CHROMIUM(VI) REDUCTION CHARACTERISTICS OF *Acinetobacter haemolyticus* IMMOBILIZED ON WOOD SHAVINGS

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This page is entirely dedicated my beloved
family and friends for their support and encouragement

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In the name of Allah s.w.t, the Most Gracious, the Most Merciful,

ABSTRACT

Hexavalent chromium contamination in the environment is a result of the extensive use of chromate and dichromate in numerous industries including electroplating, stainless-steel production and wood preservation. Cr(VI)-reducing biofilms system in the treatment of Cr(VI)-containing wastewaters has been receiving great attention due to its efficiency and cost effectiveness. In this study, a lab-scale bioreactor consisting of *Acinetobacter haemolyticus* immobilized on wood husk was used to form Cr(VI)-reducing biofilms in packed bed column. The chromium reduction process was carried out at the laboratory–scale bioreactor for 90 days using different batches of electroplating wastewater (EW) containing Cr (VI) ranging from 25-200 mg/L where parameters such as flowrate, nutrient supplementation and initial Cr(VI) concentration in the bioreactor were initially optimized using Response Surface Methodology (RSM). RSM was used to achieve optimum condition for three parameters i.e. flowrate (3-6 mL/min), initial Cr(VI) concentration (40-100 mg/L) and nutrient supplementation (10-20% v/v) for complete reduction of Cr(VI). The attached bacterial cells in the bioreactor were also quantified during the course of Cr(VI) reduction via colony forming unit (CFU/mL) and biofilm development observation in the bioreactor using Field Emission Scanning Electron Microscope (FESEM) analyses. The optimum conditions were determined as flowrate of 3 mL/min, initial Cr(VI) concentration of 100 mg/L and nutrient supplementation of 20%. Under these optimized conditions, the lab-scale bioreactor was able to reduce completely EW at concentration of 100 mg/L in single cycle while two or three cycles were needed for higher Cr(VI) concentrations (110-200 mg/L). The number of *A. haemolyticus* cells in the bioreactor decreases to 10^5 from an initial cell concentration of 10^7 after treatment with 150-200 mg/L Cr(VI). Extracellular Polymeric Substances (EPS) was secreted by the cells in the bioreactor during the course of Cr(VI) reduction as evidenced from FESEM analysis.

ABSTRAK

Pencemaran kromium heksavalen kepada persekitaran adalah disebabkan oleh penggunaan kromat dan dikromat secara meluas dalam pelbagai industri seperti pengelektrogilapan, pengeluaran keluli tahan karat dan pengawetan kayu. Penggunaan sistem biofilem yang menurunkan Cr(VI) di dalam air buangan yang mengandungi Cr(VI) telah menerima perhatian besar disebabkan kecekapan dan kos efektifnya. Dalam kajian ini, bioreaktor berskala makmal yang mengandungi *Acinetobacter haemolyticus* dipegunkan pada habuk kayu digunakan untuk membentuk biofilem yang berupaya menurunkan Cr(VI) dalam turus padat. Proses pengurangan kromium telah dijalankan pada bioreaktor berskala makmal selama 90 hari menggunakan kumpulan air buangan yang berbeza dari proses pengelektrogilapan (EW) yang mengandungi kepekatan Cr(VI) 25-200 mg/L di mana parameter seperti kadar aliran, penambahan nutrien dan kepekatan awal Cr(VI) dalam bioreaktor terlebih dahulu dioptimumkan menggunakan metodologi permukaan resapan (RSM). RSM digunakan bagi mencapai keadaan optimum untuk tiga parameter iaitu kadar aliran (3-6 mL/min), kepekatan awal Cr(VI) (40-100 mg/L) dan penambahan nutrien (10-20% v/v) untuk pengurangan lengkap Cr(VI). Sel bakteria yang dipegunkan di dalam bioreaktor juga dikuantifikasikan semasa pengurangan Cr(VI) melalui koloni membentuk unit (CFU/mL) dan pemerhatian pembangunan biofilem dalam bioreaktor menggunakan analisis FESEM. Keadaan optimum telah dikenalpasti pada kadar aliran 3 mL/min, kepekatan awal Cr(VI) 100 mg/L dan penambahan nutrien 20%. Di bawah keadaan optimum ini, bioreaktor berskala makmal mampu mengurangkan sepenuhnya EW pada kepekatan awal Cr(VI) 100 mg/L dalam kitar tunggal manakala dua atau tiga kitaran diperlukan bagi kepekatan Cr(VI) yang tinggi (110-200 mg/L). Jumlah bakteria *A. haemolyticus* di dalam bioreaktor telah berkurang kepada 10^5 daripada jumlah asal 10^7 selepas rawatan dengan 150-200 mg/L Cr(VI). Luar sel bahan polimerik (EPS) dirembeskan oleh sel bakteria dalam bioreaktor semasa pengurangan Cr(VI) dan dibuktikan melalui analisis FESEM.

TABLE OF CONTENTS

3 APPLICATION OF RESPONSE SURFACE METHODOLOGY (RSM) FOR OPTIMIZING REMOVAL OF Cr(VI) SOLUTIONS USING Cr(VI) REDUCING BIOFILM SYSTEMS

4   PERFORMANCE AND DEVELOPMENT OF Cr(VI)-REDUCING BIOFILM SYSTEM FOR TREATING Cr(VI) FROM ELECTROPLATING WASTEWATER

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

mg	-	miligram
g	-	gram
mL	-	mililiter
L	-	liter
mg/L	-	miligram per liter
g/L	-	gram per liter
ICP-MS	-	Inductively coupled plasma-mass spectrometry
AAS	-	Atomic Absorption Spectrophotometer
FESEM	-	Field Emission Scanning Electron Microscopy
EDAX	-	Energy Dispersive X-ray
NB	-	Nutrient Broth
NA	-	Nutrient agar
TSS	-	Total Suspended Solid
SS	-	Suspended Solid
COD	-	Chemical Oxygen Demand
NaOH	-	Sodium Hydroxide
Cr(VI)	-	Hexavalent Chromium
Cr(III)	-	Trivalent Chromium
A.haemolyicus	-	*Acinetobacter haemolyticus*
LPW	-	Liquid Pineapple Waste
EW	-	Electroplating Wastewater
ND	-	Not detected
REL	-	Recommended Exposure Limit
TLV	-	Threshold Limit Value
OSHA	-	Occupational Safety and Health Administration
REL	-	Recommended Exposure Limit

ACGIH   -   American Conference of Industrial Hygenists

TLV   -   Threshold Limit Value

OSHA   -   Occupational Safety and Health Administration

PEL   -   Permissible Exposure Level

TWA   -   Time weighted average

NIOSH   -   National Institute for Occupational Safety and Health

TSS   -   Total suspended solids

CFU   -   Colony forming unit

H_2SO_4 -   Sulphuric acid

HCl   -   Hydrochloric acid

v/v   -   Volume per volume

OD   -   Optical density

OD_{600} -   Optical density at 600 nm

^0C -   Degree celsius

RSM   -   Response Surface Methodology

DPC   -   1,5- diphenylcarbazide

$mL\ min^{-1}$ -   milliliter per minute

EW   -   Electroplating Wastewater

ppm   -   part per million

LIST OF APPENDICES

CHAPTER 1

GENERAL INTRODUCTION

1.1   Heavy metal and its impact on the environment

The diverse physical properties of metal have resulted in their extensive use in industry. Heavy metals have been excessively released into the environment due to rapid industrialization and have created a major global concern. Chromium, cadmium, zinc, copper, nickel, lead, mercury are often detected in industrial wastewaters, which originate from metal plating, mining activities, smelting, battery manufacture, tanneries, petroleum refining, paint manufacture, pesticides, pigment manufacture, printing and photographic industries (Ying *et al.,* 2006). These industries produce large quantities of heavy metal wastewater every year, part of which is poured into water bodies without treatment or standard treatment, which results in the pollution of the aquatic environment.

