Then the dried SCB was cut into small pieces and ground to obtain the desired particle size of 300 to 425 µm. For the analysis, 10 mg of ground SCB was mixed with 200 mg of KBr (Merck, spectroscopy grade, 119.01 g/mol) in a mortar before being pressed into 10 mm diameter disks under 6 tons of pressure. FT–IR spectra were obtained on a Perkin Elmer Spectrum One FT–IR Spectrometer. The analysis conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400–4000 cm^{-1}.

3.6.4 Morphology determination using Scanning Electron Microscopy coupled with Energy Dispersive X–Ray

The SEM–EDX was used to determine the attachment of *A. haemolyticus* on SCB and also the bacterial morphology after treatment with Cr(VI). The SCB was viewed using SEM–EDX (Philips XL–40, England) at the Material Science Laboratory, Faculty of Mechanical Engineering, UTM, Johor.

For the sample preparation, treated SCB was first cut into small pieces using a flame–sterilized knife before being pooled and fixed in 2.5% (v/v) glutaraldehyde (TED PELLA Inc.) for 1–2 h at room temperature. Fixed cells were washed twice with deionised water before post–fixed with 2.0% (v/v) osmium tetroxide, OsO_4 (Fluka) in 0.1 M phosphate buffer saline (PBS) for 1 h. The samples were then washed twice again with deionised water, dehydrated using 10–100% (v/v) of absolute ethanol (QRec) for 5 min each and left to dry at 60 °C overnight in an oven.

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The dried samples were then mounted on a sample holder with carbon–conductive adhesive tapes and coated with gold using a sputter coater (Bio–Rad) prior to viewing using a scanning electron microscope.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of agricultural wastes

This present study focuses on the suitability of agricultural wastes (AW) such as SCB, SPW and SBM to be used as sole carbon source and energy for growth of *A. haemolyticus*. As a prerequisite to the growth studies, it was considered important to characterize the SCB, SPW and SBM in terms of their physicochemical composition such as the amount of moisture, ash content and total solids, pH, α–cellulose content and lignin, heavy metals content, monosaccharide, total carbohydrate and anion content. The composition of AW tested in this study is summarized in Table 4.1.

SCB shows the highest moisture content (8.03±0.08%) while the highest ash content and total solids were determined in SBM i.e. 47.64±0.17% and 97.77±0.01% respectively. Moisture and ash content for SPW are 7.84±0.07% and 4.30±0.08%, its similar which reported by Kareem *et al.* (2010). High moisture content was favoured by microbial development (Guo *et al.*, 2010). Study by Liang *et al.* (2003) reported that moisture content even had an influence on the microbial activity greater than the temperature. As an important parameter, moisture content of the materials also reported by Zhang *et al.* (2011) to provide a medium for the transport of dissolved nutrients required for the metabolic and physiological activities of microorganisms. The low ash content in AW offers numerous advantages for usage in bioconversion processes using microbial cultures (Parameswaran, 2009).

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Table 4.1: Composition of SCB, SPW and SBM. Data shown are the mean value of three different batches

	Unit	SCB	SPW	SBM
Moisture content	%	8.03 ± 0.08	7.84 ± 0.07	2.23 ± 0.01
Ash content	%	1.96 ± 0.14	4.30 ± 0.08	47.64 ± 0.17
Total solids	%	91.97 ± 0.08	92.16 ± 0.07	97.77 ± 0.01
pH	-	3.04 ± 0.04	3.77 ± 0.08	5.64 ± 0.04
α–cellulose	%	27.70 ± 0.30	ND	ND
Lignin	%	13.60 ± 0.10	ND	ND
Heavy metals content				
Arsenic	mg/kg	1.37 ± 0.15	2.61 ± 0.37	0.13 ± 0.04
Cadmium	mg/kg	7.91 ± 0.34	0.27 ± 0.15	0.05 ± 0.00
Cobalt	mg/kg	2.94 ± 0.35	0.40 ± 0.17	0.21 ± 0.09
Chromium	mg/kg	0	0	0
Cerium	mg/kg	4.68 ± 0.68	2.71 ± 0.04	0.40 ± 0.22
Beryllium	mg/kg	0.80 ± 0.13	0.02 ± 0.00	0
Nickel	mg/kg	0.20 ± 0.07	4.35 ± 0.53	2.87 ± 0.46
Lead	mg/kg	7.28 ± 0.82	3.17 ± 0.22	3.07 ± 0.21
Monosaccharide				
Glucose	mg/kg	23.69 ± 2.36	13.62 ± 0.43	0
Xylose	mg/kg	3.44 ± 0.65	3.18 ± 0.45	0
Arabinose	mg/kg	0	0	0
Galactose	mg/kg	0	0	0
Sucrose	mg/kg	0	0	0
Fructose	mg/kg	0	0	0
Total carbohydrate	%	197.23 ± 2.97	140.73 ± 1.92	14.77 ± 0.84
Anion content				
Sulphate	%	48.43 ± 1.33	47.47 ± 1.27	65.15 ± 1.36
Phosphate	%	0.03 ± 0.00	0	0
Nitrate	%	0.36 ± 0.20	0.33 ± 0.02	0.24 ± 0.03
Chloride	%	1.39 ± 0.44	1.34 ± 0.42	14.59 ± 0.36

* SCB = sugarcane bagasse, SPW = solid pineapple waste, SBM = soybean meal, ND: not determined

All AW used were acidic in nature with pH values ranging between 3.04±0.04–5.64±0.04. The pH of SPW was reported by Abdullah and Mat (2008) and Sasaki (1991) at range value of 4.00–4.30. Moisture, pH and nutrient content are some factors that affect bacterial growth (Cheung, 2008).

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The composition obtained for the SCB was 27.70±0.30% α–cellulose and 13.60±0.10% lignin (based on the weight percent on dry basis). SCB has been reported to contain 33–45% cellulose, 23–28% hemicellulose and 6–23% lignin (Garg *et al.*, 2009; Rodrigues *et al.*, 2010). Most plant materials are made up of complex organic residues such as cellulose and lignin that contain several types of polar functional groups such as alcohols, aldehydes, ketones, carboxylic acids and ethers that facilitate metal complexation resulting biosorption of heavy metal ions (Kaur *et al.*, 2012). These groups in biomaterials can be involved in chemical bonding and are responsible for the typical cation–exchange capacity (Owamah *et al.*, 2012). Cellulose is a crystalline homo–polymer of glucose with β→1,4 glycosidic linkages and intra–molecular and inter–molecular hydrogen bonds. Hemicellulose is a heteropolymer of mainly xylose with β→1,4 linkage with other substances of acetyl feruoyl and glycouronyl groups. In view of the high carboxyl and hydroxyl groups present in the SCB, this may enhance its potential to be used as an adsorbent.

On the other hand, the amount of heavy metals in AW were less than 8 mg/kg heavy metals with Cd and Pb were measured highest concentration (7.91±0.34 mg/kg and 7.28±0.81 mg/kg respectively) while Ce and Ni were detected by 4.68±0.68 mg/kg and 4.35±0.53 mg/kg respectively. Heavy metals can be classified as essential (e.g., Co, Ni, Cu) or non–essential (e.g., Cd, Hg, Pb) depending on whether they have a biological role for microorganisms (Bruins *et al.*, 2000). Essential metals have growth stimulatory effects up to a certain concentration limit. Above this level, the heavy metal will cause inhibitory effects to bacteria. However, bacteria have adapted to the presence of heavy metals in the environment and have developed resistance mechanisms (Bruins *et al.*, 2000). Although the type of mechanisms may be more or less homologous in all species of bacteria (Ji and Silver, 1995), it is expected to obtain different responses to the same toxic concentration for several bacterial species (or bacterial groups) (Rial *et al.*, 2011).

Analysis of sugar indicates that SCB and SPW contain mainly glucose as the main component in the hydrolyzates, followed by xylose. The concentrations of both sugars in SCB and SPW (analyzed using HPLC) were 23.69±2.36 mg/kg and

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13.62±0.43 mg/kg for glucose (at retention time 6.836 min) and 3.44±0.65 mg/kg and 3.18±0.45 mg/kg for xylose (at retention time 4.036 min). Other researchers also reported that glucose and xylose as the main monosaccharide in SCB (Walford, 2008; Kim *et al.*, 2009). Glucose was reported as the preferred carbon source utilized by *A. haemolyticus* compared to sucrose and fructose when grown in 15% (v/v) liquid pineapple waste (LPW) (Zakaria *et al.*, 2007a). While Holt *et al.* (1994) reported that some strains of *A. haemolyticus* were utilized glucose, D–ribose, D–xylose, D–arabinose, ethanol, D–malate, DL–lactate and citrate during metabolism processes. All monosaccharide analyzed was not detected in SBM. This is because SBM contains components which are mainly dry matter, crude protein, non–starch polysaccharides, crude fat, ash, essential amino acids and non–essential amino acids (Overland *et al.*, 2009). SCB also gives the highest total carbohydrate content with values of 197.23±2.97%, making it as a good substrate for the development of microorganisms.

The results show that all AW contains high percentage levels of sulphate (ranging from 47–65%), followed by chloride (< 15%), nitrate and phosphate (< 0.4%). Zakaria *et al.* (2007b) reported the role of oxyanions such as PO_4^{3-}, SO_4^{2-}, SO_3^{2-} and NO_3^- as competing electron acceptors in Cr(VI) reduction where SO_4^{2-} is a particular importance as Cr(VI) is known to enter the bacterial cell via the sulphate uptake pathway. This means that the presence of anion content in the raw AW may helps in the reduction of Cr(VI) in later study. These anion content are considered as basic requirement for growth of microorganism in general particularly nitrogenous compound like nitrate play important role in the production of protein and metabolic enzyme (Lobo, 2009).

Nutrients in agricultural wastes and their proportions determine the types of microorganisms that will grow. The presence of high levels of several important micronutrients for microbial development makes these AW important and promising substrates, since it does not require supplementation. Also, microorganisms vary in their ability to use nutrients. The presence of easily utilizable nutrients will encourage faster growth of bacteria. The concentration of the carbohydrates also will determine the type and extent of growth of bacteria.

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4.2 Growth and Cr(VI) reduction of *A. haemolyticus* in agricultural wastes in a batch system

4.2.1 Screening of agricultural wastes as an alternative growth medium for *A. haemolyticus*

Batch study was conducted to screen the suitability of AW as bacterial growth medium. The feasibility of each AW to sustain the bacterial population was assessed by measuring the cell concentration (CFU/mL), total carbohydrate and pH of *A. haemolyticus* which grown in different concentration of the medium.

The profile for the growth of *A. haemolyticus* grown in three different medium of SCB, SPW and SBM are shown in Figure 4.1. *A. haemolyticus* showed the highest survival in 10% (w/v) SBM based on the cell count of 5.4±0.85 X 10^8 CFU/mL. The high cell count obtained in SBM was due to the more bioavailable substrate present in NB. However, the population of *A. haemolyticus* seems to decrease with time and this is attributed by the increased in a growth of indigenous bacteria present especially in SCB and SPW. Lowest growth of *A. haemolyticus* was observed in 2% (w/v) SCB with maximum cell concentration of 2.5±0.71 X 10^6 CFU/mL. The bacterial cell count in the liquid medium containing SCB was lower compared to in SBM. This could be due to the growth of *A. haemolyticus* in the form of biofilm or aggregates on the solid substrate.

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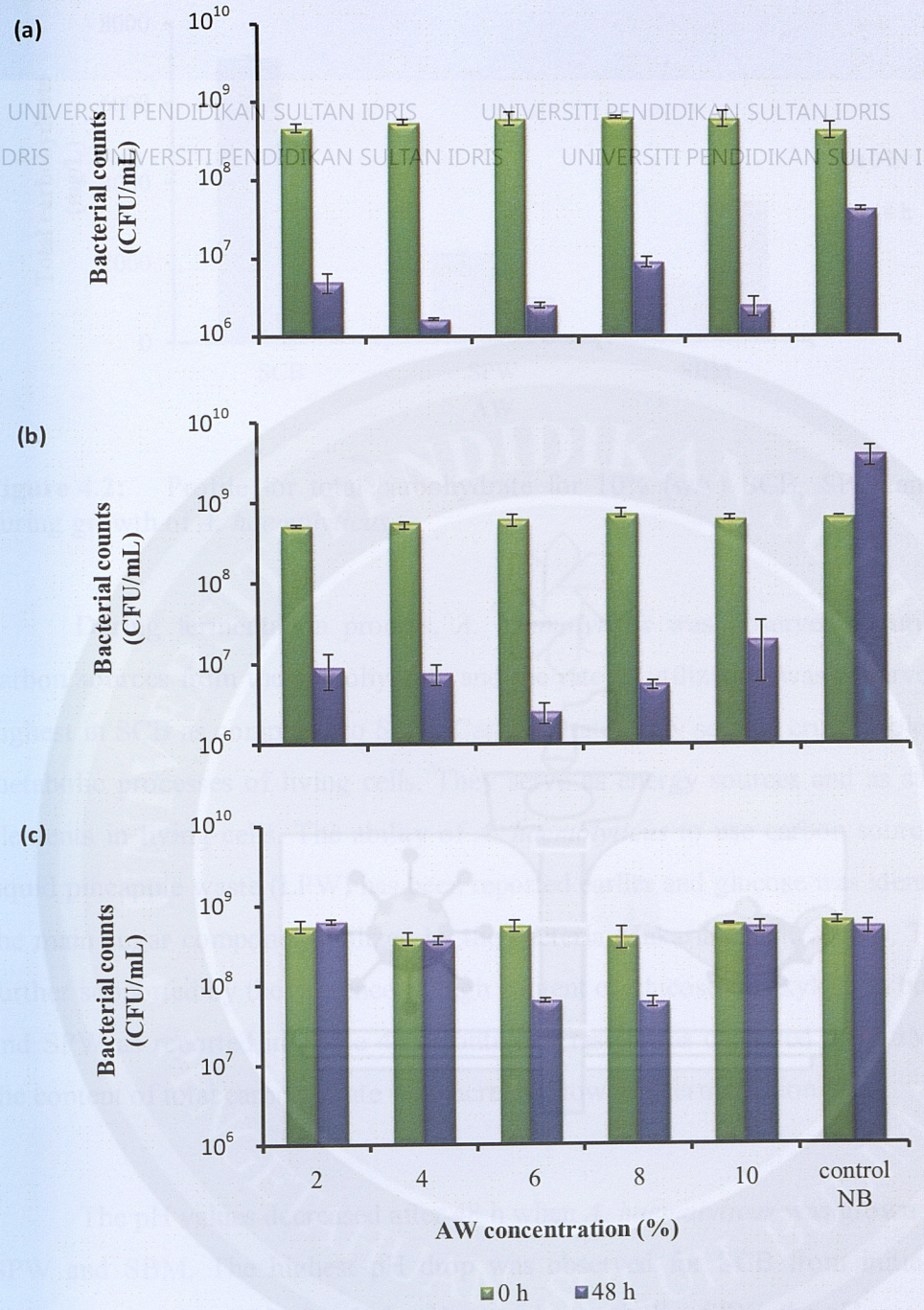


Figure 4.1: Effect of different concentration of SCB, SPW and SBM, sterilized with 5% (v/v) denatured ethanol towards bacterial counts

Low concentration of total carbohydrate remained in the liquid medium indicated to an efficient consumption of carbohydrate as source of carbon and energy for bacterial growth. SCB showed the highest initial total carbohydrate content compared to SPW and SBM (Figure 4.2).

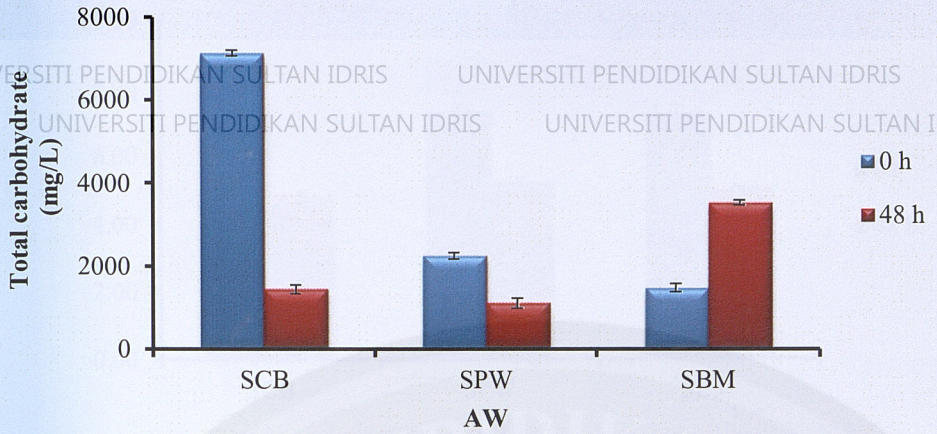


Figure 4.2: Profile for total carbohydrate for 10% (w/v) SCB, SPW and SBM during growth of *A. haemolyticus*

During fermentation process, *A. haemolyticus* was observed to utilize the carbon sources from the carbohydrate and the rate of utilization was observed to be highest in SCB as compared to SPW. Carbohydrates play several crucial roles in the metabolic processes of living cells. They serve as energy sources and as structural elements in living cells. The ability of *A. haemolyticus* to use carbon sources from liquid pineapple waste (LPW) has been reported earlier and glucose was identified as the main sugar component utilized by the bacteria (Zakaria *et al.*, 2007a). This was further supported by the presence of high content of glucose and xylose in both SCB and SPW as reported in Table 4.1. Contradict result was observed for SBM where the content of total carbohydrate was increased towards fermentation time.

The pH values decreased after 48 h when *A. haemolyticus* was grown in SCB, SPW and SBM. The highest pH drop was observed for SCB from initial pH of 7.31±0.15 to 4.82±0.25 after 48 h of bacterial growth. For SPW, the pH was reduced from 7.31±0.29 initially, to 5.21±0.35 while SBM showed the lowest pH reduction i.e. from initial values of 7.21±0.10 to 5.02±0.06 (Figure 4.3). Often during bacterial growth, organic acids are released into the medium, which lower its pH value. Under limited oxygen condition, bacteria were carried out fermentation which finally induced to the production of mixed organic acids such as acetic acid and lactic acid (Nester *et al.*, 2001). Lower pH in SCB and SPW could be related to the anaerobic metabolism of carbohydrate which if analyzed the concentration of organic acid should be higher in comparison to that in SBM.

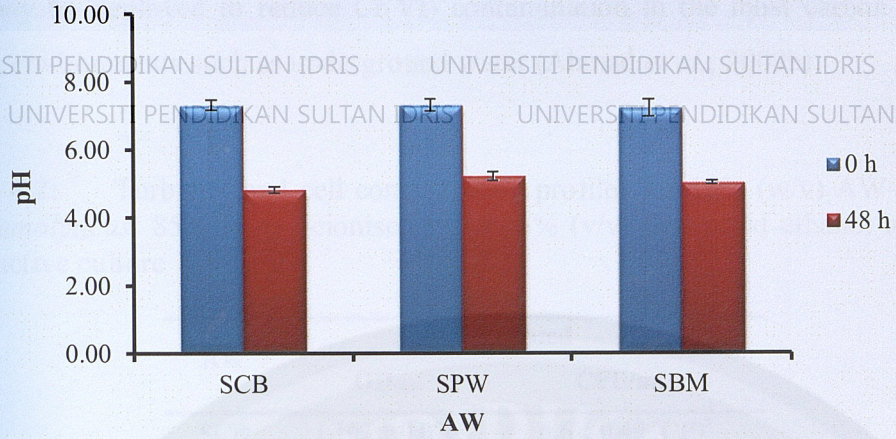


Figure 4.3: pH profiles for 10% (w/v) SCB, SPW and SBM during growth of *A. haemolyticus*

4.2.2 Adaptation of *A. haemolyticus* in agricultural wastes

Adaptation process was conducted to introduce and increase the ability of *A. haemolyticus* to tolerate and survive in AW at lower nutrient supplementation. Throughout the adaptation process, NB was replaced by deionised water to eliminate the nutrient sources available in the NB and to introduce AW as the sole source of nutrients for the bacteria to grow. Microorganisms are capable of utilizing the organic matter in wastes both as a source of energy for growth and as carbon for the synthesis of cell biomass (Mahmood *et al.*, 1998).

Table 4.2 shows the ability of *A. haemolyticus* to utilize nutrient for growth from AW. High cell concentration ranging from $2.2 \times 10^9 - 4.7 \times 10^9$ CFU/mL was obtained for control which referred to the growth of *A. haemolyticus* in rich medium of NB. Adaptation of *A. haemolyticus* in AW shows the decreasing in cell concentration to the region of 10^8 CFU/mL after 24 h of incubation. The turbidity for adapted *A. haemolyticus* was measured by OD_{600} ranging from 1.107–2.082. The ability of *A. haemolyticus* to survive in a carbon source–limiting environment, i.e. NB medium only is expected because it is known as a nontaxing organism in terms of nutrient requirement. This is also advantageous from industrial application point of view as *A. haemolyticus* can be propagated easily using various types of wastes

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and may be deployed to reduce Cr(VI) contamination in the most carbon source–taxing environments such as underground water (Ahmad *et al.*, 2009b).

Table 4.2: Turbidity and cell concentration profiles for 10% (w/v) AW–adapted *A. haemolyticus*, 85% (v/v) deionised water, 5% (v/v) denatured ethanol and 10% (v/v) active culture

AW	Adapted	
	OD_{600}	CFU/mL
SCB	1.107 ± 0.076	$6.6 \pm 0.49 \times 10^8$
SPW	1.139 ± 0.001	$4.8 \pm 0.85 \times 10^8$
SBM	2.082 ± 0.026	$2.4 \pm 0.11 \times 10^8$

4.2.3 Cr(VI) reduction study of agricultural wastes adapted and non–adapted *A. haemolyticus*

Results from the Cr(VI) reduction studies carried out using AW control and AW–adapted and non–adapted *A. haemolyticus* are shown in Table 4.3. Overall, AW–adapted *A. haemolyticus* shows high efficiency of Cr(VI) reduction as compared to non–adapted and control. Of the three AW used, SCB showed the greatest Cr(VI) reduction efficiency as compared to others. Cr(VI) reduction for non–adapted are as follows; 38–89% for SCB, 45–86% for SPW and 43–81% for SBM. Result obtained for 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 for all metal concentration tested from 10 mg/L to 100 mg/L at the adsorbent dosage of 10% (w/v). Even though SCB without bacteria (control) also performed in Cr(VI) reduction, the removal efficiency dropped gradually when the metal concentration was increased. These abiotic reductions occurred was supported by the composition of SCB itself which consist of high concentration of glucose and xylose obtained from the characterization (Table 4.1). One reason to support the use of adapted *A. haemolyticus* is to sustain the Cr(VI) reduction process during the treatment. This suggests that the reduction may have been caused by components itself since no bacteria was inoculated into the medium.

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Table 4.3: Percentage reduction of 10–100 mg/L Cr(VI) by AW–adapted and non–adapted *A. haemolyticus* after 48 h contact time

Cr(VI), mg/L	SCB			Actual Cr(VI) reduction by bacteria[a]
	Control	Non–Adapted	Adapted	
10	94 ± 6	89 ± 3	99 ± 1	5
30	75 ± 2	75 ± 1	99 ± 1	24
50	68 ± 1	52 ± 4	97 ± 1	29
70	59 ± 6	45 ± 2	95 ± 1	36
100	41 ± 1	38 ± 4	92 ± 5	51

Cr(VI), mg/L	SPW			Actual Cr(VI) reduction by bacteria[a]
	Control	Non–Adapted	Adapted	
10	93 ± 4	86 ± 1	94 ± 3	1
30	65 ± 7	75 ± 7	87 ± 3	22
50	57 ± 1	68 ± 4	60 ± 7	3
70	38 ± 3	55 ± 7	48 ± 0	10
100	31 ± 6	45 ± 4	40 ± 2	9

Cr(VI), mg/L	SBM			Actual Cr(VI) reduction by bacteria[a]
	Control	Non–Adapted	Adapted	
10	87 ± 3	81 ± 2	85 ± 2	0
30	68 ± 7	67 ± 3	75 ± 6	7
50	52 ± 8	61 ± 1	73 ± 3	21
70	51 ± 1	56 ± 3	59 ± 3	8
100	36 ± 0	43 ± 3	21 ± 1	0

[a] Adapted – Control
(Actual Cr(VI) reduction by bacteria for Non–Adapted is negligible)

During reduction, the pH was observed to reduce from pH 7.21–7.31 to pH 4.07–5.21. The decrease in reduction above pH 4 may be due to occupation of the adsorption sites by anionic species like $HCrO_4^-$, $Cr_2O_7^{2-}$ and CrO_4^{2-} which retards the approach of such ions further toward the sorbent surface (Agarwal *et al.*, 2006). The decrease in adsorption at high pH values may be due to the competitiveness of the oxyanion of chromium and OH^- ions in the bulk.

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This finding also demonstrates the potential of SCB to be used completely in place of NB for bacterial propagation. A point to note is the ability of SCB alone to carry out abiotic reduction of Cr(VI) in the absence of bacteria. SCB was reported to be able to reduce Cr(VI) to Cr(III) by other researchers. Ahmad Khan and Mohamad (2007) reported the removal efficiency was 70.2% Cr(VI) at initial metal concentration of 10 mg/L, pH 1 and 4 h contact time with the adsorbent dosage of 7 g/L. While 92% of 50 mg/L Cr(VI) was reported to be reduced to Cr(III) at pH 2 and 1 h contact time with 20 g/L adsorbent dose (Garg *et al.*, 2007).

The ability of SCB to reduce Cr(VI) is due to the presence of carbon sources, i.e. glucose as proved via composition of SCB in Table 4.1. The most common pathway for breakdown of hexose sugars such as glucose (catabolism to the final products, CO_2 and H_2O) is via formation pyruvate through glycolysis with at least two molecules of NADH released for each glucose molecule oxidized (Madigan *et al.*, 2001). The effective role of glucose carbon source in Cr(VI) reduction can be described by Equation 4.1 (Zakaria *et al.*, 2007a).

$$C_6H_{12}O_6 + 8CrO_4^{2-} + 34H^+ \rightarrow 8Cr^{3+} + 6HCO_3^- + 20H_2 \quad (4.1)$$

From Equation 4.1, complete breakdown of 1 mole of glucose would yield sufficient energy to reduce 8 mole of Cr(VI). For example, complete breakdown of 5 g/L glucose (28 mM) is expected to yield sufficient energy to reduce 500 mg/L Cr(VI) (192 mM). Bacterial species such as *Bacillus* sp. may produce a soluble reductase that Cr(VI) complexes in an enzymatic reaction in which Cr(VI) receive electrons to form Cr(III). NADH is required in the reduction of Cr(VI) to achieve maximum activity as shown in Equation 4.2 (Zakaria *et al.*, 2007a).

$$2CrO_4^{2-} + 13H^+ + 3NADH \rightarrow 2Cr^{3+} + 3NAD^+ + 8H_2O \quad (4.2)$$

$\Delta G^{o'}$ = –87 kJ/electrons transferred (calculated from reported standard electrode potential, $E^{o'}$).

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The energy required for the reduction of Cr(VI) is lower than the energy released in the complete oxidation of glucose (yielding CO_2 and H_2O) i.e. $\Delta G^{o'} = -121$ kJ/electrons transferred. The enzyme–mediated reduction of Cr(VI) involves the overall deprotonation of NADH and the release of two electrons per mole which are transferred to Cr(VI) (Ishibashi *et al.*, 1990).

Growth of *A. haemolyticus* in AW leads to a decrease in pH of the culture after 24 h fermentation. This acidic condition allows chromate ion to reduce to Cr(III) as shown in Equation 4.3 below.

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7H_2O \qquad (4.3)$$

In summary, the effectiveness of low cost agricultural wastes namely sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM) were able to support the growth of *A. haemolyticus* and indicated the efficient biosorbents for eliminating Cr(VI) from aqueous solutions. Batch study indicated that Cr(VI) was reduced following the sequence SCB>SPW>SBM. Of these, 5 g of SCB in 50 mL total volume (10% w/v) SCB was chosen for subsequent studies based on the highest cell concentration, total carbohydrate content and Cr(VI) reduction which up to 99%. Beside, choice of SCB was made due to its abundance, cheap pricing and its rich–cellulosic content which assisted in bacterial growth.

4.2.4 Growth and Cr(VI) reduction by SCB–adapted *A. haemolyticus*

4.2.4.1 Denatured and absolute ethanol as disinfecting agents

Table 4.4 shows the effect of pre–treatment of SCB using denatured and absolute ethanol. From the results obtained, 5% (v/v) denatured ethanol manage to kill all the indigenous bacteria present in the SCB. However, with time the ethanol–treated SCB is prone to contamination. This is possible due to the inability of the ethanol pre–treatment to remove the bacterial endospore problem. No indigenous

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bacterial growth was observed on NA plates that used absolute ethanol. The use of ethanol as germicide is employed because it is impractical to use heat or autoclave for sterilization as heating and high temperature can cause degradation of the sugars (Nester *et al.*, 2001). Nordin (2006; 2009) reported the effectiveness of using 5% (v/v) absolute ethanol as disinfecting agent for a period of less than 12 h only for liquid pineapple waste (LPW). Even though the use of absolute ethanol performed better results, 5% (v/v) denatured ethanol had been chosen for the pre–treatment preparation. This was based on its availability and is relatively cheaper as compared to absolute ethanol itself and other chemicals such as hydrogen peroxide or formaldehyde. The ability of denatured ethanol as disinfecting agent is due to its ability to coagulate enzyme and essential proteins as well as damaging lipid membrane via increasing the fluidity of the membrane (Nordin and Ahmad, 2008).

Table 4.4: Effect of ethanol pre–treatment on SCB

% ethanol (v/v)	Bacterial counts (CFU/mL)	
	Denatured	Absolute
1	$2.1 \pm 0.42 \times 10^5$	0
3	$3.0 \pm 1.4 \times 10^4$	0
5	0	0
7	0	0
10	0	0

4.2.4.2 Effect of different concentrations of denatured ethanol on growth of *A. haemolyticus*

The use of SCB as nutrient for the bacteria was addressed by supplemented with 5% (v/v) denatured ethanol to suppress the growth of indigenous bacteria present in the unsterile SCB. With increasing incubation time, cell turbidity was observed to increase. Otherwise, the cell concentration was observed to decrease (Table 4.5). The decrease in cell concentration was expected due to the limited source of nutrient remaining during fermentation. The increase in cell turbidity however is a measure of dead and living cells observed during fermentation. *A.*

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haemolyticus showed highest growth in the presence of 5% (v/v) denatured ethanol at 12 h agitation time with cell concentration of 8.6±0.07 X 10^7 CFU/mL and OD_{600} value of 0.603±0.021. However, cell concentration of 6.5±4.9 X 10^4 CFU/mL of indigenous bacteria was started to observed after 48 h contact time.

Table 4.5: Turbidity and cell concentration profiles for *A. haemolyticus* grown in pre–treated SCB

Time (h)	OD_{600}	CFU/mL*
0	0.267 ± 0.013	4.0 ± 4.3 X 10^8
6	0.510 ± 0.093	5.6 ± 2.4 X 10^7
12	0.603 ± 0.021	8.6 ± 0.07 X 10^7
24	0.640 ± 0.065	5.9 ± 0.85 X 10^7
48	0.732 ± 0.015	6.9 ± 1.9 X 10^6

* CFU/mL was counted for *A. haemolyticus*.

