ROLE OF RHAMNOLIPIDS IN *Pseudomonas aeruginosa* RW9 REMEDIATION OF HEXAVALENT CHROMIUM

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Faculty : Biotechnology and Biomolecular Sciences

Chromium hexavalent (Cr(VI)) pollution has become a global issue. Despite the efficiency of various bacterial species reported to treat the pollutant, the removal performance of each species and strains were found to be distinctive. The same phenomenon was also observed in *Pseudomonas aeruginosa* as a well-known efficient species in Cr(VI) remediation. The discrepancy in performance might be resulted from the removal mechanisms deployed by the strains. Thus, in this study the potential of *P. aeruginosa* RW9 in Cr(VI) remediation was assessed. The strain was subjected to a range of Cr(VI) to evaluate its tolerance. Results showed its ability to resist a high concentration of up to 40 mg/L Cr(VI). The Cr(VI) distribution analysis by a shake flask study at 30 °C, agitation of 150 rpm for 24 hours incubation with 10 mg/L Cr(VI) was conducted. It was revealed that the strain developed a complex mechanism to remove Cr(VI), based on surface-bound (0.46 mg/L), intracellularly accumulated (1.24 mg/L) and extracellular sequestration (6.74 mg/L), which accounted for approximately 85% of the removal efficiency. The extracellular sequestration was attributed to the extracellular reductase and biosurfactant with the latter discovered as the predominant metabolite. High performance liquid chromatography with a variable wavelength detector (HPLC-VW) revealed that the strain produced more biosurfactant in Cr(VI) treated experiment as compared to that of the control (0 mg/L Cr(VI)). The biosurfactant was extracted by acid precipitation coupled with ethyl acetate extraction and purified by using solid phase extraction method prior to characterisation analyses by fourier transform infra-red spectroscopy, nuclear magnetic resonance (NMR) and liquid chromatography mass spectrometry (LC-MS) analyses to confirm the rhamnolipid structure. The LC-MS analysis also exposed that cells in both control and Cr(VI) treated experiments produced more di-rhamnolipid with the main congener being Rha-Rha-C10-C10 (m/z 649). Furthermore, Cr(VI) was found to induce the synthesis of Rha-Rha-C8-C10/ Rha-Rha-C10-C8 congener (m/z 620). The Whole Genome Sequencing (WGS) verified the presence of the rhamnolipid-encoded genes (*rhlA*, *rhlB* and *rhlC*) and reductase-encoded gene (*chrR*) before conducting the one-step quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). It was discovered that Cr(VI) induced the transcriptional expression of all the target genes with the highest enhancement observed in *rhlB*. Later, the performance of the produced rhamnolipid was tested to assess its efficiency to remediate six chosen elements (Se, Cd, B, Cr, Zn and Mn) in partially treated leachate considering the metals concentration in the wastewater. The rhamnolipid, in its crude form of 10 mg/L, was applied to the wastewater and the remaining concentrations of the elements were measured using inductively coupled plasma atomic emission spectroscopy (ICP-OES). Se and Cd were completely removed after a 48-hour treatment. Meanwhile, B, Zn, Cr and Mn were reduced at 93.45%, 77.37%, 68.04% and 55.42%, respectively. The rhamnolipid exhibited preferential to the respective elements following Se > Cd > B > Cr > Zn > Mn sequence. The same pattern was also displayed by the commercial rhamnolipid which served as the control experiment. Collectively, the findings proved that the strain has a great potential for application in Cr(VI) bioremediation. In terms of application, it can be manipulated to produce rhamnolipid which exhibited various downstream applications especially in remediating metal pollutants.

