CHARACTERIZATION OF ARSENATE REDUCTION BY ARSENIC TOLERANT *MICROBACTERIUM FOLIORUM* STRAIN SZ1 ISOLATED FROM GOLD ORES

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To my beloved parents, Mohd Bahari bin Mohd Daai and Zaimah binti Zainoddin

To my wonderful eleven siblings

To my amazing husband, Ahmad Rafie bin Mohmad Tahir

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ABSTRACT

Arsenic is a metalloid of global concern that primarily exists in two inorganic forms of severe toxicity, As (III) and As (V). The reduction of As (V) to As (III) increases toxicity, mobility and bioavailability of arsenic. Understanding how microorganisms reduce As (V) is important to elucidate As (V) reduction mechanism and inevitably, discover approaches to minimise its toxic impact on the environment. This study was aimed at investigating the capability of arsenic tolerant *Microbacterium foliorum* strain SZ1 isolated from gold ores to undergo As (V) reduction to As (III). This strain demonstrated complete reduction of 1 mM As (V) achieved within 120 hours under aerobic condition indicating a possible mechanism of detoxification through regulation of *ars* operon. Further optimization of factors enhancing As (V) reduction capacity of strain SZ1 resulted in complete reduction of 1 mM As (V) achieved within 36 hours in Tris minimal medium supplemented with 10 mM sucrose and 0.1 % (w/v) tryptone at pH 7. The effect of cell adaptation or acclimation towards As (V) reduction was investigated. Well-adapted strain SZ1 recorded complete reduction of 0.5 mM As (V) to 3 mM As (V) within 18 hours to 42 hours incubation. Exopolysaccharides (EPS) was observed to be secreted during reduction of As (V) and subjected to further characterization through chemical analysis of neutral carbohydrate and protein contents and Fourier transform infra-red (FT-IR) analysis. As As (V) concentration increased, so did the protein and carbohydrates concentration of EPS, indicating that EPS played an important role in enabling strain SZ1 to resist and reduce arsenic. Haldane inhibition model was used to fit the reduction rate at different initial As (V) concentrations and the parameters μ_{max}, K_s and K_i were determined to be 0.14 h^{-1}, 0.39 mM and 35.3 mM, respectively. In addition, presence of As (III) as the final product was further confirmed by detection through high performance liquid chromatography (HPLC) analysis. Field emission scanning electron microscopy analysis (FESEM) showed that cells grown in the presence of As (V) exhibited distinct changes in cell morphology and presence of EPS. Exploration of the draft genome of *M. foliorum* SZ1 identified the presence of *ars* operon (*arsC-arsC-ACR3-arsT-arsC-arsR-arsC*) and another two stand-alone genes, *arsC* and *arsB* which further confirmed SZ1's tolerance towards high concentration of arsenic. From the screening of plant growth promoting (PGP) traits, strain SZ1 was able to produce siderophores and indole acetic acid which highlighted its potential use in microbe-assisted arsenic phytoremediation. This is the first study that elucidates the characterization of As (V) reduction by *M. foliorum* SZ1.

