Bacteriology Research Developments

Bacteria in Environmental Biotechnology

The Malaysian Case Study-Analysis, Waste Utilization and Wastewater Remediation

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Editors

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BACTERIA IN ENVIRONMENTAL BIOTECHNOLOGY: THE MALAYSIAN CASE STUDY-ANALYSIS, WASTE UTILIZATION AND WASTEWATER REMEDIATION

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PREFACE

Environmental biotechnology encompasses a wide field of science and engineering. In a nutshell, it revolves around the use of microorganisms in a well-defined process to produce something valuable to mankind. Real-time practitioners, academicians, researchers and students from different backgrounds such as chemistry, biochemistry, chemical engineering, bioprocess engineering, environmental scientists and engineers, microbiologist, statisticians and sometimes mechanical engineers, would happily stake a claim that they would somewhat contribute towards the advancement of knowledge in this exciting field.

During the last 15 or 20 years, Malaysia has put a great emphasis on the importance of biotechnology in driving the future of the country's economy and wealth. Numerous funding and research grants have been made available to the academia, researchers and biotechnologists to pursue their interests, whether in the laboratory or at the pilot-scale. The government, via the Ministry of Science, Technology and Innovation (MOSTI), always encouraged a close collaboration between researchers and industry as to ensure all research outputs can be benefited as quickly and as usefully as possible to the intended beneficiaries.

We believe that this book, written as a case study approach, will provide an insight into important aspects of Environmental Biotechnology. The content includes applications of bacteria for the removal of toxic and precious metals from solution, degradation of phenol and production of protein liquor from solid waste obtained from the fisheries industry. There are also chapters (8 and 9) that focus solely on the interactions between metals and bacteria. Even though the chapters presented in this book described work carried out at lab-scale, the experiments were designed for applications in the industry. This is

evident from the direct involvements by various industries such as electroplating enterprise, oil processing facility, Brackishwater Aquaculture Research Center and PERMINT Minerals.

We are highly indebted to the contributors for their enthusiastic support and cooperation in preparing this book. We are also grateful to the Ministry of Science, Technology and Innovation, Malaysia (MOSTI) for the financial support. This book might not serve everyone in the area of Environmental Biotechnology; however, we envisage that this book would interest academicians, practitioners, researchers, entrepreneurs, policymakers, graduate students and anyone keen in applying bacteria in various processes.

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Chapter 1

BACTERIAL REDUCTION OF Cr(VI) - CONTAINING ELECTROPLATING WASTEWATER UTILIZING AGRICULTURAL WASTES

Wan Azlina Ahmad, Salmijah Surif and Zainul Akmar Zakaria

ABSTRACT

Acinetobacter haemolyticus, a Gram-negative aerobic locally isolated bacterium, immobilized on wood husk, showed the ability to detoxify Cr(VI) to Cr(III). Wood husk, a natural cellulose-based support material, packed in an upward-flow column was used as support material for bacterial attachment. Around 97% of the Cr(VI) in wastewater containing 15 mg L^{-1} of Cr(VI) was reduced when liquid pineapple wastewater (LPW) was used as nutrient. Substitution of the LPW with brown sugar resulted in a much higher Cr(VI) reduction capacity for the bacteria, with 99.8 to 100% of the initial 237 to 320 mg L^{-1} of Cr(VI) reduced. This remarkable Cr(VI) removal capacity was largely assisted by the abiotic Cr(VI) reduction by brown sugar used. The column Cr(VI) reduction capacity increases with column length, indicating the importance of column design to ensure process efficiency. The high percentage conversion of Cr(VI) to Cr(III) suggests the feasibility of using a bacterial system as an alternative treatment for Cr(VI) contamination in the aqueous system. The use of 0.1% (v/v) formaldehyde as a disinfecting

agent inhibited growth of bacteria present in the final wastewater discharge. This finding is important in view of the ethical code regarding possible introduction of exogenous bacterial species into the environment.