According to Department of Environment, premises that discharged effluents are subjected to the control under Environmental Quality Sewage and Industrial Effluents Regulations, 1979 (Department of Environment, 2008). In 2008, compliance status of industries and other premises indicated that metal finishing and electroplating achieved 79% (Figure 1.1). Some of these industries were found to be operating without effluent treatment plants or some had effluent treatment plants that are not capable of treating the effluent to the stipulated standards. Generally, the problematic parameters are biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), heavy metal, oil and grease (Department of Environment, Malaysia, 2008).
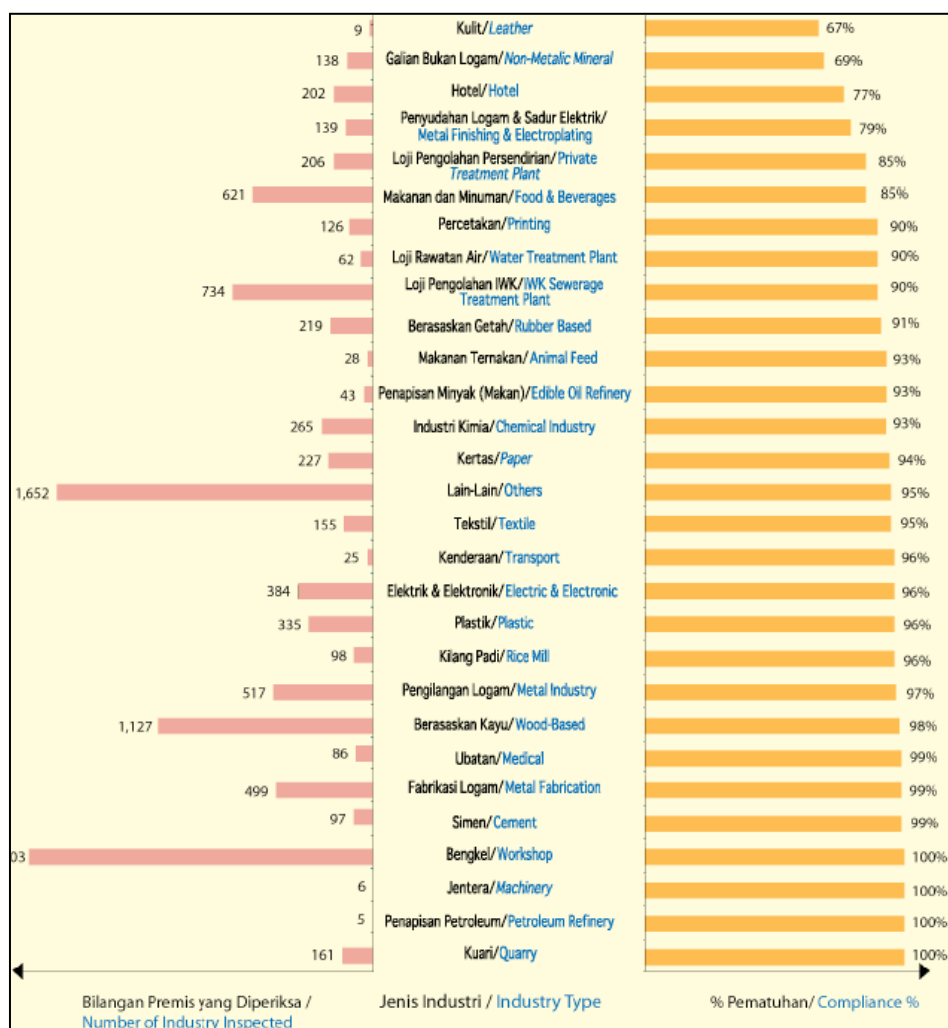
Figure 1.1: Department of Environment: Environmental Quality (Sewage and industrial Effluent) Regulations, 1979, Compliance Status, 2008.

1.1.1 Toxicity of heavy metals

Heavy metals are difficult to be biodegraded and ruined in natural conditions, they are usually ingested by aquatic animals and plants, as well as the crops on the land, and then enter into human body through food chain. They accumulate in some organs of human body and cause the chronic intoxication, which seriously endangers the health of human body. It has been reported that copper can cause stomach and intestinal distress, liver and kidney damage, and anemia. Cadmium can also damage the kidneys and lead adversely affects red blood cells, the nervous system, and the

kidneys (Brown *et al.,* 2000). Table 1.1 shows toxicity of metals as well as exposure limits to human body (Ladou, 2004).

Table 1.1: Toxicity and exposure limits of metals to human body.

Chromium	Mercury	Lead
Essentials of Diagnosis		
• Sinusitis, nasal septum perforation • Respiratory irritation, asthma • Lung cancer	• Mental disturbances • Ataxia, Spasticity • Paresthesias • Visual and auditory disturbances	• Fatigue • Hypertension • Anemia • Gout and gouty nephropathy • Chronic renal failure
Exposure Limits		
Chromium metal- ACGIH TLV:0.5 mg/m^3 TWA Cr(III) -ACGIH TLV:0.5 mg/m^3 TWA Cr(VI)- OSHA PEL: 5.0 µg/m^3 TWA ACGIH TLV: 0.05 µg/m^3 TWA (Soluble) ACGIH TLV: 0.01 µg/m^3 TWA (insoluble) NIOSH REL:1 µg/m^3 TWA	ACGIH TLV: 0.01 mg/m^3 TWA 0.03 mg/m^3 short term exposure limit Vapor- ACGIH TLV: 0.025 mg/m^3 TWA	ACGIH TLV: 0.05 mg/m^3 TWA OSHA PEL: 0.05 mg/m^3 TWA ACGIH BEL: 30 µg/dL whole blood ACGIH BEL: 0.1 mg/m^3 TWA (skin)

1.1.2   Chemistry of heavy metal

The highly electronegative metals with a density greater than 5g/cm^3 are termed heavy metal. Heavy metal includes the elements with atomic number greater than 20, excluding alkali metal, alkaline earths, lanthanides and actinides. Metallic elements are intrinsic components of the environment. From the environmental point of view, the metals that are of greatest concern are those which, either by their accumulation, can have a toxic or an inhibitory effect on living things. Metals can be dispersed, both naturally and by man's activities, into any of the earth's elements; soil, water or air. The metals which are of greatest environmental concern are

cadmium, chromium, cobalt, copper, lead, mercury, nickel, silver, tin, zinc, lanthanides/actinides (Sequiera and Moffat, 1997).

However, many heavy metals are very rare or extremely unavailable and are of no environmental concern. The toxicity of heavy metals is not due to metal itself, but to ionic forms and other chemical species (e.g. Pb^{2+} and $Cr_2O_7^{2-}$). The active and toxic form of metal usually constitutes only a small proportion of the total concentration in an environmental compartment and depends on properties of the environment as well as the metal. One of the most important influences is due to environment pH. A low pH promotes dissociation of metal complexes and may increase the fraction of metals present in ionic form without changing the total concentration (Agarwal, 2009).

