ISOLATION AND CHARACTERIZATION OF CADMIUM RESISTANT BACTERIA FROM INDUSTRIAL WASTEWATER

By

Syed Zaghum Abbas

Thesis submitted in fulfillment of the requirements for the degree of

Master of Science

September 2014

PENGASINGAN DAN PENCIRIAN BAKTERIA RINTANG KADMIUM DARIPADA AIR SISA INDUSTRI

Oleh

Syed Zaghum Abbas

Thesis yang diserahkan untuk memenuhi keperluan bagi

Ijazah Sarjana Sains

September 2014

ACKNOWLEDGEMENT

My first and foremost duty is to thanks to ALLAH, the most gracious and benevolent to mankind, WHO enabled me to carry out this work. I would like to express my sincere gratitude to my respectful supervisor, Senior Lecturer Dr. Mohd Rafatullah, School of Industrial Technology, Universiti Sains Malaysia for all his guidance, advice and encouragement during study. His kindness and generosity in sharing knowledge and experience is very much appreciated. I also would like to thank my co-supervisors Assoc. Prof. Dr. Norli Ismail, Senior Lecturer Dr. Japareng Lalung for their help and encouragement.

I am very grateful to Dr. Mohammed Danish, Dr. Mehraj Ahmad and Dr. Mohammad Asad (Post Doctoral Fellows) for their encouragement and help during my research plan and thesis writing. I am highly thankful to our environmental research group and to all my colleagues, thank you so much for all those times, advices and efforts you have spent for my work.

I can not forget the help of our division laboratory staffs, my special thanks to Mr. Ravi Vinayagamuertty, Madam Teh Siew Hong, Mr. Alfenddi bin Jamaluddin and Miss Farhana, Center of Chemical Biology, USM for their kind support and help during my experiments.

I must express my profound thanks to the University Sains Malaysia, Short Term Grant (304/PTEKIND/6312008) for the financial supports.

Last but certainly not least, to my family members particularly my father, mother, and sisters for having patient during my study. I would like to thank all of you from the bottom of my heart for being so supportive… Thank you so much.

TABLE OF CONTENTS

CHAPTER THREE-MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

CCB, USM	Center of Chemical Biology, Universiti Sains Malaysia
DOE	Department of Environment
EQA	Environmental Quality Act
IARC	International Agency for Research on Cancer
LB	Lauria Bertani
MR-VP	Methyl-Red Voges Proskaure test
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
US EPA	United States Environmental Protection Agency

LIST OF SYMBOLS

Cd	Cadmium
%	Percentage
°C	Degree (s) Celsius
µ/L	Micro per liter
µg/mL	Microgram (s) per mililiter
h	Hour
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
mg/L	Miligram (s) per liter
min	Minutes
Mn	Manganese
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nano Meter
psi	Pound per square inch
rcf	Relative centrifugal force
rpm	Revolutions per min
sec	Seconds
U	Units
X	Magnification
Zn	Zinc

PENGASINGAN DAN PENCIRIAN BAKTERIA RINTANG KADMIUM DARIPADA AIR SISA INDUSTRI

ABSTRAK

Sejak beberapa dekad yang lalu, penyingkiran Kadmium secara biologi telah mendapat perhatian yang besar. Walaupun banyak penemuan mengenai mekanisma penyingkiran Kadmium, hanya sebahagian kecil sahaja daripada kajian tersebut yang telah dilakukan di Malaysia. Enam strain bakteria telah diasingkan daripada air sisa industri di Pulau Pinang, Malaysia. Strain RZ1, RZ2, RZ3, RZ4, RZ5 dan RZ6 telah dikenal pasti sebagai *Pantoea* sp. RL32.2, *Salmonella enterica*, *Enterobacter* sp. OCPSB1, *Enterobacter mori*, *Enterobacter* sp. WS12 dan *Pseudomonas* sp. M3. berdasarkan kepada ciri ciri morfologi, biokimia, fisiologi dan analisis 16S rDNA. Kesemua enam strain tersebut menunjukkan pertumbuhan dan penyingkiran Kadmium optimum pada pH 7.0 dan suhu 35 ° C. Strain RZ1, RZ2, RZ3, RZ4, RZ5 dan RZ6 menyingkirkan masing-masing 89,89%, 82,10%, 89,14%, 87,75%, 85,11% dan 81,89% Kadmium. Kapasiti penyingkiran Kadmium oleh semua strain dipengaruhi oleh suhu dan pH. Kepekatan perencatan minimum strain RZ1, RZ2, RZ3, RZ4, RZ5 dan RZ6 adalah masing-masing 750 μg / mL, 410 μg / mL, 550 μg / mL, 450 μg / mL, 500 μg / mL dan 550 μg / mL. Strain tulen yang telah diasingkan menunjukkan ketahanan dan sensitiviti terhadap pelbagai antibiotik. Kemunculan band protein dengan berat molekul yang berbeza dalam keadaan tertekanan menunjukkan peranan penting protein ini dalam penyingkiran Kadmium. Secara keseluruhan, strain ini mungkin berguna untuk penyingkiran Kadmium daripada air sisa industri.