INTRODUCTION

Chemical reduction followed by precipitation is the most common technique used in the industry to remove Cr(VI) (Cushnie, 1985). However, this technique has its own serious disadvantages, such as the possibility of chemical spillage and the high cost of treatment chemicals, while the large generation of sludge leads to disposal problems. This prompts the need to look into safer and cheaper alternatives to carry out the Cr(VI) reduction process such as biological processes. Numerous reports have demonstrated the feasibility of using bacterial processes for the treatment of Cr(VI)-containing industrial wastewaters by use of either a pure culture or a bacterial consortium (Romanenko et al. 1976; Bopp and Ehrlich, 1988). Bacteria of various genera have been used including *Achromobacter, Aeromonas, Agrobacterium, Bacillus, Desulfovibrio, Enterobacter* and *Pseudomonas* (Wang, 2000). These bacteria showed different Cr(VI) reducing capacity depending on factors such as availability of organic compounds as electron donor, dissolved oxygen, Cr(VI) concentration, pH, redox potential, temperature, presence of other electron acceptors and inhibition effects by metallic or phenolic compounds (Wang, 2000; Ishibashi et al., 1990). In this study, wood husk was chosen as the column support material for bacterial attachment because it is a natural source for cellulose that is known for its bacterial attachment property, and it is inexpensive and stable. Another point to note is that the glucose-containing pineapple waste is readily consumed by *Acinetobacter haemolyticus*, and so is an effective substitute for expensive growth medium such as NB (Ivy, 2005). One example of a bioremediation process that was short lived due to its requirement for a high cost nutrient was the bacterial-based metal removal system developed by Advanced Mineral Technologies, Inc. in Colorado that used *Bacillus sp.* as the biosorbent (Volesky, 1990).

Bacterial reduction of Cr(VI) can be considered as a mechanism of resistance to Cr(VI). Cr(VI) is a strong oxidizing agent that can easily penetrate the cell membrane of prokaryotic cells such as bacteria. Cr(VI) uptake is carried out by the sulfate transport pathway; hence, it is competitively inhibited by sulfate (Ohtake et al., 1987). However, the role of

sulfate as inhibitor for Cr(VI) uptake is more pronounced in anaerobic cells (Komori et al., 1989) compared to aerobic cells (Wang, 2000). The ability of Cr(VI) anions to overcome the permeability barrier of a prokaryotic cell can be attributed to the chemical similarity between CrO_4^{2-} and SO_4^{2-} ions (Mabbettt and Macaskie, 2001). Bacterial resistance to Cr(VI) was reported to be plasmid-determined. Cr(VI) resistance was also related to the decrease in Cr(VI) accumulation in resistant cells compared with the sensitive cells (Cervantes, 1991). The aerobic Cr(VI) reduction is normally associated with a soluble protein fraction utilizing NADH or NADPH as electron donor (Camargo et al., 2003), whereas in anaerobic condition, Cr(VI) can act as the terminal electron acceptor through membrane-bound reductase activity, which was reported in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Enterobacter cloacae* by Wang and Xiao (1995). Aerobic reduction is considered to be a detoxification mechanism where normally the reduction of Cr(VI) by the soluble protein fraction takes place either internal or external to the plasma membrane. In the anaerobic respiration of *Enterobacter cloacae* HO1, possible involvement of the respiratory chain in the transfer of reducing equivalents to anionic Cr(VI) compounds through cytochrome c was implicated (Shen and Wang, 1993). Anaerobic reduction of Cr(VI) by six strains of Cr-resistant *Pseudomonads* was also reported. However, it was postulated that energy generated in the anaerobic respiration process is insufficient to sustain cell growth because fermentable organic compounds generated is utilized for cell metabolism (Mclean and Beveridge, 2001). Besides that, some of the bacteria were able to reduce Cr(VI) either aerobically or anaerobically. *Pseudomonas ambigua* G-1 and *Pseudomonas putida* PRS2000 were reported to reduce Cr(VI) in both conditions with higher reduction rates under aerobic conditions. However, opposite trend was observed for *Enterobacter coli* ATCC 33456 where Cr(VI) reduction proceeded at higher rate anaerobically.

This work reports on the reduction of Cr(VI) in electroplating wastewater using wood husk immobilized bacterial bioiflm. Two kinds of agricultural wastes were used as nutrient for the bacteria, namely, the liquid pineapple waste and brown sugar.

BIOREACTOR AND RAW MATERIALS

The laboratory-scale bioreactor consist of a glass column with inner diameter (I.D.) 8.0 cm, outer diameter (O.D.) 8.70 cm and height 100 cm was