The speciation and fate of metals in the natural environment as well as their separation and or control by engineered processes are ultimately governed by the electronic structures of the heavy metal. Such electronic structures also dictate the biochemical actions of metals as nutrients and toxicants.

The electronic configuration of Ca^{2+} ($1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^{6)}$ is completely filled and the octet formation is satisfied. Thus Ca^{2+} is not a good electron acceptor and hence, a poor lewis acid. Ca^{2+} is not readily deformed by electric fields and has low polarizabilities. They are referred to as "hard" cations and they form only outer sphere complexes with aquoes-phase ligands containing primarily oxygen donor atoms. In contrast, the transition metal cation, Cu^{2+} ($Cu^{2+}\ 1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^3$) has incomplete d orbitals and contains electron clouds more readily deformable by electric fields of other species. In general these ions are fairly strong lewis acid and tend to form inner sphere complexes with ligands in the aqueous phase (Sengupta, 2002).

Heavy metals, to a large extent, are dispersed in the environment through industrial effluents, organic wastes, refuse burning, transport and power generation.

Metal containing industrial effluents constitute a major source of metallic pollution of hydrosphere (Table 1.2) (Agarwal, 2009).

Table 1.2: Toxic metals in industrial effluents

Metals	Manufacturing industries
Arsenic	Phosphate and fertilizer, metal hardening, paints and textile
Cadmium	Phosphate fertiliser, electroplating, pigments and paints
Chromium	Metal plating, tanning, rubber, photographic
Copper	Plating, rayon, electrical
Lead	Paint, battery
Nickle	Electroplating, iron, steel
Zinc	Galvanising, plating, iron, steel
Mercury	Chlor-alkali, scientific instrument, chemical

1.2 Chromium

1.2.1 Chemistry and physical properties

Chromium with atomic number of 24 and atomic mass of 51.996 exists in oxidation states ranging from -2 to +6. The most common oxidation states of chromium are +2, +3, and +6, with +3 being the most stable. Oxidation state of +1, +4 and +5 is rare. Table 1.3 shows the characteristics of Cr (VI) and Cr(III) (Singh and Rudra, 2007). Chromium is a steel-gray, lustrous, hard metal that takes a high polish and has a high melting point. It is also odorless, tasteless, and is malleable. Chromium compounds of oxidation state 6 are powerful oxidants. The orbital arrangement of the electrons is $1s^2$, $2s^2$, $2p^6$, $3s^2$, $3p^6$, $3d^5$, and $4s^1$ (Nriagu and Nieboer, 1988).

Table 1.3: Characteristic of Cr (VI) and Cr (III) (Singh and Rudra, 2007).

Characteristic	Cr(VI)	Cr(III)
1. Electron configuration	$1s^2 2s^2 2p^6 3s^2 3p^6$	$1s^2 2s^2 2p^6 3s^2 3p^6 3d^3$
2. Toxicity	Highly toxic, carcinogenic and mutagenic to animals as well as human. Exposure levels above the maximum contaminant level. Short term: stomach irritation/ ulceration. Long term: Dermatitis, damage to liver, Kidney circulation, nerve tissue damage.	Less toxic, essential to human glucidic metabolism
3. Chromium compounds	$K_2CrO_4, K_2Cr_2O_7, BaCrO_4, (NH_4)_2CrO_4, CaCrO_4$ …	$CrB, CrB_2, CrBr_3, Cr_2O_3, Cr_2S_3, CrN.$
4. Mobility	Greater mobility in the environment and easily penetrate the cell wall.	Less mobile because it has a strong affinity for negative charged ions in solution. This characteristic also limits their bioavailability.
5.Solubility	Highly soluble. As solubility of metal increases, metal toxicity increases due to enchance mobility and bioavailability.	Less soluble and the solubility is limited by the formation of several oxide and hydroxide species.
6.Stability	not stable	most stable under reduced condition
7.Treatment technologies	Bioreduction, biosorption, chemical treatment process; sodium metabisulfite, synthetic resin and activated carbon	Biosorption, precipitation process using coagulant and flocculant agents, synthetic resin and activated carbon.
8.Effluents discharged limit	0.05 mg/L	1.0 mg/L

The existence of heavy metal in the environments represents a very significant and long term environmental hazard. Even at low concentrations these metals can be toxic to organisms, including humans. Chromium is a contaminant that is a known mutagen, teratogen and carcinogen (Kang and Lee, 2006).

Chromate (CrO_4^{2-}), which is the most prevalent form of Cr (VI) present in solid/liquid waste due to human activities, such as electroplating, steel and automobile manufacturing, production of pigments and dyes, wood preservation, is a hazardous contaminant because it is a serious threat to human health and it readily spreads beyond the site of initial contamination through aquatic systems and groundwater (Singh and Rudra, 2007).

1.2.2   Chromium toxicity

Chromium is an essential micronutrient in the diet of animals and humans, as it is indispensable for normal sugar, lipid and protein metabolism of mammals. Its deficiency in the diet causes alteration to lipid and glucose metabolism in animals and humans. Chromium is included in the complex named glucose tolerance factor (GTF). As reported by Nies *et al*., 1990 there is no positive effect of chromium known in plant and microorganisms. However elevated levels of chromium are always toxic although the toxicity level is related to chromium oxidation state. Cr(VI) not only is highly toxic to all forms of living organisms, mutagenic in bacteria, mutagenic and carcinogenic in humans and animals, but also it is involved in causing birth defects and the decrease of reproductive health. Cr (VI) may cause death in animals and humans if digested in large doses. The LD_{50} (dose that causes the death of 50% of a defined animal population) for oral toxicity in rats is from 50 to 100 mg/kg for Cr (VI) and 1900-3000 mg/kg for Cr (III). Cr (VI) toxicity is related to its easy diffusion across the cell membrane in prokaryotic and eukaryotic organisms and subsequently Cr (VI) reduction in cells, which gives free radicals that, may directly cause DNA alterations as well as toxic effects (Singh and Rudra, 2007).

1.3   Electroplating Industry

1.3.1   Electroplating process

Electroplating is the deposition of a metal onto a metallic surface from a solution by electrolysis process. The essential components of an electroplating process are an electrode to be plated (the cathode or substrate), a second electrode to complete the circuit (the anode), an electrolyte containing the metal ions to be deposited, and a direct current power source. Metals commonly used to plate surface are silver, chromium, cadmium, zinc, gold and copper (Cushnie, 1985).

1.3.2   Processes of Chromium Electroplating

Chromium plating can be divided into two categories; hard plating and decorative plating.

In hard plating, a thick layer of chromium is deposited directly on   the   base metal (usually steel) to provide a surface with wear resistance, a low coefficient of friction, hardness, and corrosion resistance, or to build up surfaces that have been eroded by use. Hard plating is used for items such as hydraulic cylinders and rods, industrial rolls, zinc die castings, plastic molds, engine components, and marine hardware.

Decorative chrome plating is sometimes called nickel-chrome plating because it always involves electroplating nickel onto the object before plating the chrome (it sometimes also involves electroplating copper onto the object before the nickel). The nickel plating provides the smoothness, much of the corrosion resistance, and most of the reflectivity. In decorative plating of metals, the base material generally is plated with layers of copper and nickel followed by a relatively thin layer of chromium to provide a bright surface with wear and tarnish resistance. It is used in

automotive trim, metal furniture, bicycles, hand tools, and plumbing fixtures (Horner, 1994).