ISOLATION AND CHARACTERIZATION OF CADMIUM RESISTANT BACTERIA FROM INDUSTRIAL WASTEWATER

ABSTRACT

In the last few decades, cadmium removal by biological ways has received great attention. Nevertheless, for the growing of microorganisms that harbor many mechanisms for cadmium sequestration and may have great cadmium removal capacities, only small number of these studies employed in Malaysia. Six bacterial strains were isolated from industrial wastewater of Penang, Malaysia. The strains RZ1, RZ2, RZ3, RZ4, RZ5 and RZ6 were identified as *Pantoea* sp. RL32.2, *Salmonella enterica*, *Enterobacter* sp. OCPSB1, *Enterobacter mori*, *Enterobacter* sp. WS12 and *Pseudomonas* sp. M3 respectively, based on morphological, biochemical, physiological observation and 16S rDNA sequence analysis. All the six strains showed optimum growth and cadmium removal at 7.0 pH and 35 °C temperature. The strains RZ1, RZ2, RZ3, RZ4, RZ5 and RZ6 removed 89.89%, 82.10%, 89.14%, 87.75%, 85.11% and 81.89% of cadmium, respectively. The cadmium removal capacities by all the strains were affected by temperature and pH. The minimum inhibitory concentrations of the strains RZ1, RZ2, RZ3, RZ4, RZ5 and RZ6 were 750 µg/mL, 410 µg/mL, 550 µg/mL, 450 µg/mL, 500 µg/mL and 550 µg/mL respectively. The purified strains showed resistance and sensitivity against some of antibiotics. The appearance of induced protein bands with different molecular weights in the stress condition which points out an important role of these proteins in cadmium removal. Overall, these strains could be useful for the removal of cadmium from industrial wastewater.

CHAPTER ONE

INTRODUCTION

1.1 General

Cadmium is a metallic element and in periodic table placed in group II B (relative atomic mass: 112.41 and atomic number: 48). This element normally exists in white silver and soft form. It is normally not present in pure form in the environment it usually forms complex oxides with copper ore, lead, zinc, carbonates and sulfides. This metal cannot be detected by taste and odor. Cadmium sulphate and cadmium chloride are more soluble in water than cadmium oxide (elemental cadmium) (Li & Wong, 2006).

Cadmium is a harmful metal due to its high toxicity and stability. It is also a heavy metal contaminant in the environment. Extensive data prove that this metal is the most widespread, toxic heavy metal and now many international agreements included cadmium in the blacklist that monitor the input of cadmium into the environment (Hu *et al.,* 2007). The release of the metal into the environment is by smelting, electroplating, alloy manufacturing, deposition from metallurgical and petrochemical industries as byproduct, land application of sewage sludge, fossil fuel burning, tobacco smoke, chemical fertilizers, nickel-cadmium batteries, chlor-alkali industries, wood pulping, plastic, mining, pigment plants, paints and refining processes (Deng *et al.,* 2007). Due to its low market price, new ways of its applications and emission has been increasing. The range of cadmium in wastewaters ranged from 10 mM to 100 mM (Jabbari *et al.,* 2010). The natural waters normally

polluted by these industrial wastewaters, so aquatic ecosystem at a great risk (Chen *et al.,* 2006a).

Cadmium is nonessential and even at low dosage the metal has fatal effects on plants, animals and humans. In plants, it affects the shoot and root growth, inhibits homeostasis and nutrient uptake and often accumulated in important agriculture crops. Cadmium enters into human and animal body through food chain and can cause serious diseases (Zeng *et al.,* 2009). The metal is embryotoxic, carcinogenic, mutagenic, teratogenic and may cause anaemia, hyperglycemia, osteomalacia, renal damage, lung cancer, damage DNA, vertebral osteoporosis and fractures, peripheral arterial disease, aging, toxicity to neuron, damage the liver, cardiovascular system, reproductive system and reduced immunopotency due to its interference with iron metabolism (Hartwig, 2010; Abyar *et al.*, 2012). It also affects apoptosis, differentiation and proliferation and increases the chances of oncogene activation. The biological half life of cadmium is extremely high and declared as human carcinogenic by the International Agency for Research on Cancer (IARC, 1993) and Maximale Arbeitsplatz-Konzentration Commission (DFG 2006). Thus, throughout world, cadmium pollution got the most attention of environmentalists. A recent list of substances that are human carcinogens by IARC provided sufficient evidences about cadmium induced-lung tumor and some data about cadmium induced-prostrate and kidney tumors. Furthermore, new data prove that endometrial and female breast cancer caused by exposure to cadmium (Amzal *et al.*, 2009). In the animals it induces cancer of testis, prostrate and carcinomas of lung after injection or inhalation of cadmium (Gallagher *et al.*, 2010).

Cadmium particularly accumulates in renal, lung, bone, pancreas, liver and damages them. Nishijo *et al.,* (2006) studied the people living in cadmium contaminated Kakehashi River Basin in Japan upto 15 year. Based on the results, cadmium induced renal tubular dysfunction, nephritis, increased the rate of cerebral infarction, heart failure and nephrosis among inhabitants of cadmium polluted area.