Keywords: *Pseudomonas aeruginosa; chromium hexavalent; mechanisms; removal efficiency; rhamnolipid*

SDG: GOAL 9: Industry, Innovation and Infrastructure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERANAN RHAMNOLIPIDS OLEH *Pseudomonas aeruginosa* RW9 DALAM PEMULIHAN KROMIUM HEKSAVALEN

Oleh

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Pencemaran kromium heksavalen (Cr(VI) merupakan isu global. Walaupun terdapat pelbagai spesies bakteria dilaporkan mampu menyingkirkan pencemaran tersebut, keupayaan setiap spesies dan strain didapati berbeza. Hal ini juga dapat dilihat pada *Pseudomonas aeruginosa* yang merupakan spesies yang terkenal dalam perawatan Cr(VI). Perbezaan ini disebabkan oleh mekanisma berlainan yang digunakan oleh setiap strain yang berbeza. Oleh yang demikian, potensi *P. aeruginosa* RW9 dalam pemulihan Cr(VI) telah diuji dalam projek penyelidikan ini. Strain tersebut telah dikultur dalam Cr(VI) berkepekatan berbeza untuk menilai tahap kerintangannya. Hasil dapatan menunjukkan kebolehannya untuk merintangi Cr(VI) pada kepekatan sehingga 40 mg/L. Analisis taburan Cr(VI) melalui kaedah aruhan kelalang pada suhu 30 °C dengan kekuatan aruhan 150 rpm selama 24 jam dengan menggunakan Cr(VI) pada kepekatan 10 mg/L. Dapatan menunjukkan strain ini mempunyai mekanisma penyingkiran yang kompleks iaitu pengikatan ion pada permukaan (0.46 mg/L), pengumpulan dalam sel (1.24 mg / L) dan pengasingan luar sel (6.74 mg / L), yang menyumbang kira-kira 85% daripada kecekapan penyingkiran. Pengasingan luar sel adalah disebabkan oleh perembesan reduktase ektrasel dan biosurfaktan yang merupakan metabolit utama. Analisis kromatografi cecair berprestasi tinggi dengan pengesan jarak gelombang boleh ubah (HPLC-VW) menunjukkan strain yang dirawat dengan Cr(VI) menghasilkan lebih banyak biosurfaktan berbanding strain dalam eksperimen kawalan (0 mg/L Cr(VI)). Biosurfaktan tersebut telah diekstrak dan ditulenkan dengan kaedah masing-masing iaitu kaedah pemendakan asid beserta ekstrasi pelarut etil asetat dan kaedah ekstrasi fasa pejal sebelum analisis pencirian menggunakan Spektroskopi inframerah fourier transformasi (FTIR), gema nuklear berdaya magnet (NMR) dan kromatografi cecair dengan spektrometri jisim (LC-MS) untuk pengesahan struktur. Analisis LC-MS juga menunjukkan bahawa sel-sel dalam eksperimen kawalan dan dirawat menghasilkan lebih di-rhamnolipid dengan kongener utama merupakan Rha-Rha-C10-C10 (m/z 649). Walau bagaimanpun, Cr(VI) telah mendorong sintesis kongener Rha-Rha-C8-C10 / Rha-Rha-C10-C8 (m/z 620). Ekspresi transkripsi gen-gen dalam rhamnolipid dan reduktase sintesis telah dinilai untuk menentukan kesan Cr(VI). Penjujukan Genom Keseluruhan (WGS) mengesahkan kehadiran gen-gen dalam sintesis rhamnolipid (*rhlA*, *rhlB* dan *rhlC*) dan reduktase (*chrR*) sebelum menjalankan analisis rantaian polimerase-transkripase membalik kuantitatif (qRT-PCR) selangkah. Cr(VI) didapati mendorong ekspresi transkripsi semua gen sasaran dengan peningkatan tertinggi diperhatikan dalam *rhlB.* Seterusnya, prestasi rhamnolipid yang dihasilkan telah diuji untuk menilai kecekapannya untuk menyingkirkan 6 unsur (Se, Cd, B, Cr, Zn and Mn) yang berkepekatan tinggi dalam bahan larut lesap yang separuh terawat. Rhamnolipid, dalam bentuk mentah berkepekatan 10 mg/L digunakan untuk menyingkirkan unsur-unsur yang terpilih di dalam air sisa dan kepekatan unsur-unsur selepas rawatan diukur menggunakan Spektrometer Pancaran Optik Terganding Plasma Beraruh (ICP-OES). Se dan Cd didapati telah disingkirkan sepenuhnya selepas rawatan selama 48 jam. Manakala, B, Zn, Cr dan Mn masing-masing disingkirkan sehingga 93.45%, 77.37%, 68.04% dan 55.42%. Rhamnolipid mempamerkan keutamaan kepada unsur-unsur mengikut jujukan Se > Cd > B > Cr > Zn > Mn. Polar yang sama juga ditunjukkan oleh rhamnolipid komersil yang bertindak sebagai eksperimen kawalan. Secara keseluruhan, hasil dapatan dalam kajian ini mencadangkan bahawa strain ini mempunyai potensi besar untuk digunakan dalam bioremediasi Cr(VI). Dari segi aplikasi, ia boleh dimanipulasi untuk menghasilkan rhamnolipid yang mempamerkan pelbagai aplikasi hiliran terutamanya dalam memulihkan bahan pencemar logam.

