# THE REMOVAL OF CHROMIUM (VI) AND PHENOL FROM INDUSTRIAL WASTE WATERS MEDIATED BY *Acinetobacter haemolyticus*

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**This page is dedicated to**
**my beloved family and friends.**
**Love you all.**

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***In The Name Of Allah, Most Gracious, Most Merciful***

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## ABSTRACT

Contamination of the environment due to heavy metals and organic waste is a serious problem nowadays. These types of contaminants are toxic and can be dangerous to human health. Heavy metal contaminants include copper, lead and hexavalent chromium while phenol, benzene and toluene are examples of organic pollutants. A treatment of these contaminated waste chemicals must be carried out before they can be released into the environment. Conventional techniques for the treatment of these contaminants are not cost - effective as they are chemical and energy - intensive. Thus, the emerging biological methods for treating these wastes i.e. bioremediation, is more favorable. In this study, a locally isolated bacterium, *Acinetobacter haemolyticus* was chosen for the removal of Cr (VI) and phenol using batch and column systems. Cr (VI) and phenol removal studies were carried out separately and simultaneously using batch and column systems. When treated separately, about 90% and 95% of Cr (VI) could be removed using the batch and column system, respectively. However, for phenol, 30 – 65% and 50 – 80% of the organic compound could be removed using the batch and column system, respectively. In the simultaneous treatment process, the mixed waste effluent of Cr (VI) and phenol was also treated using the same system. About 70% and 85% of Cr (VI) could be removed in the batch and column system respectively while for phenol, about 30 – 40% was removed using the batch system and 50 – 60% for the column system. The batch system also required a longer retention time compared to the column system. This study has demonstrated the ability of *Acinetobacter haemolitycus* to remove Cr (VI) and phenol both from separate and mixed effluent, thus offering a promising alternative method for the detoxification of both pollutants prior to releasing the effluent into the environment.

## ABSTRAK

Pencemaran yang disebabkan oleh sisa – sisa logam berat dan organik adalah satu masalah serius ketika ini. Bahan – bahan pencemar seperti ini adalah berbahaya dan membimbangkan berikutan kesan toksiknya ke atas kesihatan manusia. Bahan pencemar logam berat termasuk kuprum, plumbum dan kromium heksavalen manakala fenol, benzena dan toluena pula merupakan contoh sisa – sisa organik. Rawatan untuk sisa air yang mengandungi bahan – bahan pencemar mesti dijalankan sebelum ianya dilepaskan ke alam sekitar. Teknik konvensional yang digunakan untuk rawatan bahan – bahan pencemar ini adalah tidak kos efektif disebabkan penggunaan bahan kimia dan tenaga yang intensif. Oleh itu, kemunculan teknik biologikal di dalam merawat sisa – sisa ini seperti bioremediasi adalah lebih diutamakan. Dalam kajian ini, bakteria pencilan tempatan, *Acinetobacter haemolyticus* telah dipilih untuk menyingkirkan Cr (VI) dan fenol menggunakan sistem kelompok dan kolum. Kajian penyingkiran Cr (VI) dan fenol dijalankan secara berasingan dan serentak menggunakan sistem yang sama. Untuk Cr (VI), 90% dan 95% Cr (VI) telah disingkirkan menggunakan kedua – dua sistem tersebut. Dalam kajian penyingkiran fenol, 30 – 65% dan 50 – 80% fenol telah disingkirkan menggunakan sistem yang sama. Dalam proses rawatan secara serentak, sisa campuran Cr (VI) dan fenol juga dirawat menggunakan sistem ini. Dengan rawatan secara serentak, 70% dan 85% Cr (VI) boleh disingkirkan melalui kedua – dua sistem manakala untuk fenol, 30 – 40% telah disingkirkan menggunakan sistem kelompok dan 50 – 60% pula untuk sistem kolum. Sistem kelompok memerlukan masa remediasi yang lebih lama jika dibandingkan dengan sistem kolum. Dari kajian ini, *Acinetobacter haemolyticus* menunjukkan kemampuan untuk mengasingkan Cr (VI) dan fenol secara berasingan atau serentak, lalu memberikan kaedah alternatif untuk penyahtoksikan kedua – dua pencemar ini.

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| | | |
|---|---|---|
| *A. haemolyticus* | - | *Acinetobacter haemolyticus* |
| cm | - | centimeter |
| Cr (VI) | - | hexavalent chromium |
| Cr (III) | - | trivalent chromium |
| CFU | - | colony forming unit |
| DPC | - | 1,5-diphenylcarbazide |
| g | - | gram |
| kPa | - | kilopascal |
| L | - | liter |
| M | - | molar |
| mg | - | milligram |
| mL | - | milliliter |
| mM | - | millimolar |
| N | - | normality |
| Nm | - | nanometer |
| $OD_{600}$ | - | optical density at 600 nm |
| ppb | - | part per billion |
| ppm | - | part per million |
| rpm | - | revolution per minute |
| µg | - | microgram |
| µm | - | micrometer |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Water Pollution

Water pollution can be described as a range of adverse effects on lakes, rivers, oceans and also ground water which is caused by human activities. Many factors can contribute to water pollution. Industrial waste is one of the sources of water pollution when the waste water is released into the environment. Industrial discharge contains a variety of pollutants such as heavy metals, organic waste, oil and solids.

### 1.1.1 Heavy Metal Pollution

Exposure to heavy metals is of concern as it can cause deleterious effects to human. Heavy metals are transition elements with incompletely filled *d* orbital with a density above 5 $g/cm^3$, which provides heavy metals cation the ability to form complex compounds. At higher concentration, heavy metal ions can form unspecific complex compounds in cell, which will lead to the toxic effects (Nies, 1999).

Heavy metals and their compounds can be adsorbed through the air passage and also alimentary canal with food and drinking water. These heavy metals are generated by certain industries such as textile, electronic and fertilizer industries which needed to be treated before being released into the environment. Unlike organic contaminants which are easily degradable, heavy metal contaminants are

stable and will still remain in the industrial effluent until it has been treated. These heavy metals have an etiological effect on hypertension, cancer and lung diseases. Examples of the heavy metals that are dangerous to humans are Hg, Co, Cd, Cr and others (Thacker *et. al*., 2006a).

### 1.1.2 Organic Waste Contamination

Contamination caused by organic material is frequently observed in the water system. Organic contamination can originate from many sources such as household (detergent, washing liquid), cosmetic industry, petroleum industry, agriculture, mining industry and many more (Zawala *et. al*., 2007).

Some organic compounds are dangerous and can be harmful to organisms because of the toxicity effect. Also, a few of the organic chemicals are mutagenic and carcinogenic such as residues of polycyclic aromatic hydrocarbon (PAH) (Chen *et. al*., 2005).

Apart from PAH, organochlorine pesticides (OCPs), phtalic acid ester (PAEs), hexachlorocyclohexanes (HCHs) and dichlorodiphenyltrichloroethane (DDTs) are called the persistent organic pollutants (POP) and are always used in the pesticides industry. Other organic pollutants that can be found in the water system are phenol, benzene, tetrahydrofuran and many more (Ma *et. al.*, 2003).

### 1.1.3 Water Quality

To prevent contamination of the water system, many authorities around the world have introduced laws and regulations. Enforcements such as laws and regulation is necessary to ensure that contamination from waste water or industrial effluent will not occur in the water systems.

In Malaysia, local authorities have a set of by - laws which regulate industries to meet the limits of effluent discharged before discharging the waste water. Table 1.1 shows the parameter limits of effluents for standard A and B (Environmental Quality Act and Regulation Handbook, 1996).

**Table 1.1**: Parameter Limits of Effluent for Standard A and B.

| | Parameter | Unit | Standard | |
|---|---|---|---|---|
| | | | A | B |
| (i) | Temperature | $^{o}C$ | 40 | 40 |
| (ii) | pH value | - | 6.0 – 9.0 | 5.5 – 9.0 |
| (iii) | $BOD_5$ at $20^{o}C$ | mg/L | 20 | 50 |
| (iv) | COD | mg/L | 50 | 100 |
| (v) | Suspended solids | mg/L | 50 | 100 |
| (vi) | Mercury | mg/L | 0.005 | 0.05 |
| (vii) | Cadmium | mg/L | 0.01 | 0.02 |
| (viii) | Chromium, hexavalent | mg/L | 0.05 | 0.05 |
| (ix) | Arsenic | mg/L | 0.05 | 0.10 |
| (x) | Cyanide | mg/L | 0.05 | 0.10 |
| (xi) | Lead | mg/L | 0.10 | 0.5 |
| (xii) | Chromium, trivalent | mg/L | 0.20 | 1.0 |
| (xiii) | Copper | mg/L | 0.20 | 1.0 |
| (xiv) | Manganese | mg/L | 0.20 | 1.0 |
| (xv) | Nickel | mg/L | 0.20 | 1.0 |
| (xvi) | Tin | mg/L | 0.20 | 1.0 |
| (xvii) | Zinc | mg/L | 2.00 | 2.0 |
| (xviii) | Boron | mg/L | 1.00 | 4.0 |
| (xix) | Iron | mg/L | 1.00 | 5.0 |
| (xx) | Phenol | mg/L | 0.001 | 1.0 |
| (xxi) | Free chlorine | mg/L | 1.0 | 2.0 |
| (xxii) | Sulphide | mg/L | 0.50 | 0.5 |
| (xxiii) | Oil and grease | mg/L | Not detectable | 10.0 |

Besides the limits in industrial discharge, the Malaysian government also formulated a water quality standard for marine water according to ASEAN Marine Quality Criteria as shown in Table 1.2 in order to protect aquatic life.

**Table 1.2**: ASEAN Marine Quality Criteria

| **Parameter** | **Criteria Values** | **Note** |
|---|---|---|
| Ammonia ($NH_3$-N) | 70 µg/L | |
| Cadmium | 10 µg/L | |
| Chromium (VI) | 50 µg/L | Criteria value proposed by CPMSII is 48 µg /L. The Meeting recommended to adopt 50 µg /L, following the existing national standards of member countries |
| Copper | 8 µg/L | As the proposed value 2.9 µg/L is too stringent, the Meeting agreed to use round-up value of 7.7 µg /L, the product of the lowest LOEC from a chronic study 77 µg /L for reproduction for *Mysidopsis bahia* and a safety factor of 0.1 |
| Temperature | Increase not more than 2°C above the maximum ambient temperature | |
| Cyanide | 7 µg/L | |
| Dissolved oxygen | 4 mg/L | |
| Lead | 8.5 µg/L | |
| Mercury | 0.16 µg/L | |
| Nitrate ($NO_3$-N) | 60 µg/L | A single criteria value should be derived for nitrate and nitrite combined in future. |
| Nitrite ($NO_2$-N) | 55 µg/L | |
| Oil and grease | 0.14 mg/L | Other related parameter, e.g. PAH, should be proposed in the future |
| Total phenol | 0.12 mg/L | |
| Phosphate | 15 µg/L (Coastal<br>45 µg/L (Estuarine) | |
| Tributyltin | 10 mg/L | |
| Total suspended solids | Permissible 10% maximum increase over seasonal average concentration | |

For Human Health Protection

| **Parameter** | **Criteria Values** | **Note** |
|---|---|---|
| Bacteria | 100 faecal coliform/100 mL<br>35 enterococci/100 mL | Coastal water quality for recreational activities |

## 1.2 Chromium

Chromium is one of the metals which are essential for growth of many organisms. Pure chromium is a steel-gray, lustrous and hard crystalline metal which occupies the $24^{th}$ position in the Periodic Table and belongs to group VIB. Chromium has a melting and boiling point of $1875^{o}C$ and $2680^{o}C$ respectively (U.S EPA, 1984).

Chromium has nine valency states, ranging from -2 to +6. Amongst these valency states, only the trivalent chromium and hexavalent chromium show significant occurance in the environment. Trivalent chromium is the most stable form of the element as it forms kinetically inert complexes with water, ammonia and some other materials. Hexavalent chromium is the most commercially and environmentally important state for chromium and always linked to oxygen, which gives the property of an oxidizing agent. The ground state electron configuration for chromium is $[Ar].3d^5.4s^1$ (Guertin *et al*., 2005).

Trivalent chromium is important as it is required for the metabolism of fat and glucose and also for proper functioning of insulin (Thacker *et. al.*, 2006b). Even though chromium is defined as a micronutrient, at higher concentration it is considered toxic. This metal is considered as a priority pollutant by the US EPA (Thacker *et. al.*, 2006a).

### 1.2.1 Sources of Chromium

Pure chromium is not naturally found. Chromium occurs primarily in nature as a member of spinel mineral group in the form of chromite ore or chrome iron ore. The ideal chromite ore is the one with the composition of $FeO.Cr_2O_3$ which contains about 46% of chromium (US EPA, 1984).

World's chromite supply comes from South Africa, Philippines, Finland and Russia. Chromite ore deposits are also found in other countries such as United States of America, but the concentration is so low and is not economically feasible for the mining process.

### 1.2.2 Chromium in the Environment

Chromium is being released into the environment through 2 major routes. The first route is via direct processes that either produce or consume chromium or use chromium compounds to manufacture products. Examples of such processes are like leather tanning, refractory production, chromium plating, steel production and others (US EPA, 1984).

The second route is the indirect emission through processes that do not produce chromium or its compounds but chromium was present as an impurity in the raw or materials used. For example, the chromium released during combustion of fossil fuels because it was present as a constituent of the fuel burned (US EPA, 1984). Other examples of indirect processes are cement production, asbestos mining and sewage sludge incineration.

Nowadays, chromium has been widely used in the production of pigments, fungicides, magnetic tapes, catalysts and in leather tanning. Significant amount of this heavy metal is released into the environment by these industries (Thacker *et. al.*, 2006a).

### 1.2.3 Hexavalent Chromium and Its Toxicity

Hexavalent chromium is one of the valence states of chromium. Hexavalent chromium is considered a dangerous pollutant, as it is believed to be mutagenic and carcinogenic. Compared to trivalent chromium which is less soluble, hexavalent

chromium is easily dissolved and in one hundred times more toxic than the trivalent state (Liu *et. al.*, 2006). When dissolved in water, hexavalent chromium is present in the forms of divalent oxyanions, $CrO^{2-}_4$ or dichromate, $Cr_2O^{2-}_7$ depending on the pH of the solution (Shen and Wang, 1994). According to US EPA, hexavalent chromium is one of the 17 chemicals posing the greatest threat to humans (Cheung and Hu, 2007).

Due to leakage, poor storage and improper disposal, hexavalent chromium has become one of the most frequently detected contaminants at waste dumping sites. This has led to increased awareness about the toxicity of this metal and its danger to humans. Hexavalent chromium may cause lung cancer, chromate ulcer, perforation of nasal septum and kidney damage (Thacker *et. al.*, 2006a).

The permissible levels of hexavalent chromium especially in industrial effluents and water systems are set by the authorities to meet the regulated standards. In Malaysia, the level of chromium in industrial discharge is set at 0.20 mg/l (standard A) and 1.0 mg/l (standard B) as shown in Table 1.1 (page 3). In marine water enviroment, the presence of hexavalent chromium is limited to 50 µg/L as shown in Table 1.2 (page 4).

## 1.3 Phenol

Phenol is an aromatic compound with an OH group attached to the benzene ring structure. It is also known as carbolic acid or hydroxybenzene. Phenol can be found in both solid and liquid form. In crystal form, phenol forms is white or colorless and sometimes pink. It can also form a thick liquid (ATSDR, 2006). Phenol has a characteristic acrid smell and sharp burning taste. In liquid state, phenol is clear, colorless, and has a low viscosity. Its melting point is 43$^{o}$C while the boiling point is 182$^{o}$C. Phenol is highly soluble in water and in most organic solvents. The structure of phenol is shown in Fig. 1.1.

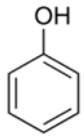


**Figure 1.1**: Structure of phenol

Phenol is usually used as solvent and starting material in industries such as refineries, pesticides, pharmaceutical, pulp and paper mills and others.

### 1.3.1 Sources of phenol

There are three sources of phenol in the environment, i.e. natural source, man made source and endogenous source.

In nature, phenol can be produced from the decomposition of organic material such as burnt woods, animal manure and others. Phenol also can occur as a constituent of coal tar in the soil environment (Murray, 1977).

Phenol can be produced from certain activities such as fossil fuel extraction and chemical manufacturing process. Oil refineries, petrochemical plants, coking plants and phenol resin plant are among the industries that produce and release phenol together with other related aromatic compounds into the environment (Haleem *et al*., 2002).

Endogenous source is an important additional source of phenol. Phenol can be formed from several xenobiotics such as benzene under the influence of light. (Hoshino and Akimoto, 1978).

### 1.3.2 Phenol in the environment

Phenol and its derivatives are among the most common pollutants in rivers, industrial effluents and landfill runoff water.

Phenol can also be released into the atmosphere mainly from the processing facilities. During manufacturing, phenol is released from storage tank vents during transportation. Phenol released into the atmosphere are also linked to wood burning, car exhaust, emission from waste incinerator plant and cigarette smoke (Haleem *et al.*, 2002).

Processing facilities are also major sources of phenol that eventually end up in the water system. Most industries such as resins, plastics, fiber and paper industries release phenol into the water system through waste effluents which are slightly treated or untreated. Many reports conclude that phenol can be detected in certain effluents discharged from industries (Tuah, 2006).

In soil, phenol is believed to have come from manufacturing processes. During production, transportation and loading of phenol, spills can occur which will contaminate the soil environment. Phenol also can be released into the soil when it leaches from hazardous chemical dumping wastes site or from landfill (Xing *et al.*, 1994).

### 1.3.3 Toxicity of phenol

Phenol and its derivatives when released into the environment through industrial effluents are toxic and persistent. They accumulate in the environment and affect organism living in the surrounding areas. Phenolic compounds are dangerous and toxic upon inhalation, contact or ingestion, even at low concentration (Yang and Lee, 2001).

At concentrations higher than 50 ppb, phenol is toxic to some of the aquatic life. For human, an oral dose of 1 g may be lethal. Exposure and continuous ingestion of phenol can cause mouth sores, diarrhea, excretion of dark urine and impaired vision (Goldfrank, 2002). The chemical also affects the nervous system and organs. In some reports, people living near rivers contaminated with phenol have been shown to suffer from some health problems such as headache, nausea, vomiting, diarrhea and abdominal pain (ATSDR, 1998).

Repeated exposure of phenol on skin may result in onychronosis (yellowing of the skin), skin irration and eruption as well as dermal inflammation and necrosis. Inhalation may lead to gastrointestinal effects such as anorexia, weight loss, excess production of saliva, muscle pain and general weakness (ATSDR, 1998).

As phenol is considered to be toxic to many living things and receives attention from various authorities, the discharge limit for phenol must be set. For Malaysia, the limit for phenol in industrial discharge is 0.001 mg/l (standard A) and 1.0 mg/l (standard B) as shown in Table 1.1 (page 3), while based on Table 1.2 (page 4), the limit of phenol level in marine water is 0.12 mg/l (Environmental Quality Act and Regulation Handbook, 1996).

## 1.4 Treatment of heavy metal contaminants

### 1.4.1 Conventional method

Heavy metal pollution is a critical environmental issue. The treatment of potential pollutants is carried in several ways such as precipitation, ion exchange and adsorption on alum, kaolinite and ash. Conventional physical and chemical methods for treating heavy metals are not cost effective and are not recommended for full scale treatment. Treating metal-contaminated industrial effluents using physico-chemical methods are relatively chemical and energy intensive. The maximum achievable chromium removal is not sufficient to meet the desired level of treated effluent quality standards for the disposal by the industries. Treatment of industrial

effluent using conventional method will also generate chemical sludge as a by - product, which also can cause contamination (Zhu *et al.*, 2008).

### 1.4.2 Bioremediation

The use of biological methods to treat heavy metal-contaminated waste is an emerging area of environmental conservation. Biological treatments for example bioremediation and biosorption could reduce the cost for chemicals and energy used compared to conventional methods.

Bioremediation is a process which uses living microorganisms to eliminate or reduce environmental hazard caused by toxic chemicals or waste. The basis of bioremediation is the utilization of naturally occurring or genetically engineered bacteria to transform organic and inorganic compounds to less harmful by - products.

For example, in the remediation of hexavalent chromium in contaminated environments, the reduction of chromium from hexavalent state to the trivalent state is important. Bacterial reduction of hexavalent chromium offers a potential cost effective remediation strategy (Rege *et. al*., 1997). Several bacterial species have been shown to have the chromate reducing ability, which can lead to an economical and environmentally treatment process.

#### 1.4.2.1 Bacteria used in bioremediation.

Extensive research has been conducted using several types of bacteria to treat industrial waste especially waste containing heavy metals. Among the bacteria used are species from the genera *Arthrobacter*, *Bacillus*, *Enterobacter* and *Escherichia* (Zhu *et. al*., 2008).

#### 1.4.2.2 Bacteria used in remediation of hexavalent chromium.

Many workers have reported the ability of bacteria to reduce hexavalent chromium to trivalent chromium (Table 1.3).

**Table 1.3**: Example of hexavalent chromium reducing bacteria.

| Bacteria | Reference |
|---|---|
| *A. haemolyticus* | Zakaria *et al*., 2006 |
| *Achromobacter* sp. Strain Ch 1 | Zhu *et al*., 2008 |
| *Bacillus* sp. | Liu *et al*., 2006 |
| *Providencia* sp. | Thacker *et al*., 2006b |

*Providencia* sp., a gram negative bacteria, can grow and reduce Cr (VI) at concentrations ranging from 100 – 400 mg/L. The other reported strains are *Bacillus* sp. which can reduce 0.33 mM $CrO_4^{2-}$ within 22 hours, *Achromobacter* sp. strain Ch 1 with a maximum reduction capacity of 54.2 mM and *A. haemolyticus,* which can tolerate and reduce Cr (VI) up to 100 mg/L.

### 1.4.3 Microbial reduction of hexavalent chromium

The other dominant form of chromium which is less toxic and soluble is Cr (III). The insolubility of Cr (III) can facilitate its precipitation and removal, thus the biotransformation of Cr (VI) to Cr (III) has been considered as an alternative way for the treatment of Cr (VI) – containing waste water (Cheung and Hu, 2007).

After the discovery of microbes with the ability to reduce $Cr^{6+}$ in the 1970s, studies on $Cr^{6+}$ reduction has been intensified. These studies include the search for $Cr^{6+}$ reducing bacteria in both aerobic and anaerobic conditions.

### 1.4.3.1 Aerobic Cr (VI) reduction

Many reports have been published on the aerobic reduction of Cr (VI) by bacteria such as *Arthrobacter* sp., *Bacillus* sp. (Megharaj *et al*., 2002), *Streptomyces* sp. (Laxman and More, 2002) and *Brucella* sp. (Thacker *et al*., 2006a).

The mechanism of aerobic reduction is usually associated with the soluble fraction of the cell which utilizes NADH as the electron donor (Thacker *et al*., 2006b). In aerobic condition, $Cr^{6+}$ reduction occurs as two or three step processes. It starts with $Cr^{6+}$ reduced to short lived intermediates $Cr^{5+}$ and/or $Cr^{4+}$ before further reduction to a stable end product, $Cr^{3+}$. But it is unclear whether the reduction of $Cr^{5+}$ to $Cr^{4+}$ and $Cr^{4+}$ to $Cr^{3+}$ were spontaneous or enzyme - mediated (Czako-Ver *et al*., 1999).

From the report by Cheung and Hu (2007), Cr (VI) reductases ChrR (identified from *Ps. Putida* MK 1) reduces $Cr^{6+}$ with one electron shuttle to generate $Cr^{5+}$, followed by two electron transfer to form $Cr^{3+}$. Reducing enzyme, YieF (identified from *E. coli* chromosome), a unique enzyme which can catalyze the reduction of $Cr^{6+}$ to $Cr^{3+}$ through four electron transfer, where three electrons were consumed in reducing $Cr^{6+}$ and one electron transferred to oxygen.

### 1.4.3.1 Anaerobic Cr (VI) reduction

A number of bacteria were found to have the ability to reduce Cr (VI) in the absence of oxygen. *E. cloacae*, a reducing facultative anaerobe, is an example where in the presence of oxygen, the Cr (VI) reduction is inhibited. However, the reduction process is resumed when the oxygen content is removed (Laxman and More, 2002). Other reported strains for the anaerobic reduction are *Shewanella putrefaciens* MR -1 (Myers *et. al*., 2000), *Desulfovibrio desulfuricans* (Tucker *et al*., 1998) and *Shewanella oneidensis* (Daulton *et al*., 2006).

Anaerobic Cr (VI) reduction, was initially considered as a fortuitous process since it provides no energy for the microbial growth. However, a SRB isolate was discovered later, which can utilize the energy generated from the anaerobic reduction for its growth. In the absence of oxygen, Cr (VI) can act as the terminal electron acceptor in the respiratory chain for a large array of electron donors such as protein, carbohydrate and hydrogen (Cheung and Hu, 2007).

## 1.5 Organic waste treatment

### 1.5.1 Conventional method

Like heavy metals, organic wastes are also physic – chemically treated using filtration, flotation, ion exchange and adsorption. However, these techniques are costly and energy intensive, beside used large amount of chemical and create secondary pollution.

### 1.5.2 Biodegradation

Biodegradation can be described as a natural way of recycling waste. It involves the breaking down of organic matter into nutrients that can be used by other organisms. "Degradation" means decay while "bio" prefix means that the decay process is carried out by assortment of bacteria, fungi, algae, insect and other organisms which consume certain materials and will be recycled into another form.

Biodegradation processes vary greatly in extent, but in the end, the final product of degradation is almost the same, which is carbon dioxide or methane. Organic material can be degraded in both aerobic and anaerobic conditions.

The term biodegradation is often related to certain aspects such as ecology, waste management and environmental remediation. Biodegradation is the aspect of waste management and can be defined as the breaking down of organic contaminants into smaller compounds which can also reduce toxicity and leads to a solution in solving pollution problems. Biodegradation is a key process in the natural attenuation of contaminants in areas contaminated with hazardous chemicals.

### 1.5.2.1 Microorganisms involved in biodegradation

Biodegradation process can be carried out by many types of microorganisms including bacteria, fungi, algae and some organisms such as worms. In natural environment, the rates of degradation processes depend on certain factors; physical, chemical and biological factors which vary among the different ecosystems (van Agteren et al., 1998).

### 1.5.2.2 Microorganisms in degradation of phenol

Phenol degrading microorganisms have been reported as early as 1908 (Evans, 1947). Phenol can be degraded by many species belonging to various genera. Many reports have been published regarding the degradation of phenol by microorganisms including bacteria, fungi, algae and yeast (Table 1.4).

**Table 1.4**: Examples of phenol degrading microorganisms

| Microorganism | Reference |
|---|---|
| A. Bacteria | |
| *A. calcoaceticus* | Nakamura and Sawada, 2000 |
| *Brevibacillus spp. Strain P-6* | Yang and Lee, 2001. |
| *Pseudomonads spp.* | De Lipthay *et al*., 1999 |
| *Rodococcus spp* | Margesin *et al*., 2004 |
| B. Fungi | |
| *Coprinus spp.* | Guiraud *et al.*, 1999 |
| *Geotrichum candidum* | Garcia *et al.*, 2000 |
| C. Yeast | |
| *Candida tropicalis* | Bastos *et al*., 2000 |
| D. Algae | |
| *Ochromonas danica* | Semple and Cain, 1995 |

### 1.5.3 Phenol Biodegradation

Phenol can be degraded by bacteria under two conditions, aerobic or anaerobic. In aerobic condition, phenol is degraded into carbon dioxide while under anaerobic condition is degraded into carbon dioxide or methane (Tay and Show, 2006). During the degradation process, many intermediates are produced in the pathway before the final product, carbon dioxide or methane is produced. Intermediates in the phenol degradation process are benzoate, catechol, cis, cis – muconate, β – ketodipate, succinate, acetate (Knoll and Winter, 1987).

Phenol can be degraded in its free form or after adsorption onto soil or sediment, which may result in lower rate of degradation process (Knoll and Winter, 1987). According to a report by Howard (1989), the highest rate for aerobic phenol degradation is in sewage (>90% in 8 hours of retention time), followed by fresh water (biodegradation less than 1 day), soil (complete degradation in 2 – 5 days) and sea water (50% degradation in 9 days). In anaerobic degradation, the rate is lower than in aerobic condition (Baker and Mayfield, 1980).

#### 1.5.3.1 Aerobic degradation

In aerobic condition, oxygen will be used as the electron acceptor in electron transfer process. During the transfer of electron between the electron donor and acceptor, a substrate is essential to create and maintain biomass. In this case, phenol is the main substrate and must be available in order to create active biomass in the degradation process. From the study by Ritmann and Sàez (1993), once the active biomass is present, any biotransformation activity can take place with the microorgasnism which possess enzyme that can catalyze the reaction. These enzymes usually define the range of substrates that can be transformed through certain metabolic pathways (Pieper and Reineke, 2000).

In aerobic metabolism, the first step is the hydroxylation of phenol into catechol by phenol hydroxylase, an NADPH dependent flavoprotein. During the hydroxylation, the aromatic ring will be incorporated with one oxygen atom to form catechol. The second step is the catalyzation process by catechol 1,2 – dioxygenase or catechol 2,3 – dioxygenase. After a series of subsequent steps, the product will be incorporated into the Kreb cycle or tricarboxylic acid cycle (TCA). It has been established that the degradation process of phenolic compound is metabolized by various strain in either the *meta* or *ortho* cleavage pathways (Shingler, 1996). The aerobic degradation of phenol is shown in Figure 1.2.

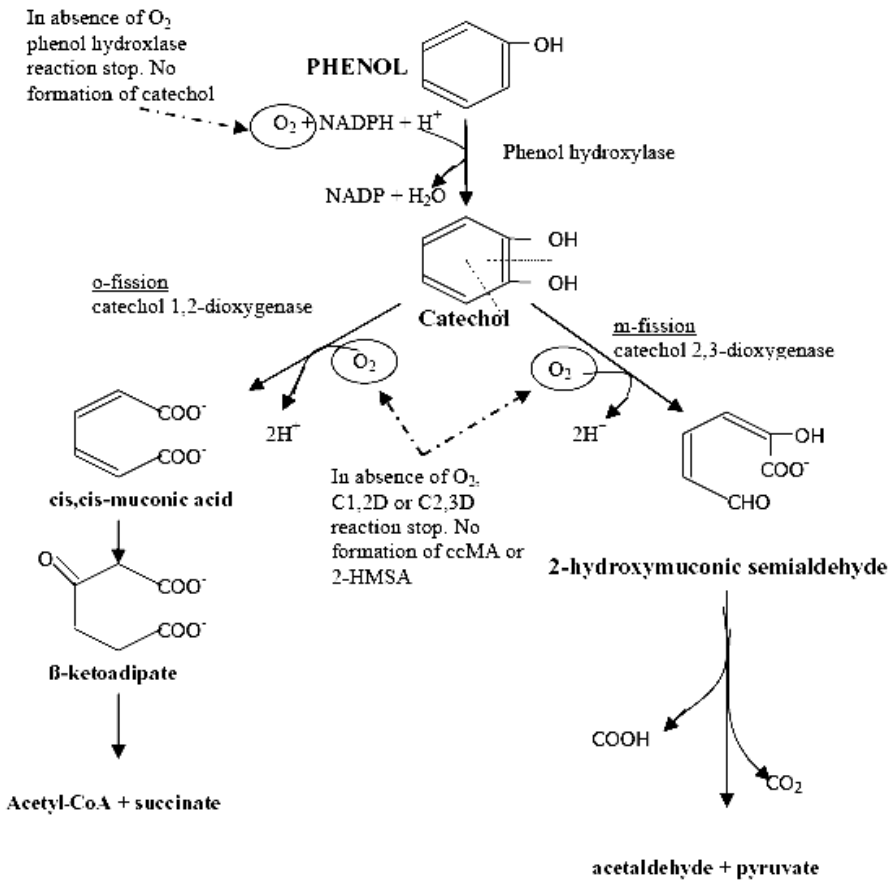


**Figure 1.2**: The main pathway of phenol degradation under aerobic condition (*ortho* and *meta* fission of the benzene ring) (Krug and Ziegler, 1985). (Note: C1, 2D – catechol 1, 2 – dioxygenase and C2, 3D – catechol 2, 3 – dioxygenase).

Bacteria normally degrade phenol via the *meta* pathway; however some bacteria can degrade phenol via both the *meta* and *ortho* pathway. An example of this is *Pseudomonas* sp. (De Lipthay *et al*., 1999).

### 1.5.3.2 Anaerobic degradation

Phenolic compounds are one of the most common pollutants in landfill. Landfill is believed to be a habitat for anaerobic microbial species which can degrade many compounds such as toluene and phenol. In landfill area, anaerobic condition

occurs during decomposition of the waste materials and carbon dioxide will be the electron acceptor (Barlaz, 1996).

Various bacteria have been reported to degrade phenol anaerobically (van Schie and Young, 1998; Shinoda *et al*., 2000). Phenol is carboxylated to 4 – hydroxybenzoate and growth of the bacteria on phenol was dependent on the presence of carbon dioxide (Tschech and Fuchs, 1987). Benzoate is a key intermediate for the degradation process for aromatic compounds such as phenol.

The carboxylation process of phenol is a 2 step process involving the formation of phenylphosphate as the first intermediate. Phenylphosphate is postulated as the substrate for second enzyme (E2 – phenylphosphate carboxylase) which requires $Mn^{2+}$ and catalyze the carboxylation of phenylphosphate to 4 – hydroxybenzoate (Lack and Fuch, 1992). The overall anaerobic degradation process of aromatic compound by denitrifying bacteria is shown in Figure 1.3.

