HEAVY METAL TOLERANCE OF *LYSINIBACILLUS FUSIFORMIS* ZB2

ISOLATED FROM TEXTILE EFFLUENT

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A special dedication to my beloved family and friends
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ABSTRACT

Heavy metal pollution has always been considered as one of the major threats to the environment and human health since these metals can accumulate in the food chain, inactivate cellular enzymes and may cause cancer related diseases. Conventional physiochemical methods do not provide economical treatment for the removal of heavy metals from heavy metal polluted environment. An effective and economical alternative method that has been widely reported is microbial bioremediation. In this study, the minimal inhibitory concentration (MIC) of *Lysinibacillus fusiformis* ZB2 for selected heavy metals, namely, cadmium (Cd), zinc (Zn), lead (Pb) and chromium (Cr(VI)) were determined. This bacteria was isolated previously from the textile effluent. It was grown in low phosphate medium (LPM) with glucose and tryptone as its carbon and nitrogen source respectively. *L. fusiformis* ZB2 reached its exponential growth within 48 hours of incubation in the LPM. The MIC of the bacteria for Cd, Zn, Pb and Cr(VI) were determined in solid and liquid media. The MIC obtained was relatively higher when using the liquid media. The MIC for Cd, Zn, Pb and Cr(VI) were 25, 75, 150, and 3500 ppm, respectively as compared to using the solid media MIC for Cd, Zn, Pb and Cr(VI) were 10, 75, 250, 3000 ppm, respectively. The order of toxicity of heavy metals towards *Lysinibacillus fusiformis* ZB2 was Cd>Zn>Pb>Cr(VI). The bacteria was found to be tolerant towards Zn, Pb and Cr(VI) with maximum tolerance towards Cr(VI).

ABSTRAK

Pencemaran logam berat sentiasa dianggap sebagai satu daripada ancaman utama terhadap alam sekitar dan kesihatan manusia kerana logam ini boleh berkumpul dalam rantaian makanan, menyahakif enzim sel dan boleh dikaitkan dengan penyakit kanser. Kaedah penyingkiran logam berat dari persekitaran yang tercemar dengan logam berat menggunakan kaedah fizik kimia konvensional adalah tidak ekonomik. Satu kaedah alternatif yang berkesan dan ekonomik yang telah dilaporkan secara meluas adalah bioremediasi mikrob. Dalam kajian ini, *minimal inhibitory concentration* (MIC) daripada *Lysinibacillus fusiformis* ZB2 untuk logam berat terpilih, iaitu, kadmium (Cd), zink (Zn), plumbum (Pb) dan kromium (Cr(VI)) telah ditentukan. Bakteria ini telah diasingkan sebelum ini dari efluen tekstil. Ia dikultur dalam *low phoshate medium* (LPM) dengan glukosa dan tripton sebagai sumber karbon dan nitrogen masing-masing. *L. fusiformis* ZB2 mencapai pertumbuhan fasa eksponen dalam tempoh 48 jam pengeraman dalam LPM. MIC bakteria untuk Cd, Zn, Pb dan Cr(VI) ditentukan dalam media pepejal dan cecair. MIC yang diperolehi adalah lebih tinggi apabila menggunakan medium cecair. MIC untuk Cd, Zn, Pb dan Cr(VI) dalam medium cecair adalah 25, 75, 150, dan 3500 ppm masing-masing berbanding dengan medium pepejal MIC untuk Cd, Zn, Pb dan Cr(VI) adalah 10, 75, 250, 3000 ppm masing-masing. Urutan kesan ketoksikan logam berat terhadap *Lysinibacillus fusiformis* ZB2 adalah Cd> Zn> Pb> Cr(VI). Bakteria ini didapati toleran terhadap Zn, Pb dan Cr(VI) dan mempunyai toleransi maksimum terhadap Cr(VI).

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LIST OF ABBREVIATIONS

ADMI	-	American Dye Manufacturers Institute
$CaCl_2$	-	Calcium chloride
Cd(II)	-	Cadmium (2^+) ion
$Cd(NO_3)_2 \cdot 4H_2O$	-	Cadmium nitrate tetrahydrate
COD	-	Chemical Oxygen Demand
Cr(VI)	-	Chromium (6^+) ion
HCl	-	Hydrochloric acid
$K_2Cr_2O_7$	-	Potassium dichromate
KCl	-	Potassium chloride
LPA	-	Low phosphate agar
LPM	-	Low phosphate medium
MIC	-	Minimal inhibitory concentration
Na_2SO_4	-	Sodium sulphate
NaCl	-	Sodium chloride
NADH	-	Nicotiamide adenine dinucleotide (reduced)
NADPH	-	Nicotiamide adenine dinucleotide phosphate (reduced)
NH_4Cl	-	Ammonium chloride