4.2.4.3 Growth profile of SCB–adapted *A. haemolyticus*

Adaptation of *A. haemolyticus* in 10% (w/v) SCB was carried out by increasing ratio of deionised water in place of NB. Bacterial counts for *A. haemolyticus* in 100% NB was 2.2±0.91 X 10^9 CFU/mL with OD_{600} value of 1.265±0.052. When 20% of deionised water was used to replace NB, the cell concentration was dropped to 1.5±0.28 X 10^7 CFU/mL and this concentration was observed to maintain in the range of 10^7 CFU/mL when *A. haemolyticus* was grown in 10% (w/v) SCB in fully deionised water (Table 4.6). This indicates the suitable role of SCB in supporting the growth of *A. haemolyticus* in deionised water medium.

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Table 4.6: Turbidity and cell concentration profiles for *A. haemolyticus* grown overnight in increasing volumes of deionised water in the presence of 10% (w/v) SCB

(DI : NB) ratios (%)	OD_{600}	CFU/mL
0 : 100	1.265 ± 0.052	$2.2 \pm 0.91 \times 10^9$
20 : 80	1.121 ± 0.095	$1.5 \pm 0.28 \times 10^7$
40 : 60	0.783 ± 0.083	$2.6 \pm 0.21 \times 10^7$
80 : 20	0.873 ± 0.043	$1.6 \pm 0.19 \times 10^7$
100 : 0	1.123 ± 0.022	$6.3 \pm 0.07 \times 10^7$

* DI = deionised water, NB = nutrient broth, indigenous bacteria was not taken into consideration

The sugars present in the SCB play a role in providing carbon source to support the growth of *A. haemolyticus* but this is not enough to give maximum yield of cells. However, this still offers a significant advantage economically because the function previously performed by nutrient broth can now be substituted by the deionised water and SCB.

Figure 4.4 shows the growth profile of SCB–adapted *A. haemolyticus* in deionised water medium where around 16 h was needed to reach stationary phase. Cell viability was used as CFU/mL rather than OD_{600} as culture turbidity for the bacterial growth profile to reflect the actual cell concentrations present in the medium. Initial cell concentration was counted as $1.7 \pm 0.35 \times 10^7$ CFU/mL. From 2–16 h, the cell concentrations increased gradually from $8.1 \pm 2.0 \times 10^5$ CFU/mL to $1.2 \pm 0.57 \times 10^7$ CFU/mL. After 48 h, cell concentration was counted to decrease to $9.5 \pm 4.9 \times 10^6$ CFU/mL.

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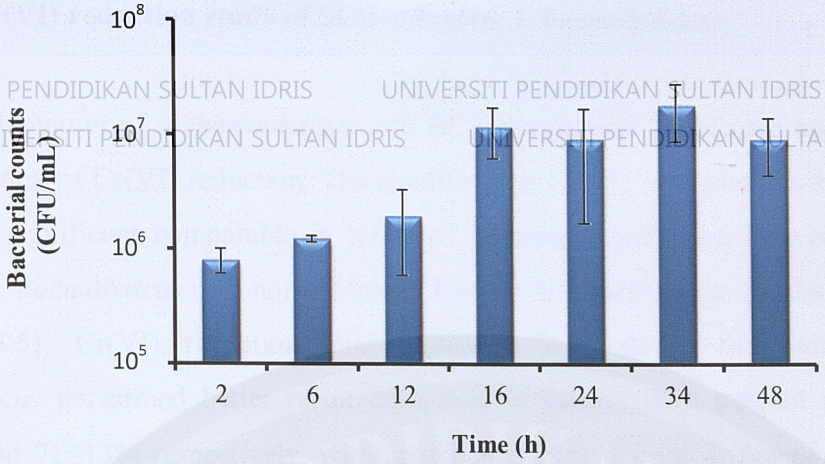


Figure 4.4: CFU/mL of *A. haemolyticus* grown in 10% (w/v) SCB in deionised water

Glycolysis is the principal pathway for catabolism of glucose (the major element of sugar in SCB) and other simple sugars. These reactions occur in the cytosol (cytoplasm) of all cells and can be summarized by Equation 4.4.

$$\text{Glucose} + 2NAD^+ + 2ADP + 2P_i \rightarrow 2 \text{ pyruvate} + 2NADH + 2ATP + \text{energy} \quad (4.4)$$

Cells derive energy from the oxidation of nutrients such as glucose. The oxidation of glucose to pyruvate occurs through a series of steps called glycolysis. The energy released during these oxidation reactions is used to form adenosine triphosphate (ATP). The initial steps in glycolysis are the additions of two phosphates to the glucose molecule, at the expense of two molecules of ATP. The result is a 6–carbon sugar diphosphate molecule and two low energy adenosine diphosphate molecules (ADP). This 6–carbon sugar diphosphate molecule is then split into two 3–carbon molecules. Each of the 3–carbon molecules is converted through a series of steps, to pyruvate. During these reactions, electrons are transferred to the coenzyme NAD^+ to form NADH and ATP. When the oxygen is available in the cell, aerobic catabolism occurs and converts pyruvate to acetyl–CoA. The acetyl–CoA is in turn oxidized to CO_2 and water. When the oxygen is not available, the anaerobic process known as lactate fermentation occurs. In this process, pyruvate is reduced to lactate in a reaction using the NADH produced by glycolysis (Armold, 2001).

4.2.4.4 Cr(VI) reduction study of SCB–adapted *A. haemolyticus*

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Adaptation of *A. haemolyticus* into SCB has proved to enhance and sustain the efficiency of Cr(VI) reduction. The results of the Cr(VI) reduction performances show the significant comparable in terms of percentage reduction between SCB–adapted *A. haemolyticus* than non–adapted (Table 4.3). Based on the results obtained (Figure 4.5), Cr(VI) reduction study towards SCB shows that adapted *A. haemolyticus* performed better result compared to control with percent reduction 83±3% and 71±13% respectively, with actual contribution from *A. haemolyticus* of 12%, at initial metal concentration of 100 mg/L and 10% (w/v) adsorbent dosage after 48 h contact time.

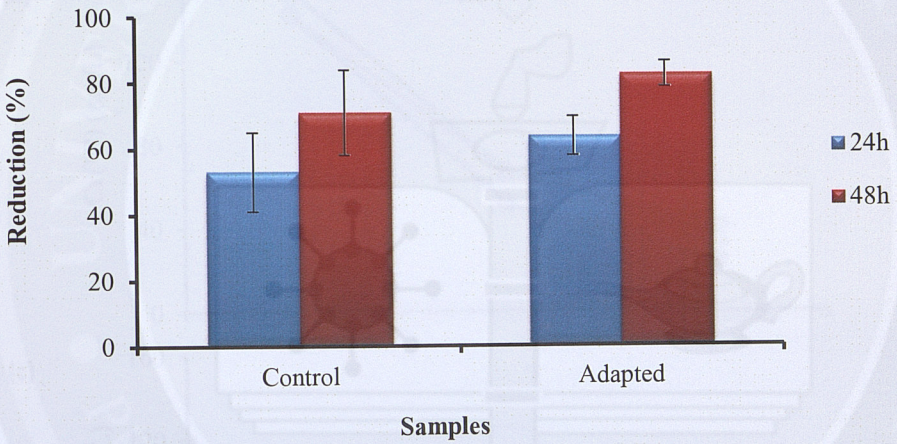


Figure 4.5: Percentage reduction of 100 mg/L Cr(VI) by SCB control and SCB–adapted *A. haemolyticus*

Reduction of SCB–adapted *A. haemolyticus* displayed a very high percentage of Cr(VI) removal capacity. Abiotic reduction of Cr(VI) by 10% (w/v) SCB used, which was most probably carried out by glucose and other reducing sugars, helped lowers Cr(VI) reduction by *A. haemolyticus*. This is evident from the Cr(VI) (Figure 4.6b) and the total Cr profiles (Figure 4.6a). With reference to the control profiles, from the initial 142–154 mg/L Cr(VI), around 71–83% of Cr(VI) were spontaneously reduced leaving around 24–42 mg/L Cr(VI) (Figure 4.6b) to be treated using SCB control, SCB–non adapted and SCB–adapted *A. haemolyticus*. The difference between total chromium and Cr(VI) values was taken as Cr(III) as shown in Figure

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4.6c. SCB control showed the ability to remove 71% of Cr(VI) as shown from the difference between Cr(VI) concentrations at the initial (142.093±4.731 mg/L) and final (41.597±0.719 mg/L) fractions as depicted in Figure 4.6.

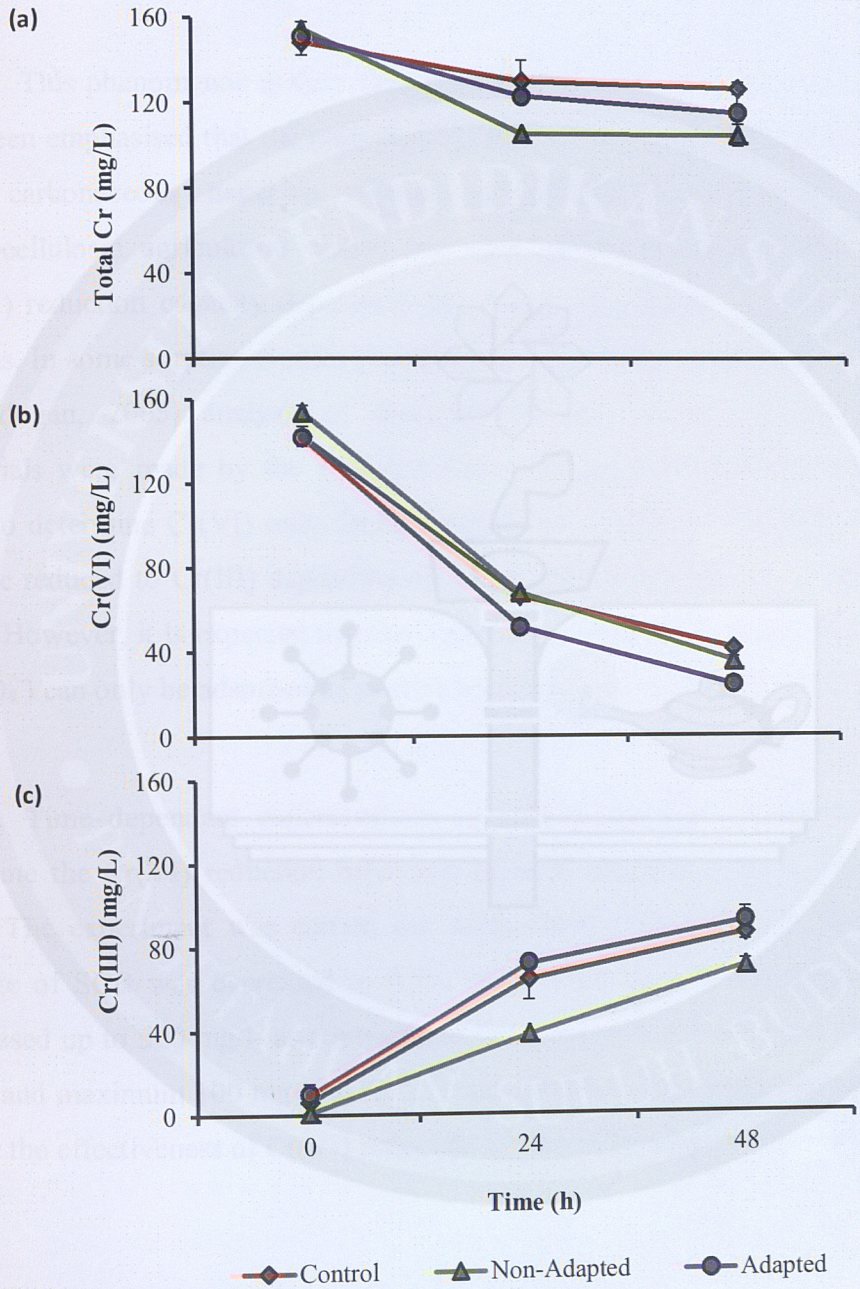


Figure 4.6: Cr(VI) reduction profile of SCB control, SCB–adapted and non–adapted *A. haemolyticus* of (a) total Cr, (b) Cr(VI) and (c) Cr(III)

Preliminary tests showed that the adsorption of Cr(VI) by SCB–non adapted and SCB–adapted *A. haemolyticus* occurs together with a reduction phenomenon.

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Cr(VI) reacts with organic matters leached out from SCB to produce Cr(III) and oxidized products. Reduction reaction can be illustrated as Equation 4.5 below:

$$HCrO_4^- \text{ or } CrO_4^{2-} + \text{Organic matter} + H^+ \rightarrow Cr^{3+} + H_2O + CO_2 \text{ (and/or oxidises products)} \quad (4.5)$$

This phenomenon is very important for evaluation of experimental results. It has been emphasised that the reduction of Cr(VI) occurs together with its adsorption onto carbonaceous materials (Cimino *et al.*, 2000; Lakatos *et al.*, 2002). Lignocellulosic agricultural wastes and their modified products may have high Cr(VI) reduction capacity depending on variety and amount of functional organic groups. In some sorption studies (Unnithan and Anirudhan, 2001; Selvi *et al.*, 2001; Altundogan, 2005) analysis of chromium in the solutions containing organic materials were made by the complexometric diphenylcarbazide method, which is able to determine Cr(VI) only. In fact, significant portion of Cr(VI) in the solution can be reduced to Cr(III) depending on the conditions such as pH, temperature and time. However, it is expected that the negatively charged Cr(VI) species (CrO_4^{2-} and $HCrO_4^-$) can only be adsorbed to positively charged surfaces in the acidic pH region.

Time–dependent concentrations of Cr(VI) and total Cr was conducted to examine the Cr(VI) reduction behaviour of SCB–adapted *A. haemolyticus* (Figure 4.7). The experiment was carried out using batch system. But in this study, the dosage of SCB was decreased to 0.5% (w/v) while the Cr(VI) concentration was increased up to 500 mg/L as compared to the previous study that used 10% (w/v) of SCB and maximum 100 mg/L of Cr(VI) concentration. This study was carried out to study the effectiveness of Cr(VI) reduction for the SCB–adapted *A. haemolyticus*.

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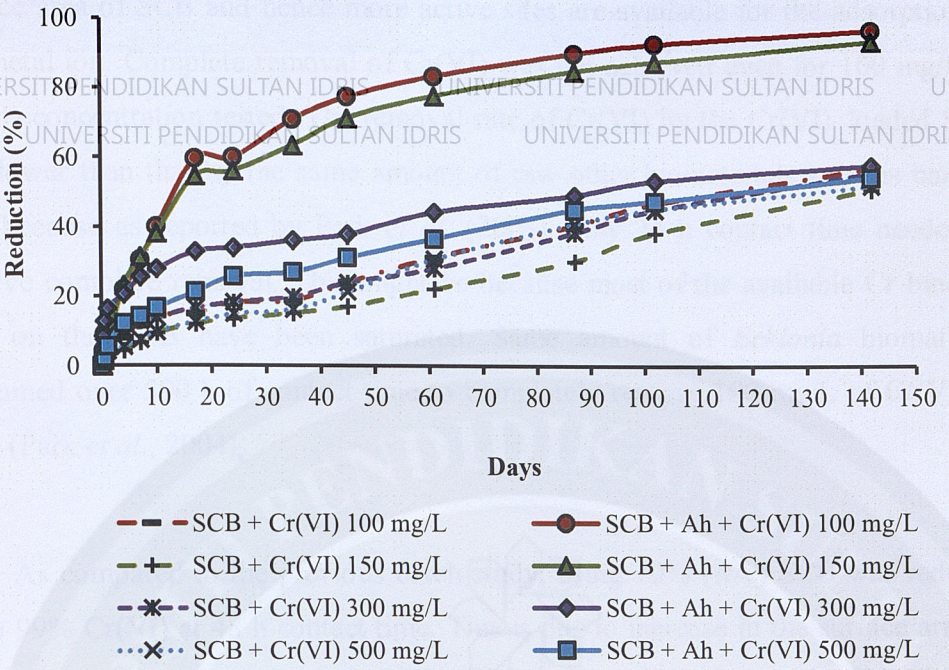


Figure 4.7: Time–dependent concentrations profile of SCB–adapted *A. haemolyticus* based on percentage of reduction achieved

Figure 4.7 shows the effect of different initial Cr(VI) concentration towards percentage Cr(VI) reduction by SCB and SCB–adapted *A. haemolyticus*. For all Cr(VI) concentrations analyzed (100, 150, 300 and 500 mg/L), results showed the highest reduction were 97%, 94%, 58% and 55% respectively after 142 days of incubation. Percentage removal efficiency of total Cr by SCB was in the range of 57–95%, while Cr adsorbed by SCB increases with increasing Cr(VI) concentration (99.150–324.000 mg/L). In the previous study conducted by Park *et al.* (2007), sixteen natural biomaterials were used to study the Cr(VI) biosorption characteristics, where the removal rate of Cr(VI) depended on the types of biomaterials. Park *et al.* (2007) concluded that the removal efficiency of total Cr by each biomaterial was not related with the removal rate of Cr(VI). The existence of total Cr in the aqueous phase implies the occurrence of Cr(VI) reduce to Cr(III) when brought into contact with the biomaterials. Regardless of the types of biomaterials, Cr(VI) was completely removed from aqueous phase and the Cr bound to its surface was in trivalent form.

In previous discussion, it was noticed that percentage removal of Cr(VI) increased with an increase in adsorbent concentration. This is due to increase in the

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surface area of SCB and hence more active sites are available for the adsorption of the metal ion. Complete removal of Cr(VI) was not achieved even for 100 mg/L of Cr(VI) concentration tested. The removal rate of Cr(VI) by the Cr(VI)–loaded SCB was lower than that by the same amount of raw other biomaterials such as banana skin because as reported by Park *et al.* (2008), only 24 h contact time needed to achieve complete removal. This might be because most of the available Cr binding sites on the SCB have been saturated. Same amount of *Ecklonia* biomaterial consumed over 500 h of contact time to completely remove 100 mg/L of Cr(VI) at pH 5 (Park *et al.*, 2004).

As compared to the previous batch study, using 10% (w/v) SCB was reduced up to 99% Cr(VI) at 48 h contact time. This is due to increase in the surface area of SCB and hence more active sites are available for the adsorption on the Cr(VI). This clearly showed the effect of adsorbent dosage towards the percentage removal. Similar observation on the effect of adsorbent dosage also reported by Ahmad Khan and Mohamad (2007).

Based on the results obtained from the batch study, SCB–adapted *A. haemolyticus* show the highest percent of reduction (83±2%) as compared to Cr–loaded SCB (71±13%) for 100 mg/L of initial Cr(VI) concentration (Figure 4.5). SCB–adapted *A. haemolyticus* showed removal efficiency of total Cr of 31±12% followed by 19±2% removal for SCB. Removal efficiency of total Cr refer to the maximum capacity of SCB to adsorb aqueous Cr(VI) solution during the incubation time. The detection of total Cr in aqueous phase means the occurrence of reduction reaction of Cr(VI) to Cr(III). It is natural that Cr(VI) can be easily and spontaneously reduced to Cr(III) by contact with biomaterials under acidic conditions because of its high redox potential value (Park *et al.*, 2008). Li *et al.* (2009) reported that Cr(VI) ions are probably in solution as $HCrO_4^-$ and $Cr_2O_7^{2-}$ species when the pH values of the solution between 2.0 and 6.0. In this study, the pH range was observed between pH 3.29–4.36.

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Murphy *et al.* (2009) identified that the optimum pH for Cr(III) sorption was pH 4.5 while for Cr(VI) was pH 2. At pH values < 3, competition from H^+ ions for biomass binding sites contributes to decrease Cr(III) binding. However, as pH increases, fewer H^+ ions are present in solution thus resulting in less competition for binding sites and hence increasing Cr(III) uptake. Sheng *et al.* (2004) reported that at pH > 3.5, dissociation of carboxyl groups and sulphonate groups lead to increase negative charge on the biomass, so, while anionic Cr(VI) species are repelled at these pHs, cationic Cr(III) experiences increased attraction to the biomass resulting in improved metal binding. As solution pH decreases, functionalities such as amino ($–NH_2$) and carboxyl groups may become protonated, thus making the biomass more positively charged and hence creating an electrostatic attraction with Cr(VI) species.

4.2.5 Chromium desorption study

Sorption of solute on any sorbent can either be by physical bonding, ion–exchange or combination of both. If the adsorption is by physical bonding, then the loosely bound solute can be easily desorbed with distilled water in most of the cases. However, if the mode of sorption is by chemical bonding or ion–exchange or combination of the both, then the desorption can be effected by stronger desorbents like acid or alkali solutions. Besides, desorption process was studied to evaluate suitable management of spent adsorbent. Regeneration of SCB is an important step in order to make the more economical for industrial applications.

Attempts were made to desorb Cr from previously Cr–loaded SCB and Cr–loaded SCB adapted *A. haemolyticus* using distilled water and followed by 0.015 M NaOH. It was found that Cr(VI) desorption was about 44–63% and 44–83% respectively when desorption was carried out using 50 mL distilled water at 30 min contact time. This followed by another desorption using alkali solutions (0.015 M NaOH) as suggested by Selvaraj *et al.* (2003). The results indicated that the desorption was in the range of 29–48% and 33–62% with 50 mL NaOH at 2 h agitation (Table 4.7). From this desorption results, it shown that chemical bonding

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involved between Cr(VI) and SCB used. Even though higher percentage of desorption was achieved using distilled water as compared to NaOH, it's not covered 100% desorption of Cr. In this study, desorption calculation is based on desorption of Cr(III) because as mention earlier, the reduction percentage was achieved up to 99% (from the previous data shown in Table 4.3). This result indicated that hydroxide ions (OH^-) may release Cr(III) from the SCB following an ion exchange mechanism.

Other solution for the desorption study also used by other researcher such as by using 0.1 M HCl (Gupta *et al.*, 2012; Saueprasearsit, 2011; Dos Santos *et al.*, 2012) and 0.2 M HCl (Ahalya *et al.*, 2008) and also higher concentration of NaOH i.e. 0.2 M NaOH (Ahalya *et al.*, 2008) and 2 M NaOH (Das *et al.*, 2013).

Table 4.7: Percentage of Cr desorption

	Cr adsorbed (mg/L)	Cr desorbed by distilled water (%)	Cr desorbed by 0.015M NaOH (%)
Cr–loaded SCB	10	63 ± 1	48 ± 2
	30	50 ± 3	38 ± 2
	50	48 ± 10	34 ± 5
	70	44 ± 7	29 ± 2
	100	51 ± 4	38 ± 1
Cr–loaded SCB adapted *A. haemolyticus*	10	83 ± 2	62 ± 2
	30	48 ± 8	37 ± 7
	50	53 ± 6	38 ± 4
	70	44 ± 5	33 ± 4
	100	49 ± 7	36 ± 5

4.3 Continuous Cr(VI) reduction process in packed–bed reactor

4.3.1 Immobilization of adapted *A. haemolyticus* onto SCB

During immobilization, SCB and *A. haemolyticus* culture are brought in contact. Using dislodging methods, the cells concentration of *A. haemolyticus* at the effluent decreased from 6.15±1.06 X 10^9CFU/mL (OD_{600} = 1.599±0.298) to 9.75±1.34 X 10^8 CFU/mL (OD_{600} = 1.214±0.079) after 72 h of continuous

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immobilization process (Figure 4.8a). Similar trend was also observed for the *A. haemolyticus*–immobilized SCB where the cells concentration was decreased after supplementation. Besides, it was noticed that the cells were not homogeneously distributed, being denser at the top of the reactor and the rates of deposition rapidly decrease with depth (Figure 4.8b). The accumulation and activity of biofilms varies from point to point along individual pore channels and thus are considered to be micro scale phenomena. Similar observation were also demonstrated by Gargiulo *et al.* (2007) who was reported that retained concentrations were highest near the reactor inlet where nearly all the bacteria (97%) were retained adjacent to the reactor inlet. The nutrient supplementation was required to assure the cell concentration was high enough for efficient biological removal. Also, the nutrient supplementation was utilized for maintenance of microbial activity. Study conducted by Mohammed (2011) reported that for reduction of Cr(VI) efficiency, nutrient supplementation was found to have greatest effect on the response evaluated using Response Surface Methodology (RSM) as compared to both the flow rate and initial Cr(VI) concentration.

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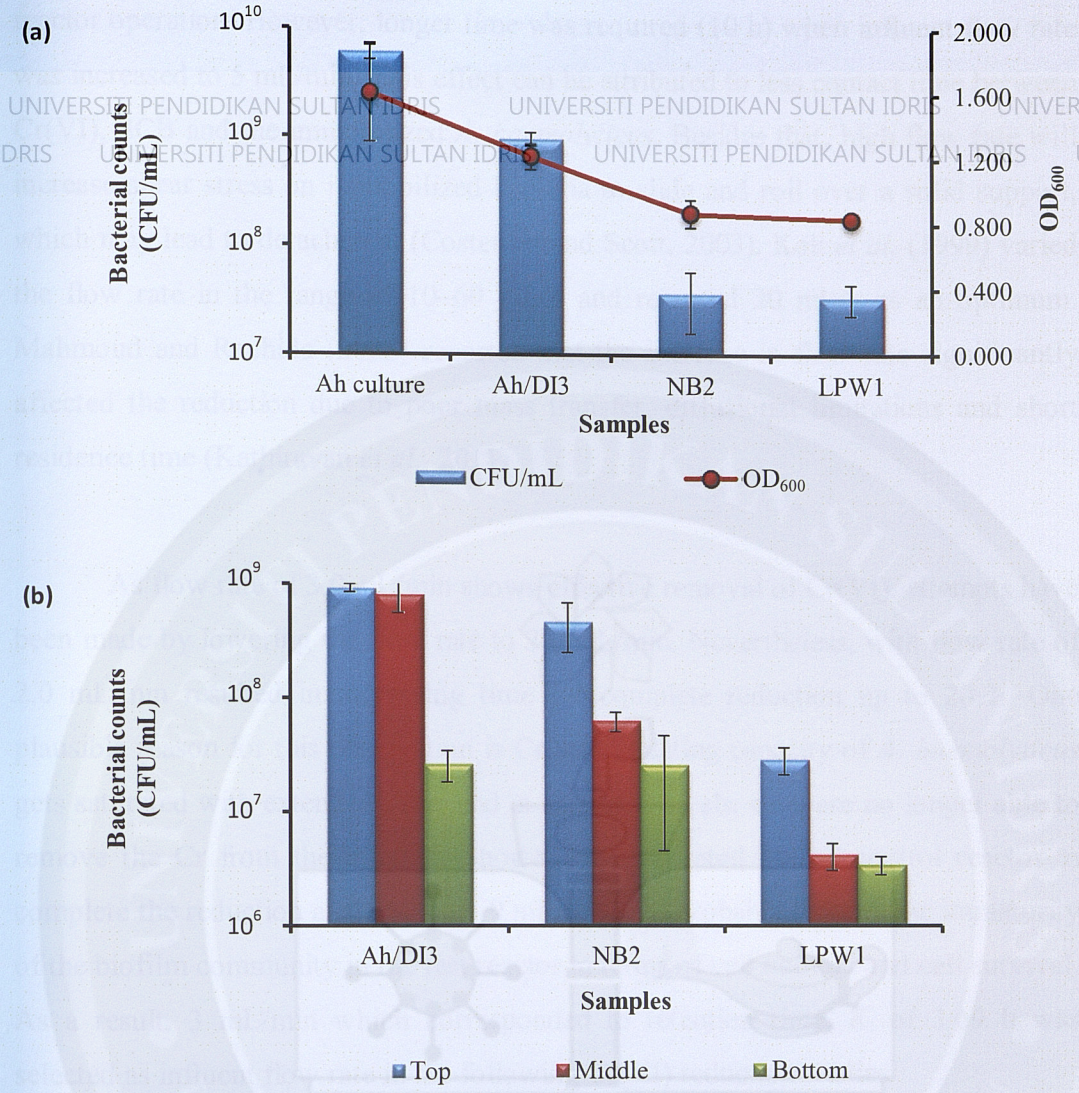


Figure 4.8: Distribution of *A. haemolyticus* cells in (a) effluent and (b) SCB. Ah culture = adapted *A. haemolyticus* culture, Ah/DI3 = adapted *A. haemolyticus* culture with continuous circulation for 3 days, NB2 = supplementation with NB for 2 days and LPW1 = supplementation with LPW for 1 day

4.3.2 Cr(VI) reduction using Cr(VI) stock solution

4.3.2.1 Effect of influent flow rate

The effect of influent flow rate on Cr(VI) reduction was carried out using the following parameters; flow rate of 2.0, 3.0 and 5.0 mL/min at initial Cr(VI) concentration of 50 mg/L. The experiment was started first with the flow rate of 3.0 mL/min. As can be seen on Figure 4.9, complete reduction was achieved after 4 h

reactor operation. However, longer time was required (10 h) when influent flow rate was increased to 5 mL/min. This effect can be attributed to less contact time between Cr(VI), SCB and the immobilized *A. haemolyticus*. Besides that, high flow rate will increase shear stress on immobilized bacteria to slide and roll over a solid support, which may lead to detachment (Costerton and Scott, 2003). Kok *et al.* (1999) varied the flow rate in the range of 10–60 mL/h and reported 20 mL/h as an optimum. Mahmoud and Rachida (2003) reported that the increase in flow rate significantly affected the reduction due to poor mass transfer, diffusional limitations and short residence time (Kathiravan *et al.*, 2011).

As flow rate of 3.0 mL/min shows effective removal of Cr(VI), attempts have been made by lowering the flow rate to 2.0 mL/min. Nevertheless, with flow rate of 2.0 mL/min resulted in increasing time for complete reduction up to 20 h. One plausible reason for this observation is Cr accumulating capacity of *A. haemolyticus* gets saturated with extended time, and at saturated levels, cells are no longer able to remove the Cr from the influent. Shorter time indicated by the control reactor to complete the reduction at flow rate 5.0 mL/min was probably reflects the immaturity of the biofilm community in the test reactor in terms of cell number and cell survival. As a result, 3 mL/min which corresponded to retention time, R_t of 3.69 h was selected as influent flow rate in the following Cr(VI) reduction studies.

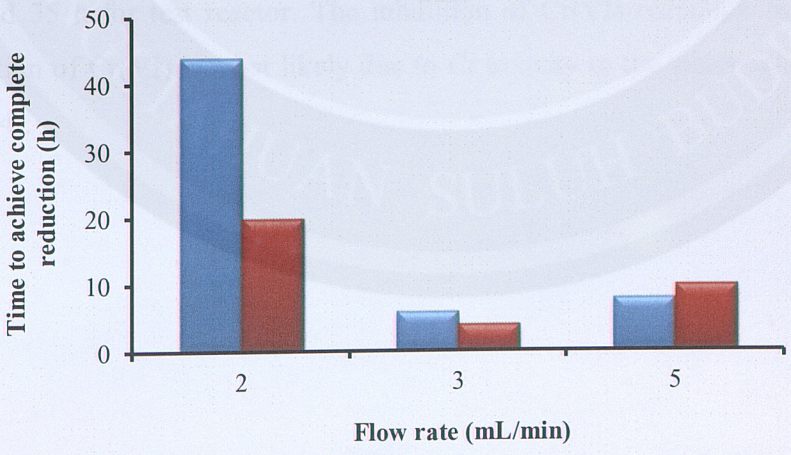


Figure 4.9: Time to achieved complete reduction towards different of influent flow rate

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4.3.2.2 Effect of influent Cr(VI) concentration

Figure 4.10 shows the effect of Cr(VI) concentration on Cr(VI) reduction by immobilized *A. haemolyticus*. The Cr(VI) removal efficiency was higher at low influent Cr(VI) concentration. At the start of reactor operation which was after immobilization and supplementation (7 days of reactor operation), 25 mg/L initial Cr(VI) concentration was introduced at flow rate of 3 mL/min. Lower Cr(VI) concentration was chosen to encourage the formation of biofilm and to prevent the toxic effects of Cr towards the bacterial cells (Chirwa and Wang, 1997). Results showed that complete reduction occurred shortly after 2 h operation for both control and test reactors. This fact is probably associated to the availability of reaction sites around or inside the surface of *A. haemolyticus* as well as SCB (positively charged group and electron donor group), able to capture Cr(VI) and sufficient retention time and becomes very efficient in the initial steps of the process.