The process involved in hard plating consists of pretreatment, alkaline cleaning, acid dipping, chromic acid anodizing, and chromium electroplating. The pretreatment step may include polishing, grinding, and degreasing. Degreasing consists of either dipping the part in organic solvents, such as trichloroethylene or perchloroethylene, or using the vapors from organic solvents to remove surface grease. Alkaline cleaning is used to dislodge surface soil with inorganic cleaning solutions, such as sodium carbonate, sodium phosphate, or sodium hydroxide. Acid dipping, which is optional, is used to remove tarnish or oxide films formed in the alkaline cleaning step and to neutralize the alkaline film. Acid dip solutions typically contain 10 to 30 percent hydrochloric or sulfuric acid. Chromic acid anodic treatment, which also is optional, cleans the metal surface and enhances the adhesion of chromium in the electroplating step. The final step in the process is the electroplating operation itself (Dennis and Such 1993).

Whereas, in decorative plating the initial process until acid dipping step is same as in hard plating, followed by strike plating of copper, copper electroplating, nickel electroplating, and chromium electroplating.

The copper strike plating step consists of applying a thin layer of copper in a copper cyanide solution to enhance the conductive properties of the base metal. Following the copper strike plate, the substrate is acid dipped again, and then electroplated with an undercoat of copper to improve corrosion resistance and cover defects. Either a copper cyanide or acid copper solution is used in this step. The substrate then is plated with nickel in two layers (semibright nickel and bright nickel) to further improve corrosion resistance and activate the surface metal for chromium electroplating. The final step in the process is the electroplating operation itself (Dennis and Such 1993).

1.3.3   Sources and regulations of Electroplating Wastewater

Any or all of the substances used in electroplating (such as acidic solutions, toxic metals, solvents, and cyanides) can be found in the wastewater, either via rinsing of the product or due to spillage and dumping of process baths. The solvents and vapors from hot plating baths result in elevated levels of volatile organic compounds (VOCs) and in some cases, volatile metal compounds (when may contain chromates).

It is reported by Wang *et al.,* (2007) that one of the critical pollution problems arising from the electroplating industry is the generation of rinse water for electroplated parts. The rinse water contains a certain amount of heavy metals, which are major causes of water and soil pollution. Also, metal surface treatment is one of the major metal working processes that generates a large amount of liquid and solid (sludge) wastes containing heavy metals (Cavavo *et al.,* 2007). Due to their high toxicity, the industrial wastewaters containing heavy metals are strictly regulated and must be treated before being discharged in the environment.

According to the Department of Environment, electroplating industry is classified as non-prescribed premise. Non-prescribed premises discharging effluents are subjected to the Environmental Quality (Sewage and Industrial Effluents) Regulations, 1978.   Department of Environment has been conducted 5190 inspections on 4402 industrial premises and other non-prescribed premises that were subjected to the Environmental Quality (Sewage and Industrial Effluents) Regulations, 1979. The overall compliance by these non prescribed premises was 79 %. Non-compliance included exceeding effluent discharge standards and installation of effluent treatment plant and factory expansion without written approval. Chemical-based, textile, metal fabrication, edible oil refinery, leather, food and beverages, paper, electroplating, water treatment plants and rubber-based industries had only less than 85% compliance. Some of these industries were found to be operating without effluent treatment plants or inefficient effluent treatment plants. Specifically, the electroplating industries had difficulty with parameters such as chemical oxygen demand, biological oxygen demand, suspended solid, nickel,

copper, zinc, iron, chromium, arsenic and cyanide (Department of Environment, Malaysia, 2008)

Under the environmental Quality Act, 1974 contravention licenses may be granted for a specific time frame to allow industries with genuine difficulties complying with stipulated discharge or emission standards to contravene acceptable conditions of effluent discharges into watercourses. These contravention licenses would provide problematic industries with adequate time to install or upgrade their pollution control systems. Justification for such contravention licenses would depend on the assessment of the socio-economic situation, the existing environmental quality and management commitment and sincerity to comply with the requirement. Of the 63 approved contravention licenses, 5 % was for electroplating industry. Among parameters under contravention licenses were chemical oxygen demand, biological oxygen demand, suspended solid, nickel, copper, zinc, iron, chromium, arsenic and cyanide, oil and grease, boron, lead, phenol, manganese and pH (Department of Environment, Malaysia, 2008).

1.4 Treatment Technologies for Cr (VI)

1.4.1 Conventional Treatments

Many industries have employed conventional treatment for Cr (VI) wastewater. In conventional treatment, chromate wastewater is typically treated in 2 stages (Figure 1.2).
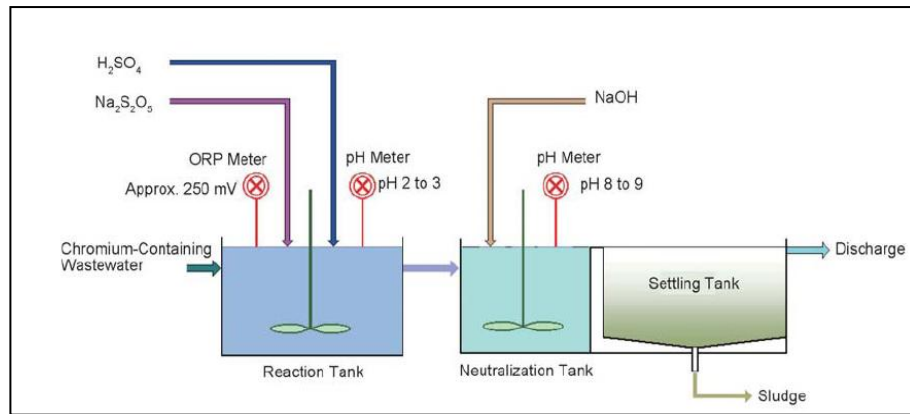
Figure 1.2: Electroplating Industry Conventional Wastewater Treatment

First, hexavalent chromium, either in the form of chromate or dichromate, is reduced to trivalent chromium. Wastewater flows into the first reaction tank, where the pH is measured and sulfuric acid is automatically brought into the process until a pH set point value in the acidic range is achieved. The reaction time is just a few minutes, and a lower pH for an even faster reaction would require considerably more acid. At the same time, the oxidation reduction potential (ORP) of the solution is measured, and sulfur dioxide (SO_2), sodium sulfite, or sodium metabisulfite is automatically injected until an ORP value of approximately 280 mV is achieved. The following equation (Eq 1.1) illustrates the reaction that takes place when sulfur dioxide is used.

$$3SO_2 + 2H_2CrO_4 + 3H_2O <---> Cr_2 (SO_4)_3 + 5H_2O \qquad\qquad (Eq\ 1.1)$$

Then in the second tank, the pH is raised to 8.5 by the addition of an alkaline solution such as ammonia, lime (CaOH) or caustic (NaOH), where it is converted to chromium hydroxide. It is reported by Wase *et al.,* 1997, NaOH often produces a bulky, poor settling sludge whereas lime gives a dense sludge with good settling properties. The precipitate, although heavier than the water, does not drop to the bottom due to agitation in the tank. The mixed slurry flows to a settling tank, where the trivalent Cr(III) chrome settles to the bottom and the clear chromium-free water flows over the tank for further treatment. The following equation 1.2 illustrates this precipitate reaction (Cushnie 1985).

$$Cr_2(SO_4)_3 + 3Ca(OH)_2 \longleftrightarrow 2Cr(OH)_3 + 3CaSO_4 \qquad \text{(Eq 1.2)}$$

In sludge disposal, it must also be considered that sludge dewatering is advisable before disposal, either to reduce transportation costs or to comply with disposal requirements. This is usually achieved by filtration. This conventional treatment provides for an effective removal of metals and if coagulation/flocculation stage is also used, removals of up to 99 % can be achieved. However, as increasingly more stringent standards are being required, the disposal of sludge may pose problems and their drawbacks like excessive chemicals consumption, sludge production, and impossibility of directly reusing heavy metals are obvious (Wang *et al.,* 2007). Thus alternative technologies must be considered.