Kata kunci: *Pseudomonas aeruginosa; kromium heksavalen; mekanisma; kebolehan penyingkiran; rhamnolipid*

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α	Alpha
δ^E	*rpoE*
µL	Microliter
µm	Micrometer
°C	Degree Celsius
%	Percentage
1D	One dimensional
2D	Two dimensional
3OC12HSL	3-oxo-C12-homoserine lactone
AHL	Acyl homoserine lactone
Al(III)	Aluminium ion
APHA	American Public Health Association
ATP	Adenosine triphosphate
ATR	Attenuated total reflection
B	Boron
BLAST	Basic Local Alignment Search Tool
BOD_5	Biochemical oxygen demand at 5-days of incubation
Bp	Base pair
Ca	Calcium
C4HSL	Butanoyl homoserine lactone
CCR	Carbon catabolite repression
Cd	Cadmium
ChrR	Chromate reductase

CH_2	Methylene functional group
Co	Cobalt
COD	Chemical oxygen demand
Cr	Chromium
Cr_2O_3	Chromite ore
Cr(VI)	Chromium hexavalent
Cr(III)	Chromium trivalent
Cu	Copper
DAF	Dissolve air floatation
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy Dispersive X-ray
EPS	Extracellular polymetric substance
ESI-MS	Electrospray ionisation mass spectrometry
ESR	Envelope stress response
EU	European Union
FAD	Flavin adenine dinucleotide
FASTA	Fast alignment
Fe	Iron
$FeO.Cr_2O_3$	Ferrous chromite
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared
g	Acceleration due to the gravity

g	Gram
GC-MS	Gas chromatography mass spectrometry
HAA	3-(3-hydroxyalkanoyloxy) alkanoic acids
HCl	Hydrochloric acid
Hg	Mercury
HLB	Hydrophilic-lipophilic balance
HNO_3	Nitric acid
H_3PO_4	Phosphoric acid
HPLC-VW	High performance liquid chromatography-variable wavelengths
H_2SO_4	Sulphuric acid
ICP-OES	Inductively coupled plasma optical emission spectroscopy
K	Potassium
kb	Kilobase
kDA	Kilodaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Litre
LC-MS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharide
M	Molar
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
Mg	Magnesium
$MgSO_4$	Magnesium sulphate
Mn	Manganese