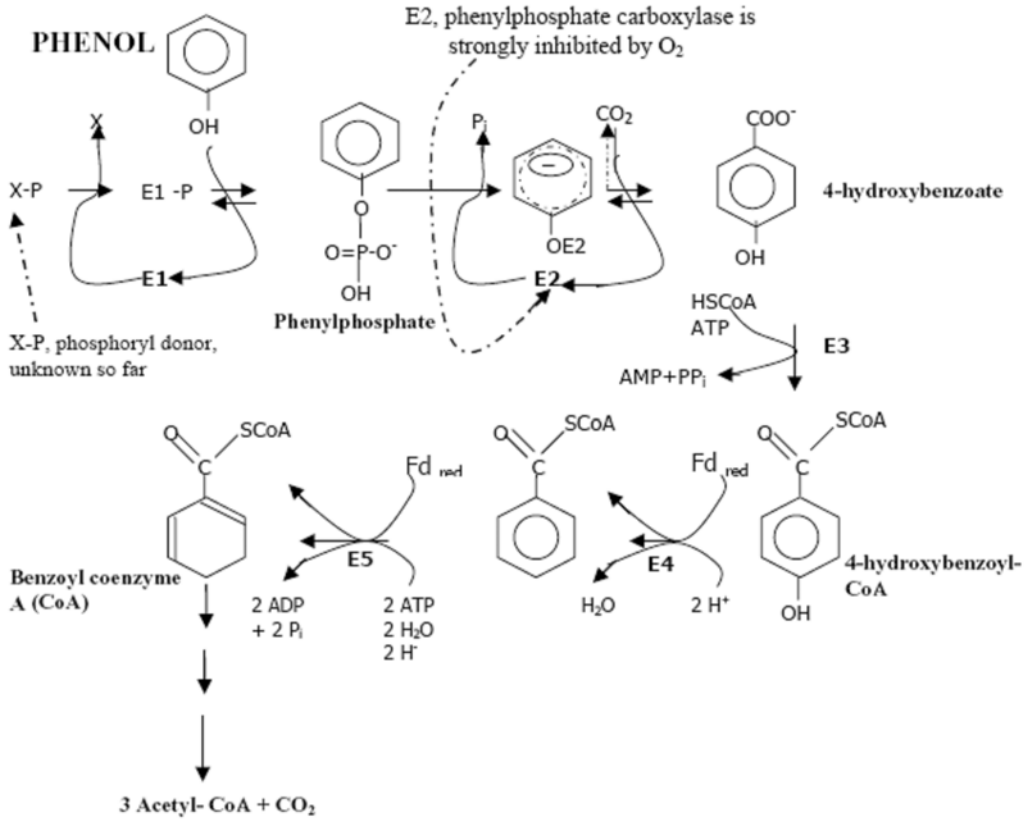


**Figure 1.3**: Anaerobic phenol degradation process of aromatic compound by the denitrifying bacteria *T. aromatic*. E1 – phenylphosphate synthesis; E2 – phenylphosphate carboxylase; E3 – 4 – hydroxybenzoate CoA ligase; E4 – 4 – hydroxybenzoyl CoA reductase; E5 – benzoyl CoA reductase; Fd – ferredoxin; X – P – phophoryl donor (Breinig *et al.*, 2000).

## 1.6 Bioaccumulation

### 1.6.1 Overview of the bioaccumulation process

Bioaccumulation can be defined as a process by which organisms accumulate chemicals both directly from the abiotic environment such as soil, water and soil or from dietary sources (Hodgson, 2004). It is commonly known that the organisms

living in the environment have the ability to adsorb chemicals from their surrounding via their gills, outer protective layer or direct ingestion of contaminated food or water. Bioaccumulation occurs either because the chemicals are taken up faster than it can be used, or due to the inability to break down or metabolize the chemicals. Usually, the level of such chemical contaminants in the environment is quite low. These adsorbed chemicals may be metabolized, excreted or stored within the fatty acid tissue layer of the organism (Robinson and Thorn, 2001).

In bioaccumulation, several steps are involved including direct partitioning between air and water with the living organisms, or more complex transfer processes where the compound is taken up with food and transport internally to other parts of the organism (Schwarzenbach *et al*., 2002). There are several steps involved in the uptake of chemicals by the microorganisms. The process involves the passage of compound through biological membrane, mediated by a carrier or as a single solute. The major uptake process for many organic compounds and metals is the passive diffusion, where the driving force involves the fugacity difference between water and organism (van Leeuween and Vermeire, 2007). Fugacity can be described as the tendency of a substance to prefer one phase (liquid, solid or gas) over the other phase.

Therefore, bioaccumulation is best described through the concept of fugacity. Organisms have the tendency or higher capacity to store xenobiotic compound per unit volume than water. For example, metals which can bind to protein (metallothionein), can be stored in relatively high concentration within the organism. Usually, organic compounds are stored in lipids whereas organometals, can be stored in either lipids or protein. Thus the bioaccumulation may reach high concentrations in the organism (van Leeuween and Vermeire, 2007).

### 1.6.2 Bioaccumulation of toxic compounds

In the environment, there are various sources of toxic pollutants, which living organisms can accumulate directly or indirectly. One example of chemical

contaminant that can be accumulated by organisms is pesticide. Besides that, many organic compounds originating from certain industries such as automobile, agriculture and pulp and paper industries can also be released into the environment. These contaminants can spread into the soil or small creeks, and eventually find their way into water bodies such as rivers, estuaries and the ocean. Once the toxic pollutants are in the soil or water, they can easily enter the food chain. There are several examples of toxic compounds that can accumulate in living organisms e.g. mercury, silver as well as insecticide such as DDT and pentachlorophenol (Connell, 1990).

### 1.6.3 Bioaccumulation of phenolic compounds

The presence of organic pollutants in the environment which originated from various sources could affect living organisms. Some of these contaminants have the potentials to be accumulated by aquatic organisms, either directly from the water or via the dietary route of aquatic food chains (Newsted, 2004). Examples of the pollutants which can be accumulated by organisms include phenol and its related compounds. Previous studies reported the bioaccumulation of phenolic compounds, such as the accumulation of phenol by green algae, *Selenastrum capricornutum* (Newsted, 2004) or 2, 4 – dichlorophenol by fresh fingernail clam, *Sphaerium corneum* (Guerrero *et al*., 2007)

## 1.7 Biosorption

### 1.7.1 Overview of biosorption

Biosorption can be defined as the passive uptake of toxicants by dead or inactive biological materials or materials derived from biological sources (Vijayaraghavan and Yun, 2008). It can also be described as a property of certain types of inactive, dead microbial biomass which can bind and concentrate metals

from aqueous solution. The special surface properties of the biomass enable them to adsorb different kinds of contaminants from the solution (Aksu, 2005).

Biosorption process holds some advantages over the conventional methods such as low cost operation, high efficiency and selectivity, minimization of chemical or biological sludge, possible metal recovery besides the reuseability of the biosorbent used (Kratochvil and Volesky, 1998). The use of dead cells in the biosorption process itself has their own advantages as they do not require continuous supply of nutrients, not affected by the toxic waste, and their reusable nature. Dead cells can also be stored or used for extended periods at room temperature, besides its abilities to accumulate pollutants to the same or greater extent in comparison to resting or growing cells (Aksu, 2005).

### 1.7.2 Biosorbent

Studies on the binding capacity by several types of biomass has been conducted since 1985, where some of the biomass types were found to be very effective in accumulating heavy metals and organic substances (Vieira and Volesky, 2000). In general, there are many types of biosorbents that can be used for the removal of heavy metals or organic materials. They can be divided into several categories including bacteria, fungi, algae, industrial waste, agriculture waste and polysaccharide materials.

Several factors should be considered in selecting the biosorbent for clean up purposes such as the availability and the origin, cost, selectivity and reusability besides the effectiveness of the biomass. The examples of the biosorbents that have been used in previous research are listed in Table 1.5.

**Table 1.5**: Examples of biosorbents used in the biosorption process.

| Name | References |
|---|---|
| A) Bacteria | |
| *Bacillus* sp | Tunali *et al*., 2006 |
| *Pseudomonas* sp | Ziagova *et al*., 2007 |
| B) Fungal | |
| *Aspergillus niger* | Rao and Viraraghavan, 2002 |
| *Penicilium* | Tan and Cheng, 2003 |
| C) Algae | |
| Red seaweed | Davis *et al*., 2003 |
| Blue seaweed | Davis *et al*., 2003 |

### 1.7.3 Mechanism in the biosorption process

The mechanism of biosorption is a bit complex, as the process may be one or a combination of complexation, ion exchange, coordination, adsorption, electrostatic interaction and microprecipitation. The complex cellular structure of the microorganism provides many ways for the contaminant to be taken up by the microbial cell. The biosorption mechanisms are various and depend on the biomass used, and they are still not fully understood (Ahalya et al, 2003).

In biosorption, as dead biomass was used, a non – metabolic process is involved. The pollutant uptake is mediated by the physicochemical interaction between the metal/organic materials with the functional groups present on the microbial cell surface. This process is based on the physical adsorption, chemical sorption and ion exchange, which are not dependent on cell metabolism. As the uptake process by the inactive cell is extracellular, the chemical functional groups of the cell wall play important roles in the biosorption process. Due to the nature of the cellular components, several functional groups are present on the microbial cell wall,

including the carbonyl, sulphate, carboxyl, amine, phosphate and hydroxyl groups (van der Wal *et al*., 1997).

### 1.7.4 Biosorption isotherm

The adsorption capacity of a biosorbent can be calculated using an equation. Equation 1.1 is the equation for the measurement of uptake by the biosorbent, where the unit is expressed as milligram of solute sorbed per gram of the biosorbent material (mg/g) (Vijayaraghavan and Yun, 2008).

$$Q = (V_oC_o - V_fC_f)/ M \qquad \text{Equation 1.1}$$

Where, $Q$ - solute uptake (mg/g)

$C_o$ - initial solute concentration in solution (mg/L)

$C_f$ - equilibrium solute concentration in solution (mg/L)

$V_o$ - initial solution volumes (L)

$V_f$ - final solution volumes (L)

$M$ - mass of the biosorbent (g)

### 1.7.5 Equilibrium modeling of biosorption

Adsorption isotherms are the basic requirement for the designation of biosorption system for the removal of the targeted compound. These empirical models are simple mathematical relationships, characterized by a set of limited number of adjustable parameters. These models give a good description of the experimental behavior over a large range of operating conditions. Some models which are frequently employed and established, usually involves two, three or more parameters to model the isotherm data. Langmuir, Freundlich, Langmuir – Freundlich, Redlich Peterson and Brunauer – Emmet – Teller (BET) and Radke – Prausnitz are amongst the models that are usually used in the biosorption study

which describe the non linear equilibrium between the sorbed materials on the cell ($q_{eq}$) and the materials in the solution ($C_{eq}$) at a constant temperature.

### 1.7.6 Biosorption of organic contaminants

Organic contaminants found in waste water can be degraded or detoxified using several techniques including physical, chemical and biological treatment before being released into the environment. Even though the stated methods can degrade some of the organic pollutants, the byproducts formed during the degradation process could be harmful and dangerous. Some compounds are nondegradable and require alternative removal technique. From current studies, these types of contaminants can be removed from the wastewater using the biosorption process (Aksu, 2005).

Biosorption has emerged as an alternative or supportive technique as a removal process in dealing with organic contaminants in waste water. The examples of organic pollutants which cause great concern are dyes, pesticides and phenolic compounds due to their extreme toxicity and persistency in the environment. Many types of biomass, including live and dead microorganisms have been investigated and used in the biosorption study (Ahalya *et al*, 2003).

#### 1.7.6.1 Phenol biosorption

Phenol is one of the organic contaminants which can be removed using either the conventional or biological methods. Biological methods which can be applied for the removal of phenol from the waste water are biodegradation, bioaccumulation and biosorption.

Many microorganisms have been found to adsorb phenol and phenolic compound including algae, fungi and bacteria. The examples of microorganisms

used for the biosorption of phenol and its related compound are *Aspergillus niger* (Rao and Viraraghavan, 2002), *Rhizopus arrhizus* and *Phanerochaete chrysosporium* (Juan and Yu, 2005). Besides the single type microorganism, the use of a bacterial consortium in biosorption has also been investigated as reported by several researchers (Aksu *et al*., 1999; Antizar – Ladislao and Galil, 2004), where the consortium bacteria was used for the biosorption of phenol with nickel or chlorophenol, respectively.

## 1.8 *Acinetobacter haemolyticus*

*Acinetobacter* is a Gram negative bacteria belonging to the phylum Proteobacteria. *Acinetobacter* species are oxidase – negative and occur in pairs when seen under the microscope.

*Acinetobacter haemolyticus* is strictly aerobic. This species occurs as rods in the early stage of growth and shows coccobacillary morphology in the later stage of their growth. *A. haemolyticus* grows well at a temperature range of 20 – 37$^{o}$C with the optimum at 33 – 35$^{o}$C. *A. haemolyticus* are widely distributed in nature and can be found in soil, sewage and water environment (Zakaria, 2006).

## 1.9 Objectives of the study

The aim of this research is to investigate chromium (VI) bioreduction and phenol biodegradation using the batch and the column systems.

## 1.10 Scope of the study

The research will focus on five main aspects:

1. Determination of the presence of both contaminants; Cr (VI) and phenol in selected industrial waste

2. Assessing the adaptability of bacteria in simulated waste solution; i) Cr (VI) waste, ii) phenol waste and iii) Cr (VI) and phenol mixed waste.

3. Evaluation of the suitability of a growth medium for the cultivation of bacteria i.e. liquid pineapple waste.

4. Determining the ability of bacteria in treating both contaminants separately and simultaneously using the batch and column system.

5. Investigation on other mechanism for phenol removal from the waste solution by the bacteria i.e. the bioaccumulation or biosorption process.

# CHAPTER II

# BATCH SYSTEM STUDY FOR THE REMOVAL OF HEXAVALENT CHROMIUM AND PHENOL

## 2.1 Introduction

Cr (VI) waste water is mainly produced by industrial activity, as Cr (VI) is a widely used metal in certain industries such as electroplating, alloy preparation, dye production, wood preservation and leather tanning (Thacker *et al*., 2006a). As Cr (VI) is well known for its carcinogenic and mutagenic properties, hence the treatment for Cr (VI)-contaminated waste water must be carried out to reduce the Cr (VI) concentrations to the levels that meet the standard regulation. The common treatment for Cr (VI) waste water includes chemical reduction or physicochemical methods such as removal using ion exchange and adsorption. These methods, however, have their own disadvantages. The implementation of conventional method is usually costly, as it is relatively energy and chemical - intensive. Moreover, the production of sludge and secondary pollutants is also a concern for selecting the treatment method. Nowadays, the reduction of Cr (VI) to Cr (III) as a means to remediate the Cr (VI) waste is receiving much attention. Although the reduction of Cr (VI) can be done using chemical or biological methods, the biological approach is more preferred as it is more cost effective and environmentally friendly process. Many species of microorganism have been isolated and identified such as *Pseudomonas*, *Bacillus*, *Shewanella* and *Achromobacter* that have shown the ability to reduce Cr (VI) (Yun - Guo *et al*., 2008).

Another type of pollutant is phenol, which is considered as a major and toxic contaminant in the environment. Phenol and other organic compounds such as naphthalene, trichloroethylene and benzene have been found at certain concentrations in Cr (VI) waste water discharged from petroleum refining industry, leather tanning and metal finishing industry (Yun – Gou *et al*., 2008). Currently, there are many methods that can be employed for the removal of phenol from the waste water including ionic exchange, flotation, filtration and adsorption using activated carbon. These conventional methods are less preferred nowadays as most of it usually consumes a large amount of chemical and energy, expensive and creates another problem such as secondary pollution. As an alternative, biological method via biodegradation process using microorganism is receiving attention nowadays. The use of microorganism for the degradation of phenol has been reported as early in 1900 and since then, many studies have been established and documented on phenol biodegradation. *Pseudomonas sp., Rodococcus sp., Brevibacillus sp.* and *Coprinus sp.* are amongst the reported microorganisms that can degrade phenol (Margesin *et al*., 2005 and Yang and Lee, 2007).

This chapter will focus on the batch study for the removal of Cr (VI) and phenol using a locally isolated bacteria, *Acinetobacter haemolyticus* (*A. haemolyticus*). The removal process of Cr (VI) and phenol by *A. haemolyticus* will be carried out separately and simultaneously using simulated and real waste water.

## 2.2 Materials and methods

### 2.2.1 Waste water

#### 2.2.1.1 Waste water collection and characterization

Waste water for the treatment purposes was collected at different sampling locations. For Cr (VI), the waste water was collected from a local plating factory, Perstima Sdn. Bhd., located in Pasir Gudang, Johor Bahru. For phenol waste water, an oil refinery, located in Port Dickson, Negeri Sembilan was selected for collection of the waste water.

The waste water collected was analyzed for its metal contents using the Inductively Coupled Plasma Mass Spectroscopy, ICP – MS at the Chemical Engineering Pilot Plant (CEPP), UTM. The phenol content in the waste water was determined using the 4 – aminoantipyrine method (Martin, 1949).

### 2.2.2 Materials

All glassware used in the experiment was washed with 10% v/v $HNO_3$, followed by rinsing with tap water for five times before rinsing with deionized water for three times for the preparation of 'metal free' condition. The flasks were autoclaved at 121$^{o}$C, 126 kPa for 15 minutes (Hirayama HVE-50 Autoclave).

### 2.2.3 Bacteria

The bacterial strain used in this research was *A. haemolyticus*, isolated from a local batek manufacturing premise located in Kota Bharu, Kelantan, Malaysia

(Zakaria, 2006). It was identified using 16srRNA technique and deposited in Gene Bank with an accession number EF369508.

### 2.2.4 Growth medium

#### 2.2.4.1 Nutrient broth

Nutrient broth (NB) medium was used as the growth medium for the bacteria. It was prepared by weighing 8 g of nutrient broth powder (Merck) and dissolving in 1 L of deionized distilled water followed by autoclaving at 121$^{o}$C, 126 kPa for 15 minutes (Hirayama HVE-50 Autoclave).

#### 2.2.4.2 Liquid pineapple waste medium

The liquid pineapple waste (LPW) medium used was obtained from Lee Pineapple Sdn. Bhd., a local pineapple canning company located in Tampoi. The pineapple waste was filtered and centrifuged at 4$^{o}$C, 7000 rpm (4K15, Sigma Laborzentrifugen) for 10 minutes to obtain a clear solution and kept in the refrigerator until further use.

### 2.2.5 Preparation of Cr (VI) stock solution

A 1000 mg/L of Cr (VI) stock solution was prepared by dissolving 0.1414 g of $K_2Cr_2O_7$ (BDH, Analytical Grade) in 50 mL of deionized distilled water. The stock solution was then filter sterilized with a cellulose acetate membrane filter paper (Milipore) with a pore size of 0.45 µm.

### 2.2.6 Preparation of phenol stock solution

A 5000 mg/L stock solution of phenol was prepared by dissolving 5 g of phenol crystal in 1000 mL of deionized distilled water. The solution was then filter sterilized with a cellulose acetate membrane filter paper (Milipore) with a pore size of 0.45 µm. The stock solution was kept in the fridge until prior use.

### 2.2.7 Active culture

A loopful of *A. haemolyticus* cell was aseptically transferred from the nutrient agar (NA) stock culture and inoculated into 250 mL Erlenmeyer flask which contained 25 mL of NB medium. The flask was then shaken at 30$^{o}$C, 200 rpm for 12 hours in an orbital shaker (Certomat R).

### 2.2.8 Serial dilution for the active culture

A tenfold ($10^{-1}$) of dilution for the active culture was done by adding 9.0 mL of sterilized deionized water to 1.0 mL of bacterial culture. A 100 fold ($10^{-2}$) dilution was obtained by pipetting 0.1 mL of the sample culture and mixing with 9.9 mL of sterilised deionized water.

### 2.2.9 Spread plate method

The $10^{-2}$ diluted sample (0.1 mL) was spread on the surface of the nutrient agar (NA) plate using a glass spreader. The plates were then incubated at 30$^{o}$C for 24 hours in a Memmert INB 400 incubator. After 24 hours, the colony count was carried out to determine the colony forming unit (CFU).

### 2.2.10 Growth profile of *A. haemolyticus*

#### 2.2.10.1 Growth profile of *A. haemolyticus* in NB and LPW medium

Growth profile for *A. haemolyticus* was monitored in both NB and LPW medium. For the LPW medium, the solution was sterilized by adding 5% (v/v) of ethanol (95%, Merck) and the pH was adjusted to 7 using 1 M NaOH (Baker). The medium was then inoculated with 10% (v/v) of the active culture ($OD_{600}$ = 1.2) and the flasks were shaken at 200 rpm, 30$^{o}$C for 24 hours (Certomat – R).

At various intervals of time, the culture was aseptically pipetted (3 mL) for turbidity measurement at 600 nm using the Genesys 20, Thermo Spectronic spectrophotometer. From the results, the absorbance at 600 nm versus time was plotted to obtain the bacteria growth profile.

### 2.2.11 Cr (VI) reduction study by *A. haemolyticus*

#### 2.2.11.1 Growth profile of *A. haemolyticus* in the presence of Cr (VI)

The growth of *A.haemolyticus* in both medium in the presence of 30 ppm Cr (VI) was monitored. Stock solution (1000 ppm) of Cr (VI) was prepared by dissolving 0.1414 g of $K_2Cr_2O_7$ in 50 ml of deionized distilled water. The stock solution was filter sterilized as described in section 2.2.5.

The medium containing 30 ppm Cr (VI) was inoculated with 10% v/v of active culture and shaken at 30$^{o}$C, 200 rpm (B. Braun, Certomat – R) for 24 hours. The growth profile was obtained by plotting the turbidity measurement ($OD_{600}$) of the culture versus time of growth.

**2.2.11.2 The effect of various Cr (VI) concentrations on growth of *A. haemolyticus***

The growth of *A. haemolyticus* in NB medium containing 0 – 90 ppm of Cr (VI) was monitored (Table 2.1). Active culture (10% v/v) was inoculated into the mixture and incubated at 30°C, 200 rpm (B.Braun, Certomat – R) for 24 hours. Culture was aseptically pipetted (3 mL) at certain time intervals for the turbidity measurement ($OD_{600}$) and growth profiles were plotted from the values obtained.

**Table 2.1**: Cr (VI) and NB medium used for growth profile monitoring

| Concentration of Cr (VI) (ppm) | Volume of Cr (VI) (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 10 | 1 | 89 | 10 |
| 30 | 3 | 87 | 10 |
| 60 | 6 | 84 | 10 |
| 90 | 9 | 81 | 10 |

For each of the Cr (VI) concentrations used, colony forming unit (CFU) count was carried out (Table 2.2). Dilution of sample was carried out as described in section 2.2.7 and the diluted sample was spread on NA plates as described in section 2.2.8. The NA plates were then incubated at 30°C for 24 hours in the incubator (Memmert, INB 400) before the CFU was obtained.

**Table 2.2**: The amounts of Cr (VI) and NB medium used for CFU count.

| Concentration of Cr (VI) (ppm) | Volume of Cr (VI) (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 10 | 0.250 | 22.250 | 2.5 |
| 30 | 0.750 | 21.750 | 2.5 |
| 60 | 1.500 | 21.000 | 2.5 |
| 90 | 2.250 | 20.250 | 2.5 |

### 2.2.11.3 Cr (VI) reduction by *A. haemolyticus* in NB medium using simulated Cr (VI) waste

Cr (VI) reduction studies using *A. haemolyticus* culture were carried out as follows. The culture (10% inoculum) was incubated in the Erlenmeyer flask (1 L) containing the NB medium and Cr (VI) solution at the desired Cr (VI) concentration. The contents of the mixture are shown in Table 2.3. The mixture was then incubated at 30$^{o}$C, 200 rpm (B.Braun, Certomat – R) for 3 - 5 days. At various time intervals, samples (7 mL) were pipetted aseptically for the determination of Cr (VI) concentration using the diphenylcarbazide (DPC) method and also for the $OD_{600}$ readings.

**Table 2.3**: Cr (VI) and NB medium used for the Cr (VI) reduction

| Concentration of Cr(VI) (ppm) | Volume of Cr(VI) (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 10 | 1 | 89 | 10 |
| 30 | 3 | 87 | 10 |
| 60 | 6 | 84 | 10 |
| 90 | 9 | 81 | 10 |

### 2.2.11.4 Cr (VI) reduction by *A. haemolyticus* in NB medium using real Cr (VI) waste

The study on real Cr (VI) waste was conducted using waste water from Perstima Sdn. Bhd., an electroplating factory in Pasir Gudang, Johor. Culture (10% inoculum) was introduced into the Erlenmeyer flask (1 L) which contains NB medium (10%) and Cr (VI) – containing waste water (80%). The mixture of NB medium and waste water without the cell acted as the control. The mixture was incubated for 3 days, and at different time intervals, samples (7 mL) were pipetted for the Cr (VI) analysis. The experiment was carried out in duplicates.

#### 2.2.11.5 Cr (VI) reduction by *A. haemolyticus* in LPW medium using simulated Cr (VI) waste

The Cr (VI) reduction studies were also carried out using LPW. The experiment set up was as mentioned in section 2.2.11.1 except for the replacement of NB medium with sterilized LPW (section 2.2.9.1). The mixtures were incubated at 30$^{o}$C, 200 rpm for 16 hours. The control set was the mixture of LPW and Cr (VI) solution without the bacteria. Samples (7 mL), duplicates were collected at different time intervals for the analysis of Cr (VI) and for $OD_{600}$ nm reading.

#### 2.2.11.6 Cr (VI) reduction by *A. haemolyticus* in LPW medium using real Cr (VI) waste

Cr (VI) reduction using real Cr (VI) waste was conducted as described in section 2.2.11.2. LPW was used instead of NB medium for the growth of the bacterial cells. The mixture was then incubated at 30$^{o}$C, 200 rpm for 16 hours and samples (7 mL) were collected for Cr (VI) analysis at specific time intervals. Samples were complimented with untreated Cr (VI) waste as the control. This experiment was conducted in duplicates.

### 2.2.12 Phenol removal study by *A. haemolyticus*

#### 2.2.12.1 Growth profile of *A. haemolyticus* in the presence of phenol

*A. haemolyticus* was tested for its survival in the presence of 100 ppm phenol. The phenol stock solution (1000 ppm) was prepared by dissolving 1 g of phenol crystal (Riedel – de Haën, Analysis Grade) in 1000 mL of deionized distilled water and filter sterilized using a cellulose acetate membrane filter paper (Milipore) with a pore size of 0.45 µm. The medium with 100 ppm of phenol was inoculated with

10% v/v of active culture and incubated at 30°C, 200 rpm (B.Braun, Certomat - R) for 24 hours. The turbidity measurement ($OD_{600}$) was recorded using spectrophotometer and from the values obtained, growth profiles were plotted.

### 2.2.12.2 The effect of various phenol concentrations on the growth of *A. haemolyticus*

The effect of various phenol concentrations on the growth of *A. haemolyticus* was carried out in the NB and LPW medium. The phenol stock solution was prepared at the concentration of 5000 ppm by dissolving 5 g of phenol (Riedel – de Haën, Analysis Grade) in 1000 mL of deionized distilled water. The phenol concentration used was from the range of 10 to 1500 ppm.

The NB was pipetted into the Erlenmeyer flask before added with phenol to the desired final concentration (Table 2.4). The mixtures were inoculated with 10% v/v of active culture and incubated at 30°C, 200 rpm (B.Braun, Certomat - R) for 24 hours. At various time intervals, culture was aseptically pipetted for the turbidity measurement ($OD_{600}$) using the spectrophotometer. From the values obtained, growth profiles were plotted.

**Table 2.4**: The amount of phenol and medium used for growth profile monitoring

| Concentration of phenol (ppm) | Volume of phenol (mL) | Volume of growth medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 100 | 2 | 88 | 10 |
| 300 | 6 | 84 | 10 |
| 600 | 12 | 78 | 10 |
| 900 | 18 | 72 | 10 |
| 1200 | 24 | 66 | 10 |
| 1500 | 30 | 60 | 10 |

Colony forming unit (CFU) count for *A. haemolyticus* was carried out for every phenol concentration used. The details of the mixture used are shown in Table

2.5. Sample from each flask was diluted and spread on NA plate as described in section 2.2.8 and section 2.2.9. Plates were incubated at 30$^{o}$C for 24 hours in the oven (Memmert, INB 400) and the CFU was counted.

**Table 2.5**: The amount of phenol and NB medium used in the CFU count.

| Concentration of phenol (ppm) | Volume of phenol (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 100 | 0.50 | 22.00 | 2.5 |
| 300 | 1.50 | 21.00 | 2.5 |
| 600 | 3.00 | 19.50 | 2.5 |
| 900 | 4.50 | 18.00 | 2.5 |
| 1200 | 6.00 | 16.50 | 2.5 |
| 1500 | 7.50 | 15.00 | 2.5 |

#### 2.2.12.3 Phenol removal by *A. haemolyticus* in NB medium using simulated waste

Phenol removal by *A. haemolyticus* was tested using the batch system in sterile condition. Active culture (10% v/v) was inoculated into the Erlenmeyer flasks containing nutrient broth medium and phenol solution at the desired concentrations. The amount of nutrient broth and phenol used in the experiment is shown in Table 2.6. The flasks were then incubated at 30$^{o}$C, 200 rpm (B.Braun, Certomat – R) for 3 -5 days. Samples were collected at different time intervals during the incubation period for the determination of phenol concentration using the 4 – aminoantipyrine method.

**Table 2.6**: Phenol and NB medium used for phenol removal

| Concentration of phenol (ppm) | Volume of phenol (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| 10 | 1 | 89 | 10 |
| 50 | 5 | 85 | 10 |
| 100 | 10 | 80 | 10 |
| 500 | 50 | 40 | 10 |

#### 2.2.12.4 Phenol removal by *A. haemolyticus* in NB medium using real waste

For the removal of phenol using real waste, waste water sample from an oil refinery was used for the study. Active culture (10%) was inoculated into the Erlenmeyer flasks which contains NB medium (10%) and the phenolic waste water (80%). The mixture was incubated at 30$^{o}$C, 200 rpm for 2 days. Samples were collected at selected time for phenol analysis. NB medium and waste water without cells acted as the control.

### 2.2.13 Simultaneous removal study of Cr (VI) and phenol by *A. haemolyticus*

#### 2.2.13.1 Growth of *A. haemolyticus* in NB in the presence of Cr (VI) and phenol

The combined effect of Cr (VI) and phenol on growth of *A.haemolyticus* was determined by adding the two chemicals at different amounts as indicated in Table 2.7. The growth of *A. haemolyticus* in the presence of both Cr (VI) and phenol was monitored, followed by colony forming unit (CFU) count. The flasks were incubated at 30$^{o}$C, 200 rpm in a Certomat R, B Braun orbital shaker. At suitable time intervals, 5 ml samples were withdrawn for the optical density measurement ($OD_{600}$) and CFU count. Serial dilution and spread plate techniques were performed as described in section 2.2.8 and 2.2.9.

**Table 2.7**: The amount of phenol, Cr (VI) and NB medium used in the mixtures for the growth profile monitoring and CFU count.

| Concentration [Cr (VI) + phenol] (ppm) | Volume of [Cr (VI) + phenol] (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| [10 + 10] | [1 + 1] | 88 | 10 |
| [10 + 50] | [1 + 5] | 84 | 10 |
| [10 + 100] | [1 + 10] | 79 | 10 |
| [10 + 150] | [1 + 15] | 74 | 10 |
| [30 + 10] | [3 + 1] | 86 | 10 |
| [30 + 50] | [3 + 5] | 82 | 10 |
| [30 + 100] | [3 + 10] | 77 | 10 |
| [30 + 150] | [3 + 15] | 72 | 10 |
| [60 + 10] | [6 + 1] | 83 | 10 |
| [60 + 50] | [6 + 5] | 79 | 10 |
| [60 + 100] | [6 + 10] | 74 | 10 |
| [60 + 150] | [6 + 15] | 69 | 10 |

#### 2.2.13.2 Simultaneous removal of Cr (VI) and phenol using simulated waste

The study on the simultaneous removal of Cr (VI) and phenol was also conducted using the batch system. Bacterial culture (10% inoculum) was pipetted into Erlenmeyer flask (1 L) containing nutrient broth medium in the presence of Cr (VI) and phenol at selected concentrations. The amounts of medium, Cr (VI) and phenol used in the mixture is shown in Table 2.9. The mixtures were then incubated at 30$^{o}$C, 200 rpm (B.Braun, Certomat – R) for 5 days. At different time intervals, samples were collected for the analysis of Cr (VI) and phenol. The control set was the mixture of medium with Cr (VI) and phenol without the bacteria.

