INCREASE OF EFFICIENCY OF PHYTOREMEDIATION FOR HEAVY METAL REMOVAL BY PLANT GROWTH-PROMOTING RHIZOBACTERIA

Ekkasit Aksorn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

Academic Year 2010

การเพิ่มประสิทธิภาพของพฤกษบำบัดในการกำจัดโลหะหนักโดยใช้ไรโซแบคทีเรียที่ส่งเสริมการเจริญของพืช

นายเอกสิทธิ์ อักษร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2553

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เอกสิทธิ์ อักษร : การเพิ่มประสิทธิภาพของพฤกษบำบัดในการกำจัดโลหะหนักโดยใช้ไรโซแบคทีเรียที่ส่งเสริมการเจริญของพืช (INCREASE OF EFFICIENCY OF PHYTOREMEDIA- TION FOR HEAVY METAL REMOVAL BY PLANT GROWTH-PROMOTING RHIZOBACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เบ็ญจมาศ จิตรสมบูรณ์, 180 หน้า.

แบคทีเรียกลุ่มส่งเสริมการเจริญของพืช (plant growth promoting rhizobacteria; PGPR) 6 สายพันธุ์ ที่คัดแยกจากดินบริเวณที่ปนเปื้อนด้วยแคดเมียมสูงจากจังหวัดตากในประเทศไทย คือ 1RER2 25R463 27RER3 39SHR 53S462 และ 65R471 ถูกนำมาศึกษาผลกระทบของแคดเมียม ตะกั่ว และสังกะสี ต่อความสามารถสร้างสารต่าง ๆ ที่ส่งเสริมการเจริญเติบโตของพืช คือ indole-3-acetic acid (IAA) gibberellins ammonia siderophore การใช้ 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase และความสามารถในการละลายฟอสเฟต ผลการทดลองแสดงว่าสายพันธุ์ 53S462 และ 65R471 มีความสามารถสร้าง IAA และ siderophore ได้สูง แต่การสลายฟอสเฟต และการสร้าง ACC deaminase มีค่าลดลง ในสภาวะที่มีโลหะหนัก ($p < 0.05$) จากการทดลองปลูกหญ้าแฝกพันธุ์ราชบุรี ร่วมกับ 53S462 และ 65R471 ในดินสังเคราะห์ที่ผสมแคดเมียม 40 มิลลิกรัมต่อกิโลกรัม ตะกั่ว 600 มิลลิกรัมต่อกิโลกรัม และสังกะสี 400 มิลลิกรัมต่อกิโลกรัม พบว่า แบคทีเรีย 65R471 ช่วยเพิ่มการสะสมของโลหะในส่วนราก และเพิ่มการสังเคราะห์คลอโรฟิลล์ เมื่อเปรียบเทียบกับ 53S462 และกลุ่มควบคุมที่ไม่มีจุลินทรีย์ ทั้งนี้ อาจสืบเนื่องจาก 65R471 มีประสิทธิภาพสูงในการสร้าง siderophores และ IAA ช่วยละลายแคดเมียม และสังกะสีในดิน ส่งผลให้หญ้าแฝกสามารถทนต่อโลหะหนักได้สูง แบคทีเรีย 65R471 ที่คัดเลือกซึ่งมีคุณสมบัติในการจับโลหะหนัก และส่งเสริมการเจริญเติบโตของพืชได้ จึงน่าจะสามารถนำมาใช้ประโยชน์ร่วมกับหญ้าแฝกในเชิงพฤกษบำบัดเพื่อกำจัดโลหะหนักในดินที่ปนเปื้อนต่อไปในอนาคต

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

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EKKASIT AKSORN : INCREASE OF EFFICIENCY OF PHYTOREMEDIATION FOR HEAVY METAL REMOVAL BY PLANT GROWTH-PROMOTING RHIZOBACTERIA. THESIS ADVISOR : ASST. PROF. BENJAMART CHITSOMBOON, Ph.D. 180 PP.

PLANT GROWTH PROMOTING RHIZOBACTERIA/HEAVY METAL/VETIVER GRASS

Six strains of plant growth-promoting rhizobacteria (PGPR) isolated from heavy metal contaminated sites in Tak Province, Thailand, were 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471. The effects of 0.5 mM Cd, 2.5 mM Pb, and 5.0 mM Zn on the plant growth promoting (PGP) traits namely indole-3-acetic acid (IAA) productions, gibberellins productions (GA_3), ammonia productions, siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase utilization as the sole N source, and insoluble phosphate solubilization were assessed in selected bacteria. The results revealed that 53S462 and 65R471 strains have significantly increased ($p < 0.05$) the IAA and siderophores productions while decreased the ACC deaminase production and phosphate solubilization. In the pot experiment, the metal resistant 53S462 and 65R471 strains were inoculated into vetiver grass ecotype *V. zizanioides* grown in soil spiked with 40 mg/kg Cd, 600 mg/kg Pb, and 400 mg/kg Zn. The results showed that 65R471 increased the heavy metal root uptake and total chlorophyll production of the *V. zizanioides*, when compared to 53S462 strain and uninoculated control. This effect can be attributed to the more efficiency of 65R471 in enhancing siderophores production and IAA production, Cd and Zn solubilization and

hence the more tolerance to heavy metal. Owing to its wide multifarious action, the metal resistant 65R471 might serve as an effective metal sequester and growth-promoting bioinoculant for vetiver in phytoremediating the heavy metal contaminated soil in the future.

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Academic Year 2010

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ACKNOWLEDGEMENTS

The success of this thesis can be attributed to the extensive support and assistance for the National Research Council of Thailand for partial financial support during my study. Laboratory facilities were supported by Suranaree University of Technology, Nakhon Ratchasima, Thailand.

I really thank to my thesis advisor, Asst. Prof. Dr. Benjamart Chitsomboon, who giving me the opportunity, valuable guidance, as well as sacrifice her time throughout my study. I would like to express the special gratitude to my co-advisor, Assoc. Prof. Dr. Neung Teaumroong who giving me a valuable guidance suggestions, Asst. Prof. Dr. Saowanit Tongpim who give me a great opportunity to do some parts of my thesis at Khon Kean University, Khon Kean, Thailand, and Dr Pittayakorn Limthong who supported the vetiver grass.

My sincere thanks are extended to all the staffs of the Land Development Department and the Royal Thai Army Chemical Department for their kindness to vetiver tissue culture supporting.

I really grateful to all the staffs of the Schools of Biology and Microbiology, Suranaree University of Technology, Nakhon Ratchasima, Thailand and special thanks to my best colleagues for their encouragement, friendship and helpfulness.

My heartfelt gratitude goes to my grandfather, grandmother, father and mother for their infinite financial support, understanding and continuously provided me with unconditional love and support.

Ekkasit Aksorn

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LIST OF ABBREVIATIONS

ACC	=	1-aminocyclopropane-1-carboxylic acid
AVG	=	Aminoethoxyvinylglycine
BLAST	=	Basic Local Alignment Search Tool
bp	=	Base pair
°C	=	Degree Celsius
CAS	=	Chrom azural S
CFU	=	colony forming unit
dATP	=	Deoxyadenosine triphosphate
dCTP	=	Deoxycytidine triphosphate
dGTP	=	Deoxyguanosine triphosphate
dNTPs	=	Deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
dTTP	=	Deoxythymidine triphosphate
DFB	=	Desferrioxamine B mesylated
DNA	=	Deoxyribonucleic acid
DW	=	Dry weight
et al.	=	et alia (and others)
GA_3	=	Gibberellic acid
GFAAS	=	Graphite Furnace Atomic Absorption Spectrophotometry
(m, μ) g	=	(milli, micro) Gram
hr	=	Hour

LIST OF ABBREVIATIONS (Continued)

IAA	=	Idole-3-acetic acid (Auxins)
(m, μ)l	=	(milli, micro) Litter
(m, μ)M	=	(milli, micro) Molar
(n, c, m)m	=	(nano, centri, milli) Metre
min	=	Minute
(m, μ)mol	=	(milli, micro) Mole
(m, μ)S/cm	=	(milli, micro) Siemen per centimeter
OD_{600}	=	optimum density at 600 nm
PCR	=	Polymerase chain reaction
rDNA	=	Ribosomal deoxyribonucleic acid
rpm	=	Round per minute
s	=	Second
SEM	=	Scanning Electron Microscopy
sp.	=	Species
TAR	=	Total accumulation rate
TF	=	Transport index
V/V	=	Volume by volume
W/W	=	Weight by weight

CHAPTER I

INTRODUCTION

1.1 Introduction

Cadmium (Cd) contamination is a worldwide environmental concern due to its high persistence in the environment and highly toxic on living organisms through food chain. Long term exposure of Cd contaminated diet causes osteomalacia and proximal tubular renal dysfunction called Itai-Itai disease. The numerous distribution of Cd associated with a byproduct of zinc (Zn) mining and industrial operations. The remediation of heavily heavy metal contaminated soils is complicated since it requires a developing system that can remove or neutralize the metal contamination. The system generally involves excavation and removal of soil to secured landfills, a technology that is expensive and requires site restoration.

Alternative technology of hazardous substance removal should be concerned about cost-effectiveness and eco-friendly techniques. The use of plants to clean hazardous substances from the environmental contamination sites may now be an alternative solution for remediation. Phytoremediation is a term applied to a group of technologies that use plants to reduce, remove, degrade or immobilize xenobiotics from contaminated environment. The advantage of phytoremediation is appealing because it is relatively inexpensive and aesthetically pleasing to the public compared to other remediation strategies. However, plants are confronted with certain limitations including the inappropriate growth conditions at the selected sites, the poor

of plant roots, and the high toxicity of contaminants. These hostile conditions make the plant establishment at the polluted sites difficult and slow.

It has been known that plant exudates in the rhizosphere such as amino acids and sugars, provide an enriched source of energy and nutrients which suitable for bacterial growth. A utilization of plant growth-promoting rhizobacteria (PGPR) technology is based on the ability of bacteria to colonize plant root systems and promote plant growth. Recent studies in this area include a growth promotion of plants for phytoremediation by adding PGPR to stimulate plant growth under various stress conditions. Having a deep and dense root system, vetiver is a good candidate for phytoremedial work. Vetiver has long been recognized for its effectiveness in soil erosion and sediment control, highly tolerance to extreme environmental variations including prolonged drought, flood, submergence, extreme fluctuation of temperature (22-60°C), soil pH (3.0-10.5) and most importantly high resistance to heavy metal toxicities (พีระพัฒน์ ชูกำเนิด, 2544).

The aims of this study were to isolate and characterize Cd-resistant bacteria from Cd contaminated soils, select appropriate PGPR strains which increased plant biomass production and improve the efficiency of plants for Cd, Zn, and lead (Pb) removal from the contaminated soil.

1.2 Research objectives

The objectives of this research were:

1.2.1 To study the effect of various growth parameters on vetiver grass under Cd, Zn, and Pb stress in hydroponic culture.

1.2.2 To identify and characterize PGPR, from Cd-contaminated soil, that can tolerate to toxicity of Cd, Zn, and Pb.

1.2.3 To study the effect of the selected PGPR on promoting vetiver growth and improving the efficiency of heavy metal uptake under pot experiments.

1.2.4 To study the relationship between root colonization of selected PGPR.

1.3 Research hypothesis

The selected PGPR strains from Tak Province, Thailand could increase vetiver grass biomass production and improve the efficiency of vetivers for Cd, Zn, and Pb removal. The capability of selected PGPR in promoting plant growth and enhancing effeciancy of heavy metals uptake of vetivers should help to overcome some major limitations in phytoremediation work.

1.4 Scope and limitation of the research

This thesis focused on the effect of PGPRs on growth promoting and heavy metal (Cd, Pb, and Zn) accumulation in vetiver grass. The PGPRs were selected, identified and characterized from highly Cd polluted soil at Mae Sot, Tak province. The tissue culture of vetiver grass was kindly provided from the Department of Land Development, the Ministry of Agriculture and the Royal Thai Army Chemical Department. In addition, the growth promoting of vetiver grass by PGPR was investigated using pot experiments. The specific ecotype of vetiver grass was selected based on high capability of heavy metal uptake *in vitro*.

1.5 Expected results

1.5.1 Remediation of heavy metal contaminated soil by application of PGPR to enhance plant growth and heavy metal biosorption.

1.5.2 Providing basic information for improving the efficiency of vetiver grass in phytoremediation.

1.5.3 Conservation and increasing the value of PGPR in Thailand.

1.5.4 Cost reduction of remediation by avoiding imported chemicals and/or technologies from foreign countries.

1.5.5 Avoiding the use of hazardous chemicals in the treatment of heavy metal contaminated sites.

CHAPTER II

LITERATURE REVIEW

2.1 Sources, fates and contamination problems of Zn in soil

Heavy metal soil contamination is a major problem for Thai people especially in Tak province. The activity of Zn mining and smelting exacerbate the contamination situation of Zn, Cd, and Pb, especially in the agricultural system. At present, Phatat Pha Daeng sub-district, Mae Sot, Tak Province, Thailand is facing the economic problem due to the excessive amount of Cd, Zn, and Pb residues in crops which negatively affects the villager health and suspends the agricultural production.

2.1.1 Cadmium

Cd is released to the biosphere from both natural and anthropogenic sources, while volcanoes and weathering of rocks are the major natural sources for mobilizations of Cd from the earth's crust which containing about 0.2 mg/kg (WHO, www, 2000), and released to soil and aquatic systems. This process plays a significant role in the global Cd cycle, but rarely results in elevated concentrations in any environmental compartment (Nordic council of ministers, www, 2003). A tremendous anthroprogenic point source of Cd currently being recovered around the world is a by product of Zn smelting and refining because Cd is closely associated with Zn in its similar ionic structures and electronegativities and both are strongly chalcophile.

Cd also has a high affinity for sulfur. It is associated with the Zn and sulfur in concentrates of sphalerite (ZnS), secondary minerals such as $ZnCO_3$ (smithsonite) and related sulfide ore minerals. In addition, Cd is recovered during the beneficiation and refining of some Pb ores and complex copper-Zn ores as a "guest" metal in Pb, copper and Zn mineralization (WHO, 1992; Sanita di Toppi and Gabbrielli, 1999). The percentage of Cd in Zn concentrates varies from mine to mine, ranging from 0.07% to 0.83% with an average of 0.23% (OECD, 1994). It is extracted from the earth's crust by man and brought into mining process, conversed an impure Zn oxide (calcine) into pure Zn sulfate solution. In the leaching tanks, calcine reacts with sulfuric acid, producing impure Zn sulfate solution and insoluble metal residues, including Cd, that settle on the bottom of the tank. In order to more fully understand the dynamics of the Cd in soil, it is important to be able to identify the forms of Cd in soil.

Generally, the free ion Cd^{2+} is more likely to be adsorbed on the surfaces of soil solids than other species, such as neutral or anionic species. The principal species of Cd in the soil solution is Cd^{2+} but the metal can also form the following complex ions: $CdCl^+$, $CdOH^+$, $CdHCO_3^+$, $CdCl_3^-$, $CdCl_4^{2-}$, $Cd(OH)_3^-$, and $Cd(OH)_4^{2-}$ together with organic complexes (Kabata-pendias and Pendias, 1992). Mattigod and Sposito, (1970) postulated that the amount of chemical species of Cd in acid soils decreases in the order as Cd(II), $CdSO_4(0)$, and $CdCl_4^+$. While in alkaline soils the Cd(II), $CdCl^+$, $CdSO_4(0)$, and $CdHCO^+$ are predominated. Cd compounds and their solubility in soil are summarized in Table 1. The dynamic of absorbed Cd in soil solution on the solid phases of soil depends on the pH, the chemical nature of the

metal species, the stability of Cd complex, the binding power of functional groups, the ionic strength of solutions and also competing ions (Table 2) (Pickering, 1980).

As a result of wide-spread use, a very large amount of Cd is released into the environment. About 7,500-29,500 tons of Cd are directed to landfills per year and are deposited in the form of discarded products and production wastes (Nordic council of ministers, www, 2003). The rest of the Cd is released through other various human activities. Cd waste from the industries such as Zn production, phosphate ore implication and bio industrial manure are distributed into streams and mainly end up in soils. Another important source of Cd emission is the production of artificial phosphate fertilizers. Part of the Cd ends up in the soil after the fertilizer is applied on farmland, which causes rising Cd levels in some foods, and the rest of Cd usually ends up in surface waters when waste from fertilizer productions is dumped by production companies.

Cd exposure may occur through occupation, smoking of tobacco, diet, and drinking water. However, food is the main route of exposure for the non-smoking population (Lauwerys, 1994). The classic evident of Cd toxicity in human is itai-itai disease discovered in Japan since 1912. The episode caused by long-term consumption of Cd-contaminated rice through irrigation with Cd-contaminated water from Jinzu River. The crisis stimulates several concerns about the effects of Cd toxicity on human being. Recently, an assessment of Cd and Zn contamination in soil and associated rice field, located downstream of an actively mined zone of Zn mineralization in western Thailand, was undertaken. The results showed that total soil Cd and Zn concentrations in the rice-based agricultural system were ranging from 0.5 to 284 mg/kg and 100 to 8,036 mg/kg, respectively. Cd concentrations of rice grain

samples in 524 fields were in the range of 0.05 to 7.7 mg/kg. Over 90% of the collected rice grain samples contained exceeding Cd concentration over 0.2 mg/kg, the maximum permissible level of Codex Committee on Food Additives and Contaminants (CCFAC). In addition, as a function of demographic group, estimated Weekly Intake (WI) values ranged from 20 to 82 μg Cd per kg Body. This poses a significant public health risk to local communities in Tak province, Thailand (Simmons, Pongsakul, Saiyasitpanich, and Klinphoklap, 2005).

2.1.2 Zinc

In 2001, the world Zn production was 9.24 million tons by China, Canada, and Japan sharing about 22.5%, 7.1%, and 7.0% of world product, respectively (ILZSG, 2002). Total Zn content in soil commonly ranges from 10-300 mg/kg with an average of 50 mg/kg, depending on the composition of the parent rock materials (Lindsay, 1979; Kabata-pendias and Pendias, 1992). The most occurrence of natural sources of Zn are the ZnS minerals, sphalerite and wurtzite, and to a lesser extent minerals such as $ZnCO_3$ (smithsonites), Zn_2SiO_4 (willemite), ZnO (zincite), $ZnSO_4$ (xinkosite), $ZnFe_2O_4$ (franklinite), and $Zn_3(PO_4)_2.4H_2O$ (hopeite) (Lindsay, 1972). However, the major discharge of Zn to soil is anthropogenic source, such as electroplating, smelting and ore processors, drainage from active and inactive mining operations, domestic and industrial sewages, combustion of fossil fuels and solid wastes, road surface runoff, corrosion of Zn alloys and galvanized surfaces, and erosion of agricultural soils (Spear, 1981; Mirenda, 1986). Metalliferous mining activities, agricultural use of sewage sludge and composted material, and the use of agrochemicals such as fertilizers and pesticides are the main pollutant sources. Mining discharge activities usually contain 75-165 μg/l of Zn and sometimes can be up to 882

μg/l for active mining (Buhl and Hamilton, 1990). Similar to other heavy metals, Zn generally deposits in soil.

The understanding of chemical behaviour of Zn in soil helps researchers monitor and appropriately choose remediation strategy for Zn. In nature, Zn content in soil depends on the soil type, for example, 40 mg/kg in acid rocks (granites), 100 mg/kg in basaltic rocks, 80-120 mg/kg in shales and clayey sediments, and 10-30 mg/kg in sandstones, limestones and dolomites (Lindsay, 1991; Kabata-pendias and Pendias, 1992). The total amount of Zn in soil is distributed among the following forms: (i) free ions (Zn^{2+}) and organo-zinc complexes in soil solution, (ii) adsorbed and exchangeable Zn in colloidal fraction of soil, composition of clay particles, humic compounds, Fe and Al hydroxides, and (iii) secondary minerals and insoluble complex in the solid phase of soil. The solubility of Zn in soil is very low. However, Zn chlorate, Zn-chloride, Zn sulfates and nitrates are readily soluble in water, whereas free Zn^{2+} in soil solution when reacted with hydroxides, carbonates, phosphates, sulphides, molybdates and with several other anions, including humates, fulvates and other organic ligands will be precipitated. The solubility of Zn increases at decreasing pH values of soil. At pH below 7.7, Zn^{2+} is the predominant species while $ZnOH^{+}$ is more prevalent above this pH. At pH above 9, the neutral species $Zn(OH)_2$ is predominant (Alloway, 1995). Zn compounds and their solubility in soil are summarized in Table 1 and the absorption characteristics are briefly described in Table 2.1.

In Thailand, Simmons, Pongsakul, Chaney, Saiyasitpanich, Klinphoklap, and Nobuntou (2003) reported that total and extractable concentration of Zn in rice-based agricultural system located nearby mined Zn at mineralized zone ranged from

100 to 8,036 mg/kg and 70 to 470 mg/kg, respectively. In addition to mining discharge and sewage sludge, fertilizers and pesticides may also increase Zn concentration in soils. Zn concentrations in inorganic phosphate fertilizers and in manure ranged from 50-1,450 mg/kg and 15-250 mg/kg, respectively (Kabata-pendias and Pendias, 1992).

However, Zn plays an importance role as an essential trace element for human, but the excessive Zn consumption is also toxic to human health. Ingestion a large dose of Zn in human causes a serious damage in the upper alimentary tract, followed by severe shock symptoms. Moreover, excessive Zn intake interferes with copper metabolism, aggravates marginal copper deficiency (Patterson, Winkelmann, and Perry, 1985) and also impairs immune responses by reducing stimulatory response of lymphocytes as well as chemotaxis and phagocytosis of granulocytes (Zelicoff and Thomas, 1998). Inhalation of Zn oxide fumes, another of Zn exposure, is the main cause of metal fume fever. The symptom is characterized by sore throat, cough, myalgias, malaise, and fever (Merian, Anke, Ihnat, and Stoeppler, 2004). Humans are sensitive to Zn toxicity, adverse effects occur in man at the diet level higher than 80 mg/kg, or at daily intakes more than 2.3 mg/kg body weight.

2.1.3 Lead

Lead (Pb) has long been known as cumulative metabolic poison since continuous exposure to low concentrations of Pb may result in adverse effects. The occurrence of Pb distribution in the environment consists of two main point sources, natural and anthropogenic sources. In natural source, Pb is a comparatively rare metal, with an average abundance in the earth's crust of 16 mg/kg (USEPA, 1979). It is also a major constituent of more than 200 identified minerals, of which only three are

sufficiently abundant to form mineral deposits (USEPA, 1979): galena (PbS), angeleside ($PbSO_4$), and cerusite ($PbCO_3$). Galena, the primary form of Pb in the natural state, is often associated with sphalerite (ZnS), pyrite (FeS_2), chalcopyrite ($CuFeS_2$), and other sulfur salts. Each year, about 3 x 10^6 tons of Pb is mined worldwide (Gerhardsson, 2004). For anthropogenic source, Pb are widely use in several activities. For industrial process, Pb is divided into two groups as inorganic and organic Pb compounds. About 4 million tons of Pb is refined annually worldwide. Domestic Pb consumption is 1.3 million tons annually, of which about half is used in battery manufacture and, until recently, about 20% in the manufacture of gasoline antiknock additives such as tetramethyllead (TML) and tetraethyllead (TEL). New usages of Pb that seem environmentally innocuous include protection shielding against radiation exposure in computers, televisions, certain medical instruments, Pb alloy solders, super conductors, certain ceramics and precision glass products, and in energy generation.