ABSTRAK

Arsenik adalah metaloid perhatian dunia yang wujud terutamanya dalam dua bentuk bukan organik bertoksik tinggi, iaitu As (III) dan As (V). Penurunan As (V) kepada As (III) meningkatkan ketoksikan, pergerakan dan bioketersediaan arsenik. Memahami bagaimana mikroorganisma menurunkan As (V) adalah penting untuk menerangkan mekanisme penurunan As (V) dan seterusnya, mencari pendekatan untuk meminimumkan kesan toksiknya terhadap alam sekitar. Kajian ini bertujuan untuk menyiasat keupayaan *Microbacterium foliorum* strain SZ1, bakteria berketahanan tinggi terhadap arsenik yang dipencilkan daripada bijih emas untuk menjalani penurunan As (V) kepada As (III). Strain ini menunjukkan penurunan lengkap 1 mM As (V) yang dicapai dalam masa 120 jam di bawah keadaan aerobik berkemungkinan mekanisme detoksifikasi adalah melalui aturan operon *ars*. Faktor-faktor peningkatan kapasiti strain SZ1 yang dioptimumkan menghasilkan penurunan lengkap 1 mM As (V) dicapai dalam masa 36 jam dalam medium Tris minimal dilengkapi dengan 10 mM sukrosa dan 0.1 % (w/v) tryptone pada pH 7. Kesan adaptasi atau penyesuaian sel terhadap penurunan As (V) telah disiasat. Strain SZ1 yang telah beradaptasi dengan baik merekodkan penurunan lengkap 0.5 mM As (V) hingga 3 mM As (V) dalam masa 18 jam hingga 42 jam eraman. Eksopolisakarida (EPS) diperhatikan telah dirembes sewaktu penurunan As (V) dan tertakluk kepada pencirian lanjut melalui analisis kimia karbohidrat neutral dan kandungan protein serta analisis spektroskopi inframerah (FT-IR). Semakin kepekatan As (V) meningkat, semakin tinggi kepekatan protein dan karbohidrat EPS menunjukkan EPS memainkan peranan penting dalam memastikan strain SZ1 merintang dan menurunkan arsenik. Model perencatan Haldane telah digunakan untuk menyesuaikan kadar penurunan pada kepekatan As (V) yang berbeza dan parameter μ_{max}, K_s dan K_i telah ditentukan pada 0.14 h^{-1}, 0.39 mM and 35.3 mM, masing-masing. Di samping itu, kehadiran As (III) sebagai produk terakhir telah dipastikan lebih lanjut melalui pengesanan analisis kromatografi cecair prestasi tinggi (HPLC). Mikroskop imbasan elektron emisi medan (FESEM) menunjukkan sel yang bertumbuh dalam kehadiran As (V) memaparkan perubahan morfologi dan kehadiran EPS. Penerokaan draf genom *M. foliorum* SZ1 mengenalpasti kehadiran operon *ars* (*arsC-arsC-ACR3-arsT-arsC-arsR-arsC*) dan dua lagi gen yang berdiri sendiri, *arsC* dan *arsB* mengesahkan ketahanan tinggi strain SZ1 terhadap arsenik. Daripada penyaringan kriteria menggalakkan pertumbuhan tumbuhan, strain SZ1 didapati menghasilkan siderophores dan asid indola asetik yang berpotensi untuk diaplikasikan dalam fitopemulihan arsenik dibantu mikrob. Ini adalah kajian pertama yang menjelaskan perincian penurunan As (V) oleh *M. foliorum* SZ1.

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%	-	Percentage
μ_{max}	-	Maximum specific growth rate
µ	-	Specific growth rate
µg/g	-	Microgram per gram
µL	-	Microlitre
µM	-	Micromolar
µm	-	Micron or micrometer
µmol	-	Micromole
ACC	-	1-amino-1-cyclopropane-1-carboxylic acid
$AgNO_3$	-	Silver Nitrate
As (III)	-	Arsenite
As (V)	-	Arsenate
BLAST	-	Basic Local Alignment Search Tool
CFU/mL	-	Colony Forming Unit per mililiter
Cl^-	-	Chloride
cm	-	Centimeter
DF	-	Dworkin Foster
DMA	-	Dimethylarsinic
DNA	-	Deoxyribonucleic acid
dw	-	Dry weight
EDX	-	Energy Dispersive X-Ray Spectroscopy

EPS	-	Exopolysaccharides
FESEM	-	Field Emission Scanning Electron Microscopy
FTIR	-	Fourier Transform Infra-Red
g	-	Gram
g/L	-	Gram per litre
g/mol	-	Gram per mole
h	-	Hour
HCl	-	Hydrochloric acid
HPLC	-	High Performance Liquid Chromatography
IAA	-	Indole acetic acid
ICP-MS	-	Inductively coupled plasma mass spectrometry
kb	-	Kilobase
K_i	-	Inhibition coefficient
K_s	-	Half saturation coefficient
L	-	Litre
LB	-	Luria Bertani
LBA	-	Luria Bertani Agar
M	-	Molarity
M.	-	*Microbacterium*
mg	-	Miligram
mg/L	-	Miligram per litre
MIC	-	Minimum Inhibitory Concentration
min	-	Minutes
mL	-	Mililiter
mM	-	Milimolar
MMA	-	Monomethylarsonic acid
NaCl	-	Sodium Chloride
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate

NCBI	-	National Centre of Biotechnology Information
NGS	-	Next Generation Sequencing
nm	-	Nanometer
^{o}C	-	Degree Celsius
OD	-	Optical density
OFAT	-	One-Factor-at-a-Time
ORF	-	Open reading frame
PCR	-	Polymerase Chain Reaction
PGP	-	Plant Growth Promoting
pKa	-	Acid dissociation constant
R^2	-	Coefficient of determination
rpm	-	Rotation per minute
rRNA	-	Ribosomal ribonucleic acid
s	-	Second
sp.	-	Species
TMM	-	Tris Minimal Medium
TMMST	-	Tris Minimal Medium Sucrose Tryptone
Tris	-	Tris Hydroxylaminomethane
tRNAs	-	Structural ribonucleic acid
TSA	-	Tryptic Soy Agar
TSB	-	Tryptic Soy Broth
UV-Vis	-	Ultra Violet-Visible
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Contamination of the environment with arsenic as a result of natural and anthropogenic sources has occurred in many parts of the world and is acclaimed as a global problem. Amongst major industrial processes that contribute to elevated arsenic concentration in air, water and soil is mining industry with mine tailings and effluents usually containing high arsenic concentration (Wang and Mulligan, 2006). In Malaysia, the famous Tasik Biru, formed from an open cast gold mining pit is declared unsafe by Natural Resources and Environmental Board of Sarawak due to high arsenic content exceeding class IIA/IIB limit of 0.05 mg/L set by National Quality Water Standards for Malaysia. A news report in the New Straits Times in August 2015 revealed very high arsenic content in fishes caught in Sungai Pengorak, a consequence of unregulated bauxite mining in Pahang that led to an alarmingly high and widespread pollution (Aliza Shah, 2015). The adverse effects resulting from arsenic contamination are severe to the environment and ultimately to the organisms living within it. Arsenic poisoning has been reported in many areas of the world spanning more than 21 countries with Bangladesh recording the worst hit case of mass arsenic poisoning in the world (Ghosh *et al*., 2008). It is an established fact that exposure even to low doses of arsenic lead to carcinogenesis not only in human, but in most other forms of life (Mandal and Suzuki, 2002). In view of the global problem

associated with arsenic and its impact on society, removal of arsenic from soil and water is indeed a major environmental need and concern.

Arsenic naturally exists in four oxidation states, 0 (elemental), -3 (arsine), +3 (arsenite) and +5 (arsenate), with the most common forms occurring as arsenate (As (V)) and arsenite (As (III)). Both inorganic forms are toxic to the majority of living organisms with As (III) considered to be ten times more toxic than As (V) (Squibb and Fowler, 1983). As (III) exists as a neutral species at most pH range in natural water causing it to be more mobile than As (V) and subsequently one of the most problematic metalloids in the environment. Therefore, in arsenic contaminated environment, a major concern is the potential for the reduction of As (V) to As (III) which may initiate the mobilization of arsenic in aqueous environment. Arsenic resistant bacteria that reduce As (V) to As (III) via detoxification mechanism have been implicated as possible catalyst of arsenic mobilization in oxic conditions especially in mine tailings (Drewniak *et al*., 2008; Guo *et al*., 2015; Inskeep *et al*., 2002; Macur *et al*., 2001). These types of bacteria are numerous in the environment, however its role in mobilizing arsenic is largely ignored to date (Drewniak *et al*., 2008).

Reduction of As (V) and subsequent methylation of As (III) are thought to be two key steps in detoxifying inorganic arsenic compounds (Dhanker *et al*., 2006; Qin *et al*., 2009). A number of arsenic resistant bacteria capable of reducing As (V) to As (III) have been successfully isolated and identified from arsenic contaminated sites (Anderson and Cook, 2004; Patel *et al*., 2007; Bachate *et al*., 2008; Archour-Rokbani *et al*., 2010; Giudice *et al*., 2013). Arsenic transformation, arsenic accumulation, arsenic resistance genes, arsenate reductase enzyme activity and its role in arsenic detoxification were investigated as it can build a practical guidance on ways of avoiding and reducing arsenic contamination. Hence, it is very important to screen the diverse microbial populations in the environment for more arsenic resistance microorganisms. Isolation and characterization of arsenic resistant bacteria capable of reducing As (V) from the environment would provide the fundamental

studies for improving the biological treatment of arsenic generated from mining effluent or polluted soil.

1.2 Problem Statement

Generally, arsenic bioremediation focuses on the application of As (III) oxidizing bacteria as it transforms the more toxic and mobile form of As (III) to less toxic, less mobile As (V) which is an ideal transformation. However, this approach is suitable where As (III) is the main pollutant and limited for water system. In the case of remediation of arsenic contaminated soil, As (V) reduction mechanism is proven to be a better approach (Drewniak and Sklodowska, 2013; Wang and Zhao, 2009). This is due to the fact that As (V) is the major species detected in soil and usually found adsorbed onto soil mineral (Drewniak and Sklodowska, 2013). Therefore, As (V) reducing bacteria could transform As (V) into the mobile and less sorptive form of As (III), promoting arsenic removal from the soil. Following that, As (III) can be completely removed from solution by its precipitation or complexation with sulfide or sulfide containing minerals (Newman *et al*., 1997; Rochette *et al*., 2000) as well as adsorption to Fe (II) based solid (Nishimura and Umetsu, 2000; Roberts *et al*., 2004).