**Table 2.8**: The amount of phenol, Cr (VI) and NB medium used in the mixtures for the simultaneous removal of Cr (VI) and phenol.

| Concentration [Cr (VI) + phenol] (ppm) | Volume of [Cr (VI) + phenol] (mL) | Volume of NB medium (mL) | Volume of active culture (mL) |
|---|---|---|---|
| [10 + 10] | [1 + 1] | 88 | 10 |
| [10 + 50] | [1 + 5] | 84 | 10 |
| [10 + 100] | [1 + 10] | 79 | 10 |
| [10 + 150] | [1 + 15] | 74 | 10 |
| [30 + 10] | [3 + 1] | 86 | 10 |
| [30 + 50] | [3 + 5] | 82 | 10 |
| [30 + 100] | [3 + 10] | 77 | 10 |
| [30 + 150] | [3 + 15] | 72 | 10 |
| [60 + 10] | [6 + 1] | 83 | 10 |
| [60 + 50] | [6 + 5] | 79 | 10 |
| [60 + 100] | [6 + 10] | 74 | 10 |
| [60 + 150] | [6 + 15] | 69 | 10 |

#### 2.2.13.3 Simultaneous Cr (VI) and phenol removal from real waste by *A. haemolyticus*

The treatment for real mixed waste of Cr (VI) and phenol was carried out using waste from an oil refinery as it contains both targeted pollutants. Active culture (10%) was inoculated into the Erlenmeyer flasks which contains NB medium (10%) and the waste water (80%). The mixture was incubated at 30$^{o}$C, 200 rpm for 2 days. Samples were collected at selected time for Cr (VI) and phenol analysis. The mixture of NB medium and waste water without cells acted as the control.

### 2.2.14 Analytical method

#### 2.2.14.1 The Diphenylcarbazide (DPC) method

Cr (VI) concentration was determined using the diphenylcarbazide (DPC) method with a detection limit of 5 µg/L (Greenberg *et al*., 1985). The procedure was carried out using 10 mL of volumetric flasks, where 1 mL of sample was mixed with

9 mL of $H_2SO_4$ (0.2 M) and added with 0.2 mL of freshly prepared 0.25% (w/v) DPC (Fluka, HPLC Grade) in acetone. The mixture was vortexed (Maxi Mix-II Thermolyne) for 10 – 15 seconds and was left for full color development (in about 10 – 15 minutes). The purple color mixture was measured at 540 nm (Genesys 20, Thermo Spectronic) with distilled water acting as the blank. The spectrophotometer was calibrated with a series of Cr (VI) solution ranging from 0.4 – 2.0 mg/L which were prepared using the Cr (VI) stock solution.

#### 2.2.14.2 The 4 – aminoantipyrine method.

Phenol content was analyzed using a colorimetric method with 4 – aminoantipyrine as the color reagent (Martin, 1949). The method is as follows: 0.3 mL of 2% 4 – aminoantipyrine (Sigma, AR grade) and 1 mL of 2 N ammonium hydroxide (QRëc) was added into 50 ml of sample. The content was mixed thoroughly and 1 mL of 2% potassium ferricyanide (Merck, AR grade) was then added to the sample. The mixture will form a red indophenol dye under alkaline condition and measured at 460 nm (Genesys 20, Thermo Spectronic). The phenol concentration was calculated using the phenol calibration curve, prepared from phenol with concentrations of 0.2 – 2.0 mg/L and treated with the above reagents.

## 2.3 Results and discussion

### 2.3.1 Waste water analysis

The result of the Cr (VI) waste water sample analysis is shown in Table 2.10. From the analysis, it was found that the total chromium content in the pre - treated waste water was quite high, 58.909 ppm which exceeded the limit of discharge (Standard B) (Environmental Quality Act and Regulation Handbook, 1996).

**Table 2.9**: Composition of metals in the pre – treated Cr (VI) waste water from Perstima Sdn. Bhd.

| Element | *Pre – treated Cr (VI) waste water (ppm) | **Standard B (ppm) |
|---|---|---|
| Cr | 58.909 ± 9.321 | 0.05 |
| Cd | 0.0145 ± 0.013 | 0.02 |
| Ni | 0.1395 ± 0.005 | 1.00 |
| Fe | 3.7025 ± 0.034 | 5.00 |
| As | 0.0075 ± 0.007 | 0.10 |
| Zn | 0.1305 ± 0.022 | 2.00 |
| Cu | 0.0557 ± 0.009 | 1.00 |
| Pb | 0.0135 ± 0.003 | 0.50 |

* Values shown are means of duplicate samples
** Standard B – permissible discharge limit for industrial waste water outside the catchment area in Malaysia.

The characterization of phenol waste water obtained from the oil refinery gave the following values: color, greenish; pH, 9.5 – 9.9; temperature, 32.4 – 32.9$^{o}$C; dissolve oxygen, 12.95 – 13.45, and phenol content 1.3 – 2.1 mg/L, which exceeded the limit discharge (Standard B – 2.0 mg/L). The profile of the metal concentrations for the waste water is shown in Table 2.11. From the table, it can be observed that the iron (Fe) and zinc (Zn) content were high and exceeded the discharge limit compared to other metals detected in the waste water. The iron (Fe) and zinc (Zn) amounts detected were 25.9 ppm and 4.99 ppm, respectively.

**Table 2.10**: Composition of metals in oil refinery waste water.

| Element | ***Oil refinery waste water (ppm) | Standard B (ppm) |
|---|---|---|
| Cr | 0.2145 ± 0.0327 | 0.05 |
| Cd | 0.0051 ± 0.0014 | 0.02 |
| Ni | 0.3528 ± 0.1332 | 1.00 |
| Fe | 25.883 ± 14.359 | 5.00 |
| As | 0.0409 ± 0.0121 | 0.10 |
| Zn | 4.9874 ± 4.8654 | 2.00 |
| Cu | 0.3439 ± 0.2242 | 1.00 |
| Pb | 0.0825 ± 0.0425 | 0.50 |

*** Values shown are means of duplicate samples

### 2.3.2 Growth of *A. haemolyticus*

#### 2.3.2.1 Growth profile of *A. haemolyticus* in NB and LPW medium

The purpose of growing *A. haemolyticus* in NB and LPW medium separately was to compare growth of the bacterium in a complex medium, nutrient broth and a cheap medium, i.e. liquid pineapple waste. As shown in Figure 2.1, growth of *A. haemolyticus* was slightly better in NB medium ($OD_{600}$ = 1.4) as compared to LPW medium ($OD_{600}$ = 1.2). From the profile, the highest turbidity measurement ($OD_{600}$) obtained was 1.4 for NB medium and 1.2 for the LPW medium, respectively. In the LPW medium, after 16 hours of incubation, the turbidity reading increased rapidly. This may be due to the presence of indigenous bacteria in the LPW. The growth of *A. haemolyticus* in the LPW was also monitored through streaking technique on agar plates as shown in Figure 2.2 (a – f). Indeed, when the culture was streaked on NA plate, the presence of more than 1 type of bacteria was observed indicating contamination by other bacteria. From the plate, it was found that on the 24$^{th}$ hour plate, other bacteria have started to appear. Thus, growth monitoring of *A. haemolyticus* in liquid pineapple waste medium was restricted to 16 hours only, where the highest $OD_{600}$ was 0.52.

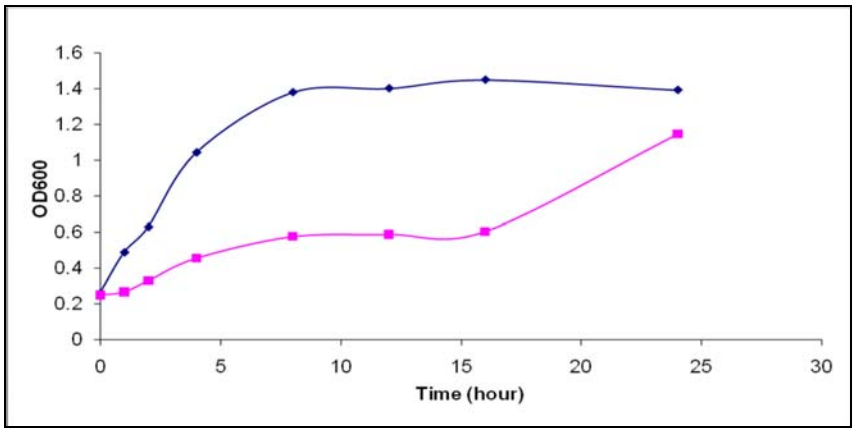


**Figure 2.1**: Growth profile of *A. haemolyticus* in NB and LPW medium, shaken at 30$^{o}$C, 200 rpm for 24 hours (♦: *A. haemolyticus* in NB medium, ■: *A. haemolyticus* in LPW medium).

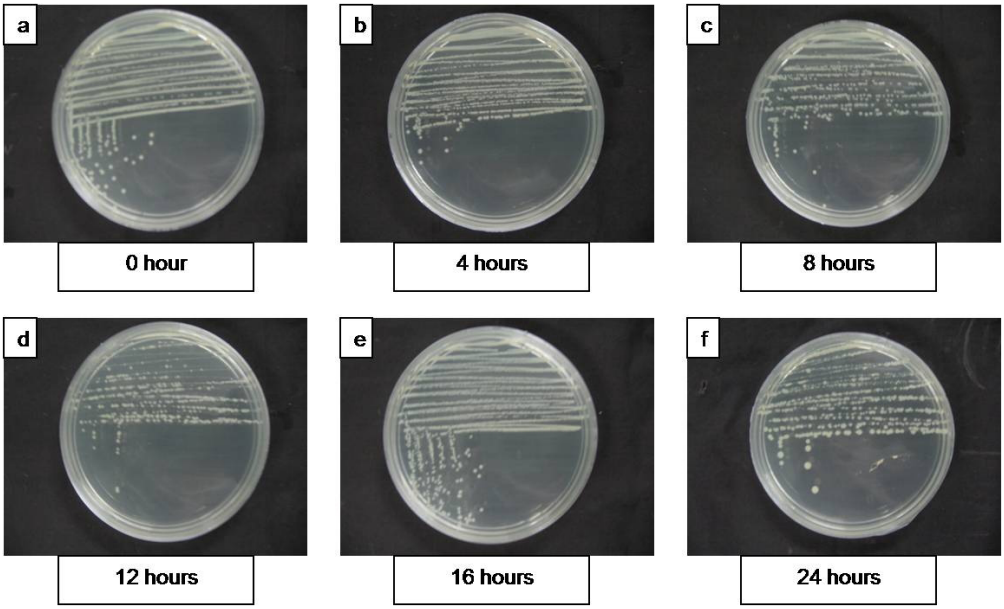


**Figure 2.2 (a – f)**: The growth of *A. haemolyticus* in LPW, monitored from 0 – 24 hours.

Poor growth observed in the LPW was a result of physiological adaptation of cells in cheap growth medium. Lack of important nutrients in the pineapple waste to support the bacterial growth may result in poor growth of the cell. The cells will synthesize new enzymes for the production of essential metabolites which are not present in the liquid pineapple waste that are crucial for their growth (Madigan and Martinko, 2006). Apart from that, the sterilization of LPW with ethanol may contribute to a longer lag phase of the cell in the liquid pineapple medium (Nordin, 2006), which could be due to the property of ethanol to lyse the cell.

The sterilization of pineapple waste using ethanol (95%) in disinfecting indigenous bacteria in the pineapple waste has certain disadvantages. Bacterial endospore could not be killed with the addition of ethanol as it is more resistant towards germicides due to their low water content and metabolism (Black, 2002). Other sterilization techniques for the liquid pineapple waste for example autoclaving or heat treatment, can lead to a decreased sugar content in the pineapple medium.

Although the growth of *A. haemolyticus* in LPW medium was poor, the use of LPW for bacterial growth can still be considered as it is a cheap medium, easily available and abundance, besides containing high sugar content which is essential for bacterial growth (Nordin, 2006).

### 2.3.3 The study on Cr (VI) reduction by *A. haemolyticus*

#### 2.3.3.1 Growth profile of *A. haemolyticus* in the presence of Cr (VI)

The effect of Cr (VI) on growth of *A. haemolyticus* is shown in Figure 2.3. For this experiment, the concentration of Cr (VI) was set at 30 ppm because from previous studies, it was shown that at this concentration, the bacteria can still show good growth even in the presence of a toxic compound (Zakaria *et al*., 2006 and Quek *et al*., 2009). In the presence of Cr (VI), the growth profile of *A. haemolyticus* was altered significantly compared to the growth in the absence of Cr (VI). From the profile, the highest turbidity measurement ($OD_{600}$) recorded for *A. haemolyticus* in the absence and presence of 30 ppm Cr (VI) was 1.41 and 0.83 in the NB medium, respectively. For the LPW medium, the highest $OD_{600}$ measured was 0.61 and 0.58, in the absence and presence of Cr (VI). In terms of growth percentage, the growth of *A. haemolyticus* was reduced to 58% and 95% in nutrient broth and LPW in the presence of Cr (VI), respectively.

From the growth curve, a longer time was needed to reach the early stationary phase of the bacterial growth in both medium which contains 30 ppm Cr (VI). The cell growth in NB medium only reached the early stationary phase after 4 hours, as where in the presence of Cr (VI), 8 hours was required. A similar observation was reported by Zakaria (2006), where the cell of *A. haemolyticus* needed 8 hours to reach the early stationary phase in the presence of Cr (VI). The growth of *A. haemolyticus* in both medium in the presence of Cr (VI) is poor as Cr (VI) is a toxic substance which can inhibit the growth of the cell (Megharaj *et al*., 2002 and Li *et. al*., 2007). Poor growth rate of *A. haemolyticus* was observed in the liquid pineapple

waste due to lack of important nutrients such as nitrogen sources to support the bacterial growth (Madigan and Martinko, 2006).

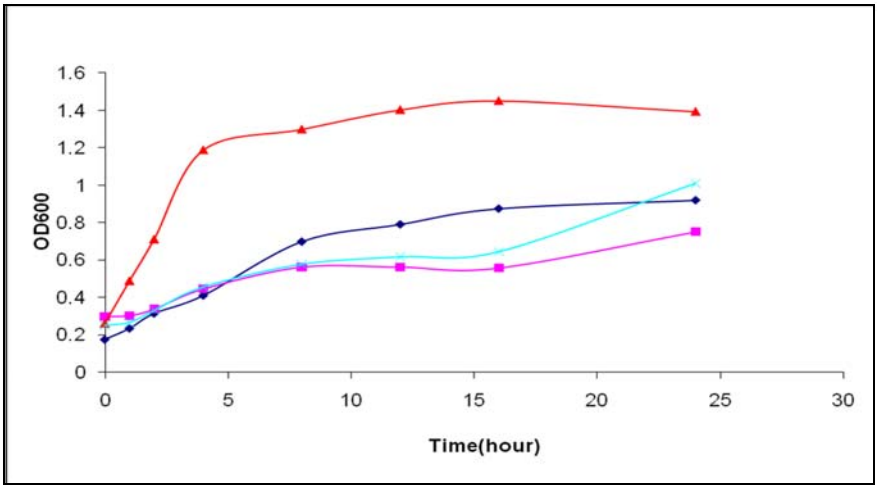


**Figure 2.3**: Growth profile of *A. haemolyticus* in the presence of Cr (VI) (♦: NB medium containing 30 ppm Cr (VI), ▲: NB medium only, ■: LPW medium containing 30 ppm Cr (VI), ×: LPW medium only)

### 2.3.3.2 Cr (VI) tolerance test for *A. haemolyticus* in NB and LPW medium

The Cr (VI) tolerance test for *A. haemolyticus* was carried out using different Cr (VI) concentrations. This step is important to determine the maximum concentration of Cr (VI) in the waste which the cell could tolerate and this will be applied to the reduction system by the bacteria. The turbidity measurement at 16 hours was selected to calculate the growth percentage as it showed the highest turbidity during the growth phase.

From Figure 2.4 (a), it was found that the growth percentage of *A. haemolyticus* decreased as the Cr (VI) concentration increased. From 100% in NB medium, the growth of *A. haemolyticus* was reduced to 62%, 60%, 40% and 29% in 10, 30, 60 and 90 ppm of Cr (VI), respectively. The toxicity of Cr (VI) towards the bacteria may be due to its oxidizing property, which can easily penetrate the cell

membrane of prokaryotic cell and shown to be mutagenic in a number of bacterial systems (Laxman and More, 2002).

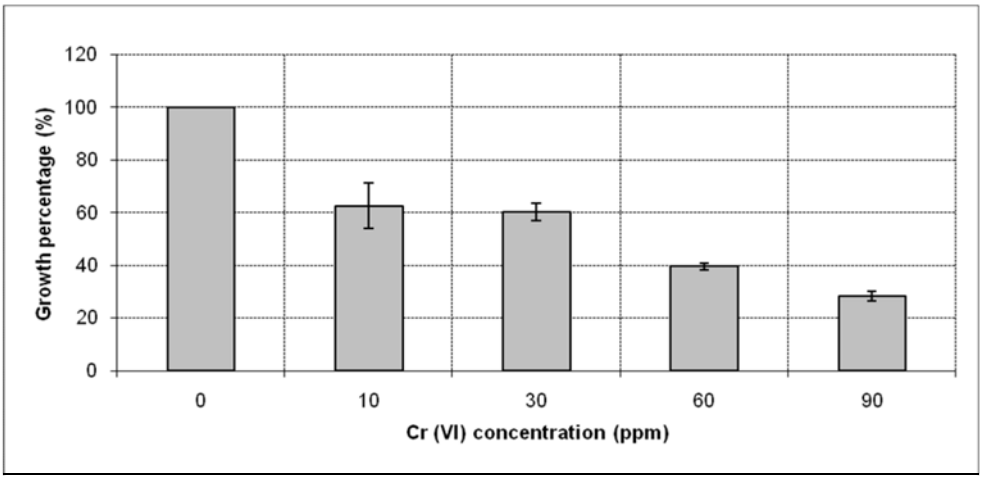


**Figure 2.4 (a)**: Cr (VI) tolerance for *A. haemolyticus* in NB medium

The colony forming units (CFU) count of *A. haemolyticus* grown in the presence of Cr (VI) at various concentrations is shown in Table 2.12. From the results, as the Cr (VI) concentration increased, the number of CFU was reduced. In 10 ppm of Cr (VI), about 10% reduction in CFU was observed. The cell number reduced by 100 and 1000 - fold at 30 ppm and 60 ppm of Cr (VI), respectively. At the highest Cr (VI) concentration used, i.e. 90 ppm, the cell number reduced by 10 000 fold, indicating the toxic effect of Cr (VI) to bacteria (Asatiani *et al*., 2004).

**Table 2.11**: The CFU of *A.haemolyticus* grown in Cr (VI)

| Cr(VI) concentration (ppm) | CFU/mL |
|---|---|
| 0 (control) | $5.3 \times 10^8 \pm 1.0$ |
| 10 | $4.8 \times 10^8 \pm 2.0$ |
| 30 | $6.0 \times 10^6 \pm 1.0$ |
| 60 | $6.0 \times 10^4 \pm 3.0$ |
| 90 | $5.1 \times 10^4 \pm 2.0$ |

A similar trend was observed as in the LPW, where the growth percentage decreased in the presence of Cr (VI). From Figure 2.4 (b), in the presence of Cr (VI), the growth percentage of the cell decreased to 98%, 78%, 67% and 46% in 10, 30, 60 and 90 ppm, respectively.

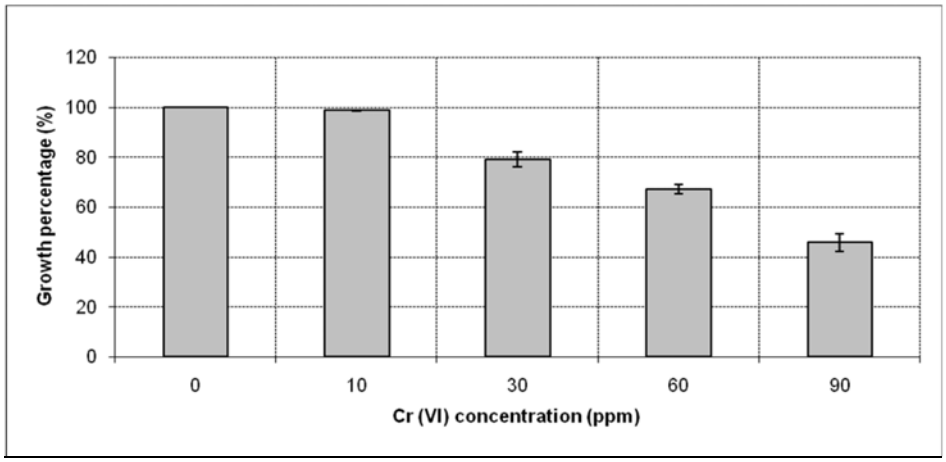


**Figure 2.4 (b)**: Cr (VI) tolerance for *A. haemolyticus* in LPW medium

### 2.3.3.3 Cr (VI) reduction by *A. haemolyticus* in NB medium using simulated and real Cr (VI) waste

The study on Cr (VI) reduction by *A. haemolyticus* was carried out in NB medium using simulated waste at different Cr (VI) concentrations. Figure 2.5 (a – d) shows the percentage Cr (VI) reduced by *A. haemolyticus* at different Cr (VI) concentrations. *A. haemolyticus* can fully remove 10, 30 and 60 ppm of Cr (VI) in 36, 60 and 120 hours, respectively. For the highest Cr (VI) concentration used, 90 ppm, the percentage reduction was about 75% in 144 hours. Longer time was needed for the reduction process as the initial Cr (VI) concentration increased. A similar observation was reported by Shen and Wang (1994) and Philip et al. (1998).

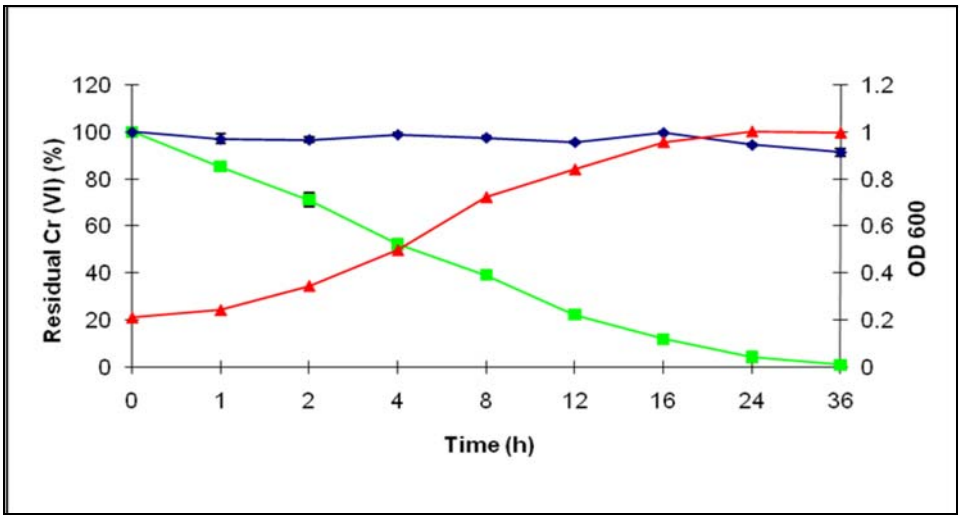


**Figure 2.5 (a)**: Cr (VI) reduction by *A. haemolyticus* grown in NB medium containing 10 ppm of Cr (VI) (■: 10 ppm of Cr (VI) with bacterial inoculum, ♦: 10 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

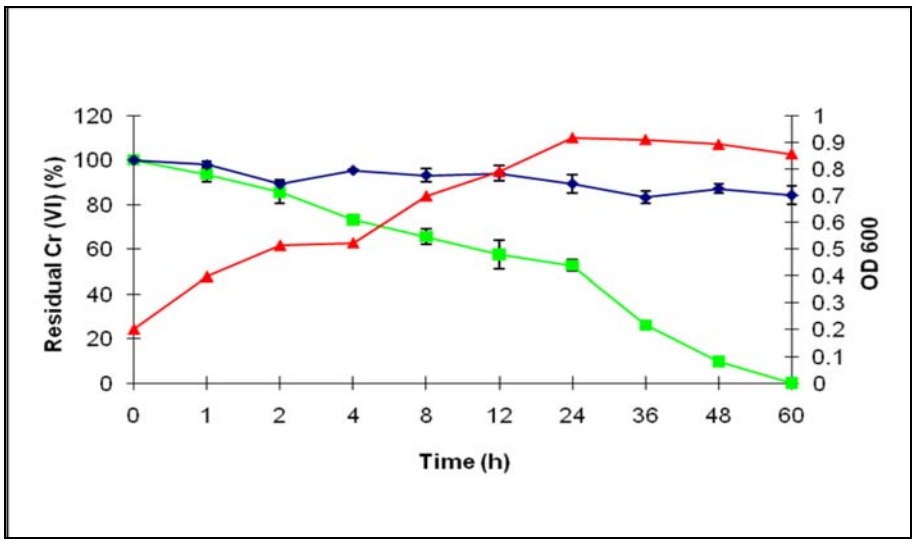


**Figure 2.5 (b)**: Cr (VI) reduction by *A. haemolyticus* grown in NB medium containing 30 ppm of Cr (VI) (■: 30 ppm of Cr (VI) with bacterial inoculum, ♦: 30 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

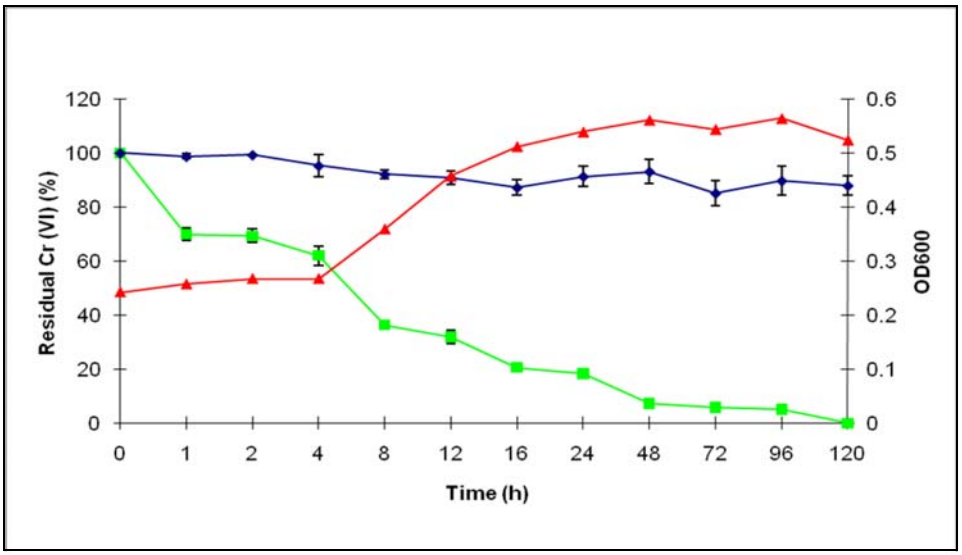


**Figure 2.5 (c)**: Cr (VI) reduction by *A. haemolyticus* grown in NB medium containing 60 ppm of Cr (VI) (■: 60 ppm of Cr (VI) with bacterial inoculum, ♦: 60 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

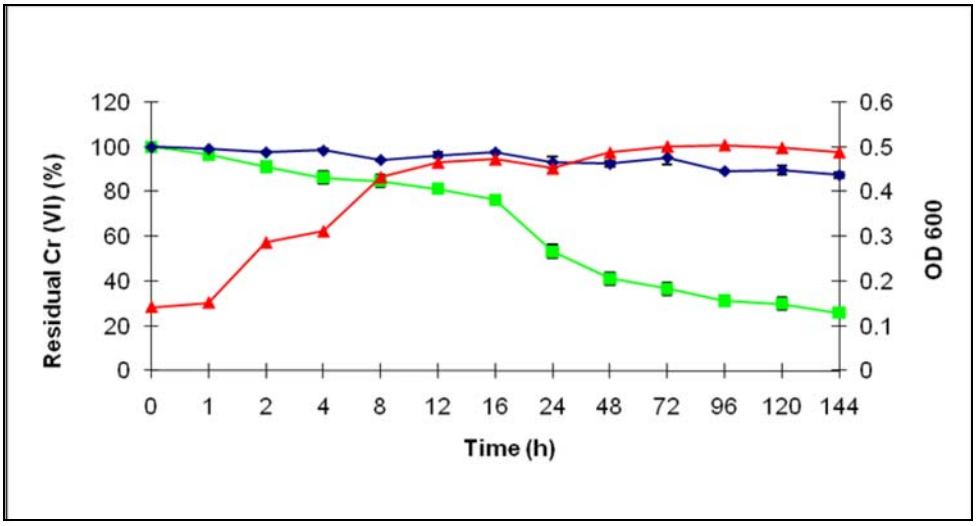


**Figure 2.5 (d)**: Cr (VI) reduction by *A. haemolyticus* grown in NB medium containing 90 ppm of Cr (VI) (■: 90 ppm of Cr (VI) with bacterial inoculum, ♦: 90 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

The study on the reduction of real Cr (VI) waste was conducted using waste water obtained from Perstima Sdn. Bhd., an electroplating company based in Pasir Gudang, Johor (Figure 2.6). The initial concentration for the Cr (VI) waste water used was 58.91 ppm. In the control experiment, only a slight reduction of Cr (VI) content was observed, i.e. 4% in 3 days. In the presence of *A. haemolyticus*, 97% of the Cr (VI) was removed indicating that the bacteria played a role in reducing and removing the Cr (VI) in the waste.

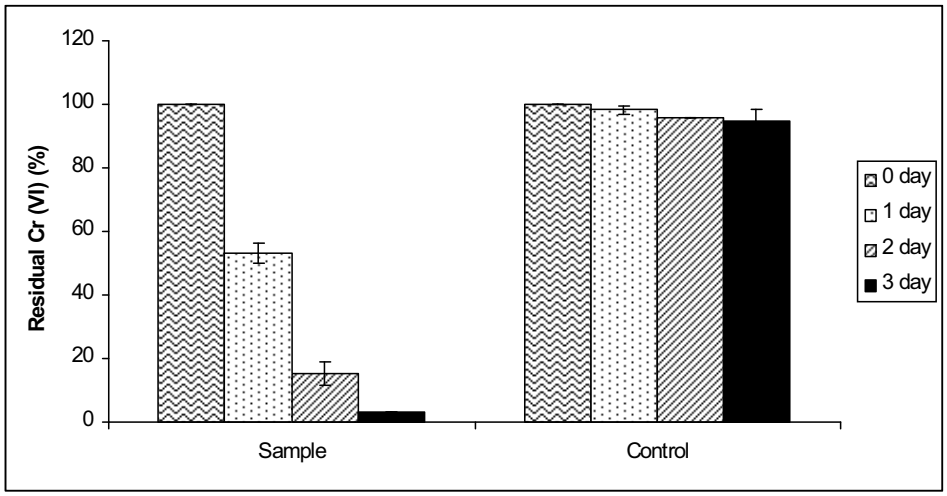


**Figure 2.6**: Cr (VI) reduction by *A. haemolyticus* using real Cr (VI) waste in NB medium.

### 2.3.3.4 Cr (VI) reduction by *A. haemolyticus* in LPW medium using simulated and real Cr (VI) waste

Besides NB medium, the Cr (VI) reduction study was also conducted using LPW medium as the bacteria showed the ability to grow in the medium in the presence of Cr (VI). Cr (VI) reduction profile by *A. haemolyticus* using simulated Cr (VI) waste in the LPW is shown in Figures 2.7 (a – d). The maximum time for the Cr (VI) reduction process in the liquid pineapple was set at 16 hours due to the contamination risk (see section 2.2.10.1).

From Figures 2.7 (a – d), the percentage reduction of Cr (VI) by *A. haemolyticus* in liquid pineapple waste medium was significantly high even after 16 hours of growth. The percentage of Cr (VI) reduced by *A. haemolyticus* in liquid pineapple waste was 95%, 78%, 75% and 61% for 10, 30, 60 and 90 ppm Cr (VI). The high amount of Cr (VI) reduced in a short time may be due to the composition of the liquid pineapple waste, where it contains several types of sugars and organic acids, besides mixture of cations and anions. The sugars present in the liquid pineapple waste include glucose, fructose and sucrose (Idris and Suzana, 2005).