Similar to other metals, soil is a sink for anthropogenic Pb, and there are several noteworthy recognized major sources, namely, mining and smelting activities, manures, sewage sludge usage in agriculture and contamination from vehicle exhausts. When Pb is released into the environment it has a long residence time compared with most other pollutants, due to its persistence in the environment, their low solubility, and relative freedom from microbial degradation. In soil, Pb slowly converts to more insoluble forms such as sulfate, sulfide, oxide, and phosphate salts (Rotkittikhun *et al*., 2006). Three processes act to limit Pb availability to plants from soil: (i) adsorption at mineral surfaces or on hydrous iron oxides; (ii) formation of stable organic complexes; (iii) precipitation of sparingly soluble Pb compounds

(sulfates, phosphates, carbonates) (Gerhardsson, 2004). Pb compounds and factors influencing in soil and soil adsorption characteristics are summarized in Tables 2.1 and 2.2, respectively.

As a consequence of widespread environmental contamination, Pb has been known for centuries to be a cumulative metabolic poison. Continuous exposure to low concentrations of the metal may result in adverse health effects (Nriagu, 1978). Pb is a mutagen and teratogen when absorbed in excessive amounts, has carcinogenic or cocarcinogenic properties, impairs reproduction, liver and thyroid functions, and interferes with resistance to infectious diseases (USEPA, 1979). In the United States, about 9% of the children aged 1 to 5 years have blood Pb levels > 100 µg/l, high enough to produce adverse health effects (Ronis, Gandy, and Badger, 1998). Among non-Hispanic black children in this age group, about 21% had blood Pb levels > 100 µg/l and 4.2% had > 200 µg/l. In some undeveloped Asiatic and eastern European countries, blood Pb concentrations > 1000 µg/l occurs in children living near Pb smelters (Ronis *et al.*, 1998). In Thailand, Rotkittikhun *et al*. (2006) collected soil from Bo Ngam Pb mine area and reported that total and extractable Pb concentrations in surface soil ranged from 325 to 142,400 mg/l and 16 to 47,940 m/kg, respectively. Consequently, the Pb distribution through environmental phases causes drinking water, food, and crop contamination. As a result, people are at particular risk to Pb contaminated diet. Moreover, exposure to Pb may also occur in the workplace, such as Pb smelting and refining industries, steel and iron factories, gasoline stations, and battery manufacturing plants (ATSDR, 1992; 1997).

Table 2.1 Metal compounds and factors influencing soil solubility factors (modified from Lindsay, 1979).

Metal	Likely soil compounds	Factors controlling solubility
Zn	$ZnCl$, $ZnOH$, $ZnNO_3$	All too soluble to be present in soil.
	$Zn(OH)_2$, $ZnSO_4$	Too soluble to be present in soil even at high $[SO_4]$.
	$ZnCO_3$, Zn_2SiO_4	Solubilities controlled by $[CO_2]$ and $[H_4SiO_4]$ respectively. Solubilities of all above minerals decrease 100-fold for each unit increase in pH.
	$ZnFe_2O_4$	Solubility controlled by $[Fe^{3+}]$ in solution, which in turn is controlled by iron minerals such as goethite and maghemite.
	$Zn_3(PO_4)_2.4H_2O$	Solubility controlled by phosphate minerals which control the soil $[H_2PO_4]$ at low and high pH.
Cd	CdO, $CdOH_2$, $CdSO_4$,$CdSiO_3$,	All are too soluble to be present in soil.
	$Cd_3(PO_4)_3$	Solubility controlled by the soil minerals which control the concentration of soil $[H_2PO_4]$: at pH < 6, strengite ($FePO_4.2H_2O$) at pH > 8, tricalcium phosphate, calcite and CO_2 at intermediate pH, TCP, and soil-Ca

Table 2.1 (Continued) Metal compounds and factors influencing soil solubility factors (modified from Lindsay, 1979).

Metal	Likely soil compounds	Factors controlling solubility
Pb	$PbSO_4$, $PBCO_3$, PbO,	All are generally too soluble to be important in soil.
	$PbCO_3.2PbO$	at pH < 6, $PbSO_4$ is stable, at pH > 6, $PbCO_3$ is stable.
	Pb_2SiO_4, $PbSiO_3$, PbO, PbO_2,	Minerals are too soluble to be expected in soils. Solubilities
	Pb_3O_4, Pb phosphates	all increase with decreasing redox.
		All solubilities are controlled by the minerals in soils which control soil $[H_2PO_4]$.
	Pb halides	PbI^+, $PbBr^+$, $PbCl^-$, and PbF^- may all contribute to total soluble Pb in soil, where they are locally important.

Table 2.2 Summary of factors influencing soil adsorption characteristics of trace metals (modified from Ross, 1994).

Metal	Adsorption characteristics
Cd	**Influencing factors:** - Increased pH = increased Cd sorption - Increased CEC = increased Cd sorption - Increased organic matter (OM) = increased Cd sorption - Increased $CaCO_3$ = increased Cd sorption **Competing cations**: Ca^{2+}, Co^{2+}, Cr^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+} can inhibit Cd sorption **Organic Cd complexes:** - Cd-humic acid complexes are less stable than Pb or Cu
Zn	**Influencing factors:** - Increased pH = increased Zn sorption - Increased CEC = increased Zn sorption - Increased clay and soil organic matter = increased Zn sorption **Competing cation:** Ca^{2+} inhibits Zn^{2+} sorption Phosphate enhances Zn sorption on variable charge colloids (Fe/Mn oxides) **Organic Zn complexes:** Soluble Zn-fulvates are important Zn species in the soil solution

Table 2.2 (Continued) Summary of factors influencing soil adsorption characteristics of trace metals (modified from Ross, 1994).

Metal	Adsorption characteristics
Pb	**Influencing factors:** - Increased pH = increased Pb retention, but probably mainly by precipitation as Pb carbonate at high pH or as adsorption of the hydrolysed species: $PbOH^+$ at intermediate pH - Increased CEC = increased Pb sorption at intermediate pHs **Competing cation:** Ca^{2+} inhibits Pb sorption at intermediated pHs **Organic Pb complexes:** - Stronger association of Pb with organic matter at high pHs - Humic and fulvic-Pb complexes more stable at high pH - Soluble Pb-chelates are important Pb species in the soil solution

2.2 Phytoremediation as a solution to heavy metal contamination

The wide use of Cd products and also residues from metal mining activities cause an excessive amount of heavy metal contents in soils. High level of Cd concentration in soils frequently poses the high risk to human and animal health, decreased plant growth and ground cover, and has negative impacts on soil microorganisms. The remediation of heavily contaminated soils is one of the most difficult tasks for environmental engineering. The techniques presently involve excavation and removal of soil to secured landfills, an expensive technology that consumes about $10 and $1000 per cubic meter, requires site restoration and destructs

the soil structure (Cunnngham, Shann, Crowley, and Anderson, 1997). Therefore, the technique is not suitable for cleaning-up large area. Furthermore, heavy metal trapped in contaminated soil waste may merely leach to environment passing problems in future generations. Alternatively, heavy metal contaminated soil may be dealt with phytoremediation, which is the use of plants to remove, destroy, or sequester hazardous substances from the environment (Suthersan, 1999). Phytoremediation offers the benefits of being *in situ*, low cost which estimated to be about $0.05 per cubic meter (Cunningham *et al*., 1997), and environmentally sustainable (McGrath, Shen, and Zhao, 1997; Burd, Dixon, and Glick, 2000).

Phytoremediaton technology can be subdivided into different categories based on their underlying process and applicability. The 6 main groups of phytoremediation applications (Saxena, KrishnaRaj, Dan, Perras, and Vettakkorumakankav, 1999) are summarized in Table 2.3.

Table 2.3 Major groups of phytoremediation and their applications (Schnoor, 2003).

Application	Contaminants	Typical Plants	Performance
1. Phyto-transformation	- Chlorinated aliphatics - Ammunition waste - Nutrients - Herbicides	- Phreatophytic trees (Salix family, poplar, willow, cottonwood) - Grasses (rye, fescue, bermuda grass,sorghum, switchgrass, Reed canary grass) - Legumes (clover, alfalfa, cowpeas)	- Proven for nutrients, in groundwater - Experimental for ammunition wastes
2. Rhizosphere Bioremediation	- Biodegradable organics	- Grasses with fibrous roots (Bermuda, wheat, fescue, rye) - Phenolics releasers (mulberry, apple, osage orange) - Phreatophytic trees	- Proven for organic compounds - Research ongoing for pesticides
3. Phytostabilization	- Metals (Pb, Cd, Zn, arsenic (As), copper (Cu), chromium (Cr), selenium (Se) -Hydrophobic organics	- Phreatophytic trees for hydraulic control - Grasses with fibrous roots for erosion Control	- Proven for soil stabilization and risk reduction

Table 2.3 (Continued) Major groups of phytoremediation and their applications (Schnoor, 2003).

Application	Contaminants	Typical Plants	Performance
4. Phytoextraction	- Metals (Pb, Cd, Zn, Cu)	- Indian mustard (*Brassica juncea*) - Sunflowers (*Helianthus* spp.) - *Thlaspi carulescens*	- Proven for low levels of Pb contamination - Proven for Cd, Zn, Ni (low levels) w/chelate addition
5. Rhizofiltration	- Metals (Pb, Cd, Cu, nickel (Ni), (Zn) - Radionuclides - Hydrophobic organics - Explosives	- Aquatic plants: - Emergents (bullrush, cattail, coontail, pondweed, arrowroot) - Submergents (algae, stonewort, parrot feather, *Hydrilla* spp.)	- Proven technology in constructed wetlands
6. Phytovolatilization	- Se, As, mercury (Hg)	- *Brassica juncea* - Wetlands plants - Phreatophytic trees for groundwater capture	- Pilot and Demonstration scale

2.2.1 Advantages and limitations of phytoremediation

The use of plants to remedy hazardous substances overcomes the limitation of the other technologies such as landfill and chemical sedimentation. Phytoremediation technology shows both multifold direct and indirect advantages. Direct advantages are: i) large scale application, as plants can be grown in large area; ii) growing plants is relatively inexpensive, not required expensive equipments or highly specialized personnels; iii) plants provide aesthetic value to the landscape of contaminated sites; iv) phytoremediation process is environmentally friendly and ecologically safe; v) large scale of phytoremediation can have potential economic returns which would offset the cost of the technology; vi) plants concentrate the contaminants within their tissues, thereby reducing the amount of hazardous waste which required smaller reclamation facilities.

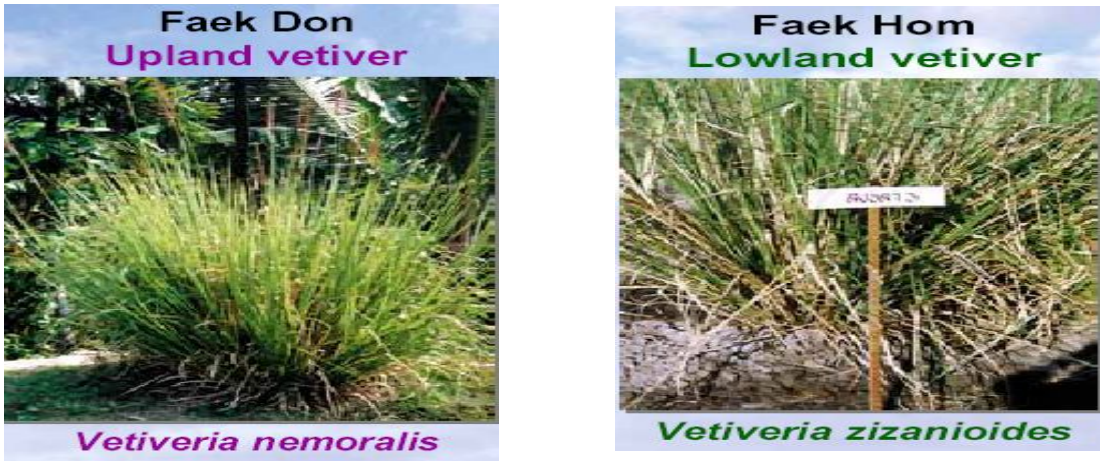
In addition to direct advantages, plants also provide indirect benefits to the contaminated sites and its by-product can be utilized in several prospectives, such as i) increase aeration of soil which in turn enables microbial degradation of substances and microbe–assisted uptake of contaminants and increase soil fertility; ii) reduce top soil erosion due to plant stand; and iii) enhance rhizopheric micro-fauna and flora for maintaining a healthy ecosystem; and iv) utilize the harvested biomass for thermal energy production in specialized facilities; and v) extract the precious metals as bio-ores (Peuke and Rennenberg, 2005).

However, the performances of plants in phytoremediation have several limitations: i) phytoremediation is most effective only at sites with shallow water table and low contamination in the soils; ii) it can be applied only under warm climates and take up several years to remedy; iii) it may not be applicable for highly

hydrophobic contaminants due to the tendency of the contaminants to remain adsorbed to soil particles; iv) the question as to whether the contaminants can accumulate in leaves and be released as litter or accumulate in the wood and mulch has not been resolved; v) the possibility of binding or complexity of some contaminants with the exudates and subsequently transported by groundwater; vi) the evaluation and development of proper handling and disposal methods for the harvested hyperaccumulator plants should be concerned; vii) the feasibility of cost-effective metal recovery techniques should be further evaluated. (Suthersan, 1999; Ghosh and Singh, 2005).

2.2.2 Vetiver grass

Vetiver grass, Faek or Ya Faek in Thai language as shown in Figure 2.1 is a magic plant for environmental protection. It has strong ecological adaptability, large biomass, extensive root system, easy management and very tolerant to extreme soil conditions. Consequently, it has high potential for various applications such as soil erosion control on sloping farmlands and on floodplain, rehabilitation of saline and acid sulphate soils, phytoremediation and biological pest control. According to their different ecological habitats, vetiver grass in Thailand can be taxonomically classified into 2 dominant species namely *Vetiveria nemolaris* as upland vetiver and *Vetiveria zizanioides* as lowland vetiver. The origin of both species and some morphological characteristics showed some major differences (Table 2.4) (Office of the Royal Development, 1997).



(A) (B)

Figure 2.1 (A) Faek Don and (B) Faek Hom (Chomchlow, 1998).

Table 2.4 Comparison of some major differences between *Vetiveria zizanioides* and *Vetiveria nemoralis* (Office of the Royal Development, 1997).

Vetiveria zizanioides	***Vetiveria nemoralis***
Origin	
- In mid Asia, presumably Indis	- Southeast Asia: Thailand, Laos, Cambodia and Vietnam
- Generally used for planting and multiplication	- Widely distributed in natural conditions, hardly cultivated
General morphology	
- Clumpy with long, erect leaves	- Tufted with leaves bending down like lemongrass
- 150-200 cm tall	- 100-150 cm tall

Table 2.4 (Continued) Comparison of some major differences between *Vetiveria zizanioides* and *Vetiveria nemoralis* (Office of the Royal Development, 1997).

Vetiveria zizanioides	***Vetiveria nemoralis***
- Capable of rattooning and aerial branching	- Normally incapable of rattooning and aerial branching
Leaf	
- 45-100 cm long and 0.6-1.2 cm wide	- 35-80 cm long and 0.4-0.8 cm wide
- Dark green, curved upper surface, white lower surface with a septum, texture clearly seen when held against sunlight	- Pale green, upper surface flapped with a triangular ridge, lower surface paler than upper septum not clearly seen when held against sunlight
- Smooth texture, with wax coated giving soft and waxy appearance	- Coarse texture, with thin coated wax rough appearance
Influorescence and Spikelet	
- 150-250 cm long	- 100-150 cm long
- Mostly purple color	- Color varies from creamy white to purple
- Most florets without awn	- Florets with awn
Seeds	
- Relatively large	- Realtively small

Table 2.4 (Continued) Comparison of some major differences between *Vetiveria zizanioides* and *Vetiveria nemoralis* (Office of the Royal Development, 1997).

	Vetiveria zizanioides	***Vetiveria nemoralis***
Roots		
	- Can anchor 100-300 cm deep	- Can anchor 80-100 cm deep
	- Mild fragrance containing 1.4-1.6% volatile oils in dry weight	- No fragrance

Both species of Thai vetiver possess distinct ecotype variation which is used to designate the variant originating in the province from which it was first found. Upland vetiver or Feak Don (*V. nemolaris*) consists of 17 ecotypes. Lowland vetiver or Feak Hom (*V. zizanioides*) consists of 11 ecotypes. Each ecotype differs in their adaptability to soil type. Upland veterver consists of several ecotypes, namely, Kamphaeng Phet 2, Chiang Rai, Songkhla 1, Songkhla 2, Songkhla 3, Surat Thani, Trang 1, Trang 2, Sri Lanka, Mae Hong Son, and Chiang Mai, whereas lowland vetiver consisting of Udon Thani 1, Udon Thani 2, Nakhon Phanom 1, Nakhon Phanom 2, Roi Et, Chaiyaphum, Loei, Saraburi 1, Saraburi 2, Huai Kha Khaeng, Kanchnaburi, Nakhon Sawan, Prachuap Khiri Khan, Ratchaburi, Chantaburi, Phitsanulok, and Kamphaeng Phet 1 (Office of the Royal Development, 1997).

2.2.3 Vetiver as a potential plant for Zn, Cd, and Pb remediation

The concept of soil remediation through plant extraction consists of two processes, phytostabilization and phytoextraction. Implementation of phytostabilization of heavy metal at originated sites involves the reduction in mobility

of heavy metals by minimizing soil erosion, decreasing the potential for wind-blown dust, and reduction in contaminant solubility by addition of soil amendments (Suthersan, 1999). The suitable plants for phytostabilization should be tolerant to high level of metals in soil as well as other extreme environmental conditions such as soil pH, salinity, soil structure, water content, lack of major nutrients, organic material, and poor translocators of metal contaminants to above ground plant tissues that could be consumed by humans or animals. In addition, the plants must grow quickly to establish ground cover, have dense rooting systems and canopies. An approach of phytoextraction or phytoaccumulation depends on two main factors (i) plants must be able to take up and accumulate high amount of metal and (ii) they must be able to produce high biomass. Unfortunately, most of the growth of metal resistant and metal accumulating plants can be severely inhibited when the concentration of available metal in the contaminated soil is very high (Burd, Dixon, and Glick, 2000).

The process of metal accumulation involves several processes consisting of solubilization of metals from soil matrixs, uptaking into roots, transporting to leaves, detoxification or chelation, and sequestration or volatilization as shown in Figure 2.2. The methods of desorbing metals from soil matrix involve acidification of the rhizosphere through the action of plasma membrane proton pumps and the secretion of ligands capable of chelating the metals. After that, soluble metals enter the root endodermal cells or the root apoplast through the space between cells. If the metal is translocated to aerial tissues, then it must enter the xylem where it must cross the Casparian strip, a waxy coating which is impermeable to solutes, unless they pass through the cells of endodermis, probably through the action of a membrane pump or channel. Then the flow of xylem sap will transport the metal to leaves, where it must

be loaded into the cells of leaf and stored or sequestered in various cell compartments such as cell wall, cytosol, and vacuoles. The metals can be converted into less toxic forms through chemical conversion (Peer, Baxter, Richards, Freeman, and Murphy, 2006).

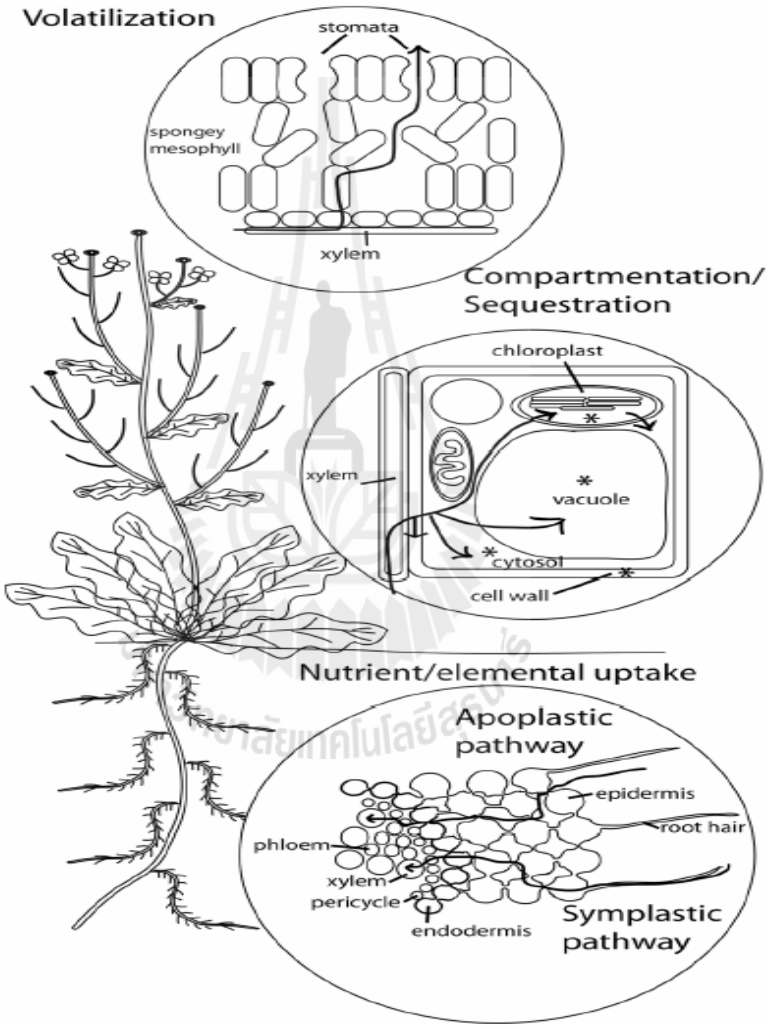


Figure 2.2 Pathways of metal/nutrient uptake in plants.

Vetiver grass may be a suitable plant for soil remediation as it can be used for both phytostabilization and phytoextraction. The most conspicuous characteristics

of vetiver grass for phytostabilization include a high massive, finely structured root system, reaching 3-4 m in the first year, high resistance to pests, diseases, and fire, and tolerance to extreme climatic variations such as extreme temperatures from -15 to 55°C. In addition, it also has high tolerance to acidity, alkalinity, salinity, magnesium (Truong and Baker, 1998), herbicides, pesticides (Pinthong, Impithuksa, and Ramlee, 1998), and several heavy metals such as Al, Mn, As, Cd, Cr, Ni, Pb, Hg, Se, and Zn in the soils (Truong and Baker, 1998; Truong, 1999; Lai and Chen, 2003). Similarly, Yang, Shu, Ye, Lan, and Wong (2003) reported that *V. zizanioides* is used for revegetation of Pb/Zn mine tailings containing high concentrations of Pb, Zn, Cu, and Cd at 4,164, 4,377, 35, and 32 mg/kg, respectively. Most of heavy metals in *V. zizanioides* were accumulated in roots and the translocation of metals from roots to shoots was restricted. Moreover, vetiver shows a very fast growth even after being affected by the above adverse conditions. All of these demonstrate that vetiver is the plant with many phytoremediation properties and has great potential for application phytoremediation of heavy metal contaminated soils and water, Furthermore, vetiver can also be used for phytoextraction because of its large biomass and high efficiency in absorbing dissolved N, P, Hg, Cd, and Pb in polluted water (Pinthong, Impithuksa, and Ramlee, 1998).

At present, there are several studies that investigate the use of vertiver as phytoremediation and phytostabilization for soil remediation. The capability of vetiver to absorp and tolerate heavy metals has been investigated in order to elucidate the mechanism of heavy metal toxicity in vetiver. For example, Meepring (2003) who studied the accumulation ability of different vetiver grass ecotypes, Monsanto, Ratchaburi, and Surat Thani, on As, Cd, and Pb from contaminated soil showed that

all of vertiver grass ecotypes could accumulate heavy metals and the highest heavy metal accumulation was in roots. Chen, Shen, and Li (2004) investigated the uptake and transport for Pb by *V. zizanioides* from high Pb concentrated soil with EDTA added. The results showed that V. zizanioides exhibited lower risk of heavy matal (Pb, Cu, Zn, and Cd) poisoning with EDTA addition.

พีระพัฒน์ ชูกำเนิด (2544) investigated the ability of 5 vetiver ecotypes consisting of Sri Lanka, Monto, Surat Thani, Kumphang Phet 1 and Nakhon Sawan, on the absorption of Pb and Cd. The results showed that *V. namoralis* could uptake and accumulate Pb and Cd better than *V. zizaioides*. Nakhon Sawan and Kumphang Phet 1 exhibited higher absorption of Pb and Cd from soil than Sri Lanka, Monto and Surat Thani.