Due to awareness about toxic effect of cadmium on ecological system and increasing value of cadmium, studies on cadmium accumulation, removal and recovery have been carried out (El-Sayed *et al.,* 2011). Various conventional methods are used to remove cadmium from industrial wastewater like electrochemical treatment, chemical precipitation, ion exchange, reverse osmosis, membrane technology, phytoremediation, oxidation and reduction are very expensive and not environmentally acceptable (Mohamed, 2001; Pandey *et al,*. 2010). The uses of these techniques are very limited due to economical constraints, production of toxic sludge, partial removal and technical issues. For example in chemical precipitation no guarantee of cadmium removal according to the standard and difficult to treat the waste produces at the end. On the other hand ion exchange is very effective method but it requires very expensive adsorbent materials (Mohamed, 2001). Therefore, there is need of eco-friendly, low cost, substitute and effective techniques to remove the cadmium from industrial wastewater. Many studies have explained that bacteria, algae, yeast, fungi, molds, protozoa can remove the cadmium from the wastewater or contaminated water (Bamatraf & Omar, 2013). The reason behind that special chemical compounds like carboxyl, carbonyl, hydroxyl and sulfydryl groups present in the cell wall of biomass of microorganisms that can bind with cadmium ions. Beside this, microorganisms also adopt variety of mechanisms to

remove cadmium ions from wastewater. They remove cadmium ions via complexation by exopolysaccharides, adsorption to cell surfaces, binding with bacterial cell envelopes, biosynthesis of metallothioneins, intracellular accumulation, precipitation, transformation to volatile compounds and formation of other proteins that trap the cadmium (Chen *et al.*, 2011b).

Microorganisms play an important role in removal of cadmium from the environment. In the past years many studies have been carried out for the isolation of cadmium resistant bacterial strains. In this regard, cadmium accumulation ability among bacteria e.g, *Pseudomonas putida, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas syringae, Pseudomonas fluorescens, Comamonas testosterone, Staphylococcus aureus, Alcaligenes eutrophus, Gluconobacter oxydans, Bacillus subtilis, Staphylococcus lugdunensis, Alcaligenes xylosoxidans, Ralstonia metallidurans, Lactobacillus plantarum, Serratia liquefaciens, Klebsiella planticola, Paenibacillus* sp. and *Bacillus thuringiensis* have been studied most (Sabdono, 2011; Amoozegar *et al.,* 2012).

1.2 Problem Statement

Higher amount of cadmium released in water by many industries in Penang (Farah *et al.,* 2012). This released amount of cadmium is more than standard value that is given by US EPA (United States Environmental Protection Agency) is 0.005 mg/L. In addition, According to the Malaysian Environmental Quality Act 1974, (EQA 1974, Regulation 2009) the allowable amounts of cadmium into industrial effluent are 0.01 mg/L (standard A, upstream catchment) and 0.02 mg/L (standard B, downstream catchment). The cadmium is toxic, has long biological half-life, has long residence times and is a major problem for public health. Cadmium is a potent

oxidative agent. Cadmium causes the breaking of DNA into single strand by inhibiting the replication of DNA (Zhang *et al.,* 2009). In addition, the genes for cadmium and antibiotics resistance are mostly located on transposons and plasmids, has suggested that through horizontal transfer genes have probably been transfer (Chovanová *et al.*, 2004).

This directly affects the health, ecology and environment of Penang. The physico-chemical methods like precipitation, reverse osmosis and ion exchange are being used for removal of cadmium from wastewater but because biological methods are eco-friendly and less expensive than conventional techniques so now a days it get more attention (Sabdono, 2011). Although a lot number of bacterial species are able to remove cadmium (refer section 2.5), but none of them not characterized from Penang industrial wastewater. It is important to characterize from local industrial wastewater in Penang because contamination of metal from industrial effluents is a big problem in the state (Azrina *et al.*, 2011).

1.3 Objectives of Study

- To isolate the cadmium tolerant bacteria from industrial wastewater and to determines their cadmium removal capacity.
- To study the bacterial tolerance capacity against cadmium.
- To characterize the bacterial strains from industrial wastewater containing high amount of cadmium.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Environment and Cadmium Contamination

Environmental contamination is one of the most important factors contributing to the destruction of the biosphere. Cadmium plays a key role in this destruction. The total amount of cadmium in the water is distributed over some fractions. The soluble and insoluble fractions of cadmium are the main cause of water pollution that affects the plant nutrition. The movement of cadmium into water depends upon composition of water (Li & Wong, 2006).

Anthropogenic sources, including industrial effluents and emissions, fertilizations and industries byproducts can contribute to the increasing amount of cadmium in water. The cadmium pollution depends upon the industrial activities in particular area and properties of water. Therefore, knowledge of the migration of cadmium and influence of cadmium on water is very important (Czech's submission 2010).

Industrial wastewater creates environmental problems because of their demand of disposal space and water pollution effect through leaching. The environmental pollution caused by toxic cadmium has gained great attention in most of the major metropolitan cities. Cadmium entering the ecosystem may cause geo-accumulation, bioaccumulation and bio-magnification. Therefore, a better understanding of cadmium source, its accumulation in water, its effect on water and

in plant systems is particularly important issues of present day research on risk assessment (Åkesson *et al*., 2005).