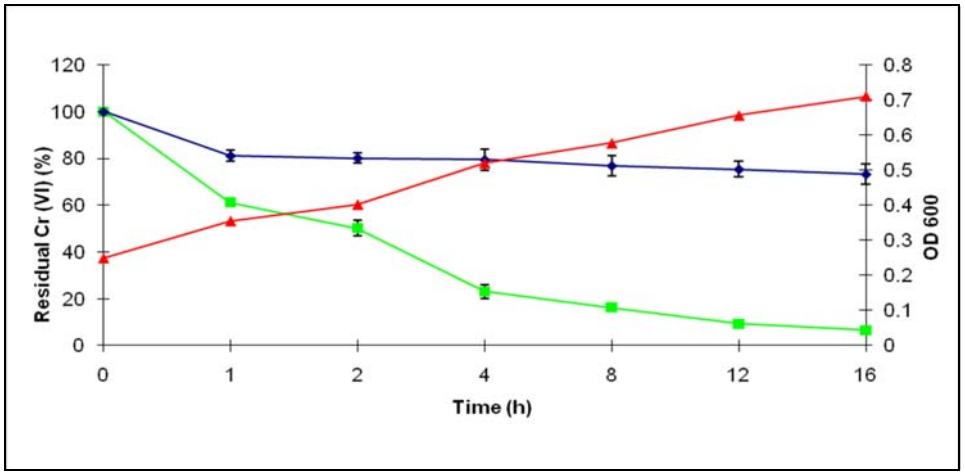


**Figure 2.7 (a)**: The percentage of Cr (VI) reduced by *A. haemolyticus* in LPW medium containing 10 ppm of Cr (VI) (■: 10 ppm of Cr (VI) with bacterial inoculum, ♦: 10 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

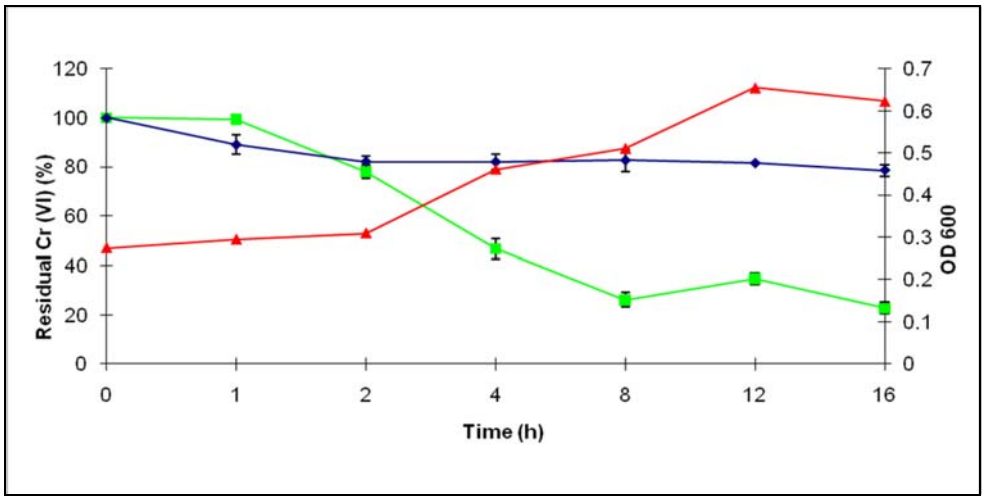


**Figure 2.7 (b)**: The percentage of Cr (VI) reduced by *A. haemolyticus* in LPW medium containing 30 ppm of Cr (VI) (■: 30 ppm of Cr (VI) with bacterial inoculum, ♦: 30 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

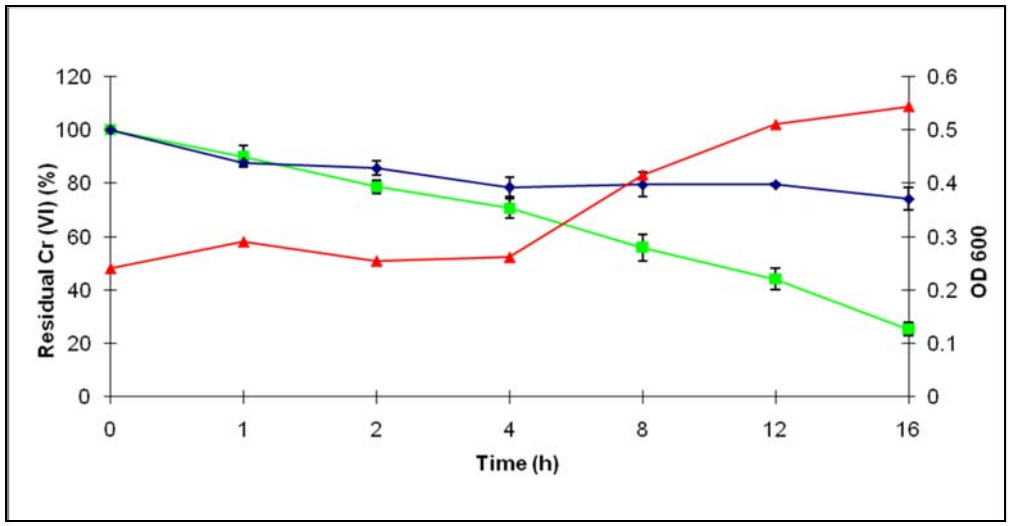


**Figure 2.7 (c)**: The percentage of Cr (VI) reduced by *A. haemolyticus* in LPW medium containing 60 ppm of Cr (VI) (■: 60 ppm of Cr (VI) with bacterial inoculum, ♦: 60 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

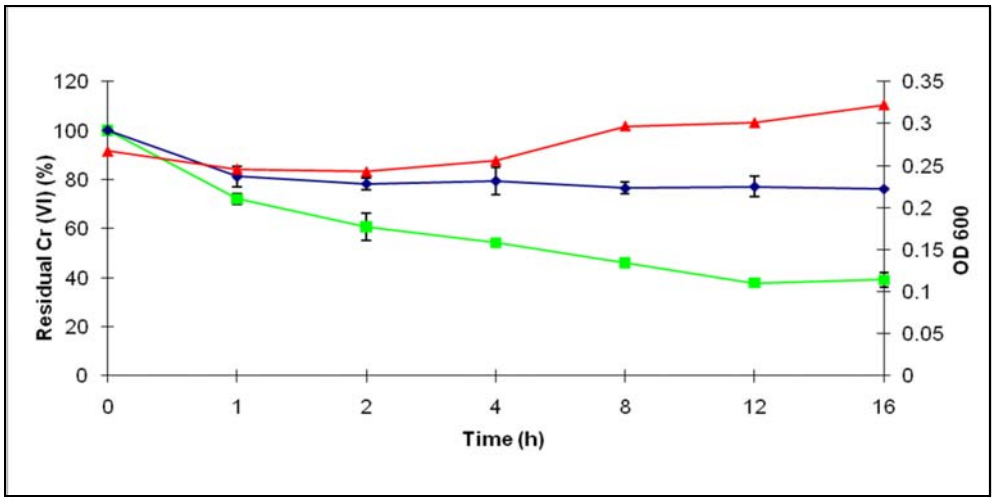


**Figure 2.7 (d)**: The percentage of Cr (VI) reduced by *A. haemolyticus* in LPW medium containing 90 ppm of Cr (VI) (■: 90 ppm of Cr (VI) with bacterial inoculum, ♦: 90 ppm of Cr (VI) without bacterial inoculum, ▲: bacterial growth profile).

Carbon compounds such as malate, citrate, lactate and glucose, was shown to play an important role in the Cr (VI) reduction process. These compounds act as the electron donor which can enhance the reduction of Cr (VI) (Philip *et al*., 1998 and Zhu *et al*., 2008). Therefore, the slight reduction of Cr (VI) concentration observed in the control (i.e. without bacteria) may be due to the presence of sugar in the liquid pineapple waste. Based on the report by Liu *et. al*. (2006), chromate - reducing microorganisms may utilize certain organic compounds as the electron donor for Cr (VI) reduction. Cr (VI) concentration was reduced from 40 to 4.42 mg/L when glucose was applied, while in the absence of glucose, the concentration dropped only to 16.42 mg/L after an incubation time of 72 hours using *Bacillus* sp (Liu *et al*., 2006). It can be concluded that the presence of glucose in the medium significantly promoted bacterial growth and Cr (VI) reduction.

Based on the report by Ang (2005), *A. haemolyticus* can use the LPW as the carbon source to grow. Amongst the organic compounds present, glucose was identified as the main sugar component utilized by the bacteria compared to other sugars (Zakaria, 2006). This could be due to the high concentrations of glucose in

the pineapple waste (Idris and Suzana, 2005) plus the substantial needs of glucose solution for the bacteria growth.

Equation 2.1 described the role of the glucose as the carbon source in the Cr (VI) reduction process (Chirwa and Wang, 1997):

$$C_6H_{12}O_6 + 8CrO_4^{2-} + 34H^+ \longrightarrow 8Cr^{3+} + 6HCO^{3-} + 2OH^- \quad \text{(Eq 2.1)}$$

From the equation, glucose plays a role in Cr (VI) reduction as the breakdown of the compound (1 mole) produces sufficient energy to reduce 8 moles of Cr (VI) to Cr (III).

Figure 2.8 shows the reduction of Cr (VI) waste (58.91 ppm) obtained from Perstima Sdn. Bhd. by *A. haemolyticus* in LPW as growth medium. Based on the result, 73% and 12% of Cr (VI) was reduced by *A. haemolyticus* in the sample and control experiment, respectively. In the control experiment, slight reduction of the Cr (VI) concentration was observed due to the sugar present in the liquid pineapple waste acting as electron donors.

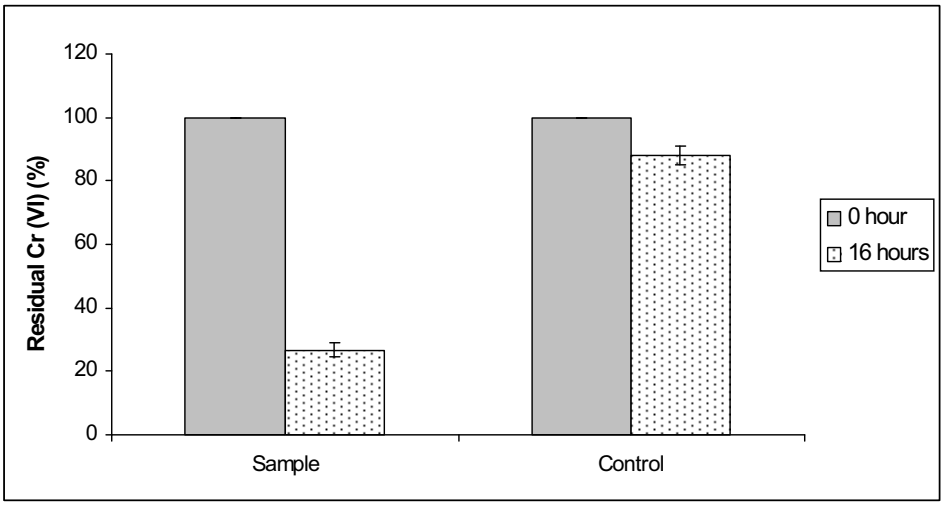


**Figure 2.8**: Cr (VI) reduction by *A. haemolyticus* using Cr (VI) waste obtained from Perstima Sdn. Bhd.

### 2.3.4 The study on phenol removal by *A. haemolyticus*

#### 2.3.4.1 Growth profile of *A. haemolyticus* in the presence of phenol

The study on the effect of the organic contaminant, phenol on the growth of *A. haemolyticus* was conducted as outlined in section 2.2.12.1. Figure 2.9 shows the effect of phenol on the growth of *A. haemolyticus* in both nutrient broth and LPW medium. Growth of *A. haemolyticus* in NB only and NB with 100 ppm phenol was quite similar, with OD readings of 1.44 and 1.22, respectively. Even though phenol is acknowledged as a toxic substance, microorganism may utilize phenol or phenol - related compound as their energy and carbon source (Margesin *et al*, 2004). Bacterial strains such as *Desulfovibrio sp.* (Knoll and Winter, 1989), *Alcaligenes feacalis* subsp. (Rehfuss and Urban, 2005) and *Pseudomonas sp.* (Tuah, 2006) are amongst the bacteria that can utilize phenol as a source of carbon and energy.

Poor bacterial growth was observed in LPW medium added with phenol where the highest $OD_{600}$ recorded was around 0.3 as compared to 0.6 in the absence of phenol. The growth of *A. haemolyticus* in LPW medium was poor compared to nutrient broth medium as it is not a rich medium. Even though LPW medium contains several sugars such as glucose, it still lacks certain important nutrients which needed to support the bacterial growth (Madigan and Martinko, 2006)

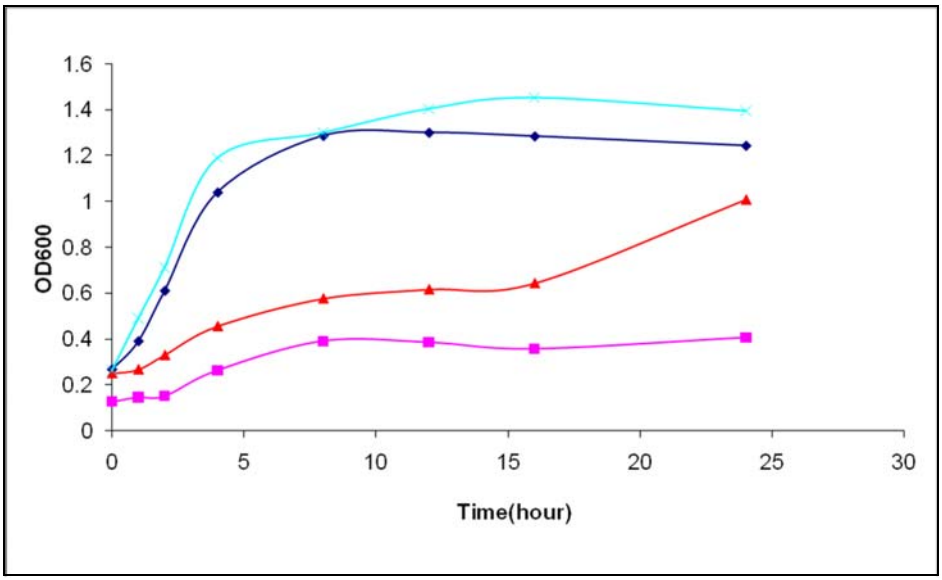


**Figure 2.9**: Growth profile of *A.haemolyticus* in the presence of phenol in NB and LPW medium (♦: NB medium containing 100 ppm phenol, ▲: LPW medium only, ■: LPW medium containing 100 ppm phenol, ×: NB medium only).

### 2.3.4.2 Phenol tolerance test for *A. haemolyticus* in NB and LPW medium

Phenol tolerance test for *A. haemolyticus* was conducted in order to determine the level or concentration of phenol which the bacteria could tolerate in the phenol removal studies. The effect of different phenol concentrations on growth of *A. haemolyticus* in nutrient broth medium is shown in Figure 2.10. From the profile, the growth percentage of *A. haemolyticus* was reduced as the concentration of phenol increased. In the presence of phenol, the growth percentage of *A. haemolyticus* was reduced to 92%, 85%, 64%, 60%, 41% and 21% in 100, 300, 600, 900, 1200 and 1500 ppm of phenol, respectively. The cell growth was minimal at 1500 ppm, i.e. the highest phenol concentration used.

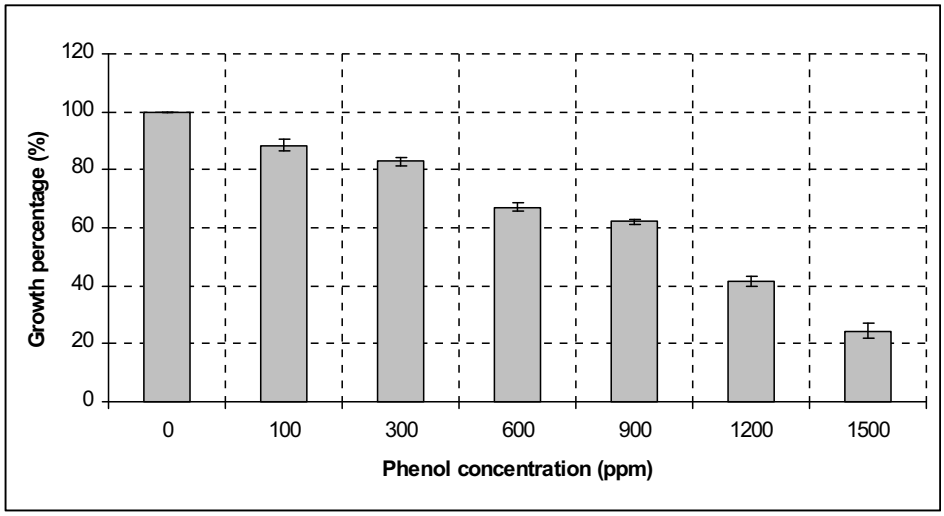


**Figure 2.10**: Phenol tolerance test for *A. haemolyticus* in NB medium.

The number of colonies formed on the NA plate for *A.haemolyticus* grown in the presence of phenol is shown in Table 2.13. From the data obtained, it was found that *A. haemolyticus* was able to grow in the presence of 100 ppm to 1500 ppm phenol. However, as the phenol concentration increased from 600 ppm to 1500 ppm, the CFU counts of *A. haemolyticus* were reduced. The viable cell counts were reduced to $1.58 \times 10^9$, $1.21 \times 10^9$, $9.2 \times 10^8$ and $2.2 \times 10^8$ in 600 ppm, 900 ppm, 1200 ppm and 1500 ppm of phenol, respectively. The reduction in the cell numbers could be due to the high phenol concentrations, where the toxic effect was very dominant and could contribute to the inhibition of the biological activity of the cell (Saravanan *et al*., 2008).

**Table 2.12**: CFU of *A.haemolyticus* grown in various concentration of phenol.

| Concentration (ppm) | CFU/mL |
|---|---|
| 0 (Control) | $2.12 \times 10^9 + 3.0$ |
| 100 | TNTC |
| 300 | TNTC |
| 600 | $1.58 \times 10^9 \pm 9.0$ |
| 900 | $1.21 \times 10^9 \pm 1.0$ |
| 1200 | $9.2 \times 10^8 \pm 11.0$ |
| 1500 | $2.2 \times 10^8 + 2.0$ |

TNTC – Too numerous to count

Phenol tolerance test for *A. haemolyticus* in the liquid pineapple waste medium is shown in Figure 2.11. The growth percentage recorded for *A. haemolyticus* was 25%, 24%, 21%, 11%, 8% and 7% in 100 ppm, 300 ppm, 600 ppm, 900 ppm, 1200 ppm and 1500 ppm of phenol, respectively.

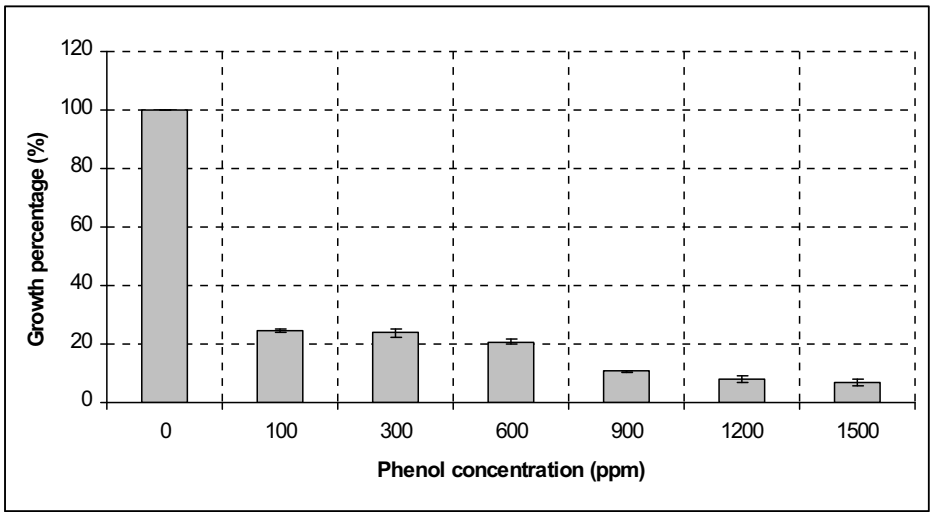


**Figure 2.11**: Phenol tolerance test for *A. haemolyticus* in LPW medium.

In the LPW medium with higher phenol concentrations (≥ 600 ppm), a more pronounced lag phase for the bacteria growth was observed (Figure 2.12). This could be due to some products being formed in the medium as the colour of the

pineapple waste grew darker with increasing phenol concentrations (Fig 2.13a and b).

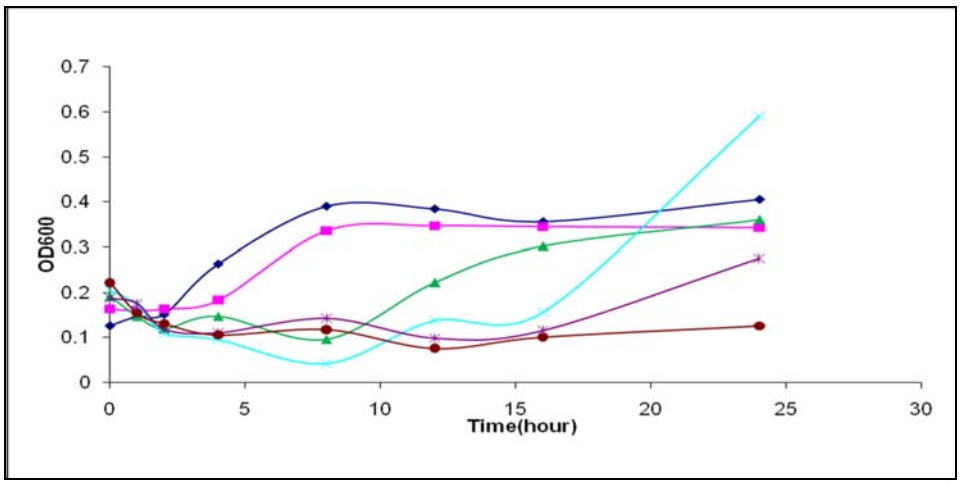


**Figure 2.12**: The effect of different phenol concentrations on growth of *A. haemolyticus* in LPW medium, shaken at 30°C, 200 rpm for 24 hours (♦: 100 ppm phenol,■: 300 ppm phenol, ▲: 600 ppm phenol, ×: 900 ppm phenol, *: 1200 ppm phenol, ●: 1500 ppm phenol).

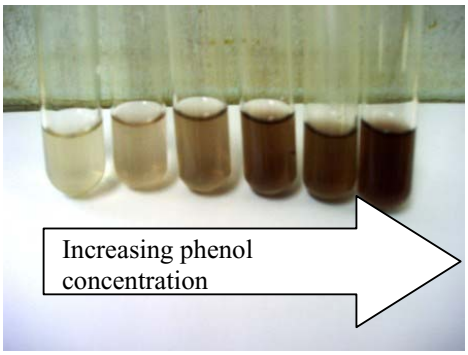


**Figure 2.13 (a)**: *A. haemolyticus* grown in the LPW medium containing phenol.

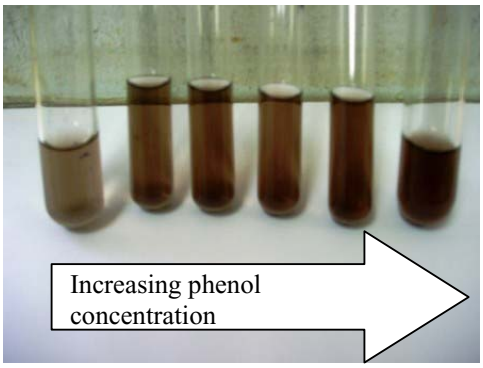


**Figure 2.13 (b)**: Control experiment consisting of LPW and phenol (in the absence of *A. haemolyticus*) which shows changes in the colour of the medium.

This phenomenon is known as the Maillard browning, which is a type of non-enzymatic browning involving the reaction of carbonyl moiety of sugar molecule with biological amine, either an amino acid or lysine residue of a protein which form a Schiff base and undergoes rearrangement to form aminoketose (Amadori product). These products will react further to form brown nitrogenous polymer and copolymers called melanoidins (Ang, 2005). Hence, the liquid pineapple waste medium was not used in the phenol removal study as the formation of the dark colour in the medium interferes in phenol analysis.

### 2.3.4.3 Phenol removal by *A. haemolyticus* in NB medium using simulated and real phenol waste

Phenol removal study by *A. haemolyticus* was carried out in the NB medium only using the simulated phenol waste at different concentrations. Liquid pineapple waste medium was not used in the phenol removal study as the growth of *A. haemolyticus* in the medium was very poor, besides the formation of dark colour in the medium which will interfere during phenol analysis. Figure 2.14 (a – d) shows bacterial growth and phenol removel by *A. haemolyticus* from the simulated waste. The removal percentages are 50.3%, 44.8%, 38.8% and 18.63% for 10, 50, 100 and 500 ppm of phenol, respectively. The removal percentage was low at 500 ppm of phenol despite good growth shown by the bacteria (Figure 2.14 c and 2.14 d). A possible explanation is that the reduction of phenol may not be due to the degradation process only (Jiang *et al*., 2007). The reduction of phenol in the mixture could be due to the adsorption of phenol on the surface of the cell. Normally, degradation of phenol by microorganism leads to the formation of carbon dioxide and water, with several intermediates such as benzoate, catechol, cis, cis – muconate, β – ketodipate, succinate and acetate being formed during the process (Tuah, 2006). Although many microorganisms can utilize and degrade phenol as their carbon and energy source, the mechanism of phenol removal in *A. haemolyticus* has not been elucidated.

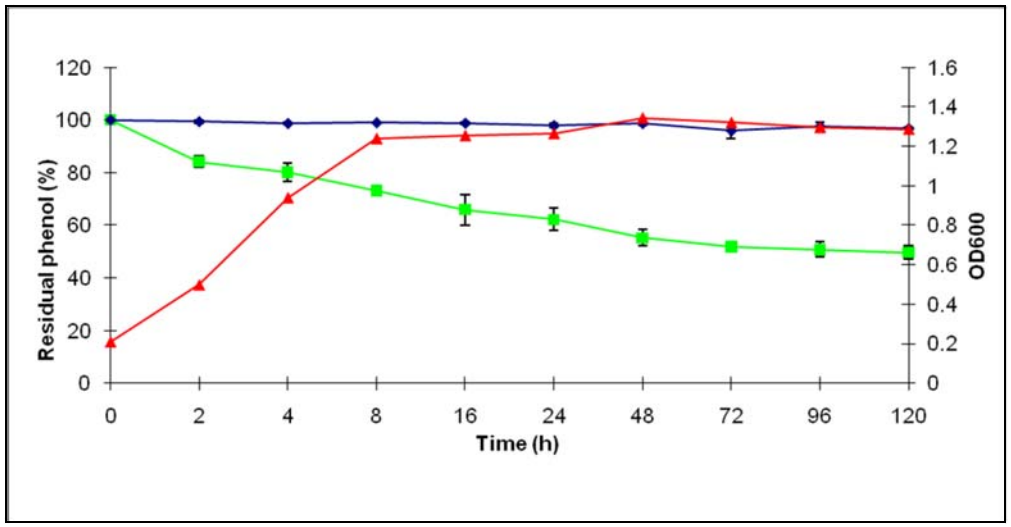


**Figure 2.14 (a)**: Phenol removal by *A. haemolyticus* in NB medium containing 10 ppm of phenol (■: 10 ppm of phenol with bacterial inoculum, ♦: 10 ppm of phenol without bacterial inoculum, ▲: bacterial growth profile).

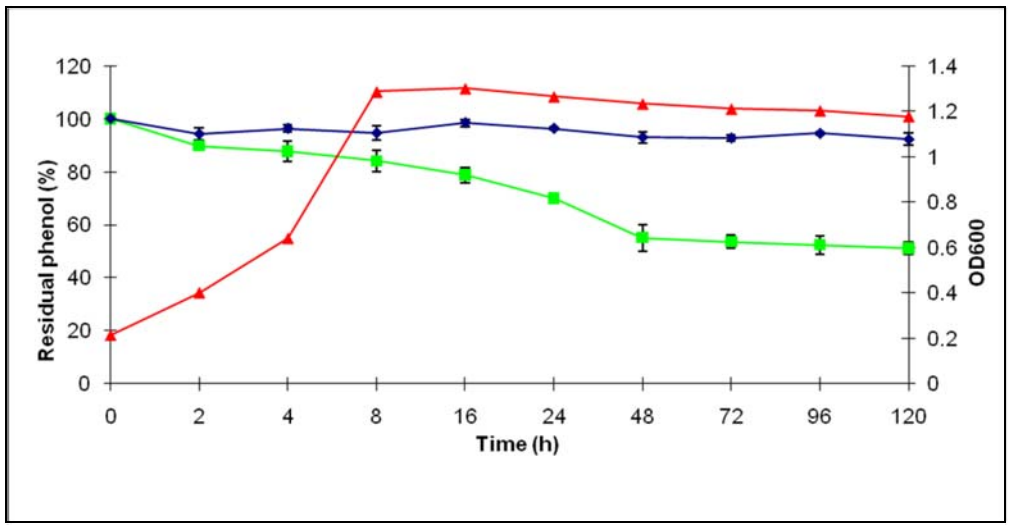


**Figure 2.14 (b)**: Phenol removal by *A. haemolyticus* in NB medium containing 50 ppm of phenol (■: 50 ppm of phenol with bacterial inoculum, ♦: 50 ppm of phenol without bacterial inoculum, ▲: bacterial growth profile).

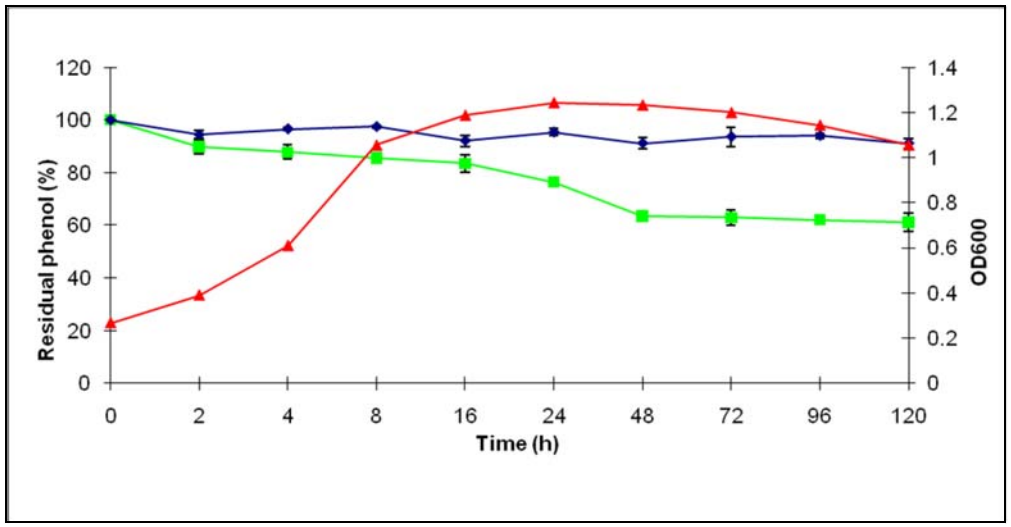


**Figure 2.14 (c)**: Phenol removal by *A. haemolyticus* in NB medium containing 100 ppm of phenol (■: 100 ppm of phenol with bacterial inoculum, ♦: 100 ppm of phenol without bacterial inoculum, ▲: bacterial growth profile).

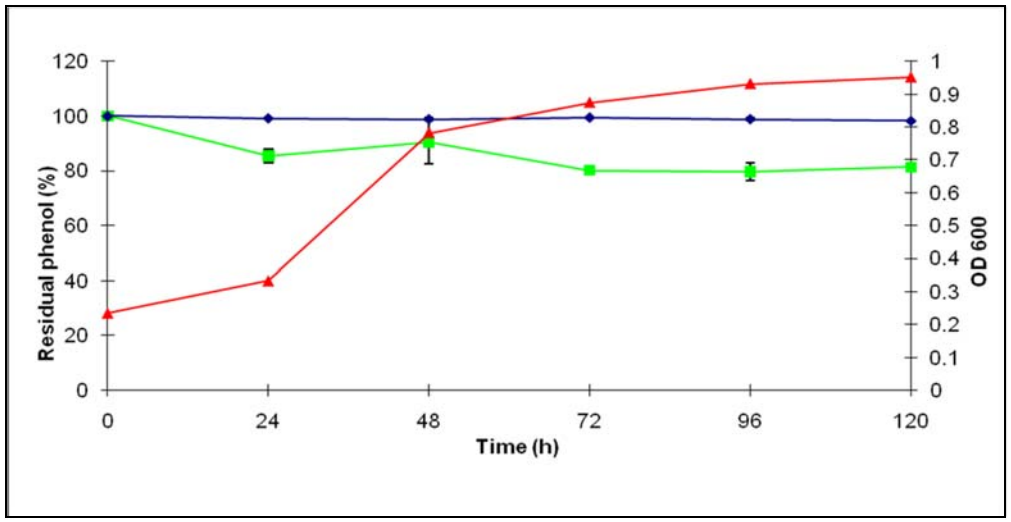


**Figure 2.14 (d)**: Phenol removal by *A. haemolyticus* in NB medium containing 500 ppm of phenol (■: 500 ppm of phenol with bacterial inoculum, ♦: 500 ppm of phenol without bacterial inoculum, ▲: bacterial growth profile).

For the treatment of phenol from industrial waste water, the oil refinery waste water obtained from Port Dickson was used. The phenol concentration in the waste water was 2.1 ppm. Figure 2.15 shows the result for the removal of real phenol

waste by *A. haemolyticus*, where 100 % of phenol content was removed in 2 days. In the control experiment, just a slight removal of phenol was observed, indicates that *A. haemolyticus* plays a role in the phenol removal process.