2.3 Plant growth-promoting rhizobacteria

In nature, soil contains diverse types of microorganism including fungi, actinomycetes, protozoa, algae, and bacteria. Despite large number of bacteria around roots of plants calling rhizosphere, only 7-15% of the total root surface is generally occupied by microbial cells (Pinton, Varanni, and Nannipieri, 2001). Generally, bacterial density at rhizosphere is greater than the rest of soil (Lynch and Brown, 1997) because of the presence of high levels of nutrients especially small molecules such as amino acids, sugars and organic acids that are exuded from the roots of most plants. The exudates can support large and active bacterial populations which may exert beneficial, neutral, or detrimental effects on plant growth and metabolism (Gray and Smith, 2005).

The breakthrough of manipulating rhizosphere microbial populations in 1978 are the inoculation of beneficial bacteria namely plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Special properties of PGPRs include the ability to colonize in developing root system, survive inoculation onto seed, multiply in the spermosphere (region surrounding the seed) in response to seed exudates, and attach to the root surface (Kloepper and Schroth, 1978).

PGPR can be divided into two general types based on association of bacteria and root plant: intracellular PGPR (iPGPR) and extracellular PGPR (ePGPR). iPGPR resides inside plant cells, produces nodules and is localized inside specialized structures. ePGPR is free-living bacteria residing outside the plant cells, does not produe nodules, but enhances plant growth through production of signal compounds that directly stimulate plant growth, improve plant disease resistance, or improve mobilization of soil nutrients (Gray and Smith, 2005). ePGPR consists of three types based on degree of association with plant roots: those living in soil, near but not in contact with the roots, and those colonizing the root surface (rhizoplane), and those living in spaces between cells of the root cortex (Gray and Smith, 2005) (Figure 2). The symbiotic bacteria have been developed as a “biological” means of increasing crop yields (Vance, 1983; Sharma, Kundu, and Dogra, 1993). Beneficial free-living soil bacteria are generally referred to as PGPR and are found in association with the roots of many different plants (Kloepper, Lifshitz, and Zablotowixz, 1989).

As the effect of PGPR on plant growth promotion was reported, the concept of PGPR is wide spread. A large number of bacterial strains have been identified and evaluated for plant growth promotion such as *Pseudomonas*, *Azospirillum*,

Burkholderia, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, and *Flavobacteria* (Rodriguez and Fraga, 1999).

2.4 The mechanism of growth promotion by plant growth-promoting rhizobacteria for growth promoting

The mechanisms of plant growth-promotion may facilitate either indirect or direct process. The indirect mechanisms of plant growth-enhancement involve the decreasing or preventing some deleterious effects of phytopathogens such as antibiotic production, depletion of iron from the rhizosphere, induction of systemic resistance, synthesis of antifungal metabolites, production of fungal-cell wall lysing enzymes and competition for inhabitants on the roots (Weller and Cook, 1986; Glick, Patten, Holguin, and Penrose, 1999; Glick, 2010). On the other hand, the direct mechanisms of plant growth are nitrogen fixation, synthesis of siderophores which solubilize and sequester iron from soil, production of phytohormones such as auxins and cytokinins which enhance plant growth, and solubilization of minerals such as phosphorus.

As previously decribed, some PGPRs contain enzyme 1-amino-cyclopropane-1-carboxylic (ACC) deaminase, a pyridoxal 5-phosphate (PLF)-dependent enzyme that degrades a cyclopropanoid amino acid, ACC, alpha-ketobutyrate, and ammonia. ACC deaminase cleaves plant ethylene precursor ACC, and lower the level of ethylene in a developing or stressed plant. ACC deaminase may act to insure that the ethylene level does not impair root growth, facilitate the formation of longer roots, and enhance seedling survival (Burd, Dixon, and Glick, 2000). ACC deaminase plays an important role in plant growth promotion by reducing the activities of ethylene synthesis. The

plant hormone ethylene, explored by Neljubov since late nineteenth century, is of great importance in plant growth and development (Abeles, Morgan, and Saltveit, 1992). Increased concentration of endogenous ethylene in plants or an enhanced sensitivity of certain plant species, mainly dicots, to ethylene can result in inhibition of seed germination and root growth in stress conditions. Therefore, lower of ethylene level will stimulate plant growth and tolerate environmental stress. PGPRs serve as plant growth enhancer by lowering the ethylene levels within plants (Hall, Peirson, Ghosh, and Glick, 1996).

Several ACC deaminase synthesizer of PGPR strains are identified since 1957 such as *Pseudomonas* sp., *Enterobacter* sp., and *Kluyvera acorbata* (Glick, Patten, Holguin, and Penrose, 1999). ACC deaminase in cytoplasm was homogenously purified from *Pseudomonas* sp. (Glick, Penrose, and Li, 1998) proposed that in some plant ACC is exuded from roots or seeds and then taken up by PGPR and cleaved by ACC deaminase to ammonium and α-ketobutyrate. The ammonium, liberated from ACC can be utilized by bacteria as a nitrogen source. The reduction of external and internal ACC levels could result in decreasing ethylene level and subsequently eliminate the inhibitory effect of ethylene on plant growth as shown in Figure 2.3 (Burd, Dixon, and Glick, 1998).

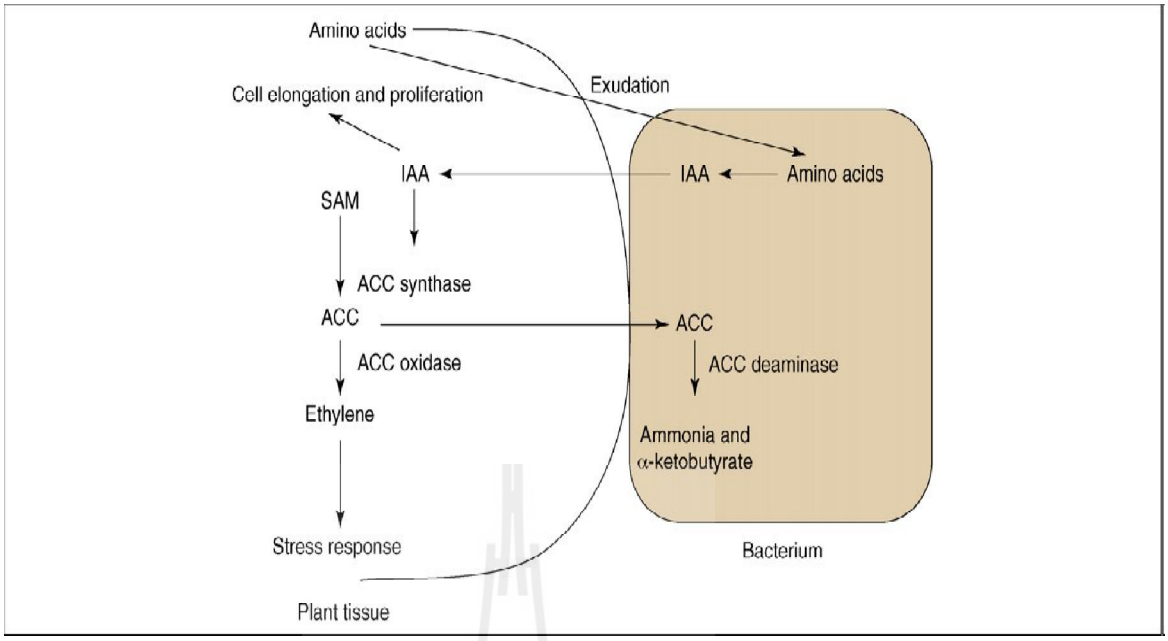


Figure 2.3 Perspectives of bacterial ACC.

The capacity of phytohormone production is another process for plant growth promotion. Plant growth hormones consist of auxin, gibberellin, cytokinin and abscisic acid. Not only higher plants but also bacteria can synthesize these hormones. The naturally occurring auxin or notably indole-3-acetic acid (IAA) has broad physiological effects (Lambrecht, Okon, Broek, and Vanderleyden, 2000), including the regulation of plant growth and development (Thimann, 1935). Auxins have been implicated in cell enlargement, cell division, root initiation, root growth inhibition, increased growth rate, phototropism, geotropism and also apical dominance (Frankenberger and Arshad, 1995). Enormous auxins producing bacteria which found in 80% of rhizosphere are *Azobacter*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Azospirillum*, *Rhizobium*, and *Bradyrhizobium*. These bacteria secrete auxins as endogenous pool for plant growth.

Gibberellins are a group of endogenous phytohormones that enhance plant development processes including seed germination, stem elongation, and induction of physiological responses such as flowering and fruit setting (Hedden and Kamiya, 1997), they also mediators of certain environmental signals e.g. light quality and photoperiod. In 1950s, there was an application of gibberellins acid (GAs) from cultures of fungus *Gibberella fujikuroi* to restore dwarf mutants of pea and maize. To date, only 4 GAs (GA_1, GA_2, GA_3, and GA_4) have been identified in bacteria (MacMillan, 2002) including *Acetobacter diazotropicus* (Bastian *et al.*, 1998), *Azospirillum lipoferum* (Bottini, Fulchieri, Pearce, and Pharis, 1989), *Azospirillum brasilense* (Janzen, Rood, Dormaar, and McGill, 1992), *Bacillus licheniformis* (Gutierri-Manero *et al*., 2001), *Bacillus pumilus* (Gutierri-Manero *et al*., 2001), *Herbospirillum seropedicae* (Bastian *et al*., 1998), and *Rhizobium phaseoli* (Atzhorn, Crozier, Wheeler, and Sandberg, 1988).

Cytokinins are N^6 substituted aminopurines that act as endogenous plant growth regulators (PGR). They are organic substances that influence physiological and developmental processes of plants at very low concentrations, less than 1 μM, and presented in plants as a free base form or a t-RNA constituent. The drastic effects of cytokinin suggest that cytokinin plays a crucial role in controlling cell division and cell-fate in plants. Furthermore, cytokinin has diverse effects on growth and development of intact plants, including axillary bud outgrowth, accumulation of chlorophyll, leaf expansion, delay of senescence, and control of nutrient metabolism (Miller, Skoog, Saltza, Okumura, and Strong, 1955; Mok, and Mok, 1994). Up to date, several cytokinin producing bacteria was identified and characterized in cultures such as *Agrobacterium* sp., *Pseudomonas* sp. (Akiyoshi, Regier, and Gordon, 1987),

Azotobacter, *Azospirillum*, *Rhizobium*, *Bacilllus*, and *Paenibacillu polymyxa* (Nieto and Frankenberger, 1989; Arshard and Frankenberger, 1993; Timmusk, Nicander, Granhall, and Tllberg, 1999). Jacqmard, Houssa, and Bemier (1994) insisted that exogenous supply of cytokinins could stimulate cell division and increase the level of endogenous cytokinins. Arshard and Frankenberger (1993) also reported the influence of rhizophere microorganisms in plant growth and development.

The plant growth promoting factor is a very complex phenomenon resulting from the combination of many mechanisms. Increasing of nutrient solubilization, nitrogen, phosphorus, and iron uptake are known to trigger plant growth. Nitrogen is one of the major nutrients for plant growth. It becomes an integral component of proteins, nucleic acids, and other biomolecules (Bockman, 1997). However, Plants can not directly uptake nitrogen in the atmosphere nitrogen, must be converted into utilizing forms by three processes; i) conversion of atmospheric nitrogen into oxides of nitrogen in the atmosphere; ii) conversion of nitrogen to ammonia and iii) fixation of nitrogen as nitrogenase by microorganisms using a complex enzyme system. The most intensive application of plant associated nitrogen-fixation bacteria is used as biofertilizers for the alternative of inorganic nitrogen fertilizer (Vessey, 2003). The variety of nitrogen fixing bacteria such as *Bacillus fusiformis* (Park *et al*., 2005), *Azospirillum barasilense* Sp-245 (Boddey and Bobereiner, 1988), *Azospirillum lipoferum* CRT1, *Azotobacte*r sp., *Bacillus* sp., *Enterobacter* sp., *Xanthobacte*r sp. (Alam, Cui, Yamagishi, and Ishii, 2001), *Pseudomonas corrugate*, *Azotobacter chrococcum* (Pandey, Durgapal, Joshi, and Palni, 1999), *Pseudomonas fluorescens*, *Pseudomonas tolaasii*, *Pseudomonas veronii*, and *Sphingomonas trueperi* (Adhikari,

2004), have been isolated from various crop rhizospheres which increase plant nitrogen content and grain yield.

Besides nitrogen, phosphorus is also an essential plant mineral fertilizer for plant growth and development, and it composts of 0.2% of plant dry weight. Consequently, the growth of plants is inhibited when lacking of phosphorus supply because phosphorus is a component of several molecules involved in enzyme reactions controlling and regulating metabolic pathways (Theodorou and Plaxton, 1993). Although, phosphorus is abundant in soil at levels of 400-1200 mg/kg but more than 80% of phosphorus becomes immobile and unavailable for plants because of its adsorption, precipitation, or conversion to other organic forms (Holford, 1997). The low solubility of phosphates is a severe limitation on phosphorus availability for plant metabolism. However, some soil microbes can promote phosphorus mobilization either by mineralization of organic phosphates or by solubilization of inorganic phosphates in soil solution by producing acids that make phosphorus forms available for plant uptake (Lifshitz *et al*., 1987).

The mechanism of phosphate solubilization induced by phosphate solubilizing bacteria consists of 2 pathways, the first pathway involves the action of organic acid in releasing phosphate from mineral phosphates (Goldstein, 1994). Examples of secreted organic acids are gluconic acid, glycolic, oxalic, malonic, and succinic acid (Illmer and Schinner, 1992). The other mechanism is organic phosphorus solubilization which strongly depends on several environmental factors, such as alkalinity, physicochemical, and biochemical properties of phosphorus molecules (Paul and Clark, 1988). Organic phosphorus such as nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates, and

phosphonates are decomposed more slowly (Ohtake *et al*., 1996). The mineralization of these compounds is carried out by means of the action of several phosphatases (also called phosphohydrolases). These dephosphorylating reactions involve optimal catalytic activity at acidic to neutral pH values, the hydrolysis of phosphoester or phosphoanhydride bonds. Several researchers have postulated the ability of bacteria, including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, and *Erwinia* to solubilize insoluble inorganic phosphorus compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986; Rodrigues and Fraga, 1999).

Then, PGPR may be appropriated to overcome the problem because some strain of PGPR has the ability to solubilize inorganic and/or organic phosphorus from soil after their inoculation (Kucey, Janzen, and Leggett, 1989). Chabot, Hani, and Cescas (1996) reported that two strains of *Rhizobium leguminosarum*, phosphorus-solubilizing bacteria, could promote root growth and significantly increased the phosphorus concentration in lettuce and maize. In addition, other PGPRs, *Pseudomonas putida* (Lifshitz *et al*., 1987), *Azospirillum lipoferum* strain 34H (Murty and Ladha, 1988), and *Bacillus* sp, have been shown to stimulate phosphorus uptake and promote root and shoot growth in several crops. Co-inocculation of 2 types of PGPR could have synergistic effect on plant growth as suggested by Belimov, Kojemiakov, and Chuvarliyev (1995). They showed that phosphate-solubilizer *Agrobacterium radiobacter* combined with nitrogen fixer *Azospirillum lipoferum* produced the improvement of grain yield of barley compared with single inoculation

of each organism in pots in a field experiment. This was due to the enhancement of nitrogen and phosphorus uptake.

Iron (Fe) is another nutrient for promoting plant growth. It serves as a cofactor of several enzymes which enhance many cellular functions in plants, including chlorophyll biosynthesis, photosynthesis, and respiration (Brittenham, 1994; Miller, Huang, Welkie, and Pushmik, 1995). However, it is not readily available to plants because it is most of presente as insoluble form of Fe^{3+} (Guerinot and Yi, 1994). Therefore, plants have evolved two distinct strategies to take up Fe from the rhizosphere. Strategy I used by all plants except graminaceous plants, involves the release of protons into the rhizosphere to lower the soil pH, the induction of Fe^{3+}-chelate reductase expression to reduce Fe^{3+} to more soluble Fe^{2+} form, and the uptake of the resulting Fe^{2+} form. Strategy II, confined to graminaceous plants, involves the release of mugineic acid family phytosiderophores (MAs) from their roots to form complex with Fe^{3+} and the Fe^{3+}-MAs complexes are taken up via the Fe^{3+}-MAs transporter (Marschner, Romheld, and Kissel, 1986). However, phytosiderophores typically have a lower affinity for iron than siderophores produced by microorganisms. Consequently, in stress condition, plants are unable to accumulate sufficient amonts of iron, unless bacterial siderophores are also present.

Bacterial siderophores is low-molecular mass iron chelators with high association constants for complexing iron. The siderophores are generally form 1:1 complexe with Fe^{3+} and converted to Fe^{2+} in the cell membrane of bacteria. Klepper, Leong, Teintze, and Schroth (1980) demonstrated that the high level of iron translocation into cucumber was induced by microbial siderophores (Ismande, 1998; Wang, Brown, Crowley, and Szaniszlo, 1993). Many different siderophore producing

PGPR could be observed in both Gram-positive and Gram-negative bacteria, such as *Pseudomonas* sp. (Sharma, A., Johri, Sharma, A.K., and Glick, 2003), *Azobacter*, *Bacillus*, *Enterobacter*, *Azospirillum*, and *Rhizobium* (Glick, Patten, Holguin, and Penrose, 1999). The ability of plants to uptake microbial siderophores (Figure 2.4) has been confirmed by using radiolabeled ferric-siderophores as a sole iron source. The results showed that the plants were able to uptake the labeled iron from microbial siderophores (Bar-ness, Chen, Hadar, Marschner, and Romheld, 1991). In addition, Jurkevitch, and Hadar (1992) reported that cotton roots could take up and translocate ferric-pseudobactin to shoot. Siderophores may also help to alleviate the stress on plants. Burd, Dixon, and Glick (1998) proposed that *Kluyvera ascorbata*, a siderophore producing PGPR was able to protect plants form nickel, Pb and Zn toxicity. A siderophore overproducing mutant of *K. ascorbata* could enhance chlorophyll content in three different plants (Canoa, Indian mustard, and tomato) cultivated in nickel contaminated site.

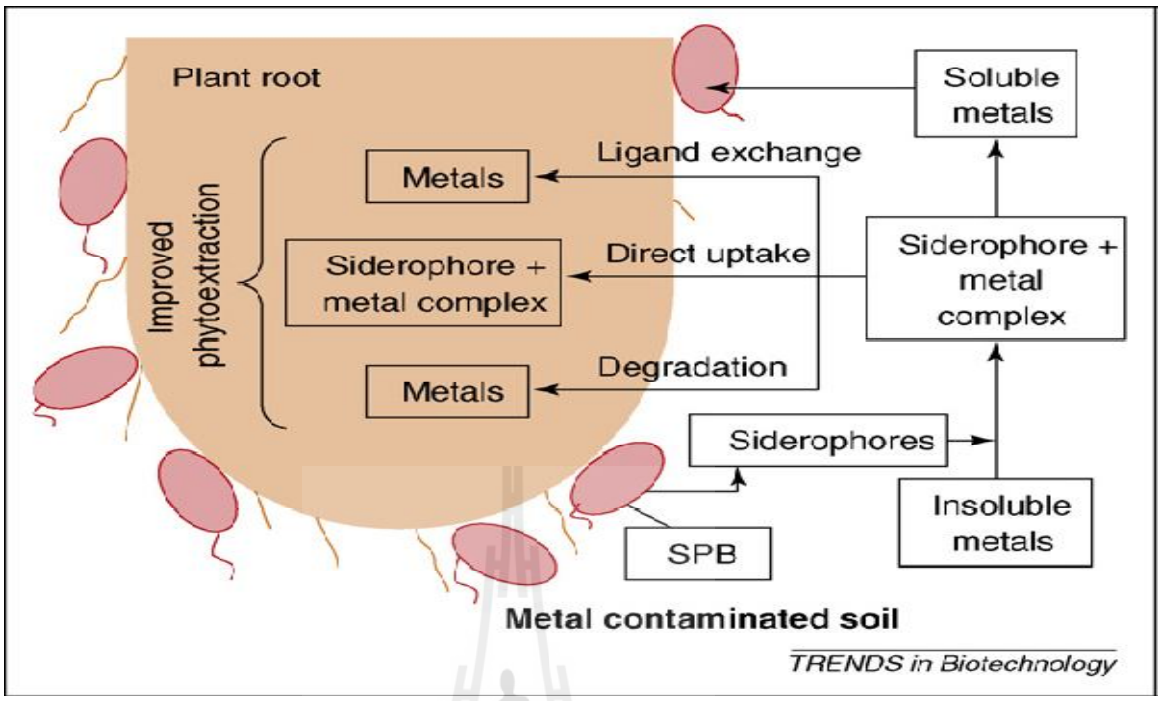


Figure 2.4 Role of heavy metal uptake into plants by bacterial siderophore complexation (Rajkumar *et al*., 2008).

2.5 Plant growth-promoting rhizobacteria for phytoremediation

The efficiency of phytoremediation for removal of heavy metal contamination sites depends on (i) the bioavailability of the target metals and (ii) the ability of various plants to accumulate metals within their aboveground biomass (Raskin and Ensley, 2000). Therefore, the improvement of plant growth under stress conditions is critical for the improvement of phytoremediation. The use of PGPR in phytoremediation is now being considered to play an important role as adding PGPR can aid plant growth on contaminated sites. PGPR may reduce heavy metals toxicity to plants in different ways. demonstrated the beneficial activities of PGPR as list: (i) IAA promotes plant growth, (ii) ACC deaminase prevents inhibitory effect of ethylene on plant growth and (iii) siderophores help plants to acquire sufficient iron in the presence of overwhelming amounts of other metals (potentially competing). The

mechanisms that PGPR used to increase the efficiency of phytoremediation are summarized in Figure 2.5.

The use of ACC deaminase-containing PGPR would be expected to decrease the level of stress ethylene, and promote plant growth in contaminated site. Under metal stress condition, IAA is released and resulte in increased uptake of metal ions as decribed by Wang, Shan, and Wen (2007) who proposed that exogenous IAA significantly increased Pb accumulation in roots.

In addition to ACC deaminase production, bacterial producing siderophores might facilitate the uptake of iron by plants. The low iron uptake into plants grown in the presence of heavy metals could result in chlorotic since iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis (Ismande, 1998). Base on these ideas, the siderophore overproducing bacteria might serve as an iron source for plants that grow under stress of heavy metals and prevent plants from becoming chlorotic by providing a sufficient amount of soluble form of iron to the plants (Suthersan, 1999). However, siderophores can also form stable complexes with other metals such as Al, Cd, Cu, Ga, In, Pb, and Zn.

