CHROMATE REDUCTASE ACTIVITY IN WHOLE CELLS AND CRUDE CELL FREE EXTRACT OF *Acinetobacter haemolyticus*

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This thesis is dedicated to:

Special dedication to my beloved father and mother, Abdul Karim bin Mohd Said and Jermiah bt. Haji Ariff, who have been with me every step of the way, through good time and bad. Thanks for all the tremendous love, prayer, guidance and support that you have always given me.

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ABSTRACT

Extensive use of hexavalent chromium, (Cr(VI)) in various industrial applications is a threat to human health, living resources and ecological system due to its high solubility, toxicity and carcinogenic effects. Previously, one locally isolated Cr(VI) reducing-resistant bacteria, *Acinetobacter haemolyticus* was used in the ChromeBac™ system, to remove toxic Cr(VI) from industrial wastewater. However, this process required long retention time which was primarily due to the toxicity of Cr(VI) towards immobilized whole cells used. The use of enzymes can be a suitable option for the effective Cr(VI) reduction as compared to whole cells. In view of this, this study was conducted to assess in vitro characterization of the enzymatic chromate reductase activity in cell free-extract (CFE) for maximum activity of Cr(VI) reduction. Cr(VI) resistance and reduction of *A. haemolyticus* was evaluated in Luria-Bertani (LB) medium supplemented with various Cr(VI) concentrations. From the results, *A. haemolyticus* can resist up to 200 mg/L Cr(VI) in LB broth compared to 100 mg/L Cr(VI) in LB agar. The FTIR and FESEM-EDX analysis suggested Cr deposition onto the bacterial cells surface via complex formation between Cr species and either carboxyl, hydroxyl or amide groups. TEM analysis showed that Cr(III) is also distributed in membrane and cytosolic fractions of bacteria. ESR analysis revealed that chromium accumulated on bacterial surface and mostly as Cr(III). The enzyme activity was optimal at 30°C and pH 7 in the presence of 1 mM Co^{2+}. The Michaelis-Menten constants, K_m and maximum reaction rate, V_{max} obtained from the Lineweaver-Burke plot were 184.47 µM and 33.3 nmol/min/mg protein in the presence of 1 mM Co^{2+}. Optimum Cr(VI) reduction by immobilized CFE-alginate was determined at initial pH 3, 100 rpm and 5 g wet weight beads dosage. Although immobilized enzyme system was able to reduce Cr(VI), the performance was not as good as the free enzyme. This study showed higher Cr(VI) reduction performance by free CFE compared to the use of whole cells demonstrate its potential for industrial application.

ABSTRAK

Penggunaan kromium heksavalen (Cr(VI)) dalam pelbagai aplikasi perindustrian telah mengancam kesihatan manusia, sumber hidupan dan sistem ekologi disebabkan keterlarutannya dalam air, kesan toksik dan bersifat karsinogenik. Sejenis bakteria berdaya-tahan Cr(VI), *Acinetobacter haemolyticus*, telah digunakan dalam sistem ChromeBac™ untuk penyingkiran Cr(VI) daripada air sisa industri. Walau bagaimanapun, proses ini memerlukan masa yang lama untuk melengkapkan proses penurunan Cr(VI) menggunakan sel tersekat jerap disebabkan ketoksikan Cr(VI). Penggunaan enzim menjadi pilihan yang sesuai untuk penurunan Cr(VI) secara berkesan berbanding dengan sel lengkap. Oleh itu, kajian ini telah dijalankan untuk menilai pencirian enzim kromat reductase secara 'in vitro' dalam ekstrak sel bebas (CFE) untuk memaksimumkan aktiviti penurunan Cr(VI). Dalam kajian ini, *A. haemolyticus* telah dibiakkan dalam media Luria-Bertani (LB) yang ditambah dengan kepekatan Cr(VI) yang berbeza. *A. haemolyticus* telah didapati mempunyai daya-tahan sehingga 200 mg/L Cr(VI) dalam media LB berbanding dengan 100 mg/L Cr(VI) dalam media pepejal LB. Analisis FTIR dan FESEM-EDX telah menunjukkan pemendapan Cr pada permukaan sel-sel bakteria melalui kompleks antara spesies Cr melalui kumpulan karboksil, hidroksil atau amida. Analisis TEM menunjukkan bahawa Cr(III) juga disebarkan dalam membran dan pecahan sitosolik bakteria. Analisis ESR menunjukkan bahawa kromium yang terkumpul pada permukaan bakteria kebanyakannya adalah Cr(III). Aktiviti enzim adalah optimum pada suhu 30 °C dan pH 7 dengan kehadiran 1 mM Co^{2+}. Pemalar Michaelis-Menten, K_m dan had laju maksimum, V_{max} diperolehi dari plot Lineweaver-Burk dengan nilai 184.47 μM dan 33.3 nmol/min/mg protein dalam kehadiran Co^{2+} (1 mM). Penurunan Cr(VI) oleh CFE tersekat gerak-alginat adalah optimum pada pH 3, kelajuan penggoncangan 100 rpm dan 5 g berat basah dos alginat. Walaupun sistem tersekat-gerak enzim dapat menurunkan Cr(VI) ke Cr(III), prestasinya tidak sebaik enzim bebas. Kajian ini menunjukkan prestasi penurunan Cr(VI) yang lebih tinggi dalam enzim bebas berbanding sel lengkap menunjukkan potensinya dalam kegunaan industri.