1.4.2   Adsorption techniques

Adsorption is the process where molecules are concentrated on the surface of the sorbent. The molecules go from the bulk phase to being adsorbed in the pores in a semiliquid state. The driving force for adsorption is the ratio of the concentration to the solubility of the compound. Adsorption is used widely to remove chromium metals from waters and industrial wastewaters. A variety of natural and synthetic materials has been used as Cr(VI) sorbents, including activated carbons, biological materials, zeolites, chitosan, and agricultural-based wastes (Owlad *et al.,* 2009).

1.4.2.1 Activated carbon

Activated carbon is a crude form of graphite with a random or amorphous structure, which is highly porous, exhibiting a broad range of pore sizes, from visible cracks, crevices and slits of molecular dimensions. Active carbons have been prepared from coconut shells, wood char, lignin, petroleum coke, bone char, peat, sawdust, carbon black, rice hulls, sugar, peach pits, fish, fertilizer waste, waste rubber tire (Mohan and Pittman 2006).

Activated carbons adsorptive properties are due to such factors as surface area, a micro-porous structure, and a high degree of surface reactivity. The starting material and the activation method used for activated carbon production determine surface functional groups (Mohan and Pittman 2006). There are two classification of activated carbon namely L and H carbon. Table 1.4 lists the description between L and H carbon (Owlad *et al.,* 2009).

Table 1.4: Comparison between L and H carbon.

L carbon	H carbon
Activated at 200–400 ∘C	Activated at 800–1000 ∘C
Develop acidic surface oxides	Develop basic surface oxides
Lower solution pH values	Raise solution pH.
Adsorb bases	Adsorb acids
Exhibit a negative zeta potential	Exhibit a positive zeta potential.

Due to the high surface areas, porous sorbent and fast kinetics, activated carbon has been chosen for Cr (VI)-containing wastewater. However, their drawbacks like high cost; the higher the quality, the greater the cost and high reactivation cost that results in a loss of the carbon has limited the wide application of activated carbon in treating Cr (VI) - containing wastewater.

1.4.2.2 Low-cost adsorbent

Recently, the applicability of agricultural residues as low-cost adsorbents has received great attention. There is a growing interest in using cheap agricultural by-products, such as sugarcane baggase (Sousa *et al.,* 2009; Garg *et al.,* 2009), coconut coir (Gonzalez *et al.,* 2008; Namasivayam *et al.,* 2008), banana skin (Park *et al.,* 2008), grape waste (Chand *et al.,* 2009) for remediation of Cr (VI)-containing wastewater.

Advantages of low-cost adsorbent are it is able to minimize chemical and/or biological sludge regeneration of adsorbent, no additional nutrient requirement, and the possibility of metal recovery (Ahalya *et al.,* 2003). However, the application of untreated adsorbents can also bring several problems such as high chemical oxygen demand and biological oxygen demand as well as total organic carbon due to release of soluble organic compounds contained in the baggase materials. Therefore sugarcane baggase needs to be treated before being applied for the removal of heavy metals (Owlad *et al.,* 2009).

Agricultural-based wastes mainly consist of lignin, cellulose, hemi-cellulose and some proteins which make them effective adsorbent for heavy metal cations (Garg *et al.,* 2009). Metals cations were bound by carboxylic acid and phenolic groups (Mohan *et al.,* 2006).

1.4.2.3 Microorganism as biosorbent

Biosorption is capable of removing traces of heavy metals from dilute aqueous solutions by living systems. Dead biomass can also be used. Algae, fungi and bacteria are examples of biomass-derived metal sorbents. Adsorption studies on several metals have produced encouraging results. Heavy metal ion uptake into the cellular structure is followed by sorption onto biomolecule binding sites. This uptake is independent of biological metabolism and is known as "biosorption" or "passive uptake". Metal uptake can also involve active metabolic passage across the cell membrane into the cell. This is referred to as "active uptake". The combination of active and passive modes is called "bioaccumulation". Metal uptake by dead cells takes place only by the passive mode. Living cells employ both active and passive modes for heavy metal uptake (Mohan *et al.,* 2006).

The use of non-living cells has advantages over growing and resting cells due to the absence of both toxicity limitations and requirements of growth media and

nutrients. Moreover, adsorbed metal ion can be easily desorbed and regenerated biomass can be reused.

1.4.3 Physical Treatments

Physical treatments such as membrane filtration, ion exchange, electrochemical are becoming alternative techniques for Cr (VI) wastewater treatment. Various types of membrane filtration such as inorganic, polymeric and liquid membrane can be employed for Cr(VI) removal. Membranes can treat inorganic effluent with a high Cr (VI) concentration. Depending on membrane characteristics, membrane filtration system can remove chromium at a wide range of operational conditions. However, the operational cost is the major problem. In addition to membrane filtration, ion exchange is also one of the most frequently applied treatment techniques for chromium uptake. Despite the advantages, ion exchange also has some limitations in treating wastewater laden with heavy metals such as Cr(VI). Prior to ion exchange, appropriate pre-treatment systems for secondary effluent such as the removal of suspended solids from wastewater are required. In addition, suitable ion exchange resins are not available for all heavy metals, the capital and operational cost is high. Another technique that was discussed for removal of Cr(VI) was electrochemical treatment with the advantage of low-cost and high selectivity (Owlad *et al.,* 2009).

1.5 Bacterial biofilm

Bacteria generally exist in one of two types of population: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface or within the confines of a biofilm. A biofilm consists of cells immobilised at a substratum and frequently embedded in an organic polymer matrix of microbial origin. Biofilms are a biologically active matrix of cells and extra-cellular substances in association with a solid surface. Biofilms are sessile microbial communities growing on surfaces,

frequently embedded in a matrix of extracellular polymeric substances. A biofilm may be described as a microbially derived sessile community characterised by cells that attach to an interface, embedded in a matrix of exo-polysaccharide which demonstrates an altered phenotype. Microcolonies are discrete matrix enclosed communities of bacterial cells that may include cells of one or many species. Depending on the species involved, the micro-colony may be composed of 10–25% cells and 75–90% extracellular polymeric substances (EPS) matrix (Garrett *et al.,* 2008).

1.5.1 Mechanism of biofilm formation

There are four stages to the development of a mature biofilm: initial attachment, irreversible attachment by the production of EPS, early development and maturation of biofilm architecture (Qureshi *et al.,* 2005).