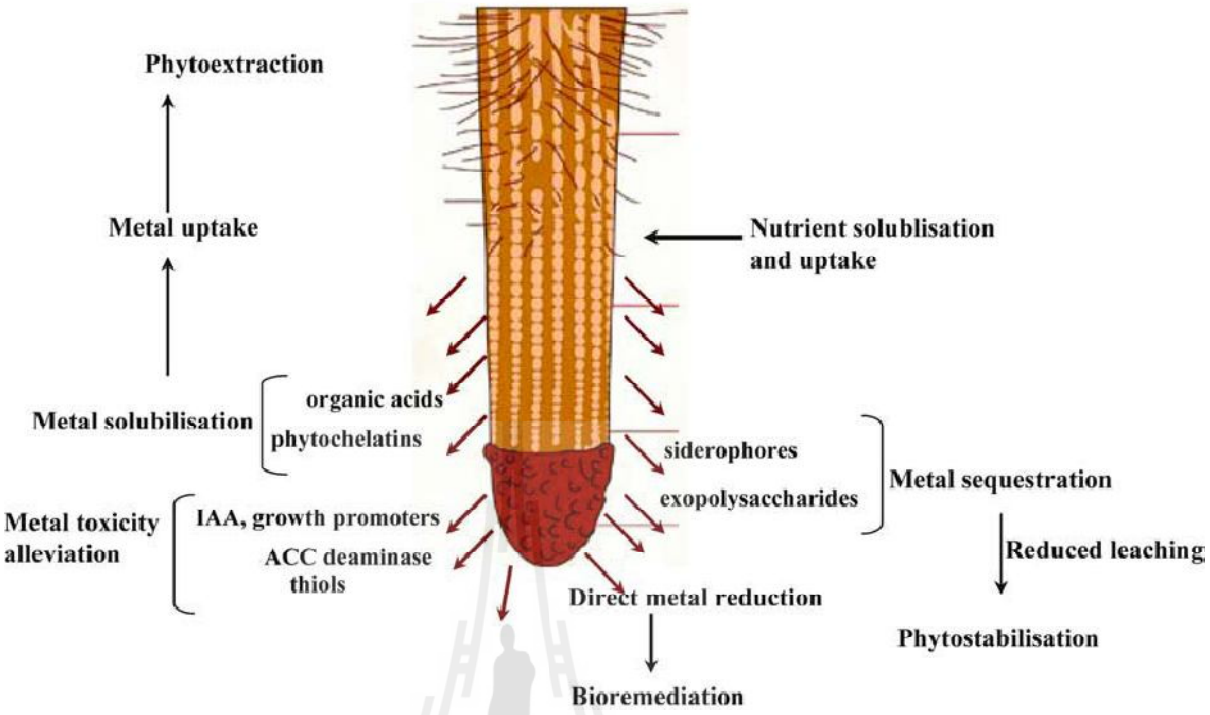


Figure 2.5 The mechanism of PGPR used to enhance phytoremediation (Kamaludeen and Ramasamy, 2008).

A numerous research on the capabilities of PGPR in increasing the effeciency of phytoremediation is summarized in Table 5. In addition, Hoflich and Metz (1997) reported that *Aqrobacterium* sp., *Pseudomonas* sp., and *Stenotrophomas* sp. promote plant growth and increase metal uptake, e.g., Zn, Cd, Pb, copper, nickel, and chromium in maize, rye, and pea. Moreover, rhizosphere bacteria enhanced the increase of Zn, nickel, Cd, and selenium uptake in *Thalaspi caerulescens*, *Alyssum murale*, *Brassica napus*, and *Brassica juncea*, respectively (Sheng and Xia, 2006). In addition, Amico, Cavlaca, and Andreoni (2005) suggested that *Pseudomonas tolaasii* RP23 and *Pseudomonas fluorescens* RS9 are also resistant to Cd and Zn in contaminated soil. *Kluyvera ascorbata* SUD165 has been shown to improve the

growth of canola, tomato, and Indian mustard seedlings treated with toxic concentrations of nickel, Pb, and Zn (Burd, Dixon, and Glick, 2000). However, PGPR may reduce heavy metal uptake and increase plant tolerance in stress condition, for example, *Azospirillum lipoferum* 137 significantly reduced the amount of radiolabelled cesium uptake per gram of plant dry weight (Belimov *et al*., 1998) while *Brevibacillus* significantly reduced Zn accumulated in *Trifolium repens* (Vivas, Biro, Ruiz-Lozano, Barea, and Azcon, 2006). *Kluyvera ascorbata* SUD165 produced a siderophore and displayed 1-aminocyclopropane-1-carboxylic acid deaminase activity which reduce nickel uptake by canola seeds (Burd, Dixon, and Glick, 1998). Notably, it is important to ensure that the bacteria used do not cause a reduction in metal uptake when choosing a PGPR to increase metal uptake by plants for phytoremediation.

Table 2.5 Examples of free-living plant growth-promoting rhizobacteria tested for phytoremediation technologies (Lucy, Reed, and Glick, 2004).

Bacteria	Plant(s)	Contaminant(s)	Conditions	Results of pairing of bacteria and plant
Agrobacterium radiobacter 10 *Arthrobacter mysorens* 7 *Azospirillum lipoferum* 137 *Flavobacterium* sp. L30	Barley	Cd, Pb	Pot experiments in greenhouse	- *Flavobacterium* sp. L30 is very negatively sensitive to Cd - *Flavobacterium* sp. L30 and *A.mysorens* 7 increase grain yield - Enhanced Pb accumulation by plants inoculated with *A. radiobacter* 10 and *A. mysorens* 7 - Significant growth improvement seen in plants inoculated by all strains at higher Cd concentrations
Agrobacterium radiobacter 10 *Arthrobacter mysorens* 7 *Azospirillum lipoferum* 137 *Flavobacterium* sp. L30	Barley	^{134}Cesium	Pot experiments in greenhouse	- *Flavobacterium* sp. L30 increases ^{134}Cs uptake by barley, but not significantly, due to increased plant biomass - *A. lipoferum* 137 significantly decreases the total accumulation of ^{134}Cs
Agrobacterium radiobacter 10 *Arthrobacter mysorens* 7 *Azospirillum lipoferum* 137 *Flavobacterium* sp. L30	Barley	Cd	Pot experiments in growth chamber	- Increased absorption of essential nutrients from contaminated growth medium - Slight stimulation of root length and biomass in contaminated growth medium - *A. lipoferum* 137 increased concentration of Cd in roots, but no change in Cd uptake by plants inoculated with other strains

Table 2.5 (Continued) Examples of free-living plant growth-promoting rhizobacteria tested for phytoremediation technologies (Lucy, Reed, and Glick, 2004).

Bacteria	Plant(s)	Contaminant(s)	Conditions	Results of pairing of bacteria and plant
Kluyvera ascorbata SUD165/26, SUD165	Indian mustard, Canola, Tomato,	Nickel, Pb, Zn	Pot experiments in growth chamber	- Both strains decrease some plant growth inhibition by the metals, but not always significantly - SUD165/26 decreases plant growth inhibition best - No increase of metal uptake with strain over noninoculated plants
Enterobacter cancerogenes *Microbacterium saperdae* *Pseudomonas monteilii*	*Thlaspi caerulescens* *Thlaspi arvense*	Zn	Pot experiments in growth chamber	- *T. caerulescens* has two-fold increase of Zn concentration in roots after inoculation and four fold increase of Zn accumulation in shoots - *T. caerculescens* has higher shoot biomass with inoculation - *T. arvense* has no increased growth or metal accumulation with inoculation
Enterobacter cloacae CAL2	Canola	Arsenate	Pot experiments in growth chamber	- Slight inhibitory effect of CAL2 on germination of canola in presence of arsenate - Partnered with transgenic plants, bacteria induced significantly higher root and shoot weights in plants

Table 2.5 (Continued) Examples of free-living plant growth-promoting rhizobacteria tested for phytoremediation technologies (Lucy, Reed, and Glick, 2004).

Bacteria	Plant	Contaminant(s)	Conditions	Results of pairing of bacteria and plant
Kluyvera ascorbata SUD165	Canola, Tomato	Nickel	Pot experiments in growth chamber	- For tomato and canola, both roots and shoots protected from toxicity with inoculation - Significant decrease in ethylene production by plants - No increase or change in nickel uptake in plant material with inoculation

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1 Chemicals and reagents for microbial growth and analysis

The microbiological media preparations were based on conventional methods, and given in Appendix A. The chemicals and reagents used for identification of bacterial isolates were Gram staining reagents, nitrate test reagents, Kovács' reagent, 1% solution of dimethyl-p-phenylenediamine dihydrochloride, 3% hydrogen peroxide, Methyl red test reagent, and Voges-Proskauer reagents. The preparation of these reagents was given in section 1, Appendix A.

3.1.2 Reagents for plant growth promoting traits

The media for bacterial growth in heavy metal stress condition were typticase soy broth (TSB), DF salts minimal medium, CAS (Chrome Azural S) agar, and MM9 medium. The plant growth promoting properties were determined by the ability of bacteria to produce tryptophan, IAA, gibberellins, ACC deaminase, tri-calcium phosphate, 2,4 dihydroxyphenylalanin, and hydroxylamine HCl. All the chemicals and reagents involved were shown in section 2, Appendix A.

3.1.3 Reagents for nucleic acid analysis

The reagent preparations for nucleic acids analysis are explained in section 3.11. The reagents used for amplification of 16S rDNA were PCR buffer without $MgCl_2$ (100 mM tris-HCl, pH 8.3; 500 mM KCl), $MgCl_2$ solution (25 mM

$MgCl_2$ in sterile water), dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), primers (fD1 and rP2), and *Taq* DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems, U.S.A.).

3.2 Plant growth traits on enhancing of vetiver grasses growth under heavy metal stress

Tissue culture of vetiver grasses both *V. zizanioides* (Ratchaburi ecotype) and *V. nemoralis* (Suratthani ecotype) which grown in Musashige and Skoog agar medium were transferred to a sterile 30 cm length glass tube as described in Figure 3.1. The glass tube contained 0.7% agar medium of Musashige and Skoog, supplemented with different heavy metals as follows: 20 mg/l Cd, 500 mg/l Zn, or 500 mg/l Pb. Each supplemented heavy metal was spiked with different constituents, consisting of 30 μM desferrioxamine B mesylated (DFB), 30 μM pyrocatechol, 2.8 μM kinetin riboside (zeatin), 3 μM gibberellic acid (GA_3), 5 μM IAA, 10 μM aminoethoxyvinylglycine (AVG), 9.0 mM KNO_3, and 1.5 mM KH_2PO_4. The tube were closed with sterile cotton and kept in a growth chamber at 23-28^oC for 7 days. Each treatment and blank in all experiments was tested in triplicates. At the end of each experiment, plants were weighed for biomass determination, and then heavy metal concentration was determined as described by Farwell *et al.* (2006). Briefly, plants were extensively rinsed with several changes of 0.01 M EDTA and followed by distilled water to remove some non-specifically bound heavy metal. Afterwards, the separated shoot and root of grass were dried in hot air oven at 65^oC for 1 day and were finely cut. The dried plant samples were digested with 0.8 ml of purified HNO_3

in incubators at 65°C for 3 h. The supernatants were determined for heavy metals by Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS).

The absorption of heavy metal from the medium by plants expressed as total accumulation rate (TAR) and transport index (TF), were calculated by the following formula as described by Solıs-Domınguez, Gonzalez-Chavez, Carrillo-Gonzalez, and Rodrıguez-Vazquez (2007):

$$TAR = \frac{(\text{Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{((\text{Shoot dry weight (g)+Root dry weight (g))x days of harvest})}$$

$$TF = \left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$$

HM conc. = heavy metal concentration

Figure 3.1 Aseptic vetiver growth system.

3.3 Sampling sites of soil and rhizosphere bacteria

The study area, Phadaeng Industry Company Limited, is located in Moo 4 and 8, Pha Taad Phadaeng Subdistrict, Mae-Sod District, Tak Province, the northwestern

part of Thailand. It covered around 40 hectares (250 rais). The company has high capacity for Zinc smelting process. The sampling sites of soil and rhizosphere bacteria were collected at different sites which divided into 5 plots according to the difference in ecological systems as described in Table 3.1.

Table 3.1 Details of 5 sampling plots at Phadaeng Industrial Company Limited.

Sampling site code	Source	Description
SP	Sedimentation pond supported water was drained from mining activity	Sediment
46 and 47	Re-vegetation area in year 2003 and 2004, respectively	Soil
ER	Mining area	Soil
LR	Low zinc ore and contaminated with lead ore	Soil
HR	High zinc ore is used for zinc smelting process	Soil

3.4 Sampling of soil and rhizosphere bacteria

Soil and rhizosphere bacteria were completely sampled from 5 different sites at Phadaeng Industry Company Limited, Tak Province in April 2007. The 1 kilogram of soil sample was collected at 10 cm depths by a sterile spatula, and stored moist in sterile plastic bags in darkness. About 10 g of rhizophere soil was collected carefully from the different hot spots of cadmium contaminated sites as described in Table 3.1.

The plants were carefully uprooted and removed non-rhizophere soil. Plants were gentle shaken whereas the residual soil was referred to as rhizophere soil. Then, the separated rhizophere soil and non-rhizophere soil were kept in polyethylene bags, stored in cool box at 4°C. The samples were kept in ice-box at 4°C during transportation and immediately brought to the laboratory at the Instrument Buildings of the Centre for Scientific and Technological Equipment, Suranaree University of Technology, Thailand.

3.5 Physical and chemical properties of soil analysis

The soil samples were air-dried, ground and then passed through 2 mm mesh sieve. Then, the physical and chemical properties of soil samples were determined using the methods as summarized in Table 3.2 and the details were shown in appendix B.

Table 3.2 Soil properties determination.

Parameters	Extractable methods	Detectors	Reference
1. pH	Soil:H_2O (1:1)	pH meter	Allen (1989)
2. Soil Texture	Hydrometer	Hydrometer	กรมพัฒนาที่ดิน (2535)
3. EC	Satwration water extract	Electric conductivity meter	Allen (1989)
4. Total N	Semi microkjeldahl	Titration	Allen (1989)
5. Available P	Bray II	UV-spectrophotometry	Bray and Kurtz (1945)
6. Available K	NH_4OAc extraction	GFAAS*	Allen (1989)
7. Total Pb, Cd, and Zn	4 M HNO_2	GFAAS	Liphadzi and Kirkham (2006)
8. Extactable Pb, Cd, and Zn	0.1M $(NH_4)_2SO_4$	GFAAS	Wenzel *et al*., (2001)

* GFAAS = Graphite furnace atomic absorption spectroscopy.

3.6 Isolation of cadmium tolerant rhizobacteria

Depending on PGPR strain, three different media were used to isolate rhizophere and non-rhizophere bacteria. The tryptic soy broth (TSB), Pseudomonads isolation broth (PSB), and Azotobacter basal broth (ABB) were used for general soil bacteria, *Pseudomonads* species and *Azotobacter* species, respectively. After autoclaving, each medium (TSB, PSB, and ABB) containing the antifungal, 100 μg/ml of cycloheximide was added separately to 1 g of homogenized soil and incubated at 30°C for 2 h in a rotary shaker. The suspensions were allowed to stand for about 1 h, and serial 10-fold dilutions of soil suspension were prepared by transferring 0.1 ml supernatant into 0.9 of appropriate medium. 100 μl aliquot of each dilution (10^{-6}-10^{-8}) was spread onto tryptic soy agar (TSA), Pseudomonas isolation agar (PSA) and Azotobacter basal agar (ABA) containing 100 μg/ml cycloheximide and 50 mg/l Cd (Carlot, Giacomini, and Casella, 2002). After incubation for 24 hr at 30°C, each colony with distinct types of bacterial community in high dilution plate was picked for further isolation and purification. The distinct type of bacteria was judged on differences in colony morphology including form, elevation and pigment production. Pure cultures were obtained by a streak plate isolation at least 2 rounds of selection. The pure culture was stored in 15% sterile glycerol at -80°C. The methods for screening PGPR were performed as described in section 3.10.1. Afterward, the selected PGPR strains were quantified as described in section 3.10.2. Phynotypic and genotypic characterization of selected PGPR were conducted as described in section 3.10.3.

3.7 Physiological characterization of plant growth-promoting rhizobacteria

3.7.1 ACC deaminase determination

The evaluation of ACC deaminase activity was based on the ability of PGPR to use ACC as N source and was done as described by Dell'Amico, Cavalca, and Andreoni (2005) and Belimov *et al*., (2005). Briefly, 30 µl of each bacterial culture were transferred into each well of a 96-well microtitre plate, which already contained 150 µl of Dworkin and Foster' s (DF) medium and 3.0 mM ACC as N source or 0.1 mM $MgSO_4$ as control. The cultures were incubated at 30^{o}C for 120 h. The absorbance of the cultures were measured either 0 or 120 hr at 405 nm. Growth was positive when the cultures developed turbidity.

3.7.2 Siderophore production

The assay was performed in 96-well microtitre plates and utilized the ternary complex chrome azurol S/iron (III)/hexadecyltrimethyammonium bromide as an indicator (section 2, Appendix B). Change in the dye color from blue to orange indicated production of siderophore. A loopfull of each bacterial culture was transferred to 3 ml of TSB medium (section 1, Appendix A) and further cultured for 24 h at room temperature with shaking at 150 rpm. 75 µl of each supernatant culture, 75 µl of CAS solution and 30 µl of Mineral salt medium were added to each well of microlitre plate and mixed with gentle tapping. The entire plate was incubated at room temperature for 30 min. An appearance of an orange color in the wells was scored as positive for siderophore production. Two replications of each bacterial strain were used and the experiment was performed at least twice. 30 µl of desferrioxamine mesylate (DFB) were spiked instead of the bacterial culture as the positive control.

3.7.3 Indole-3-acetic acid (IAA) production

Production of IAA by PGPR strains was assayed as described by Patten and Glick (2002). Each of bacterial culture was propagated in TSB medium for 24 h at room temperature. 50 µl of each bacterial inoculum were transferred to 96-well microtitres plates containing 50 µl TSB supplemented with or without 500 µg/ml L-tryptophan obtained from a filter-sterilized 2 mg/ml of stock solution using worm water as solvent. After incubation for 72 h in dark condition, the cultures were completely mixed with 150 µl Salkowski's reagent (Appendix B) and incubated at room temperature for 20 min. The appearance of a pink color in the wells was scored as positive for IAA production. Two replications of each bacterial strain were used and the experiment was performed twice. 100 µl IAA were spiked instead of the bacterial culture as the positive control.

3.7.4 Phosphate solubilization

All isolates were screened on National Botanical Research Institute's phosphate growth medium (NBRIP) agar plates for phosphate solubilization as described by Nautiyal (1999). Briefly, each of bacterial culture was propagated in TSB medium for 24 h at room temperature. Afterward, 10 µl of each bacterial culture were spotted in NBRIP agar for seven strains per plate and incubated at 30^{o}C for 14 days. The halo and colony diameters were measured after incubation. The development of a clear zone around the spot after incubation was an index of phosphate solubilization.

3.7.5 Ammonia production

Bacterial isolates were detected for ammonia production as described by Dey, Bhatt, and Chauhan (2004). 50 µl of all isolates were grown in test tubes

containing 2 ml peptone water. The tubes were further inoculated at 30^{o}C for 4 days. The accumulation of ammonia was detected by adding 0.1 ml Nessler's reagent. Development of a faint yellow color indicated a small amount of ammonia while deep yellow to brownish color indicated maximum production of ammonia.

3.7.6 Cytokinin production

Cytokinin product of each bacterial culture was conducted using OLCHEMIM Enzyme Immonoassay Kits which is based on the principle of competitive antibody binding. Briefly, each of bacterial culture was propagated in TSB medium for 24 h at room temperature. After incubation, 50 µl of each culture were inoculated in 10 ml minimal media as prepared in section 2, Appendix A. The cultures were incubated by shaking at 150 rpm, 30°C for 168 h. The bacterial supernantant were collected and determined for cytokinin production as described by the kit instruction. A competitive binding reaction is set up between a constant amount of alkaline phosphate (tracer), a limited amount of the antibody and the unknown sample containing cytokinin. The color produced after substrate addition was inversely proportional to the amount of cytokinin in the sample. The intensity of the color was read at 405 nm using a 96 ELISA plate reader and related to cytokinin concentrations by means of a zeatin riboside standard provided by the kit.

3.8 Heavy metal resistance and minimum inhibitory concentration

Minimum inhibitory concentration of the selected isolates against increasing concentration of Cd, Pb, and Zn on TSA was evaluated by the method of Raja, Anbazhagan, and Selvam (2006). The mid log phase as 10^{8} CFU/ml of selected bacterial cells were grown in TSB broth and spread all over the surface of TSA using the

three dimension swab technique. The 6 mm sterile paper disks were placed on the inoculated plates using a sterile forcep. Six disks were placed with space evenly on one 100 mm plate, approximately 15 mm from the edge of the plate. Then, each disc was gently pressed down to ensure contact with the medium. The serial dilutions of $CdCl_2$ (0.1, 0.5, 1, 1.5, 2.0, 2.5, 3, 3.5, 4 mM), $Pb(NO)_3$ (1, 3, 5,7, 9, 11, 13, 15 mM) and $ZnSO_2.7H_2O$ (1, 5, 10, 15, 20, 25, 30, 35 mM) were prepared. The 20 µl of various heavy metal concentrations were spotted on sterile paper discs and incubated at 30°C for 24 h. Tests were performed in duplicate. In this study, the MIC was defined as the lowest heavy metal concentration that induced the clear zone at the end of incubation.

3.9 Quantification of plant growth-promoting bacteria attributes in heavy metal stress

3.9.1 ACC deaminase determination

ACC deaminase activity was assayed (Penrose and Glick, 2003) by measuring the amount of alpha-ketobutyrate, the product from the cleavage of ACC by ACC deaminase. The quantity of α-ketobutyrate was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate. A series of standard α-ketobutyrate concentrations (0.01 mM-1mM) was aliquoted of 200 µl, and 300 µl 2,4-dinitrophenyl hydrazine reagent (0.2% 2,4-dinitrophenyl hydrazine in 2 M HCl) was added, and then the content was vortexed and incubated at 30°C for 30 min. The colour was developed by the addition of 2 ml 2 M NaOH and the absorbance readings were taken at 540 nm.

ACC deaminase activity is measured in bacterial extracts prepared in the following manner. The 10^8 CFU/ml of selected bacteria was cultured in TSB. Cultures

were incubated overnight in a shaker at 150 rpm at room temperature. The biomass accumulated was harvested by centrifugation at 4500 rpm for 10 min at 4°C. The supernatant was removed and the cells were washed with 5 ml DF salts minimal medium (Dworkin and Foster, 1958). Following an additional centrifugation for 10 min at 4500 rpm at 4°C, the cells were suspended in 7.5 ml DF salts minimal medium alone or the media with 0.5 mM $CdCl_2$, 2.5 mM $Pb(NO)_3$ or 5 mM $ZnSO_2.7H_2O$. Just prior to incubation, the frozen 0.5 M ACC solution (the solution was filter-sterilized through a 0.2 µm membrane, aliquoted and stored frozen at -20°C) was thawed, and an aliquot of 45 µl was added to the cell suspension to obtain a final ACC concentration of 3 mM. The bacterial cells were returned to the shaker at room temperature for overnight incubation. The bacteria were harvested by centrifugation at 4500 g for 10 min at 4°C. The supernatant was removed and the cells were washed by suspending the cell pellet in 1 ml of 0.1M Tris-HCl, pH 7.6 and transferred to a 1.5 ml microcentrifuge tube. The contents were centrifuged at 12,000 g for 10 min and the supernatant was removed. The pellet was suspended in 600 µl 0.1 M Tris-HCl, pH 8.5. 30 µl of toluene were added to the cell suspension and vortexed at the highest setting for 30 s, and 100 µl aliquot of the toluenized cells is set aside and stored at 4°C for protein assay at a later time. The remaining toluenized cell suspension was aliquoted for assay of ACC deaminase activity. 200 µl microlitres of the toluenized cells were placed in a fresh 1.5 ml microcentrifuge tube. 20 µl of 0.5 M ACC and sterile water (control tube) were added to the suspension, briefly vortexed and further incubated at 30°C for 15 min. Then 1 ml 0.56 M HCl was added the mixture was vortexed and centrifuged for 5 min at 12,000 g at room temperature. After centrifugation, 1ml the supernatant was placed into 800 µl of 0.56 M HCl and the

mixture was vortexed. Subsequently, 300 µl of the 0.2% 2,4-dinitrophenyl hydrazine reagent was added to the glass tube, the contents were vortexed and then incubated for 30 min at 30°C. Following the addition and mixing of 2 ml of 2 N NaOH in the final step, the absorbance was measured at 540 nm.

3.9.2 Protein determination

The protein concentration of cell suspensions was determined by the method of Bradford (1976)(Appendix B). Briefly, 1 ml of protein reagent were added to 100 µl of sample, waited for 2-60 min, and the absorbance was measured at 595 nm. Bovine serum albumin ranging from (0-1600 µg/l) was used to establish a standard curve.