In a second stage, with the gradual occupancy of these sites, the adsorption becomes less effective. A longer time, 8 h was needed when the Cr(VI) concentration was increased to 50 mg/L. At these 25 and 50 mg/L influent Cr(VI) concentration, both reactors were observed to give the same reduction rate performance of achieving complete reduction. However, increasing Cr(VI) concentration up to 100 mg/L, shows the increasing time to achieve complete reduction up to 25 h for control reactor and 35 h for test reactor. The inhibition of Cr(VI) reduction by increasing concentration of Cr(VI) is most likely due to Cr toxicity to the intact cells (Camargo *et al.*, 2004).

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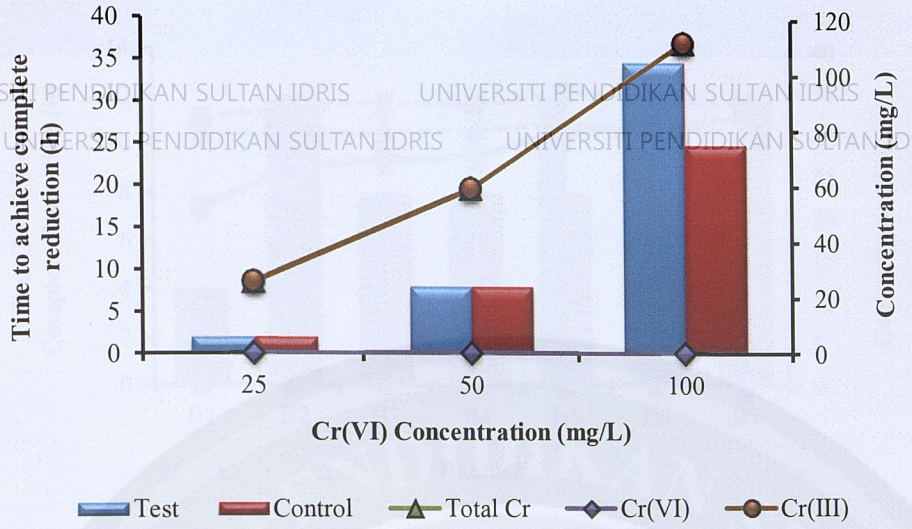


Figure 4.10: Time to achieved complete reduction towards different of influent Cr(VI) concentration

4.3.2.3 Packed–bed reactor performance study

Reduction of Cr(VI) from pure Cr(VI) solution was carried out for 32 days and the profile obtained is represented in Figure 4.11. Overall, the reactor system showed the ability to achieve complete reduction of 25 mg/L Cr(VI) within 4–12 h. The reactor efficiency was observed using seventh batches of Cr(VI) at a flow rate of 3 mL/min. These results showed the performances of reactor study 4 times faster as compared to batch study. The fastest Cr(VI) reduction can be observed for the first batch after 8 days reactor operation i.e. after LPW supplementation. Complete Cr(VI) reduction was achieved at the first batch of Cr(VI) influent as the early stage of reactor operation. This shows fast start up for the reactor i.e. complete Cr(VI) reduction was achieved immediately after supplementation without the need to adapt the reactor's content to the Cr(VI). The rate of percent Cr removal is higher at the beginning. That is probably due to larger surface area of the SCB as well as *A. haemolyticus* that being available at beginning for the adsorption of metals. As the surface adsorption sites become exhausted, the removal rate is controlled by the rate at which the adsorbate is transported from the exterior to the interior sites of the adsorbent particles (Agarwal *et al.*, 2006).

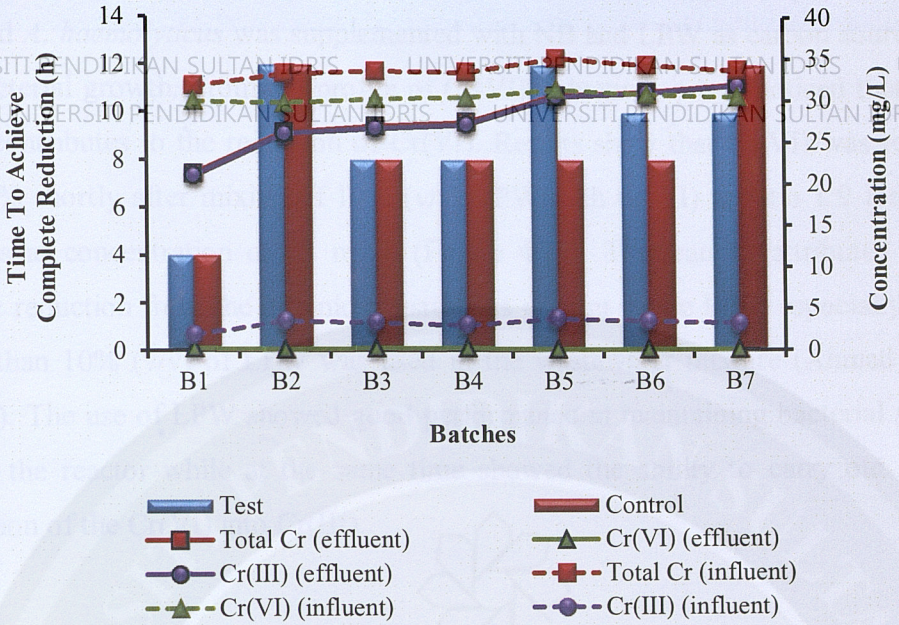


Figure 4.11: Profile of chromium concentration during Cr(VI) reduction using seventh batches of Cr(VI) (B1–B7) at a flow rate of 3 mL/min and 25 mg/L Cr(VI) concentration.

However, longer time was needed to completely reduce the second and the other batches of Cr(VI). It was noticed that the finding from this study contradicts that of Mohamed (2011) and Ahmad *et al.* (2010b) where with time, the number of cycles needed for Cr(VI) reduction was reduced which eventually leads to a single cycle of treatment only. In other words, shorter time was needed to completely reduce the influent Cr(VI) concentration. As a result, fresh cells are needed to re–supply the reactor or nutrient supplementations to increase and promote bacteria growth. Besides, more bacterial colonies were present in the reactor effluent and also at the SCB. However, this condition was fully expected due to the unsterile operating condition used. The invasion of other microorganisms could originate from the LPW and indigenous microbes present on the SCB. This problem has to give attention for long period of reactor operation. Analysis for total Cr concentration was also carried out using AAS for the influent and effluent fractions to determine simple mass balance of Cr inside the system. Results show that removal efficiency of total Cr in the range of 5–33% which refer to Cr adsorption from 2–11 mg/L for all batches.

During immobilization which is after bacterial attachment, the immobilized adapted *A. haemolyticus* was supplemented with NB and LPW as carbon sources for the bacterial growth. From the profile of Cr(VI) reduction, it was noticed that LPW itself contributes to the reduction of Cr(VI). Results show that Cr(VI) was reduced 34–40% shortly after mixing of 10% (v/v) LPW with Cr(VI) at ratio 1:9 for every batches at concentration of 25 mg/L (Figure 4.12). This can be attributed to the abiotic reduction from the organic constituents present in the LPW especially when more than 10% (v/v) of LPW was used in the wastewater mixture (Ahmad *et al.*, 2010b). The use of LPW showed good performance in maintaining bacterial growth inside the reactor while at the same time showed the ability to carry out abiotic reduction of the Cr(VI) into Cr(III).

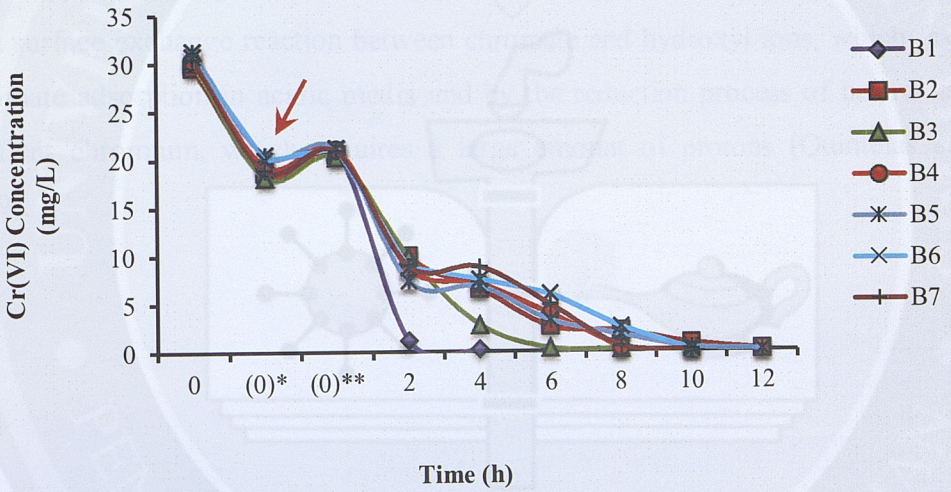


Figure 4.12: Profiles of abiotic reduction of LPW for all batches studied (B1–B7). 0 = Cr(VI), (0)* = Cr(VI):LPW, (0)** = Cr(VI):LPW pH 7

For the Cr(VI) reduction, results showed that Cr(VI) was completely converted to Cr(III) for all batches. Cr(VI) reduction–resistance performance analysis obtained showed decreasing total carbohydrate content and COD up to 95% (from initial concentration of 5855 mg/L to 291 mg/L) and 42% (from initial concentration of 7300 mg/L to 4250 mg/L) respectively. This final COD concentration is much higher when compared to concentration of COD value at the final discharge before modification of post treatment using the ChromeBac™ system (2624 mg/L) and still exceeded the permissible discharge limit (< 200 mg/L). Decreasing total carbohydrate content was expected to be caused by its use as carbon

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sources for the bacteria to grow. Kathiravan *et al.* (2011) reported that Cr(VI) reducing microorganisms utilize a variety of organic compounds as electron donors and majority of the known electron donors are low molecular weight carbohydrates. Cr(VI) reduction rate mainly depends on the growth of *Bacillus* sp. and the nature of carbon sources. One plausible reason for the high COD concentrations at the effluent is the high contents of organic matters contributed by the use of LPW and SCB itself. Therefore, post–treatment is recommended. Besides, increasing values of OD_{600} from 0.020 A to 0.062 A and pH from 2.63 to 4.79 was measured after 32 days of treatment operation (Figure 4.13). It has been postulated by Garg *et al.* (2007) that Cr(VI) could be reduced to Cr(III) in the presence of an adsorbent under acidic conditions. More recently, Krishnani *et al.* (2008) affirm that the reduction of Cr(VI) mainly depends on the proton concentration in solution, as the higher the proton concentration, the higher the efficiency of Cr(VI) reduction. This can be explained by a surface exchange reaction between chromate and hydroxyl ions, which favours chromate adsorption in acidic media and by the reduction process of hexavalent to trivalent chromium, which requires a large amount of protons (Quintelas *et al.*, 2009).

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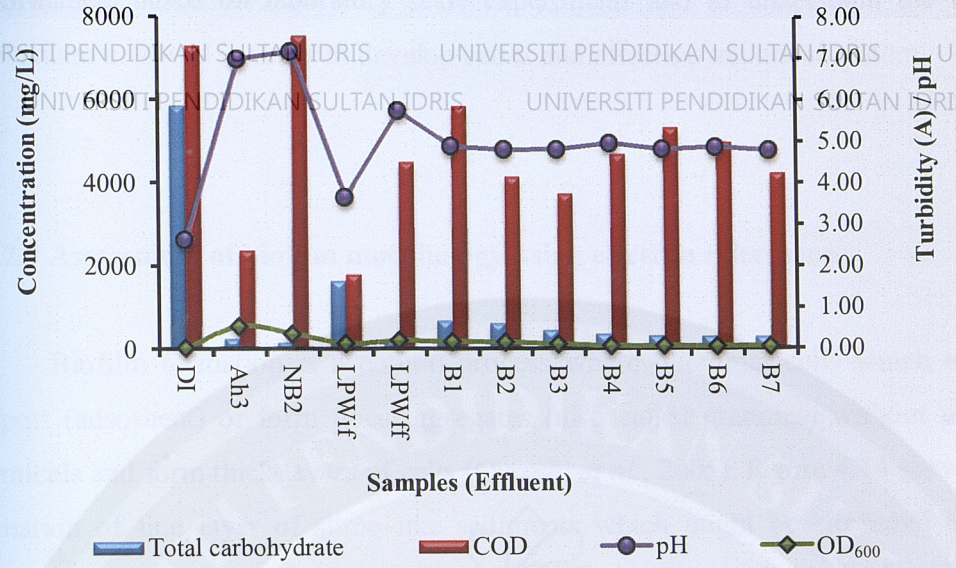


Figure 4.13: Profile of total carbohydrate, COD, OD_{600} and pH during Cr(VI) reduction of test reactor. DI = effluent after rinsed with deionised water, Ah3 = adapted *A. haemolyticus* culture with continuous circulation for 3 days, NB2 = supplementation with NB for 2 days, LPWif = initial reading for filtered LPW, LPWff = final reading for filtered LPW and B1–B7 = Cr(VI) of 25 mg/L for first batch to seventh batches

This study shows the feasibility of using the reactor system consisting of immobilized living bacteria (*A. haemolyticus*) onto and biosorbent (SCB) as a useful alternative treatment for Cr(VI) contamination in the aqueous system. Combination of this adapted *A. haemolyticus*–immobilized SCB resulted in a Cr(VI) removal capacity of 100% at initial Cr(VI) concentration of 25 mg/L. This remarkable Cr(VI) removal capacity was largely assisted by the abiotic Cr(VI) reduction by LPW used. The high percentage conversion of Cr(VI) to Cr(III) suggests the feasibility of using SCB as an alternative treatment for Cr(VI) contamination in the aqueous system with both biosorption and bioreduction were identified as the main mechanism occurred at the same time. The ability of *A. haemolyticus*, as a primary Cr(VI)–reducing microorganisms was obviously significant during the batch system, but reactor system shows that performance of native biosorbent (without immobilization of bacteria) is better than the immobilized biosorbent. However, various optimization parameters and immobilization techniques have yet to be thoroughly investigated for ease, efficacy and cost effective. The development of a packed–bed reactor would be helpful for evaluating industrial–scale biosorption and bioreduction reactor

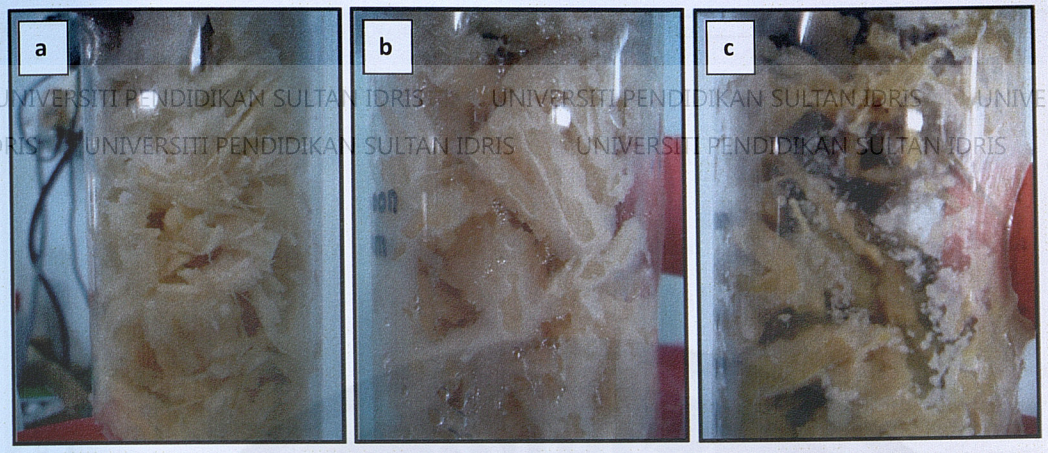
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performance, based on laboratory scale experiments and to understand the basic mechanism involved in order to develop better and effective treatment system.

4.3.2.4 Assessment of biofilm morphology using electron microscopy

Biofilm formation is a natural process where microbial cells attach to the support (adsorbent) or form flocs/aggregates (also called granules) without use of chemicals and form thick layers of cells (Qureshi *et al.*, 2005). Figure 4.14 shows the formation of fine layer of slime–like sediments which might be attributed to the presence of slime–containing water–borne bacteria throughout the study. Besides, *A. haemolyticus* biofilm formed may be subjected to colonization by ubiquitous Cr(VI) resistant bacteria. At this stage, the invading microorganisms were beginning to have a role in the biofilm as evident from its good survival ability. During biofilm development, portions of biofilm peel away from the surface and are entrained in the fluid flow. Detachment is a process of continuous biofilm removal and is highly dependent on hydrodynamic conditions such as flow rate and toxicity. The presence of toxic substances or the limitations on by–product diffusion from the biofilm further aggravate the situation and would lead to death or lysis of the bacteria (Zakaria *et al.*, 2007a). Figure 4.14c clearly showed uneven distribution of biofilm on the SCB surface together with the change of SCB colour from pale yellow to light brown.

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Figure 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)

Formation of *A. haemolyticus* biofilm on the surface of SCB is shown by FESEM micrographs (Figure 4.15). After 7 days of reactor operation which relates to complete supplementation with LPW, rod shape cells observed may be representative of log phase cells of *A. haemolyticus* clumped to one another (Figure 4.15b). The same observation also reported by Ahmad *et al.* (2009b) where the bacteria formed a chain–like structure or formed in pairs. Since the reactor operation was carried out under unsterile condition, the biofilm developed a stable microbial unit consisting of not just cells of *A. haemolyticus*, but also other organisms which originated from the unsterile LPW and Cr(VI) and also SCB itself. Formation of EPS was noticed on SCB surface after immobilization process in the reactor (Figure 4.15d). Figure 4.16 shows the presence of a massive EPS in the 61 days of reactor operation. The EDX profiles of biofilm on SCB of the reactor after 8 days of operation revealed small but prominent Cr peaks of 0.30–0.60 weight % and 0.28–0.49 weight % for control and test reactors respectively (Figure 4.15c, d). Higher Cr weight percentage were detected (14.45–15.13 weight % and 11.57–11.97 weight %) which referred to the sample after seventh batches of Cr(VI) treatment.

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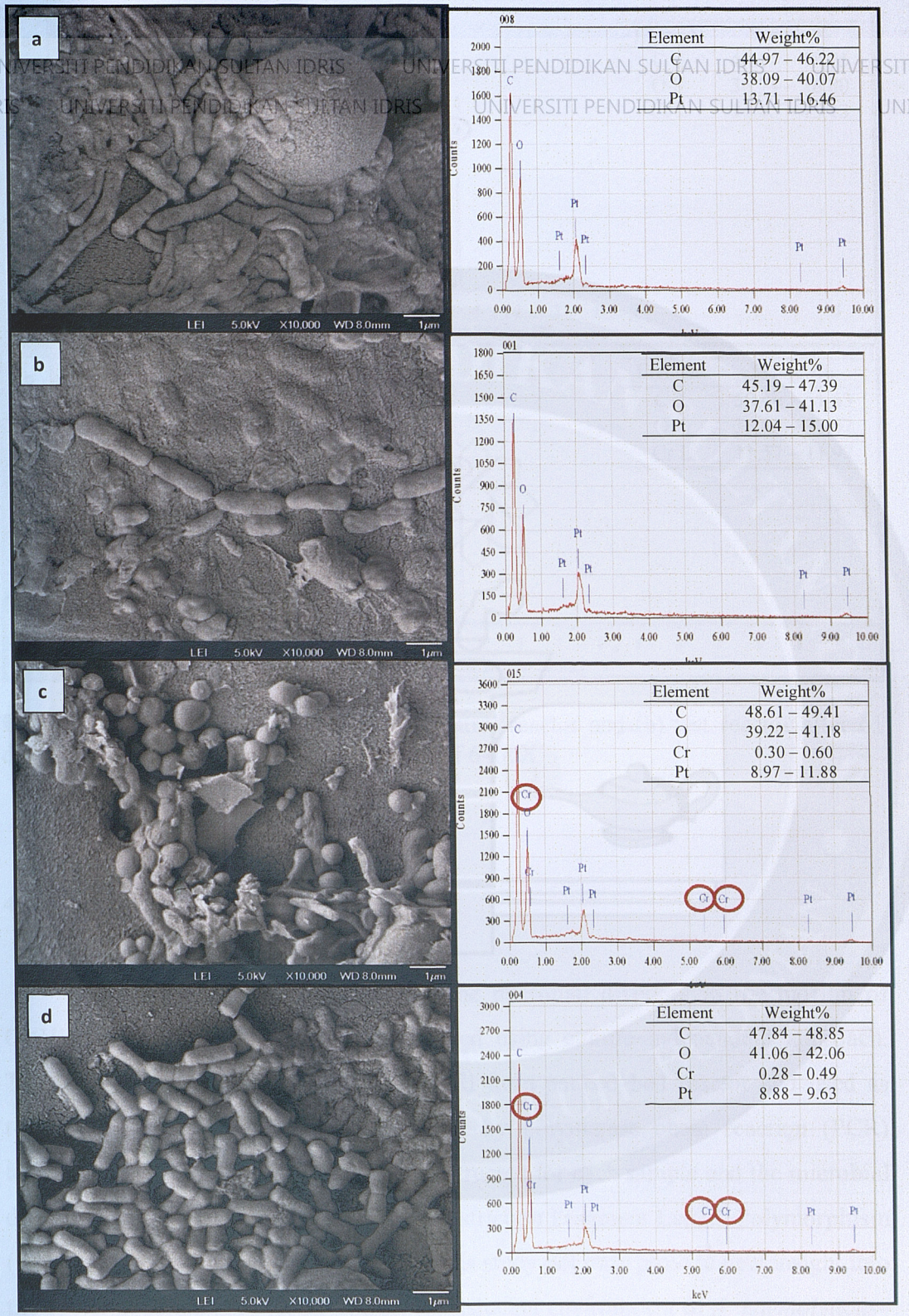


Figure 4.15: FESEM micrographs and EDX spectra of (a) control reactor and (b) test reactor after supplementation with LPW (7 days reactor operation), while (c) control reactor and (d) test reactor after first batch tr eatment of Cr(VI) (8 days reactor operation) at magnification of 10, 000X. Pt peak is associated with platinum sputter coating

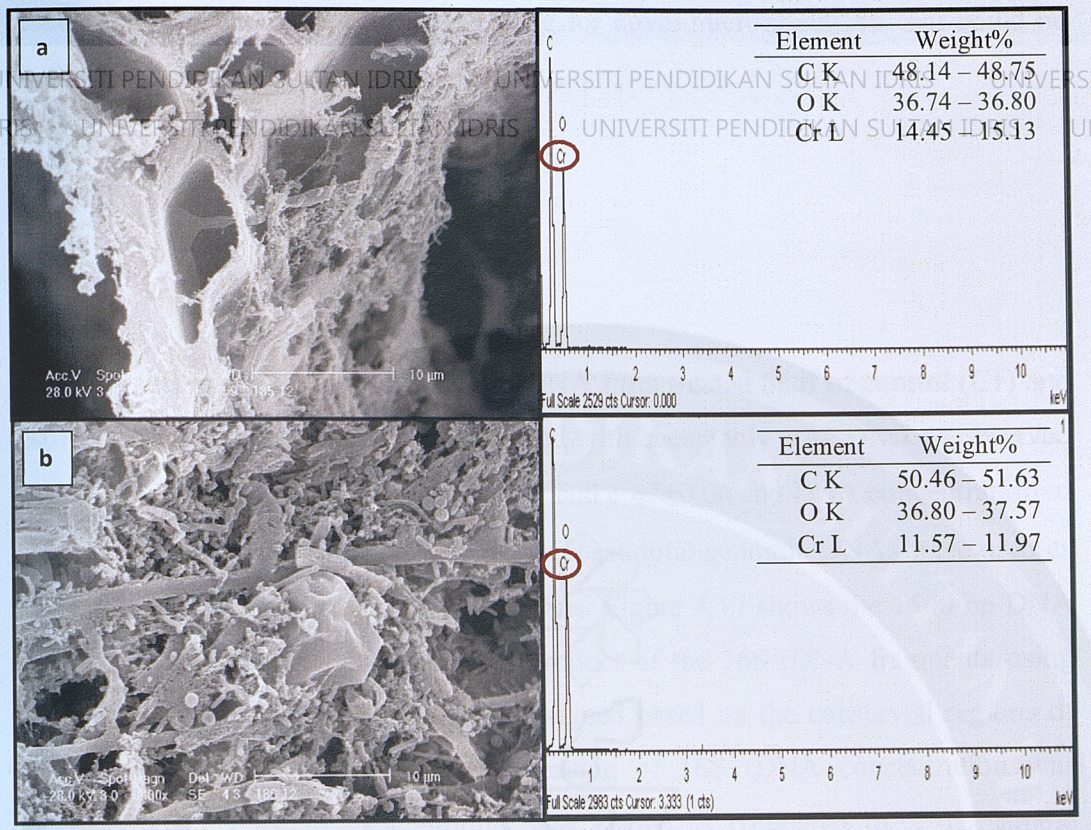


Figure 4.16: SEM micrographs of (a) control reactor and (b) test reactor after 61 days of reactor operation at magnification of 6400X

4.4 Biofilm microbial diversity study

In this microbial diversity study, the microbial diversity in the biofilm of Cr(VI) reducing environment was identified using culture–independent approach. The total genomic DNA was first extracted from packed–bed reactor and used as template for the 16S rDNA amplification by polymerase chain reaction (PCR) technique. The 16S rDNA library was constructed for each sample and the microbial diversity was determined by performing Restriction Fragment Length Polymorphism (RFLP) analysis. The finding obtained in this study not only provide new insight into the bacterial community formed in Cr(VI) reducing environment of *A. haemolyticus*–immobilized sugarcane bagasse (SCB) treatment system, but generating useful information that might assist in future studies and further enhancement of metal–microbe interaction in the presence of SCB as support medium. Furthermore, the

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results obtained may be useful in searching for novel microorganisms that could be useful in Cr(VI) reduction.

4.4.1 16S rDNA sequence analyses

In this experiment, total genomic DNA from treated SCB of control (C1) and test (T1) reactor were successfully extracted with acceptably pure DNA as observed from the ratio of A_{260}:A_{280} being 1.83±0.08 and 1.87±0.06 and DNA concentration of 31.4±0.8 and 29.0±0.5 ng/μL respectively. These total genomic DNAs were used as template for amplification of 16S rRNA gene. Figure 4.17 shows the 1500 bp DNA fragment represents the PCR–amplified product of the 16S rDNA fragments using the universal primers, 27F and 1492R, designed based on the conserved regions of the *E. coli* 16S rDNA sequences. Increasing of 16S rDNA concentration was observed when 5 μL of PCR product was loaded as compared to 2 μL sample volume. The single DNA band at high sample concentration also indicates the specificity of the PCR reactions.

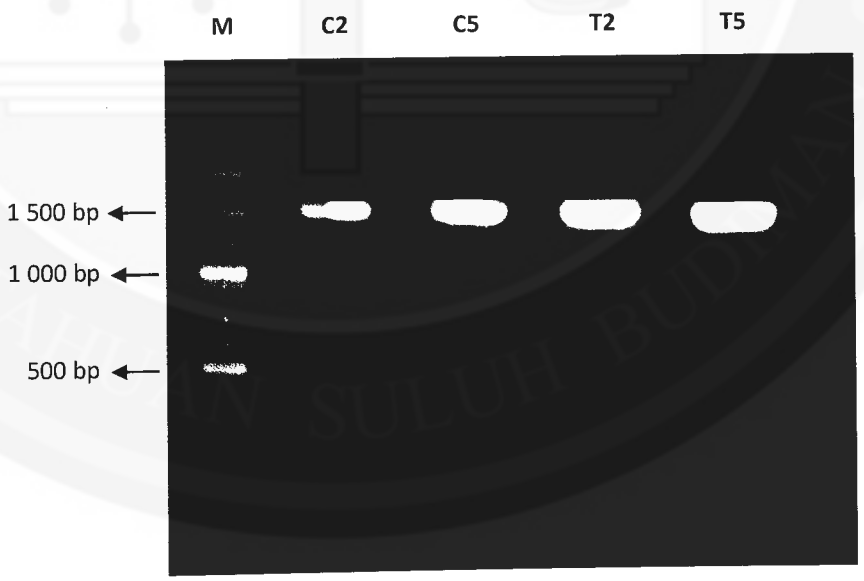


Figure 4.17: Agarose gel electrophoresis of PCR–amplified product of 16S rDNA. M: 100 bp plus marker; C: Control sample; T: Test sample, 2 and 5: Volume of DNA used (μL)

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Purified 16S rDNA fragments were then cloned into cloning vector, yT&A cloning vector (Yeastern Biotech Co. Ltd., Taiwan) and transformed into *E. coli* DH5α competent cells. A total of 693 white positive colonies of control sample (C5) and 422 of test sample (T5) were screened respectively. Out of these, 145 and 186 positive clones respectively which carry the desired insert of approximately 1500 bp were selected for subsequent analysis. Figure 4.18 shows the example of positive and negative clones of colony PCR obtained from test sample (T5).

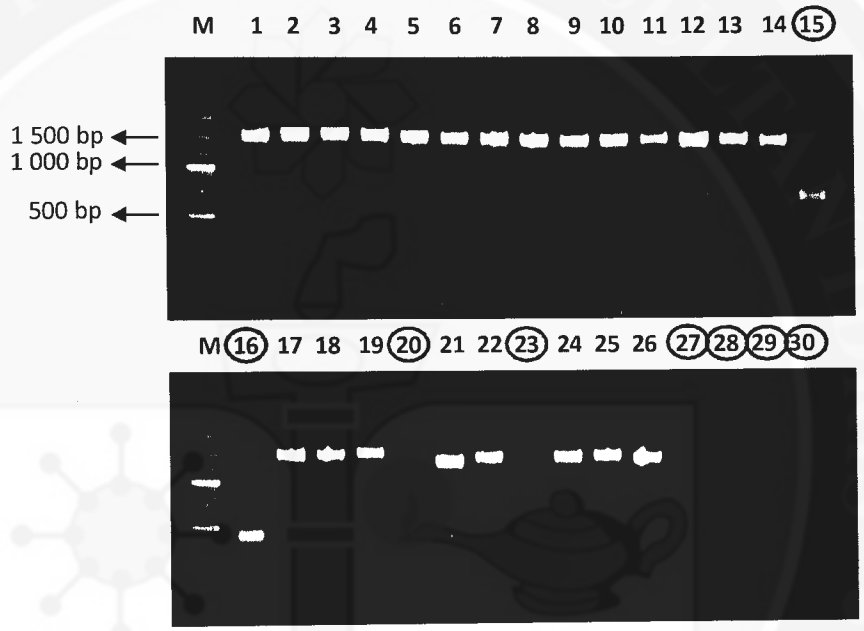


Figure 4.18: Agarose gel electrophoresis of positive and negative colony PCR band. Numbers in red circle show the negative colony. M: 100 bp plus marker

To differentiate the identity of these positive clones, RFLP analysis was carried out. The analysis revealed that the control and test samples consisted of 81 and 114 RFLP patterns, respectively. Clones carrying the identical RFLP pattern were presumptively the same bacteria. Nevertheless, none of these patterns resemble similar RFLP pattern for *A. haemolyticus*. This might be because the portion of the sample did not cover the whole reactor content. As mentioned earlier, the total genomic DNA of treated SCB was extracted from the middle section of reactors for both control and test. So, the DNA of *A. haemolyticus* might not exist at the sampling portion. Ideally, the bacterial population is expected to be the highest at point closest to the inlet and gradually decreasing with increase of the reactor length. This is because the bacterial population nearer to the inlet section would receive the highest

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concentrations of dissolved oxygen and nutrients which is crucial for bacterial growth and maintenance. In this study, bacterial populations at the top section was expected to give the highest Cr(VI) reduction. However, it should also be noted that it also received the highest incoming Cr(VI) concentration, hence greater chance for Cr(VI) toxicity (Ahmad *et al.*, 2009b). The number of *A. haemolyticus* cells was observed to decrease during the seventh batches of treatment process (starting from the 10^7 CFU/mL after supplementation until too numerous to count, TFTC), showing the toxicity effect of Cr(VI) towards the bacteria. The aforementioned reasons were prompted the selection of the middle section of the reactor for subsequent microbial diversity study.