Figure 1.3 shows developments model for biofilm formation. After initial attachment to the surface, the cells then undergo surface colonization using mechanism such as cell division, migration along the surface and recruitment from the medium. As the biofilm develops an organized structure, the mature biofilm is formed. The mature biofilm is characterized as pillars of cells surrounded by an extracellular matrix material. The mature biofilm then releases cells into the medium (Jordan *et al.,* 2004).
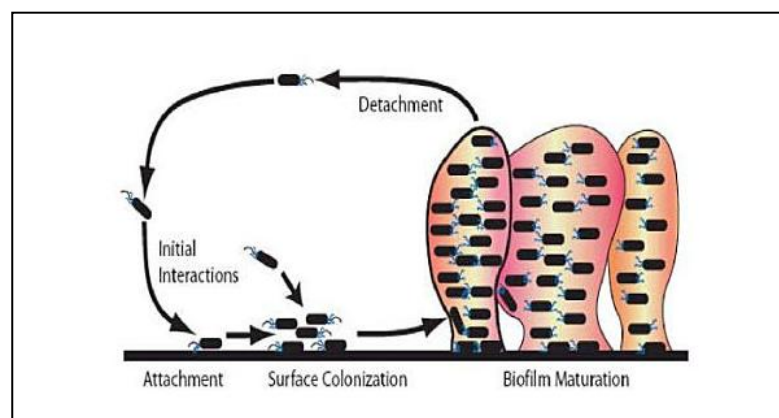
Figure 1.3: Development model for biofilm formation

1.5.2   Extracellular Polymeric Substances (EPS)

EPS are biopolymers resulting from active bacterial secretion, shedding of cell surface material, cell lysis materials and from adsorption of organics from the environment. They are composed of a variety of organic substances: carbohydrates and proteins being major constituents with humic substances, uronic acids and nucleic acids in smaller quantities (Comte *et al.,* 2006). EPS may account for 50% to 90% of the total organic carbon of biofilms and can be considered the primary matrix material of the biofilm (Donlan, 2002).

Yields, composition and properties of EPS can vary spatially and temporally in response to the availability of nutrients and other environmental condition. EPS production is affected by the availability and composition of nutrients (Wuartz *et al.,* 2003).

EPS can be divided into two types; bound (sheaths, capsular, polymers condensed gel, loosely bound polymer, attached organic material) and soluble (soluble macromolecules, colloids, slimes) as illustrated in Figure 1.4.
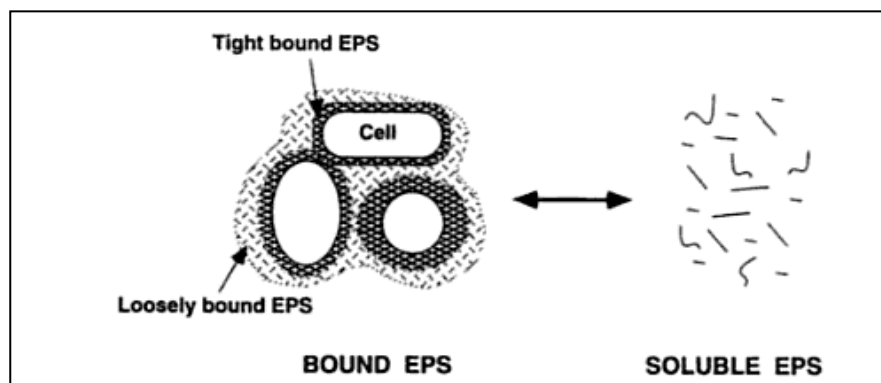
Figure 1.4: Bound and Soluble EPS.

1.5.3. Function of EPS

The formation of EPS has been regarded as the most important general function allowing microorganisms to live in aggregated communities. This primary function of EPS seems to represent an important survival advantage to immobilization process. Hence, it is important to use the EPS-producing bacteria in the bioreactor. However there are several functions of EPS as summarized in Table 1.5.

Table 1.5: Functions of EPS (Wuertz *et al.,* 2003)

Function	Relevance
Mechanical stability to biofilm	Once bacteria are at the surface they begin to secrete EPS, which binds both the bacteria and other EPS into a matrix.
Accumulation of organic nutrients	Serve as a nutrient reserve
Adhesion of surfaces	Colonization of inert and tissue surface, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments.
Protective effect	EPS layer delays or prevent biocides from reaching target microorganisms within the biofilm by diffusion limitation

1.5.4 Observation of EPS

Scanning electron microscopy (SEM) has been used to visualize the formation of EPS during biofilm development. Ploux *et al.,* 2007 has been used SEM to confirm the ability of *E.coli* PHL628 to produce curli as well as EPS Figure 1.5 (a) while Figure 1.5 (b) shows dense EPS formation when forming biofilm (Malcova *et al.,* 2008).
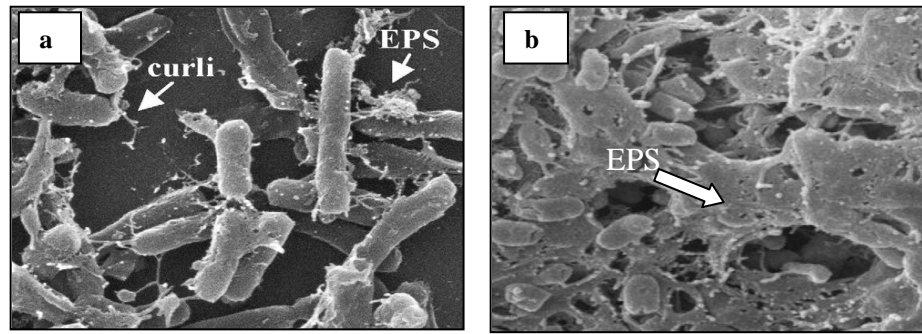
Figure 1.5: *E. coli* PHL628 observed by SEM. Arrows indicate some curli and EPS (a) dense EPS formation (b).

1.5.5 Factors enhancing biofilm formation

There are several factors which influence the formation of biofilm including surface properties of support material, bacterial cell surface, hydrodynamics (fluid flow) and also environment factors which are temperature and pH.

1.5.5.1 Surface properties of support material

The colonization of solid surfaces is ruled by the surface properties of both interacting bodies (surface charge, surface hydrophobicity, surface tension, wettability, composition, porosity and roughness) (Bryers, 2000). There are many reports about the advantages of using porous and rough supports for biofilm development. Apart from displaying a high surface area, a rough surface and internal pore space may provide a more hydrodynamically quiescent environment; thereby reducing the detachment of immobilized cells by hydraulic shearing forces (Wuertz *et al.,* 2003). It is assumed that bacteria preferentially stick to rougher surfaces for three reasons; higher surface areas available for attachment, protection from shear forces and chemical changes that cause preferential physicochemical interactions.

From Figure 1.6 shear forces are lower near a rough and porous surface, and there is a larger surface area to which cells can adhere. Also, pores provide a protected environment for cells to attach and grow (Bryers, 2000). The presence of divalent cations such Ca^{2+} and Mg^{2+} has also considered important in the adhesion process by promote the establishment of ionic bridges resulting in an attractive interaction (Wuertz *et al.,* 2003).
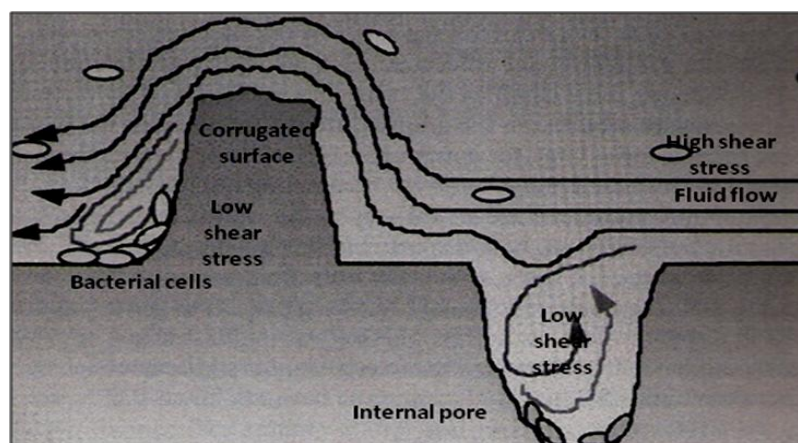
Figure 1.6: Prevalent concept regarding the effect of substratum roughness on bacterial adhesion. Pits and appendages were considered to provide bacteria place of reduced fluid shear (Bryers, 2000).