2.2 Cadmium Contamination in Penang

In 2012 eight locations (Sungai Air Hitam 3, Sungai Jelutong, Sungai Dondang, Sungai Air Hitam 2, Sungai Pinang Sungai Air Hitam, Sungai Air Putih and Sungai Air Terjun) of Penang (Malaysia) were used for sampling of industrial wastewater and noted concentrations of cadmium, lead, zinc and copper. Overall ranges of concentrations of these metals in these locations were cadmium (2.0-51.96 mg/L), lead (0.01-2.98 mg/L), zinc (0.01-48.05 mg/L) and copper (37.18-47.43 mg/L) (Farah *et al.*, 2012). The concentrations of cadmium in these locations was higher than the standard value given by Malaysian EQA, 1974 that is 0.01 mg/L (standard A, upstream catchment) and 0.02 mg/L (standard B, downstream catchment).

The tap water samples of Peninsular Malaysia were analyzed in 2011 and found that cadmium concentration in tap water of Penang range from 0.36-0.86 mg/L (Azrina *et al.*, 2011), which was 280 times higher than standard value given the National Standard for Drinking Water Quality, Ministry of Health Malaysia that is 0.003 mg/L. This finding has to be taken cautiously because Nalatambi (2009) reported that the concentration of cadmium into tap water of Peninsular Malaysia was 0.00025 mg/L. According to Department of Environment (DOE) Malaysia the waters of west coast of peninsular Malaysia has high concentrations of cadmium. Some water samples contain cadmium above than standard value. A recent survey found that concentrations of cadmium were higher than those determined indicating an increase in cadmium contamination identified in the coastal waters of Perak and

Penang. It was also reported that most of the rivers along the west coast of peninsular Malaysia contained high concentrations of cadmium which exceeded the interim standards (Norzatulakma, 2010).

2.3 Hazardous Effect of Cadmium on Humanity

For a biological function in human cadmium is non-essential. The kidney is considered to be a target organ in both occupational and general cadmium exposed populations. Many studies indicate that renal tubular damage occurs in smokers because smoking is additional source of cadmium accumulation in the body. After kidney damage, the secondary response is skeletal damage in which cadmium directly damage the bone cells. Some studies indicated that cadmium alter the mechanism of calcium that results in osteoporosis. Lung cancer has been reported by inhalation of cadmium but until there is no evidence that cadmium cause cancer by oral route (WHO Chemical Fact Sheet, 2012).

The IARC (1993) classified the cadmium and cadmium compounds in the groups of human carcinogen, having evidences that cadmium causes lung cancer in man and animals by inhalation of cadmium (WHO, 2000). Some publication concluded the link between cadmium and prostate cancer (Menke *et al.*, 2009), breast cancer (McElroy *et al.*, 2006) and renal cancer (Il'yasova & Schwartz, 2005) in the human but the evidences are not clear.

The evidences from the experiments on the animals suggested that cadmium interfere with the production of testosterone, ovarian steroids that participated in the mammalian development and increased uterine weight (Johnson *et al*., 2003). The cadmium exposure on maternal stage causes lower birth weight and spontaneous

abortion. From in vitro study of animals it is now clear that cadmium also affects the endocrine system and hypothalamus-pituitary (Den Hond & Schoeters, 2006).

2.4 Industrial Wastewater Treatment Technologies

Different methods are used to treat the cadmium contaminated industrial wastewater. The wastewater treatment methods divided into conventional and non-conventional methods.

Conventional methods include (**i**) Electrochemical treatment in which electricity and chemicals are used to treat polluted water. (**ii**) For the removal of dissolved metals from wastewaters mostly chemical precipitation is used. The metal ions normally converted into insoluble particles through chemical reaction between the precipitating reagent and soluble metal compounds. These insoluble metal ions removed from solution through filtration and/or settling. (**iii**) Ion-exchange processes are reversible chemical reactions for removing dissolved ions from water and replacing them with other similarly charged metal ions. It is primarily used in water treatment for softening where magnesium and calcium ions are removed from water (**iv**) Reverse osmosis in which cellophane-like membranes are used to separate purified water from contaminated water. In reverse osmosis on the concentrated side of the membrane a pressure is applied, on the dilute side purified water move, on the concentrated side the rejected water washed away the remaining impurities. (**v**) Phytoremediation technique used to remove of metal ions through different plants. (**vi**) Oxidation and reduction convert toxic forms of metals into non-toxic forms or soluble forms of metals converted into non-soluble forms.

There are many disadvantages of conventional methods e.g.,

(i) They are not eco-friendly.

(ii) High maintenance.

(iii) During secondary treatment they produce pollutants.

(iv) They cannot remove dissolve gaseous pollutants like reverse osmosis.

So there is need of low cost, innovative and eco-friendly methods.

Non-conventional methods include removing cadmium by using microorganisms. In this context, environmental reclamation through microbes has been a promising aspect. Many strategies used by microorganisms to adjust with cadmium-stress like cadmium accumulation, enzymatic detoxification, active efflux of cadmium and cadmium ions sequestration. The use of different cadmium resistant bacteria has raised high hopes for cost-effective and eco-friendly methods toward remediation of cadmium from industrial wastewater.

2.5 Removal of Cadmium by Using Bacteria

The risk of accumulation of cadmium in the environment as a result of industrial activity has led to a growing need to find solution to clean up the environment and remove poisonous effects from all life forms on the ground.

Pardo *et al.* (2003) characterized the *Pseudomonas putida* which showed more than 80% cadmium removal in less than 5 min contact time at pH values ranging from 5.0 to 7.5.