Sequencing analysis of these unique RFLP patterns revealed a large bacteria community in the sample studied. For control sample, out of the 81 patterns, 5 dominant patterns represent 26.2% (38 clones) of the total library clones. While for test sample, 8 dominant patterns out of 114 patterns represent 21.0% (39 clones) of the total library clones (Figure 4.19 and Figure 4.20).

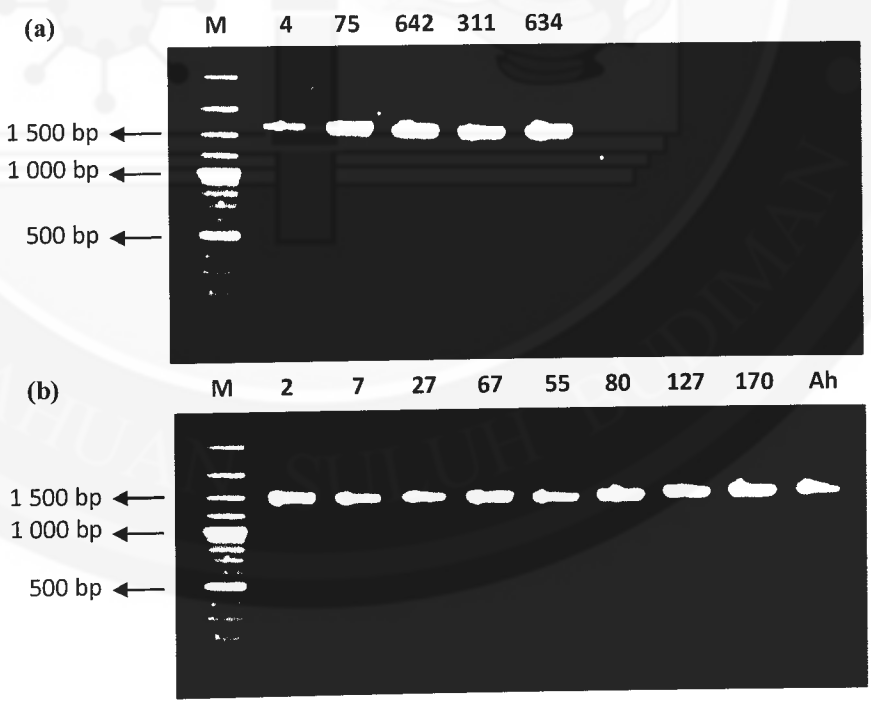


Figure 4.19: Agarose gel electrophoresis of purified PCR products. M: 100 bp plus marker; (a): Control sample, C5; (b): Test sample, T5

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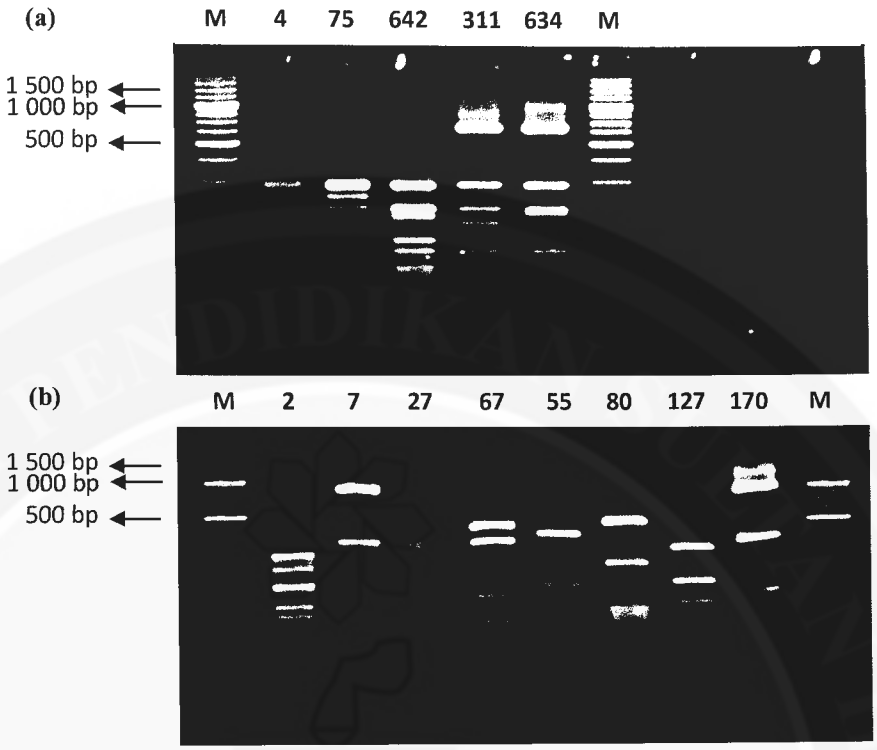


Figure 4.20: Agarose gel electrophoresis of RFLP bands of purified PCR products. M: 100 bp plus marker; (a): Control sample, C5 (5 clones with different RFLP patterns); (b): Test sample, T5 (8 clones with different RFLP patterns)

To determine the identity of these clones, plasmid DNAs of selected clones were isolated for DNA sequencing analysis. The purity and concentration of the plasmid DNAs were measured prior to DNA sequencing analysis to ensure the quality of the analysis. In this study, the isolated plasmid DNAs produced relatively pure DNA with A_{260}:A_{280} ratio of 1.60–1.82 for control sample (C5) and 1.67–1.85 for test sample (T5), respectively. Meanwhile, DNA concentration ranging from 41.4–59.2 ng/μL and 36.8–56.7 ng/μL were obtained from samples C5 and T5, respectively (Table 4.8). *A. haemolyticus* shows DNA purity with A_{260}:A_{280} ratio of 1.80 and DNA concentration of 87.8 ng/μL.

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Table 4.8: The plasmid DNA concentration and purity of each selected clone for (a) control (C5) and (b) test (T5) samples

(a)

C5			
Clone No.	Total Clones	DNA concentration (ng/μL)	Purity (A_{260}/A_{280})
4	4	41.1 ± 7.5	1.60 ± 0.0
75	7	48.8 ± 1.1	1.78 ± 0.0
311	12	47.0 ± 6.0	1.70 ± 0.1
634	10	54.3 ± 1.3	1.80 ± 0.0
642	5	59.2 ± 1.2	1.82 ± 0.0

(b)

T5			
Clone No.	Total Clones	DNA concentration (ng/μL)	Purity (A_{260}/A_{280})
2	4	55.7 ± 1.0	1.79 ± 0.0
7	6	39.2 ± 0.4	1.77 ± 0.0
27	4	36.8 ± 2.6	1.76 ± 0.0
55	9	40.1 ± 0.4	1.72 ± 0.0
67	4	56.0 ± 3.8	1.85 ± 1.0
80	4	50.4 ± 3.0	1.67 ± 0.0
127	4	42.6 ± 0.7	1.75 ± 0.0
170	4	56.6 ± 3.5	1.73 ± 1.0

The 16S rDNA sequences were aligned with sequences from other bacteria in the GenBank database of NCBI via BLASTn. Sequencing analysis of the nearly complete 16S rDNA sequences of the predominating pattern (22 clones, 6.6%) showed highest sequence homology to the *Chitinophaga terrae* strain KP01 (GenBank Accession No. NR041540), originally isolated from soil in South Korea (Kim and Jung, 2007) (Table 4.9). The second most abundant pattern as represented by 11 clones (3.3% abundance) was most closely related (94% sequence homology) to *Laribacter hongkongensis* strain HKU1 (GenBank Accession No. NR025167). The biochemical profile of *L. hongkongensis* strain HKU1 shows it can produce catalase, cytochrome oxidase, urease, arginine dihydrolase and reduces nitrate. The bacterium was initially isolated from the blood and empyema of a cirrhotic patient (Yuen *et al.*, 2001).

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The third (10 clones, 3.0%) and the fourth (9 clones, 2.7%) most abundant occurrences were related to uncultured bacterium clone WW2_146 (GenBank Accession No. GQ264320) and *Ottowia thiooxydans* strain K11 (GenBank Accession No. NR029001) with 94% and 97% sequence similarities, respectively. *O. thiooxydans* strain K11 was reported as facultatively anaerobic bacteria which reduces nitrate and nitrite and produces N_2O as sole end product of nitrate respiration. It has also been reported to utilize carboxylic acids and amino acids as substrates. The type strain, K11 was originally isolated from activated sludge of a high–load basin of a municipal wastewater treatment plant (Spring *et al.*, 2004).

Other patterns were matched with 96% sequence similarity (9 clones, 2.7%) to the uncultured bacterium clone WW3_39 (GenBank Accession No. GQ264495) followed by 93% sequence (4 clones, 1.2% abundance) to *Rhizobium cellulosilyticum* strain ALA10B2 (GenBank Accession No. NR043985), Candidate division OP10 bacterium Gsoil 348, 96% (GenBank Accession No. GQ339893), *Pedobacter* sp. oral clone AV100, 94% (GenBank Accession No. AF385531) and uncultured bacterium clone GI5–005–F01, 95% (GenBank Accession No. FJ192729). *R. cellulosilyticum* strain ALA10B2 was reported to actively produce cellulases on media containing CM–cellulose as substrate utilizes glucose, L–arabinose, mannose, mannitol, N–acetylglucosamine and malate as carbon sources and also grows on glutamate and methionine as carbon and nitrogen sources. The type strain ALA10B2 was isolated from sawdust of *Populus alba* in Spain (Garcia–Fraile *et al.*, 2007). Meanwhile, 16S rDNA sequence analysis of Candidate division OP10 bacterium Gsoil 348 indicated that the strain is related to members of the phylum *Armatimonadetes*. 16S rDNA sequences representing the phylum *Armatimonadetes* have been identified by culture–independent methods from various environments, including aerobic and anaerobic wastewater treatment processes, the rhizosphere, hypersaline microbial mats and subsurface geothermal water streams. Strain Gsoil 348 could not grow in high–strength nutrients (higher than 1.5 g/L of total organic carbon) such as nutrient agar and showed slow assimilation of limited carbon substrates (Im *et al.*, 2012).

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The taxonomic affiliations of the nearly complete 16S rRNA gene sequences of control and test samples are shown in Table 4.9. Among the selected dominant patterns for both control and test samples, 54 (16.3%) were affiliated to cultivated counterparts whereas 23 (6.9%) were affiliated to yet–uncultivated or uncultured counterparts in the GenBank databases. It is interesting to observe that phylum of *Bacteroidetes*, *Proteobacteria* and *Armatimonadetes* were the most abundant group of bacteria observed in the SCB biofilm with 7.9% (26 clones), 7.3% (24 clones) and 1.2% (4 clones) occurrences, respectively. In addition, *Bacteroidetes* could also contributes significantly to lignocelluloses constituents, including cellulose, hemicelluloses and pectin in the soil and some of the order *Sphingobacteriales* have been reported as cellulolytic taxa (Rattanachomsri *et al.*, 2011; Coughlan and Mayer, 1992). A phylogenetic tree was constructed based on the nearly complete 16S rDNA sequences of predominant clones (Figure 4.21).

The detection of *Rhizobium* in the test reactor is interesting. Being a known nodules formed nitrogen–fixing bacteria of the legume, the bacteria were able to hydrolyze plant materials (Shetta *et al.*, 2011). Besides, several studies reported the availability of using microbial exopolysaccharides produced by *Rhizobium* and *Chryseomonas* in metal biosorption (Mamaril *et al.*, 1997; Ozdemir *et al.*, 2005). The metal–microbe interaction in natural environment is complex and is influenced by pH or organic matter content. The ability to grow at concentration of metals is however, found in many plant growth–promoting *Rhizobacteria* including symbiotic nitrogen–fixing bacteria (Lakzian *et al.*, 2002) and may be the result of intrinsic or induced mechanism (Giller *et al.*, 1998). There are reports that have shown the high level tolerance to heavy metals by *Rhizobia* (Wani and Khan, 2012). While in the *Armatimonadetes* group, the presence of Candidate division also observed in the test reactor. Candidate division OP10 was first reported predominance community in Horestooth Reservoir, Colorado, a metal–rich lake that becomes fully stratified in late summer (Stein *et al.*, 2002). Metal oxides from the underlying sandstone are reduced by anaerobic respiration in the anoxic hypolimnion and reprecipitate as metal oxides when they diffuse into oxic surface waters. Candidate division OP10 comprised 50% of the bacteria detected in a 16S rRNA gene library constructed from these metal precipitates. This sheer predominance makes certain speculation

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reasonable: perhaps these bacteria have a role in redox metal cycling, either as lithotrophs, anaerobic metal respirers or anoxygenic phototrophs. OP10 strains have been detected in other metal–rich sites as well, particularly mine tailings (Dunfield *et al.*, 2012). The presence of these bacteria (*R. cellulosyliticum* and Candidate division OP10) in the test reactor consisting treated SCB–immobilized bacteria may enhance the performance of the reactor during Cr(VI) treatment.

Besides the well–known cultivated bacteria, the analysis has revealed a rather large community portion of uncultured bacteria in the SCB biofilm. It was observed that almost 7% of the library is constituted by still–unknown or yet–uncultivated bacterial members. These clones are mainly associated with uncultured bacterial clones from environment samples of simulated low level radioactive waste site, including cellulosic waste in the form of paper towels, cardboard boxes or wood contaminated with heavy metals and radionuclides such as chromium and uranium (Field *et al.*, 2010) and spacecraft assembly clean room (La Duc *et al.*, 2009).

The predominating by *Bacteroidetes* and *Proteobacteria* in the treatment reactors are in good agreement with Rattanachomsri *et al.* (2011). The research group conducted culture–independent phylogenetic analysis of the microbial community in industrial sugarcane bagasse feedstock piles and revealed that the microbial community was composed of nine major bacterial lineages dominated by *Firmicutes*, *Acidobacteria*, *Proteobacteria* and *Bacteroidetes* with > 85% abundance. Other minor phyla include *Actinobacteria*, *Planctomycetes*, *Sphirochaetes*, *Deinococcus–Thermus* and Candidate division TM7 (Rattanachomsri *et al.*, 2011). *Actinobacteria* was reported to play important roles, especially in the degradation of macromolecules in waste material, such as hemicelluloses, lignin and cellulose, at the later stages of composting (Liew *et al.*, 2009; Steger *et al.*, 2007).

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Table 4.9: Taxonomic affiliations and abundance of 16S rRNA sequence types as defined by RFLP analysis

Phylogenetic group	Number of clones (% abundance)	Representative sequence	Accession no.	% Identity	Description of closest relative in GenBank database (name, accession number)	Source, Country
Bacteroidetes	4 (1.2)	T5–27	KC295709	94	*Pedobacter* sp. oral clone AV100, AF385531	Advanced noma lesions, USA
	12 (3.6)	C5–311	KC295704	99	*Chitinophaga terrae* strain KP01, NR041540	Soil, South Korea
	10 (3.0)	C5–634	KC295705	99		
Proteobacteria	4 (1.2)	C5–4	KC295702	94	*Laribacter hongkongensis* strain HKU1, NR025167	Blood culture, China
	7 (2.1)	C5–75	KC295703	94		
	5 (1.5)	C5–642	KC295706	97	*Ottowia thiooxydan* strain K11, NR029001	Activated sludge, Germany
	4 (1.2)	T5–2	KC295707	97		
	4 (1.2)	T5–80	KC295712	93	*Rhizobium cellulosilyticum* strain ALA10B2, NR043985	Sawdust of *Populus alba*, Spain
Armatimonadetes	4 (1.2)	T5–67	KC295711	96	Candidate division OP10 bacterium Gsoil 348, GQ339893	Ginseng field soil, Korea
Uncultured	9 (2.7)	T5–55	KC295710	96	Uncultured bacterium clone WW3_39, GQ264495	Simulated low level radioactive waste site, USA
	4 (1.2)	T5–127	KC295713	95	Uncultured bacterium clone GI5–005–F01, FJ192729	Spacecraft assembly clean room, USA
	6 (1.8)	T5–7	KC295708	94	Uncultured bacterium clone WW2_146, GQ264320	Simulated low level radioactive waste site, USA
	4 (1.2)	T5–170	KC295714	94		

* C5 = Control samples, T5 = Test samples

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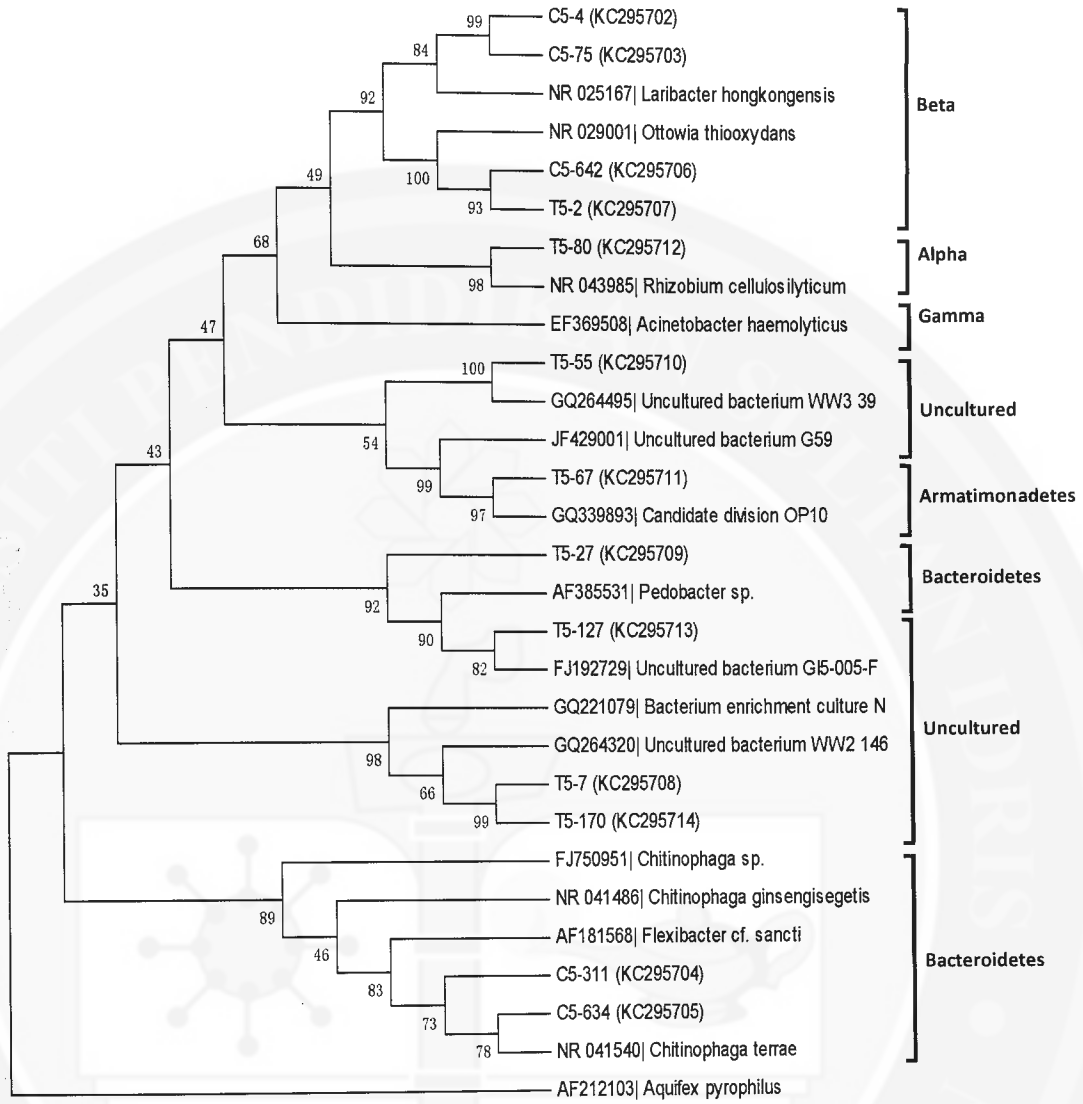


Figure 4.21: Phylogram shows phylogenetic relationships of selected 13 dominant patterns

Study conducted by Tekerlekopoulou *et al.* (2010) reported the effect of the carbon source (sodium acetate and sucrose) on microbial community structure in batch cultures derived from industrial sludge and Cr(VI) reduction. Results showed that the dominant communities were bacterial species *Acinetobacter iwoffii*, *Defluvibacter lusatiensis*, *Pseudoxanthomonas japonensis*, *Mesorhizium chacoense* and *Flavobacterium suncheonense* when sodium acetate was used as carbon source. While fungal strains such as *Trichoderma viride* and *Pichia jadinii* were dominated when sodium acetate was replaced by sucrose. A change of carbon source completely switched the structure of the microbial community from bacteria–dominated to fungus–dominated. These results indicate that the carbon

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source is a key parameter for microbial dynamics and enhanced Cr reduction and should be taken into account for efficient bioreactor design.

4.5 Cr(VI) reduction mechanism study

4.5.1 Oxidation state determination using X–Ray Photoelectron Spectroscopy

The oxidation state of Cr bound on the Cr–loaded SCB and Cr–loaded SCB–adapted *A. haemolyticus* were performed by X–Ray Photoelectron Spectroscope (XPS) and Electron Spin Resonance (ESR). In this study, XPS was employed to ascertain the oxidation state of the Cr bound on the surface of SCB. For example, if only Cr(III) was present, it can be concluded that Cr(VI) was completely reduced to Cr(III) by the SCB. However, if both trivalent and hexavalent forms of Cr exist on the surface of SCB, it can be concluded that both Cr(VI) adsorption and Cr(VI) reduction contributed to the removal of Cr(VI) from aqueous solution. Therefore, XPS was employed to verify the oxidation state of the Cr bound to the SCB.

While the EDX results in section 4.5.4 offer a qualitative estimation of the elemental changes after Cr(III) binding, no such information is provided for Cr(VI) binding. XPS offers a more quantitative information after Cr(VI) binding and acts as a support for the results previously obtained by FT–IR analysis as discussed in section 4.5.3. It also identifies the functionalities involved in binding as well as facilitating elucidation of Cr binding mechanisms to the SCB–adapted *A. haemolyticus*.

Low resolution survey of XPS spectra shows small Cr peak was detected on the Cr–loaded SCB and Cr–loaded SCB–adapted *A. haemolyticus* at the binding energy range of 0–1200 eV used (Figure 4.22). The elemental composition of the Cr–loaded SCB and Cr–loaded SCB–adapted *A. haemolyticus* was found in survey spectra are summarised in Table 4.10 with only the elements

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of interest in binding shown. From Table 4.10, it is clearly seen that the SCB had relatively large amounts of Cr associated with their surface after exposure. Results indicated that SCB–adapted *A. haemolyticus* had comparable amounts of Cr bound to their surfaces for initial Cr(VI) concentration used, 100 mg/L (1.82±0.49%) while a lower quantity was bound to Cr(VI)–loaded SCB (0.51±0.37%). Similar amounts of Cr bound to the seaweeds surface (0.74–1.14%) were reported by Murphy *et al.* (2009). A decreased in the carbon content of all samples from 71.47–73.67% to 64.59–68.56% were noted after Cr–loading thus indicating some biomass leaching during the experiment, while an increased in oxygen atomic concentration was observed from 25.52–27.78% to 30.67–33.60%. This increased concentration may potentially be due to the adsorption of chromium hydroxides during the metal–binding process (Murphy *et al.*, 2009). Both study conducted by Raize *et al.* (2004) and Yang and Chen (2008) found that, for *Sargassum* sp., higher levels of oxygen (38.40–42.03%) were present which may be due to increase quantities of carboxyl functionalities in this species. A lower carbon atomic concentration (47.44–47.60%) was also observed.

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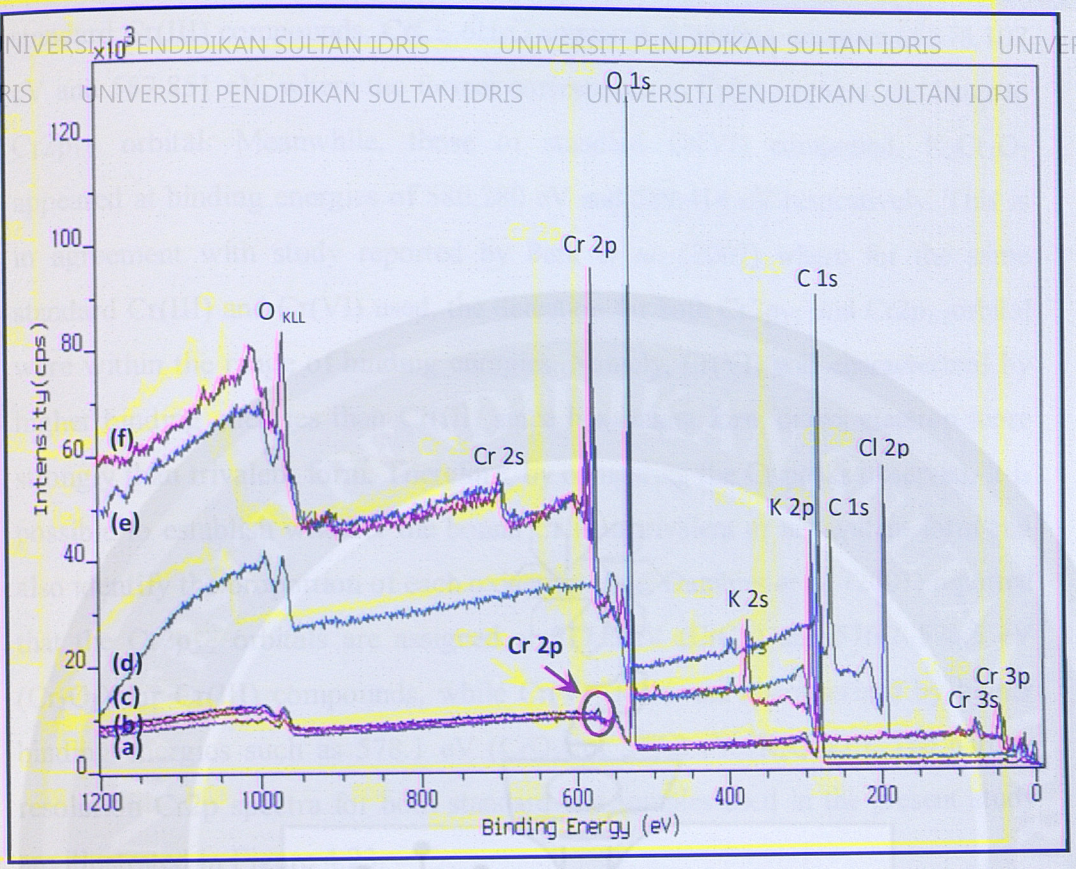


Figure 4.22: Wide scan of XPS spectra for (a) SCB adapted *A. haemolyticus*, (b) SCB in the present of 100 mg/L Cr(VI), (c) SCB adapted *A. haemolyticus* in the present of 100 mg/L Cr(VI), (d) SCB, (e) standard Cr(III) and (f) standard Cr(VI)

Table 4.10: Elemental composition of raw and Cr–loaded SCB as determined using XPS

Elements	Atomic concentration (%)			
	Raw		100 mg/L Cr(VI)–loaded SCB	
	SCB	SCB + Ah	SCB + Cr(VI)	SCB + Ah + Cr(VI)
C 1s	71.47 ± 4.82	73.67 ± 3.83	68.56 ± 8.33	64.59 ± 4.67
O 1s	27.78 ± 3.74	25.52 ± 2.68	30.67 ± 7.57	33.60 ± 4.19
Cr 2p	-	-	0.51 ± 0.37	1.82 ± 0.49
K 2p	-	-	-	-
Cl 2p	-	-	-	-

* SCB (Sugarcane bagasse), SCB + Ah (*A. haemolyticus* cells grown on SCB), SCB + Cr(VI) (Sugarcane bagasse with presence of 100 mg/L Cr(VI) and SCB + Ah + Cr(VI) (*A. haemolyticus* cells grown in sugarcane bagasse with the presence of 100 mg/L Cr(VI)

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Narrow resolution survey of XPS spectra show that significant bands of standard Cr(III) compounds, $CrCl_3.6H_2O$ appeared at binding energies of 578.107 eV and 587.851 eV, where the former corresponds to $Cr2p_{3/2}$ orbital, the latter to $Cr2p_{1/2}$ orbital. Meanwhile, those of standard Cr(VI) compound, $K_2Cr_2O_7$ appeared at binding energies of 580.280 eV and 589.414 eV respectively. This is in agreement with study reported by Park *et al.* (2007) where for the same standard Cr(III) and Cr(VI) used, the detection for both $Cr2p_{3/2}$ and $Cr2p_{1/2}$orbital were within the range of binding energies. Namely, Cr(VI) was characterized by higher binding energies than Cr(III) since hexavalent form draws electron more strongly than trivalent form. Therefore, by comparing the Cr peaks observed, it is possible to establish whether the bound Cr is in trivalent or hexavalent form and also identify the proportion of each oxidation state. Dambies *et al.* (2001) reported that the $Cr2p_{3/2}$ orbitals are assigned at 577.2 eV ($CrCl_3$) and 576.2–576.5 eV (Cr_2O_3) for Cr(III) compounds, while Cr(VI) forms are characterized by higher binding energies such as 578.1 eV (CrO_3) or 579.2 eV ($K_2Cr_2O_7$). The narrow resolution Cr2p spectra for both standard and samples used in the present study are illustrated in Figure 4.23.

As shown in Figure 4.23b, narrow resolution of $Cr2p_{3/2}$ and $Cr2p_{1/2}$ XPS spectra for Cr–loaded SCB and Cr–loaded SCB adapted *A. haemolyticus* were well matched with that of standard Cr(III). This result implies that the Cr bound to the surface of SCB were mostly in trivalent form. Quantitative curve fitting was applied to the XPS spectrum (Figure 4.24) and the results reveal that SCB after adsorption of Cr(VI) ions shows $Cr2p_{3/2}$ peaks appear at 574.960 eV and 577.496 eV while the $Cr2p_{1/2}$ peaks appear at 583.514 eV and 587.094 eV with about 49.65% of the Cr species on the surface is in the Cr(III) state, while about 16.00% is in the Cr(VI) state. For SCB–adapted *A. haemolyticus*, $Cr2p_{3/2}$ peaks appear at 574.257 eV and 575.706 eV while the $Cr2p_{1/2}$ peaks appear at 584.224 eV and 585.577 eV with about 46.30% of the Cr species on the surface is in the Cr(III) state, while about 22.75% is in the Cr(VI) state (Table 4.11).