However, much of the research up to now have only focused on the role of dissimilatory As (V) reducing bacteria as the potential agent for bioremediation of arsenic contaminated soil rather than As (V) reducing bacteria (Drewniak *et al*., 2014; Kudo *et al*., 2013; Sierra-Alvarez *et al*., 2005; Soda *et al*., 2009; Yamamura *et al*., 2003; Yamamura *et al*, 2005). A key issue is the irrelevant role of As (V) reducing bacteria in mobilizing arsenic (Zobrist *et al*., 2000). Nevertheless, there have been well documented reports suggesting As (V) reducing bacteria plays an important role in mobilization of arsenic in oxic soil and surface water (Cullen and Reimer, 1989; Drewniak *et al*., 2008; Guo *et al*., 2015; Macur *et al*., 2004; Macur *et al*., 2001; Sohrin *et al*., 1997). Although As (V) reducing bacteria enhanced arsenic

mobilization, little attention has been given on its bioremediative potential. In recent years, there has been an increasing interest in the application of As (V) reducing bacteria in assisting arsenic hyperaccumulator plant for removal of arsenic from soil or water as As (III) is more desirable form of arsenic for plant's uptake due to its mobility and inhibition of As (V) uptake by phosphate (Cavalca *et al*., 2010; Yang *et al*., 2012). Moreover, the abundance of As (V) reducing bacteria and the ease of handling indicate the feasibility of this type of bacteria in comparison to dissimilatory As (V) reducing bacteria, to which its applicability is limited under anoxic condition.

Therefore, it is of paramount importance to understand the knowledge of the physiology and the underlying mechanism of As (V) reducing bacteria for providing insights into the potential of arsenic bioremediation.

1.3 Objectives of the Study

This study was carried out to investigate the capability of arsenic tolerant bacterium isolated from gold ores to undergo As (V) reduction to As (III). The specific objectives of the study were:

i) To isolate, screen and characterize arsenic resistant bacteria from a gold mining environment

ii) To characterize and optimize As (V) reducing properties of isolated As (V) reducing bacteria

iii) To elucidate As (V) reducing pathway via biochemical and whole genome analysis and evaluate the bioremediative potential of isolated As (V) reducing bacteria.

1.4 Scope of the Study

In this study, bacteria were isolated from arsenic contaminated sources originated from a gold mining environment with the aim of obtaining arsenic resistant bacteria. Following isolation and screening, identification of arsenic resistant bacteria was conducted using 16S rRNA analysis. Characterization of the isolates in terms of tolerance towards As (III) and As (V) and growth rates in the absence and presence of arsenic was conducted. After that, the isolates were screened for arsenic transformation capabilities with only one isolate demonstrating As (V) reducing trait, hence, selected for further studies. Parameters (effect of carbon sources and its concentration, effect of nitrogen sources and its concentration, influence of pH, effect of cell adaptation or acclimation, effect of initial As (V) concentrations) influencing As (V) reduction were optimized conventionally using one factor at a time (OFAT) method. Then, Haldane inhibition model was used to fit the reduction rate at different initial As (V) concentrations for determination of biokinetics parameters. The final reduction products and the possible presence of methylated arsenic were investigated using high performance liquid chromatography (HPLC). Characterization of cells morphology in the absence and presence of arsenic was evaluated using FESEM-EDX. In addition, whole genome sequence of isolate was analysed using Next Generation Sequencing (NGS) to explore the presence of arsenic resistance mechanism. At the end of the study, the potential of isolate for bioremediation of arsenic was elucidated.

1.5 Significance of Study

The present study was focused on the characterization of As (V) reduction by locally isolated arsenic tolerant *Microbacterium foliorum* strain SZ1. Important environmental parameters enhancing As (V) reduction capacity of strain SZ1 were provided in this study. In addition, Haldane substrate inhibition model was employed for the estimation of biokinetic parameters for As (V) reduction. To the best of our knowledge, the application of Haldane substrate inhibition model to describe growth kinetics of As (V) reducing bacteria that reduced As (V) through detoxification mechanism has yet to be reported. Apart from that, the availability of genome sequences of strain SZ1 determined from Next Generation Sequencing method allowed the identification of its arsenic resistance mechanism. The presence of two important plant growth promoting traits accompanied with the capability to produce extracellular polysaccharides (EPS) and high arsenic resistance highlighted this strain potential use in microbe – assisted arsenic phytoremediation. The findings of this study allow better understanding of the role played by As (V) reducing bacteria in arsenic transformation. This is the first study ever reported on the characterization of As (V) reduction by *Microbacterium foliorum* strain SZ1.

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