Mahvi and Diels (2004) recorded cadmium removal upto 90% at pilot-plant scale by using Based on polysulfone, *Alcaligenes eutrophus CH34* was grown on a composite membrane. The membrane was casted on a polyester support. The outer side of biological membrane was in contact with cadmium containing wastewater

about 120 mg/L and inside of membrane was in continuous contact with nutrients. The cadmium removal could be recovered from the recuperation column by acid treatment without damaging the bacteria.

Chovanová *et al.* (2004) isolated eight cadmium-resistant bacteria from cadmium contaminated sludge sewage, were characterized by biochemical tests and physiological terms. After biochemical characterizations, out of 8 bacterial strains, 6 strains were closely related to *Serratia liquefacien, Comamonas testosterone, Pseudomonas fluorescens, Alcaligenes xylosoxidans, Pseudomonas putid, Klebsiella planticola*. All strains were able to remove cadmium but removal efficiency depends upon the production of induced proteins in the cells. The plasmid analysis showed that only two strain of *K. planticola* contain plasmids.

Malik (2004) isolated three species of *Pseudomonas aeruginosa* from active sludge of a food factory in the city of Kerman. Out of three species, only one species was able to grow on Muller-Hinton agar medium containing 6 mM of cadmium ions. Therefore this species was selected for further study. This isolate were mutated by using 2 mutagenic agents (Acriflavine and Acridine Orange) using gradient plate and sub-inhibitory concentration techniques. The minimum inhibitory concentration of selected strains against cadmium was increased up to 7mM after mutation. Removal of cadmium was observed in wild type and mutated strains of this bacterium at different time intervals (10-300 min). The 94.7% of cadmium was removed in 30 mg/L of cadmium solution in 60 min. However, in 60 mg/L of cadmium solution, only 53.58% and 38.68% cadmium removal was observed in mutated and wild type bacteria, respectively.

Selatnia *et al.* (2004) studied dead biomass of *Streptomyces rimosus* treated with 0.1 M NaOH, was an efficient adsorbent of cadmium in dilute solutions. Up to 63.3 mg of cadmium can be fixed by each gram of NaOH-treated biomass. The cell wall of *Streptomyces rimosus* consist anionic groups such as amino, carboxyl, amide, hydroxyl and phosphate whose cadmium adsorbent ability is high. Adsorption was also depends on various parameters such as initial cadmium concentration, initial pH, stirring speed and biomass concentration. Based on the results, it may be concluded that this method for removal of cadmium was better than conventional methods.

Zouboulis *et al.* (2004) removed cadmium from water using *Baccilus licheniformis and Baccilus laterosporus* because microorganisms normally use biosorption for the removal of toxic metals from streams and waters. The 60 ºC did not affect the removal of cadmium by *Baccilus licheniformis* and *Baccilus laterosporus.* The maximum cadmium removal capacities of the non-living cells of *Baccilus licheniformis* and *Baccilus laterosporus* were 142.7 and 159.5 mg/g, respectively. Yilmaz and Ensari (2005) checked cadmium removal capacity of *Bacillus circulans* EB1 about 6.7 mg cadmium/g biomass.

Lu *et al.* (2006) removed cadmium by using *Enterobacter* sp. J1, which exhibited good cadmium uptake capacity and high resistance against cadmium. The biosorbent was able to adsorb cadmium with a capacity of 46.2 mg/g dry cell. In this study a new kinetic model was developed which predicts the cadmium biosorption kinetics of *Enterobacter* sp. J1. This strain recovered 90% of cadmium due to adjustment of pH with HCl. The regenerated biosorbent can achieve 75-90% of its original adsorption capacity after repeated adsorption and desorption operations for four times. The results of this study showed that for the removal and recovery of cadmium from industrial wastewater, *Enterobacter* sp. J1 appears an effective

adsorbent because it has advantages of high cadmium uptake capacity, high cadmium tolerance and satisfactory recovery efficiency. Li and Yuan (2006) noted cadmium removal capacity of *Rhodotorula* sp. Y11 was 11.38 mg/g. By using *Pseudomonas pseudoalkaligenes* PTCC 1666, Shirdam *et al.* (2006) removed cadmium up to 40-50%.

Deng *et al.* (2007) studied a cadmium transport system in genetically engineered *Escherichia coli* JM109 and metallothionein due to its cadmium accumulation ability from aqueous solutions. *Escherichia coli* JM109 showed resistance against cadmium and could accumulate cadmium more than one times than original host strain 63.26 mg/g cadmium. Against pH variation *Escherichia coli* JM109 was resistant. Cadmium uptake by strain M4 severely inhibited by Cu^{2+}, Pb^{2+} and Zn^{2+}, whereas cadmium bioaccumulation gently decreased by Ni^{2+} and Mn^{2+}. Ziagova *et al.* (2007) studied the cadmium removal capacity of *Staphlococcus xylosus* and *Pseudomonas* sp. which were 278 and 250 mg/g, respectively. Green-Ruiz *et al.* (2008) recorded the cadmium uptake capaity of *Bacillus jeotgali* U3 was high at pH 7.0 and 35 ºC.