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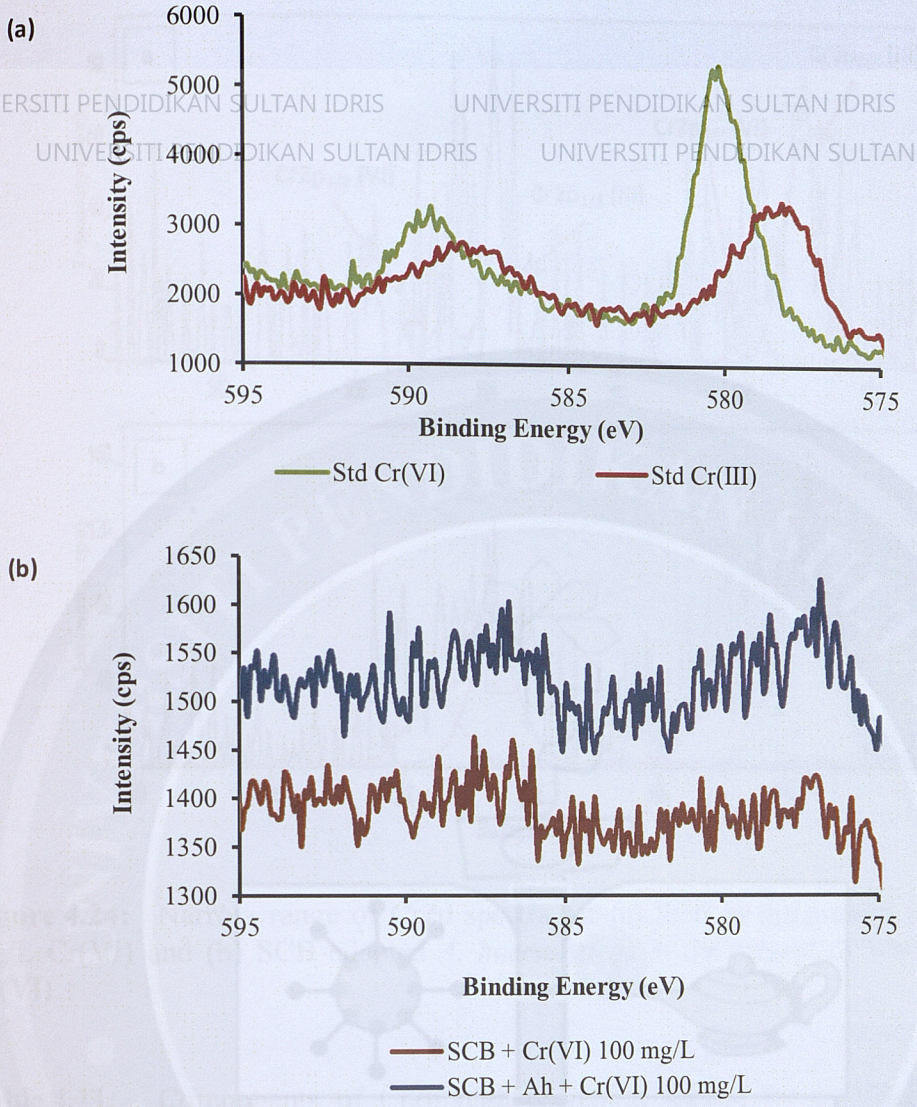


Figure 4.23: Narrow–resolution spectra collected from the Cr2p core region; (a) standard Cr(VI) and Cr(III) used and (b) Cr–loaded SCB and Cr–loaded SCB adapted *A. haemolyticus*

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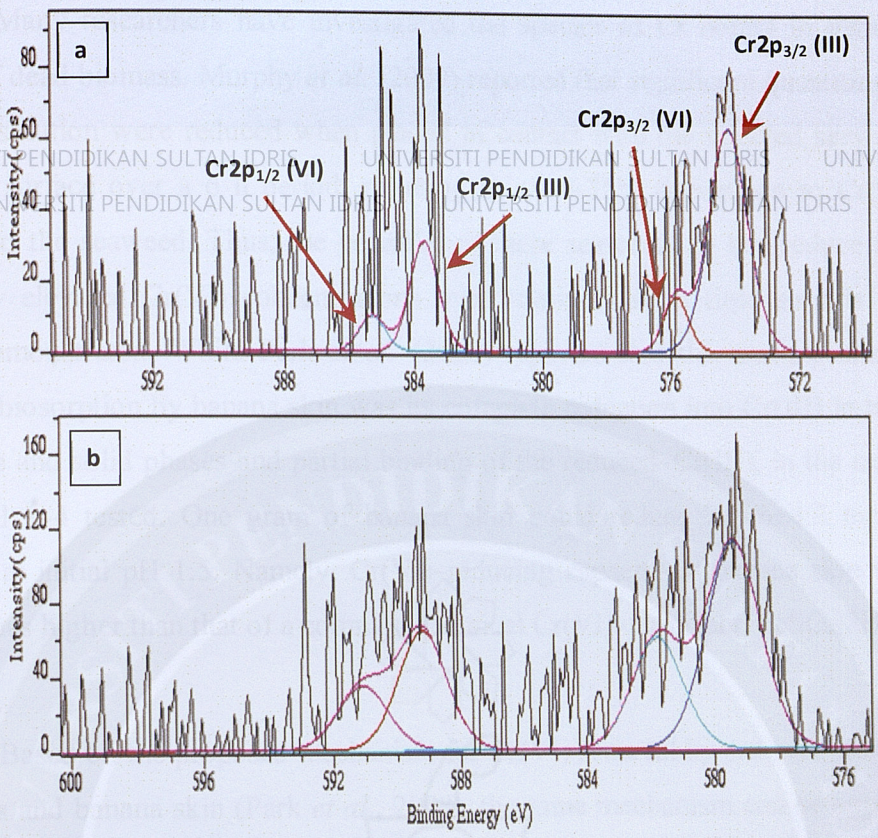


Figure 4.24: Narrow range of Cr2p spectra for (a) SCB in the present of 100 mg/L Cr(VI) and (b) SCB adapted *A. haemolyticus* in the present of 100 mg/L Cr(VI)

Table 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)

Samples	Peak	Energy position (eV)	Relative proportion (%)	Cr(III) / Cr(VI) ratio
SCB + Cr(VI)	$Cr2p_{1/2}$ (III)	583.514	49.65	3.10
	$Cr2p_{3/2}$ (III)	574.960		
	$Cr2p_{1/2}$ (VI)	587.094	16.00	
	$Cr2p_{3/2}$ (VI)	577.496		
SCB + Ah + Cr(VI)	$Cr2p_{1/2}$ (III)	584.224	46.30	2.04
	$Cr2p_{3/2}$ (III)	574.257		
	$Cr2p_{1/2}$ (VI)	585.577	22.75	
	$Cr2p_{3/2}$ (VI)	575.706		

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Many researchers have investigated the species of Cr bound in various types of dead biomass. Murphy *et al.* (2009) reported that significant quantities of Cr(VI) solution were reduced when placed in contact with the selected seaweed species surface over a 6 h period, resulting in a 64–75% conversion to Cr(III) bound to the seaweed. Thus, the potential of these seaweeds to bio–reduce and detoxify elevated Cr(VI) concentrations over relatively short time periods has been demonstrated. While Park *et al.* (2008) reported that the mechanisms of Cr(VI) biosorption by banana skin was its complete reduction into Cr(III) in both aqueous and solid phases and partial binding of the reduced–Cr(III), in the range of pH 1.5–4 tested. One gram of banana skin could reduce 249.6±4.2 mg of Cr(VI) at initial pH 1.5. Namely, Cr(VI)–reducing capacity of banana skin was four times higher than that of a common chemical Cr(VI)–reductant, $FeSO_4.7H_2O$.

Based on the proposed mechanism for Cr(VI) removal by seaweed, fungal biomass and banana skin (Park *et al.*, 2008), the same mechanism can be applied to this study. Cr(VI) can be removed from the aqueous solution by non–living biomass via two mechanisms. The first mechanism involves direct reduction of Cr(VI) to Cr (III) in the aqueous phase while the second mechanism involves indirect reduction. Amino and carboxyl groups may take part in the binding of anionic Cr(VI) of indirect reduction mechanism. As the pH of the aqueous phase decreased, a large number of hydrogen ions can easily coordinate with the amino and carboxyl groups present on the SCB surface. Thus, a low pH makes the SCB surface more positive. The more positive the surface charge of the SCB, the faster the rate of Cr(VI) removal from the aqueous phase, since the binding of anionic Cr(VI) ion species with the positively–charged groups is enhanced. A low pH also accelerates the redox reactions in both direct and indirect mechanisms, since the protons take part in this reaction. Meanwhile, if there are a small number of electron–donor groups in the SCB or protons in the aqueous phase, the Cr bound onto the SCB surface may remain in the hexavalent state. Therefore, a portion of direct and indirect mechanisms depend on the adsorption system (solution pH, temperature and species on the SCB), as well as the SCB dosage and Cr(VI) concentrations.

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While the SCB and SCB adapted *A. haemolyticus* were exposed to Cr(VI) solution, there is evidence of both Cr(III) and Cr(VI) on the SCB surface (Figure 4.24). This indicated that both Cr(VI) adsorption and Cr(VI) reduction are involved in binding to these SCB (indirect mechanism). An estimation of the Cr(III)/Cr(VI) ratio reveals that, in the case of Cr–loaded SCB, the proportion of Cr(III) on the SCB surface is more than triple that of Cr(VI) (3.10) while for Cr–loaded SCB–adapted *A. haemolyticus*, this value is more than double (2.04) (Table 4.11).

Even though various studies have shown the effectiveness of the biomaterials in reducing toxic Cr(VI) to less toxic Cr(III) (Dupont and Guillon, 2003; Deng and Ting, 2005; Suksabye *et al.*, 2007; Sawalha *et al.*, 2007), but to date, no work on the reduction of Cr(VI) by SCB adapted any bacteria has been found in the literature. Dupont and Guillon (2003) studied the removal mechanism of Cr(VI) by a lignocellulosic substrate extracted from wheat bran where results showed that the adsorption reaction consumed a large amount of protons, which is consistent with the reduction of Cr(VI) to Cr(III); the oxidation of lignin moieties occurred concurrently to the Cr(VI) reduction and led to the formation of hydroxyl and carboxyl functions. XPS results of Deng and Ting (2005) indicated that redox reaction occurred on the surface of polyethylenimine–modified fungal biomaterial, and whether the converted Cr(III) ions were released to solution or adsorbed on the biomaterial depended on the solution pH. Desorption study of Cr(VI) on coir pith and XANES analysis suggested that most of the Cr bound on the coir pith was in Cr(III) form and the reduced Cr(III) might be bound with C=O groups and O–CH_3 groups of the coir pith (Suksabye *et al.*, 2007). Sawalha *et al.* (2007) also observed that some of the Cr(VI) bound to saltbush biomass (*Atriplexcanescens*) was reduced to Cr(III). These studies reach the same conclusion, that is, the Cr bound to the natural biomaterials was mostly or totally in Cr(III) form. In contrast to the results obtained in this study, where both Cr(VI) and Cr(III) were detected with the adsorbed Cr predominantly existed in trivalent form. As previously discussed, because both Cr(VI) and Cr(III) were present on the SCB surface, this indicated that complete reduction had not occurred within the contact time of 48 h.

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4.5.2 Oxidation state determination using Electron Spin Resonance

The oxidation state of Cr adsorbed on SCB and in the aqueous phase after Cr(VI) reduction was measured by ESR. ESR is a physical method for observing resonance adsorption of microwave power by unpaired electron spins states in a magnetic field. Cr(III), Cr(IV) and Cr(V), containing unpaired electrons are ESR positive, while Cr(VI) with no unpaired electron is ESR negative, which is diamagnetic. Figure 4.25 shows the ESR spectra of referred standard Cr(III) and Cr(VI) in solid and aqueous phase.

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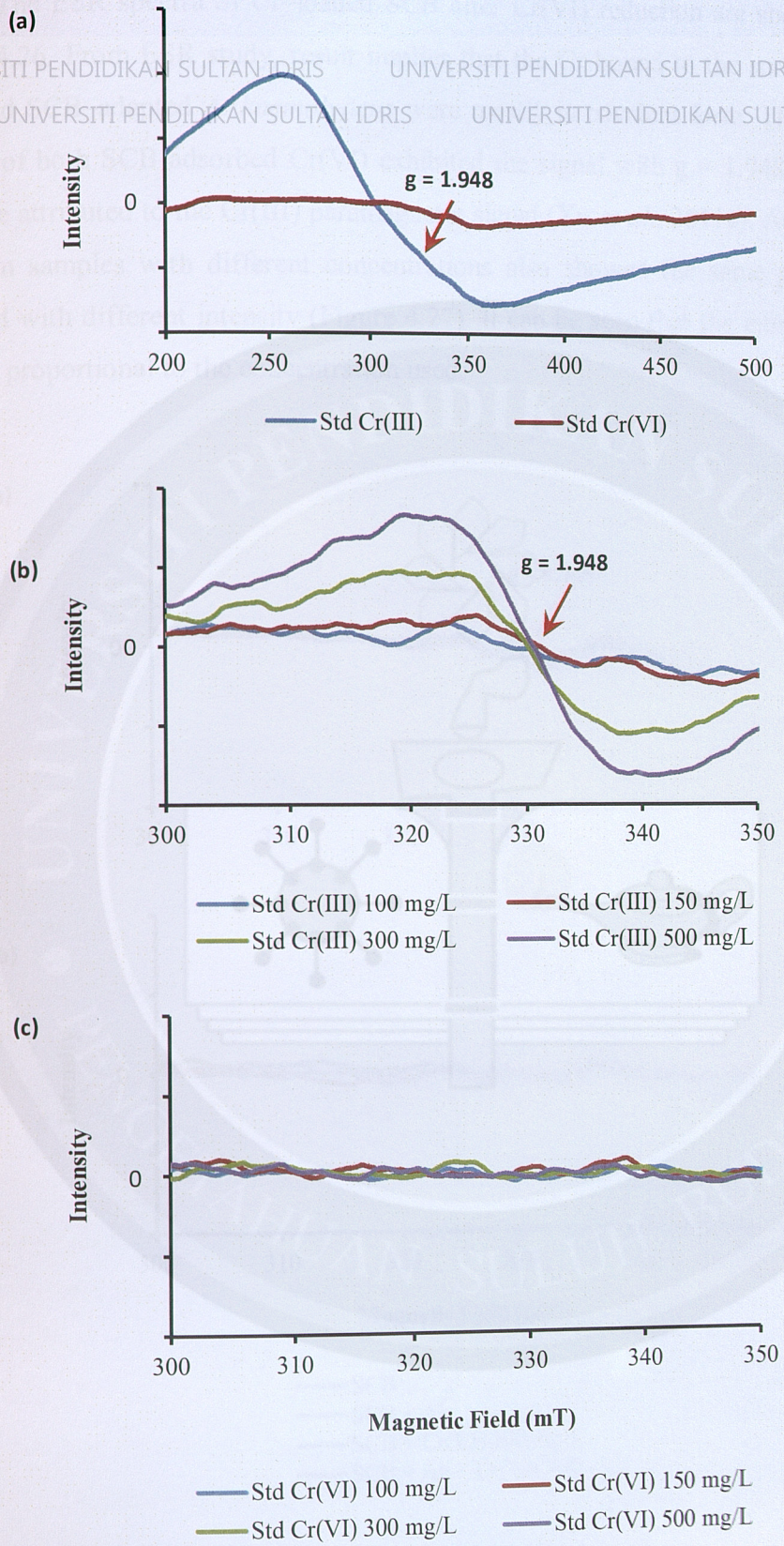


Figure 4.25: ESR spectra of standard Cr(III) and Cr(VI) at wide range for (a) solid and narrow range for (b) aqueous Cr(III) and (c) aqueous Cr(VI) at concentration of 100, 150, 300 and 500 mg/L

The ESR spectra of Cr–loaded SCB after Cr(VI) reduction are shown in Figure 4.26. From ESR study, result implies that the Cr bound to the surface of SCB and SCB–adapted *A. haemolyticus* were mostly in trivalent form. The ESR spectra of both SCB adsorbed Cr(VI) exhibited the signal with g = 1.948 which could be attributed to the Cr(III) paramagnetic signal (Xu *et al.*, 2011a). Attempts made on samples with different concentrations also showed the same g value obtained with different intensity (Figure 4.27). It can be seen that the intensity is directly proportional to the concentration used.

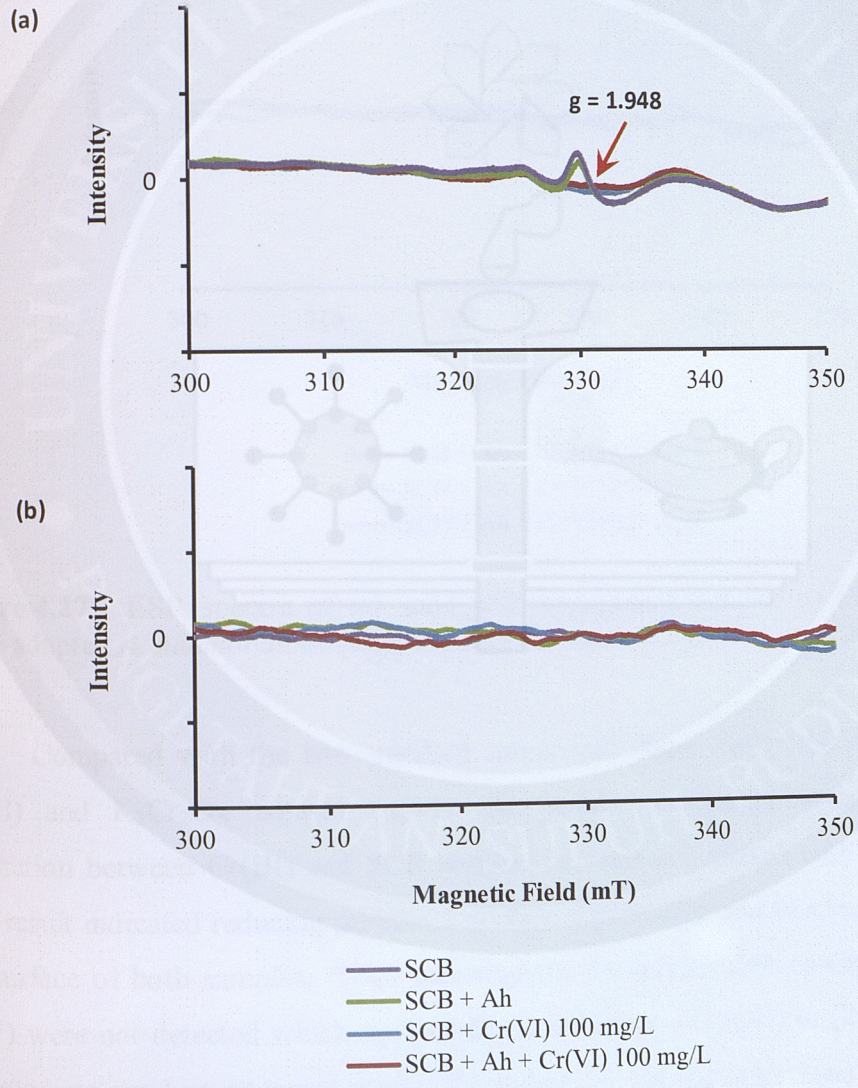


Figure 4.26: ESR spectra of (a) solid and (b) liquid samples

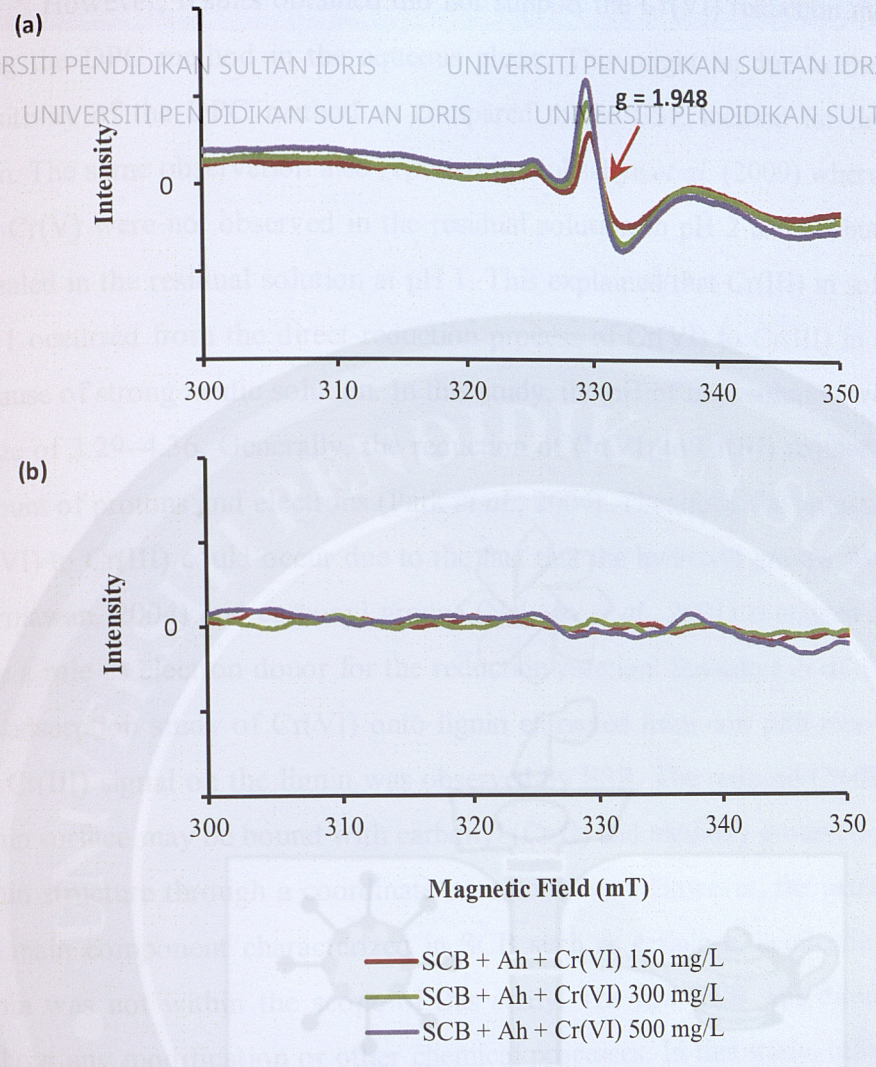


Figure 4.27: ESR spectra of (a) solid and (b) aqueous samples of Cr–loaded SCB–adapted *A. haemolyticus* at Cr(VI) concentration of 150, 300 and 500 mg/L

Compared with the two standard compounds name $CrCl_3 \cdot 6H_2O$, control Cr(III) and $K_2Cr_2O_7$, control Cr(VI), this condition strongly indicates the interaction between Cr(III) and SCB surfaces as well as bacterial cells surface. This result indicated reducing process, Cr(VI) to Cr(III) exist and was bound with the surface of both samples. Other paramagnetic Cr species such as Cr(IV) and Cr(V) were not detected which showed direct reduction of Cr(VI) to Cr(III). No intermediate product occurred during the reduction. On the other hand, Cr(III), Cr(IV) and Cr(V) were not observed in the residual solution based on the planar profile which were due to the absence of paramagnetic signal for Cr(VI).

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However, results obtained did not support the Cr(VI) reduction measured using the DPC method in the aqueous phase. This might be due to the higher sensitivity of the DPC method as compared to the ESR method for the liquid form. The same observation also reported by Suksabye *et al.* (2009) where Cr(III) and Cr(V) were not observed in the residual solution in pH 2 and 10 but Cr(III) revealed in the residual solution at pH 1. This explained that Cr(III) in solution at pH 1 occurred from the direct reduction process of Cr(VI) to Cr(III) in solution because of strong acidic solution. In this study, the pH of final solution was in the range of 3.29–4.36. Generally, the reduction of Cr(VI) to Cr(III) requires a large amount of protons and electrons (Park *et al.*, 2004). Therefore, the reduction from Cr(VI) to Cr(III) could occur due to the fact that the hydroxyl groups (Babel and Kurniawan, 2004) and carbonyl groups (Nakano *et al.*, 2001) contained in lignin play a role as electron donor for the reduction reaction. Suksabye *et al.* (2009) in the adsorption study of Cr(VI) onto lignin extracted from coir pith reported that the Cr(III) signal on the lignin was observed by ESR. The reduced Cr(III) on the lignin surface may be bound with carbonyl (C=O) and methoxy group (O–CH_3) in lignin structure through a coordinate covalent bound. However, the extraction of the main component characterized in SCB such as cellulose, hemicellulose and lignin was not within the scope of this study. The raw SCB was directly used without any modification or other chemical processes. In this study, effect of pH was not carried out because the preferential initial pH for *A. haemolyticus* growth was reported in the range of 6.5–7.5 (Sharma *et al.*, 2009). If the acidic pH condition was prepared as reported by other researchers, the growth of the *A. haemolyticus* is retarded.

4.5.3 Functional group determination using Fourier Transform–Infra Red

The FT–IR spectra of *A. haemolyticus* grown onto SCB with and without Cr(VI) is shown in Table 4.12. The analysis was carried out to assess the main functional group responsible for the binding of Cr(VI) anions. The spectrum of the SCB displays a number of absorption peaks, reflecting the complex nature of

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the studied SCB. The polysaccharides found in SCB are biopolymers having many hydroxyl and/or phenolic groups (Navarro *et al.*, 1996).

A. haemolyticus cells grown on SCB shows the broad absorption peak around 3400 cm^{-1} which is indicative of the existence of the stretching of O–H groups which refers to the hydroxyl group in glucose (Liu *et al.*, 2006; Sun *et al.*, 2004; Garg *et al.*, 2007). The absorption peaks in the 2924 cm^{-1} can also be attributed to C–H stretching vibration from carboxylic acid. The band at 1634 cm^{-1} corresponds to the carbonyl stretching of aromatic ring that may be attributed to the lignin aromatic groups (Sun *et al.*, 2004; Garg *et al.*, 2007). The absorption peaks at 1605 cm^{-1} and 1516 cm^{-1} is due to C=C stretching of aromatic ring of lignin (Viera *et al.*, 2007). The absorption of 1376 cm^{-1} is due to the C–H deformation while the band at 1164 cm^{-1} relates to C–O antisymmetric bridge stretching, which is one of the fingerprint region for polysaccharides. The C–O–C pyranose ring skeletal vibration occurs in the region 1076–1023 cm^{-1}. The peak at 898 cm^{-1} originated from glycosidic C_1–H deformation with ring vibration contribution and O–H bending, which is characteristic of β–glycosidic linkage between glucose in cellulose.

Upon contacting with 100 mg/L of Cr(VI), band shifts were observed from 3400 cm^{-1} to 3422 cm^{-1} indicative of –OH stretching, probably due from the oxidation of sugar moieties present in SCB during the Cr(VI) reduction process. Apart from this, the C–O absorption peaks were also shifted from 1252 cm^{-1} to 1261 cm^{-1} and 1048 cm^{-1} to 1037 cm^{-1}. A similar observation was reported by Garg *et al.* (2007) reporting on the involvement of carboxyl groups from sugarcane bagasse for Cr(VI) reduction.

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Table 4.12: FT–IR analysis of *A. haemolyticus* grown onto SCB with and without Cr(VI) after 48 h contact time

Frequency (cm^{-1})						Band Assignments
SCB	SCB + *A. haemolyticus*	SCB + Cr(VI)	SCB + *A. haemolyticus* + Cr(VI)	Viera *et al.* (2007)	Liu *et al.* (2006)	
3410	3400	3400	3422	3426	3420	O–H stretching.
2920	2924	2921	2925	2922	2926	C–H stretching of CH_2 and CH_3 groups
1735	1732	1735	1734	1730	-	C=O stretching of acetyl or carboxylic acid
1635	1634	1634	1631	1634	1631	Carbonyl stretching of aromatic ring
1606	1605	1605	1605	1620, 1595	-	C=C stretching of aromatic ring (lignin)
1515	1516	1515	1515	1512	-	C=C stretching of aromatic ring (lignin)
1425	1425	1425	1426	1429	-	CH_2 bending
1377	1376	1377	1373	1376	1382	C–H deformation
1327	1328	1330	1334	1335	-	O–H in plane bending
1250	1252	1250	1261	1250	-	C–O stretching of ether linkage
1161	1164	1161	1161	1166	1161	C–O–C antisymmetric bridge stretching
1047	1048	1047	1037	1062	1046	C–O symmetric stretching of primary alcohol
899	898	898	899	904	896	β–Glycosidic linkages between glucose and cellulose

Notes: SCB (Sugarcane bagasse), SCB + *A. haemolyticus* (*A. haemolyticus* cells grown on SCB), SCB + Cr(VI) (Sugarcane bagasse with presence of 100 mg/L Cr(VI) and SCB + *A. haemolyticus* + Cr(VI) (*A. haemolyticus* cells grown in sugarcane bagasse with the presence of 100 mg/L Cr(VI)

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4.5.4 Morphology determination using Scanning Electron Microscopy coupled with Energy Dispersive X–Ray

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In the current study, SEM analysis was carried out to observe any morphological changes to *A. haemolyticus* upon exposure to Cr(VI) and its attachment to SCB while EDX was used to estimate the elemental composition of both *A. haemolyticus* and SCB as well as to determine the presence of Cr element. The bacterial attachment on the surface of raw SCB can be seen clearly from Figure 4.28. The nature structure of SCB with high content of cellulose fibres and pores make bacteria easier to attach.

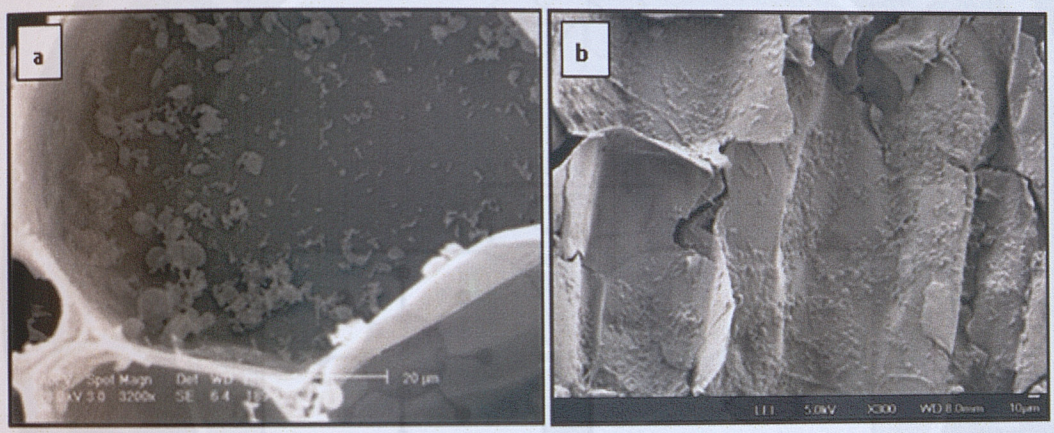


Figure 4.28: Bacterial attachments (a) in the pore and (b) at the surface of SCB

A. haemolyticus is a microaerophilic rod at log phase and will change to cocci at stationary phase (Brenner *et. al.*, 2005). The cocci shape was observed when *A. haemolyticus* cells grown on SCB (Figure 4.29b). The cells could be in the form of cocci shape as the cells were harvested during stationary phase with rough surface and cells diameter ranging from 0.648–0.782 µm. However, bacterial cells in contact with 100 mg/L Cr(VI) clearly showed shorter diameter (0.156–0.240 µm) with some surface having wrinkled appearance (Figure 4.29d) due to the environmental stress from Cr(VI). The effect of Cr(VI) on inducing wrinkle appearance on the bacterial cells surface was also reported for *Arthrobacter K–2* (Lin *et al.*, 2006) while its effect on reducing the size of bacteria was reported for *Streptomyces* sp. *MC1* (Polti *et al.*, 2009) and *B. cereus SJ1* (He *et al.*, 2010).