used. Inlet and outlet points were set at 2 cm from the bottom and top of column, respectively. Teflon tubing with I.D of 2.0 mm and O.D. of 4.0 mm was fitted to the inlet and outlet points, respectively. The teflon tubes were sterilized by soaking in 100% ethanol before use. Inert stones were packed to 75 cm^3 at the bottom of the column to ensure good flow distribution inside the column and to retain the column content. Following this, wood husk was packed into the column to a volume of 825 cm^3. This volume is considered as the working volume of the column. Inert stone was then packed on top of the working volume for 50 cm^3. A headspace of around 30 cm^3 was allowed in the column. The total volume of the column is, therefore, 1000 cm^3. A modified procedure from Von Canstein et al. (1999) was used during the immobilization of *Acinetobacter haemolyticus* cells onto wood husk packed in the column. The column was first rinsed with deionised water to prevent clogging by large particulate substances on the support material and to allow the wood husk surface material to acquire necessary charge for bacterial attachment. Then, 1 L of the *Acinetobacter haemolyticus* culture (grown for 24 h in NB) was pumped using the same flow rate as the rinsing step. The wastewater collected was recycled back into the column and pumped continuously for 6 h to allow bacterial attachment. A mixture of 20% (v/v) NB in 1 L pineapple waste (final pH of mixture 7.00) was pumped into the column using the same flow rate as *Acinetobacter haemolyticus* cells for 24 h to ensure initial formation of biofilm by the attached bacteria.

Acinetobacter haemolyticus was used as the primary strain inoculated inside the bioreactor. It was isolated from the Cr(VI)-containing wastewater from a batek (textile-related) manufacturing premise in Kota Bharu, Kelantan, Malaysia. *Acinetobacter haemolyticus* was cultivated in NB (8 g L^{-1}, Merck) at 200 rpm and 30 °C (Certomat, B. Braun). It was identified via the 16S rRNA gene sequencing analysis carried out by First BASE Laboratories Sdn. Bhd., Malaysia where a 99.5% similarity with *Acinetobacter haemolyticus* (AY586400 and X81662) was obtained from the nucleotide sequence of 597 bp. The nucleotide suquence was deposited to GenBank, where it was given the accession number EF369508.

Brown sugar used was obtained from local sundry shops. In this chapter, unsterilized stock solution of brown sugar (200 g L^{-1} in deionized water) was used. Results from the analysis on brown sugar are as follows: total sugar content of 154.2 ± 10.9 g L^{-1}, total nitrogen at 383.00 ± 12.73 g L^{-1}, ammonia at 0.4645 ± 0.0148 and nitrate at 1.696 ± 0.055 g L^{-1}, elemental compositions in mg L^{-1}; Pb (0.03), Cu (0.13), Zn (2.94), As (0.03), Fe (8.26), Hg (not detected), Ni (0.26), Cd (0.01) and Cr (13.46).

Table 1.1. Survival of indigenous microorganisms in LPW after treatment with 1 to 5% (v/v) ethanol

Pre-treatment of LPW	(*CFU mL^{-1})
1% (v/v) ethanol	1×10^5
3% (v/v) ethanol	3×10^4
5% (v/v) ethanol	0

* CFU – colony forming unit; counted based on two distinct colonies formed on NA plate, LPW without any treatment acted as control.

Table 1.2. Profile of metal concentrations in pre- and post-treated electroplating wastewater (EW)*

Element	Pre-treated EW, mg L^{-1}	Post-treated EW, mg L^{-1}	Standard B, mg L^{-1}
Pb	1.97 ± 0.43	1.34 ± 0.22	0.50
As	0.29 ± 0.13	0.29 ± 0.07	0.10
Hg	0.02 ± 0.01	0.03 ± 0.02	0.05
Cu	0.63 ± 0.11	0.66 ± 0.07	1.00
Fe	1.49 ± 0.18	1.48 ± 0.07	5.00
Ni	4.76 ± 1.67	4.62 ± 0.72	1.00
Cd	0.08 ± 0.01	0.06 ± 0.02	0.02
Cr	28.77 ± 2.38	0.18 ± 0.49	0.05

*values shown are means of triplicate sample; Standard B – permissible discharge limit for industrial wastewater outside the catchment area in Malaysia.

Rubber wood sawdust (RWS) used in this study was collected from the compound of a wood finishing factory in Skudai, Malaysia. Sawdust collected originated from the rubber wood treated with the chromated copper arsenate (CCA). The specific surface area was determined using the Surface Area Analyzer ASAP 2010 (Micromeritics, USA). The values for specific surface areas obtained by BET, Langmuir and Single Point (at P/ Po = 0.2002) methods were 3.0025, 5.8345 and 1.9806 m^2g^{-1}, respectively, while the average pore diameter was 694.03 nm. In this study, the RWS was used without any other physical or chemical treatment, hence the term URWS (untreated rubber wood sawdust).