OD_{600nm}	-	Optical density at 600nm
Pb(II)	-	Lead (2^+) ion
$Pb(NO_3)_2$	-	Lead (II) nitrate
rpm	-	Revolutions per minute
Tris	-	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Zn(II)	-	Zinc(2^+) ion
$ZnSO_4$	-	Zinc sulfate

LIST OF SYMBOLS

%	-	percent
℃	-	degree Celsius
μm	-	micrometre
g	-	gram
g/L	-	gram per litre
h	-	hour
kPa	-	kilopascal
L	-	Litre
mg/L	-	milligram per litre
mL	-	millilitre
mM	-	millimolar
nm	-	nanometer
ppm	-	parts per million
v/v	-	volume per volume
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Heavy metals are elements with atomic weight ranging from 63.5 to 200.6 and have specific gravity more than 5.0 (Srivastava and Majumder, 2008). Toxic metals such as mercury (Hg), chromium (Cr), lead (Pb), zinc (Zn), copper (Cu), nickel (Ni), cadmium (Cd), arsenic (As), cobalt (Co) and tin (Sn); precious metals, such as palladium (Pd), platinum (Pt), silver (Ag), gold (Au) and ruthenium (Ru); and radionuclides such as uranium (U), radium (Ra) and americium (Am). These three types of heavy metals are rising concerns because of their negative impact towards the environment and human health (Wang and Chen, 2006).

Heavy metal pollution is known as a critical environmental problem as a result of the metals toxic effect and their accumulation throughout the food chain can cause severe problems to the ecology and human health (Malik, 2004). Wastewater containing heavy metals are discharged directly and indirectly into the environment particularly in developing countries (Fu and Wang, 2011). Coal, natural gas, paper and chlor-alkali, metal plating, mining, fertilizer, tanneries, batteries and pesticides industries are known to be the source for heavy metal pollution (Matlock *et al.*, 2002; Fu and Wang, 2011).

Growing interests among researchers in bioremediation of heavy metals by microorganisms in recent years are possibly due to its potential application in industry and scientific novelty of the microorganism (Singh *et al.*, 2013). Metal ions can be readily adsorbed and accumulated by bacteria, algae and fungi (Abbott *et al*., 2005; Volesky and Holan, 1995). In the study for the heavy metal tolerance, the medium composition affects interactions between metal ions and microbes in terms of bioavailability due to accumulation and precipitation of metal ions (Kumar *et al.*, 2013). As a result of low carbon source and negligible phosphate, low phosphate medium (LPM) provide more reliable results as compared to complex medium such as the Mueller-Hinton (MH) medium (Kumar *et al.*, 2013). Minimal medium also provide conditions that is more similar to those found in the environmental sample compared to that of rich medium (Karelova *et al.*, 2011).

Heavy metals are often used in textile processes (Rybicki *et al.*, 2004) and eventually found in the textile wastewater in the form of free ionic metals or complex metals (Hill *et al.*, 1993). Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu) and iron (Fe) are present in the textile dye of the textile effluent (Halimoon and Goh, 2010; Siddiqui *et al*., 2011). In previous study by Kee *et al*. (2015), *Lysinibacillus fusiformis* strain ZB2 together with *Bacillus pumilus* strain ZK1, *Bacillus cereus* strain ZK2, *Brevibacillus panacihumi* strain ZB1 was used to treat real textile wastewater successfully for decolourization purpose and *L. fusiformis* ZB2 was identified as the dominant species in the mature granules.

Bacterial strains are potential candidates for simultaneous removal of metals from wastes as they have high tolerance to different metals (Malik, 2004). Since heavy metal pollution is becoming one of the major threats to the environment and possess many health hazards, bioremediation potential of bacterial isolates should be assessed by preliminary study in terms of their resistance level and the minimal inhibitory concentrations (MICs). This is fundamental in order to check for the tolerance of the bacterial strains towards different heavy metals to develop suitable heavy metal waste remediation.

1.2 Problem Statement

Heavy metal tolerance test of bacteria in complex medium results in higher tolerance behavior by the bacteria. This is because the metals chelate with constituents of the complex medium resulting in metal precipitation and non-uniform availability of metals in the medium. Low phosphate medium (LPM) is preferred over complex medium as the metal's precipitation is reduced due to negligible phosphate and low carbon source, thus, more metal ions are available to the bacteria (Kumar *et al.*, 2013). However, the suitability of LPM as *Lysinibacillus fusiformis* ZB2 growth medium is still yet to be tested.