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The EDX peaks observed in this study for *A. haemolyticus* grown onto SCB with and without Cr(VI) are also shown in Figures 4.29b and 4.29d. Similar results were obtained for SCB with and without Cr(VI) (Figures 4.29a and 4.29c). The EDX profiles showed higher intensity ratios between C:O that is in the range of 49.80–65.63:30.36–47.69 weight percent due to the higher carbon content in SCB (Rodrigues *et al.*, 2010; Lee *et al.*, 2009). No significant differences was observed between Cr content in Cr(VI)–loaded SCB and Cr(VI)–loaded SCB–adapted *A. haemolyticus*. The results show the detection of 5.44–7.14% (Figure 4.29c) and 5.88–6.81% (Figure 4.29d) respectively. This suggest the possible direct contribution from SCB on Cr(VI) reduction (abiotic) besides contribution from attached *A. haemolyticus* on Cr(VI) reduction capabilities. Zakaria *et al.* (2007a) reported the presence of fine electron–dense particles which is indicative of Cr precipitation for *A. haemolyticus* grown in 50 mg/L Cr(VI) by using transmission electron microscope (TEM) analysis. Hsiao Pei *et al.* (2009) also reported the appearance of microprecipitates in the cytoplasmic region of the cells which suggesting the transportation of Cr(VI) into the cells.

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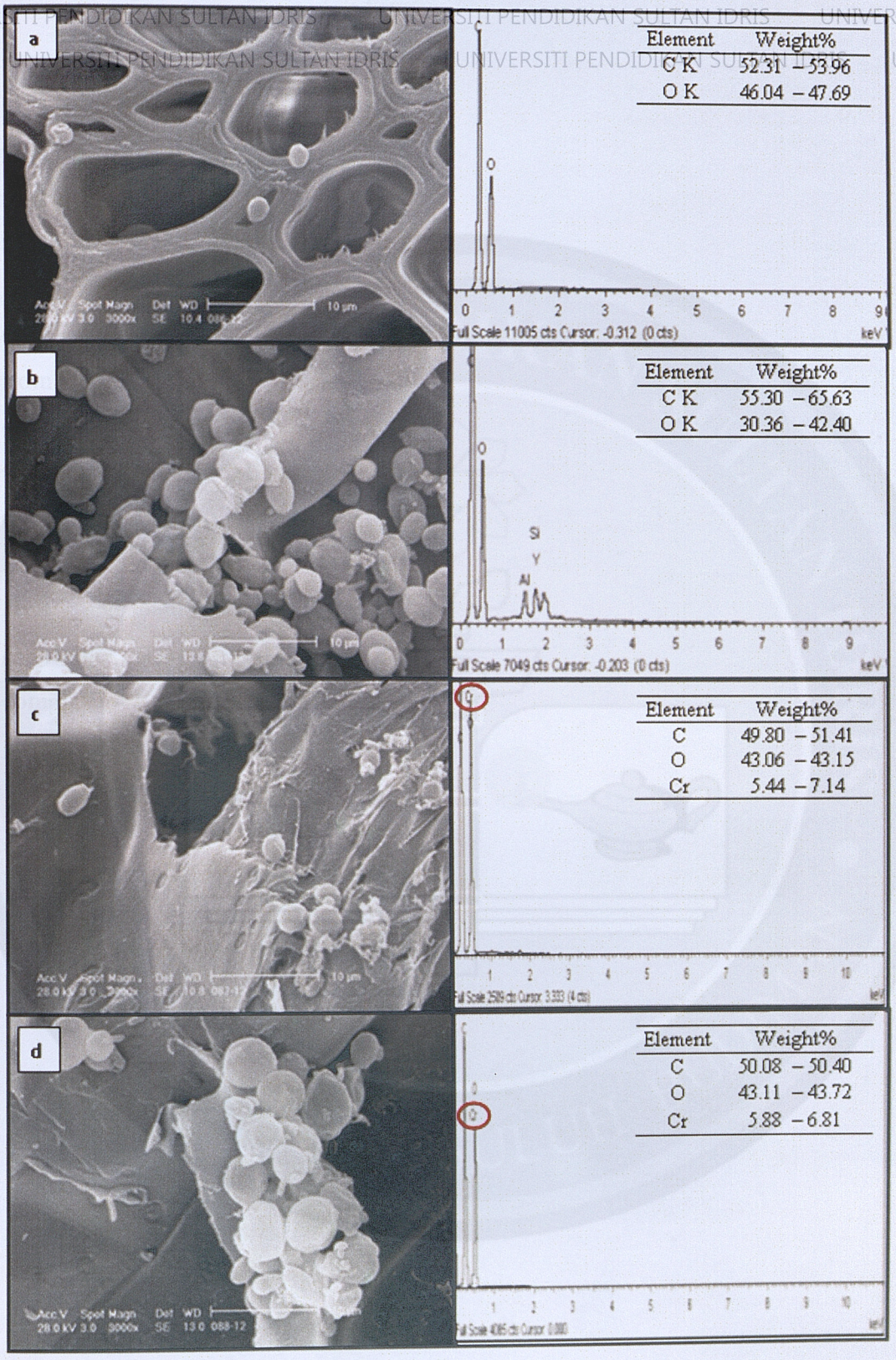


Figure 4.29: SEM micrograph and EDX spectra of (a) raw SCB, (b) *A. haemolyticus* cells grown on SCB, (c) SCB in the presence of 100 mg/L Cr(VI) and (d) *A. haemolyticus* cells grown on SCB in the presence of 100 mg/L Cr(VI) at magnification of 3000X

Therefore, based on X–Ray Photoelectron Spectroscopy (XPS), Electron Spin Resonance (ESR), Fourier Transformed–Infra Red (FT–IR) and Scanning Electron Microscopy coupled with Energy Dispersive X–Ray (SEM–EDX) studies, it is proposed that the mechanism of Cr(VI) binding was adsorption coupled with reduction to Cr(III). Cr(VI) was abiotically removed from an aqueous Cr(VI) system by SCB through both direct and/or indirect reduction mechanisms. Direct reduction mechanism occurred in the aqueous phase (effluent). Cr(VI) was directly reduced to Cr(III) in the aqueous phase by contact with electron–donor groups leaching out from the SCB, and the reduced–Cr(III) remained in the aqueous phase or formed complexes with Cr–binding groups of it. However, indirect reduction mechanism which occurred in the sugarcane bagasse (SCB) consisted of three steps; starting with the binding of anionic Cr(VI) to positively–charged groups present on the surface of SCB, then the reduction of Cr(VI) to Cr(III) takes place by adjacent electron–donor groups and finally, the release of the reduced–Cr(III) into the aqueous phase due to electronic repulsion between the positively–charged groups and the Cr(III) ion, or the complexation of the reduced–Cr(III) with adjacent groups capable of Cr–binding groups (Park *et al.*, 2007).

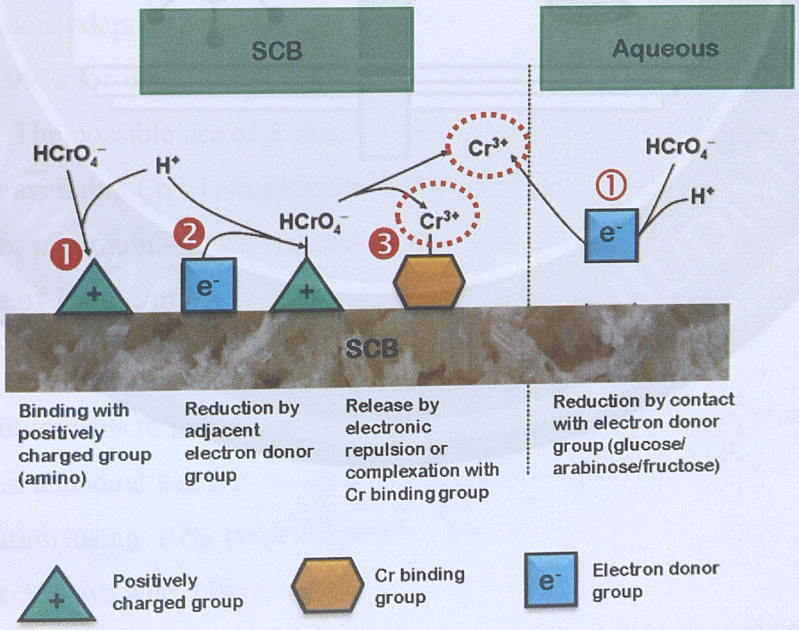


Figure 4.30: Proposed mechanism of Cr(VI) reduction (modified from Park *et al.*, 2007)

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CHAPTER 5

CONCLUSION AND SUGGESTION

5.1 Conclusion

SCB, SPW and SBM have been evaluated as possible growth medium for Cr(VI) removal from aqueous solutions. This study shows that SCB can serve as an alternative and cost–effective growth medium for cultivation of *A. haemolyticus*. Adapted *A. haemolyticus* showed the highest growth in 10% (w/v) SCB with OD_{600} of 1.053 and cell concentration of 6.2 X 10^7 CFU/mL. Also SCB–adapted *A. haemolyticus* is the most feasible for the Cr(VI) reduction process compared to control and non–adapted bacteria after 48 h incubation. The reduction was in the range of 92–99% for initial metal concentration ranging from 10 mg/L to 100 mg/L respectively. The possible use of *A. haemolyticus* immobilized–SCB as alternative to the currently available Cr(VI) treatment was demonstrated. At 25 mg/L initial Cr(VI) concentration, minimum 4 h was required for complete reduction of Cr(VI) to Cr(III) at a flow rate of 3.0 mL/min.

Uncultured bacterium clone WW2_146 and *Ottowia thiooxydan* strain K11 were the most abundant bacteria isolated from the SCB biofilm from the test reactor. Supplementation using 10% (v/v) LPW into unsterile SCB in the reactor system operation for 32 days were effected *A haemolyticus* colonization by other indigenous bacteria. The proposed mechanisms of Cr(VI) reduction involved adsorption coupled with reduction at the SCB and direct reduction in aqueous phase.

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5.2 Suggestion

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In the present microbial community analysis, the results suggested that the bacterial populations of uncultured bacterium clone WW2_146 and *Ottowia thiooxydan* isolated from the test reactor, might have a significant role in the reduction of Cr(VI). Based on the packed–bed reactor performance study, 4–12 h are required to achieve complete reduction of 25 mg/L Cr(VI). However, the capacity of these two bacterial strains for reduction of Cr(VI) still not yet been reported. Further analysis will be carried out to investigate the contribution of reductase capable of reducing Cr(VI) in all bacteria isolated from the reactor study. To the best of our knowledge, this study is the first to examine the microbial diversity of the Cr(VI) reduction by bacteria immobilized–SCB in a reactor treatment system.

As the reactor treatment system was carried out in unsterile conditions, the capability of indigenous bacteria in reducing Cr(VI) to Cr(III) are to be quantified. Besides, the optimal conditions are to be defined in order to improve the ability of specific bacterial strains to play their role under stressful conditions as well as those in polluted environments.

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REFERENCES

Abdulkadir, M. I. (2009). Optimization and application of plant–based waste materials for the remediation of selected trace metals (Cd, Pb and Mn) and oxyhalides (BrO_3^-, ClO_3^- and IO_3^-) in a aqueous system. MSc Thesis. University of South Africa.

Abdullah, M. B. and Mat, H. (2008). Characterisation of solid and liquid pineapple waste. *Reaktor*. 12(1): 48–52.

Acar, F. N. and Malkoc, E. (2004). The removal of chromium (VI) from aqueous solutions by *Fagus orientalis L. Bioresource Technology*. 94: 13–15.

Acharya, J., Sahu, J. N., Sahoo, B. K., Mohanty, C. R. and Meikap, B. C. (2009). Removal of chromium (VI) from wastewater by activated carbon developed from Tamarind wood activated with zinc chloride. *Chemical Engineering Journal*. 150: 25–39.

Agarwal, G. S., Bhuptawat, H. K. and Chaudhari, S. (2006). Biosorption of aqueous chromium (VI) by *Tamarindus indica* seeds. *Bioresource Technology*. 97: 949–956.

Agrawal, A., Pal, C. and Sahu, K. K. (2008). Extractive removal of chromium (VI) from industrial waste solution. *Journal of Hazardous Materials*. 159: 458–464.

Ahalya, N., Kanamadi, R. D. and Ramachandra, T. V. (2008). Biosorption of chromium (VI) by *Tamarindus indica* pod shells. *Journal of Environmental Science Research International*. 1(2): 77–81.

Ahluwalia, S. S. and Goyal, D. (2007). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource Technology*. 98: 2243–2257.

Ahmad Khan, N. and Mohamad, H. (2007). Investigations on the removal of chromium (VI) from wastewater by sugarcane bagasse, *Water & Wastewater Asia*. January / February 2007. 37–41. Kuala Lumpur: University of Malaya.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA
DRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PEN

Ahmad, W. A., Zakaria, Z. A. and Nordin, N. (2009a). Cr(VI) reduction by *Acinetobacter* sp. grown in liquid pineapple waste. In (Eds.) Pandey, A., Laroche, C., Soccol, C. R. and Gussap, R. *New Horizons in Biotechnology*. 212–227. New Delhi: Asiatech Publisher Inc.

Ahmad, W. A., Zakaria, Z. A., Khasim, A. R. and Shaik Ismail, S. M. H. (2010a). Removal of chromium from industrial wastewater using microorganisms. *The 1st IWA Malaysia Young Water Professionals Conference (IWAYP2010)*. 1–4 March 2010, Kuala Lumpur, Malaysia. ISBN 983–9805–86–4.

Ahmad, W. A., Zakaria, Z. A., Khasim, A. R., Alias, M. A. and Shaik Ismail, S. M. H. (2010b). Pilot–scale removal of chromium from industrial wastewater using the ChromeBac™ system. *Bioresource Technology*. 101: 4371–4378.

Ahmad, W. A., Zakaria, Z. A., Razali, F. and Samin, J. Evaluation of the combined Cr(VI) removal capacity of sawdust and sawdust–immobilized *Acinetobacter haemolyticus* supplied with brown sugar. (2009b). *Water, Air and Soil Pollution*. 204: 195–203.

Aiello, C., Ferrer, A. and Ledesma, A. (1996). Effect of alkaline treatments at various temperatures on cellulase and biomass production using submerged sugarcane bagasse fermentation with *Trichoderma reesei* QM 9414. *Bioresource Technology*. 57: 13–18.

Akbal, F. and Camci, S. (2011). Copper, chromium and nickel removal from metal plating wastewater by electrocoagulation. *Desalination*. 269: 214–222.

Almaguer–Busso, G., Velasco–Martinez, G., Carreno–Aguilera, G., Gutierrez–Granados, S., Torres–Reyes, E. and Alatorre–Ordaz, A. (2009). A comparative study of global hexavalent chromium removal by chemical and electrochemical processes. *Electrochemistry Communications*. 11: 1097–1100.

Altundogan, H. S. (2005). Cr(VI) removal from aqueous solution by iron (III) hydroxide–loaded sugar beet pulp. *Process Biochemistry*. 40: 1443–1452.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Reviews*. 59: 143–169.

American Society for Testing and Materials. (1972). ASTM D1107–56, Reapproved 1972.

American Society for Testing and Materials. (1977). ASTM D1103–60, Reapproved 1997.

UNIVERSITI PENDIDIKAN SULTAN IDRIS

American Society for Testing and Materials. (1978). ASTM D1104–56, Reapproved 1978.

American Society for Testing and Materials. (2007). ASTM D1106–96, Reapproved 2007.

American Society for Testing and Materials. (2007). ASTM E1755–01, Reapproved 2007.

American Society for Testing and Materials. (2007). ASTM E1758–01, Reapproved 2007.

American Society for Testing and Materials. (2008). ASTM E1756–08.

Armold, M. T. (2001). Essentials of general, organic and biological chemistry. 519–521. Harcourt: USA.

Asatiani, N. V., Abuladze, M. K. Kartvelishvili, T. M., Bakradze, N. G., Sapojnikova, N. A., Tsibakhashvili, N. Y., Tabatadze, L. V., Lejava, L. V., Asanishvili, L. L. and Holman, H. Y. (2004). Effect of chromium (VI) action on *Arthrobacter oxydans*. *Current Microbiology*. 49: 321–326.

Azizullah, A., Khattak, M. N. K., Richter, P. and Hader, D. P. (2011). Water pollution in Pakistan and its impact on public health – a review. *Environment International*. 37: 479–497.

Babel, S. and Kurniawan, T. A. (2004). Cr(VI) removal from synthetic wastewater using coconut shell charcoal and commercial activated carbon modified with oxidizing agent and/or chitosan. *Chemosphere*. 54: 95–967.

Bailey, S. E., Olin, T. J., Bricka, R. M. and Adrian, D. D. (1999). A review of potentially low – cost sorbents for heavy metals. *Water Resources*. 33(11): 2469–2479.

Banaszkiewicz, T. (2011). Nutritional Value of Soybean Meal, Soybean and Nutrition. In El–Shemy, H. (Ed.) ISBN: 978–953–307–536–5.

Barakat, M. A. (2011). New trends in removing heavy metals from industrial wastewater. *Arabian Journal of Chemistry*. 4: 361–377.

Barrera–Diaz, C. E., Lugo–Lugo, V. and Bilyeu, B. (2012). A review of chemical, electrochemical and biological methods for aqueous Cr(VI) reduction. *Journal of Hazardous Materials*. 223– 224: 1– 12.

Bartholomew, D. P., Paull, R. E. and Rohrbach, K. G. (2003). The pineapple botany, production and uses. 1: 1–29, 281–288. London, CABI publishing.

UNIVERSITI PENDIDIKAN SULTAN IDRIS

Bhatnagara, A. and Sillanpaa, M. (2010). Utilization of agro-industrial and municipal waste materials as potential adsorbents for water treatment – a review. *Chemical Engineering Journal.* 157: 277–296.

Bo, S., Mei, L., Tongbin, C., Yuanming, Z., Yunfeng, X., Xiaoyan, L. and Ding, G. (2009). Assessing the health risk of heavy metals in vegetables to the general population in Beijing, China. *Journal of Environmental Sciences.* 21: 1702–1709.

Brenner, D. J., Krieg, N. R. and Staley, J. T. (2005). *Bergey's manual of systematic bacteriology: Vol 2 – The proteobacteria.* Part B: The gamma proteobacteria. 435. United States: Bergey's manual trust.

Bruins, M. R., Kapil, S. and Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety.* 45:198–207.

Camargo, F., Okeke, B., Bento, F. and Frankenberger, W. (2004). Hexavalent chromium reduction by immobilized cells and the cell–free extract of *Bacillus* sp. *ES 29. Bioremediation Journal.* 8(1): 23–30.

Chatterjee, S., Sau, G. B. and Mukherjee, S. K. (2009). Plant growth promotion by a hexavalent chromium reducing bacterial strain, *Cellulosimicrobium cellulans* KUCr3. *World Journal of Microbiology and Biotechnology.* 25: 1829–1836.

Chen, Y. and Gu, G. (2005). Preliminary studies on continuous chromium (VI) biological removal from wastewater by anaerobic–aerobic activated sludge process. *Bioresource Technology.* 96: 1713–1721.

Cheung, H. N. B. (2008). Bacterial growth inhibition during composting of food waste: effects of organic acids. MSc Thesis. University of Regina.

Cheung, K. H. and Gu, J. D. (2007). Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. *International Biodeterioration and Biodegradation.* 59: 8–15.

Cheung, K. H. and Ji–Dong, G. (2007). Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. *International Biodeterioration and Biodegradat*ion. 59: 8–15.

Chirwa, E. M. N. and Wang, Y. T. (1997). Chromium (VI) reduction by *Pseudomonas fluorescens* LB 300 in fixed–film bioreactor. *Journal of Environmental Engineering.* 123(8): 760–766.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA
DRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PEN

Cimino, G., Passerini, A. and Toscano, G. (2000). Removal of toxic cations and Cr(VI) from aqueous solution by hazelnut shell. *Water Resources*. 34(11): 2955–2962.

Contreras, E. M., Ferro Orozco, A. M. and Zaritzky, N. E. (2011). Biological Cr(VI) removal coupled with biomass growth, biomass decay and multiple substrate limitation. *Water Resources*. 45: 3034–3046.

Costerton, J. W. and Scott, H. M. L. (2003). *Microbial Biofilm*. United Kingdom. Cambridge University Press.

Coughlan, M. P. and Mayer, F. (1992). *The prokaryotes: a handbook on the biology of bacteria*. (2nd ed.). In Balows, A., Truper, H. G., Dworkin, M., Harder, W. and Schleifer, K. H. (Ed.) 460–516. New York: Springer–Verlag.

Couto, S. R. and Sanroman, M. (2006). Application of solid–state fermentation to food industry – a review. *Journal of Food Engineering*. 76: 291–302.

Cronje, K. J., Chetty, K., Carsky, M., Sahu, J. N. and Meikap, B. C. (2011). Optimization of chromium (VI) sorption potential using developed activated carbon from sugarcane bagasse with chemical activation by zinc chloride. Desalination. 275: 276–284.

Dambies, L., Guimon, C., Yiacoumi, S. and Guibal, E. (2001). Characterization of metal ion interactions with chitosan by X–Ray Photoelectron Spectroscopy. *Colloids and Surfaces A*. 177: 203–214.

Das, B., Mondal, N. K., Roy, P. and Chattaraj, S. (2013). Equilibrium, kinetic and thermodynamic study on chromium (VI) removal from aqueous solution using *Pistia stratiotes* biomass. *Chemical Science Transactions,* 2(1): 85–104.

Davis, K. E. R., Joseph, S. J. and Janssen, P. H. (2005). Effects of growth medium, inoculums size and incubation time on culturability and isolation of soil bacteria. *Applied and Environmental Microbiology.* 71: 826–834.

DeLeo, P. C. and Ehrlich, H. L. (1994). Reduction of hexavalent chromium by *Pseudomonas fluorescens* LB300 in batch and continuous cultures. *Applied Microbiology and Biotechnology*. 40: 756–759.

Deng, S. B. and Ting, Y. P. (2005). Polyethylenimine–modified fungal biomass as a high–capacity biosorbent for Cr(VI) anions: Sorption capacity and uptake mechanisms. *Environmental Science and Technology*.39: 8490–8496.

Department of Environment. (2009). Annual Report. Ministry of Natural Resources and Environment, Malaysia.

Dermou, E., Velissariou, A., Xenos, D. and Vayenas, D. V. (2007). Biological removal of hexavalent chromium in trickling filters operating with different filter media types. *Desalination.* 211: 156–163.

Dhal, B., Thatoi, H. N., Das, N. N. and Pandey, B. D. (2013). Chemical and microbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste: A review. *Journal of Hazardous Materials.* 250– 251: 272– 291.

Dos Santos, V. C. G., Andrade Salvado, A. D. P., Cardoso Dragunski, E. D. Clausen Peraro, D. N., Teixeira Tarley, E. C. R. and Caetano, J. (2012). Highly improved chromium (III) uptake capacity in modified sugarcane bagasse using different chemical treatments. *Quim Nova.* 35(8): 1606–1611.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. *Analytical Chemistry*. 28(3): 350–356.

Dunfield, P. F., Tamas, I., Lee, K. C., Morgan, X. C., McDonald, I. R. and Stott, M. B. (2012). Electing a candidate: a speculative history of the bacterial phylum OP10. *Environmental Microbiology*. 14(12): 3069–3080.

Dupont, L. and Guillon, E. (2003). Removal of hexavalent chromium with a lignocellulosic substrate extracted from wheat bran. *Environmental Science and Technology*.37: 4235–4241.

Dursun, A. Y., Uslu, G., Cuci, Y. and Aksu, Z. (2003). Bioaccumulation of copper (II), lead (II) and chromium (VI) by growing *Aspergillus niger. Process Biochemistry.* 38: 1647–1651.

Eaton, A. D., Clesceri, L. S., Rice, E. W. and Greenberg, A. E. (2005). Standard methods for the examination of water and wastewater. 21st ed. New York. *American Public Health Association.*

Elangovan, R. and Philip, L. (2009). Performance evaluation of various bioreactors for the removal of Cr(VI) and organic matter from industrial effluent. *Biochemical Engineering Journal.* 44: 174–186.

Fendorf, S., Wielinga, B. W. and Hansel, C. M. (2001). Reduction of chromium in surface and subsurface environments, contributions of biological and abiological processes. *Eleventh Annual V. M. Goldschmidt Conference.*

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA
DRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PEN

Ferro Orozco, A. M., Contreras, E. M. and Zaritzky, N. E. (2010). Cr(VI) reduction capacity of activated sludge as affected by nitrogen and carbon sources, microbial acclimation and cell multiplication. *Journal of Hazardous Materials*. 176: 657–665.

Field, E. K., D'Imperio, S., Miller, A. R., VanEngelen, M. R., Gerlach, R., Lee, B. D., Apel, W. A. and Peyton, B. M. (2010). Application of molecular techniques to elucidate the influence of cellulosic waste on the bacterial community structure at a simulated low–level–radioactive–waste site. *Applied and Environmental Microbiology*. 76(10): 3106–3115.

Flogeac, K., Guillon, E., Marceau, E. and Aplincourt, M. (2003). Speciation of chromium on a straw lignin: Adsorption isotherm, EPR and XAS studies. *New Journal of Chemistry*. 27: 714–720.

Food and Agricultural Organization online. (2013). Retrieved on January 31, 2013, from *http://faostat.fao.org/site/339/default.aspx*

Fu, F. and Wang, Q. (2011). Removal of heavy metal ions from wastewaters: A review. *Journal of Environmental Management*. 92: 407–418.

Fude, L., Harris, B., Urrutia, M. M. and Beveridge, T. J. (1994). Reduction of Cr(VI) by a consortium of sulphate–reducing bacteria (SRB III). *Applied and Environmental Microbiology*. 60(5): 1525–1531.

Garcia–Fraile, P., Rivas, R., Willems, A., Peix, A., Martens, M., Martinez–Molina, E., Mateos, P. F. and Velazquez, E. (2007). *Rhizobium cellulosilyticum* sp. nov., isolated from sawdust of Populus alba. *International Journal of Systematic and Evolutionary Microbiology*. 57: 844–848.

Gardea–Torresdey, J. L., Tiemann, K. J., Armendariz, V., Bess–Oberto, L., Chianelli, R. R., Rios, J., Parsons, J. G. and Gamez, G. (2000). Characterization of Cr(VI) binding and reduction to Cr(III) by the agricultural byproducts of *Avena monida* (Oat) biomass. *Journal of Hazardous Materials*. B 80: 175–188.

Garg, U. K., Kaur, M. P., Garg, V. K. and Sud, D. (2007). Removal of hexavalent chromium from aqueous solution by agricultural waste biomass. *Journal of Hazardous Materials*. 140: 60–68.

Garg, U. K., Kaur, M. P., Sud, D. and Garg, V. K. (2009). Removal of hexavalent chromium from aqueous solution by adsorption on treated sugarcane bagasse using response surface methodological approach. *Desalination*. 249: 475–479.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA

Gargiulo, G., Bradford, S., Simunek, J., Ustohal, P., Vereecken, H. and Klumpp, E. (2007). Bacteria transport and deposition under unsaturated condition: the role of the matrix grain size and the bacteria surface protein. *Journal of Contaminated Hydrology*. 92(3–4): 255–273.

Geets, J., Vangronsveld, J., Diels, L., Taghavi, S. and van der Lelie, D. (2008). Microbial activities, monitoring and application as part of a management strategy for heavy metal–contaminated soil and ground water. In Naidu, R. (Ed) *Developments in Soil Science*. 32: 521–559.

Ghosh, G. and Bhattacharya, P. K. (2006). Hexavalent chromium ion removal through micellar enhanced ultrafiltration. *Chemical Engineering Journal*. 119: 45–53.

Gilbride, K. A., Lee, D. Y. and Beaudette, L. A. (2006). Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens and real–time process control. *Journal of Microbiological Methods*. 66: 1–20.

Giller, K. E., Witter, E. and Mcgrath, S. P. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biology and Biochemistry*. 30(10/11): 1389–1414.

Goyal, N., Jain, S. C. and Banerjee, U. C. (2003). Comparative studies on the microbial adsorption of heavy metals. *Advances in Environmental Research*. 7: 11–319.

Grasshoff, K., Ehrhardt, M. and Kremling, K. (1983). Methods of seawater analysis (2nd ed). 340. Germany: Verlag Chemie.

Guo, X. M., Trably, E., Latrille, E., Carrere, H., Steyer, J. P. (2010). Hydrogen production from agricultural waste by dark fermentation: a review. *International Journal of Hydrogen Energy*. 35: 10660–10673.

Gupta, V. K., Pathania, D., Agarwal, S. and Sharma, S. (2012). Removal of Cr(VI) onto *Ficus carica* biosorbent from water. *Environmental Science and Pollution Research*. DOI: 10.1007/s11356–012–1176–6.

Gupta, V. K., Rastogi, A. and Nayak, A. (2010). Adsorption studies on the removal of hexavalent chromium from aqueous solution using a low cost fertilizer industry waste material. *Journal of Colloid and Interface Science*. 342: 135–141.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA
DRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PEN

Hamilton, M., Heersink, J., Buckingham–Meyer, K., and Goeres, P. L. (2003). *The biofilm laboratory, step–by–step protocols for experimental design, analysis and data interpretation*. Montana University.

Harvey, D. (2008). Modern Analytical Chemistry. ISBN 0–07–237547–7. McGraw–Hill Companies.

He, M., Li, X., Guo, L., Miller, J. S., Rensing, C. and Wang, G. (2010). Characterization and genomic analysis of chromate resistant and reducing *Bacillus cereus* strain SJ1. *BMC Microbiology*. 10: 221–231.

He, M., Li, X., Liu, H., Miller, S. J., Wang, G. and Rensing, C. (2011). Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain *Lysinibacillus fusiformis* ZC1. *Journal of Hazardous Materials*. 185: 682–688.

Hill, G. T., Mitkowskia, N. A., Aldrich–Wolfe, L., Emelea, L. R., Jurkonie, D. D., Ficke, A., Maldonado–Ramireza, S., Lyncha, S. T. and Nelsona, E. B. (2000). Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology*. 15: 25–36.

Hirsch, P. R., Mauchline, T. H. and Clark, I. M. (2010). Culture–independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry*. 42: 878–887.

Holt, J. G., Krieg, N. R., Sneath, P. H. a., Stanley, J. T. and William, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*. (9th ed.) New York. Williams and Wilkins.

Hou, Y., Liu, H., Zhao, X., Qu, J. and Chen, J. P. (2012). Combination of electroreduction with biosorption for enhancement for removal of hexavalent chromium. *Journal of Colloid and Interface Science*. 385: 147–153.