1.5.5.2 Bacterial cell surface

Bacterial cell surface hydrophobicity is one of the most important factors that govern bacterial adhesion to various surfaces such as the air/water interface, oil/water interface, biomaterials, teeth, animal cells, activated sludge, and different solid surfaces. As reported by Wuertz *et al.,* 2003 several techniques have been used to determine the degree of hydrophobicity of bacterial cells or particulate materials. For material that can be obtained in a flat plate shape, hydrophobicity has very often been expressed in terms of the contact angle formed by a sessile drop of water. In the case of bacterial cells, one of the most frequently used techniques to

assess hydrophobicity is the so called Microbial adherence to hydrocarbons (MATH).

The MATH test is extremely simple in essence: a microbial suspension is mixed with a small volume of hydrocarbon in an appropriate buffer and the subsequent decrease in optical density of the suspension due to adhesion of the microorganisms to the hydrocarbon is measured. Strains adhering well to the hydrocarbon are considered to be "hydrophobic" and strains adhering poorly are considered "hydrophilic". The MATH test is most often performed using n-hexadecane, n-octane, p-xylene and toluene as a hydrocarbon (Busscher *et al.,* 1995).

1.5.5.3 Hydrodynamics (fluid flow)

Fluid flow is an important factor in microbial immobilization. An increase in fluid flow velocity will in the first instance yield increased microbial transport towards a substratum surface (convective diffusion), but at the same time cause an increase in hydrodynamic detachment forces. In principle, two critical shear rates can be distinguished based for: a critical shear rate to prevent adhesion and a critical shear rate to stimulate detachment of already adhering organisms (Bryers, 2000).

1.5.5.4 Temperature and pH

Qureshi *et al.,* 2005 reported that temperature can have an effect on biofilm formation. Temperatures at the high end of a culture's growth range can enhance biofilm formation. Depending upon the species involved, high temperature increases the rate of cell growth, EPS production, and surface adhesion, all of which enhance biofilm formation.

Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes.

Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria (Garrett *et al.,* 2008).

1.5.5.5 Types and amount of nutrients

Bacterial biofilm require a range of nutrients for growth and activity to carry out bioremediation of pollutants. The amount of nutrients present in the medium can affect the rate of biofilm formation. Biofilms tend to form more readily in the presence of ample nutrients (Qureshi *et al.,* 2005). When nutrients are limited, new bacterial is formed at low rates and excessive growth occurs when nutrients are continually supplied. Besides that, clogging due to extensive biomass formation is common with inert support material where nutrient solution is fed at regular interval (Shareefdeen and Singh 2008).

1.5.6   Biofilm reactors in Cr(VI) wastewater treatment

Treatment of Cr (VI)-containing wastewater using biofilm reactors is one of the most active research fields in recent years (Table 1.6). These systems employed potential Cr(VI)-reducing microorganisms immobilized on support material. Biofilm reactors is able to remove Cr(VI) continuously without the intermittent addition of fresh Cr(VI) reducing cells to the system. In these reactors, electron donors were supplied externally to the wastewater depending upon the requirement (Elangovan *et al.,* 2009). Moreover, the biofilm systems have the advantages over the non-living cells that the simultaneous removal of metal and cells can be maintained biochemically active.

Table 1.6: Lists the treatment of Cr(VI)-containing wastewater using biofilm reactors

Types of reactor	Packing material	Types of bacteria	Maximum Cr(VI) reduction and External carbon source	Reference
Packed bed (PVC) Length (L): 40 cm Diameter (D):3cm	River gravel	• *Arthrobacter* CR47 grown in Luria broth. • Cells harvested and resuspended in glucose-minimal medium. • Recirculated for 1 week in reactor	• Cr(VI): 30 ppm • Percentage removal:100 % • External carbon source: glucose	Cordoba *et al.,* 2008
Packed bed (glass) L:12.5cm D: 2.45 cm	Spherical Pyrex glass beads	• *Bacillus* sp grown in Nutrient broth. • Cells harvested and resuspended in NaCl (0.85%). • Recirculated for 6 days with Vogel Bonner broth in reactor.	• Cr(VI): 100 ppm • Percentage removal:100 % • External Carbon source: glucose	Chirwa and Evans, 1997
Packed bed (cilindric tank) L:100cm D: 14.2 cm	Granular activated carbon	• *Arthrobacter viscosus* was grown in Nutrient broth. • Recirculated for 24h at flowrate 250 ml/min in reactor.	• Cr(VI): 10 ppm and 100 ppm • Percentage removal: 100 % and 72% in 24h. • No external carbon source.	Quintelas *et al.,* 2009

Packed bed ;(glass) L:100cm D:8cm	Wood husk	• *Acinetobacter haemolyticus* grown in Nutrient broth. • Recirculated for 24h at flowrate 3 ml/min in reactor.	• Cr(VI): 15 ppm • Percentage removal: 97% in 4.58 h. • External carbon source: Liquid pineapple waste. • Cr(VI): 237 and 320 ppm. • Percentage removal: 99.8–100%. • External carbon source: brown sugar	Zakaria *et al.,* 2007 Ahmad *et al.,* 2009
Packed bed L:60cm D:5 cm	Spiral shaped plastic media	• *Arthobacter rhombi* RE (MTCC7048) grown in nutrient medium consisted of peptone, beef extract, yeast extract, sodium chloride and distilled water. • Cells harvested and recirculated for 24h at flowrate 0.69 ml/min.	• Cr(VI): 20 ppm • Percentage removal: 99% in 24h • External carbon source: molasses	Elangowan *et al.,* 2009

25

1.5.6.1   Support material

In biofilm reactors, selection of the supports for bacterial immobilization is of great importance to obtain a stable biofilm leading to high overall reactor efficiency. The support must favour microbial adhesion, must hard if subjected to high hydrodynamic shear stress, must have a low cost and must be easily available (Wuetz *et al.,* 2003).

The supports can be either natural bioactive or inert (natural and synthetic). The natural bioactive supports are soil, peat, compost and bark which retain water and generally contain enough mineral nutrients to support an initial active microbial population. The natural supports may degrade with time and loose the structure and water-retaining capacity, inducing channelling and the loss of performance

Inert natural and synthetic such as activated carbon, ceramics, sintered glass, wood chips, lava rock, polyurethane foam, vermiculite and perlite do not contain the required nutrients to sustain microbial activity and hence it is necessary to provide them intermittently. On the other hand they are not degraded (Shareefdeen and Singh., 2008).

A rubber wood shaving is a waste by-product of the timber industry that is either used as cooking fuel or a packing material. It is brown and cut to irregular lengths of between 0.35 and 0.21 cm. It is also can be used as a low-cost adsorbent of heavy metals, largely due to its lignocellulosic composition. It is mainly composed of cellulose (45–50%) and lignin (23–30%), both with a capacity for binding metal cations due to hydroxyl, carboxylic and phenolic groups present in their structure. Together with its high surface areas and high concentration of active functional groups, the abundance and availability of rubber wood shavings makes it economically feasible (Zainul *et al.,* 2009).