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LIST OF ABBREVIATIONS

%	-	Percentage
[Cr]	-	chromium Concentration
°C	-	Degree Celcius
µg	-	Microgram
CFE	-	Cell free extract
CFU	-	Colony forming unit
DPC	-	1,5 - diphenylcarbazide
EDTA	-	Ethylenediaminetetraacetic acid
g	-	Gram
h	-	Hour
K_i	-	Dissociation constant for inhibitor binding
K_m	-	Michaelis–Menten constant
kPa	-	kilopascal
kWh	-	kilo Watt hour
L	-	Liter
M	-	Molar
m^3	-	Meter Cubic
mg	-	Miligram
NADH	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate
ng	-	Nanogram
OD_{600}	-	Optical density at 600 nm
ppm	-	Part Per Million
rpm	-	Rotation per minute
v	-	Volume
v/v	-	Volume per volume
V_{max}	-	maximum rate or maximum velocity

V_o	-	initial velocity
w	-	Weight
w/v	-	Weight per volume
λ	-	wavelength

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The contamination of our water system by the toxic hexavalent chromium, Cr(VI) is of concern. Cr(VI) originates from various anthropogenic sources such as alloy manufacturing, dyes and pigments, electroplating, metal finishing, petroleum refining, leather tanning, wood preservation and as corrosion inhibitor in conventional and nuclear power plants (Elangovan *et al.*, 2010). Its high solubility makes it a very toxic and carcinogenic element, hence its compulsory removal from water/ wastewater prior to discharging into the environment. Conventional methods for Cr(VI) removal involves physico-chemical techniques which is highly expensive, inappropriate at low Cr(VI) concentration, high reagent consumption, energy requirements and generation of toxic sludge. Therefore, development of an effective system for Cr(VI) bioremediation is highly desirable.

Microbial reduction of Cr(VI) is considered as an alternative remediation technique for Cr(VI) contamination due to its lower cost and less sludge production (Lloyd, 2002). Various studies were reported on the ability of microbial species to carry out both the Cr(VI) resistant-reducing reaction including *Pannonibacter phragmitetus* LSSE-09 (Xu *et al*., 2011), *Staphylococcus* sp.(Ilias *et al*., 2011), *Lysinibacillus fusiformis* ZC1 (He *et al*., 2011), *Serratia* sp. (Zhang and Li, 2011), *Ochrobactrum* sp. (Francisco *et al*., 2010; Yangjian *et al*., 2009; Sultan and Hasnain, 2007), *Bacillus* sp. (Campos *et al*., 1995; Camargo *et al*., 2003; Cheng and Li, 2009; Rehman *et al*., 2008); *Leucobacter* sp. (Zhu *et al*., 2008a), *Pseudomonas* sp. (DeLeo and Ehrlich, 1994; Badar *et al*., 2000; McLean *et al*., 2000), *Exiguobacterium* sp. (Okeke, 2008), *Acinetobacter haemolyticus* (Zakaria *et al*., 2007a; 2007b) and others. Most of the biological systems for the treatment of Cr(VI)-containing wastewater using microbial Cr(VI) reduction are operated in batch mode (Elangovan *et al.*, 2010). However, this system is not fully effective (compared to continuous or fixed film bioreactor system) with an eventual loss of active biomass. This was mainly due to metal toxicity and stage of biofilm development which has not fully matured. Several improvements are needed both at the enzymatic as well as cellular levels for bacteria to work efficiently as agents for chromate bioremediation (Arkerley *et al*., 2004b)