Zeng *et al.* (2009) removed cadmium from indsutrial wastewater by using cadmium resistant *Pseudomonas aeruginosa* strain E1, isolated from wastewater. The size of bacterium became smaller when this bacterial strain incubated in cadmium medium. Both non-living and living bacterial cells of this strain can remove cadmium from solution. Cadmium concentrations in both non-living cell and living cell, decrease by 25.0 mg/L (from 110.2 to 85.2 mg/L) after 24 h incubation, 47.7 mg/L (from 110.2 to 62.5 mg/L), and finally cadmium removal rates reach 22.6% and 43.3%, respectively.

Raja *et al.* (2009) isolated four strains based on high level of antibiotics and cadmium resistances, *Pseudomonas aeruginosa* (BC2), *Proteus vulgaris* (BC1), *Pseudomonas aeruginosa* (BC5), *Acinetobacter radioresistens* (BC3). Theses bacterial strains showed maximum biomass at pH 7.0 and 30 °C. The identified strains were capable to remove the cadmium up to 4-7 mM.

Sinha and Mukherjee (2009) isolated the strain KUCd1 that was closely related to *Pseudomonas aeruginosa* after characterization. They studied the cadmium tolerance capacity, cadmium removal mechanism and cadmium-induced siderophore. This bacterial strain could remove the cadmium by intracellular accumulation and could resist up to 8 mM of cadmium. The cadmium-induced siderophores were observed in this strain at 1.75 mM of cadmium concentration. The *Pseudomonas aeruginosa* strain KUCd1 showed ability to remove more than 75% and 89% of cadmium. Zeid *et al.* (2009) noted that cadmium biosorption capacity of *Chryseomonas luteola* and *Pseudomonas mendocina* were 0.72 and 0.67 mg/g dry wt., respectively.

Pandey *et al.* (2010) isolated a cadmium-resistant bacterium named as CdSP9 from the slag disposal site of IISCO, Burnpur, West Bengal, India. On the basis of molecular phylogenetic approach and phenotypic characteristics the isolate was identified as *Ochrobactrum* sp. It was a short rod (0.5-1.0 μ), Gram negative, aerobic bacterium, growing well at 2-6% NaCl, between pH 6.0-9.0 and temperature ranged 10-42 °C. This bacterial strain removed 0.214 mg/g of the dry weight of cadmium at exponential phase determined by atomic absorption spectroscopy. At optimum growth conditions, the cadmium removal capacity was high.

Sarin and Sarin (2010) studied Immobilized biosurfactant producing *Bacillus subtilis TP8 and Pseudomonas fluorescens* G7 for survival in cadmium contaminated soil and for their ability to remove cadmium contaminated soil. Due to high minimum inhibitory concentrations against cadmium *P. fluorescens* G7 was considered to be a good candidate for bioremediation of cadmium. The immobilized biosurfactant produced by bacteria removed 16.7% of cadmium after incubation for 2 weeks. Patel *et al.* (2010) removed the cadmium from wastewater by using recombinant strain.

Bhatia *et al.* (2011) studied statistical modelling and optimization of substrate composition for bacterial growth and cadmium removal using response surface methodology by *Achromobacter xyloxidans*. The maximum responses were 1.74 mg/mL of protein content, cadmium removal 65.65%, 0.265 for bacterial growth and incubation time 60 h. This model demonstrated 99.83%, 89.85% and 82.57% removal of cadmium ions, protein content and growth of bacterial cells, respectively.

Sabdono (2011) studied cadmium removal by a bioreducpiun coral bacterium *Pseudoalteromonas* sp. strain CD15 isolated from the tissue of coral *goniastrea aspera*, jepara waters and selected 17 cadmium-resistant bacterial symbionts. These 17 bacteria strains showed cadmium removal by range of 68-90%. One of these strains, CD15 was selected further to examine and on the base of molecular and physiological characteristics it closely related to *Pseudoalteromonas* sp. This was the first report on the natural cadmium metal tolerance levels of coral bacteria.

Abd-Alla *et al.* (2012) studied freeze-dried biomass of the cadmium resistant bacteria *Rhizobium leguminosarum* bv. viciae isolated from industrial wastewater in Egypt, identified as a low-cost and effective biosorbent for removal of cadmium

from aqueous solution. The optimum pH for removal of cadmium was 6. When the contact time was 30 min at room temperature, cadmium adsorption maximum capacity was maximum 135.3 mg/g.

Krishna *et al.* (2012) studied bioaccumulation of cadmium by *Pseudomonas* sp. isolated from metal polluted industrial region and out of 164 strains the mostly bacterial strains showed low resistance (<500 μg/mL), while the rest of strains demonstrated high resistance (>1500 μg/mL). 11 bacterial strains isolated from water, 10 bacterial strains were selected from soil and only 5 bacterial strains collected from sediment samples. *Enterobacter*, *Pseudomonas* and *Bacillus* were found in water samples, sediment and soil. According to results, the biomass of *Pseudomonas* sp. was increase with time so cadmium removal rate was also high with time. After 74 h the cadmium rate was reached up to 40% in experimental flask than control flask in which reduction rate was only 5%. Comparatively cadmium removal rate was high at pH 7.