Hsiao Pei, Q., Shahir, S., Santhana Raj, A. S., Zakaria, Z. A. and Ahmad, W. A. (2009). Chromium (VI) resistance and removal by *Acinetobacter haemolyticus*. *World Journal of Microbiology and Biotechnology*. DOI: 10.1007/s11274–009–9989–2.

Hsu, H. T., Chen, S. S. and Chen, Y. S. (2011). Removal of chromium (VI) and naphthalenesulfonate from textile wastewater by photocatalysis combining ionic exchange membrane processes. *Separation and Purification Technology*. 80: 663–669.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA

Im, W. T., Hu, Z. Y., Kim, K. H., Rhee, S. K., Meng, H., Lee, S. T. and Quan, Z. X. (2012). Description of *Fimbriimonas ginsengisoli* gen. nov., sp. nov. within the *Fimbriimonadia* class nov., of the phylum *Armatimonadetes*. *Antonie van Leeuwenhoek*. 102: 307–317.

Imandi, S. B., Bandaru, V. V., Somalanka, S. R., Bandaru, S. R. and Garapati, H. R. (2008). Application of statistical experimental designs for the optimization of medium constituents for the production of citric acid from pineapple waste. *Bioresource Technology*. 99: 4445–4450.

Ishibashi, Y., Cervantes, C. and Silver, S. (1990). Chromium reduction in *Pseudomonas putida*. *Applied and Environmental Microbiology*. 56: 2268–2270.

Jeyasingh, J. and Philip, L. (2005). Bioremediation of chromium contaminated soil: optimization of operating parameters under laboratory conditions. *Journal of Hazardous Materials B*. 118: 113–120.

Ji, G. and Silver, S. (1995). Bacterial resistance mechanisms for heavy metals of environmental concern. *Journal of Industrial Microbiology*. 14:61–75.

Jianlong, W., Zeyu, M. and Xuan, Z. (2004). Response of *Saccharomyces cerevisiae* to chromium stress. *Process Biochemistry*. 39: 1231–1235.

Jin, D., Wang, P., Bai, Z., Wang, X., Peng, H., Qil, R., Yu, Z. and Zhuang, G. (2011). Analysis of bacterial community in bulking sludge using culture–dependent and –independent approaches. *Journal of Environmental Sciences*. 23(11): 1880–1887.

Jong, B. C., Kim, B. H., Chang, I. S., Liew, P. W. Y., Choo, Y. F. and Kang, G. S. (2006). Enrichment, performance and microbial diversity of a thermophilic mediatorless microbial fuel cell. *Environmental Science and Technology*. 40: 6449–6454.

Kamaludeen S. P. B., Arunkumaer, K. R., Avudainayagam, S. and Ramasamy, K. (2003). Bioremediation of chromium contaminated environments. *Indian Journal of Experimental Biology*. 41: 972–985.

Kareem, S. O., Akpan, I. and Alebiowu, O. O. (2010). Production of citric acid by *Aspergillus niger* using pineapple waste. *Malaysian Journal of Microbiology*. 6(2): 161–165.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA
DRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PEN

Kargi, F. and Eker, S. (2001). Rotating–perforated–tubes biofilm reactor for high–strength wastewater treatment. *Journal of Environmental Engineering*. 127: 959–963.

Kathiravan, M. N., Karthick, R. and Muthukumar, K. (2011). Ex situ bioremediation of Cr(VI) contaminated soil by *Bacillus* sp.: Batch and continuous studies. *Chemical Engineering Journal*. 169: 107–115.

Kaur, R., Singh, J., Khare, R. and Ali, A. (2012). Biosorption the possible alternative to existing conventional technologies for sequestering heavy metal ions from aqueous streams: a review. *Universal Journal of Environmental Research and Technology*. 2(4): 325–335.

Khan, N. A., Ibrahim, S. and Subramaniam, P. (2004). Elimination of heavy metals from wastewater using agricultural wastes as adsorbents. *Malaysian Journal of Science*. 23: 43–51.

Kim, M. K. and Jung, H. Y. (2007). *Chitinophaga terrae* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*. 57: 1721–1724.

Kim, M., Aita, G. and Day, D. F. (2009). Compositional changes in sugarcane bagasse on low temperature, long–term diluted ammonia treatment. *Applied of Biochemistry and Biotechnology*. DIO 10.1007/s12010–009–8827–1.

Kiran, B., Kaushik, A. and Kaushik, C. P. (2007). Biosorption of Cr(VI) by native isolate of *Lyngbya putealis (HH–15)* in the presence of salts. *Journal of Hazardous Materials*. 141: 662–667.

Kok, F. N., Arica, M. Y., Hahcigil, C., Alaeddinoglu, G. and Hasirci, V. (1999). Biodegradation of aldicarb in a packed–bed reactor by immobilized *Methylosinus*. *Enzyme and Microbial Technology*. 24(5–6): 291–296.

Konovalova, V. V., Dmytrenko, G. M., Nigmatullin, R. R., Bryk, M. T. and Gvozdyak, P. I. (2003). Chromium (VI) reduction in a membrane bioreactor with immobilized *Pseudomonas* cells. *Enzyme and Microbial Technology*. 33: 899–907.

Kotas, J. and Stasicka, Z. (2000). Chromium occurrence in the environment and methods of its speciation. *Environmental Pollution*. 107: 263–283.

Krishnani, K. K., Meng, X., Christodoulatos, C. and Boddu, V. M. (2008). Biosorption mechanism of nine different heavy metals onto biomatrix from rice husk. *Journal of Hazardous Materials*. 153: 1222 – 1234.

Kumar, S., Dudley, J., Nei, M. and Tamura, K. (2008). MEGA: A biologist–centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*. 9: 299–306.

La Duc, M. T., Osman, S., Vaishampayan, P., Piceno, Y., Andersen, G., Spry, J. A. and Venkateswaran, K. (2009). Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. *Applied And Environmental Microbiology*. 75(20): 6559–6567.

Lakatos, J., Brown, S. D., Snape, C. E. (2002). Coals as sorbents for the removal and reduction of hexavalent chromium from aqueous waste streams. *Fuel*. 81: 691–698.

Lakzian, A., Murphy, P., Turner, A., Beynin, J. L. and Giller, K. E. (2002). *Rhizobium leguminosarum* bv. *viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance. *Soil Biology and Biochemistry*. 34: 519 – 529.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In (Eds.) Stackebrandt, E. and Goodfellow, M. *Nucleic acid techniques in bacterial systematics*. 115–175. New York: John Wiley and Sons.

Laxman, R. S. and More, S. (2002). Reduction of hexavalent chromium by *Streptomyces griseus*. *Minerals Engineering*. 15: 831–837.

Lazarova, V. and Manem, J. (2000). Innovative biofilm treatment technologies for water and wastewater treatment. In Bryers, J. D. (Ed.) *Biofilm II: Process analysis and applications*. 159–206. New York: Wiley–Liss Press.

Lee, Y. J. (2005). Oxidation of sugarcane bagasse using combination of hypochlorite and peroxide, M. Sc. Thesis. Lousiana State University and Agricultural and Mechanical College.

Lee, Y. J., Chung, C. H. and Day, D. F. (2009). Sugarcane bagasse oxidation using a combination of hypochlorite and peroxide. *Bioresource Technology*. 100: 935–941.

Li, J. P., Lin, Q. Y., Zhang, X. H. and Yan, Y. (2009). Kinetic parameters and mechanisms of the batch biosorption of Cr(VI) and Cr(III) onto *Leersia hexandra Swartz* biomass. *Journal of Colloid and Interface Science*. 333: 71–77.

UNIVERSITI PENDIDIKAN SULTAN IDRIS

Liang, C., Das, K. C. and McClendon, R. W. (2003). The influence of temperature and moisture contents regimes on the aerobic microbial activity of a biosolids composting blend. *Bioresource Technology*. 86: 131–137.

Liew, P W. Y., Jong, B. C., Goh, C. M. and Ahmad, M. (2009). Bacterial diversity associated with empty oil palm fruit bunch compost as revealed by cultivation–independent analyses of PCR–amplified 16S rRNA genes. *Journal of General and Applied Microbiology*. 55: 233 – 240.

Lin, Y. K., Chen, K. H., Ou, K. L. and Liu, M. (2011). Effects of different extracellular matrices and growth factor immobilization on biodegradability and biocompatibility of macroporous bacterial cellulose. *Journal of Bioactive and Compatible Polymers*. 26(5): 508–518.

Lin, Z., Zhu, Y., Kalabegishvili, T. L., Tsibakhashvili, N. Y. and Holman, H. Y. (2006). Effect of chromate action on morphology of basalt inhabiting bacteria. *Materials Science and Engineering: C*. 26: 610–612.

Lindsay, D. and Von Holy, A. (1997). Evaluation of dislodging methods for laboratory–grown bacterial biofilms. *Journal of Food Microbiology*. 14(4): 383–390.

Liu, C. F., Sun, R. C., Zhang, A. P., Ren, J. L. and Geng, Z. C. (2006). Structural and thermal characterization of sugarcane bagasse cellulose succinates prepared in ionic liquid. *Polymer Degradation and Stability*. 91: 3040–3047.

Liu, G., Yang, H., Wang, J., Jin, R., Zhou, J. and Lv, H. (2010). Enhanced chromate reduction by resting *Escherichia coli* cells in the presence of quinone redox mediators. *Bioresource Technology*. 101: 8127–8131.

Liu, L., Yuan, Y., Li, F. B., Feng, C. H. (2011b). In situ Cr(VI) reduction with electrogenerated hydrogen peroxide driven by iron–reducing bacteria. *Bioresource Technology*. 102(3): 2468–2473.

Liu, Y. X., Yuan, D. X., Yan, J. M., Li, Q. L. and Ouyang, T. (2011a). Electrochemical removal of chromium from aqueous solutions using electrodes of stainless steel nets coated with single wall carbon nanotubes. *Journal of Hazardous Materials*. 186: 473–480.

Llobat–Estelles, M., Mauri–Aucejo, A. R. and Lopez–Catalan, M. D. (2001). Spectrophotometric determination of chromium with diphenylcarbazide in the presence of Vanadium, Molybdenum and Iron after separation by solid–phase extraction. *Fresenius Journal of Analytical Chemistry*. 371: 358–363.

Llovera, S., Bonet, R., Simon–Pujol, M. D. and Congregado, F. (1993). Effect of culture medium ions on chromate reduction by resting cells of *Agrobacterium radiobacter*. *Applied Microbiology and Biotechnology*. 39: 424–426.

Lobo, S. A. L. (2009). Anaerobic bacteria: an investigation of metabolic important enzymes: A novel type of oxygen reductase and enzymes of the tetrapyrrole biosynthesis. PhD Thesis. Universidade Nova de Lisboa.

Lovley, D. R. and Phillips, E. J. P. (1994). Reduction of Chromate by *Desulfovibrio vulgaris* and its c_3 cytochrome. *Applied and Environmental Microbiology*. 60(2): 726–728.

Lutpi, N. A., Hui Yin, T., Yee Shian, W. and Kamarudzaman, A. N. (2011). Removal of methylene blue using pineapple peel powder as adsorbent. *3rd CUTSE International Conference (CUTSE 2011)*. 8–9 November. Miri, Sarawak.

Madigan, M. T., Martinko, J. M. and Parker, J. (2001). *Brock: Biology of Microorganisms*. (9th ed.) New York, USA: Prentice–Hall.

Mahmood, A. U., Greenman, J. and Scragg, A. H. (1998). Orange and potato peel extracts: analysis and use as *Bacillus* substrates for the production of extracellular enzymes in continuous culture. Enzyme and Microbial Technology. 22: 130–137.

Mahmoud, A. S. and Rachida, M. (2003). Biodegradation of naphthalene by free and alginate entrapped *Pseudomonas* sp. *Z. Naturforsch*. 58c: 726–731.

Malik, A. (2004). Metal bioremediation through growing cells. *Environment International*. 30: 261– 278.

Mamaril, J. C., Paner, E. T. and Alpante, B. M. (1997). Biosorption and desorption studies of chromium (III) by free and immobilized *Rhizobium* (BJVr 12) cell biomass. *Biodegradation*. 8: 275–285.

Mary Mangaiyarkarasi, M. S., Vincent, S., Janarthanan, S., Subba Rao, T. and Tata, B. V. R. (2011). Bioreduction of Cr(VI) by alkaliphilic *Bacillus subtilis* and interaction of the membrane groups. *Saudi Journal of Biological Sciences*. 18: 157–167.

Masood, F. and Malik. A. (2011). Hexavalent chromium reduction by *Bacillus* sp. strain FM1 isolated from heavy–metal contaminated soil. *Bulletin of Environmental Contamination and Toxicology*. 86: 114–119.

Maukonen, J. and Saarela. M. (2009). Microbial communities in industrial environment. *Current Opinion in Microbiology*. 12: 238–243.

Mendes, R., Pizzirani – Kleiner, A. A., Araujo., W. L. and Raaijmakers, J. M. (2007). Diversity of cultivated endophytic bacteria from sugarcane: Genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. *Applied and Environmental Microbiology*. 73(22): 7259–7267.

Mishra, S., Das, A. P. and Seragadam, P. (2009). Microbial remediation of hexavalent chromium from chromite contaminated mines of Sukinda Valley, Orissa (India). *Journal of Environmental Research and Development*. 3(4): 1122–1127.

Mohamed, A. R., Mohammadi, M., Darzi, G. N. (2010). Preparation of carbon molecular sieve from lignocellulosic biomass: A review. *Renewable and Sustainable Energy Reviews*. 14: 1591–1599.

Mohammed (2011). Chromium (VI) reduction characteristics of *Acinetobacter haemolyticus* immobilized on wood shavings. MSc Thesis. Universiti Teknologi Malaysia.

Mohan, D. and Pittman Jr., C. U. (2006). Activated carbons and low cost adsorbents for remediation of tri– and hexavalent chromium from water. *Journal of Hazardous Materials*. 137: 762–811.

Mohan, D. and Singh, K. P. (2002). Single– and multi–component adsorption of cadmium and zinc using activated carbon derived from bagasse – an agricultural waste. *Water Research*. 36: 2304 – 2318.

Mohd Zain, N. A., Suhaimi, M. S. and Idrid, A. (2010). Hydrolysis of liquid pineapple waste by invertase immobilized in PVA–alginate matrix. *Biochemical Engineering Journal*. 50: 83–89.

Mona, S., Kaushik, A. and Kaushik, C. P. (2011). Biosorption of chromium (VI) by spent cyanobacterial biomass from a hydrogen fermentor using Box–Behnken model. *International Biodeterioration and Biodegradation*. 65: 656–663.

Morales–Barrera, L. and Cristiani–Urbina, E. (2006). Removal of hexavalent chromium by *Trichoderma viride* in an airlift bioreactor. *Enzyme and Microbial Technology*. 40: 107–113.

Murphy, V., Tofail, S. A. M., Hughes, H. and McLoughlin, P. (2009). A novel study of hexavalent chromium detoxification by selected seaweed species using SEM–EDX and XPS analysis. *Chemical Engineering Journal*. 148: 425–433.

Nakano, Y., Takeshita, K. and Tsutsumi, T. (2001). Adsorption mechanism of hexavalent chromium by redox within condensed–tannin gel. *Water Resources.* 35: 496–500.

Navarro, R. R., Sump, K., Fujii, N. and Matsumura, M. (1996). Mercury removal from wastewater using porous cellulose carrier modified with polyethyleneimine. *Water Resources.* 30(10): 2488–494.

Neagu, V. and Mikhalovsky, S. (2010). Removal of hexavalent chromium by new quaternized crosslinked poly (4–vinylpyridines). *Journal of Hazardous Materials.* 183: 533–540.

Nemec, A., Dijkshoorn, L., Cleenwerck, I., Baere, T. D., Janssens, D., Reikden, T. J. K., Jezek, P. and Vaneechoutte, M. (2003). *Internatiol Journal of Systematic and Evolutionary Microbiology.* 53: 1563–1567.

Nester, E. W., Anderson, D. G., Robert, C. E., Pearsall, N. N. and Nester, M. T. (2001). *Microbiology – A Human Perspective.* (3rd ed.) Toronto, Canada: Mc–Graw–Hill Companies, Inc.

Ng, T. W., Cai, Q., Wong, C. K., Chow, A. T. and Wong, P. K. (2010). Simultaneous chromate reduction and azo dye decolourization by *Brevibacterium casei*: azo dye as electron donor for chromate reduction. *Journal of Hazardous Materials.* 182: 792–800.

Nordin, N. (2006). Biosorption of chromium (VI) from electroplating wastewater using *Acinetobacter calcoaceticus* genospecies 3 grown in liquid pineapple waste. B. Sc. Thesis. Universiti Teknologi Malaysia, Skudai.

Nordin, N. (2009). Two-stage copper (II) and nickel (II) removal using rubber wood shavings and strontium alginate immobilized bacteria. M. Sc. Thesis. Universiti Teknologi Malaysia, Skudai.

Nordin, N. and Ahmad, W. A. (2008). Biosorption of Cr(VI) using *Acinetobacter calcoaceticus* grown in liquid pineapple waste. *International Conference on Environmental Research and Technology (ICERT 2008).* 896–900.

Ofomaja, A. E. and Ho, Y. (2007). Effect of pH on cadmium biosorption by coconut copra meal. *Jounal of Hazardous Materials. B* 139: 356–362.

Ohtake, H., Fujii, E. and Toda, K. (1990). Reduction of toxic chromate in an industrial effluent by use of a chromate–reducing strain of *Enterobacter cloacae*. *Environmental Technology.* 11:663–668.

Olmsted, J. and Williams, G. M. (2006). Chemistry. (4th ed.) USA: John Wiley and Sons, Inc.

Omojasola, P., Folakemi, J., Omowumi, P. and Ibiyemi, S. A. (2008). Cellulase production by some fungi cultured on pineapple waste. *Natural Sciences*. 6(2): 64–79.

Overland, M., Sorensen, M., Storebakken, T., Penn, M., Krogdahl, A., Skrede, A. (2009). Pea protein concentrate substituting fish meal or soybean meal in diets for Atlantic salmon (*Salmo salar*) – effect on growth performance, nutrient digestibility, carcass composition, gut health and physical feed quality. Aquaculture. 288: 305–311.

Owamah, H. I., Izinyon, O. C. and Asiagwu, A. K. (2012). Sorption model and kinetic assessment of ultramarine blue removal using modified cassava peels biomass. *Journal of Civil and Environmental Engineering*. 2: 5.

Ozdemir, G., Ceyhan, N. and Manav, E. (2005). Utilization of an exopolysaccharide produced by *Chryseomonas luteola* TEM05 in alginate beads for adsorption of cadmium and cobalt ions. *Bioresource Technology*. 96: 1677–1682.

Paepatung, N., Nopharatana, A. and Songkasiri, W. (2009). Bio–methane potential of biological solid materials and agricultural wastes. *Journal of Energy and Environment.* 10: 19–27.

Parameswaran, B. (2009). Sugarcane bagasse. In Nigam, P. S. and Pandey, A. (Eds.), *Biotechnology for Agro–Industrial Residues Utilisation*. 239–252.

Park, C. H., Keyhan, M., Wielinga, B., Fendorf, S. and Matin, A. (2000). Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Applied and Environmental Microbiology*. 66(5): 1788–1795.

Park, D., Lim, S. R., Yun, Y. S. and Park, J. M. (2008). Development of a new Cr(VI)–biosorbent from agricultural biowaste. *Bioresource Technology*. 99: 8810–8818.

Park, D., Lim, S. R., Yun. Y. S. and Park, J. M. (2007). Reliable evidences that the removal mechanism of hexavalent chromium by natural biomaterials is adsorption–coupled reduction. *Chemosphere*. 70: 298–305.

Park, D., Yun, Y. -S. and Park, J. M. (2004). Reduction of hexavalent chromium with the brown seaweed *Ecklonia* biomass. *Environmental Science and Technology*. 38: 4860–4864.

Park, D., Yun, Y. -S. and Park, J. M. (2006). Comment on the removal mechanism of hexavalent chromium by biomaterials or biomaterial based activated carbons. *Industrial and Engineering Chemistry Research.* 45: 2405–2407.

Patra, R. C., Malik, S., Beer, M., Megharaj, M. and Naidu, R. (2010). Molecular characterization of chromium (VI) reducing potential in Gram positive bacteria isolated from contaminated sites. *Soil Biology and Biochemistry.* 42: 1857–1863.

Pattanapipitpaisal, P., Brown, N. L. and Macaskie, L. E. (2001). Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)–contaminated site. *Applied Microbiology and Biotechnology.* 57: 257–261.

Paul, M. L., Samuel, J., Chandrasekaran, N. and Mukherjee, A. (2012). Comparative kinetics, equilibrium, thermodynamic and mechanistic studies on biosorption of hexavalent chromium by live and heat killed biomass of *Acinetobacter junii* VITSUKMW2, an indigenous chromite mine isolate. *Chemical Engineering Journal.* 187: 104– 113.

Pinon–Castillo, H. A., Brito, E. M. S., Goni–Urriza, M., Guyoneaud, R., Duran, R., Nevarez–Moorillon, G. V., Gutierrez–Corona1, J. F., Caretta, C. A. and Reyna–Lopez, G. E. (2010). Hexavalent chromium reduction by bacterial consortia and pure strains from an alkaline industrial effluent. *Journal of Applied Microbiology.* 109: 2173–2182.

Polti, M. A., Garcia, R. O., Amoroso, M. J. and Abate, C. M. (2009). Bioremediation of chromium (VI) contaminated soil by *Streptomyces* sp. MC1. *Journal of Basic Microbiology.* 49: 285–292.

Pust, C. (2004). Basic on COD measurement. HACH technical sales support EU. Retrieved on July 25, 2010, from *www.multilab.com.lb/images/water.pps*

Qaiser, S., Saleemi, A. R. and Ahmad, M. M. (2007). Heavy metal uptake by agro based waste materials. *Electronic Journal of Biotechnology.* 10: 409–416.

Quintelas, C., Fonseca, B., Silva, B., Figueiredo, H. and Taraves, T. (2009). Treatment of chromium (VI) solutions in a pilot–scale bioreactor through a biofilm of *Arthrobacter viscosus* supported on GAC. *Bioresources Technology.* 100: 220–226.

Qureshi, N., Annous, B. A., Ezeji, T. C., Karcher, P. and Maddox, I. S. (2005). Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. *Microbial Cell Factories. 4:* 24.

Raize, O., Argaman, Y. and Yannai, S. (2004). Mechanisms of biosorption of different heavy metals by brown marine macroalgae. *Biotechnology and Bioengineering*. 87(4): 451–458.

Ramirez–Ramirez, R., Calvo–Mendez, C., Avila–Rodriguez, M., Lappe, P., Ulloa, M., Vazquez–Juarez, R. and Gutierrez–Corona, J. F. (2004). Cr(VI) reduction in a chromate–resistant strain of *Candida maltose* isolated from the leather industry. *Antonie van Leeuwenhoek*. 85(1): 63–68.

Ramrakhiani, L., Majumder, R. and Khowala, S. (2011). Removal of hexavalent chromium by heat inactivated fungal biomass of *Termitomyces clypeatus*: Surface characterization and mechanism of biosorption. *Chemical Engineering Journal*. 171: 1060– 1068.

Rani, A., Porwal, S., Sharma, R., Kapley, A., Purohit, H. J. and Kalia, V. C. (2008). Assessment of microbial diversity in effluent treatment plants by culture dependent and culture independent approaches. *Bioresource Technology*. 99: 7098–7107.

Rattanachomsri, U., Kanokratana, P., Eurwilaichitr, L, Igarashi, Y. and Champreda, V. (2011). Culture–independent phylogenetic analysis of the microbial community in the industrial sugarcane bagasse feedstock piles. *Bioscience, Biotechnology and Biochemistry*. 75(2): 232–239.

Reeder, R. J., Schoonen, M. A. A. and Lanzirotti, A. (2006). Metal speciation and its role in bioaccessibility and bioavailability – Reviews. Mineralogy and Geochemistry. 64: 59–113.

Rengaraj, S., Joo, C. K., Kim, Y. and Yi, J. (2003). Kinetics of removal of chromium from water and electronic process wastewater by ion exchange resins: 1200H, 1500H and IRN97H. *Journal of Hazardous Materials*. B102: 257–275.

Rial, D., Vazquez, J. A. and Murado, M. A. (2011). Effects of three heavy metals on the bacteria growth kinetics: A bivariate model for toxicological assessment. *Applied Microbiology and Biotechnology*. 90: 1095–1109.

Rodgers, M. and Zhan, X. M. (2003). Moving–medium biofilm reactors. *Review in Environmental Science and Bio/Technology*. 2: 213–224.

Rodrigues, R. C. L. B., Rocha, G. J. M., Rodrigues Jr., D., Filho, H. J. I., Felipe, M. G. A. and Pessoa Jr., A. (2010). Scale–up of diluted sulphuric acid hydrolysis for producing sugarcane bagasse hemicellulosic hydrolysate (SBHH). *Bioresource Technology*. 101: 1247–1253.

Saha, B. and Orvig, C. (2010). Biosorbents for hexavalent chromium elimination from industrial and municipal effluents. *Coordination Chemistry Reviews*. 254: 2959–2972.

Saitou, N. and Nei, M. (1987). The neighbour–joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. 4: 406–425.

Sasaki, K., Noparatnaraphorn, N. and Nagai, S. (1991). Use of photosynthetic bacteria for the production of SCP and chemicals from agro industrial waste. In Martin, A. M. *Bioconversion of waste material to industrial product*. 225–233. London: Elviser Applied Science.

Saueprasearsit, P. (2011). Adsorption of chromium (Cr^{+6}) using durian peel. *International Conference on Biotechnology and Environment Management (IPCBEE)*. 18. IACSIT Press, Singapore.

Sawalha, M. F., Peralta–Videa, J. R., Saupe, G. B., Dokken, K. M. and Gardea–Torresdey, J. L. (2007). Using FTIR to corroborate the identity of functional groups involved in the binding of Cd and Cr to saltbush (*Atriplex canescens*) biomass *Chemosphere*. 66: 1424–1430.

Selvaraj, K., Manonmani, S. and Pattabhi, S. (2003). Removal of hexavalent chromium using distillery sludge. *Bioresource Technology*. 89: 207–211.

Selvi, K., Pattabhi, S. and Kadirvelu, K. (2001). Removal of Cr(VI) from aqueous solution by adsorption onto activated carbon. *Bioresource Technolology*. 80: 87–89.

Sharma, A., Thakur, I. S. and Dureja, P. (2009). Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP–3 from effluent discharge site. *Biodegradation*. 20: 643–650.

Sharma, D. C. and Forster, C. F. (1993). Removal of hexavalent chromium using Sphagnum moss peat. *Water Resources*. 27(7): 1201–1208.

Sharma, S. and Adholeya, A. (2011). Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by *Paecilomyces lilacinus* fungi. *International Biodeterioration and Biodegradation*. 65: 309–317.

Shen, H. and Wang, Y. T. (1993). Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Applied of Environmental Microbiology*. 59: 3771–3777.

Sheng, P. X., Ting, Y. P. Chen, J. P. and Hong, L. (2004). Sorption of lead, copper, cadmium, zinc and nickel by marine algal biomass: characterization of biosorptive capacity and investigation of mechanisms. *Journal of Colloid and Interface Science*. 275: 131–141.

Shetta, N. D., Al–Shaharani, T. S. and Abdel–Aal. (2011). Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition. American–*Eurasian Journal of Agricultural and Environmental Science*. 10(3): 410–418.

Silva, C. F., Azevedo, R. S., Braga, C., da Silva, R., Dias, E. S., Schwan, R. F. (2009). Microbial diversity in a bagasse–based compost prepared for the production of *Agaricus brasiliensis*. *Brazilian Journal of Microbiology*. 40: 590–600.

Singh, R., Kumar, A., Kirrolia, A., Kumar, R., Yadav, N., Bishnoi, N. R. and Lohchab, R. K. (2011). Removal of sulphate, COD and Cr(VI) in simulated and real wastewater by sulphate reducing bacteria enrichment in small bioreactor and FTIR study. *Bioresource Technology*. 102: 677–682.

Somasundaram, V., Philip, L. and Bhallamudi, S. M. (2011). Laboratory scale column studies on transport and biotransformation of Cr(VI) through porous media in presence of CRB, SRB and IRB. *Chemical Engineering Journal*. 171: 572–581.

Spring, S., Jackel, U., Wagner, M. and Kampfer, P. (2004). *Ottowia thiooxydans* gen. nov., sp. nov., a novel facultatively anaerobic, N_2O–producing bacterium isolated from activated sludge, and transfer of *Aquaspirillum gracile* to *Hylemonella gracilis* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. 54: 99–106.

Srivastava, H. C. P., Mathur, R. P. and Mehrotra, I. (1986). Removal of chromium from industrial effluents by adsorption on sawdust. *Environmental Technology Letters*. 7: 55–62.

Stasinakis, A. S., Thomaidis, N. S., Mamais, D., Papanikolaou, E. C., Tsakon, A. and Lekkas, T. D. (2003). Effect of chromium (VI) addition on the activated sludge process. *Water Research*. 37: 2140–2148.

Steger, K., Jarvis, A., Vasara, T., Romantschuk, M. and Sundh, I. (2007). Effects of differing temperature management on development of *Actinobacteria* populations during composting. *Research in Microbiology*. 158: 617–624.

Stein, L. Y., Jones, G., Alexander, B., Elmund, K., Wright–Jones, C. and Nealson, K. H. (2002). Intriguing microbial diversity associated with metal–rich particles from a freshwater reservoir. *FEMS Microbiology Ecology*. 42: 431–440.

Suksabye, P., Nakajima, A., Thiravetyan, P., Baba, Y., Nakbanpote. (2009). Mechanism of Cr(VI) adsorption by coir pith studied by ESR and adsorption Kinetic. *Journal of Hazardous Materials*. 161: 1103–1108.

Suksabye, P., Thiravetyan, P., Nakbanpote, W. and Chayabutra, S., (2007). Chromium removal from electroplating wastewater by coir pith. *Journal of Hazardous Materials*. 141: 637–644.

Sun, J. X., Sun, X. F., Zhao, H. and Sun, R. C. (2004). Isolation and characterization of cellulose from sugarcane bagasse. *Polymer Degradation and Stability*. 84: 331–339.

Tekerlekopoulou, A. G., Tsiflikiotou, M., Akritidou, L., Viennas, A., Tsiamis, G., Pavlou, S., Bourtzis, K. and Vayenas, D. V. (2013). Modelling of biological Cr(VI) removal in draw–fill reactors using microorganisms in suspended and attached growth systems. *Water Resources*. 47(2): 623–36.

Teterlekopoulou, A. G., Tsiamis, G., Dermou, E., Siozios, S., Bourtzis, K. and Vayenas, D. V. (2010). The effect of carbon source on microbial community structure and Cr(VI) reduction rate. *Biotechnology and Bioengineering*. 107(3): 478–487.

Thacker, U. and Madamwar, D. (2005). Reduction of toxic chromium and partial localization of chromium reductase activity in bacterial isolate DM1. *World Journal of Microbiology and Biotechnology*. 21: 891–899.

Thacker, U., Parikh, R., Shouche, Y. and Madamwar, D. (2006). Hexavalent chromium reduction by *Providencia* sp. *Process Biochemistry*. 41: 1332–1337.

Thacker, U., Parikh, R., Shouche, Y. and Madamwar, D. (2007). Reduction of chromate by cell–free extract of *Brucella* sp. isolated from Cr(VI) contaminated sites. *Bioresource Technology*. 98: 1541–1547.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position–specific gap penalties and weight matrix choice. *Nucleic Acids Research*. 22: 4673–4680.