3.9.3 Siderophore production

The selected cultures in TSB broth were incubated at 30°C with shaking (150 rpm) for overnight. Throughout the incubation, 0.1ml of 10^8 CFU/ml of population density, monitored turbimetrically at 600 nm, was inoculated in 10 ml MM9 medium containing with or without as 0.5 mM $CdCl_2$, 2.5 mM $Pb(NO)_3$ or 5 mM $ZnSO_2.7H_2O$. The culture was dispensed in three replicate and grown aerobically in 150 rpm of orbital shaker at 30°C for 24 h. The cells were removed from growth media by centrifugation, and 10 ml volumes of the supernatants were assayed for the presence of iron chelators using hydroxamate- and catechol-type siderophore assays as described in Section 3.9.3.1 and Section 3.9.3.2, respectively.

3.9.3.1 Hydroxymated determination

The presence of hydroxamate compounds in the supernatants was evaluated by colorimetric assays and detected by the ferric perchlorate method (Duhan, Khetarpaul, and Bishnoi, 1998) using deferriferrioxamine mesylate, the

synthetic iron chelator, as standard. Briefly, the culture supernatant was taken in 2 ml aliquots in 10 ml glass tubes and 2 ml sulfanilic acid (1%, w/v, in 30% acetic acid) and 1 ml iodine solution (1.3%, w/v, in 100% acetic acid) were added. The mixture was swirled with every addition and finally kept for 5 min. Excess iodine was removed by adding 2 ml of 2% (w/v) sodium arsenite solution and 2 ml 0.5% N-l-naphthylethylenediamine solution. The reaction mixture was left to stand for complete color development for 30 min, was diluted to 50 ml and absorbance was measured at 500 nm on spectrophotometer (Benchmark Plus).

3.9.3.2 Catecholate determination

The presence of catechol compounds in supernatant was investigated by colorimetric assays. Catechols were detected by Arnow's method. The 2, 3-dihydroxybenzoic acid was used as a standard. Briefly, 1 ml culture filtrate, 0.1 ml 5 mol/l HCl, 0.5 ml ammonium molybdate reagent containing 10 g each $NaNO_2$ and $Na_2MoO_4.2H_2O$ in 50 ml water was added. After the formation of a yellow color at this point, 0.1 ml 10 mol/l NaOH (a red color resulted) and enough distilled water was added to make the volume 5 ml. Absorbance was noted at 515 nm. (Benchmark Plus).

3.9.4 Hormone production

A single colony of the selected bacteria was transferred to 10 ml of TSB medium and grown overnight at 30°C in 150 rpm of orbital shaker until log phase. Afterwards, 0.1 ml innoculum size (10^8 CFU/ml of the selected bacteria) were transferred into 10 ml SMM medium supplemented with or without 500 µg/ml L-tryptophan and contained 0.5 mM $CdCl_2$, 2.5 mM $PbNO_3$ or 5.0 mM $ZnSO_2.7H_2O$.

The media alone was served as blank solution. The culture was dispensed in three replicates and grown aerobically in 150 rpm of orbital shaker at 30°C for 72 h.

3.9.4.1 Gibberellin production

Amount of GA present in the supernatant of the culture without L-tryptophan was determined by the method of Paleg (1965). After incubation, five mililitre of the supernatant was transferred to a test tube containing 0.4 ml 1.0 M zinc acetate. After 2 min, 0.4 ml 1.0 M potassium ferrocyanide was added and then centrifuged at 1000 rpm for 15 min. 1.0 ml of the supernatant was transferred to a new tube, 1.0 ml 30% HCl was added and incubated at 20°C for 75 min. The blank sample was treated with 5% HCl and the absorbance of the samples as well as blank was measured at 254 nm in a spectrophotometer (Jenway 6405 uv/vis. Spectrophotometer). The amount of GA_3 present in the extract was calculated from the standard curve. The standard curve of GA_3 was prepared by analytical grade GA_3.

3.9.4.2 Indole-3-acetic acid (IAA) production

The concentration of IAA was followed by the method of Sarwar and Kremer (1995). 150 µl the culture filtrate were dispensed into wells of 96 well microplates followed by addition of 100 µl Salkowaki reagent, allowed to react for 30 min, and color intensity was measured at 530 nm on a microplated reader. The amount of IAA present in the extract was calculated from the standard curve of analytical grade IAA.

3.9.5 Phosphate solubilization

Quantitative analysis of tri-calcium phosphate solubilization by isolated bacteria was quantified in NBRIP medium. The 1% innoculum of 10^8 CFU/ml bacterial cultures in TSB were transferred to 10 ml NBRIP medium with 0.5% tri-

calcium phosphate, supplemented with vehicle alone or with heavy metal as 0.5 mM $CdCl_2$, 2.5 mM $PbNO_3$ or 5.0 mM $ZnSO_2.7H_2O$. Each treatment was done in triplicates. Cultures were incubated at 30°C for 144 h in a 150 rpm rotary shaker. The culture supernatants were collected by centrifugation at 4500 rpm for 20 min. A soluble phosphate in the culture supernatants was determined spectrophotometry by using the ascorbic acid method (King, 1932). Briefly, 2.5 ml supernatants were mixed with 0.4 ml of combined reagents (details in section 2, Appendix A) and waited for 10-20 min until blue color developed. The absorbance of the developed blue color was read at 880 nm. The amount of soluble phosphorus was determined from the standard curve of KH_2PO_4.

3.10 Characterization of selected PGPRs

The phenotypic characteristics of all selected PGPR bacteria were determined and compared to phenotypic data of known organisms described in the Bergey's Manual of Systematic Bacteriology (Krieg *et al*., 2001), Microbiology: A Laboratory Manual (Cappuccino and Sherman, 1999), and Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The phenotypic features characterized in this study were as follows:

3.10.1 Morphological characteristics

Gram stain reaction, motility, and cell morphology of bacterial isolates were examined as described by Cappuccino and Sherman (1999).

3.10.2 Biochemical characteristics

Varieties of biochemical tests were conducted according to Krieg *et al*. (2001), Cappuccino and Sherman (1999) and Holt *et al*. (1994). The biochemical tests

were as follows: oxidase, catalase, indole, citrate utilization, methyl red, voges proskauer, O-F test, nitrate reduction, lysine, and triple sugar iron (TSI).

3.10.3 Genotypic characterization of bacterial isolates

The identification of selected PGPR strains were confirmed by determination of 16S rRNA gene sequences from cultures. Four major steps for genotypic characterization of selected PGPR isolates were used in this study: genomic DNA extraction, PCR amplification of 16S rDNA, sequencing of PCR amplicon, and 16S rDNA sequence analysis.

3.10.3.1 Extraction of genomic DNA

Genomic DNA extraction from PGPR isolates were performed as described by Rattanasuk and Ketudat-Cairns (2009). Bacterial strains were cultured overnight at 30°C on TSB agar. A single colony was suspended in 50 µl of distilled water. Bacterial cells were lysed by heating at 100°C for 5 min. The lysate was centrifuged at 4,000 rpm for 2 min and 5 µl of the supernatant was directly used for PCR amplification as described below.

3.10.3.2 Amplification of 16S rDNA of bacterial isolates

The amplification of 16S rDNA of selected bacteria was performed as follows: 1) the PCR amplification was conducted in 25 µl reaction containing 2.5 µl genomic DNA, 14.25 µl MilliQ water, 2.5 µl 10× buffer (100 mM Tris-HCl, pH 9.1; 500 mM KCl, and 0.1% Triton^{TM} X-100), 2 µl $MgCl_2$ (25 mM), 0.25 µl dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.25 µl each primer (10 µM/µl) using fD1 and rP2 (Table 3.2), and 1 µl of *Taq* DNA polymerase (*Taq* DNA Polymerase, Vivantis, Poland). 2) the PCR was performed with a ThermoHybaid PX2 (Thermo Scientific, U.S.A.) using the PCR protocol with

an initial denaturation at 94°C for 2 min, followed by 35 cycles with denaturation at 94°C (45 s), annealing at 55°C (45 s) and extension at 72°C (2 min), and a final extension at 72°C for 10 min. 3) The PCR amplified fragments were about 1,500 bp from the 16S rRNA gene and were examined by electrophoresis using a 1% agarose gel. Briefly, 5 µl of PCR-amplified product was thoroughly mixed with 6X loading buffer. The mixture was loaded into the 1% agarose gel, and electrophoresis was carried out at constant 100 volts for 5 min and 60 volts for 40 min. A 1 kbp DNA ladder (Fermentas International Inc., Canada) served as the reference for size estimation of the PCR products. After electrophoresis, the agarose gel was stained with ethidium bromide by soaking the gel in a solution containing 10 µg/ml ethidium bromide, visualized and photographed under UV transilluminator (Bio-imaging systems minibus pro). 4) The PCR products were submitted to Macrogen Inc in Korea for 16S rDNA sequencing.

Table 3.3 Oligonucleotide primers used for PCR amplification and sequencing of 16S rDNA.

Primer	Primer sequence (5' to 3')	Target region[a]	Reference
fD1	AGAGTTTGATCCTGGCTCAG	339-357	Weisburg *et al*. (1991)
rP2	ACGGCTACCTTGTTACGACTT	1392-1406	Weisburg *et al*. (1991)

[a] *Escherichia coli* numbering

3.10.3.3 16S rDNA sequence analysis

Nucleotide sequence data obtained from DNA sequencing software of ABI377 Automated DNA Sequence was interpreted and converted to single letter code in text file format by the Chromas 1.56 program. The sequence was also corrected by manual inspection of the chromatogram. The sequence was compared to local alignment search of the GenBank database using the BLAST (Basic Local Alignment Search Tool) version 2.2.9 program of the National Center for Biotechnological Information (NCBI). (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic tree was inferred using the Maximum Pasimony method with software MEGA version 3.1 (Kumar, Tamura, and Nei, 2004). The stability relationships were evaluated by a boot strap analysis of 1,000 replications.

3.11 Antibiotic sensitivity test

The susceptibility to antimicrobial agents was tested on TSA with antibiotic disks by the method of Bauer *et al*. (1966). The test was only applied to the selected isolates. The commercial 6 different antibiotic disks used were neomycin (30 μg), tetracycline (30 μg), chloramphenical (30 μg), colistin sulphate (10 μg), streptomycin (10 μg) and erythromycin (15 μg). The mid log phase as 10^8 CFU of selected bacterial cells were grown in TSB broth and spread all over the surface of TSA using the three dimension swab technique. The antibiotic disks were placed on the inoculated plates using a sterile forcep. Seven disks were placed evenly on one 100 mm plate with each disk space was approximately 15 mm from the edge of the plate, and one disk was placed in the center of the plate. Each disc was pressed down gently to ensure contact with the medium. The incubated plate was left to dry for a few minutes at room

temperature with the lid closed. Then it was incubated at 30°C for 24 hours or until bacterial growth was observed. At the end of incubation, the diameter of each zone (including the diameter of the disk) was measured and recorded in mm. The sensitivity and resistance profiles were determined based on the diameter of the inhibitory zone and the evaluation was done according to the National Committee for Clinical Laboratory Standard's (NCCLS) chart provided by Himedia antibiotic kit.

3.12 Biosorption of heavy metals by bacteria

The biosorption study was carried out as described by Kanazawa and Mori (1996) with some modifications. Bacteria were grown in 250 ml nutrient broth until reaching one unit of optical density (600 nm). Then cells were harvested by centrifugation at 7000g for 30 min and the bacterial pellets were washed twice with sterile distilled water. The three replicates of harvested cell biomass were incubated for 10 h at 30°C with an individual solution containing either tolerant concentration of 0.5 mM $CdCl_2$, 2.5 mM $PbNO_3$ or 5.0 mM $ZnSO_2.7H_2O$ as determined from the previous experiment. Afterward, the suspension was centrifuged at 10000 rpm for 20 min to separate the biomass from the supernatant. The supernatants were discarded and the pellets were washed two times with sterile water. The pellets were collected, dried, weighed, and heavy metals were extracted with HNO_3 at 65°C for 24 h. The Zn, Cd, and Pb contents were determined on microbial biomass by GFAAS.

3.13 Soil heavy metal availability

Batch studies on the effects of bacteria on the mobility of soil metals were carried out by using 50 ml polypropylene centrifuge tubes. One small part of air-dried

soil sample collected from cadmium contaminated site was autoclaved (121°C, 30 min). The selected bacterial suspension with 10^8 CFU/ml was added to 1.0 g of autoclaved soil in 50 ml centrifuge tubes, and 10 ml sterile water was added to the autoclaved soil as an axenic control. All tubes were weighed, wrapped in brown paper and placed on an orbital shaker at 200 rpm at room temperature. After one week, the tubes were weighed again to compensate for evaporation of water. 10 ml sterile water was added to each tube to extract the soil water-extractable (i.e. labile and available) metals. The soil suspensions were centrifuged at 4000 rpm for 10 min and filtered (Whatman Grade No. 41 Quantitative Filter Paper). The concentrations of Cd, Pb, and Zn in the filtrate were determined by GFAAS.

3.14 Growth curve analysis

For further experiments in section 3.16, a single colony of the selected bacteria was transferred to 10 ml TSB medium and incubated at 30°C until log phase (Garcia de Salamone *et al*., 2001). Afterwards, 1% innoculum size of the selected bacteria were transferred into 10 ml TSB medium containing 0.5 mM $CdCl_2$, 2.5 mM $PbNO_3$ or 5.0 mM $ZnSO_2.7H_2O$ using media alone as the blank solution. Cultures were grown aerobically in 150 rpm of orbital shaker at 30°C at selected time intervals of 0, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 30, and 36 h. Bacteria growth was determined by measuring the absorbance at 600 nm against blank, and pH was also determined. After each time point, 1 ml of each culture was collected for heavy metal determination.

3.15 Effect of the selected PGPR on promoting vetiver growth under heavy metal stress condition in the laboratory

Tissue cultures of vetiver grasses both *V. zizanioides* (Ratchaburi ecotype) and *V. nemoralis* (Surat Thani ecotype), which grown in 10 ml Musashige and Skoog agar medium, were transferred to sterile glass bottles containing 0.7% agar medium of Musashige and Skoog, supplemented with different heavy metals: 0.5 mM $CdCl_2$, 5.0 mM $ZnSO_2.7H_2O$, or 2.5 mM $PbNO_3$. Each heavy metal solution was spiked with 0.1 ml 10^8 CFU/ml of different bacteria cultures and incubated in a growth chamber at 23-28°C for 14 days. The treatments and blanks were performed in triplicates. At the end of each experiment, plants were weighed for biomass determination, and then heavy metal concentration was determined as described by Farwell *et al.* (2006). Briefly, plants were extensively rinsed with several changes of 0.01 M EDTA and followed by distilled water to remove some non-specifically bound heavy metal and root bacterial adherence. Afterward, the separated shoot and root of grass were dried in hot oven at 65°C for 1 day and were finely cut. The dried plant samples were digested with 0.8 ml of purified HNO_3 in incubators at 65°C for 3 h. The supernatants were determined by GFAAS.

The absorption of heavy metal from the medium by plant expressed as TAR and TF was calculated as described by Solıs-Domınguez, Gonzalez-Chavez, Carrillo-Gonzalez, and Rodrıguez-Vazquez (2007).

3.16 The colonized ability of heavy metal tolerated bacteria on vetiver grass

The selected vetiver grass was transferred to test tube containing modified Murashige and Skoog (1962) inoculated with 10^8 CFU/ml of each selected PGPR strain. The colonization of bacteria around the vetiver root and chlorophyll contents were determined after 15 days as following. Briefly, the vetiver grass was carefully up rooted. Then the roots were gently dipped in sterile water to remove the still adhering agar and obtain the rhizoplane population. The grasses were divided into 2 parts as the shoot for chlorophyll content analysis, and the roots for root colonization by colony count method and scanning electron microscopy (SEM). The best effective bacteria were chosen for subsequent study.

For determination of root colonization, roots were placed in a series of about 10 sterile polyethylene centrifuge tubes containing 10 ml sterile water each. After root dipping, each tube was vortexed for 5 min to release the rhizoplane bacteria prior transfer to the next one in the series. To determine colonizing ability, the solution from each tube was spread on TSA dishes containing appropriate anti-fungal antibiotics. The PGPR that found in the higher number of tube was considered as the better colonizer. Colony was counted and expressed as a log number of cells per root weight. Colony morphology and characteristics were also taken into consideration.

Roots were fixed in 2.5% gluteraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature, washed with the same buffer for 1 h at 4°C and dehydrated by passage through increasing acetone concentrations (50, 70, 95, and 100 %) at 4°C. The samples were then dried in a critical-point dryer, affixed to stubs with carbon

cement, coated with gold (30 nm thickness) and examined using a Hitachi S570 SEM at 15 kV.

As described by Inskeep and Bloom (1985), 1 g fine cut shoot were soaked with 1 ml *N,N*-dimethylformamide (DMF) for 48 hr at 4°C in dark. After incubation, the supernatant was read at 647 nm and 664.5 nm in 1.00 cm quart cuvettes on the Beckman model 25 spectrophotometer. The chlorophyll contents were calculated by the following formulars:

Chlorophyll b (Chl b) = $20.70\ A_{647} - 4.62\ A_{664.5}$

Chlorophyll a (Chl a) = $12.70\ A_{664.5} - 2.79\ A_{647}$

Total chlorophyll (Total Chl) = $17.90\ A_{647} + 8.08\ A_{664.5}$

3.17 Effect of the selected PGPR on promoting vetiver growth in heavy metal stress under pot experiment

Soil samples were collected from Suranaree University of Technology, Nakhonratchasima, Thailand. Soils were sieved (2 mm) and sterilized by steaming at 121°C for 1 hr on three consecutive days. After autoclaving, 1 g soil sample was thoroughly mixed with 100 ml sterile water, plated onto the medium and incubated overnight at 30°C for confirmation of sterility. Then the soils were artificially contaminated with sterile Cd (40 mg/kg), Pb (600 mg/kg), and Zn (400 mg/kg) and left in a greenhouse for a 3 week period (for metal stabilization). Before outdoor planting, plastic pots were treated with 10% sodium hypochloride for disinfection. Afterward, 10 sterilized vetiver grasses were placed in each pot at a 2 cm depth. There were three replicates of each treatment and all samples were arranged in a randomized block design in a greenhouse. Soils were maintained at 60% water holding capacity

by regular applications of deionized water to pots per day. 1.0 ml 10^8 CFU/ml/pot bacterial culture of selected strain were spotted on the root and soil surface, whereas the control group received only media. Bacterial inoculated and non-inoculated plants were grown in pots under outdoor conditions for 1 month (Hewitt, 1952). At harvest, the maturity average height, shoot or root biomass, total fresh, dry weight, and chlorophyll contents were recorded. Later, plants were separated into root, straw and grain, extensively washed twice in 0.01 M EDTA and then in distilled water to remove any non-specifically bound heavy metal, and then oven-dried (70°C), weighed and ground. The concentrations of Zn, Cd, and Pb in shoot and root were also determined after wet digestion of the air-dried plant samples with HNO_3 using open tube. Prior to digestion, plant samples were pre-digested overnight at an ambient temperature. Following uprooting, soil in pots were mixed and a subsample (200 g) were removed, air-dried and sieved (< 2 mm) for subsequent chemical analysis. Total heavy metal content of soils was determined after digestion with HNO_3 and $HClO_4$ (1:4 v/v). Then Pb, Cd, and Zn in the diluted solutions were analyzed with GFAAS technique. Extractable heavy metals were determined by tumbling soils at 180 rpm for 4 h in 4 M $(NH_4)_2SO_4$.

Population densities in the rhizosphere of each pot experiments were measured and expressed as a log number of cells/g. Rhizoplane (root surface) were taken for determining the population of the inoculant strains. During sampling, plants in each pot were uprooted along with soil adhering to it. Then, roots were shaken repeatedly to remove the loose adhered soil, namely rhizosphere soil. Thereafter, roots were gently dipped in sterile water to remove the still adhering soil particles and these roots were used for getting the rhizoplane population. The roots were placed in 10 ml sterile

water in sterile polyethylene centrifuge tubes, and vortexed for 5 min to release the rhizoplane bacteria. For PGPR isolates, appropriate dilutions were spread on TSA dishes containing 100 µl/ml of cycloheximide to inhibit the fungal growth. Colonies were counted and expressed as a log number of cells/g. Colony morphology and characteristics were also taken into consideration while counting to avoid counting of the spontaneously growing population.

3.18 Statistical analysis

All chemical experiments were analyzed in triplicate. Statistical analysis was evaluated in Completely Randomized Design (CRD). The data were analyzed with analysis of Variance (ANOVA) using SPSS 16.0. Significant differences at $P < 0.05$ were compared using the Duncan's Multiple Range Test.

CHAPTER IV

RESULTS

The purpose of this study was to investigate the growth promotion effect of plant growth promoting rhizobacteria (PGPR), collected from different sites in April 2007 in Phadaeng Industry Public Company Limited, Tak Province, Thailand. Indigenous PGPR which has high capacity to tolerate cadmium concentration at 50 mg/l was isolated from the soil of research sites based on their plant growth promoting bacteria traits as ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, siderophore production, hormone synthesis, and phosphate solubilization. The selected indigenous PGPR were tested in the greenhouse and the field for their effects on vetiver grasses biomass and heavy metal content in plant tissue.

4.1 The influence of plant growth promoting traits on heavy metal uptake by vetiver grasss

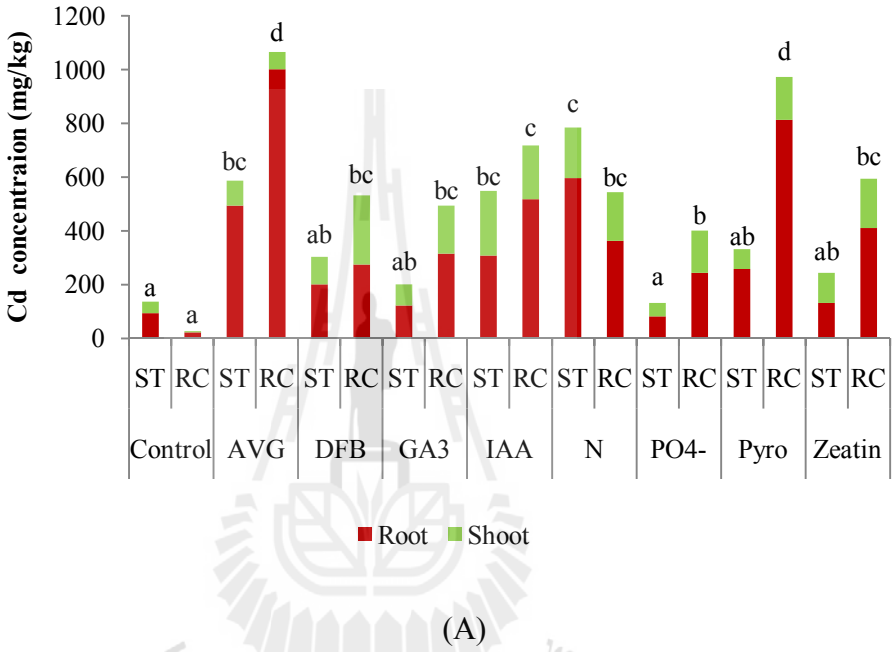
The aims of this experiment were to study the effect of various growth parameter traits on vetiver grass growth, heavy metal uptake and to select the best traits for the subsequent experiment. The plant growth promoting traits, consisted of 30 μM DFB, 30 μM pyrocatechol, 2.8 μM zeatin, 3 μM GA_3, 5 μM IAA, 10 μM AVG, 9.0 mM KNO_3, and 1.5 mM KH_2PO_4, were separately added on Musashige and Skoog agar medium in axenic culture under single concentration of 20 mg/l Cd, 500 mg/l Zn or 500 mg/l Pb. The heavy metal concentration and the percentage change

of fresh weight of two vetiver grasses ecotypes (*V. zizanioides* and *V. nemoralis*) were determined.