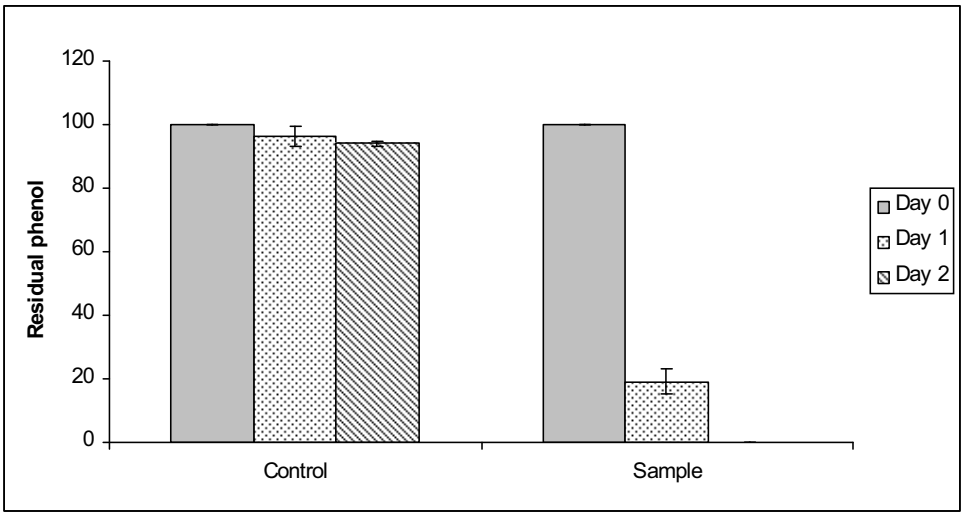


**Figure 2.15**: Removal of phenol from oil refinery waste water using *A. haemolyticus*.

### 2.3.5 Simultaneous removal of Cr (VI) and phenol by *A. haemolyticus*

#### 2.3.5.1 The effect of both Cr (VI) and phenol on growth of *A.haemolyticus*.

The simultaneous effect of Cr (VI) and phenol on *A. haemolyticus* was carried out as mentioned in section 2.2.13.1. A combination of Cr (VI) and phenol was chosen as waste effluents from various industries such as leather tanning, wood preservation and petroleum refinery were known to contain both Cr (VI) and phenol (Chirwa and Wang, 1999). The effect of various concentrations of Cr (VI) and phenol on the growth of *A. haemolyticus* is shown in Table 2.14. The growth of *A. haemolyticus* was reduced significantly when grown in the presence of both Cr (VI) and phenol. In the presence of 10 and 30 ppm Cr (VI), the growth percentage recorded for *A. haemolyticus* were around 40 - 50% at all phenol concentrations tested. However, the growth percentage decreased to less than 30% at 60 ppm Cr

(VI). This strongly suggests the more dominant role of Cr (VI) toxicity as opposed to phenol. Despite the presence of Cr (VI) and phenol, *A. haemolyticus* can still exhibit good growth and this can be attributed to the ability of *A. haemolyticus* to utilize phenol and subsequently reduce Cr (VI) to Cr (III). Moreover, a previous study reported that several types of bacteria were able to utilize phenol at low concentration (Rehfuss and Urban, 2005). However, as the concentration of both substances increases, the growth of *A. haemolyticus* is inhibited due to high toxicity of the mixture towards the cell.

Table 2.13: Growth of *A. haemolyticus* in the presence of Cr (VI) and phenol at various concentrations

| Concentration of mixed waste (ppm) | | Percentage growth of |
|---|---|---|
| Cr (VI) | Phenol | *A. haemolyticus* (%) |
| 0 | 0 | 100 |
| 10 | 10 | 43.6 ± 2.7 |
| 10 | 50 | 43.4 ± 4.8 |
| 10 | 100 | 46.6 ± 2.7 |
| 10 | 150 | 45.6 ± 4.1 |
| 30 | 10 | 38.0 ± 4.0 |
| 30 | 50 | 37.4 ± 1.2 |
| 30 | 100 | 35.8 ± 4.6 |
| 30 | 150 | 41.6 ± 1.4 |
| 60 | 10 | 35.2 ± 4.9 |
| 60 | 50 | 23.8 ± 4.0 |
| 60 | 100 | 35.0 ± 6.0 |
| 60 | 150 | 26.1 ± 2.2 |

Table 2.14 shows the colony count for *A. haemolyticus* in NB medium in the presence of both Cr (VI) and phenol at various concentrations. From the bacterial count results, Cr (VI) is more toxic to *A. haemolyticus* cells if compared to phenol. *A. haemolyticus* showed good cell survival ability in mixtures of 10 ppm Cr (VI) and 10 – 150 ppm phenol with bacterial number in the region of 7.8 ± 2.0 – 9.1 ± 2.0 x $10^8$ CFU/ mL. Within the same phenol concentrations range, increasing the initial Cr (VI) to 30 ppm resulted in the reduction of bacterial cell number to 3.1 ± 1.0 – 4.9 ± 1.0 x $10^6$ CFU/ mL. At 60 ppm Cr (VI), the bacterial cell number dwindled further to

$6.5 \pm 5.0 \times 10^4 - 1.1 \pm 6.0 \times 10^5$ CFU/ mL. The reduction of the cell number indicates the toxic effect of Cr (VI) and phenol to the cell.

**Table 2.14**: The colony count for *A.haemolyticus* in NB medium in the presence of phenol and Cr (VI) after 8 hours of incubation.

| Concentration [Cr (VI) + Phenol] (ppm) | CFU/mL |
|---|---|
| 0 (Control) | $7.8 \times 10^8 \pm 2.0$ |
| [10 + 10] | $8.9 \times 10^8 \pm 1.0$ |
| [10 + 50] | $7.2 \times 10^8 \pm 3.0$ |
| [10 + 100] | $9.1 \times 10^8 \pm 2.0$ |
| [10 + 150] | $8.5 \times 10^8 \pm 1.0$ |
| [30 + 10] | $3.9 \times 10^6 \pm 1.0$ |
| [30 + 50] | $3.1 \times 10^6 \pm 1.0$ |
| [30 + 100] | $4.9 \times 10^6 \pm 1.0$ |
| [30 + 150] | $4.3 \times 10^6 \pm 2.0$ |
| [60 + 10] | $7.9 \times 10^4 \pm 3.0$ |
| [60 + 50] | $6.5 \times 10^4 \pm 5.0$ |
| [60 + 100] | $1.1 \times 10^5 \pm 6.0$ |
| [60 + 150] | $9.3 \times 10^4 \pm 4.0$ |

### 2.3.5.2 Simultaneous Cr (VI) and phenol removal by *A. haemolyticus* using simulated waste

The performance of *A. haemolyticus* in the simultaneous removal of Cr (VI) and phenol is shown in Figure 2.17 – 2.19. For the mixture of 10 ppm Cr (VI) and 10 – 150 ppm phenol (Fig. 2.17), *A. haemolyticus* was able to completely remove the Cr (VI) with the phenol removal of 35.8%, 42.1%, 64.2% and 58.6% for 10, 50, 100 and 150 ppm phenol, respectively. Using the mixtures of 30 ppm Cr (VI) and same phenol concentration ranges (Fig. 2.18), resulted in more than 90% of Cr (VI) being removed while for phenol, the removal percentage were as follows; 29.1% (10 ppm), 29.9% (50 ppm), 56.6% (100 ppm) and 50.9% (150 ppm). Similar trend was

observed when Cr (VI) was further increased to 60 ppm and the removal percentages for both Cr (VI) and phenol were lower. From Figure 2.19, the percentage reduction for 60 ppm Cr (VI) were 40.3%, 47.2%, 61.63% and 54.1% in the presence of 10, 50, 100 and 150 ppm phenol, respectively. The removal percentage for phenol was also decreased, where only 19.9%, 20.8%, 50.7% and 38.9% of 10, 50, 100 and 150 ppm of phenol were removed, respectively.

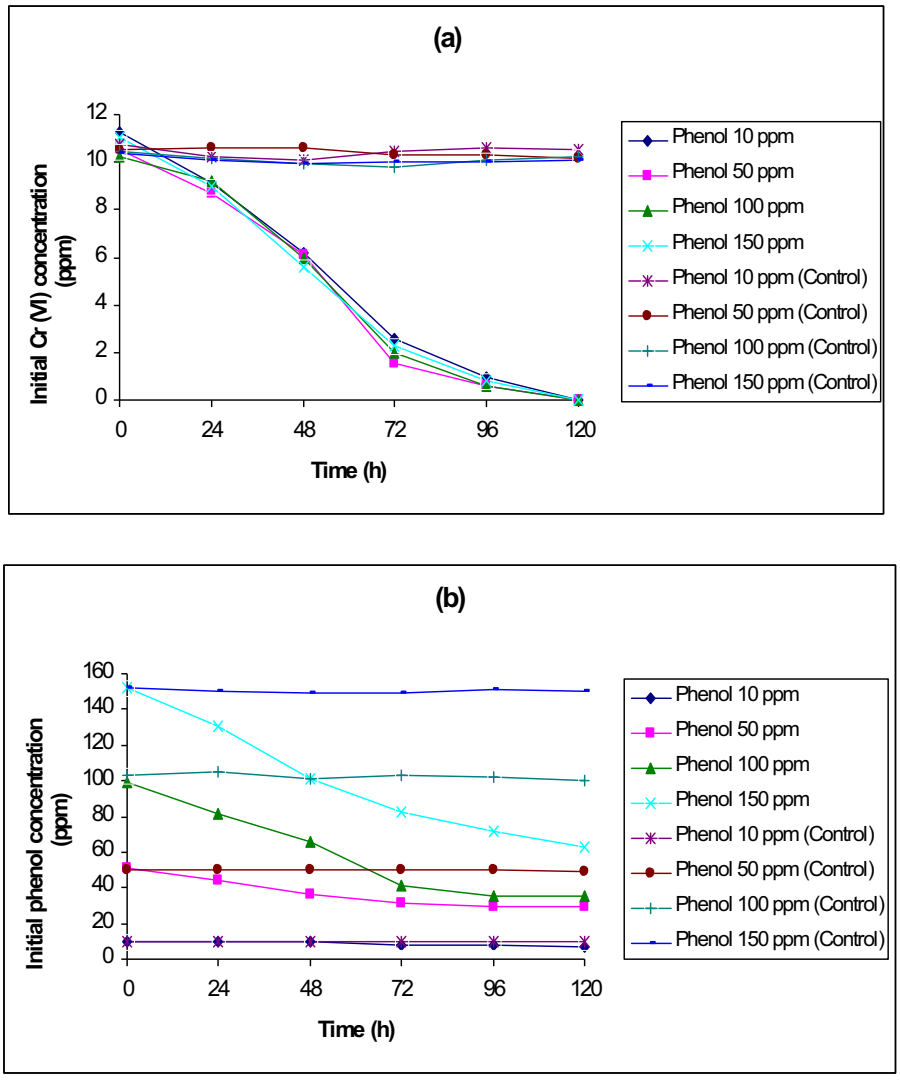


**Figure 2.16**: (a) Cr (VI) removal profile and (b) phenol removal profile of *A. haemolyticus* grown in NB containing 10 ppm Cr (VI) and varying concentrations of phenol.

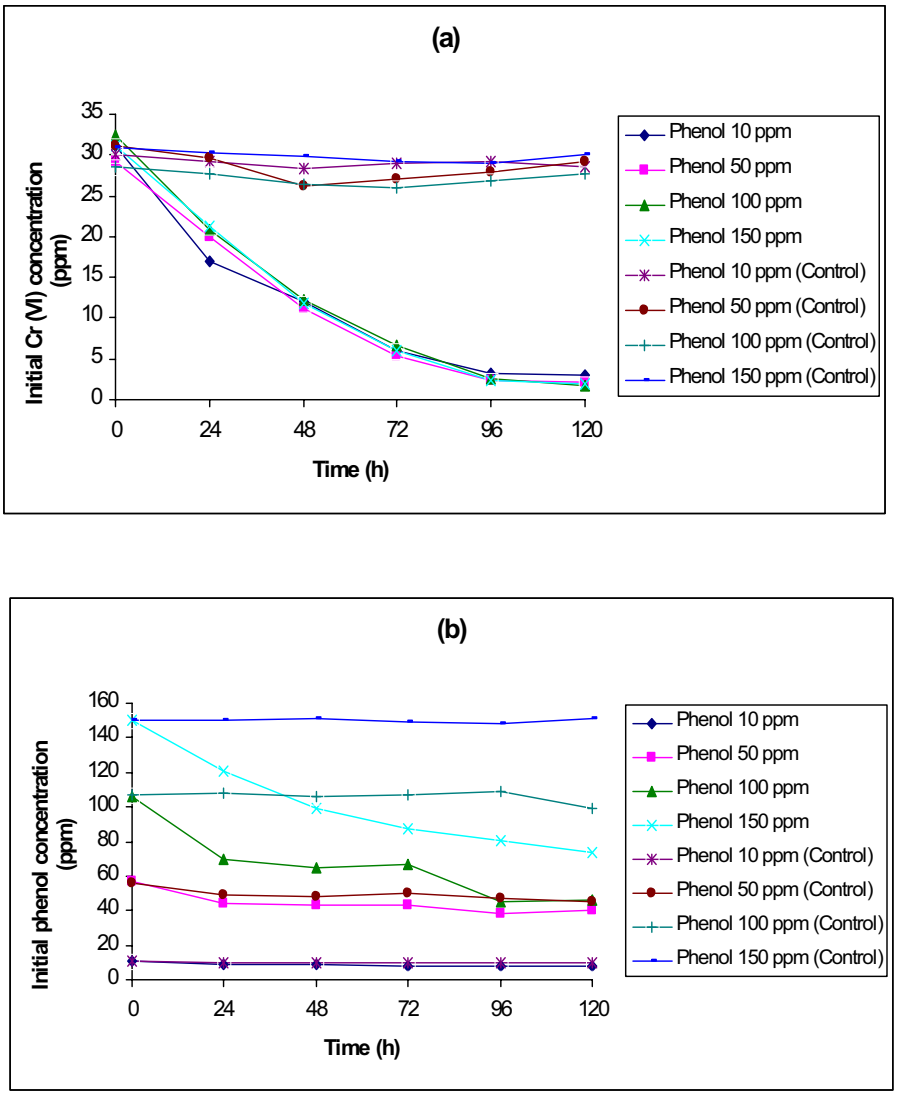


**Figure 2.17**: (a) Cr (VI) removal profile and (b) phenol removal profile of *A. haemolyticus* grown in NB containing 30 ppm Cr (VI) and varying concentrations of phenol.

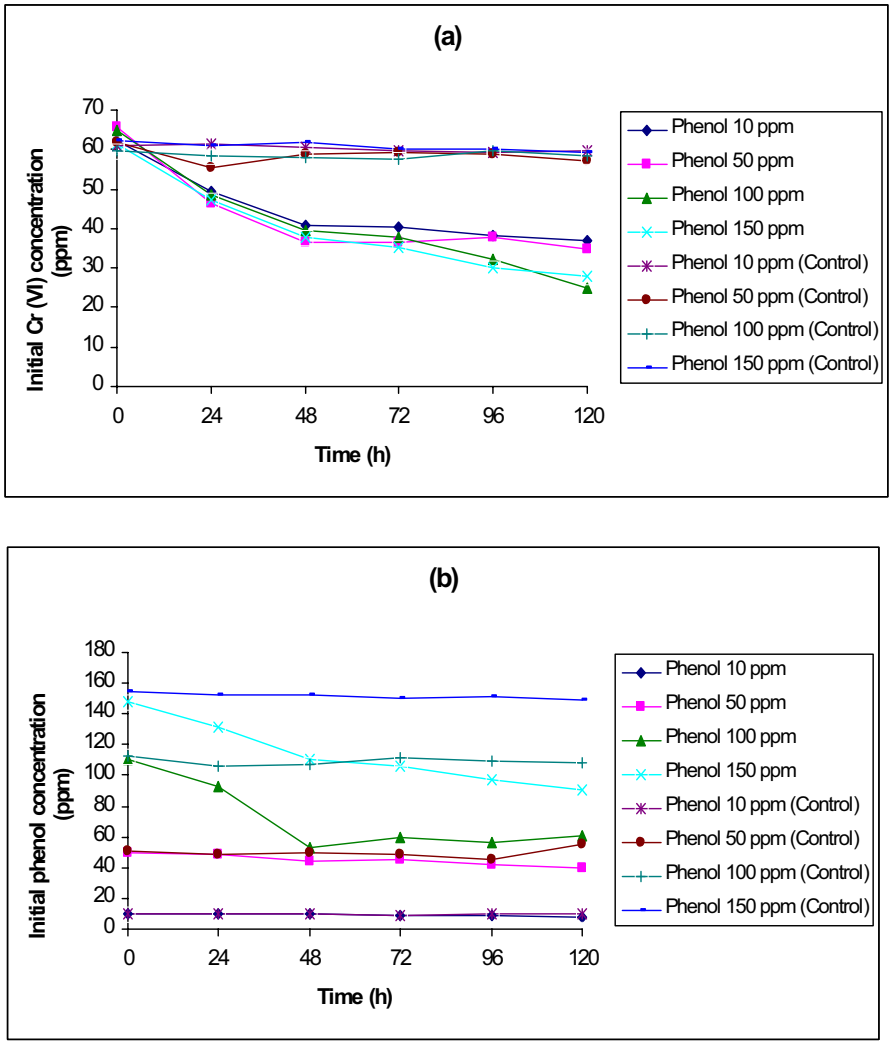


**Figure 2.18**: (a) Cr (VI) removal profile and (b) phenol removal profile of *A. haemolyticus* grown in NB containing 60 ppm Cr (VI) and varying concentrations of phenol.

From the results obtained, it was found that the Cr (VI) reduction was better in the presence of phenol at 100 ppm compared to other concentrations used in the study. A similar observation was observed by Yun – Gou *et al*. (2008), where optimum Cr (VI) reduction was observed at the phenol concentration of 100 ppm, where the reduction ratios were 57.8%, 73.47%, 84.47%, 79.67%, 57.93% and 49.60% for the initial phenol concentrations of 25, 50, 100, 150, 200 and 250 ppm, respectively. In the study, the simultaneous removal of Cr (VI) and phenol was carried out using the consortium culture of *Bacillus* sp. and *Pseudomonas putida*. Based on another report by Chirwa and Wang (1999) which uses anaerobic

consortium of bacteria (*Escherichia coli* ATCC 33456 and phenol degrader microorganism), optimum Cr (VI) reduction was achieved at the initial phenol concentration of 200 ppm during the simultaneous removal, where the phenol concentration used was from 50 – 500 ppm. The average Cr (VI) reduction rates were 0.34, 0.48, 0.48, and 0.2 (mg $L^{-1}h^{-1}$) for 50, 100, 200 and 500 ppm of phenol, respectively. From the results obtained, it showed that the phenol degradation during the simultaneous removal of Cr (VI) and phenol, could provide the main driving force for the biological activity in the culture (Chirwa and Wang, 1999).

However, the percentage of Cr (VI) reduction decreased when the initial concentration of Cr (VI) was increased from 10 ppm to 60 ppm. Also, the degradation percentage of phenol decreased when the initial Cr (VI) concentration increased. This could be due to the toxicity and oxidative potential of chromate, which may inhibit the biological activity of the bacteria at high concentrations (Chirwa and Wang, 1999).

#### 2.3.5.3 Simultaneous Cr (VI) and phenol removal by *A. haemolyticus* using real waste

The study on the removal of both Cr (VI) and phenol from an oil refinery waste water was also conducted (Figure 2.20). The initial concentration for Cr (VI) and phenol in the waste water were 0.2 ppm and 2.1 ppm. The removal percentage for Cr (VI) and phenol was 100% and 99% respectively in 2 days. From the experiment, almost complete removal for both Cr (VI) and phenol was achieved as the concentration of Cr (VI) and phenol in the sample was low, and thus can be easily degraded by the bacteria.

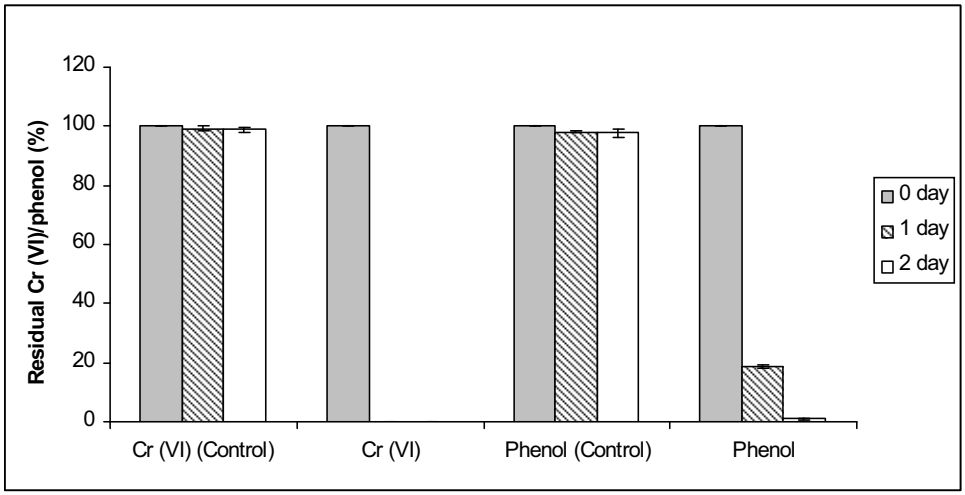


**Figure 2.19**: The percentage of Cr (VI) and phenol removed by *A. haemolyticus* using the real waste.

## 2.4 Conclusion

*A. haemolyticus* can remove both Cr (VI) and phenol, either in separate or simultaneous treatment using the batch system. This strain shows a promising ability to remove Cr (VI) and phenol and offers alternative methods for the detoxification of both pollutants. As a continuation of the study, column study for the removal of Cr (VI) and phenol by *A. haemolyticus* will be carried out and discussed in Chapter III.

# CHAPTER III

# THE REMOVAL OF HEXAVALENT CHROMIUM AND PHENOL USING A PACKED BED COLUMN

## 3.1 Introduction

This chapter will discuss the removal of Cr (VI) and phenol using the packed bed column system. The removal process of both contaminants will be conducted separately and simultaneously.

Application of immobilization techniques in the waste water treatment system is more attractive as it offers high removal efficiency for both contaminants besides good operational stability. The system also provides several advantages such as the production of high bacterial cell concentration, minimal clogging in the system and easier liquid – solid separation.

## 3.2 Materials and methods

### 3.2.1 Preparation of the packed bed column

#### 3.2.1.1 Column

In this study, a glass column with an inner diameter (ID) of 5.0 cm, outer diameter (OD) of 5.7 cm and a height of 50 cm was used. The column was first rinsed with $HNO_3$ (10%v/v) followed by deionized water and left to dry.

Inert stones were packed at the bottom and top of the column, where the stones were used to retain the column content and to distribute the water flow evenly. The mode of operation selected for the column was the down flow mode, with the use of a peristaltic pump (Eyela MP – 1000). The schematic diagram for the column used in the Cr (VI) reduction and phenol degradation process is shown in Figure 3.1.

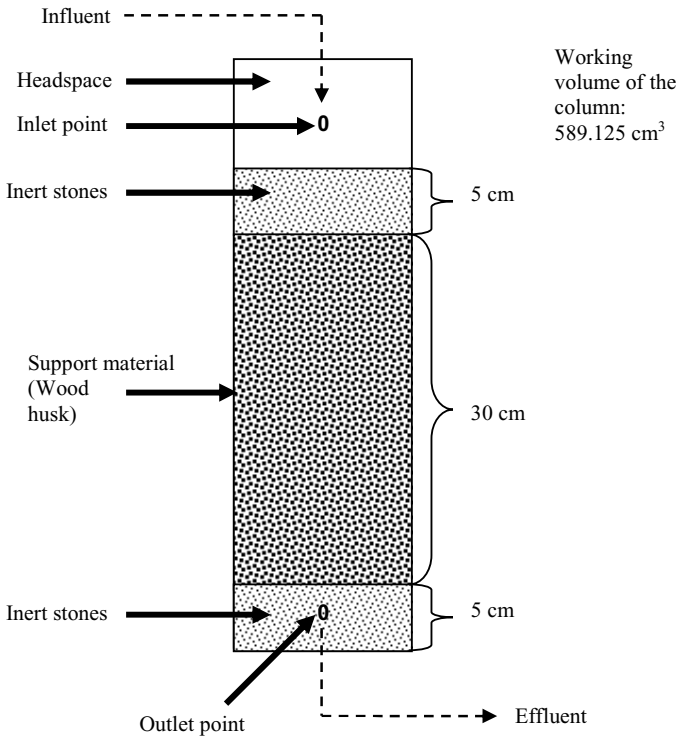


**Figure 3.1**: Schematic diagram of the column

### 3.2.1.2 Inert stones

The stone used in the column was obtained from a local aquarium store, located in Skudai. The stones were immersed in distilled water for 24 hours before rinsing several times with deionized water and dried at room temperature.

### 3.2.1.3 Support material

Wood husk, obtained from a local sawmill factory located in Pontian, was used as the support material for the immobilization of the bacteria. The wood husks

were brown to dark brown in color. The wood husks obtained were first sieved to remove dust or particles, which can clog the column.

The glass column was then packed with wood husk, followed by rinsing with deionized water using a peristaltic pump at a flow rate of 3 mL/min. The wood husk packed in the column was rinsed to prevent clogging by large particulate substances on the support materials and to allow the wood-husk surface material to acquire necessary charge for bacterial attachment (Zakaria *et al*., 2006).

Wood husk was chosen as the support material in the column because it is one of the natural sources for cellulose material. Cellulose is the most abundant natural polymer in the world and the most essential component of all plant fiber, about 40 – 50% of the plant mass (Bledzki *et al*, 2002). Cellulose, a sugar based polymer consist of glucose groups, and has the easily substituted hydroxyl group that provide a weakly basic and acidic ion exchange condition that can enhance bacterial attachment (Zainul *et al*, 2007). Besides, wood husk is also cheap, abundant and stable at operation conditions.

### 3.2.2 Immobilization of *A. haemolyticus* cell in the column

A starter culture of *A. haemolyticus* was first grown in 1 L of nutrient broth (Merck) medium at 30$^{o}$C, 200 rpm for 12 hours (Certomat R). Then, the culture was pumped into the column using a peristaltic pump (Eyela MP – 1000) at a flow rate of 2 mL/min. The culture was pumped continuously for 1 day. Next, sterilized nutrient broth was prepared and pumped into the column for another day as food supplementation for the bacteria, and to enhance the formation of biofilm by the cell.

### 3.2.3 The Cr (VI) and phenol removal system

The Cr (VI) reduction and phenol degradation system consists of the holding tank (A), outlet tank (D), peristaltic pump (B) and the column (C). The schematic diagram for the Cr (VI) reduction and phenol degradation system is shown in Figure 3.2.

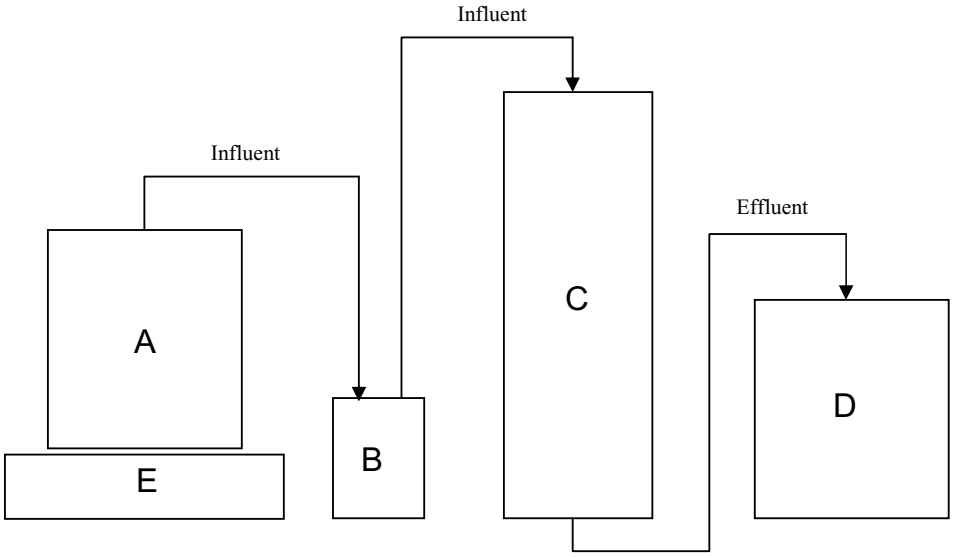


**Figure 3.2**: A – Influent/holding tank, B – peristaltic pump, C – column/bioreactor, D – effluent/receiving tank and E – magnetic stirrer.

#### 3.2.3.1 Separate removal of Cr (VI) and phenol using the column system

The influent tank was set up using a Schott bottle (1 L) which contains a mixture of supplementation medium (NB or LPW) and the waste water (simulated or real waste), where the mixture acts as the influent for the column system. The supplementation medium is necessary for the growth of bacteria in the column. For the Cr (VI) and phenol removal studies using simulated waste, the mixture in the influent tank was prepared with final Cr (VI) or phenol concentrations ranging from 10 – 150 ppm. When NB was used as the supplementation medium, the initial pH

recorded was around 6.5 – 7, thus no modification on pH was needed. However, for the mixture of LPW and Cr (VI) waste, the pH was modified to 7 as most of the bacterial systems operate optimally at this pH. For the treatment of industrial waste, waste water (20%) was incorporated into the supplementation medium and the pH of the solution was modified to 7 before being pumped into the column.

The mixture (influent) was pumped into the column (working volume = 590 $cm^3$) at a flow rate of 3 mL/min for a minimum time of 6 hours. Samples, 5 mL were collected from the holding and receiving tanks and analyzed for Cr (VI) and phenol. The effluent was recycled if the Cr (VI) or phenol waste concentration was still high after the treatment.

At the end of the column operation for the treatment of Cr (VI) waste, the column was washed with deionized water to flush out the remaining Cr (VI) before the other contaminant, phenol was pumped into the column. This step is important to remove residual Cr (VI) left in the column. The same step was repeated for the treatment of phenol waste, before proceeding with the treatment of mixed Cr (VI) and phenol waste.

#### 3.2.3.2 Simultaneous removal of Cr (VI) and phenol using the column system

The experimental setup for the simultaneous removal of Cr (VI) and phenol was as described in section 3.2.3.1, where the mixture of Cr (VI) and phenol acted as the simulated waste for the system. The mixed waste was combined with the supplementation medium (NB) before being pumped into the column. The concentrations of the mixed waste was varied and shown in Table 3.1. The pH of the mixture was unmodified as the pH for the mixture was around 6.2 – 7.0. The influent was pumped into the column at a flow rate of 3 mL/min for at least 6 hours. Then, the effluent was collected for the determination of Cr (VI) and phenol concentration.

**Table 3.1**: The concentration of Cr (VI) and phenol used as the mixed waste.

| Initial Cr (VI) concentration (ppm) | + | Initital phenol concentration (ppm) |
|---|---|---|
| 30 | + | 50/100/150/200 |
| 60 | + | 50/100/150/200 |
| 90 | + | 50/100/150/200 |
| 100 | + | 50/100/150/200 |
| 150 | + | 50/100/150/200 |

For the removal of real mixed waste of Cr (VI) and phenol, the waste was incorporated into the supplementation medium (20%) and the pH was modified to 7. Influent was pumped into the column with a flow rate of 3 mL/min for about 6 hours. Samples will be collected at the holding and receiving tank to analyze the Cr (VI) and phenol concentration.

### 3.2.4 Analytical methods

#### 3.2.4.1 The Diphenylcarbazide (DPC) method

The Cr (VI) concentration in the influent and effluent tank was analyzed using the diphenylcarbazide (DPC) method. This method was carried out as described in section 2.2.13.

#### 3.2.4.2 The 4 – aminoantipyrine method

The phenol content in the influent and effluent tank was determined using the 4 – aminoantipyrine method. Analysis for the phenol sample was carried out as described in section 2.2.14.

## 3.3 Results and discussion

### 3.3.1 Cr (VI) removal study using the column system

#### 3.3.1.1 Treatment of simulated Cr (VI) waste

The efficiency of Cr (VI) removal by immobilized *A. haemolyticus* was determined. Figure 3.3 shows the amount of Cr (VI) removed by the immobilized *A. haemolyticus* cell which has been supplemented with NB medium. Complete removal of Cr (VI) with concentrations below 100 ppm was achieved in 20 hours using the column system. For Cr (VI) concentrations higher than 100 ppm, a slightly lower removal (97%) was achieved within 20 hours. The column system showed a shorter removal time for Cr (VI) as compared to a batch system. In a column system, 90 ppm Cr (VI) was removed after 20 hours; compared to 144 hours using a batch system (section 2.3.3.3). Similar observations were reported on the effect of initial Cr (VI) concentration on the removal process, where longer time was needed to remove Cr (VI) at high concentration. As an example, *Streptomyces griseus* can fully reduce 10 ppm of Cr (VI) in less than 30 hours. However, for 50 ppm of Cr (VI), the bacteria can only remove 50% of the Cr (VI) content within 48 hours (Laxmann and More, 2002).

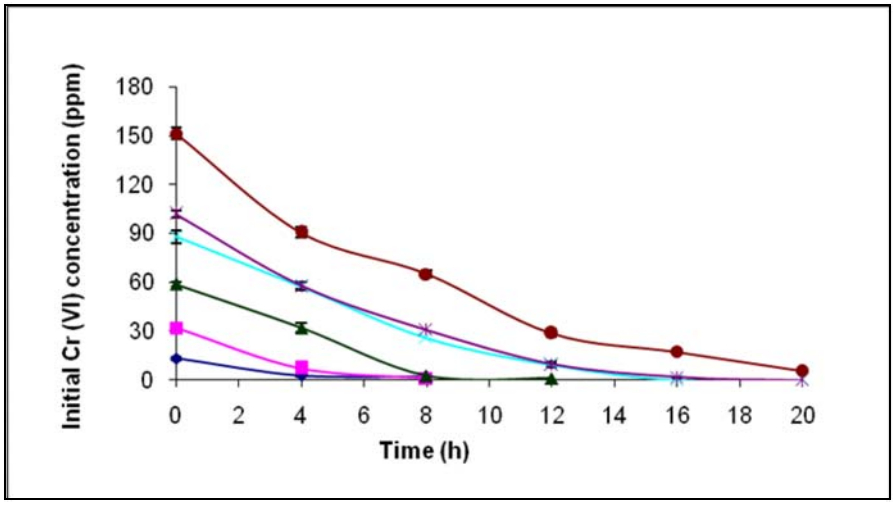


**Figure 3.3**: Cr (VI) removal by immobilized cells of *A. haemolyticus* in NB - supplemented mixture (♦: 10 ppm, ■: 30 ppm, ▲: 60 ppm, x: 90 ppm, *: 100 ppm, ●: 150 ppm).

