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

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

Faculty of Science
Universiti Teknologi Malaysia

DECEMBER 2013

## ABSTRACT

The high cost of culture growth medium is one of the problems faced in the scaling–up of biological processes involved in wastewater treatment. This makes it imperative to find a useful, cheap and easily available alternative source for culture growth medium. The possibility of using sugarcane bagasse (SCB), solid pineapple waste (SPW) and soybean meal (SBM) as alternative sources for culture medium is preferable as these agricultural wastes are easily available, cheap and abundantly grown. The present work highlights the use of SCB, SPW and SBM to sustain the bacterial population for the Cr(VI) reduction process. Growth of *A. haemolyticus* in agricultural wastes was measured by optical density ($OD_{600}$) followed by viable cell counts. Reduction of Cr(VI) was determined using diphenylcarbazide method. For all Cr(VI) concentrations tested (10–100 mg/L), SCB–adapted *A. haemolyticus* showed the highest reduction ranging from 92–99% followed by SPW and SBM with 40–94% and 21–85% reduction respectively. From the FESEM–EDX analysis, toxicity effect can be seen clearly from the shape of bacteria in the presence of 100 mg/L Cr(VI). The FT–IR analysis showed shifting of the C–O band absorption peak from 1252–1261 $cm^{-1}$ and 1048–1037 $cm^{-1}$ after Cr–loaded which was due to the binding of Cr(VI) to this functional group. In this study, down–ward biofilm packed–bed reactor was used. A minimum of 4 h was required for complete reduction of Cr(VI) to Cr(III) at the flow rate of 3.0 mL/min using 25 mg/L initial Cr(VI) concentration. Cr(VI) reduction mechanism study using XPS and ESR implies that the Cr bound to the SCB and SCB–adapted *A. haemolyticus* were mostly in trivalent form. SCB can serve as an alternative and cost–effective growth medium for cultivation of *A. haemolyticus* with high percent reduction of Cr(VI). Phylogenetic analysis revealed that the microbial community was dominated by *Chitinophaga terrae*, *Laribacter hongkongensis*, *Ottowia thiooxydans*, *Rhizobium cellulosilyticum*, *Candidate division OP10*, *Pedobacter sp.* and uncultured bacterium.

# ABSTRAK

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

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## LIST OF ABBREVIATIONS

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

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

# LIST OF APPENDICES

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the problem

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

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

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

## 1.2 Statement of the problem

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

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

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

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

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

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

## 1.3 Objectives of the study

The objectives of this work are:

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