Heavy metal determination in the root and shoot parts of *V. zizanioides* (Table 4.1 and Figures 4.1, 4.2, 4.3) revealed that most plant growth promoting traits-tested enhanced the heavy metal uptake when compared to control. Table 4.1 showed that the translocation of Cd from root to shoot was rather low in all investigated traits. This was suggested by higher Cd concentration in root than shoot, especially under the influence of AVG, the Cd accumulation in root was 10 times higher than shoot. The amount of total Cd uptake per day (TAR) by *V. zizanioides* under different PGPR traits were about the same and all were higher than control group. The total Cd accumulations (mg Cd per kg plant dry weight) of Control, AVG, DFB, GA_3, IAA, Nitrogen, Phosphorus, Pyrocatechol, and Zeatin were 26.15, 1,067.06, 533.07, 494.16, 717.67, 545.1, 400.41, 972.88, and 593.33 mg/kg, respectively. The metal TF as capacity of heavy metal translocation from root to shoot has been determined. The results showed that the TF was highest (84.33) when supplemented with DFB. A significant higher Cd concentration in root than shoot was also observed in all evaluated parameter (Table 4.1) and all concentration were hiher than control group.

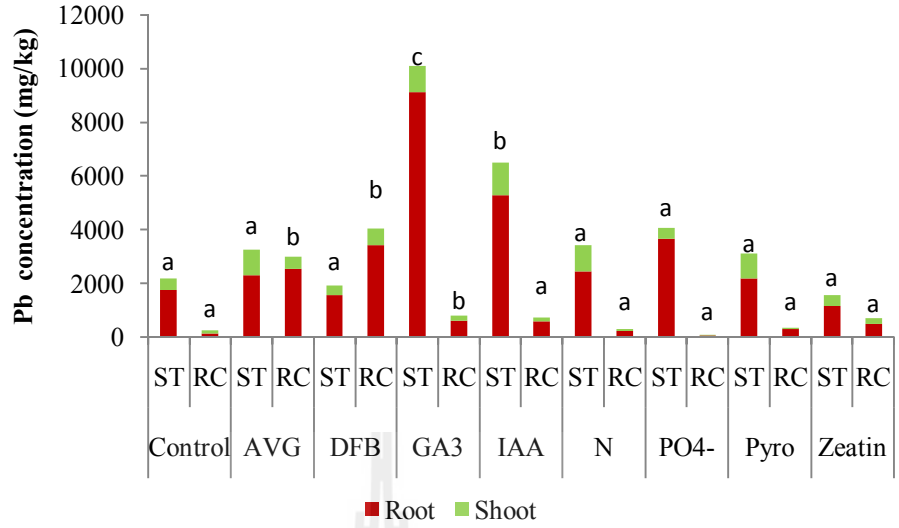
Only *V. zizanioides* grown under AVG and DFM significantly displayed higher Pb in root compared to control (Table 4.2). The total uptake of Pb was in the order of DFB, AVG, GA_3 > IAA, Zeatin, Pyrocatechol, N, control, and P, respectively (Figure 4.1B). TF analysis found no difference between group and each PGP traits in Pb translocation compared to control. The total concentrations of Zn in *V. zizanioides* were in the order of P > DFB, IAA, Pyrocatechol, control, N, AVG, GA3, and Zeatin, respectively (Figure 4.1C). The average maximum TF and TAR was observed in Zn (Table 4.3). All of the tested PGR traits, except P had no influence of Zn translocation

from root to shoot (TF) compared to the control group. Similarly, P also induced the maximum total accumulation of Zn per day (TAR) while the rest of PGR traits had no effect. Notably, the TF and TAR for Zn uptake and accumulation in control or every PGP traits were higher than the other heavy metal.

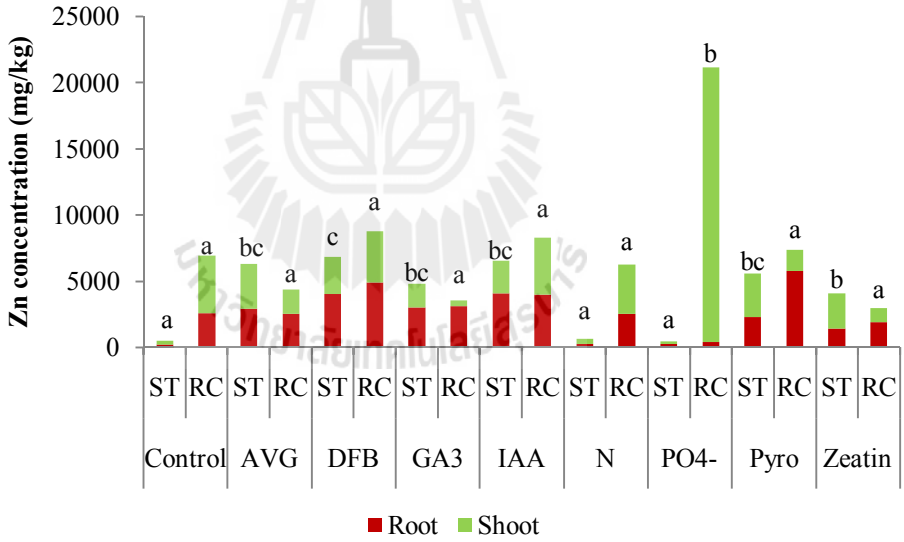


(A)

Figure 4.1 Total heavy metal uptake of *V. zizanioides* (RC)- and *V. nemoralis* (ST) inoculated with the plant growth promoting traits and grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significant difference ($p < 0.05$).



(B)



(C)

Figure 4.1 (Continued) Total heavy metal uptake of *V. zizanioides* (RC)- and *V. nemoralis* (ST) inoculated with the plant growth promoting traits and grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significant difference ($p < 0.05$).

Table 4.1 Cd concentration in *V. zizanioides* grown under various growth promoting traits of PGPR.

Parameter	Cd concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	6.21±1.79[a]	24.591±16.51[a]	35.32±21.67[a]	0.0015±0.00[a]
AVG	83.28±38.92[b]	1000.95±230.92[d]	7.73±6.35[ab]	0.03±0.00[b]
Desferosamine	128.84±35.82[bc]	237.45±50.94[bc]	84.343±62.86[c]	0.037±0.03[b]
Gibberellin	179.55±44.40[c]	314.6±73.50[bc]	58.21±15.15[bc]	0.03±0.00[b]
IAA	200.74±47.16[c]	516.92±181.71[c]	37.84±9.13[ab]	0.03±0.01[b]
Nitrogen	183.65±47.57[c]	361.45±177.88[bc]	54.32±13.99[bc]	0.02±0.01[b]
Phosphorus	123.68±90.04[bc]	243.21±40.27[ab]	52.07±37.60[bc]	0.02±0.01[b]
Pyrocatechol	159.38±46.59[bc]	813.5±296.72[d]	21.17±9.35[ab]	0.03±0.01[b]
Zeatin	182.74±57.25[c]	410.59±261.74[bc]	52.62±21.41[abc]	0.03±0.00[b]

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF= $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

Table 4.2 Pb concentration in *V. zizanioides* grown under various growth promoting traits of PGPR.

Parameter	Pb concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	42.03±14.48[ab]	164.25±75.72[a]	142.36±70.64[bc]	0.018±0.02[a]
AVG	458.04±78.17[bc]	2545.49±1593.86[b]	18.4±7.25[a]	0.11±0.10[bc]
Desferosamine	626.09±353.37[c]	3421.62±1662.75[b]	22.1±13.37[a]	0.18±0.11[c]
Gibberellin	202.4±144.03[ab]	618.52±459.30[a]	49.45±38.69[bc]	0.03±0.01[ab]
IAA	142.07±22.97[ab]	597.07±323.05[a]	28.07±12.33[a]	0.04±0.03[ab]
Nitrogen	65.46±14.95[ab]	239.5±69.22[a]	31.95±0.63[a]	0.01±0.00[a]
Phosphorus	22.2±10.45[a]	75.82±98.36[a]	99.9±10.08[c]	0.003±0.00[a]
Pyrocatechol	40.1±6.71[ab]	318.69±37.69[a]	18.78±2.01[a]	0.01±0.00[a]
Zeatin	230.48±18.45[ab]	488.18±257.59[a]	43.94±18.24[bc]	0.04±0.02[ab]

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF= $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

Table 4.3 Zn concentration in *V. zizanioides* grown under various growth promoting traits of PGPR.

Parameter	Zn concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	3747.00±1489.71[a]	1896.6±1307.32[abc]	206.18±48.90[a]	0.6±0.18[a]
AVG	1843.12±1268.82[a]	2557.12±1066.82[abc]	66.34±24.43[a]	0.33±0.02[a]
Desferosamine	3882.45±98.06[a]	4921.32±1770.06[c]	83.85±24.52[a]	0.67±0.16[a]
Gibberellin	415.48±101.84[a]	3152.14±1401.37[abc]	14.68±6.88[a]	0.18±0.12[a]
IAA	4325.5±990.88[a]	4012.14±908.50[bc]	115.46±46.21[a]	0.7±.011[a]
Nitrogen	3700.38±1900.93[a]	2579.02±217.32[abc]	132.67±93.93[a]	0.56±0.26[a]
Phosphorus	61017.33±40341.78[b]	449.23±366.35[a]	109,741.25±29,094.31[b]	16.88±20.37[b]
Pyrocatechol	1586.72±1694.43[a]	5789.62±4676.67[bc]	39.77±40.11[a]	0.4±0.34[a]
Zeatin	1035.92±1003.82[a]	1947.29±156.29[ab]	154.87±108.26[a]	0.217±0.19[a]

Value in the same column with different superscript are significantly different at $p < 0.05$.

*$TF = \left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** $TAR = \frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

Heavy metal analysis obtained from the root and shoot of *V. nemoralis* (Tables 4.4-4.6) revealed that roots Cd concentration was higher than in shoots concentration, especially in AVG, the Cd accumulation in root was about 5 time of shoot (Table 4.4). The average total Cd accumulation of control, AVG, DFB, GA_3, IAA, Nitrogen, Phosphorus, Pyrocatechol, and Zeatin, expressed as mg Cd per kg dry plant, were 135.35, 587.16, 302.89, 199.62, 547.89, 785.99, 130.79, 332.68, and 244.15 mg/kg, respectively. There were no effects of PGP traits on Cd translocation from root to shoot in *V. nemoralis*. In addition, the higher Cd accumulation rate than control group was only deserved under the influence of DFB, IAA, and N. Similar to the *V. zizanioides*, the *V. nemoralis* also exhibited higher Pb root concentration than Pb shoot concentration in all parameters-tested, including the control (Table 4.5). Compared to control, the higher Pb total accumulation was only deserved under the

influence of GA_3, IAA, and N (Figure 4.1B and Table 4.5). In addition, only AVG could increase the translocation from root to shoot higher than control. The higher total Zn accumulation than control group was only deserved under the influence of DFB, AVG, GA_3, IAA, and pyrocatechol (Figure 4.1C). Table 4.6 suggested that most of PGP traits increased the concentrations of Zn in root and shoot compared to the control group. The total rate of Zn accumulation was also higher than control under the influence of AVG, DFB, IAA, and pyrocatechol. However, there were no effects of PGP traits on Zn translocation from root to shoot.

Table 4.4 Cd concentration in *V. nemoralis* grown under various growth promoting traits of PGPR.

Parameter	Cd concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	41.2±29.16[a]	94.156±78.15[a]	108.94±164.36[a]	0.007±0.003[ab]
AVG	92.37±74.93[a]	494.79±91.48[ab]	96.94±15.51[a]	0.012±0.005[abc]
Desferosamine	102.64±33.65[a]	200.24±137.02[ab]	178.36±97.38[a]	0.018±0.005[c]
Gibberellin	78.309±47.11[a]	121.32±80.11[a]	76.8±52.99[a]	0.013±0.007[abc]
IAA	240.33±34.62[b]	307.56±84.32[ab]	88.52±48.64[a]	0.036±0.002[d]
Nitrogen	188.667±63.02[b]	597.32±288.71[b]	37.43±16.54[a]	0.040±0.01[d]
Phosphorus	48.9±39.01[a]	81.88±48.32[a]	235.79±41.68[a]	0.005±0.004[a]
Pyrocatechol	75.424±43.46[a]	257.26±51.26[ab]	81.31±9.75[a]	0.015±0.005[bc]
Zeatin	111.93±51.13[a]	132.22±82.77[a]	149.32±134.43[a]	0.015±0.007[bc]

Value in the same column with different superscript are significantly different at $p < 0.05$.

*$TF = \left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** $TAR = \frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

Table 4.5 Pb concentration in *V. nemoralis* grown under various growth promoting traits of PGPR.

Parameter	Pb concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	423.81±65.56^{a}	1757.62±572.91^{a}	24.63±8.33^{abc}	0.1±0.01^{ab}
AVG	970.4±230.08^{bc}	2303.09±1145.61^{ab}	55.56±30.66^{d}	0.17±0.009^{bcd}
Desferosamine	357.66±77.99^{a}	1571.98±436.63^{a}	22.88±1.18^{abc}	0.08±0.02^{a}
Gibberellin	968.5±285.25^{bc}	9127.426±4517.81^{c}	17.98±20.28^{ab}	0.3±0.07^{f}
IAA	1208.62±188.15^{c}	5279.9±2418.43^{b}	25.7±9.45^{abc}	0.24±0.05^{df}
Nitrogen	972.91±125.91^{bc}	2458.34±445.41^{ab}	41.25±12.71^{bcd}	0.19±0.02^{cd}
Phosphorus	309.19±310.2^{a}	3674.88±2136.17^{ab}	9.63±8.99^{a}	0.11±0.08^{abc}
Pyrocatechol	913.73±132.71^{b}	2196.55±738.54^{ab}	44.84±13.81^{cd}	0.17±0.03^{bcd}
Zeatin	409.84±78.12^{a}	1168.05±214.53^{a}	33.39±5.05^{abcd}	0.07±0.01^{a}

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF= $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

Table 4.6 Zn concentration in *V. nemoralis* grown under various growth promoting traits of PGPR.

Parameter	Zn concentration in plants (mg/kg)		TF*	TAR**
	Shoot	Root		
Control	279.25±215.36^{a}	230.37±195.23^{a}	245.51±300.53^{a}	0.03±0.008^{a}
AVG	3378.97±891.73^{cd}	2936.57±489.01^{c}	118.65±41.75^{a}	0.54±0.29^{c}
Desferosamine	5255.43±3794.9^{d}	4061.4±778.12^{d}	156.36±155.1^{a}	0.58±0.31^{c}
Gibberellin	1774.82±767.58^{abc}	3039.32±856.21^{c}	63.33±31.89^{a}	0.36±0.14^{bc}
IAA	2433.85±1390.77^{bc}	4124.45±1042.48^{d}	46.1±34.78^{a}	0.54±0.29^{c}
Nitrogen	430.01±517.2^{ab}	269.15±207.98^{a}	269.66±328.06^{a}	0.03±0.01^{a}
Phosphorus	169.76±83.9^{a}	296.53±224.8^{a}	112.41±140.03^{a}	0.03±0.02^{a}
Pyrocatechol	3263.85±660.19^{cd}	2336.626±666.6^{c}	111.82±18.13^{a}	0.4±0.15b^{c}
Zeatin	2640.51±1826.88^{c}	1461.53±769.59^{b}	157.97±85.27^{a}	0.25±0.14^{ab}

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF= $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

4.2 Soil analysis and isolation of cadmium tolerant rhizobacteria

Phadaeng Industry Public Company Limited is located in Moo 4 and 8, Pha Taad Phadaeng Subdistrict, Mae-Sod District, Tak Province located in the northwestern part of Thailand and neighboring area. The company has been confronted with leachate of heavy metal form Zinc smelting process. The sampling sites of soil and rhizosphere bacteria were collected from different sites which divided into 5 plots according to 1) Sedimentation pond supported by water drainage from mining activity, 2) Re-vegetation area in year 2003 and 2004, respectively, 3) Mining area, 4) Low zinc ore contaminated with lead ore and 5) High zinc ore as the ore for zinc smelting process.

The soil physical characterization as soil moisture content and soil texture and soil chemical properties as soil pH, organic matter, and EC were determined. The results were reported in Table 4.7.

The matrixes of soil and sediment contained clay 13% and 35%, respectively and were coarse and moderately fine. The moisture content of soil and sediment were nearly the same, 10.31 and 11.38, respectively. The pH of both soil types was slight alkaline. Total P, Cd, Pb, and Zn concentrations in soil were higher than sediment, especially the total Zn was almost 15 times of sediment. In contrast, the sediment contained about 21 times of total K than soil. The exchange capacity of soil was higher than sediment. The order exchangeable Zn in both soil and sediment was highest followed by Cd and Pb, respectively. The exchange capacity of soil was higher than sediment. Also, total P, K, and Zn concentration in soil and sediment samples were higher than their exchangeable forms.

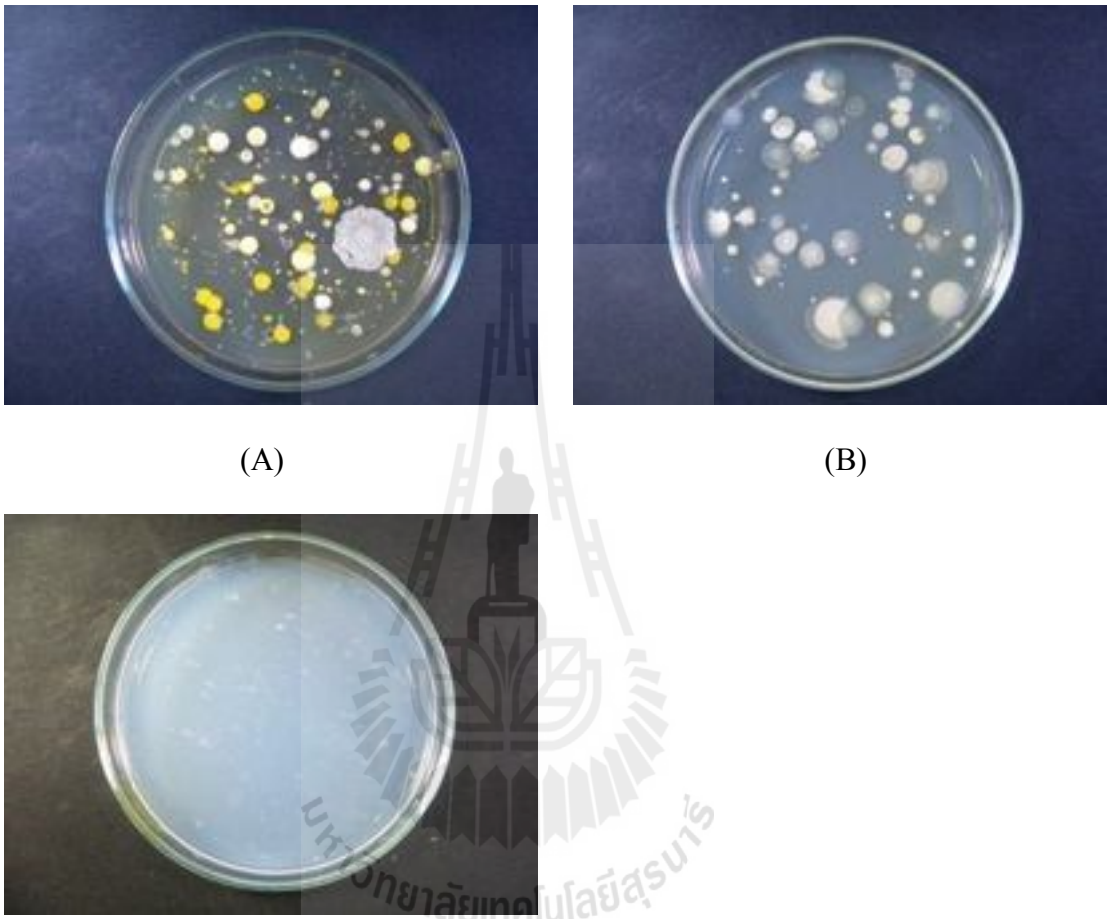
Table 4.7 The soil physical and chemical characterization.

Parameters	Soil	Sedimentation
Type of soil matrix	Sandy loam	Clay loam
Moisture content (%)	10.31	11.36
pH	7.80±0.2	7.50±0.2
EC (dS/m)	1.33	0.54
Total N (%)	0.04	0.04
Total P (mg/l)	200	100
Total K (mg/l)	620	1,390
Total Cd (mg/l)	6,250	501
Total Pb (mg/l)	1,103	114
Total Zn (mg/l)	368,856	7,223
Exchangeable Cd (mg/l)	8.15	5
Exchangeable Pb (mg/l)	0.34	0.27
Exchangeable Zn (mg/l)	25	8

The aim of the experiment was to screen the Cd-tolerated rhizobacteria from Cd-contaminated soil at Phadaeng Industry Company Limited, Tak Province, Thailand. The distinct bacterial strains grown on different types of agar media containing 50 mg/l Cd were selected for subsequent PGPR traits experiment. The criteria of selecting based on differences in colony morphology including colony form, elevation and pigment production.

Depending on Cd-tolerated strain, three different media including TSA for general soil bacteria, PSA for *Pseudomonads* species, and ABA for *Azotobacter* sp., and *Azomonas* were used to isolate rhizophere and non-rhizophere bacteria (Figure 4.2). Five hundred and twenty-one colonies were selected from initial Cd level (50

mg/l) supplemented in each medium. The number of distinguish colonies on TSA, PSA, and ABA were 207, 203, and 111, respectively.



(A) (B)

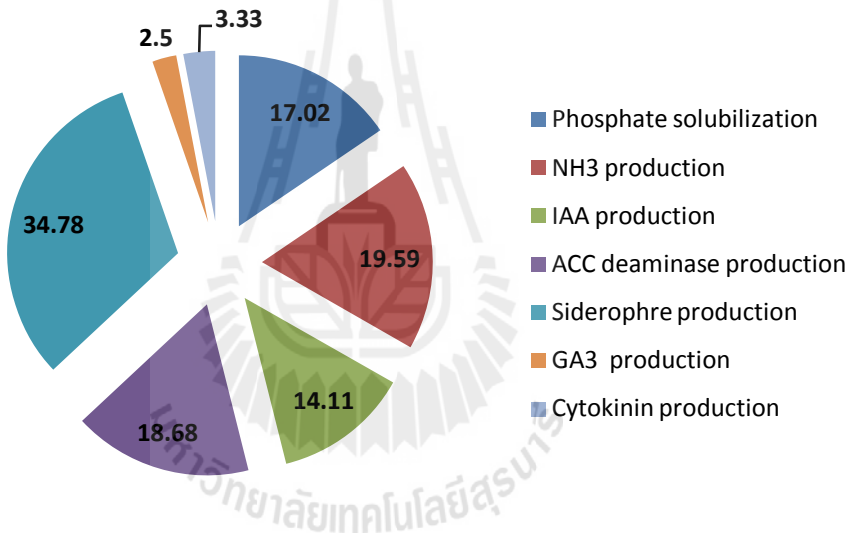
(C)

Figure 4.2 The bacterial colonies on different media (A) PSA (B) TSA and (C) AZA.