A similar finding was reported by other researchers where the performance of column system was found to be better than the batch system (Ying *et al.*, 2006 and Konovalova *et al.*, 2003). The toxicity of a compound at high concentrations could inhibit the growth of the free bacterial cells and result in lower removal efficiency. Under the same circumstances, the presence of the support materials for the immobilized cells could act as the protective shelter against the toxic effect (Ying *et al.,* 2006). It has been reported that, the immobilization of cells could alter the physiological features in metabolism such as the enhanced enzyme induction (Chung *et al.,* 2003). In addition, the immobilization of microorganism into a packed column provides high cell concentration and active microorganism are held in stationary phase, which contributes to the better performance in the removal of toxic compound (Kim *et al.,* 2001).

The reduction of Cr (VI) using the immobilized cells of *A. haemolyticus* supplemented with the LPW is shown in Figure 3.4. Complete removal of Cr (VI) with concentrations below than 100 ppm was achieved in 8 hours. For Cr (VI) concentration higher than 100 ppm, a lower removal (93%) was achieved within 8 hours.

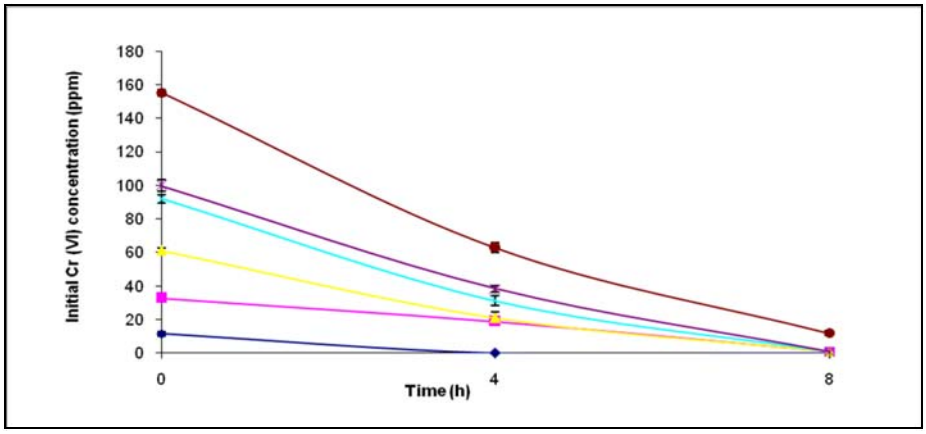


**Figure 3.4**: Cr (VI) removal by immobilized cells of *A. haemolyticus* in a mixture supplemented with LPW medium (♦: 10 ppm, ■: 30 ppm, ▲: 60 ppm, x: 90 ppm, *: 100 ppm, ●: 150 ppm).

The use of LPW in the column system contributes to better performance in terms of reduction time as the medium components play a role in Cr (VI) reduction. Several sugars (glucose, sucrose, fructose), cations, anions and organic acids are found in liquid pineapple waste (Idris and Suzana, 2005). Zhu *et al*. (2008) concluded that certain carbon compounds such as citrate, malate, lactate and glucose, play an important role in the Cr (VI) reduction process. These compounds act as the electron donors that will enhance the reduction process of Cr (VI). The specific Cr (VI) reduction activity by *E. coli* ATCC 33456 was also found to increase when certain organic substances including glucose, acetate, lactate and oxalate were added to the medium (Bae *et al*., 2000). For example, the specific Cr (VI) reduction activity of the culture increased to 0.62, 0.35, 0.36 and 0.40 (mg $g^{-1}$ $h^{-1}$) when glucose, acetate, lactate and oxalate were added respectively compared to 0.22 (mg $g^{-1}$ $h^{-1}$) where no substance was added. The evidence on the presence of glucose enhancing Cr (VI) reduction was further supported by Aravindhan *et al*. (2007), where Cr (VI) at 100 mg/L was reduced by mixed *Pseudomonas* cultures in 3 hours using glucose medium, compared to 24 hours using the glycerol medium. Thus, it can be concluded that the presence of organic compounds in the LPW especially glucose, can enhance the Cr (VI) reduction process by *A. haemolyticus*.

### 3.3.1.2 Treatment of real Cr (VI) waste

The treatment for real Cr (VI) waste water obtained from Perstima Sdn Bhd was carried out using immobilized cells of *A. haemolyticus* and the result is shown in Figure 3.5. Complete removal of Cr (VI) was observed in 12 hours when either NB or liquid pineapple waste was used as supplements. A shorter time was needed for Cr (VI) removal as the initial concentration of the waste water was only 58.91 ppm.

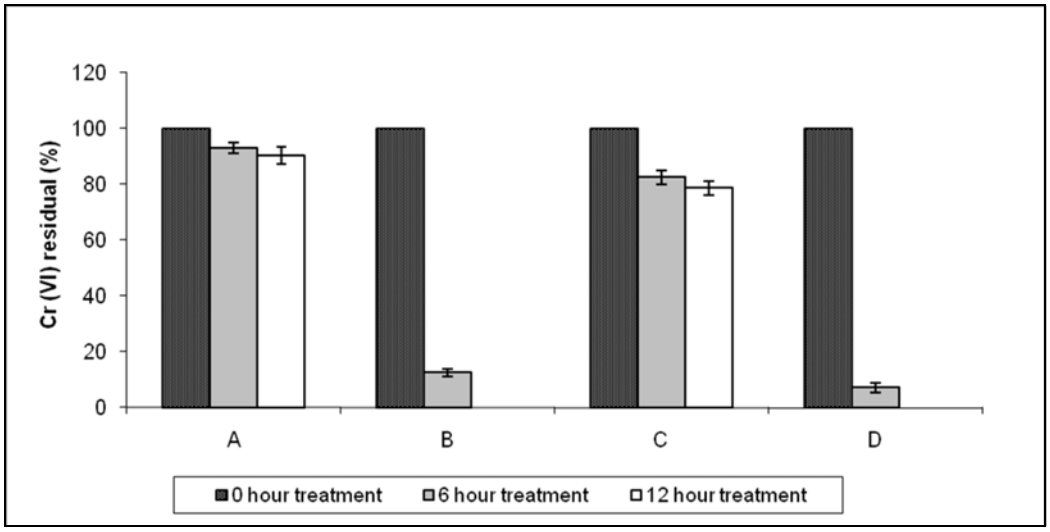


**Figure 3.5**: Removal of Cr (VI) from real Cr (VI) wastewater by *A. haemolyticus* in a column system (A – real wastewater supplemented with NB without bacteria (control), B – real wastewater supplemented with NB inoculated with bacteria, C – real wastewater supplemented with LPW without bacteria (control), D - real wastewater supplemented with LPW inoculated with bacteria).

### 3.3.2 Phenol removal study using the column system

### 3.3.2.1 Treatment of simulated phenol waste

Phenol removal study using immobilized cells of *A. haemolyticus* was conducted to monitor the ability of the bacteria to remove organic contaminants

besides treating heavy metal waste. The phenol removal profile using the column system is shown in Figure 3.6. Complete removal of phenol was observed for 10 and 30 ppm phenol in 4 and 12 hours, respectively. At higher phenol concentrations, ranging from 60 – 150 ppm, the removal percentages decreased and required longer time for the removal process. The removal percentages for phenol were as follows; 62%, 61%, 51% and 48% for 60, 90, 100 and 150 ppm, respectively, with removal time above 20 hours. The removal percentage for phenol using the immobilized cell system was low compared to other studies. A study on phenol degradation using *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel, showed that 150 ppm of phenol was fully degraded by the bacteria in only 5 hours (El – Naas *et al.*, 2009). Furthermore, from a study conducted by Tziotzios *et al.* (2005), which used a packed bed reactor immobilized with indigenous bacteria isolated from olive pulp, 2700 mg/l of phenol was fully removed in 12 hours using the system. However, it is worth mentioning that the column size used in the system was bigger.

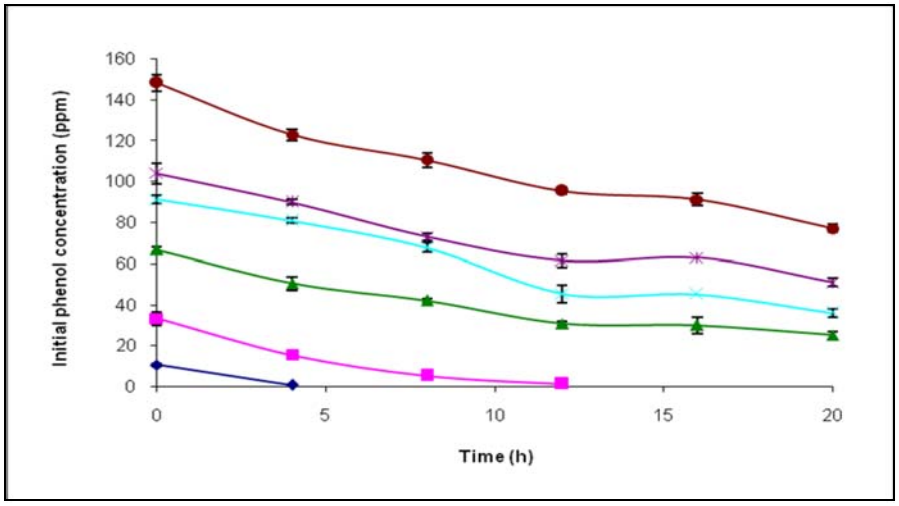


**Figure 3.6**: Phenol removal profile by the immobilized cells of *A. haemolyticus* using simulated phenol waste (♦: 10 ppm, ■: 30 ppm, ▲: 60 ppm, x: 90 ppm, *: 100 ppm, ●: 150 ppm).

It was observed that as the phenol concentration increased, the phenol removal efficiency by the column system was decreased. There are several reasons that could affect the performance of the column system, one of which is the initial

concentration of the substance used. The initial phenol concentration used in the removal system either the batch or column system is of great concern (Chung *et al*., 2003). At certain level of concentration, microbial growth can be inhibited due to the effect of phenol, which can cause bacterial cell lysis (Monteiro *et al*., 2000). Thus, at high phenol concentration, the low removal percentage of phenol obtained may be due to a reduction of viable cell number as a result of phenol – initiated lyses.

In spite of the above findings, the use of a column system for phenol removal showed great potential over other approaches such as the use of suspended or free cultures (Aksu and Bulbul, 1999 and Chen *et al.*, 2002). The immobilization technique in the column system can protect the bacteria from the toxic effect of phenol besides maintaining cell growth during the degradation process (Chung *et al*., 2003). Furthermore, the presence of support material allows the microorganisms to withstand washout and phenol toxicity as they are held on a stationary carrier surface (Kim *et al*., 2001).

#### 3.3.2.2 Treatment of industrial waste containing phenol

Phenol removal using immobilized cells of *A. haemolyticus* for industrial waste containing phenol was also investigated. The waste was obtained from an oil refinery and from the analysis, the initial phenol concentration detected in the waste water was 2.1 ppm. Figure 3.7 shows the result of the experiment, where 100% phenol was removed in 1 hour due to the low concentration of phenol. In the control experiment, the phenol was also removed from the waste, due to the support material i.e. wood husk adsorbing the phenol during the process, leaving no residual phenol in the waste. In previous studies on phenol degradation by *Pseudomonas sp.* and *Candida sp.* adsorbed on activated carbon, the activated carbon itself can adsorbed phenol to about half of its own weight. Therefore, it was difficult to distinguish between the phenol adsorption by the activated carbon and phenol degradation by the immobilized bacteria (Ehrhadt and Rehm, 1985).

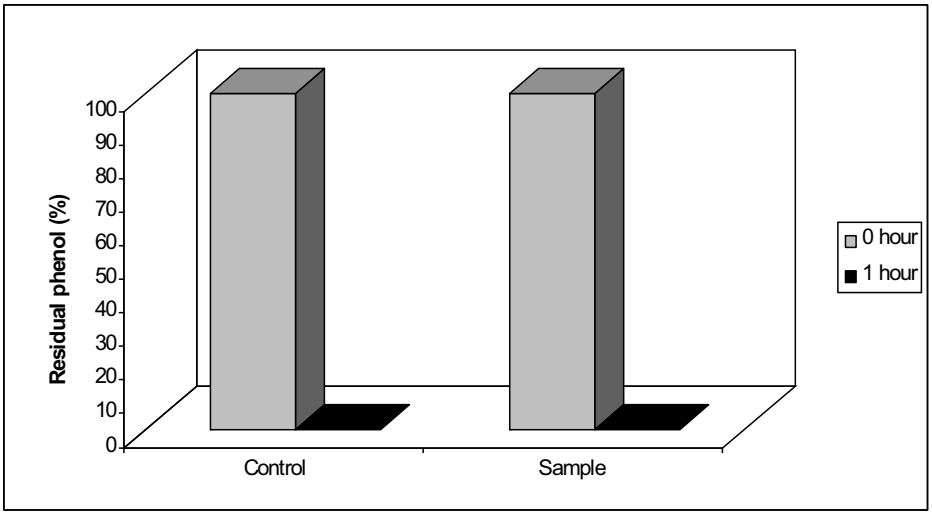


**Figure 3.7**: Phenol removal from industrial waste by immobilized cell of *A. haemolyticus*.

### 3.3.3 Simultaneous removal of Cr (VI) and phenol using the column system

#### 3.3.3.1 Treatment of simulated mixed waste of Cr (VI) and phenol

The simultaneous removal of Cr (VI) and phenol from waste mixture containing both chemicals by immobilized cells of *A. haemolyticus* was investigated and the results are shown in Figure 3.8 – 3.12. It was found that the Cr (VI) in the mixture was completely removed using the column system, with increasing removal time as the Cr (VI) concentration was increased. However, despite the high rate of removal of Cr (VI), the percentage removal for phenol was moderate.

As shown in Figure 3.8 (a), for 30 ppm of Cr (VI) in the presence of 50 – 200 ppm of phenol, the percentage removal for Cr (VI) was 100%. As for phenol, the percentage removal was 46%, 66%, 55% and 48% for 50 ppm, 100 ppm, 150 ppm and 200 ppm of phenol, respectively (Figure 3.8 (b)). Figure 3.9 (b) shows that the percentage removal for phenol was 42%, 59%, 55% and 47% for 50, 100, 150 and 200 ppm, respectively. At 60 ppm Cr (VI), 100% removal was observed (Figure 3.9

(a). For the mixture of 90 ppm Cr (VI) and 50 – 200 ppm of phenol, 100% of Cr (VI) was removed in all phenol concentrations used (Figure 3.10(a)). Figure 3.10 (b) shows that the phenol removal percentage was 37%, 45%, 41% and 40% for 50, 100, 150 and 200 ppm, respectively. For the initial Cr (VI) concentrations of 100 ppm, where complete Cr (VI) was observed, the percentage removal for phenol was 32%, 41%, 39% and 36% for 50, 100, 150 and 200 ppm, respectively (Figure 3.11 (a) and (b)). Figure 3.12 (a) and (b) shows that 150 ppm of Cr (VI) was removed in 24 hours and for phenol, the removal percentage was 27%, 36%, 34% and 29% for 50, 100, 150 and 200 ppm, respectively.

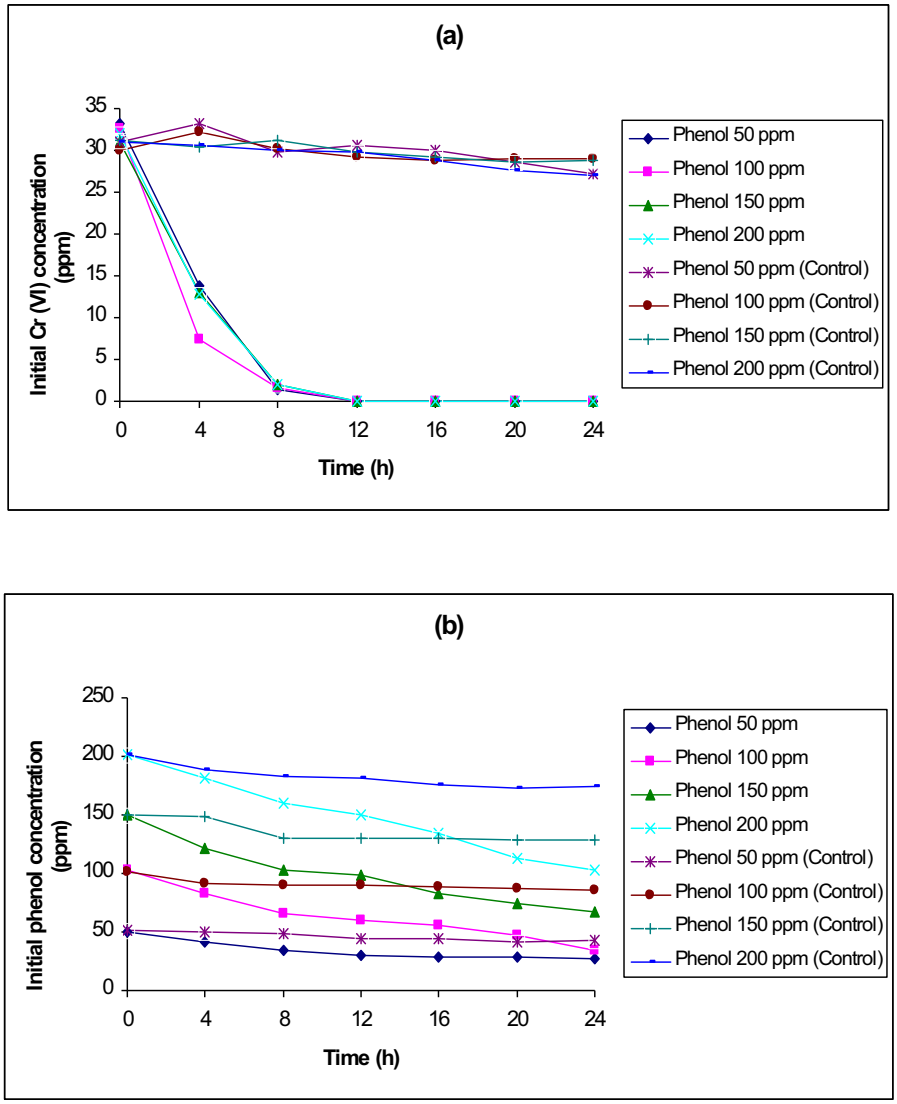


**Figure 3.8**: (a) Cr (VI) removal profile and (b) phenol removal profile by immobilized cell of *A. haemolyticus* supplemented with NB medium containing 30 ppm Cr (VI) and varying concentrations of phenol.

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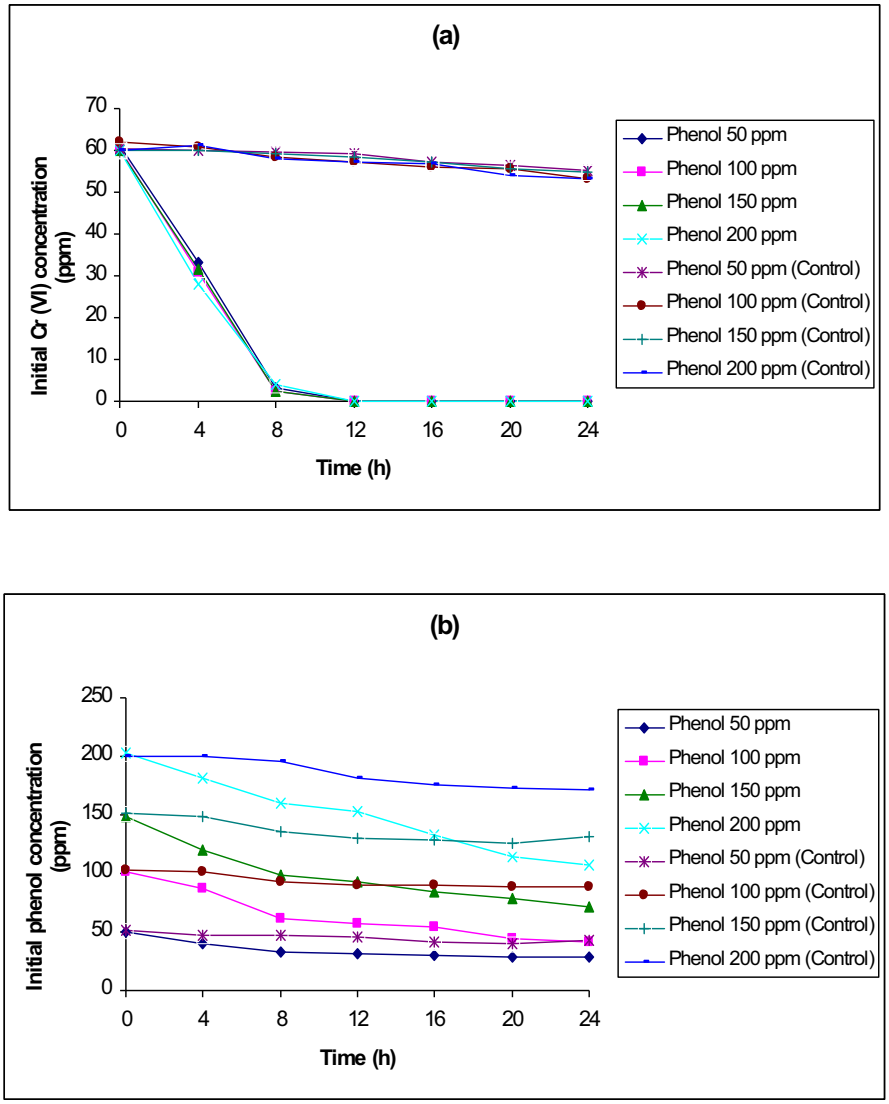


**Figure 3.9**: (a) Cr (VI) removal profile and (b) phenol removal profile by immobilized cell of *A. haemolyticus* supplemented with NB medium containing 60 ppm Cr (VI) and varying concentrations of phenol.

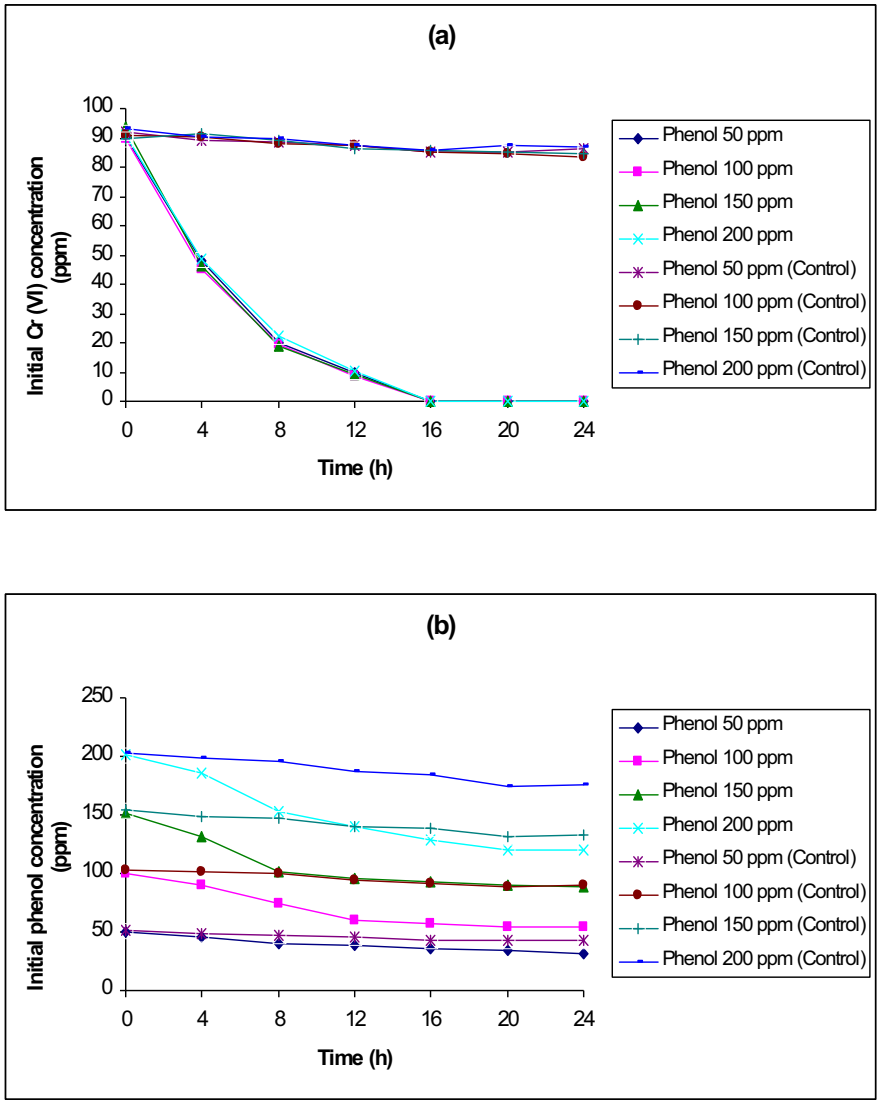


**Figure 3.10**: (a) Cr (VI) removal profile and (b) phenol removal profile by immobilized cell of *A. haemolyticus* supplemented with NB medium containing 90 ppm Cr (VI) and varying concentrations of phenol.

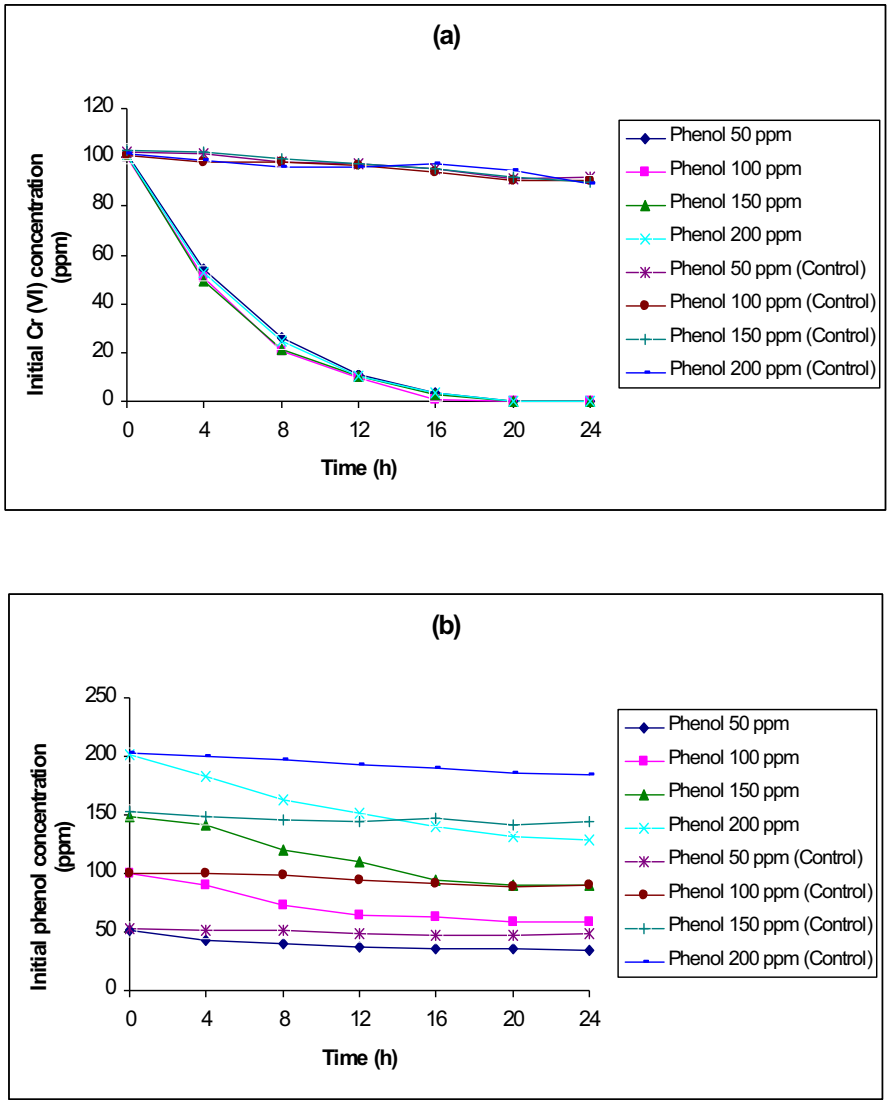


**Figure 3.11**: (a) Cr (VI) removal profile and (b) phenol removal profile by immobilized cell of *A. haemolyticus* supplemented with NB medium containing 100 ppm Cr (VI) and varying concentrations of phenol.

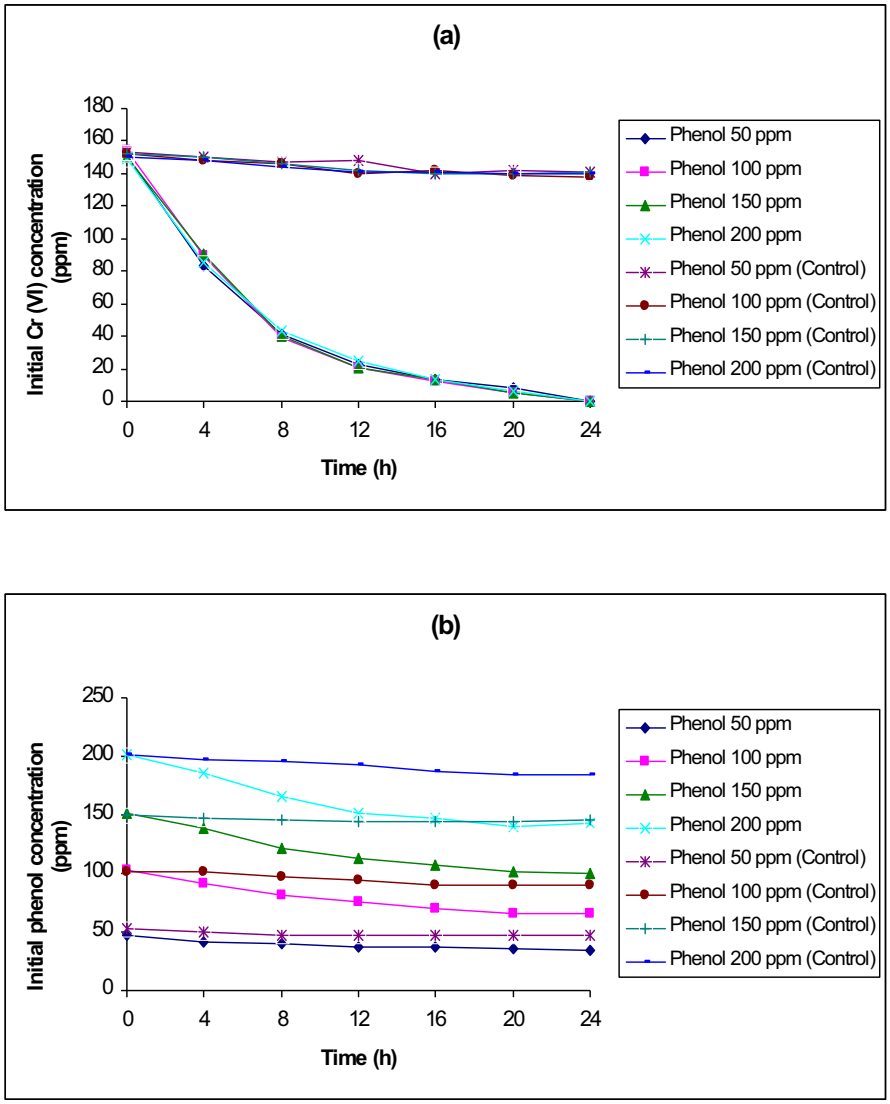


**Figure 3.12**: (a) Cr (VI) removal profile and (b) phenol removal profile by immobilized cell of *A. haemolyticus* supplemented with NB medium containing 150 ppm Cr (VI) and varying concentrations of phenol.

The results presented above show that Cr (VI) content in the mixed waste could be fully removed by the immobilized cells of *A. haemolyticus* in the column system. The immobilization technique applied for the treatment of the mixed waste therefore seems to be better than the free cell system as the bacterial cells were entrapped on the support material and remain viable for considerable duration of time (Karigar *et al*., 2006). Column systems with immobilized cells have been

increasingly used for waste water treatment, due to their high performance and stability. These advantages can be attributed to the high cell densities, stability of the cells against washout and the extended duration of biochemical or biotransformation reactions (Konovalova *et al*., 2003)

However, for phenol, the removal rate was only moderate although the same column system was used. Complete removal of phenol could not be obtained through the column system as the phenol removal mechanism by *A. haemolyticus* has not been elucidated yet. An understanding of phenol removal mechanism is important as it could contribute towards enhancing the removal process. Other non - biological mechanisms for phenol removal i.e. the adsorption of phenol to the microbial cell surface and accumulation of phenol inside the cell (Ahmaruzzaman, 2008 and Newsted, 2004) also need to be studied. These aspects will be described in Chapter 4.