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKA

Unnithan, M. R. and Anirudhan, T. S. (2001). The kinetics and thermodynamics of sorption of chromium (VI) onto the iron (III) complex of a carboxylated polyacrylamide–grafted sawdust. *Industrial and Engineering Chemistry Research*. 40: 2683–701.

Vaiopoulou, E. and Gikas, P. (2012). Effects of chromium on activated sludge and on the performance of wastewater treatment plants: A review. *Water Research*. 46: 549–570.

Vargas–García, M. C., Lopez, M. J., Suarez–Estrella, F. and Moreno, J. (2012). Compost as a source of microbial isolates for the bioremediation of heavy metals: In vitro selection. *Science of the Total Environment*. 431: 62–67.

Verma, A., Chakraborty, S. and Basu, J. K. (2006). Adsorption study of hexavalent chromium using tamarind hull–based adsorbents. *Separation and Purification Technology*. 50: 336–341.

Viera, R. G. P., Filho, G. R., Assuncao, R. M. N., Meireles, C. S., Vieira, J. G. and Oliveira, G. S. (2007). Synthesis and characterization of methylcellulose from sugar cane bagasse cellulose. *Carbohydrate Polymers*. 67: 182–189.

Virotec Global Solution. (2011). Case study treatment of chromium contaminated solid waste at the Tasman Sheepskin Tannery. Retrieved on March 25, 2013, from http://virotec.com / download / email / web_files / industrial–wastewater–remediation–viroflow / Case%20Study%20Tasman%20Solids.pdf

Von Canstein, H., Kelly, S., Li, Y. and Wagner–Dobler, I. (2002). Species diversity improves the efficiency of mercury–reducing biofilms under changing environmental conditions. *Applied of Environmental. Microbiology*. 68(6): 2829–2837.

Wagner–Dobler, I., Von Canstein, H., Li, Y., Timmis, K. N. and Deckwer, W–D. (2000). Structure and species composition of mercury–reducing biofilms. *Applied of Environmental Microbiology*. 66(10): 4559–4563 .

Walford, S. N. (2008). Sugarcane bagasse: How easy is it to measure its constituents? *Proceeding Congress of the South African Sugar Technologist Association*. 81: 266–273.

Wani, P. A. and Khan, M. S. (2012). Bioremediation of lead by a plant growth promoting *Rhizobium* species RL9. *Bacteriology Journal*. 2(4): 66–78.

UNIVERSITI PENDIDIKAN SULTAN IDRIS

Witek–Krowiak, A. and Harikishore Kumar Reddy, D. (2013). Removal of microelemental Cr(III) and Cu(II) by using soybean meal waste – Unusual isotherms and insights of binding mechanism. *Bioresource Technology*. 127: 350–357.

Wu, J., Zhang, H., He, P. J., Yao, Q. and Shao, L. M. (2010). Cr(VI) removal from aqueous solution by dried activated sludge biomass. *Journal of Hazardous Materials*. 176: 697–703.

Xu, L., Luo, M., Li, W., Wei, X., Xie, K. Liu, L., Jiang, C. and Liu, H. (2011a). Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE–09 stimulated with external electron donors under alkaline conditions. *Journal of Hazardous Materials*. 185: 1169–1176.

Xu, L., Yang, L., Luo, M., Liang, X., Wei, X., Zhao, J. and Liu, H. (2011b). Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE–09 coated with polyethylenimine–functionalized magnetic nanoparticles under alkaline conditions. *Journal of Hazardous Materials*. 189: 787–793.

Yang, L. and Chen, J. P. (2008). Biosorption of hexavalent chromium onto raw and chemically modified *Sargassum* sp. *Bioresource Technology*. 99: 297–307.

Ye, J., Yin, H., Mai, B., Peng, H., Qin, H., He, B. and Zhang, N. (2010). Biosorption of chromium from aqueous solution and electroplating wastewater using mixture of *Candida lipolytica* and dewatered sewage sludge. *Bioresource Technology*. 101: 3893–3902.

Yuen, K. Y., Woo, P. C. Y., Teng, J. L. L., Leung, K. W., Wong, M. K. M. and Lau, S. K. P. (2001). *Laribacter hongkongensis* gen. nov., sp. nov., a novel Gram–negative bacterium isolated from a cirrhotic patient with bacteremia and empyema. *Journal Of Clinical Microbiology*. 39(12): 4227–4232.

Zahoor, A. and Rehman, A. (2009). Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. *Journal of Environmental Sciences*. 21: 814–820.

Zakaria, Z. A., Zakaria, Z., Surif, S. and Ahmad, W. A. (2005). Cr(VI) reduction by bacteria isolated from batek (textile–related) effluent. In (Eds.) Harrison S. T. L., Rawlings, D. E. and Petersen, J. *Proceedings of the 16th International Biohydrometallurgy Symposium*. 519–528. Cape Town.

UNIVERSITI PENDIDIKAN SULTAN IDRIS

Zakaria, Z. A., Zakaria, Z., Surif, S. and Ahmad, W. A. (2006). Bioremediation of Cr(VI)–containing electroplating wastewater using *Acinetobacter* sp., *International Conference on Environment 2006 (ICENV 2006), Penang, Malaysia*. (13–15 November 2006).

Zakaria, Z. A., Zakaria, Z., Surif, S. and Ahmad, W. A. (2007a). Biological detoxification of Cr(VI) using wood–husk immobilized *Acinetobacter haemolyticus*. *Journal of Hazardous Materials*. 148: 164–171.

Zakaria, Z. A., Zakaria, Z., Surif, S. and Ahmad, W. A. (2007b). Hexavalent chromium reduction by *Acinetobacter haemolyticus* isolated from heavy–metal contaminated wastewater. *Journal of Hazardous Materials*. 146: 30–38.

Zhang, J., Zeng, G., Chen, Y., Yu, M., Yu, Z., Li, H., Yu, Y. and Huang, H. (2011). Effects of physic–chemical parameters on the bacterial and fungal communities during agricultural waste composting. *Bioresource Technology*. 102: 2950–2956.

Zouboulis, A. I., Loukidou, M. X. and Matis, K. A. (2004). Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal–polluted soils. *Process Biochemistry*. 39: 909–916.

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APPENDIX A
Blast results of dominant clones

No.	Clone No.	Total clones	Bacteria	Query Coverage (%)	Max Identity (%)	Bp
1	C5 4	4	*Aquaspirillum dispar* gene for 16S rRNA, partial sequence	94	99	1577
2	C5 75	7	*Aquaspirillum dispar* gene for 16S rRNA, partial sequence	94	99	1584
3	C5 311	12	*Chitinophaga terrae* Kim and Jung 2007 strain KP01 16S rRNA, partial sequence	94	99	1567
4	C5 634	10	*Chitinophaga terrae* Kim and Jung 2007 strain KP01 16S rRNA, partial sequence	94	99	1552
5	C5 642	5	*Acidovorax avenae* 16S rRNA gene, partial sequence	94	99	1569
6	T5 2	4	*Acidovorax avenae* 16S rRNA gene, partial sequence	94	99	1573
7	T5 7	6	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN122	94	96	1564
8	T5 27	4	Uncultured *Flavobacterium* sp. clone JXS1-17 16S rRNA gene, partial sequence	88	94	1576
9	T5 55	9	Uncultured bacterium clone WW3_39 16S rRNA gene, partial sequence	92	96	1511
10	T5 67	4	Candidate division OP10 bacterium Gsoil 348 16 S rRNA gene, partial sequence	92	96	1553
11	T5 80	4	*Rhizobium cellulosilyticum* strain ALA10B2 16S rRNA, partial sequence	94	93	1529
12	T5 127	4	Uncultured *Flavobacterium* sp. clone JXS1-17 16S rRNA gene, partial sequence	94	96	1569
13	T5 170	4	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN122	94	95	1564
14	*A. haemolyticus**	-	*Acinetobacter haemolyticus* 16S rRNA gene	-	-	598

* Reported by Zakaria *et al.* (2007a)

APPENDIX B
GenBank database
(Accession number: EF369508)

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS

Acinetobacter haemolyticus 16S ribosomal RNA gene, partial sequence

GenBank: EF369508.1

FASTA Graphics

Go to:

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ACCESSION   EF369508
VERSION     EF369508.1  GI:124518391
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SOURCE      Acinetobacter haemolyticus
  ORGANISM  Acinetobacter haemolyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 598)
  AUTHORS   Zakaria,Z.A., Zakaria,Z., Surif,S. and Ahmad,W.A.
  TITLE     Hexavalent chromium reduction by Acinetobacter haemolyticus
            isolated from heavy-metal contaminated wastewater
  JOURNAL   J. Hazard. Mater. 146 (1-2), 30-38 (2007)
   PUBMED   17188812
REFERENCE   2  (bases 1 to 598)
  AUTHORS   Ahmad,W.A. and Zakaria,Z.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-JAN-2007) Chemistry, Universiti Teknologi Malaysia,
            Skudai, Johor 81310, Malaysia
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APPENDIX C
GenBank database
(Accession numbers: KC295702 to KC295714)

UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS

Uncultured bacterium clone C5-4 16S ribosomal RNA gene, partial sequence

GenBank: KC295702.1

FASTA Graphics PopSet

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SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1469)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1469)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
            81310, Malaysia
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      421 tccgggagga aaccctgatc gttaatagcg gtcgggactg acagtaccgg aagaataagc
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Uncultured bacterium clone C5-75 16S ribosomal RNA gene, partial sequence

GenBank: KC295703.1

FASTA Graphics PopSet

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            Bacteria; environmental samples.
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  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
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REFERENCE   2  (bases 1 to 1470)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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Uncultured bacterium clone C5-311 16S ribosomal RNA gene, partial sequence

GenBank: KC295704.1

FASTA Graphics PopSet

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  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1459)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
            81310, Malaysia
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UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDID
N IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI F

Uncultured bacterium clone C5-634 16S ribosomal RNA gene, partial sequence

GenBank: KC295705.1

FASTA Graphics PopSet

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DEFINITION  Uncultured bacterium clone C5-634 16S ribosomal RNA gene, partial
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VERSION     KC295705.1  GI:444898186
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SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1445)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1445)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
            81310, Malaysia
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Uncultured bacterium clone C5-642 16S ribosomal RNA gene, partial sequence

GenBank: KC295706.1

FASTA Graphics PopSet

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DEFINITION  Uncultured bacterium clone C5-642 16S ribosomal RNA gene, partial
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VERSION     KC295706.1  GI:444898187
KEYWORDS    ENV.
SOURCE      uncultured bacterium
  ORGANISM  uncultured bacterium
            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1461)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1461)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
            81310, Malaysia
FEATURES             Location/Qualifiers
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Uncultured bacterium clone T5-2 16S ribosomal RNA gene, partial sequence

GenBank: KC295707.1

FASTA Graphics PopSet

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DEFINITION  Uncultured bacterium clone T5-2 16S ribosomal RNA gene, partial
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VERSION     KC295707.1  GI:444898188
KEYWORDS    ENV.
SOURCE      uncultured bacterium
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            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1461)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1461)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS

Uncultured bacterium clone T5-7 16S ribosomal RNA gene, partial sequence

GenBank: KC295708.1

FASTA Graphics PopSet

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REFERENCE   1  (bases 1 to 1453)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1453)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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N IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI F

Uncultured bacterium clone T5-27 16S ribosomal RNA gene, partial sequence

GenBank: KC295709.1

FASTA Graphics PopSet

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DEFINITION  Uncultured bacterium clone T5-27 16S ribosomal RNA gene, partial
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VERSION     KC295709.1  GI:444898190
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  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1392)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDIDIKAN SULTAN IDRIS UNIVERSITI PENDID
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Uncultured bacterium clone T5-55 16S ribosomal RNA gene, partial sequence

GenBank: KC295710.1

FASTA Graphics PopSet

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VERSION     KC295710.1  GI:444898191
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SOURCE      uncultured bacterium
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REFERENCE   1  (bases 1 to 1399)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1399)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
            81310, Malaysia
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Uncultured bacterium clone T5-67 16S ribosomal RNA gene, partial sequence

GenBank: KC295711.1

FASTA Graphics PopSet

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            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1442)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1442)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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Uncultured bacterium clone T5-80 16S ribosomal RNA gene, partial sequence

GenBank: KC295712.1

FASTA Graphics PopSet

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            Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 1414)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1414)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
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Uncultured bacterium clone T5-127 16S ribosomal RNA gene, partial sequence

GenBank: KC295713.1

FASTA Graphics PopSet

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  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1454)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
            Universiti Teknologi Malaysia, UTM Skudai, Johor Bahru, Johor
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Uncultured bacterium clone T5-170 16S ribosomal RNA gene, partial sequence

GenBank: KC295714.1

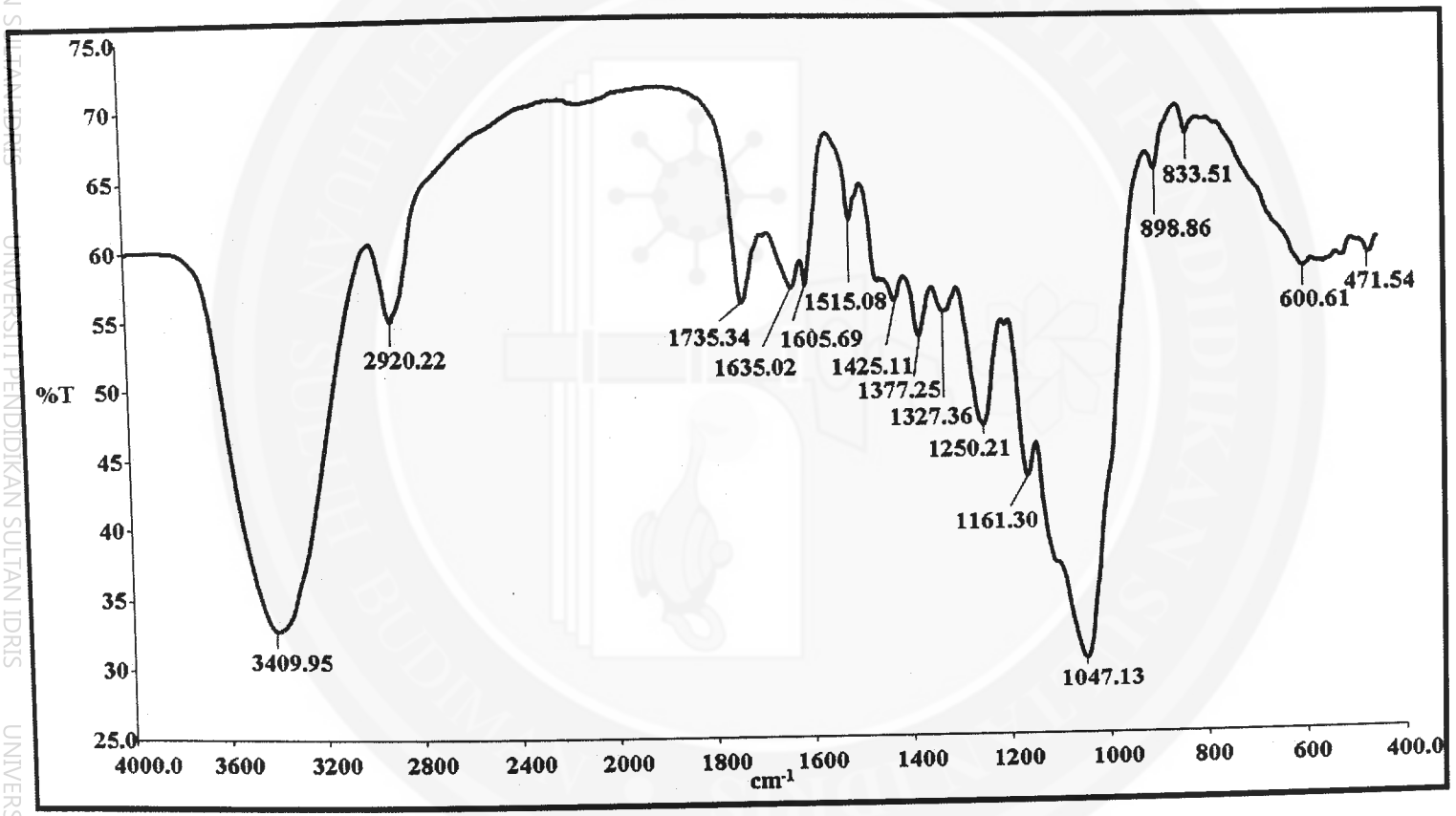
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REFERENCE   1  (bases 1 to 1453)
  AUTHORS   Wan Haslinda,W.A., Wan Azlina,A. and Jong,B.C.
  TITLE     Identification of microbial diversity present in biofilm of
            Cr(VI)-reducing environment using culture-independent approach
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1453)
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  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2012) Chemistry Department, Faculty of Science,
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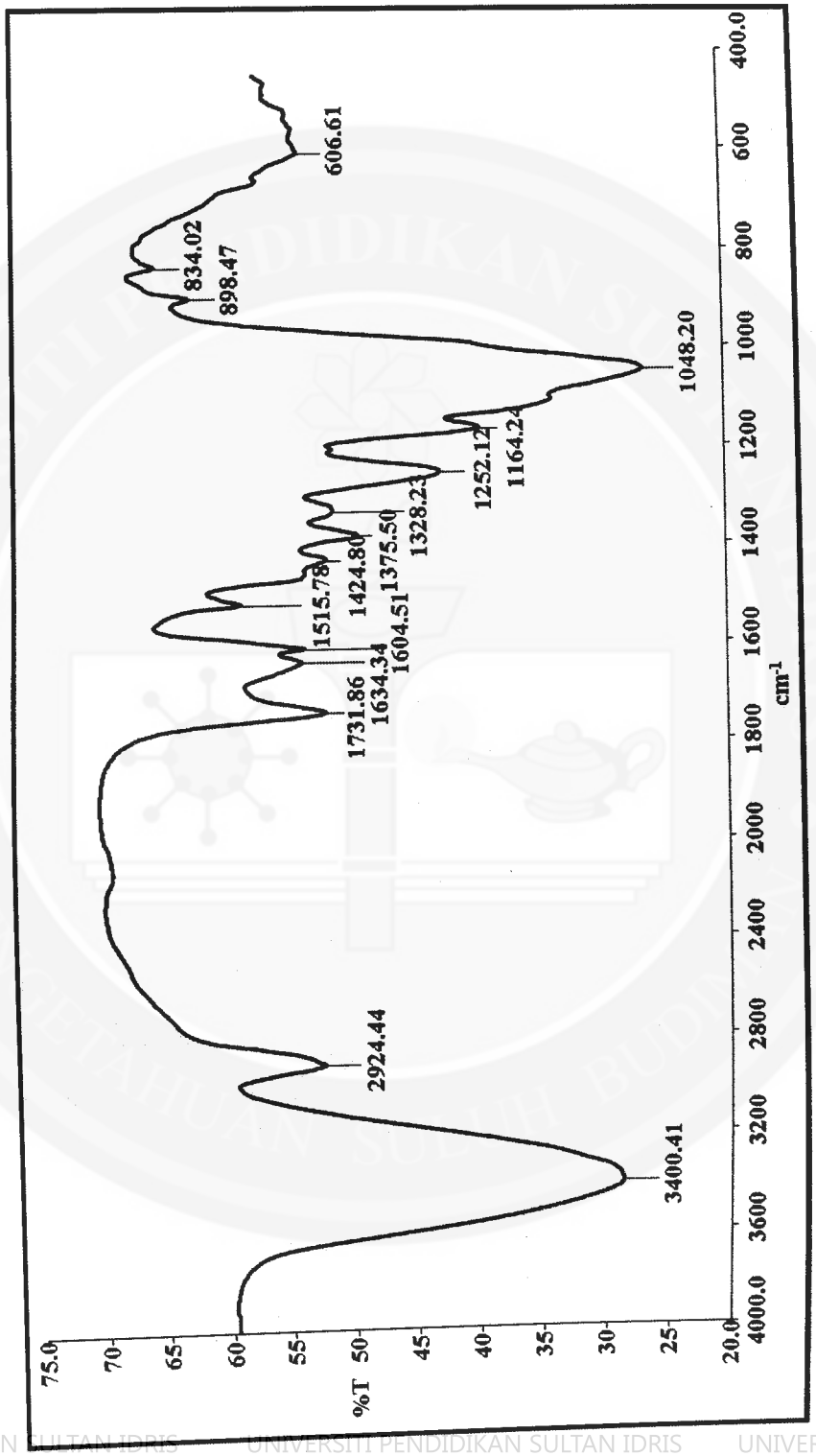
APPENDIX D
FT–IR spectra



(a) Sugarcane bagasse (SCB)

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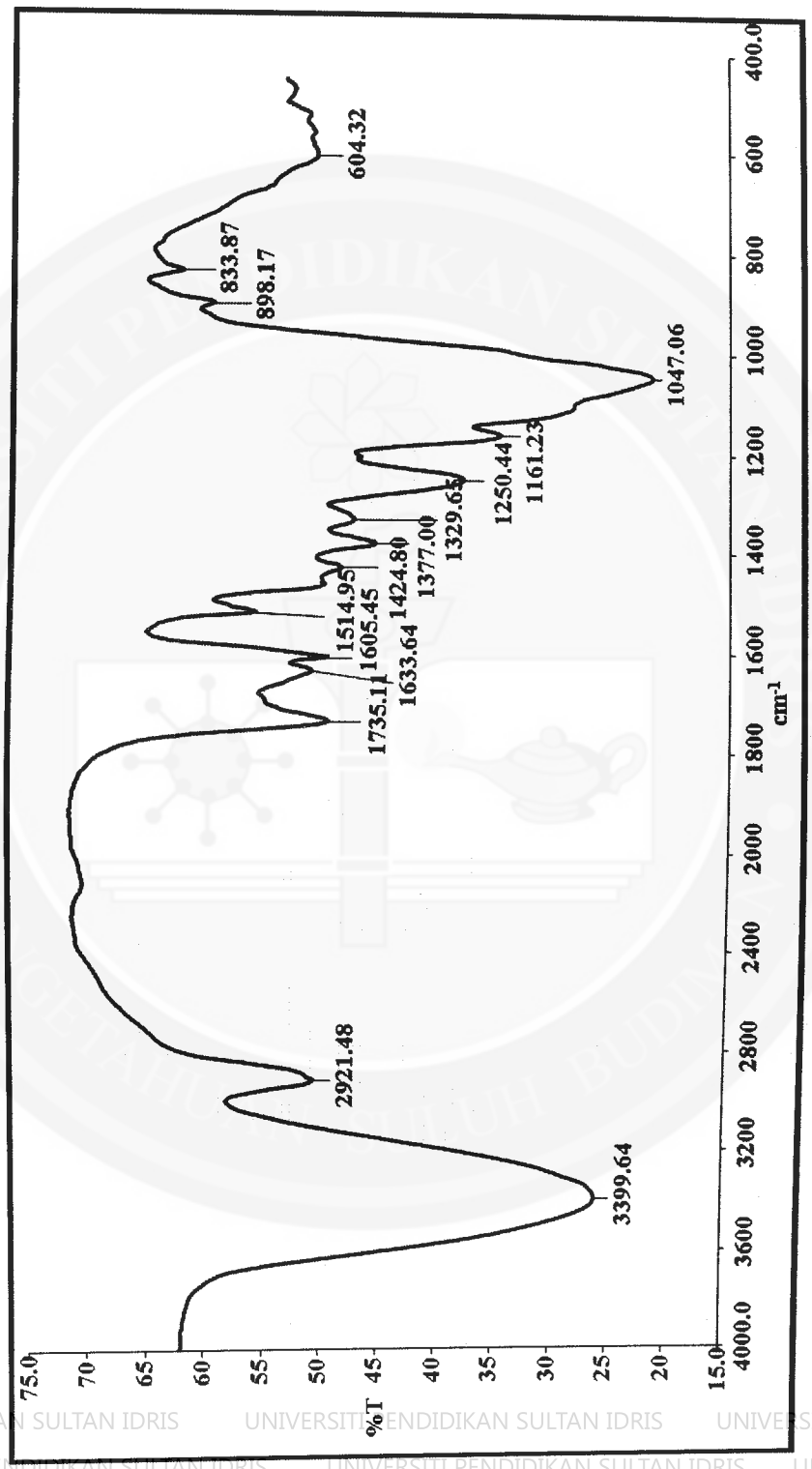
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(b) *A. haemolyticus* cells grown on SCB

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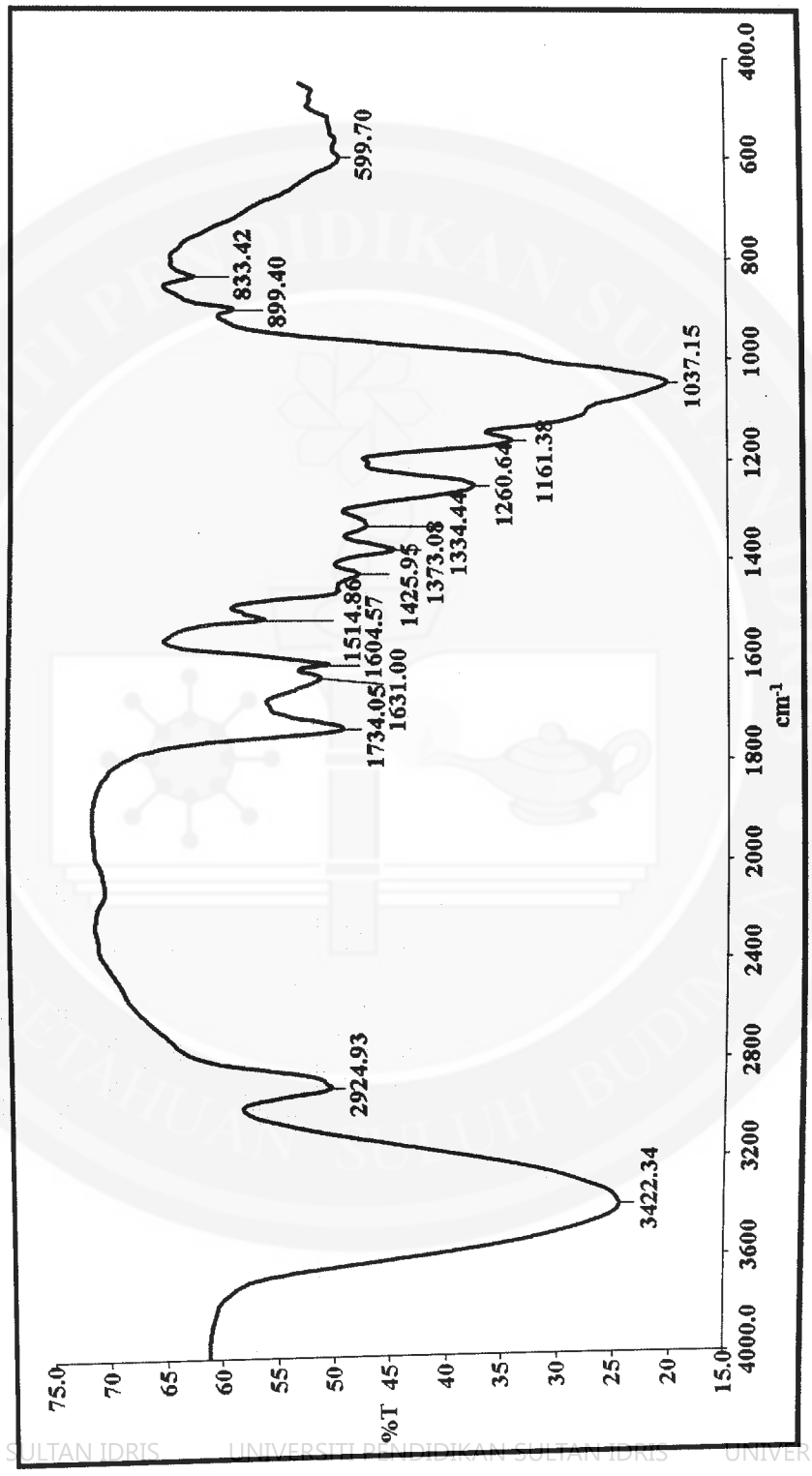
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(c) SCB in the presence of 100 mg/L Cr(VI)

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(d) *A. haemolyticus* cells grown on SCB in the presence of 100 mg/L Cr(VI)

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APPENDIX E
List of publication (journal/article) and paper presentation

Publication and Seminar

Wan Azlina Ahmad, Wan Haslinda Wan Ahmad, Norsuhada Abdul Karim, A. S. Santhana Raj and Zainul Akmar Zakaria. (2013). Cr(VI) reduction in naturally rich growth medium and sugarcane bagasse by *Acinetobacter haemolyticus*. *International Biodeterioration and Biodegradation.* 85: 571–576.

Wan Haslinda Wan Ahmad, Shaik Muhammad Hasbullah Shaik Ismail, Nor Syamimi Musa and Wan Azlina Ahmad. (2013). Utilization of local agricultural wastes in bacterial–based bioremediation application. In Zainul Akmar Zakaria, Wan Azlina Ahmad and Zainoha Zakaria (Eds.) *Biotechnology Development in Agriculture, Industry and Health.* (1–28). Skudai: Penerbit UTM Press. ISBN : 978–983–52–0900–0.

Wan Haslinda Wan Ahmad, Norsuhada Abdul Karim, Wan Azlina Ahmad and Zainul Akmar Zakaria. (2012). Cr(VI) reduction by *Acinetobacter haemolyticus*in rich and agricultural waste medium. *Proceeding ofThe 5th International Conference on the Challenges in Environmental Science and Engineering (CESE–2012).* 9–13 September 2012. Melbourne, Australia. 57. Paper was presented by Dr. Zainul Akmar Zakaria.

Wan Haslinda Wan Ahmad, Norsuhada Abdul Karim, Zainul Akmar Zakaria and Wan Azlina Ahmad. (2011). Cr(VI) Reduction behaviour in rich and agricultural waste medium by *Acinetobacter haemolyticus.Proceeding of the International Congress of the Malaysian Society for Microbiology 2011 (ICMSM 2011).*8–11 December 2011. Batu Ferringhi, Penang.123–126. ISBN: 978–983–99873–1–7.

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Other–related contribution

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Slide presentation preparation for 'The Best FRGS Research Findings Year 2009 Seminar' at Palm Garden IOI Resort Hotel, Putrajaya, 12 November 2012.

Writing FRGS final report entitled Cr(VI) Reduction Pathways of a *Acinetobacter haemolyticus*–Biofilm during the Treatment of Industrial Wastewaters Using Agricultural waste as Nutrient, 22 February 2012.

Completed attachment at Malaysian Nuclear Agency (Nuclear Malaysia) for identification of a microbial population present in a biofilm study from 21 September 2011–21 October 2011 and 8–18 November 2011.

FRGS Application. (2011). Fill in the FRGS application form for the project entitled 'Cr(VI) reduction–resistance mechanisms of *A. haemolyticus* grown in sugarcane bagasse'.

ScienceFund Application. (2011). Fill in the ScienceFund application form for the project entitled 'Application of sugarcane bagasse–immobilized *A. haemolyticus* for detoxification of Cr(VI) from industrial wastewater'.

UTM Postgraduate (Chemistry) Global Outreach Program Year 2010, 7–12th November 2010, The Halal Science Center, Chulalongkorn University and King Mongkut's University of Technology Thonburi (Remediation Laboratory and Excellent Center of of Waste Utilization and Management).

Committee member in the Industrial Wastewater Treatment Workshop, 2nd July 2010, Faculty of Science, UTM Skudai.

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