MT	Metallothionein
NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NH	Amino functional group
NMR	Nuclear magnetic resonance
Ni	Nickel
OD 600	Optical density at wavelength 600 nanometer
OH	Hydroxyl functional group
P. aeruginosa	*Pseudomonas aeruginosa*
Pb	Lead
PCR	Polymerase chain reaction
PDA	Photodiode array
PTFE	Polytetrafluoroethylene
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
QS	Quorum sensing
RhlA	3-(3-hydroxydecanoyloxy) decanoate synthase
RhlB	Rhamnosyltransferase I
RhlC	Rhamnosyltransferase II
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
rpm	Rotation per minute
RpoE	RNA polymerase extracytoplasmic E

RpoN	RNA polymerase nitrogen-limitation N
RpoS	RNA polymerase sigma S
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
Se	Selenium
SEM	Scanning electron microscopy
SPE	Solid phase extraction
TAE	Tris-acetate-EDTA
TLC	Thin layer chromatography
USEPA	United States Environmental Protection Agency
Zn	Zinc

CHAPTER 1

INTRODUCTION

1.1 Background

Chromium hexavalent (Cr(VI)) pollution has been a global issue. The extensive usage of the ion in various sectors inadvertently serves as its main entering route to the environment through improperly treated wastewater. The ion is primarily extracted by mining in the form of chromite ores (Cr_2O_3) or ferrous chromite ($FeO.Cr_2O_3$) which later undergoes a series of treatments to produce metallic chromium (Cr) (Tumolo et al., 2020). A major portion of this product is used as an alloying agent to produce stainless steel and other nonferrous metallurgical utensils (Pradhan et al., 2017) while the remaining serves as a preservative or enhancer in various processes of other industries such as leather tanning, electroplating, wood preservation, textile and pigment industries as preservative or enhancer (Pradhan et al., 2017). Stainless steel is largely used as building materials for refineries of oil and gas as well as thermal power stations (Garg et al., 2012). In addition, it is also commonly used in the pipeline system, either for wastewater sewerage or waterways for freshwater supply. Considering its wide range in industrial applications, the possibility of Cr(VI) pollution is not surprising.

The incidence of Cr(1lkanoic1n has been observed to be Increased worldwide. Cr(VI) contamination of water resources is prevalent in the United States which rendered its usage status unsafe for use as it possesses a serious threat to public health (Vengosh et al., 2016). The energy sectors are the main contributor to Cr(VI) emissions through the combustion of coal as well deteriorating of Cr(VI) coating of the energy plant and pipeline system. The ion has been widely used as steel coating to shield the metals from corrosion due to its durability. As for the European Union (EU) countries, the same sector is also highlighted as the major industry that released a significant amount of Cr(VI) to the environment which accounted for 83% of the total Cr(VI) emission in water (Tumolo et al., 2020). Meanwhile, another 10.9% of the total Cr(VI) emission in water was contributed by other industries that are related to waste and wastewater management through combustion of municipal solid waste are rich in hexavalent chromium, manufacturing of metal, paper and wood as well as tanning and dyeing.

In contrast, the mining industry has been found as the leading contributor to Cr(VI) emission in China with the Xingjiang River was reported as the most polluted river in the country (Huang et al., 2020). The concentration of Cd, As, Pb, Zn, Cu, Ni, Cr, and Co are exceptionally high in the river, signifying the worsening condition of metal pollution. Such scenario is expected considering the country's status has emerged as the top stainless-steel producer and is the leading chromium-consuming and ferrochromium-producing country (Pradhan et al., 2017). Its annual production capacities and discharge of Cr slag have reached 329,000 and 450 000 tonnes, respectively. Meanwhile, in Malaysia, rapid urbanisation has been reported as among the major contributors to Cr(VI) emissions in water (Othman et al., 2018). It has caused an increasing number of sewage treatment plants, wet markets, mining and agricultural activities which serve as the main Cr(VI) emission (Praveena et al., 2015). The increased number of the metal pollution incidence reported in rivers or estuaries that are situated in the main urbanisation zones such as in the Klang Valley, Penang and Johor (Department of Environment Malaysia, 2019) was following the rapid urbanisation trend recorded.