### 3.3.3.2 Treatment of industrial waste containing Cr (VI) and phenol

The column system or immobilized cell technique was applied for the treatment of industrial waste containing Cr (VI) and phenol. The waste water used was obtained from an oil refinery as mentioned in section 2.2.1.1. The initial concentration was 0.2 ppm and 2.1 ppm for Cr (VI) and phenol, respectively. Figure 3.13 shows the percentage removal for both contaminants. From the results, 100% removal was observed for both Cr (VI) and phenol in less than 1 hour. It seemed that, complete and fast removal was achieved using this system to treat real mixed waste of Cr (VI) and phenol. However, this could be due to the low concentrations of both contaminants in the waste, where only 0.2 ppm Cr (VI) and 2.1 ppm phenol were present in the waste mixture. In the control experiment, both Cr (VI) and phenol were also removed from the industrial waste. The possible explanation is that the support materials itself can adsorb Cr (VI) and phenol, thus resulted in no Cr (VI) or phenol residual left. It is noteworthy that at low concentrations of Cr (VI) and phenol, the adsorption of both chemicals by the support materials i.e. wood husk is more dominant than the removal process by the bacteria. However, at high

concentrations, the presence of bacteria significantly promotes the removal of both contaminants. This is supported by the findings during the removal of both compounds from simulated mixed waste by immobilized cells of *A. haemolyticus* described in section 3.3.3.1.

A similar finding on the adsorption of targeted pollutants (i.e. Cr (VI) and phenol) by the support materials used during the removal process has been reported. For example, in the removal of phenol by biological activated carbon (BAC), the removal mechanism involves two processes; adsorption and degradation by the microorganism that lives on the surface or adsorption into the pores of the activated carbon. The activated carbon binds phenol in the solution until it reached the equilibrium state before the degradation process by the microorganism takes place for the complete removal of phenol (Wolborska, 2006).

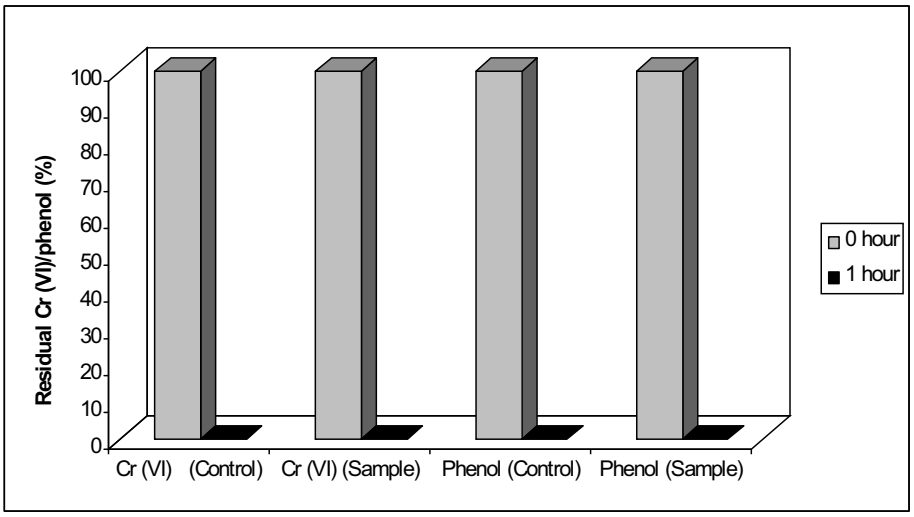


**Figure 3.13**: The percentage of Cr (VI) and phenol removed by the immobilized *A. haemolyticus* using the real mixed waste of Cr (VI) and phenol.

## 3.4 Conclusion

The use of column system containing immobilized *A. haemolyticus* for the removal of Cr (VI) and phenol, showed better removal efficiency for both wastes when compared to the batch system. For Cr (VI) with the range concentrations of 10 – 150 ppm, complete removal was achieved for both NB and LPW – supplemented mixture waste, in 20 and 8 hours, respectively. For phenol, complete removal was only achieved for low concentrations only, i.e. 10 and 30 ppm, whereas for higher concentrations, the removal percentage was around 50 -60%. In the simultaneous removal using mixed waste of Cr (VI) and phenol, complete removal was obtained for Cr (VI) while for phenol, the removal percentage was about 27 – 60%.

# CHAPTER IV

# THE EFFECT OF PHENOL ON *A. haemolyticus* AND OTHER REMOVAL METHODS FOR PHENOL

## 4.1 Introduction

In this chapter, the toxic effect of phenol on *A. haemolyticus* was monitored through the use of Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). Meanwhile the roles of the nonbiological mechanisms, i.e. bioaccumulation and biosorption that contributes to phenol removal by *A. haemolyticus* were also studied.

## 4.2 Materials and methods

### 4.2.1 The effects of phenol on the structure and morphology of *A. haemolyticus*

The effects of phenol on the cellular morphology of *A. haemolyticus* was investigated. The changes in the morphology of the cell structure after being subjected to different concentration of phenol was examined using SEM (Scanning Electron Microscope) while TEM (Transmission Electron Microscope) was used to observe the effect of phenol on cellular components.

### 4.2.2 Preparation of samples for SEM

The structure or morphology of *A. haemolyticus* before and after exposure to phenol was observed using SEM. *A. haemolyticus* grown in 0 ppm (control), 100 ppm and 1000 ppm of phenol for 24 hours, 200 rpm and 30$^{o}$C was centrifuged at 7500 rpm, 4$^{o}$C for 10 minutes to obtain the cell pellet. The pellet obtained was then washed thoroughly using sterilized deionized water, followed by immersing in glutaraldehyde solution (2.5% v/v, Fluka) for 2 hours. The cell pellet was then washed again with sterilized deionized water and subjected to osmium tetraoxide staining (2% v/v, Fluka) for 1 hour, followed by rinsing using sterilized deionized water. The cell pellet was dehydrated using 25, 50, 70 and 90% of ethanol for 5 minutes and 100% ethanol for 10 minutes. The dehydrated pellet was dried overnight in a dessicator and mounted on stainless steel stub, with a double stick carbon stub. The sample was then coated with a thin layer of platinum under vacuum to increase electron conduction and improve the quality of the micrograph (Tunali *et al*., 2005).

### 4.2.3 Preparation and growth of *A. haemolyticus* for TEM analysis

#### 4.2.3.1 Transmission Electron Microscope (TEM) Analysis

Cells of *A. haemolyticus* grown in various growth conditions; a) no phenol added b) addition of 100 ppm phenol, c) addition of 1000 ppm phenol for 24 hours, 200 rpm and 30$^{o}$C were centrifuged at 9000 rpm, 4$^{o}$C for 10 minutes. Pellet obtained was prepared for TEM examination (Philips TEM 400 Transmission Electron Microscope) using modified procedure of Bencosme and Tsutsumi (1970).

The cell pellet was fixed using 4% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline for 2 hours. It was then washed and suspended in 5 mL of the same buffer followed by staining using 1% (v/v) osmium tetraoxide in deionised water for 20 minutes. After washing in three changes of deionised water, the sample was stained using 2% (v/v) uranyl acetate in deionised water for 10 minutes. It was then

rinsed in deionised water prior to dehydration in 50%, 70%, 90% and 100% (v/v) ethanol for 3 minutes, respectively. Propylene oxide was then added for 5 minutes, mixture between epoxy resin and propylene oxide (1:1 and 3:1) for 15 minutes each and epoxy resin only (10 minutes). The sample was then embedded in a fresh epoxy resin and polymerized at 75$^{o}$C and 95$^{o}$C for 45 minutes each. The resultant resin block containing bacterial sample was allowed to cool at room temperature overnight before ultra-thin sectioning (70 – 80 nm thick) was made using LKB-IV System 2128 (Bromma, Sweden) ultramicrotome. The ultra-thin sections obtained were then viewed using TEM.

### 4.2.4 Bioaccumulation of phenol by *A. haemolyticus*

The phenol accumulation test on *A. haemolyticus* was carried out in order to investigate the other mechanism beside degradation which contributes to the phenol removal by *A. haemolyticus* during the experiment described in Chapter II.

#### 4.2.4.1 Phenol uptake experiment

An active culture of *A. haemolyticus* was inoculated into 100 mL of NB medium containing 100 ppm phenol and the mixtures were incubated at 200 rpm, 30$^{o}$C until stationary phase was reached. Aliquots of the mixture were taken at various time intervals for determination of phenol concentrations. Samples were centrifuged and resuspended in PIPES buffer and the supernatant, washed and cell digests were analyzed for their phenol content using the 4 – aminoantipyrine method.

#### 4.2.4.2 Cell digestion for *A. haemolyticus*

Cell pellet of *A. haemolyticus* grown in phenol was obtained by centrifugation at 7000 rpm, 4$^{o}$C for 5 minutes. The cell pellet was lysed using

lysozyme (10 mg/mL in 0.25 M Tris, pH 8) (Sigma, Grade I) and the liquid fraction was separated from cell debris using centrifugation at 7000 rpm for 10 minutes.

### 4.2.5 Biosorption of phenol by *A. haemolyticus*

The biosorption of phenol by *A. haemolyticus* was investigated to assess the ability of the cell to adsorb phenol. The ability of the cell to accumulate and degrade phenol was quite low, considering the high tolerance level of phenol shown by the bacteria. For this experiment, non living biomass of *A. haemolyticus* previously used for the degradation and accumulation experiments were used.

#### 4.2.5.1 Microorganism and growth condition

*A. haemolyticus* was cultivated at 30$^{o}$C in 100 mL of nutrient broth and shaken for 16 hours at 200 rpm. The bacteria was harvested when the cell growth reached stationary phase.

#### 4.2.5.2 Preparation of Non - living bacterial biomass

For the preparation of non – living bacteria biomass, the cells at the stationary phase were first harvested by centrifugation at 7500 rpm and 4$^{o}$C for 10 minutes (Sigma 4K15). The pellet obtained was washed twice with sterilized deionized water. The washed pellet was suspended in minimal volume of sterilized deionized water and was killed by autoclaving at 121$^{o}$C for 15 minutes (Hirayama).

#### 4.2.5.3 Dry weight determination

The bacterial cell dry weight was determined gravimetrically. The suspended cell (3 mL) was filtered through a pre weighed and dried membrane filter (Sartorius, Cellulose Acetate Filter, 0.45 µm pore size). The membrane filter was dried in an oven at 70$^{o}$C until constant weight was achieved. The experiment was carried out in triplicates and the dry weight was expressed as grams of dried cell in 1 mL of the suspended cells.

### 4.2.6 Preparation of phenol stock solution

For the biosorption study, phenol stock solution at 5000 ppm was prepared by dissolving 0.5 g of phenol crystal (Scharlau, Analytical Grade) in 100 mL of deionized water.

### 4.2.7 Biosorption study

Biosorption study was conducted in 250 mL conical flasks and the optimum operating conditions were determined. All the experiments were carried out using phenol solution at 100 ppm and the working volume used was 25 mL.

#### 4.2.7.1 Effect of contact time

The effect of contact time on phenol biosorption was investigated to determine the required time to reach the equilibrium state. The phenol biosorption was monitored for 48 hours, the bacterial biomass dosage used was 0.25% (w/v) and initial pH was adjusted to 7.

In this experiment, a number of flasks with the same mixture of phenol solution and bacterial biomass were prepared as described in section 4.2.7. At the end of shaking time, samples (10 mL) were centrifuged and the supernatant was analyzed for phenol content as described in section 4.2.8. The phenol solution at pH 7 without the bacteria was used as the control.

#### 4.2.7.2 Effect of biomass dosage

The study on the effect of biomass dosage for phenol biosorption was conducted using varying dosages; 0.25%, 0.50%, 0.75% and 1.0%, at the initial pH of 7. The mixture of phenol solution and bacterial biomass was prepared as described in section 4.2.7 at the biomass dosage stated above. The mixture was then shaken at 100 rpm, at room temperature for 24 hours, after which phenol analysis was conducted. Phenol solution at pH 7 without the bacteria biomass was used as the control.

#### 4.2.7.3 Effect of pH

The effect pf pH on phenol sorption by the bacterial biomass was studied at a pH range of 2 – 12. The biomass dosage used was 0.25% (w/v). Phenol solution (2.5 mL) was pipetted into a 25 mL volumetric flask and biomass was then added to give a final biomass dosage of 0.25% (w/v). The mixture was then transferred into a 250 mL Erlenmeyer flask and pH of the solution was adjusted to the desired value using $H_2SO_4$ or NaOH solution. Distilled water was then added to the mark on the 250 mL Erlenmeyer flask. The flask was then shaken at 100 rpm, room temperature for 24 hours (Certomat – R, B. Braun). The phenol solution at different pH without the bacterial biomass was used as the control set for the experiment.

After 24 hours, samples were withdrawn (10 mL) and centrifuged at 7000 rpm, 4$^{o}$C for 5 minutes (Sigma 4K15) to separate the biomass from the solution. The

supernatant collected was kept for phenol analysis using the 4 – aminoantipyrine method.

#### 4.2.7.4 Phenol biosorption isotherm study

The phenol biosorption study was conducted by varying the initial phenol concentrations. Throughout the experiments, the biomass dosage and temperature were kept constant. For this experiment, a 5000 ppm stock solution of phenol was prepared by dissolving 0.5 g of phenol crystal in 100 mL of deionized water. The initial phenol concentrations used were varied, with concentrations ranging from 25 – 1250 ppm while the bacterial biomass dosage used was 0.75% (w/v). The pH was adjusted and the mixture was then transferred into a 250 mL Erlenmeyer flask and distilled water was added to the mark. The flasks were shaken at 100 rpm, at 30$^{o}$C for 16 hours. Samples (10 mL) were centrifuged and the supernatant obtained was subjected to phenol analysis. Phenol solution with concentration ranging from 25 – 1250 ppm at pH 7 without the bacterial biomass was used as controls in the experiment.

### 4.2.8 Phenol analysis – 4 aminoantipyrine method

Phenol content was analyzed using a colorimetric method with 4 – aminoantipyrine as the color reagent (Martin, 1949). The method is as follows: 0.3 mL of 2% 4 – aminoantipyrine (Sigma, AR grade) and 1 mL of 2 N ammonium hydroxide (QRëc) was added into 50 ml of sample. The content was mixed thoroughly and 1 mL of 2% potassium ferricyanide (Merck, AR grade) was then added to the sample. The mixture which will form a red indophenol dye under alkaline condition was measured at the wavelength of 460 nm (Genesys 20, Thermo Spectronic). The phenol concentration was calculated using the phenol calibration curve, prepared from a phenol concentration ranging from 0.2 – 2.0 mg/L and

subjecting to the above procedure. The biomass adsorption capacity (uptake, q) was calculated using the following equation (Equation 4.1):

$$q = \frac{V (C_i - C_{eq})}{M} \qquad \text{Equation 4.1}$$

q – specific uptake of phenol (mg of phenol/g of biomass)
V – volume of phenol solution (L)
$C_i$ – known initial phenol concentration (mg/L)
$C_{eq}$ – residual (equilibrium) phenol concentration (mg/L)
M – known amount of biomass (g)

## 4.3 Results and discussion

### 4.3.1 The effect of phenol on *A. haemolyticus*

#### 4.3.1.1 SEM images of *A. haemolyticus* grown in the presence of phenol

The effect of phenol on the morphology of *A. haemolyticus* cells was observed using SEM technique. The SEM micrographs of *A. haemolyticus* cells grown in the absence and presence of phenol at 100 ppm and 1000 ppm are shown in Figures 4.1 – 4.3. From the micrographs, morphological changes of *A. haemolyticus* were observed. In the presence of 100 ppm and 1000 ppm of phenol (Fig 4.2 and 4.3), the cells tend to agglomerate and increased in size. As seen in Fig. 4.1, the diameter of normal cells of *A. haemolyticus* ranged between 510 – 550 nm, and when subjected with 100 ppm and 1000 ppm of phenol, the cells diameter increased to 520 – 570 nm and 780 – 915 nm, respectively.

The increase in size of the cells upon exposure to phenol has also been reported by Block (2001). The mechanism of reaction between phenol and bacteria involves the binding of active phenol species to the bacterial cell surface. When the active phenol species has bound to the outer layer of the cell, it will penetrate to its

target sites via hydrophobic lipid bilayer pathway for gram negative bacteria, or by passive diffusion for the gram positive bacteria. The inhibition of membrane bound enzyme will occur at the cytoplasmic membrane after the binding of the active phenol species to the exterior of the cell. Next, the damage on the cytoplasmic membrane by the action of phenol active species is the loss of the ability of the membrane to act as the permeability barrier. The failure of the cytoplasmic membrane to act as the barrier could explain the increase in cell size when phenol was added at high concentration (Block, 2001). Penetration of high phenol concentration into the cell would eventually disrupt the cell wall leading to agglomeration of the cells. Thus, it can be concluded that the exposure of *A. haemolyticus* to phenol can alter the morphology of the cell.

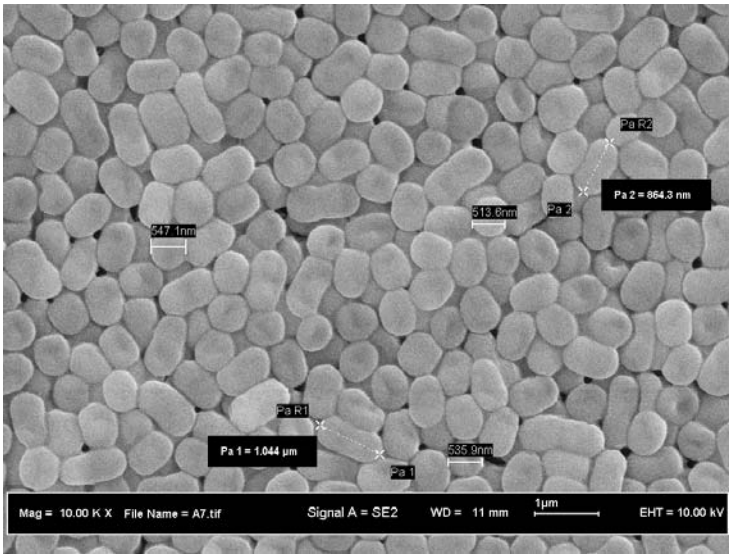


**Figure 4.1**: SEM micrograph of *A. haemolyticus* cells grown without phenol.

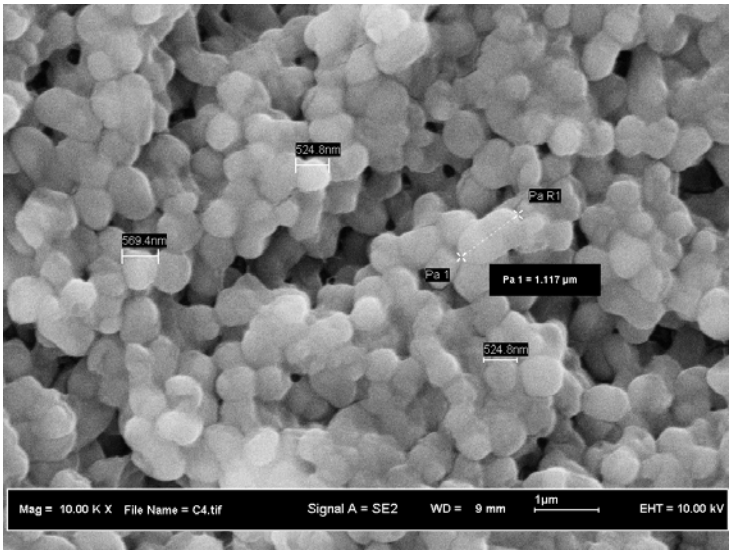


**Figure 4.2**: SEM micrograph of *A. haemolyticus* cells grown in the presence of 100 ppm phenol.

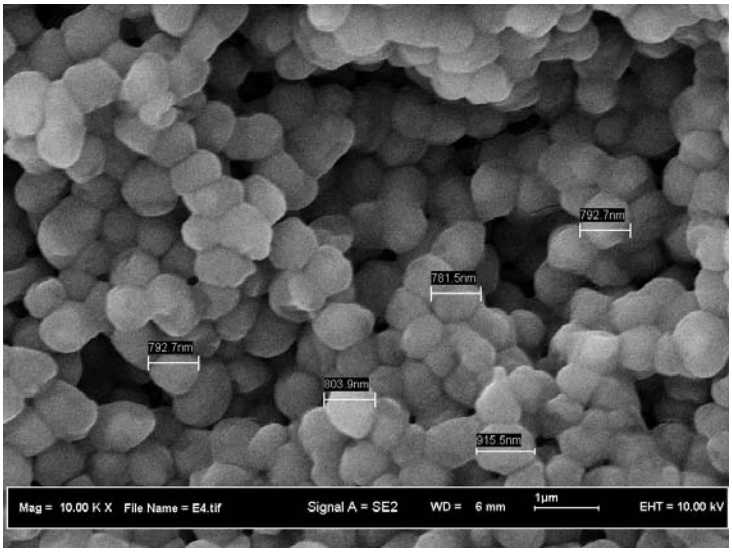


**Figure 4.3**: SEM micrograph of *A. haemolyticus* cells grown in the presence of 1000 ppm phenol.

#### 4.3.1.2 TEM images of *A. haemolyticus* grown in the presence of phenol

The effect of phenol at 100 ppm and 1000 ppm on *A. haemolyticus* was also monitored using TEM. TEM micrographs for *A. haemolyticus* grown in the absence and presence of phenol are shown in Figure 4.4 – 4.6. As indicated in Figure 4.5 and 4.6, formation of carbon storage product, poly β – hydroxybutarate (PHB) was observed in *A. haemolyticus* cell when subjected with 100 ppm and 1000 ppm phenol. Besides the formation of PHB, irregular bulges at the plasma membrane of the cell, known as bleb was also observed. The PHB granules and blebs could be clearly seen when the cell was exposed to a high concentration of phenol, 1000 ppm (Figure 4.6) and absent in cells grown without phenol.

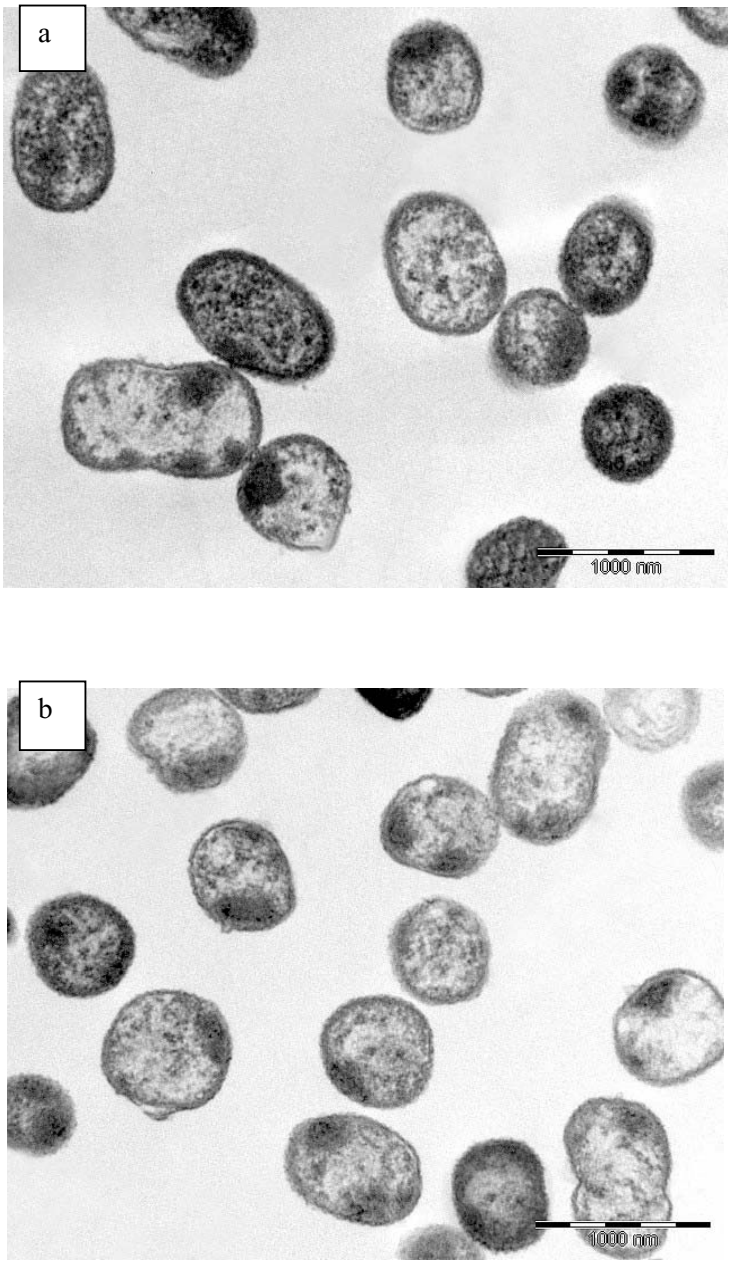


**Figure 4.4(a) and (b)**: TEM micrograph of *A. haemolyticus* cell grown without phenol.

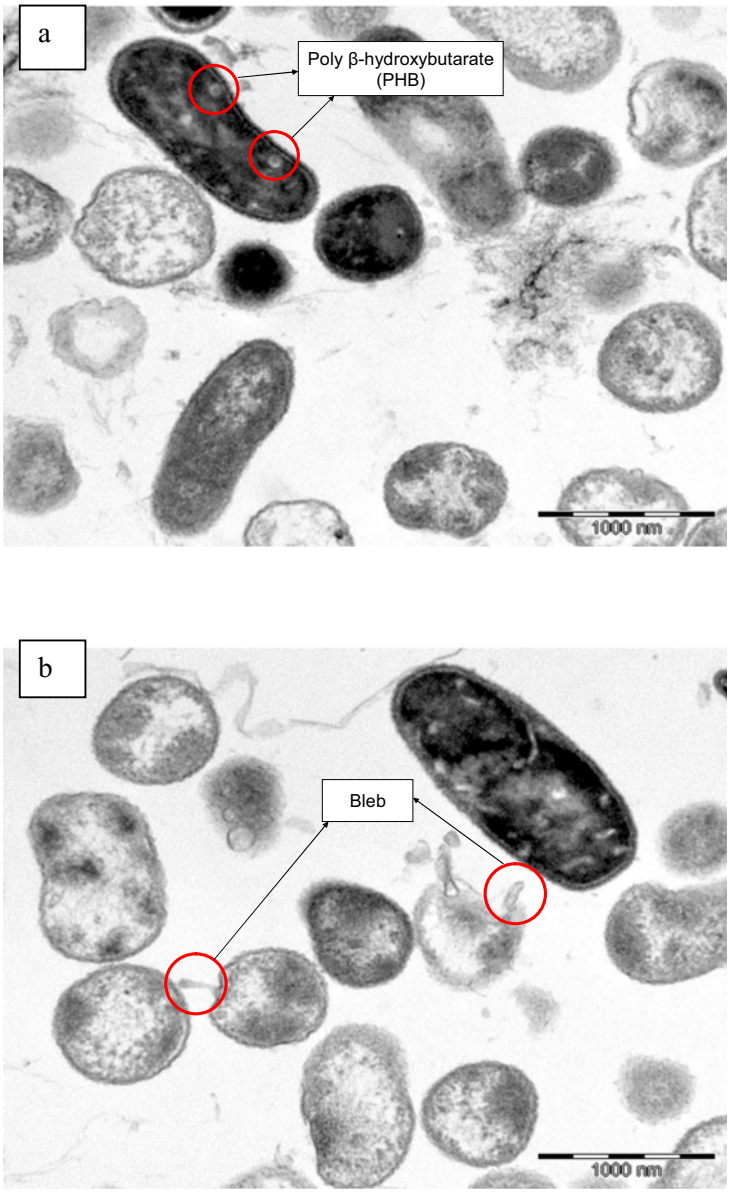


**Figure 4.5(a) and (b)**: TEM micrograph of *A. haemolyticus* cells grown with 100 ppm phenol.

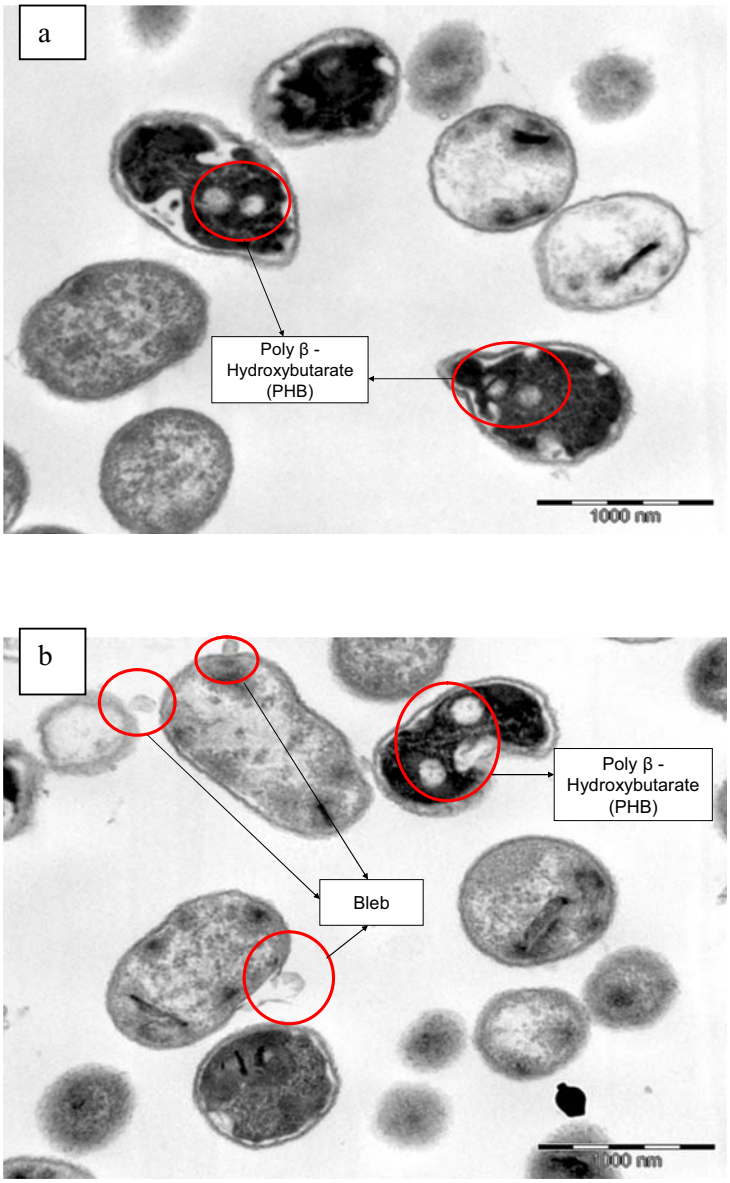


**Figure 4.6(a) and (b)**: TEM micrograph of *A. haemolyticus* cells grown with 1000 ppm phenol.

Poly β – hydroxybutarate (PHB) is a polymer which belongs to a class of polyesters also known as polyhydroxyalkanoate (PHA). PHA can be produced by many microorganisms such as *Alcaligenes eutrophus*, *Bacillus sp*. and *Rhodococcus sp*. (Ramsay *et al*., 1990). These granules were formed as a response by the bacteria to environmental stress or nutrient imbalance, besides acting as a carbon and energy sink (Lopez - Cortez *et al*., 2008). One of the main properties of the PHB is the

electron - translucent perisplasmic space or area which can be seen under the electron microscope. Similar observations regarding the electron translucent characteristic of PHB were also reported by several researchers (Berg *et al*,1979; Yakimov *et al*, 2001). The production of PHB during the growth in the presence of phenol is probably a detoxifying mechanism, where the bacteria could reduce the toxic effect of phenol and cope with the stressed environment.

The formation of blebs on plasma membrane is one of the morphological changes shown by the cell when it undergoes apoptosis. Apoptosis can be described as a series of biochemical events which leads to a variety of morphological changes including membrane blebbing, cell shrinkages and loss of membrane asymmetry and attachment (Vermeulen *et al.*, 2005). The phenomenon of membrane blebbing was shown by *A. haemolyticus* cells when the culture was exposed to phenol. This could be related to the inability of the cell to resist or detoxify the toxic effect of phenol, which leads to cell death. Phenol exhibits several types of anti - bacterial action by damaging the cell membrane and can cause the release of intracellular constituents. Phenol can also cause the intracellular coagulation of cytoplasmic constituents which could lead to cell death or inhibition of biological activities (Park *et al.*, 2001). Based on the report by Bennett (1992), phenol can also cause a leakage of intracellular components of the cell. The release of the intracellular constituents may reduce the viability of cell and lead to cell death.

As a conclusion, the addition of phenol into the growth medium has some significant effects on the bacteria. The changes in the intracellular structure of the bacteria after exposure to phenol was observed through TEM, including the formation of PHB granules and blebs.

### 4.3.2 Phenol bioaccumulation by *A. haemolyticus*

The study on the accumulation of phenol by cells of *A. haemolyticus* was carried out as described in section 4.2.4.1. After incubation for 24 hours, the cells were harvested and digested before analyzed for phenol content using 4 –

aminoantipyrine method. Table 4.1 shows the phenol concentration present in the supernatant, washed cells and cell digest of *A. haemolyticus* after the accumulation test.

**Table 4.1**: Phenol concentrations in the supernatant, washed cell and cell digestion of *A. haemolyticus* after accumulation test.

| | Phenol concentration (ppm) | |
|---|---|---|
| | Sample | Control |
| Supernatant | 93.5 $\pm$ 1.5 | 0 |
| Washed cell | 0.7 $\pm$ 0.2 | 0 |
| Cell digestion | 0.4 $\pm$ 0.3 | 0 |

The results obtained showed that low phenol concentration was present in both washed cell and cell digestion suggesting low phenol adsorption or accumulated by the cell. *A. haemolyticus* can only accumulate a very small amount of phenol into their cell as the phenol concentration in the supernatant was found to be high.