Chromate reductase activity can be found in the cell extracts of many bacteria either in crude, partial or purified fractions. Chromate reductase facilitates the reduction of Cr(VI) to Cr(III) either in aerobic or anaerobic conditions. Aerobic Cr(VI) reduction is generally associated with a soluble fraction that utilizes NADH as an electron donor (Kwak *et al.*, 2003). Conversely, anaerobic Cr(VI) reduction is mediated by membrane bound cytochrome *b*, *c* and *d*, (Bopp and Ehlirch, 1988 and Lovley and Phillips, 1994) or cytoplasmic membrane proteins (Myers *et al*., 2000). Though many reports are available on the use of whole cell reactors for the treatment of Cr(VI)-contaminated wastewater, uses of purified, partially purified or crude enzymes in bioremediation of Cr(VI) from contaminated wastewater treatment is almost unheard of. This makes it imperative to find and employ an effective way to remediate Cr(VI) by using highly active enzyme (chromate reductase) isolated from Cr(VI) resistant-reducing microorganism.

1.2 Statement of Problem

This study is a result from the Cr(VI) reduction system i.e. ChromeBac™ which has been developed at the laboratory and pilot-scale in Universiti Teknologi Malaysia, Skudai for the past 7 years. ChromeBac™ is a novel and environmental–friendly system to treat Cr(VI)–bearing water consisting of bioreactor packed with sawdust-immobilized Cr(VI) resistant-reducing bacteria. One novel-locally isolated bacterium, *Acinetobater haemolyticus* (*A. haemolyticus,* GenBank Acession 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.

Amongst the important observations made during the ChromeBac™ process is the substantial retention time needed to complete Cr(VI) reduction. This was due to the immaturity of the biofilm system (Zakaria *et al*., 2007a; 2007b; Ahmad *et al*., 2009b) and the need for intermittent reseeding of the bioreactor. Hence, the use of enzymes (chromate reductase) isolated from bacteria itself may be a suitable option for the effective Cr(VI) reduction as compared to whole cells. Previously, it was demonstrated that the Cr(VI) reduction–resistance pathways for the bacterium occured aerobically in the soluble proteins fractions (Pei *et al*., 2009). Therefore, in the present study, enzymatic reduction of Cr(VI) and optimum conditions for chromate reductase isolated from *Acinetobater haemolyticus* will be evaluated.

1.3 Objectives of Study

The objective of this study was to compare the chromate reductase activity in the whole cells and crude cell-free extract of *Acinetobacter haemolyticus*. This shall be assessed by elucidating the sub-cellular localization of chromate reductase, to assess in vitro characterization of the enzymatic chromate reductase activity in cell free-extract (cytosolic fractions) and to investigate the performance of immobilized-chromate reductase to reduce Cr(VI) by *Acinetobacter haemolyticus*.

1.4 Scope of Study

Cr(VI) resistance and reduction study of *A. haemolyticus* was assessed in LB medium supplemented with various Cr(VI) concentrations. To elucidate the role of enzymes and abiotic reduction during Cr(VI) reduction, Cr(VI) reduction assay was carried out aerobically under growth and non-growth conditions. Instrumental analysis was carried out to investigate the metal-microbe interaction during Cr(VI) reduction such as FTIR, FESEM-EDX, TEM and ESR. The chromate reductase activity was determined via in-vitro enzymatic study using sub-cellular fractions where fractions with high chromate reductase activity were assessed for protein contents, effect of temperature, pH, metal ions, metabolic inhibitors, electron donors and kinetic study (K_m, V_{max}, $K_{i)}$. The enzyme fraction was immobilized in calcium alginate and evaluated for its Cr(VI) reduction ability in batch system using parameters such as initial pH of Cr(VI), temperature, agitation speed, bead dosage and effect of contact time and initial Cr(VI) concentration. The reusability of beads immobilized-chromate reductase also was investigated.

1.5 Significance of Study

The significance of this study is to evaluate the feasibility of using enzymatic extracts from the previously isolated Cr(VI) resistant-reducing *A. haemolyticus*, in an attempt to improve the Cr(VI) reduction levels of the previously developed biofilm system.

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