Liquid pineapple waste was used as the energy source for bacteria. It was obtained from one pineapple-processing premise in Tampoi, Johor. The liquid pineapple waste (LPW) normally appears as yellowish green, slightly turbid, pH – 3.19 to 4.17 ± 0.28, 33.9 °C ± 1.61, sulphate – 58.2 ± 1.41 mg L^{-1} and

microbiological count – four colonies isolated. From the ICP–MS analysis, concentrations of Pb, As, Hg, Cu and Cr exceed the permitted level. Sugar content was as follows: glucose 4.95 g L^{-1} and fructose 4.49 g L^{-1}. Sucrose content was relatively low in comparison with the glucose and fructose, and hence was not readily detected. The reduction of the glucose and fructose concentrations to the undetectable levels after three days of storage at both room temperature and at 4 °C was due to the consumption by indigenous bacteria from LPW as supported by Tseng and Bielefeldt (2002). The effectiveness of LPW treatment using different concentrations of ethanol is shown in Table 1.1. From the results obtained, 5% (v/v) ethanol managed to kill all the indigenous microorganisms present in the LPW, hence its selection as method of choice for preserving the LPW. However after two days of treatment, the LPW showed signs of microorganisms' growth when left at room temperature that can be attributed to the rich sugar content of the LPW itself. The electroplating wastewater was obtained from the rinse-bath tank of a local electroplating company in Pasir Gudang, Johor. The electroplating wastewater showed the following characteristics: colour – yellow to dark orange, turbidity – clear, pH – between 2.30 to 2.70 ± 0.05, temperature - 30.7 °C ± 3.39, sulphate - 4.45 ± 3.32 mg L^{-1}. Microbiological count did not yield any colonies. Profile for the heavy metals content of the raw electroplating wastewater is shown in Table 1.2.

OPERATION OF THE BIOREACTOR – LPW AS NUTRIENT

The experimental setup for the Cr(VI) reduction system consists of a holding tank, peristaltic pump, column (bioreactor), precipitation tank and disinfection tank. Solution from the holding tank consisting of 15 mg L^{-1} Cr(VI) from electroplating and pineapple wastewaters was first adjusted to pH 7.0 using 15% (v/v) NaOH solution. The solution was then pumped into the *A. haemolyticus*-immobilized column at 3.0 mL min^{-1} until the Cr(VI) concentration in the effluent fraction was more than 0.5 mg L^{-1}. At this point, a decrease in the population of the Cr(VI)–reducing *Acinetobacter haemolyticus* was expected due to Cr(VI) toxicity. The column was then regenerated using NB followed by pineapple waste. Then, 200 mL of fresh *Acinetobacter haemolyticus* culture, grown for 24 h in NB, was introduced before the column was left idle for three days to allow biofilm formation (Von Canstein et al. 2001). Then, Cr(VI) reduction was continued for 30 days when the influent flow rate was varied between 3.0 to 8.0 mL min^{-1}. The following parameters

were periodically measured: Cr(VI) and total Cr concentrations, heavy metals, pH, microbiological count and dissolved oxygen (DO).

Bacterial reduction of Cr(VI) in simulated effluent and electroplating wastewater (EW) was carried out for 24 h using *A. haemolyticus* in LPW supplemented with NB. Figure 1.1 shows profiles for bacterial growth (OD_{600}) and Cr(VI) reduction in 50 mg L^{-1} simulated solution and real EW.

Figure 1.1. shows an inversely proportional relationship of *A. haemolyticus* growth with amount of Cr(VI) reduced. Rapid reduction of Cr(VI) was observed in the exponential growth phase of the bacteria i.e., 4 to 12 h. A higher Cr(VI) reduction was observed in simulated solution (99.27%) compared to in EW (95.35%). The consumption of glucose resulted in increase biomass that ultimately promotes Cr(VI) reduction. A similar finding was reported by Tseng and Bielefeldt (2002), where carbon availability was cited as the most critical factor limiting chromium biotransformation. Also, sugar addition had the greatest effect on enhancing Cr(VI) removal. Glucose as a major constituent in the LPW serves as electron donor and Cr(VI) serves as terminal electron acceptors along with oxygen (Wang and Xiao, 1995).