Limited information is available on the accumulation of phenol by microorganism, especially bacteria. The accumulation of phenol is usually shown by marine organisms such as fish, prawns and snail. In a study conducted using green algae, *Selenastrum capricornutum*, the average final phenol concentrations in the flask was approximately 5.32 mg/ L, which was about 60% of the initial concentrations measured. Thus, it can be summarized that green algae was able to accumulate phenol into their cell through several mechanisms, including abiotic processes or the one that alters their physiology and growth (Newsted, 2004).

The accumulation of phenol has antagonistic effect on the organism as reported by several researchers. The effect of phenol accumulation has been studied using blue tilapia juveniles, *Oreochromis aureus* (Abdel – Hameid, 2007). This work showed that after exposure to phenol, the internal organs of the fish such as the liver, showed histopathological symptoms, i.e. inflammation, central necrosis and cell degeneration. In addition, the levels of several metabolites in the liver including

glucose and total protein were reduced after exposure to phenol. These symptoms suggest that phenol induces liver damage and disturbs the metabolic state of *Oreochromis aureus* juveniles.

### 4.3.3 Phenol biosorption by *A. haemolyticus*

#### 4.3.3.1 Preparation of non - living bacterial biomass

In this study, non – living biomass was chosen as the biosorbent and prepared by growing *A. haemolyticus* in sterilized NB medium. The cells were harvested during the stationary phase of bacterial growth as the cell concentration was the highest at that time. The harvested cells were autoclaved at 121$^{o}$C to obtain the dead or non – living biomass. The use of non – living biomass has certain advantages compared to living system. Dead organisms are not affected by toxic wastes, do not require continuous supplementation of nutrient, and can be reused and recycled several times (Aksu, 2005).

It has been found that biosorbent derived from suitable microbial biomass can be used for the removal of phenolic compounds from solutions since certain phenolic compounds have a particular affinity for binding to microbial cells. The use of biomass for wastewater treatment is increasing because of its availability in large quantities and at low price. Besides, biomass has a high potential as an adsorbent due to its physico-chemical characteristics (Ahmaruzzaman, 2008).

#### 4.3.3.2 Effect of pH on phenol biosorption

Phenol biosorption study by *A. haemolyticus* was carried out in order to determine the ability of the cells to adsorb phenol. Several parameters on phenol biosorption such as pH, contact time, biomass dosage and initial phenol concentration were tested in order to optimize the biosorption process.

The effect of pH on phenol biosorption by *A. haemolyticus* was conducted using a set of Erlenmeyer flasks containing 25 mL of 100 ppm phenol solution. The pH range used for this experiment was from 2 – 12, with the biomass dosage of 0.25% (w/v). Figure 4.7 shows the result for the effect of pH on phenol biosorption by *A. haemolyticus*. From the results obtained, it was found that the highest uptake for phenol by the cell was 6.77 mg/g observed at pH 7.

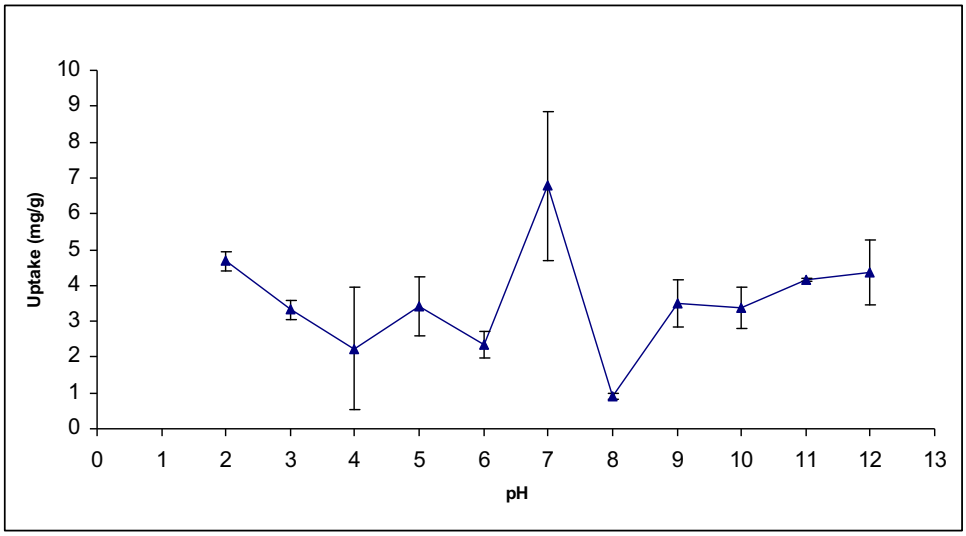


**Figure 4.7:** The effect of pH on phenol biosorption by *A. haemolyticus*.

pH is an important parameter in the adsorption of environmental contaminants in solution since it will influence the chemistry of phenol through certain processes such as hydrolysis and complexation. pH also contributes to the speciation and sorption availability for phenolic compound as well as the ionic state of functional groups at the biomass surface (Navarro *et al*., 2008). Besides phenol speciation, certain types of polarized group on bacterial surfaces such as phosphate, carboxyl, hydroxyl and amino group, which are capable of interacting with ions, are also influenced by pH (Vecchio *et al*., 1998).

Generally, phenol is weakly acidic, thus it partially dissociates to form phenate (phenoxide) ions and proton ($H^+$) (Andrews, 2004). This ion will be negatively charged and directly attracted due to the electrostatic forces by the

positively charges on biomass surface. The unionized phenol molecules can also be attracted, usually by physical force (Rao and Viraraghavan, 2002).

At high basic pH range, the presence of excess hydroxyl ions ($OH^-$) would compete with the phenol molecules for the biosorption sites. The adsorption of hydroxyl ions will change the positively charged surface into a negatively charged surface, which will repel the negatively charged phenoxide ion. This could explain the decreasing uptake in phenol biosorption at basic pH range (Aksu and Gönen, 2004). When the solution was changed to acidic pH, phenol molecules will be protonated and subsequently positively charged. This would cause repulsion between the positively charged surface and phenol molecule, thus resulting in decreased uptake (Rao and Viraraghavan, 2001).

However, Aksu and Akpinar (2001) proposed that the biosorption mechanism due to the initial pH is not sufficient to explain the efficient biosorption of both phenol and chromium (VI) observed at all pH values studied. It is thought that additional biosorption mechanisms such as ion exchange, complex formation, membrane transport and physicochemical forces such as Van der Waals and H – binding are important for the bioremoval of phenol and chromium (VI) ions by the biomass, irrespective of initial pH.

From a previous study on phenol biosorption by *Aspergillus niger* biomass, maximum phenol removal was observed at pH 5.1. A decrease or increase from this optimized pH will cause a reduction in the phenol removal. The use of activated sludge in phenol adsorption occurred best at pH 1 as studied by Aksu and Gönen (2004) and the equilibrium uptake capacity recorded at this pH was 55.6 mg $g^{-1}$. As the pH increased, the uptake capacity by the activated sludge reduced drastically. Other studies on the effect of pH on phenol biosorption is shown in Table 4.2.

**Table 4.2**: Effect of pH on phenol adsorption by various adsorbent

| Adsorbent/ Biosorbent | pH | Initial concentration | Uptake capacity/ Adsorption percentage | Reference |
|---|---|---|---|---|
| Jordanian zeolite | 10.5 | 0.15 mmol/L | 0.4 mmol/g | Yousef and Eswed, 2009 |
| Marine seaweed | 10.0 | 100 mg/L | 35 % | Navarro *et al.*, 2008 |
| Dried anaerobic activated sludge | 1.0 | 100 mg/L | 74 mg/g | Aksu and Akpinar, 2001 |
| Dried aerobic activated sludge | 1.0 | 100 mg/L | 49.8 mg/g | Aksu *et al.*, 1999 |

#### 4.3.3.3 Effect of biomass dosage on phenol biosorption

Figure 4.8 shows the uptake of phenol by different amounts of bacterial biomass dosage. The phenol uptake is as followes; 2.82, 3.09, 3.45 and 4.11 (mg/g) for the biomass dosage of 0.25%, 0.5%, 0.75% and 1.0% (w/v), respectively.

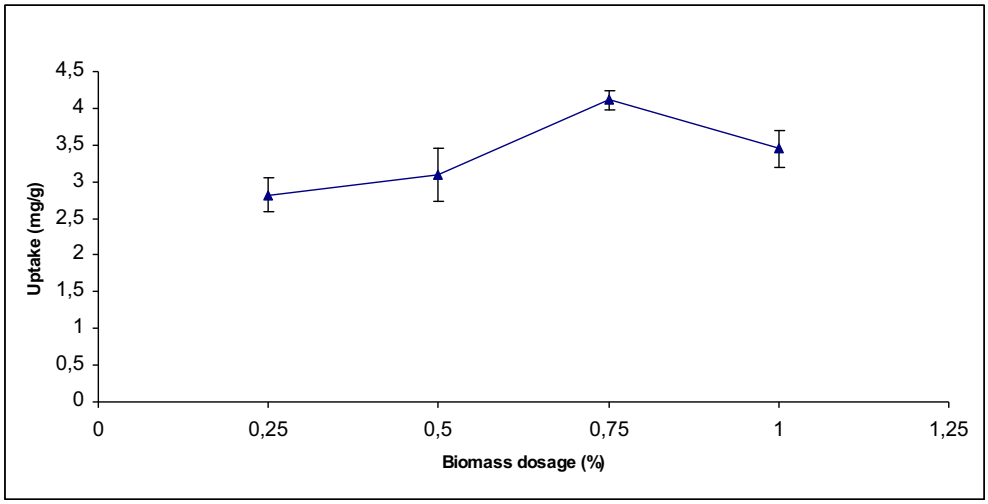


**Figure 4.8:** The effect of biomass dosage on phenol biosorption by *A. haemolyticus*.

The optimized biosorbent dosage was at 0.75% (w/v). The optimized uptake at 0.75% (w/v) of biomass dosage can be explained by the sorbate (ion)/biosorbent ratio. At lower biomass concentrations of a given sorbate concentration, the sorbate (ion)/biosorbent ratio was enhanced thus the sorbate uptake increase, provided the biosorbent is not saturated (Tsekova and Petrov, 2002).

At the highest biomass dosage, 1.0% (w/v), the uptake by the bacterial biomass decreased. A possible explanation is at high biosorbent concentration, cell aggregation may take place and this can contribute to the decrease in number of active sites on the biosorbent surface (Esposito *et al*., 2001).

#### 4.3.3.4 Effect of contact time on phenol biosorption

The effect of contact time on phenol biosorption by *A. haemolyticus* was studied. The experiment was conducted for 48 hours using a set of Erlenmeyer flasks containing 100 ppm of phenol solution and 0.25% bacterial biomass (w/v).

From the profile obtained (Fig 4.9), it was found that the biosorbent (*A. haemolyticus*) reached equilibrium state at 16 hours, where the uptake was 4.231 mg/g (Figure 4.9). After 16 hours, the uptake readings at 24, 36 and 48 hours were almost similar, around 4.2 – 4.3 mg/g.

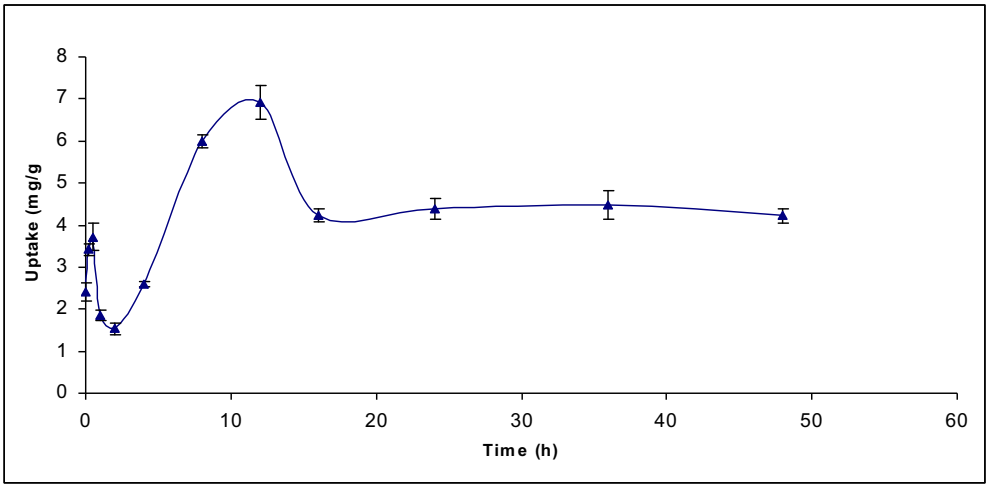


**Figure 4.9:** The effect of contact time for phenol biosorption using *A. haemolyticus*.

Different biosorbents have different adsorption capacity thus the kinetic for phenol biosorption varies amongst each other. From the report by Wu and Yu (2006), the biosorption of phenol by fungal *mycelia* showed the sorption uptake increased with increasing contact time and reached equilibrium after 90 minutes. When the contact time was prolonged to 6 hours, no significant effect was observed suggesting the surface of the fungal biomass was already saturated after 90 minutes.

Another example on the kinetic of phenol biosorption was demonstrated through the use of paper mill sludge. From the study conducted, the biosorbent needed a long time to reach the equilibrium phase, which was around 260 hours (Calace *et al*., 2002). Other biosorbents with their respective equilibrium time is listed in Table 4.3.

**Table 4.3**: Biosorbents and their equilibrium time in phenol biosorption.

| Biosorbent | Equilibrium time | Reference |
|---|---|---|
| *Aspergillus niger* | 24 hours | Rao and Viraraghavan, 2002 |
| Dried anaerobic activated sludge | 24 hours | Aksu and Akpinar, 2001 |
| Activated sludge | 50 minutes | Aksu and Yener, 1998 |

**4.3.3.5 Biosorption isotherm study for phenol biosorption**

The Langmuir and Freundlich isotherms are the most frequently used model to describe the biosorption isotherm. These models are simple, well – established and have physical meaning, besides easy to interprete the data. The Langmuir isotherm model is given by Equation 4.2 while Equation 4.3 represents the linearized form of the isotherm:

$$Q = \frac{Q_{max} b_L C_f}{1 + b_L C_f} \qquad \text{Equation 4.2}$$

Where,

Q - solute uptake (mg/g)

$Q_{max}$ - maximum achievable uptake by a system

$b_L$ - affinity between the sorbate and sorbent

$C_f$ - equilibrium solute concentration in solution (mg/L)

$$1/q = 1/Q^o + (1/bQ^o)(1/Ceq) \qquad \text{Equation 4.3}$$

From Equation 4.2, the Langmuir constant, $Q_{max}$ is used to compare the performance of biosorbent, where the other constant, $b_L$, characterized the initial slope of the isotherm. From the linearized equation, a plot of 1/q versus $1/C_{eq}$ would result in a straight line with a slope of $1/bQ^o$ and an intercept of $1/Q^o$.

The Freundlich isotherm is shown in as Equation 4.4 and the linearized form of Freundlich equation is shown by Equation 4.5.

$$Q = K_f C_f^{1/n_f} \qquad \text{Equation 4.4}$$

Where,

Q - solute uptake (mg/g)

$K_f$ - binding capacity

$N_f$ - affinity between the sorbent and sorbate

$C_f$ - equilibrium solute concentration in solution (mg/L)

$\ln q = \ln K + 1/n (\ln Ceq)$ Equation 4.5

From Equation 4.5, the plot of ln q versus ln $C_{eq}$ would result in a straight line with a slope of 1/n and intercept of ln k. The Freundlich isotherm was interpreted as the sorption to heterogenous surface or to surfaces supporting sites with different affinities. The isotherm assumed that stronger binding sites will be occupied first, with the binding strength decreasing as the degree of site occupation increased.

The effect of initial phenol concentration on the biosorption process was carried out in order to determine the biosorption isotherm. The study for biosorption isotherm was conducted at pH 7, 25$^{o}$C, 100 rpm of agitation rate, 16 hours of equilibrium time with the biomass dosage of 0.75% (w/v). As the other parameters were kept constant, the initial phenol concentration used was varied between 50 – 1250 ppm.

The uptake for phenol by *A. haemolyticus* biomass increased until the optimum uptake of 11.58 mg/g at 500 ppm of phenol (Figure 4.10). After this point, the phenol uptake by the biomass decreased slowly. The increase of loading capacity of adsorbent with increasing phenol concentration may be due to the higher probability of collision between phenol and the biosorbent (Aksu and Yener, 1998). However, increasing the phenol concentration generally caused a decrease in the adsorption capacity. This could be due to the saturation of adsorption site on the biomass surface at high phenol concentration.

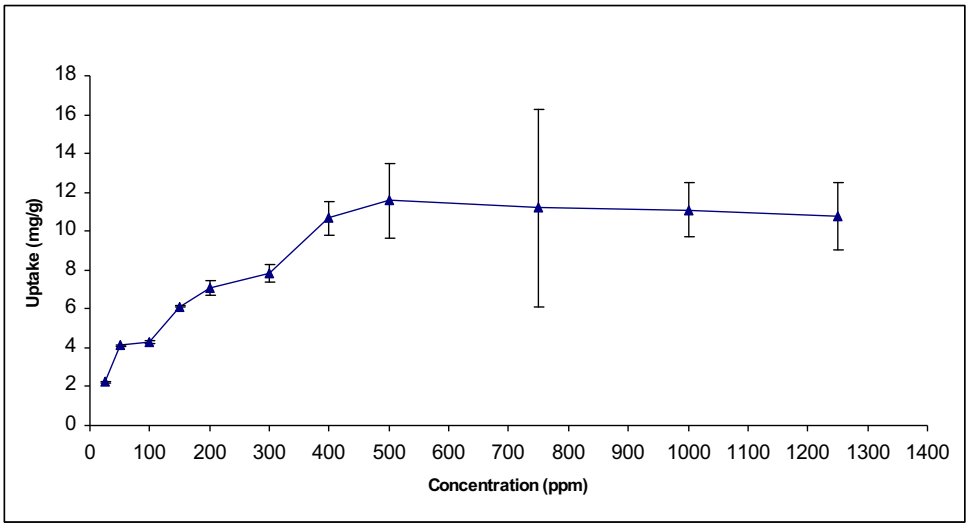


**Figure 4.10**: The effect of initial phenol concentration (ppm) for phenol biosorption by *A. haemolyticus.*

Different trends were observed by other researchers when studying the effect of initial concentration in phenol biosorption. From the investigation by Yousef and El – Eswed (2009), the loading capacity of phenol by natural zeolite increased when the initial phenol concentration increased, until the highest concentration of phenol used, 0.275 mmol. Based on a report by Aksu and Yener (1998), which investigated the biosorption of phenol and monochlorophenol by dried activated sludge, the uptake by the adsorbent increased with increasing phenol concentration until the maximum concentration of 500 mg $L^{-1}$.

The data obtained from phenol biosorption using *A. haemolyticus* was found to fit the Langmuir and Freundlich model, with the regression coefficient ($R^2$) of 0.9147 and 0.9015, respectively (Figure 4.12 and 4.13). From this, it can be concluded that the biosorption of phenol by *A. haemolyticus* is a monolayer phenomena. A similar conclusion was also reached by Kennedy *et al*., 1992, where the sorption data for phenol and a number of chlorophenol compounds by anaerobic granular sludge fitted the Freundlich equation.

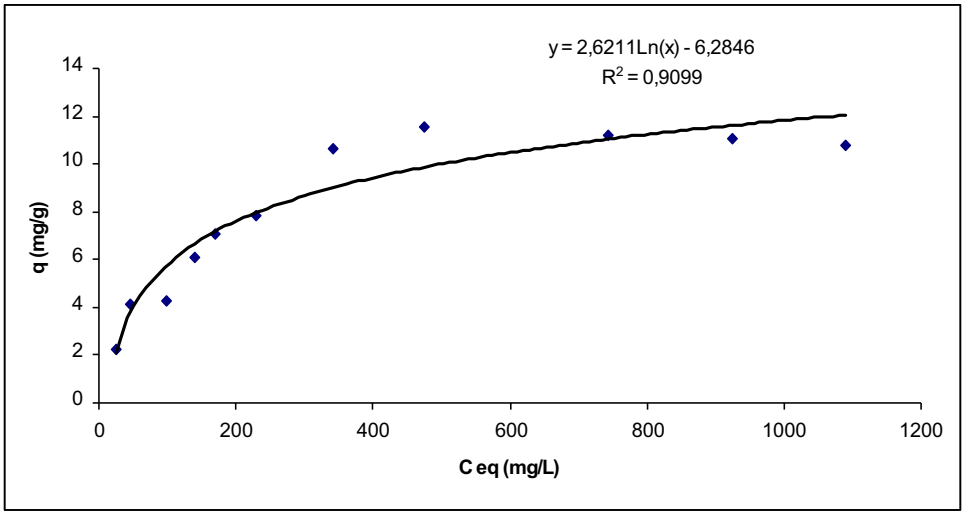


**Figure 4.11:** Langmuir isotherm for phenol biosorption by *A. haemolyticus*

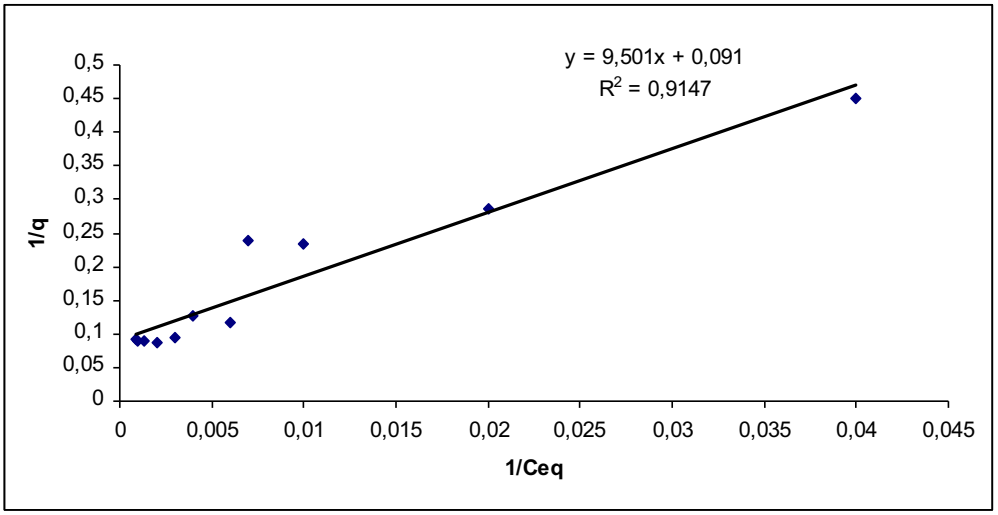


**Figure 4.12:** Langmuir isotherm (Linearized form) for phenol biosorption by *A. haemolyticus*

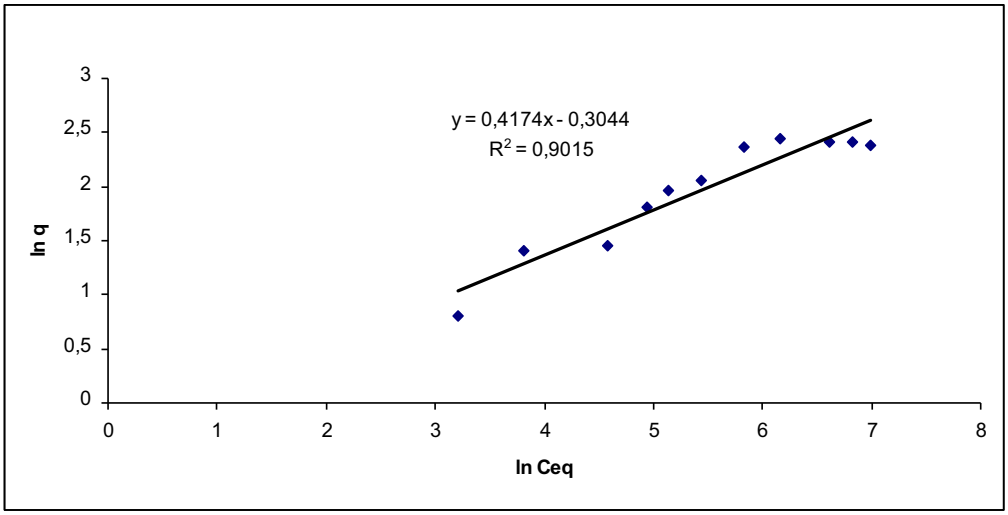


**Figure 4.13**: Freundlich isotherm for phenol biosorption by *A. haemolyticus*.

Table 4.4 shows the Langmuir and Freundlich adsorption constants calculated from the isotherm equation.

**Table 4.4**: Isotherm constants for phenol biosorption by *A. haemolyticus* biomass.

| Parameter | Langmuir isotherm | Freundlich isotherm |
|---|---|---|
| $R^2$ | 0.9147 | 0.9015 |
| $Q^o$ (mg/g) | 10.98 | - |
| b (adsorption energy, affinity) | 0.0096 | - |
| n (biosorption intensity) | - | 2.395 |
| K (biosorption capacity) | - | 0.7376 |

Another research by Aksu and Yener (2001), showed the sorption phenomena of phenol, o – chlorophenol and p – chlorophenol to the dried activated sludge were expressed by the Langmuir and Freundlich adsorption model. The adsorption isotherms showed that the equilibrium data for the pollutants fitted well to both Langmuir and Freundlich models.

## 4.4 Conclusion

In the presence of phenol, significant changes were observed either in the intracellular structure or on the cell morphology of *A. haemolyticus*. *A. haemolyticus* showed poor performance in accumulating phenol, but demonstrated a promising ability in biosorption of phenol.

# CHAPTER V

# GENERAL DISCUSSION AND CONCLUSION

## 5.1 Conclusions

Cr (VI) is considered as one of the most dangerous pollutant, largely due to its carcinogenic and mutagenic property. Another major pollutant found in the environment is phenol, which resulted from petroleum refining, leather tanning and metal finishing industries. Apart from the usual conventional methods, bioremediation has also proven to be cost effective and efficient in solving or treating wastes and environmental pollutants. Many studies have been conducted in past years on the removal of Cr (VI) and phenol using microorganisms. In this study, the ability of a locally isolated bacteria, *A. haemolyticus* to remove Cr (VI) and phenol from industrial waste waters was investigated.

In the preliminary studies using the batch system (Chapter II), *A. haemolyticus* showed the ability to grow in the presence of Cr (VI) and phenol, where growth was inhibited at 90 ppm Cr (VI) and 1500 ppm phenol. In the presence of both Cr (VI) and phenol, bacterial growth was inhibited at 60 ppm Cr (VI) and 150 ppm phenol. In experiments where removal of each pollutant was studied separately, the removal efficiency for Cr (VI) was very high but quite low for phenol. However, in simultaneous removal experiments, *A. haemolyticus* showed the ability to remove both compounds.

The use of a packed bed column system showed better removal efficiency for Cr (VI) and phenol, either separately or simultaneously. Shorter time for removal was demonstrated when the column system was used compared to the batch system.

The effect of phenol on the cellular morphology of *A. haemolyticus* was examined using SEM and TEM. Significant changes were observed in the intracellular structure and on the cell morphology of *A. haemolyticus* after exposure to phenol. The cells of *A. haemolyticus* were found to agglomerate and the size was bigger than normal cells. Exposure to phenol also led to the formation of blebs and poly β – hydroxybutarate (PHB) in *A. haemolyticus* cells.

Non – biological phenol removal methods i.e. via the accumulation and biosorption processes were also studied. The accumulation of phenol by *A. haemolyticus* was very poor, but biosorption was significant. Several parameters for phenol biosorption have been optimized, i.e. biomass dosage of 0.75% (w/v), contact time of 16 hours, pH 7 and initial phenol concentration of 500 ppm.

This study demonstrates the potential of using a biological material for simultaneous removal of phenol and Cr (VI) from wastewater contaminated with these two chemicals. The use of microorganisms such as *A. haemolyticus* offers durability under various operating conditions. The high percentage conversion of Cr (VI) to Cr (III) and high phenol removal by *A. haemolyticus* suggests that this may be an efficient and economical method to remove both pollutants form industrial wastewater

## 5.2 Suggestions for future work

Based on the study carried out in this research, *A. haemolyticus* was found to have a significant ability to remove Cr (VI) and phenol, either separately or simultaneously. However, the removal efficiency from a mixed waste water is moderate, thus an adaptation study for the bacteria in both Cr (VI) and phenol should be carried out to enhance the removal efficiency.

In this study, a single bacterial species (*A. haemolyticus*) was used for Cr (VI) and phenol removal. The use of bacterial consortium might be more effective.

This aspect could be investigated to improve the removal efficiency for both Cr (VI) and phenol from industrial waste water.

The use of NB for the cultivation of the bacteria in pilot or industrial scale is not favorable and economic. The use of a medium derived from liquid pineapple waste can be optimized to obtain better growth. Alternative sources for cultivation medium such as brown sugar can be used for the bacterial growth.

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## APPENDIX A

List of publications (journal/article), awards and seminar/paper presentation during Msc study period between December 2006 to December 2009.

### Publication and Seminar

Mohd Saufi Mohd Sidek, Shafinaz Shahir, Wan Azlina Ahmad and Zainul Akmar Zakaria. (2009). Simultaneous Cr (VI) and phenol removal by Cr (VI) – reducing *Acinetobacter haemolyticus*. Biodegradation (Submission being processed).

Mohd Saufi Mohd Sidek, Santhana Raj, Shafinaz Shahir, Wan Azlina Ahmad and Zainul Akmar Zakaria. (2009). The effect of phenol toxicity on *Acinetobacter haemolyticus*. Journal of Hazardous Materials. (Submission being processed).

Mohd Saufi Mohd Sidek, Shafinaz Shahir and Wan Azlina Ahmad (2008). Hexavalent chromium reduction and phenol degradation by *Acinetobacter haemolyticus*. Environmental Management and Technologies Towards Sustainable Development, International Conference on Environment 2008 (ICENV 2008). – Oral Presentation.

5$^{th}$ Annual Seminar of National Science Fellowship Scholarship (NSF), Universiti Putra Malaysia with paperwork entitled 'Hexavalent chromium and phenol removal by *Acinetobacter haemolyticus*' (19 – 20$^{th}$ November 2008).

### Grant

Awarded a research grant (RM 30 000) from ExxonMobil Exploration and Production Malaysia Inc. for research entitled 'Biodegradation of phenol found in oil refineries waste'.

**Awards and Acknowledgement**

Awarded the National Science Fellowship scholarship award to pursue MSc. Programme in UTM for a period of 2 years. (December 2006 – December 2008).

Featured in New Straits Times column (2008) "RM 30,000 grant to UTM". *New Straits Times*. 9 April 2008.

Featured in Utusan Malaysia column (2008) "Penyelidik UTM terima geran RM 30,000 ExxonMobil". *Utusan Malaysia*. 4 April 2008.

Featured in Kosmo column (2008) "Penyelidik UTM terima geran penyelidikan rawatan air terpakai". *Kosmo*. 4 April 2008.

**Other – related contribution**

Showcase at Hari Peladang, Penternak dan Nelayan Negeri Johor Exhibition, PERSADA Johor International Convention Centre with invention entitled 'Form – D – A rapid method of formalin detection in aquaculture products ($17^{th}$ – $20^{th}$ of August 2007).

# APPENDIX B

**CEPP LABORATORY SERVICES**
**CHEMICAL ENGINEERING PILOT PLANT**
**UNIVERSITI TEKNOLOGI MALAYSIA**
**81300, JOHOR BAHRU, JOHOR**
07-5531648 / 31665 / 31558
Fax : 07-5569706

CEPP
Chemical Engineering
Pilot Plant

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Date: 3/11/2008 page 1 of 1

| | |
|---|---|
| Sample Reference No. | CEPP/L309/08/012(03) & CEPP/L309/08/012 (04) |
| Company | Jabatan Kimia Fakulti Sains, UTM.Skudai Johor |
| Attention | Prof Dr.Wan Azlina Ahmad |
| Sample Description | C1 & C2 |
| Date of received | 25/10/2008 |
| Date of completion | 03/11/2008 |

**Sample test result**

| | C1 | C2 |
|---|---|---|
| Lead, Pb (ppm) | 0.011 | 0.016 |
| Mercury, Hg (ppm) | ND | ND |
| Copper, Cu (ppm) | 0.065 | 0.047 |
| Zinc, Zn (ppm) | 0.152 | 0.109 |
| Arsenic, As (ppm) | 0.001 | 0.014 |
| Iron, Fe (ppm) | 3.736 | 3.669 |
| Magnesium, mg (ppm) | 0.008 | 0.006 |
| Nikel, Ni (ppm) | 0.140 | 0.139 |
| Cadmium, Cd (ppm) | 0.002 | 0.027 |
| Chromium, Cr (ppm) | 49.590 | 68.229 |
| Cobalt, Co (ppm) | 0.027 | 0.001 |
| Calcium, Ca (ppm) | 7.298 | 8.068 |
| Sodium, Na (ppm) | 132.111 | 181.088 |
| Phosphorus, p (ppm) | 0.460 | 0.470 |
| Selenium, Se (ppm) | ND | ND |
| Aluminum, Al (ppm) | 0.186 | 0.389 |
| Silver, Ag (ppm) | 0.764 | 0.011 |
| Barium, Ba (ppm) | 0.001 | 0.001 |
| Potassium,K (ppm) | 11.577 | 12.291 |

Verified by,

Nooraizwani Zainol
Technical Manager
CEPP Laboratory Services