In the last few decades, the river water and sediments are receiving increasing concentration of heavy metals. Although industrialization has long been accepted as a hallmark of civilization, it is undeniable that industrial discharges have been causing negative impacts to the environment. Mining, milling, surface finishing industries are the main sources of heavy metal pollution that discharge a variety of toxic metals such as Cd, Co, Cu, Ni, Pb and Zn into the environment. The toxics and heavy metals in the industrial effluents are often discharged into the river which might be a source of drinking water for another town downstream (Moore, 1990; Ewan and Pamphlett, 1996). According to Barakat (2011), heavy metals are hazardous to human health as they may inhibit growth and development, cause organ damage and cancer, damage to nervous system and in extreme cases, death.

Furthermore, there is an increasing demand to shift to cleaner production methods in different industries and develop environmental friendly, economical and efficient treatment technique for metal contaminated effluent as many industries have adhered to more stringent environmental regulations (Malik, 2004; Fu and Wang, 2011). A variety of methods are employed for the removal of heavy metal ions such as chemical precipitation, ion-exchange, adsorption, membrane filtration, electrochemical treatment technologies, etc. (Fu and Wang, 2011). However, these methods have their disadvantages as they are expensive, not environmental friendly and involve complex processes (Karman *et al.*, 2015). Also, remediation of heavy metal contaminated sites are unfeasible as heavy metals may disperse both horizontally and vertically as they migrate (Nikolaidis *et al.*, 1999).

Since heavy metals are the environmental priority pollutants and cause one of the most serious environmental problems, removal of these toxic heavy metals is essential in order to protect the people and environment (Fu and Wang, 2011). In the past few decades, microbial mediated detoxification technologies are still being valued over physicochemical ones. Biological remediation is receiving more attention as they are more economic and have long lasting nature (Ali *et al.*, 2009). Interactions of microbes and metals have significant environmental implications and monitoring as the microbes have adapted to resist the presence of metals or utilizes the metals for their growth. One of the useful environmental implications is the use of bacteria to clean up metal polluted areas (Nithya *et al*., 2011).

Thus, in this study, the low phosphate medium (LPM) with different combinations of carbon and nitrogen source was used to grow *Lysinibacillus fusiformis* ZB2 in order to test the suitability of this medium as the growth medium for the bacteria and subsequent heavy metal testing. Furthermore, this bacteria was used to screen for its heavy metal tolerance and determine its minimal inhibitory concentration (MIC) to selected heavy metals which are Cd, Zn, Pb and Cr(VI) since these heavy metals are present mostly in the industrial effluents such as textile effluents.

1.3 Research Objectives

There are three objectives for this study:

i. To determine suitable carbon and nitrogen co-substrate for growth of *Lysinibacillus fusiformis* ZB2 in low phosphate medium (LPM)

ii. To screen resistance of *L. fusiformis* ZB2 to selected heavy metals

iii. To determine minimal inhibitory concentration (MIC) of *L. fusiformis* ZB2 to selected heavy metals

1.4 Research Significance

Heavy metals are used in many industries and this causes a lot of concerns as they are not biodegradable and can remain the environment for a long period of time. It causes harm to both environment and human health. Conventional physico-chemical methods are employed for the removal of the metals from heavy metal polluted sites, however, it has several disadvantages making remediation of heavy metal pollution a challenging task to achieve. With the emergence of biotechnology, microorganisms such as bacteria, algae and fungi are studied and are found to be capable of tolerating with heavy metals. In order to explore more potential microbe candidates for the remediation of heavy metal and overcome the limitation of physico-chemical methods, more bacteria can be studied for their heavy metal tolerance. Therefore, in this study *Lysinibacillus fusiformis* ZB2 isolated previously from textile effluent was used to test for its heavy metal tolerance.

1.5 Scope of Research

This study was mainly focused on heavy metal tolerance or resistance of *Lysinibacillus fusiformis* ZB2 in four selected heavy metals, namely, cadmium (Cd), zinc (Zn), lead (Pb), and chromium (Cr(VI)). Cd, Zn, Pb, and Cr(VI) are usually present in many industrial effluent and cause hazardous effect to the environment and living organisms. Therefore, these heavy metals were chosen for this study. *L. fusiformis* ZB2 was previously isolated from the textile effluent. The bacteria was grown in low phosphate medium (LPM) with optimisation of carbon and nitrogen source and the exponential growth phase in the best LPM was determined. The heavy metal resistance of *L. fusiformis* ZB2 was tested by spot inoculation on low phosphate agar (LPA) and by growing the bacteria in LPM supplemented with different concentrations of heavy metals. In general, the heavy metal resistance was determined in terms of minimal inhibitory concentration (MIC), which is the concentration of heavy metal that inhibit the bacteria growth in LPA and LPM. The MIC and percentage of tolerance were investigated by evaluating growth in LPA and measurement of bacteria concentration at OD_{600} nm when the bacteria was grown in LPM incorporated with different concentrations of heavy metals.

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