1.5.6.2   Bioreactor media

In attached-growth biological system using living microorganisms to reduce heavy metal i.e. hexavalent Cr (VI), it is necessary to select nutrient for microorganisms that can both function as nutrient (carbon source) and electron donor. It was also reported that the presence of suitable carbon source is necessary to enhance the Cr(VI) reduction capacity of microbial Cr(VI) reduction. Other than that, Cr(VI)-reducing activity of the microbial cells may vary in the presence of different carbon sources (Orozcoa *et al.,* 2009).

1.6   Microscopy techniques applied for monitoring the development of biofilm

Microbial biofilms associated with surfaces form a heterogeneous architecture with many microniches, which vary continually with time, depending on physicochemical and biological conditions of the environment (Wuertz *et al.,* 2003). Several microscopy techniques have been used to study biofilm development i.e. Scanning Electron Microscope (SEM), Environmental Scanning Electron Microscope (ESEM), Transmission Electron Microscope (TEM), Confocal Scanning Laser Microscope (CSLM). The explanation of microscopy techniques are focused more on SEM and other techniques are summarized in Table 1.7.

Table 1.7: The summary of microscopy techniques applied for monitoring the development of biofilm (Bozzola, 1998; Lei *et al.,* 2009; Priester *et al.,* 2007).

Types	SEM	ESEM	TEM
Specimen Thickness	Specimen can be several centimetre thick and many centimetres across.	Specimen can be several centimetre thick and many centimetres across.	Specimen must be just a few tens to hundreds of nanometres thick.
Sample preparation	Fixation, staining, drying and conductively coating.	No sample preparation. operating it in 'wet' mode—is that it is not necessary to make nonconductive	fixation, staining, drying embedded in a resin and conductively coating,
Mode operation	High vacuum.	Moderate vacuum.	High vacuum.
Application in biofilm study	View structured community of microbial biofilm. However sample preparation can cause sample distortion and leading to the imaging of unwanted artefacts.	Examination of partially hydrated microbial biofilm. Minimizes biofilm dehydration and thus preserves native morphologies including surface structures. EPS are more apparent in ESEM.	TEM is not applicable for observing the extent and form of surface associated growth. However, the use of TEM is suitable for viewing the EPS layer outside bacterial cell.

1.6.1   Scanning Electron Microscope (SEM)

SEM provides one well-developed method to get special qualitative information about morphological of biofilms, the occurrence of extracellular polymers. Lei *et al.,* (2009) studied the formation of biofilm formed on the surfaces of chalcopyrite during the bio-oxidation process with microscopy techniques; SEM and TEM. They found that SEM images showed that this type of structured community of *Acidithiobacillus ferrooxidans* and TEM images indicated that these bacteria were wrapped by EPS (Lei *et al.,* 2009). In SEM, biofilm specimens are prepared by fixation, staining, drying and conductively coating prior to imaging under high vacuum. While any pre-treatment can alter specimen morphology, drying appears to significantly alter biofilms due to EPS polymers collapsing.

In a conventional SEM the sample is placed under high vacuum and must be conductive. This means a sample must be 100 percent water free because water boils in a vacuum, a real problem for many, especially biological samples. The process of drying the samples is tedious, time consuming and may change the sample at the microscopic level. The process of making the sample conductive usually involves coating the sample with a thin metallic layer, something that may also alter the sample, and in some cases make it unusable for other investigations (Priester *et al.,* 2007).

1.7   Problem statement

A previous study has reported the ability of *Acinetobacter haemolyticus* to remove 97% of 15 mg/L Cr(VI)-containing wastewater using laboratory-scale bioreactor (Zakaria *et al.,* 2007).   However, performance of biological Cr(VI) reduction process in biofilm reactors can be affected by factors such as initial hexavalent chromium, flowrate, and appropriate nutrient supplementation.   Hence, parameters that affect Cr(VI) reduction for example initial Cr(VI) concentration, flowrate and nutrient supplementation will be studied.

1.8   Objective of thesis

The aim of this study is to investigate the ability of *Acinetobacter haemolyticus* to form Cr (VI)-reducing biofilms on wood husk in packed-bed bioreactors for bioremediation of Cr (VI)-containing wastewater.

1.9   Scope of thesis

In this study, laboratory-scale bioreactor was setup for Cr(VI)-reduction. Initially, the bioreactor system was inoculated with *A. haemolyticus* using wood husk as support material.   The Cr(VI)-reducing biofilm was used to treat Cr(VI) wastewater using two different carbon sources for Cr(VI) reduction i.e molasses and liquid pineapple waste (LPW). The performance of Cr(VI)-reducing biofilm systems was evaluated using Response Surface Methodology (RSM) to achieve optimum condition for three parameters i.e flowrate, nutrient supplementation and initial metal concentration for complete removal of Cr(VI). Under these optimized conditions, the Cr(VI)-reducing biofilms was operated to treat real Cr(VI) effluent with varying concentration. The formation of *A. haemolyticus* to form Cr(VI)-reducing biofilms and its development in bioreactor was observed using Field Emission Scanning Electron Microscopy (FESEM) together with the activity and distribution in a packed bed bioreactor using colony-forming unit (CFU/mL) measurement

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APPENDIX A

List of publications (journal/article), awards and seminar/ paper presentation during MSc study period (July 2008- June 2010).

Publication and Seminar

Nurfadilah Mohammed and Wan Azlina Ahmad. (2010). Application of Response Surface Methodology (RSM) for optimizing removal of Cr(VI) wastewater using Cr(VI)-reducing biofilm systems. Journal of Fundamental Sciences. 6. 15-21.

Zainul Akmar Zakaria, Marlini Suratman, Nurfadilah Mohammed, Wan Azlina Ahmad.(2009). Chromium (VI) removal from aqueous solution by untreated rubber wood sawdust. Journal of Desalination. 244. 109-121.

Nurfadilah Mohammed and Wan Azlina Ahamd. (2010). Application of Response Surface Methodology (RSM) for optimizing removal of Cr(VI) wastewater using Cr(VI)-reducing biofilm systems. Regional Annual Fundamental Science Sympossium (RAFSS 2010) - Oral Presentation.

Nurfadilah Mohammed and Wan Azlina Ahmad (2010). Removal of chromium from electroplating wastewater using Cr(VI)-reducing biofilm systems. Faculty of Science Postgraduate Conference 2010- Oral Presentation.

Awards and Recognitions

Awarded the National Science Fellowship scholarship to pursue MSc programme in UTM for a period of 2 years (June 2008- June 2010).

Other-related contribution

Participant in the Global Outreach Programme UTM – Halal Science Center, Chulalongkorn University and King Mongkut's Institute of Technology, Bangkok, Thailand on 7-12 November 2010.

Committee member in the Industrial Wastewater Treatment Workshop jointly organized by UTMBacTec and Department of Environment Malaysia, Johor Branch on 2nd June 2010.

Participant in the Design Expert Software Workshop. Faculty of Bioscience and Bioengineering –UTM 2010.

Participant in the Shell Inter-Varsity Paper presentation Contest (S-SPEC 2010)-Poster competition.

Participant in the Scientific Writing Workshop. Research Alliance on Biotechnology –UTM (2009).