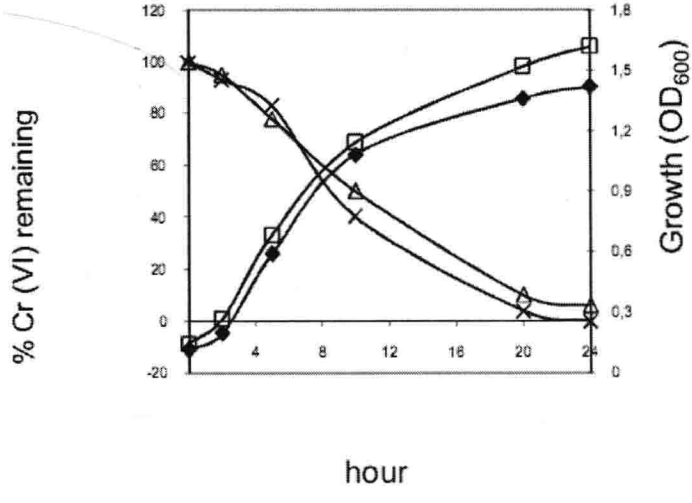

Figure 1.1. Growth and reduction of Cr(VI) by *A. haemolyticus*; (□) Growth in LPW - NB (3 : 2) and 50 mg L^{-1} simulated Cr(VI) solution, (◆) Growth in LPW - NB (3 : 2) and EW, (x)% Cr(VI) remaining in LPW – NB (3 : 2) and 50 mg L^{-1} simulated Cr(VI) solution, (●)% Cr(VI) remaining in LPW - NB (3 : 2) and EW.

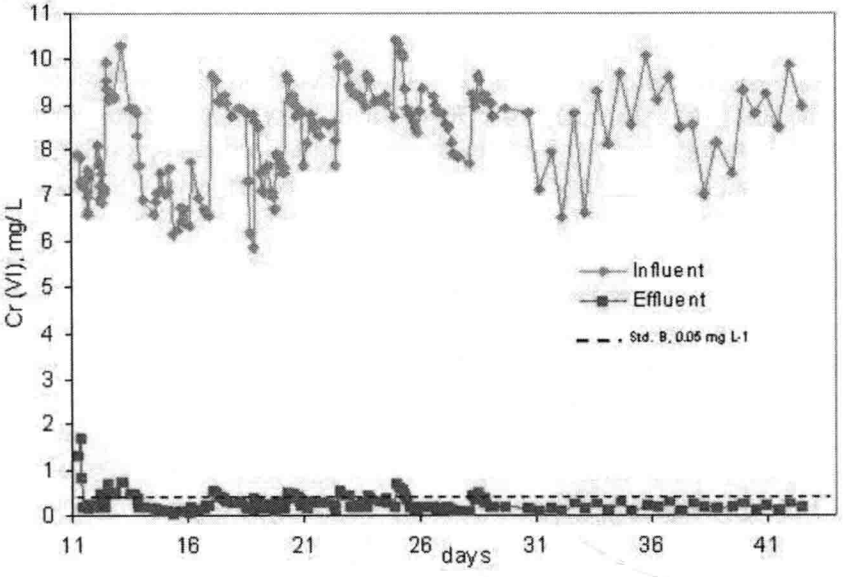

Figure 1.2. Profile for bioremediation of Cr(VI) from electroplating wastewater by wood- husk-immobilized *Acinetobacter haemolyticus* in a column system..

Rapid reduction of Cr(VI) at exponential phase of growth indicated that the rate of Cr(VI) reduction was strongly dependent on the bacterial cell density. Lower Cr(VI) reduction by *A. haemolyticus* was observed for EW compared to the simulated Cr(VI) solution. The existence of ions such as sulphate may hinder the bioreduction of Cr(VI), as it competes for the reducing capacity generated by NADH in the bacterial cell. In the presence of high concentration of sulphate, Cr(VI) reduction can be inhibited as CrO_4^{2-} and SO_4^{2-} are known to be transported by the same carrier proteins due to chemical similarities (Ganguli and Tripathi, 1999). Initial influent Cr(VI) concentration was 8.37 mg L^{-1} with Cr(VI) reduction around 55.33% (Figure 1.2).

Lowering of influent Cr(VI) concentration did not result in increased Cr(VI) reduction. More than 95% of Cr(VI) reduction was achieved after three days of column operation. However, the inability to achieve effluent Cr(VI) concentration below the discharge limit (Std. B, 0.05 mg L^{-1}) prompted the column operation to be stopped to allow a regeneration step. After regeneration, the effect of influent flow rate and column retention time on Cr(VI) reduction was investigated. Cr(VI) reduction was not affected up to influent flow rate of 8.0 mL min^{-1} at Cr(VI) concentration of 7.37 to 8.44 mg L^{-1} when more than 99% Cr(VI) was reduced. Variation of influent flow rate