2.6 Mechanisms of cadmium removal by bacteria

The eukaryotic microorganisms detoxify the cadmium as well as other heavy metals by binding with the polythiols. On the other side the bacterium also developed many mechanisms to resist against the heavy metals. These mechanisms are important in such conditions where high concentrations of heavy metals do not have any dangerous effect on cell growth of resistant strains (Bruins *et al.*, 2000). In mostly bacteria genes that involve in the metal resistant present on plasmids that harbor many genes against many metals. Due to this plasmid, bacteria can survive competitively in the presence of heavy metals. According to some findings this metal resistance associated with multiple antibiotics resistance on R plasmid. Normally R plasmid present in the clinically human isolates pathogens like *Klebsiella*

pneumonia, *Pseduomonas aeruginosa*, *Staphylococcus aureus* and others (Cloete, 2003).

In the bacterial resistance to heavy metals, the following mechanisms are involved;

1. Efflux mechanisms.
2. Enzymes and mechanisms that make the bacterial cell wall impermeable to the metals.
3. Enzymes which catalyze the transformation of metals to non-toxic forms.
4. Bindings of metal ions (O'Brien et al, 2002).

2.6.1 Efflux Mechanisms

Many efflux mechanisms have been studied. The well studied efflux mechanism present in *Staphylococcus aureus* (Howden & Grayson, 2006). In these bacteria, there are many systems that are responsible of resistance against cadmium. The cadA system involves in resistance to zinc and cadmium. It can easily be understood at the biochemical level, genetic and molecular level because it codes for an energy-dependent efflux mechanism (Dopson *et al.,* 2003). The cadA gene is present on plasmid pI258 from which a DNA fragment was isolated. Another gene cadB also located on the plasmid that involves in different resistance mechanisms like change in the binding sites. An additional chromosomal based resistant mechanism also present in the *S.aureus*. Like cadA, it involves energy dependent cadmium efflux but confers cadmium resistance only, while cadA confer resistance to both cadmium and zinc (Choudhury & Srivastava, 2001).

2.6.2 Mechanisms and Enzymes that Make the Bacterial Cell Wall Impermeable to the Metals

In the gram positive bacteria, the cadmium enters into the cell as the toxic alternative substrate of manganese transport system while in gram negative bacteria

for zinc transport system. Both systems are coded by chromosome (Lee *et al.*, 2001). This impermeable mechanism best studied in the *Bacillus subtilis* where it linked with the chromosomal mutation so after this mutation the membrane of manganese transport system block the entrance of cadmium (Pereira *et al.*, 2006).

2.6.3 Enzymes which Catalyzes Transformation of Metals to Non-Toxic Forms

Biological conversion of heavy metals is an important mechanism to detoxify the metals that is exhibited by many organisms like fungi and bacteria. As a result of biological action, conversion into organometallic compounds or metals undergoes changes in valency (Ansari & Malik, 2007). After bioconversion, the metal undergoes valency change as a result less toxic and volatile compounds have been produced in many cases, for example the oxidation of arsenite to arsenate and the reduction of mercury ions to metallic mercury. But still it is unknown, if cadmium can be converted to Cd^0 biologically (Schweizer, 2003).

Another important detoxification mechanism is methylation in which metals are converted into organometallic compounds. Lead and mercury are the only two metals that can undergo methylation. Although the products after methylation are more dangerous than free metal form but they are volatile and easily released into the air. As in the case of mercury the methylated compounds are dimethyl mercury and methyl mercury. Although methylated products undergo the chemical and microbial degradation as a result of formation of volatile compounds (Silver & Phung, 2005). Many organocadmium compounds have been synthesized like diorganocadmium is analogous to dimethyl-mercury compounds that have been shown thermolabile and light sensitive. Only one report gives evidence about the biological conversion of cadmium and lead (Abou-Shanab *et al.*, 2007).

2.6.4 Bindings of Metal Ions

The cadmium resistant bacteria have developed mechanisms to bind the cadmium at surface factors or intracellular binding. For example in some bacteria like *Klebsiella aerogenesi* and *Arthobacter viscosus*, cadmium binds to capsular surface, while some bacteria like mutant *citrobacteri* store cadmium inside the cell by binding the cadmium with insoluble cell-bound $CdHPO_4$ (Baker-Austin *et al.*, 2006).

Table 2.1: List of cadmium resistant bacteria isolated from different sources with their removal capacity

Sr no.	Bacterial strains	Cadmium removal capacity (%)	Optimum pH	Optimum temperature (˚C)	References
1.	*Alcaligenes eutrophus CH34*	99%	9	37	(Mahvi & Diels, 2004)
2.	*Alcaligenes xylosoxidans*	60.4%	7.0	37	(Chovanová *et al.*, 2004)
3.	*Alcaligenes xylosoxidans*	65.65%	7.0	35	(Divya & Kumar, 2011)
4.	*Bacillus subtilis* TP8	16.7%	6.8	30	(Sarin & Sarin, 2010)
5.	*Bacillus*	79%	6.9	37	(El-Helow