4.3 Physiological Characterization of Plant Growth-Promoting Rhizobacteria attributes

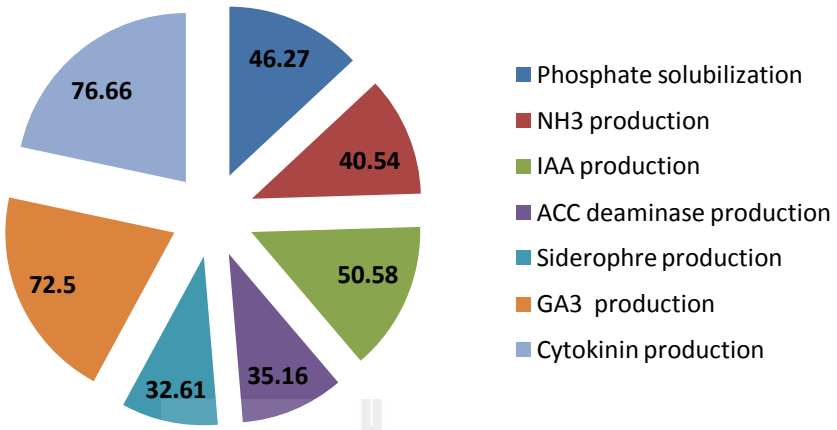
Five hundred and twenty-one colonies of rhizobacteria with high resistance to 50 mg/l Cd isolated from previous studies were collected and further screened for

PGPR traits. Screening results of PGPR traits were depicted in Figure 4.3. The selected rhizobacteria could display phosphate solubilization, IAA-, gibberellins-, and cytokinin production in all media with the highest number of positive clones were observed in the PSA medium followed by TSA and AZA medium, respectively. Especially the percent of rhizobacteria capable of producing of gibberellins and cytokinin were markedly increased in the PSA medium. In contrast, there was no effect of media on the siderophore production as evidenced by similar percentage of positive clones in AZA (34.78%), TSA (32.61%), and PSA (32.61%) media.

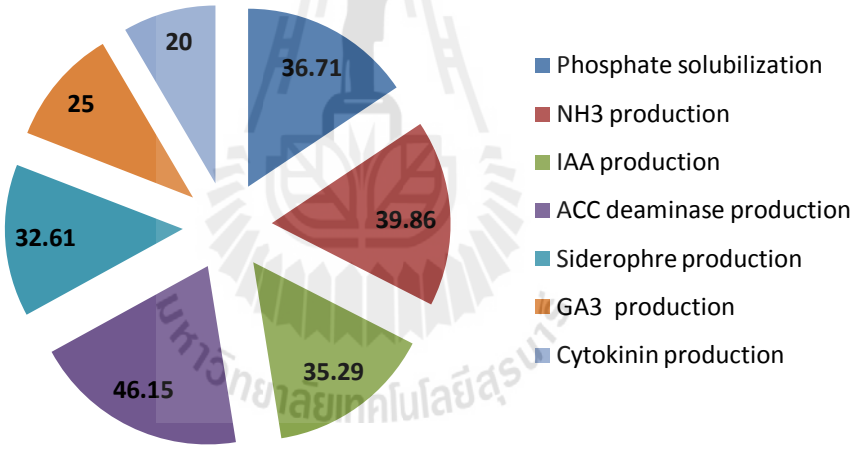


(A)

Figure 4.3 The percentage of PGP traits production in different media (A) AZA, (B) PSA, and (C) TSA.



(B)



(C)

Figure 4.3 (Continued) The percentage of PGP traits production in different media (A) AZA, (B) PSA, and (C) TSA.

4.4 Heavy metal resistance and minimum inhibitory concentration (MIC)

In order to conduct the heavy metal resistant ability, six selected bacteria were isolated from the previous experiments as follows: 1RER2, 25R463, 27RER3,

39SHR, 53S462, and 65R471. All six bacteria had capability to produce PGPRs traits including IAA, siderophore, ACC deaminase, and phosphate solubilization. Moreover, they were collected and determined for the minimum inhibitory concentration against increasing concentration of Cd, Pb, and Zn on Tryptic soy agar (TSA) using the paper discs technique. The bacterial isolates exhibited different resistance to the tested heavy metals as shown in Table 4.8.

Table 4.8 MIC of the selected bacterial strains grown in various heavy metal concentrations.

Bacteria	MIC (mM)		
	Cd	Pb	Zn
1RER2	1	3	10
25R463	3	9	5
27RER3	0.5	9	10
39SHR	0.5	3	10
53S462	1	5	10
65R471	3.5	7	5

The size of inhibition zone normally indicated chemical resistant ability of bacterial cells. For the same chemical, a bacteria strain that showed no clear zone attributed high chemical resistant. Strain 1RER2, 25R463, 53S462, and 65R471 showed highly potential cadmium resistance against 0.5 mM $CdCl_2$ because there was not any clear zone found on the plate. In contrast, 27RER3 and 39SHR showed the inhibition zone. The highest Cd resistant strain was 65R471 showing capability to tolerate Cd at 3.5 mM. Strain 1RER2, 39SHR, 53S462, 65R471, 25R463, and 27RER3 tolerated Pb concentration at 3, 3, 5, 7, 9, and 9 mM, respectively. On the contrary, 1RER2, 39SHR, and 53S462 showed the highest capability to tolerate Zn at 10 mM.

4.5 Quantification of plant growth-promoting bacteria attributes in heavy metal stress

The quantification of PGPRs traits (IAA, gibberellins, siderophore, and ACC deaminase production and phosphate solubilization) by 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 isolates were determined under each heavy metal stress by using their corresponding MIC as listed in Table 4.8.

4.5.1 ACC deaminase determination

ACC deaminase activity was assayed (Penrose and Glick, 2003) by measuring the amount of the product α-ketobutyrate formed from the cleavage of ACC by the enzyme ACC deaminase. The quantity of alpha-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to the standard curve of α-ketobutyrate (Figure 4.4). The results revealed that all the selected straines could produce ACC deaminase in the absence of heavy metal ($p < 0.05$) in the media. However in the presence of heavy metal, the selected strains could utilize ACC as the sole nitrogen source, except under Cd stress. In the presence of Zn, the rate of ACC deaminase production by 1RER2, 25R463, 27RER3, and 65R471 were 6,812, 1,651, 8,671, and 52 μmol/hr/mg, respectively, while 39SHR and 53S462 could not produce ACC deaminase. Under Pb stress, there were no significantly different of ACC deaminase production among tested strains. Figure 4.5 showed that 39SHR and 65R471 significantly decreased ACC deaminase production under heavy metal stress. In contrast, ACC deaminase production by 1RER2, 25R463, and 27RER3 were increased under Zn stress.

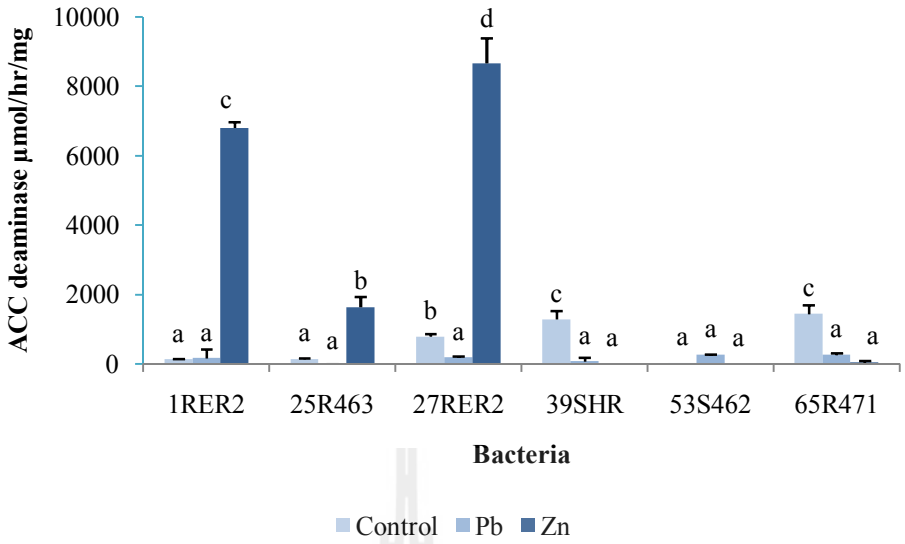


Figure 4.4 ACC deaminase production by bacteria grown under various heavy metal stress. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

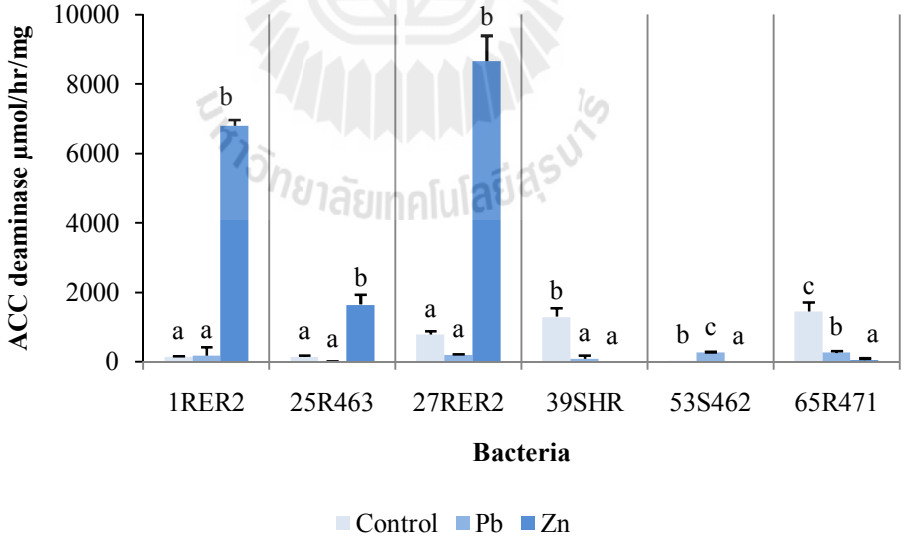


Figure 4.5 ACC deaminase production by bacteria grown under various heavy metal stress. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

4.5.2 Siderophore production

The 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 were cultured in 10 ml MM9 medium in the presence or absence of 0.5 mM $CdCl_2$, 2.5 mM $Pb(NO)_3$ or 5.0 mM $ZnSO_4$. The supernatants were assayed in the presence of iron chelators using hydroxamate- and catechol-type siderophore assay.

The production of hydroxamate compounds in the supernatants of the bacterial cultures was detected by the ferric perchlorate assay as shown in Figure 4.6. The results indicated that 53S462 and 65R471 isolates showed the highest hydroxymate compounds ($p < 0.05$) in the presence of Pb compared to control. All of selected strains, except 1RER2, significantly produced more hydroxymate compound production in the presence of Pb (Figure 4.7).

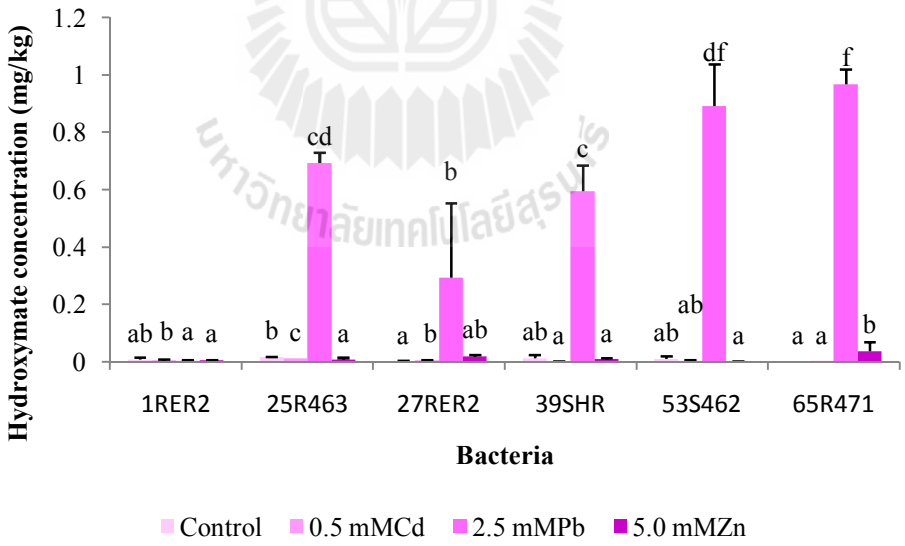


Figure 4.6 Hydroxymate compound production by bacteria grown under various heavy metal stress. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

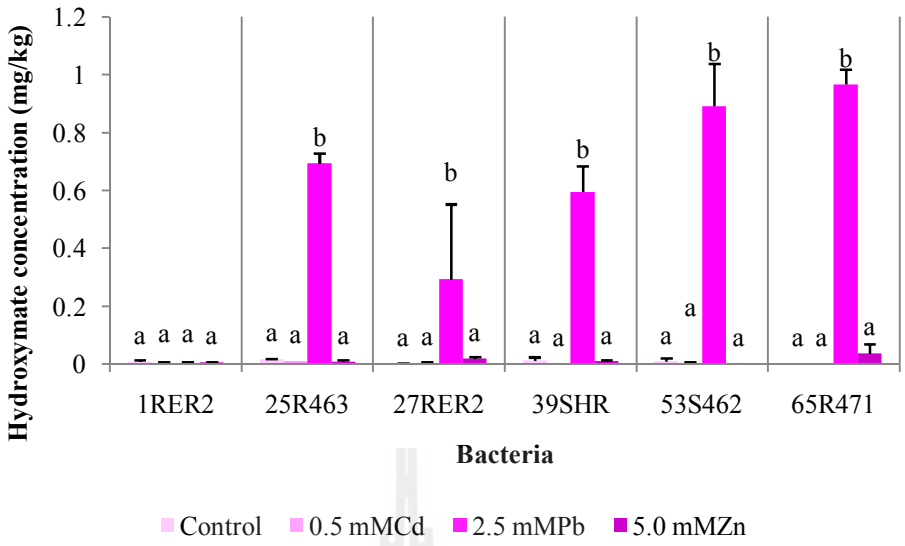


Figure 4.7 Hydroxymate compound production by bacteria grown under various heavy metal stress. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

The presence of catecholate compounds in the supernatants could be differentiated by the colorimetric assays and the result was shown in Figure 4.8. Under heavy metal stress, 1RER2, 25R463, 27RER3, and 39SHR showed the trend of increased catecholate production, but not statically significant at $p < 0.05$. Only 65R471 exhibited higher catecholate production than the other tested strains in the presence of heavy metal. The ability of the selected strain for catecholate production under Cd stress was as follow: 53S462, 65R471 > 39SHR, 25R463, 27RER3, 1RER2. 53S462 also exhibited the highest catecholate production capability in the presence of 5.0 mM Zn. Furthermore, Zn stimulated catecholate compounds production in almost all selected strains (Figure 4.9).

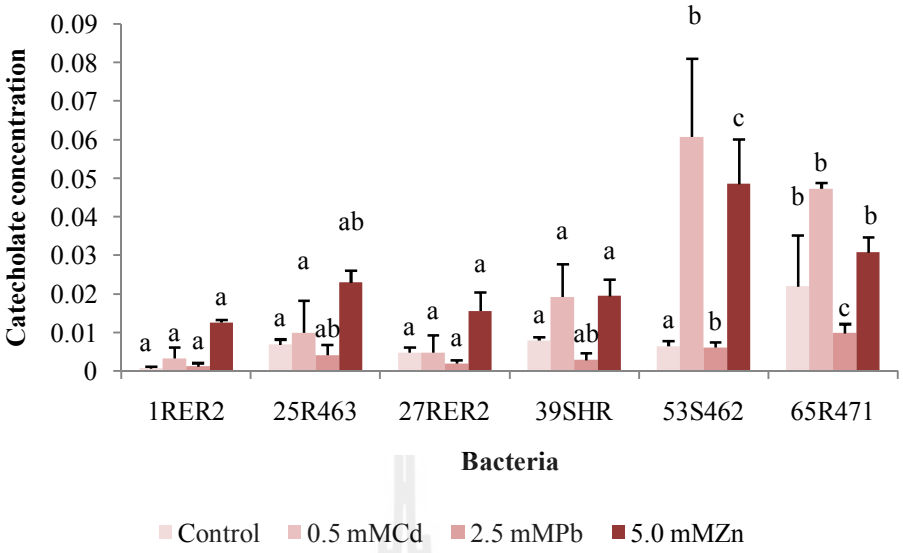


Figure 4.8 Catecholate production by bacteria grown under various heavy metal stress. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

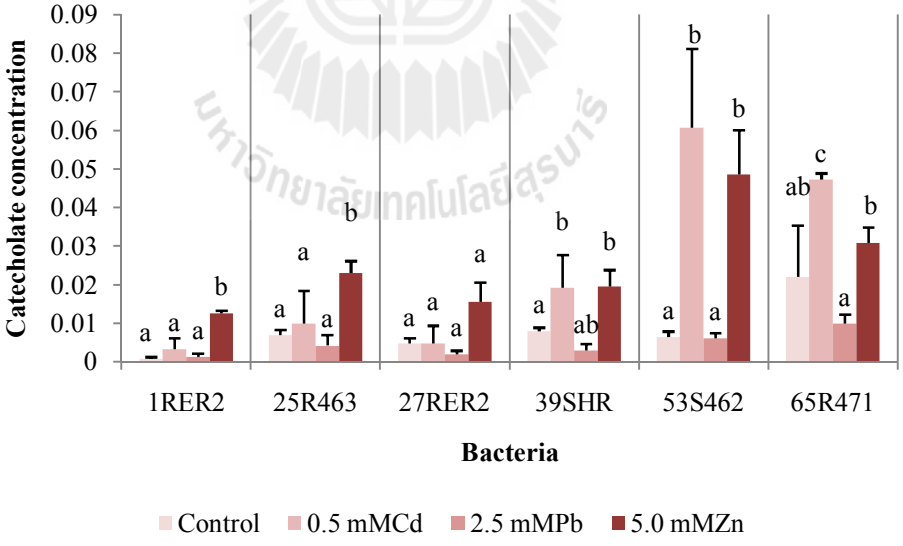


Figure 4.9 Catecholate production by bacteria grown under various heavy metal stress. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

4.5.3 Hormone production

A single colony of the 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 was transferred to 10 ml of SMM medium supplemented with or without 500 µg/ml L-tryptophan and contained either or media alone (blank solution) or 0.5 mM Cd, 2.5 mM Pb or 5.0 mM Zn. After 48 hr incubation, the culture supernatants were assayed for GA_3 and IAA production.

The amount of GA_3 presented in the culture supernatant was determined (Figure 4.10). The GA_3 production by most isolates showed the trend of decreased GA_3 production in heavy metal supplemented medium, except 1RER2 and 25R463. In the absence of heavy metal 53S462 and 65R471 showed the highest GA_3 production of 4.99±0.35, and 5.76±2.69 mg/l. 65R471 significantly showed the highest GA_3 production in the presence of heavy metal compared to the other strains. However, 53S462 and 65R471 could produce GA_3 under heavy metal stress condition. Almost all of selected strains, except 25R463, decreased GA_3 production under heavy metal stress (Figure 4.11).

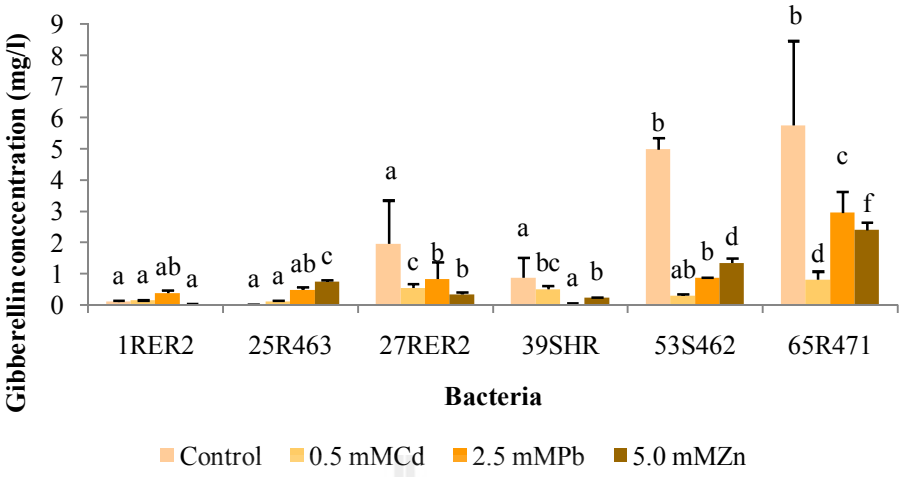


Figure 4.10 GA_3 production by bacteria grown under various heavy metal stress. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

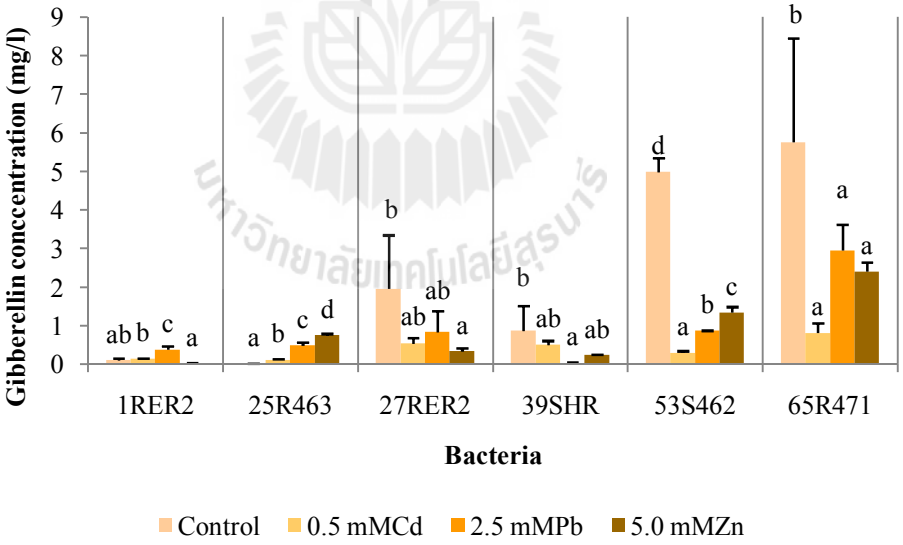


Figure 4.11 GA_3 production by bacteria grown under various heavy metal stress. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

The estimation of IAA level in bacterial culture grown under different heavy metal stress condition in the presence and absence of L-tryptophan was shown in Figures 4.12-4.15, respectively. They showed the trend of increase IAA production when the culture was supplemented with L-tryptophan. However, all selected strains could produce IAA in the absence of L-tryptophan as well.

In L-trytophan addition, the IAA production was higher than heavy metal stress condition. However, the IAA production in the absence of L-tryptophan condition tended to decrease in the presence of heavy metal. The maximum IAA production under the influence of in heavy metal stress could be observed in 53S462 and 65R471 either with or without L-tryptophan supplement. As shown in Figure 4.15, 25R463, 39SHR, and 65R471 promoted IAA production under Cd and Pb stress. In addition, IAA production by 65R471 was increased under all heavy metal stress tested. In contrast, all tested PGPRs exhibited no difference in IAA production under heavy metal stress in the media without L-tryptophan supplement (Figure 4.15).