The ecological hazard of Cr(VI) was well-known. The United States Environmental Protection Agency (USEPA) has regarded Cr(VI) as one of the primary pollutants due to its potentially hazardous effect over minimal exposure (Garg et al., 2012). Additionally, it also has not been found to benefit any cellular process in most living organisms (Prabhakaran et al., 2016). Its fatal impact is attributed to the formation of reactive oxygen species (ROS) such as hydrogen peroxide which is often produced as the by-product in the reduction process of Cr(VI) as the ion entering the cellular membrane. These molecules have been associated with damage to various cellular components, resulting in impairment of their functions which lead to numerous physiological disorders. In addition, the reduced form of Cr(VI), the chromium trivalent (Cr(III)), also is associated with the carcinogenicity of Cr(VI) (Tchounwou et al., 2012). It readily can bind to the phosphate group of the DNA and disrupt physiological function. Reflecting the hazardous threat possessed by the ion, the Department of Environment, Malaysia has set up a standard effluent discharge limit of 0.05 mg/L which requires industries to treat their wastewater prior to discharge (Department of Environment Malaysia, 2019).

To comply with the standard effluent discharge, numerous methods have been developed to treat Cr(VI) laden wastewater. In Malaysia, an integrated approach by the combination of either chemical, physical, or/and biological methods using living bacteria are commonly used by industries to treat their wastewater prior to discharge to the environment (Pang & Abdullah, 2013). In chemical treatment, coagulating agents such as aluminium sulphate, polyaluminium chloride and ferric chloride are added to the wastewater for floc formation (Mojiri et al., 2021). The efficiency of the coagulant/flocculant is much dependent on the selection of the chemical. Upon addition of the chemical, floc was let to be formed for ease or removal commonly by mechanical means. Despite being the simplest and relatively cheaper approach, it generated a large amount of sludge which required proper management and transportation for safe disposal, resulting in cost inefficiency (Barrera-Díaz et al., 2012). Moreover, the approach also was found to be more efficient only in organic pollutants removal. Due to these drawbacks, some industries have preferred to use the physical approach (Mojiri et al., 2021). Physical treatment using activated carbon is the common physical approach for industrial wastewater treatment (Pang & Abdullah, 2013). It was proven to significantly remove Cr(VI) along with other ionic pollutants. Due to its efficiency, numerous research has been conducted to develop adsorbents with better removal performance. Large interest has been observed in developing adsorbents using low-cost materials such as rice straw and wood chips (Bohli et al., 2017; Gupta & Suhas, 2009). This practice involuntarily helps to reduce agricultural waste by transforming it into value-added products. Albeit its efficiency, the same drawback was also observed in this approach which is the sludge generation that lead to disposal issue.

In contradiction to those previous approaches, remediation using living bacteria has not been associated with the generation of secondary pollution (Barrera-Díaz et al., 2012). Moreover, it is more environmentally safe as compared to other approaches. In this technique, the bacteria transform or reduce the metallic ions into less hazardous forms through their various physiological processes that are often metabolism dependent (Ahemad, 2014). This ability is attributed to the presence of various metal-resistance genes which can be induced by numerous metal species. The induction of these genes often resulted in the synthesis of either intra- or extracellular reductases and cysteine-rich low molecular weight proteins in some microbes such as *Escherichia coli*. In addition, some of the bacteria such as *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas aeruginosa* produced biosurfactants that aid to sequestrate the ions, which subsequently shields the cell wall/membrane from the hazardous ions. This condition will lessen the adsorption of the ions on the cell wall/membrane, consequently preventing their breaching into the cellular membrane and suppressing their fatal effect (Bestawy et al., 2013). However, the interaction of the biosurfactant-producing bacteria and their metabolites to ionic metals was found to be a complex process that might be different with the genetic makeup of the bacteria, the characteristics of the produced metabolites as well as the ionic behavioural of the targeted metals in a particular environment. Thus, a thorough study needs to be done to understand the intractability of the interactions.