	thuringiensis				*et al.,* 2000)
6.	Caulobacter crescentus	99%	7.0	30	(Patel *et al.,* 2010)
7.	*Enterococcus faecium*	98.1%	5.0	37	(Valls & Lorenzo, 2002)
8.	*Enterobacter* sp. J1	90%	5.0	37	(Lu *et al.,* 2006)
9.	*Halophilic bacteria*	92.74%	7.2	45	(Massadeh *et al.,* 2005)
10.	*Halomonas* sp.	50%	3.0	25	(Ali *et al*., 2009)
11.	*Pantoea* sp. TEM 18	50%	6.0	25	(Ozdemir et al., 2004)
12.	*Pseudomonas aeruginosa*	94.7%	8.0	42	(Jabbari *et al.,* 2010)
13.	*Pseudomonas aeruginosa* strain KUCd1	75%-89%	-	-	(Sinha and Mukherjee, 2008)
14.	*Pseudomonas aeruginosa*	43.3%	6.5	36	(Zeng *et al.,* 2009)
15.	*Pseudomonas pseudoalkaligenes* PTCC 1666	40-90%	7.0	30	(Shirdam *et al.,* 2006)
	Pseudomonas	99.9%	8.1	30	

	putida				
16.	*Pseudomonas putida*	80%	6.0	37	(Pardo *et al.,* 2003)
17.	*Pseudomonas* sp.	40%	7.0	37	(Bruins *et al.*, 2000)
18.	*Pseudoalteromonas* sp. strain CD15	68-90%	7.0	37	(Sabdono, 2011)
19.	*Pseudomonas veronii* 2E	50%	7.5	32	(Vullo *et al.*, 2008)
20.	*Stenotrophomonas maltophilia*	80%	7.0	37	(Chien *et al.*, 2007)
21.	*Baccilus licheniformis*	62 mg/g	-	-	(Zouboulis *et al.,* 2004)
	Baccilus laterosporus	72.6 mg/g	-	-	
22.	*Bacillus circulans* EB	15.8 mg/g	7.0	-	(Yilmaz & Ensari, 2005)
23.	*Bacillus jeotgali* U3	99.9 mg/g	-	35	(Green-Ruiz *et al.,* 2008)
24.	*Chryseomonas luteola*	0.67 mg/g	7.0	30	(Zeid *et al.,* 2009)

25.	*E. coli* JM109	63.26 mg/g	4.6	-	(Deng *et al.*, 2007)
26.	*Ochrobactrum* sp. CdSP9	0.214 mg/g	6.0-9.0	10–42	(Pandey *et al.*, 2010)
27.	*Pseudomonas* sp.	250 mg/g	7.0	-	(Ziagova *et al.*, 2007
28.	*Rhodobacter sphaeroides*	30-40 mg/g	-	35	(Bai *et al.*, 2008)
29.	*Rhodotorula* sp. Y11	11.38 mg/g	-	-	(Li & Yuan, 2006)
30.	*Rhizobium leguminosarum* bv. viciae	167.5 mg/g	-	-	(Abd-Alla *et al.*, 2012)
31.	*Staphlococcus xylosus*	278 mg/g	7.0	-	(Ziagova *et al.*, 2007)

Alcaligenes xylosoxidans had equal capacity of cadmium removal as shown in Table 2.1, but *Alcaligenes eutrophus CH34* had high cadmium removal capacity than *Alcaligenes xylosoxidans* at same temperature but pH was variable. The Ozdemir *et al.*, (2004) reported that *Pantoea* sp. TEM 18 could remove 50% of cadmium removal but in this study *Pantoea* sp. RL32.2 could remove upto 89.89% of cadmium due to different experimental conditions like pH and temperature. All strains of *pseudomonas aeruginosa* had almost same capacity of cadmium uptake but cadmium removal capacity of *Pseudomonas aeruginosa* described by Zeng *et al.*, (2009) was different because pH was variable but temperature was same in this case.

The *Pseudomonas putida* had slightly different cadmium removal capacities due to slightly difference in pH and temperatures as mentioned by Pardo *et al.*, (2003) and Shirdam *et al.,* (2006). The different bacterial species as shown in Table 2.2 had different cadmium removal capacities at different experimental conditions. At the end it can be suggested that the cadmium removal capacity are based on the bacterial species and experimental conditions.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

For the isolation of cadmium resistant bacteria, industrial wastewater samples were collected from company A and company B. From each factory two wastewater samples were stored in sterilized screw-capped bottles and brought to the microbiology laboratory of Universiti Sains Malaysia. The parameters such temperature, pH, cadmium concentration, latitude and longitude were recorded.

3.2 Preparation of Lauria Bertani Agar

For the preparation of LB agar 10 g casein enzymic hydrolysate, 5 g yeast extract, 10 g NaCl and 15 g agar were mixed i in 1000 mL of distilled water (final pH 7.5 ± 0.2). The mixture in the flasks was well mixed by using magnetic stirrer. The flasks were covered with caps loosly, autoclaved at 15 psi (pound per square inch) and 121°C for 15-20 min (Sheng *et al.,* 2008).

3.3 Preparation of LB Broth

LB broth was prepared by mixing casein enzymic hydrolysate (10 g), yeast extract (5 g) and NaCl (10 g) in 1000 mL of distilled water and final pH of medium was adjusted at 7.5 ± 0.2. These ingredients were well mixed in the distilled water by using magnetic stirrer. The medium was steam sterilized at 15 psi and 121 °C.

3.4 M9 Acetate Minimal Media

The M9 acetate minimal medium was prepared by mixing, 0.2 g $MgSO_4$, 0.5 g yeast extract, 0.001 g $FeSO_4$, 5.0 g sodium acetate, 0.001 g $CaCl_2$, 1.0 g NH_4Cl and