1.2 Problem statement and the significant of the study

The increase of Cr(VI) pollution globally has urged the development of reliable Cr(VI) treatment methods. Living bacterial Cr(VI) treatment offers environmental sustainability solutions relative to other remediation approaches. Nevertheless, it has been reported that the efficiency of this technique is much dependent on the bacterial species used. This is due to the fact that each bacterium deployed different removal/tolerance strategies to thrive in Cr(VI)-laden environments. The advancement in previous studies was merely on the isolation of more potentially viable bacteria for Cr(VI) treatment with limited understanding of the mechanisms involved in their removal. As a result, effective bioremediation strategies cannot be developed to fully exploit their potential applications. Furthermore, some of the bacteria species used in biological treatment are either non-native or pathogenic which executed their application in in-situ treatment, especially in large-scale systems.

Due to such issues, some researchers have suggested the use of metabolites from the bacteria as Cr(VI) adsorbent which is commonly observed in biosurfactant-producing bacteria such as *P. aeruginosa* and *Bacillus* sp. In *P. aeruginosa* which is a notable Cr-tolerant microorganism, less attention was given to the role of rhamnolipid in metals removal, especially in Cr(VI). Previous research has identified two distinct roles of rhamnolipid in divalent metals tolerance/remediation which served as either a sole protective barrier for the cell to shield it from the hazardous effect of the ions (González et al., 2010; Kenney & Fein, 2011) or metabolite that induced its synthesis in the presence of ions and sequesters them extracellularly (Chien et al., 2013; Izrael-Živković et al., 2018).

Nevertheless, earlier studies have shown the distinguished metal removal efficiency of rhamnolipid produced by different producing strains. In nature, the rhamnolipid existed as a mixture of diverse group of molecules with different hydroxy fatty acid chain length and rhamnose which referred as congener. Abdel-Mawgoud et al. (2010) suggested that the distribution of various congeners might explain the efficiency of the produced rhamnolipid and its role in other applications. The distribution also exhibited different affinity of the produced rhamnolipid to different metal species (Wei et al., 2017). The synthesis of different congeners was proven to be associated with transcriptional expression of the main rhamnolipid-encoded genes, *rhlA*, *rhlB* and *rhlC*. Thus, it was recognised that the congeners distribution can be altered depending on targeted pollutants of interest to yield better performance. Nevertheless, limited information was available on these niches. Some recent works only focus on several divalent metal species with Cd(II), Pb(II) and Fe (II/III) as the leading, leaving other ions, especially those with higher valency unexplored.

1.3 Hypothesis

In this study, a locally isolated strain identified as *P. aeruginosa* RW 9 which is a rhamnolipid producer was used to remediate Cr(VI). The strain was hypothesised to be able to remediate a variety of metal species based on the earlier studies through its various removal strategies which includes rhamnolipid synthesis. It was conjectured that the presence of Cr(VI) might enhance the synthesis of the rhamnolipid and affect its congeners distribution of the produced rhamnolipid. The ion was hypothesised to upregulating the transcriptional expression of rhamnolipid-encoded genes. In terms of performance, the produced rhamnolipid was expected to have a good pollutants removal efficiency which might exhibited different affinity towards various elements species.

1.4 Objectives

This study was aim to assess the potential application of *P. aeruginosa* RW9 in Cr(VI) remediation. To achieve the goal, the following objectives were set:

1) To determine the Cr(VI) tolerance of *P. aeruginosa* RW9 and its removal mechanisms.
2) To characterise biosurfactant produced by *P. aeruginosa* RW9 in Cr(VI) remediation.
3) To determine the effect of Cr(VI) on the transcriptional expression of rhamnolipid- and reductase-encoding genes.
4) To assess the pollutants' removal efficiency of rhamnolipid in leachate from a sanitary landfill.

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