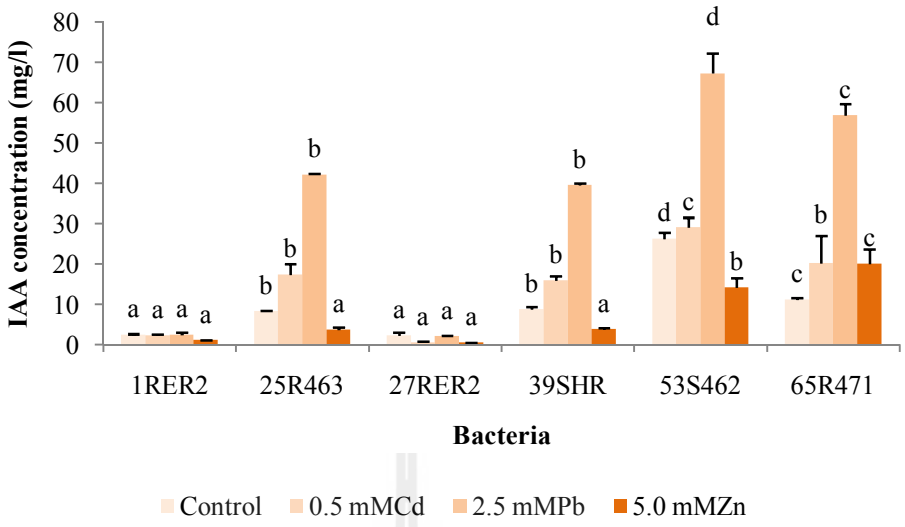


Figure 4.12 IAA production by bacteria grown under various heavy metal stress in L-tryptophan supplemented media. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

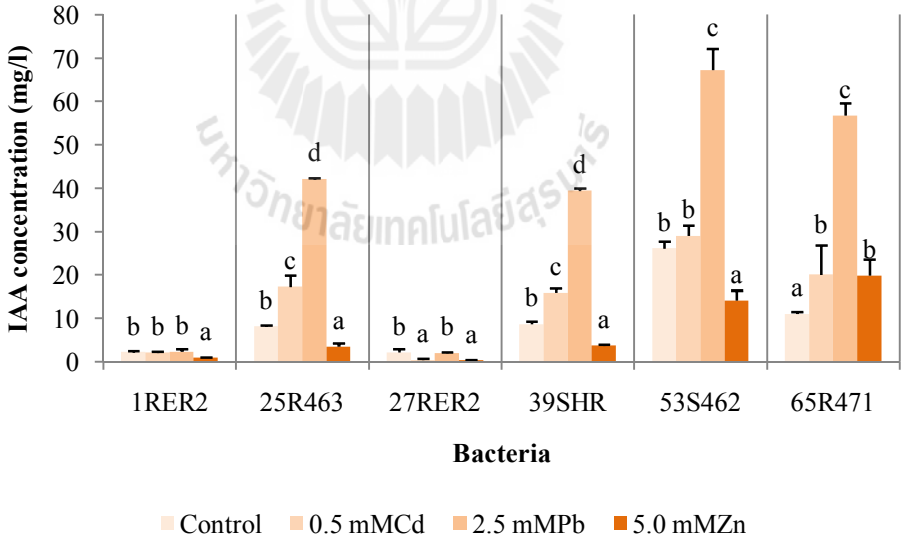


Figure 4.13 IAA production by bacteria grown under various heavy metal stress in L-tryptophan supplemented media. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

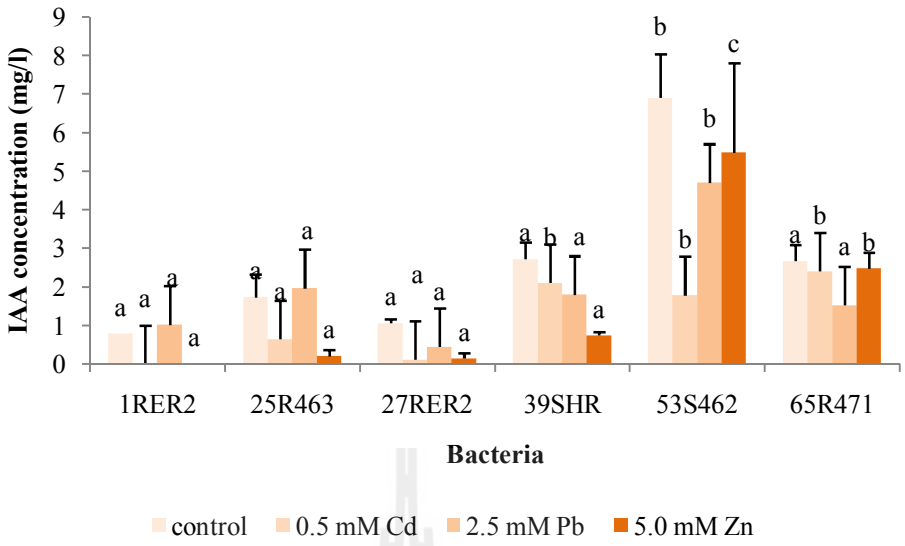


Figure 4.14 IAA production by bacteria grown under various heavy metal stress in the media without L-tryptophan. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

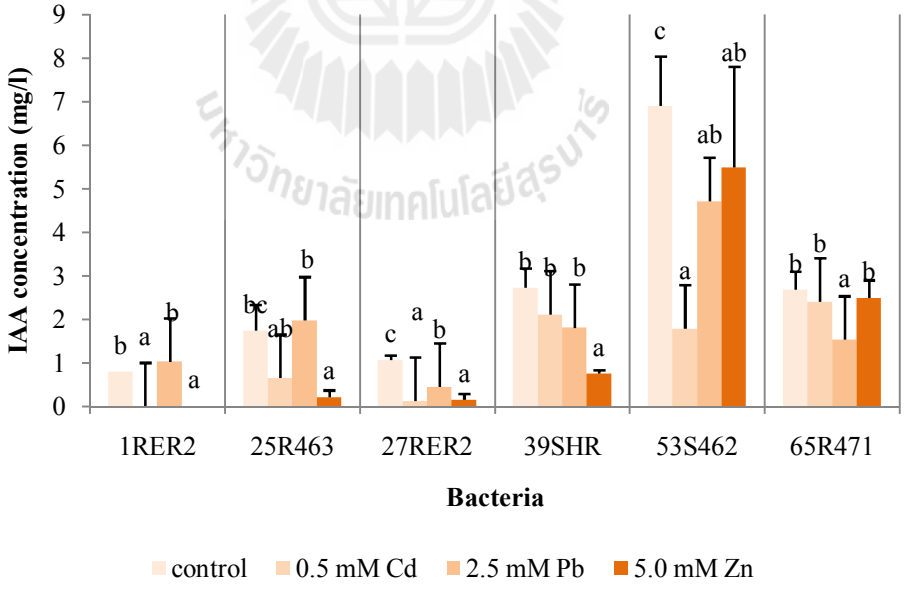


Figure 4.15 IAA production by bacteria grown under various heavy metal stress in the media without L-tryptophan. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

4.5.4 Phosphate solubilization

The quantitative analysis of tri-calcium phosphate solubilization by isolated bacteria was quantified in NBRIP medium, supplemented with or without heavy metal as 0.5 mM $CdCl_2$, 2.5 mM $PbNO_3$ or 5.0 mM $ZnSO_4$ (Figure 4.10). Furthermore, the growth of strain 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 in NBRIP medium containing heavy metal demonstrated the potential for phosphate solubilization. The amount of soluble phosphate released in liquid medium due to solubilization of tri-calcium phosphate in control condition has been observed in 53S462 and 25R463. Under Zn condition, 65R471 exhibited the maximum amount of phosphate solubilization. There was no effect of heavy metal stress on phosphate solubilization by tested PGPR (Figure 4.17). In addition, the data showed the increased amount of phosphate solubilization which exhibited a slightly inverse relationship with the pH of the medium in all conditions (Figure 4.18).

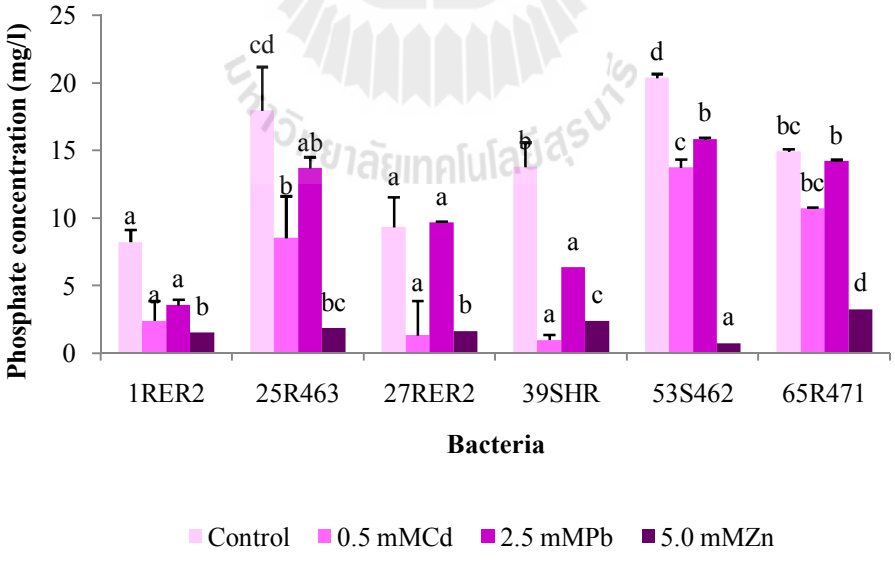


Figure 4.16 Capability of bacteria to exhibit the property of phosphate solubilization under heavy metal stress condition. Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

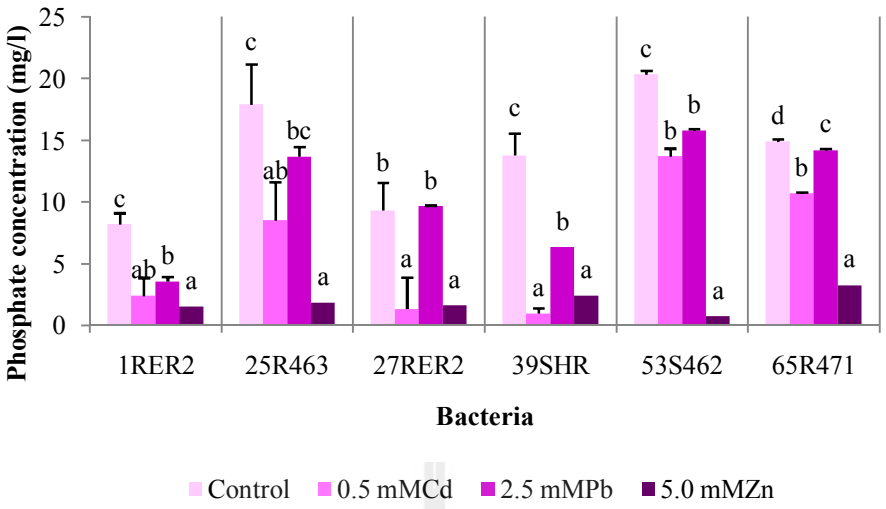
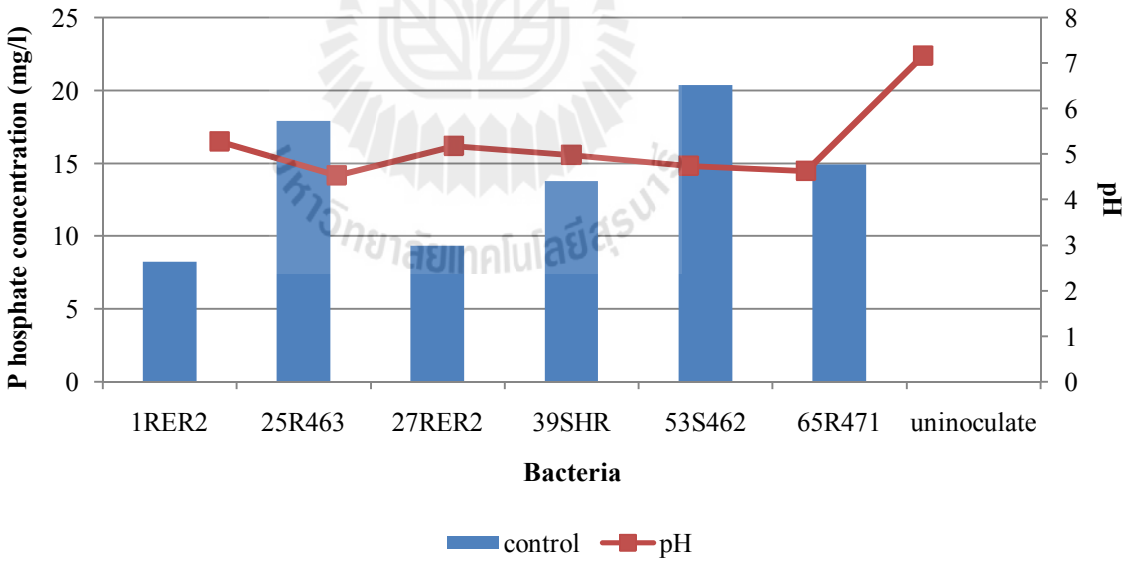
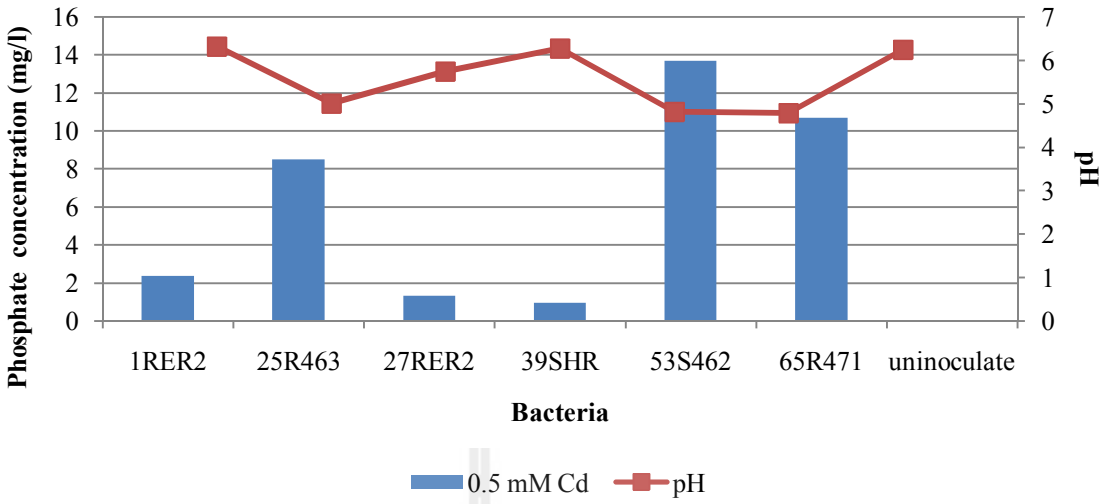


Figure 4.17 Capability of bacteria to exhibit the property of phosphate solubilization under heavy metal stress condition. Bars of each bacteria with different superscript are significantly different at $p < 0.05$.

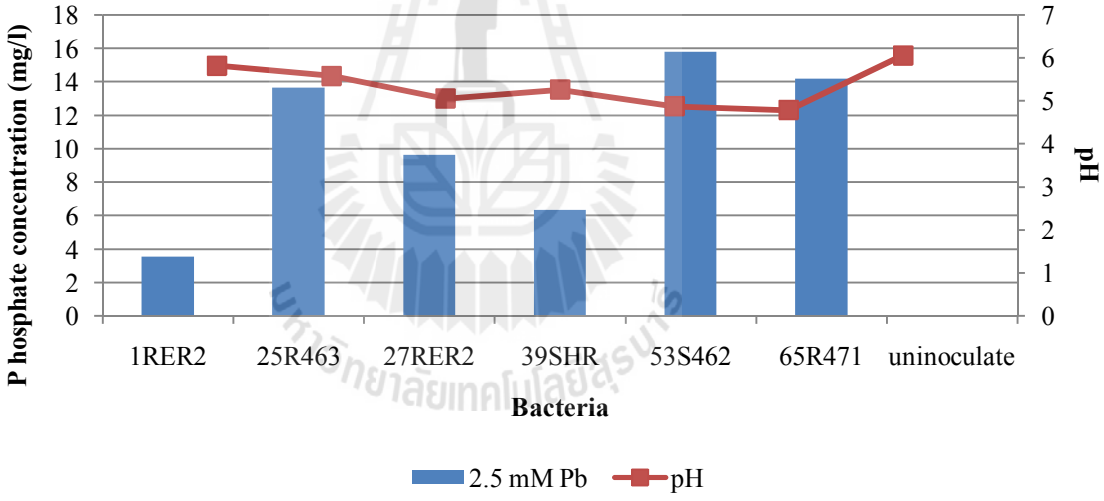


(A)

Figure 4.18 The relationship between PO^{4-} concentration and pH in (A) control, (B) Cd, (C) Pb, and (D) Zn condition supplemented media.

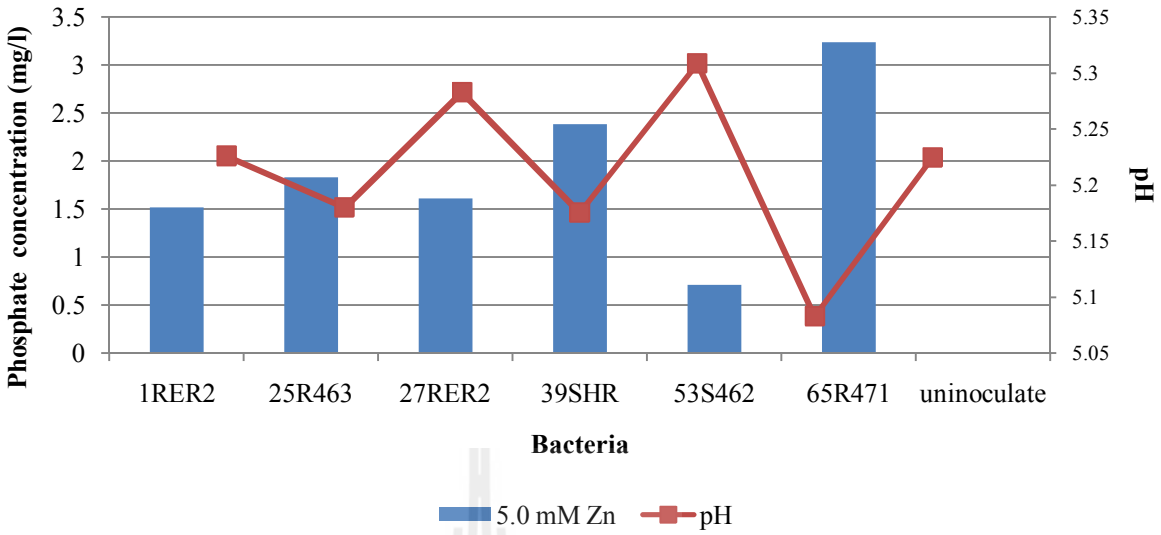


(B)



(C)

Figure 4.18 (Continued) The relationship between PO^{4-} concentration and pH in (A) control, (B) Cd, (C) Pb, and (D) Zn condition supplemented media.



(D)

Figure 4.18 (Continued) The relationship between PO^{4-} concentration and pH in (A) control, (B) Cd, (C) Pb, and (D) Zn condition supplemented media.

4.6 Characterization of selected PGPR bacteria

The morphological and physiological characteristics of 6 strains were determined. Colony morphology of the selected strains were shown in Table 4.9. All isolates were Gram-negative, rod shape, non-sporing, catalase positive and non-motile (Table 4.9).

Table 4.9 The distinguished types of the selected bacteria.

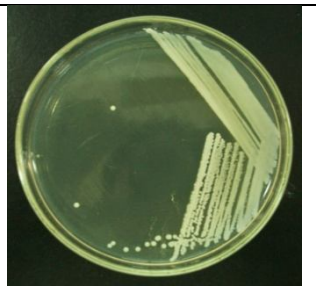
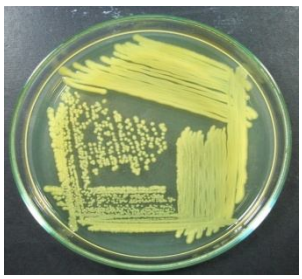
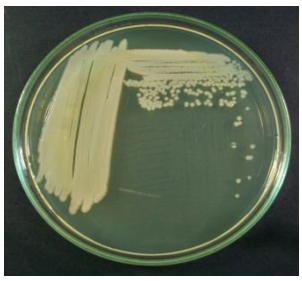
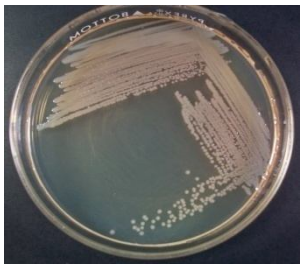
Strain	Cell shape	Gram strain	Colony morphology on TSA plate	
1RER2	Rod	Positive	White, Circular, Entire, Smooth, Convex	

Table 4.9 (Continued) The distinguished types of the selected bacteria.

Strain	Cell shape	Gram strain	Colony morphology on TSA plate	
25R463	Rod	Negative	White, Circular, Entire, Smooth, Umbonate	
27RER3	Rod	Positive	White, Circular, Entire, Smooth, Convex	
39SHR	Rod	Positive	Yellow, Circular, Entire, Smooth, Convex	
53S462	Rod	Negative	White, Circular, Entire, Smooth, Umbonate	
65R471	Rod	Negative	White, Circular, Entire, Smooth, Convex	

The identification of selected PGPR strains shown in Table 4.9 were also confirmed by the determination of 16S rDNA gene sequences from the cultures. Four major steps for genotypic characterization of selected PGPR isolates in this study were as follows: genomic DNA extraction; PCR amplification of 16S rDNA; sequencing of PCR amplicon; and 16S rDNA sequence analysis. Based on the 16S rDNA gene sequences (Figure 4.19), 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 was identified as *Arthrobacter* sp., *Aeromonas* sp., *Arthrobacter* sp., *Microbacterium* sp., *Pseudomonas* sp., and *Pseudomonas* sp, respectively. Partial sequence of 16S rDNA of 1RER2 (1456 bp) showed 95% homology with *Arthrobacter* sp. EH66. 25R463 (1716 bp) showed 99% homology with *Aeromonas tacta* CECT7082. 27RER3 (1578 bp) showed 97% homology with *Arthrobacter* sp MTR-44. 39SHR (1422 bp) showed 98% homology with *Microbacterium paraoxydans* strain M2. 53S462 (1722 bp) showed 99% homology with *Pseudomonas plecoglossicida* GBA738. 65R471 (1714 bp) showed 99% homology with *Pseudomonas putida* J3121.

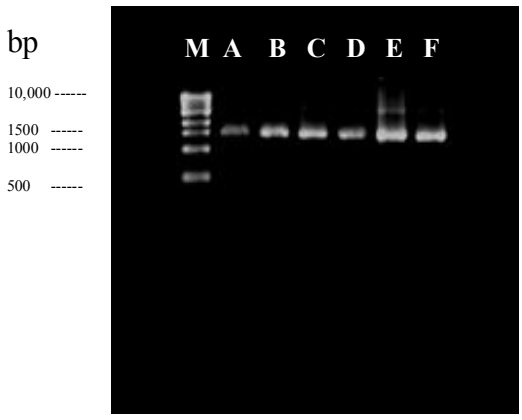


Figure 4.19 PCR products of (M) marker, (A) 1RER2, (B) 25R463, (C) 27RER3, (D) 39SHR, (E) 53S462, and (F) 65R471.

Table 4.10 Bacterial identification.

Strain	Closest described relative	Accession no	bp	Similarity (%)
1RER2	*Arthrobacter* sp. EH66	GU339291.1	1456	95
25R463	*Aeromonas tacta* CECT7082	HQ832416.1	1716	99
27RER3	*Arthrobacter* sp MTR-44	EU034524.1	1578	97
39SHR	*Microbacterium paraoxydans* strain M2	EU714377	1422	98
53S462	*Pseudomonas plecoglossicida* GBA738	HM209783.1	1722	98
65R471	*Pseudomonas putida* J3121	EF203210.1	1714	99

4.7 Antibiotic sensitivity test

Only the selected isolates obtained from previous experiment as 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 were used in antibiotic sensitivity test. The experiment used 6 commercialized different antibiotic disks namely neomycin (30 μg), tetracycline (30 μg), chloramphenical (30 μg), colistin sulphate (10 μg), streptomycin (10 μg), and erythromycin (15 μg). The sensitivity and resistance profile to antibiotics was based on the diameter of the inhibition zone and the evaluation followed the National Committee for Clinical Laboratory Standard's (NCCLS) chart provided with the antibiotic kits by Himedia.

The antibiotic profiles of the isolates indicated the sensitivity of the strains to different antibiotics (Table 4.11). All the isolates were resistant to colistin sulphate. 27RER3 was quite sensitive to all antibiotics, while 53S462 and 65R471 were resistance to most of the antibiotics tested except neomycin, which was intermediate.

Table 4.11 Antibiotic sensitivity profile of the selected bacteria.

Antibiotic	Concentration (µg)	Diameter of inhibition zone (mm) of the selected bacteria					
		1RER2	25R463	27RER3	39SHR	53S462	65R471
Chloramphenicol	30	47 (S)	30(S)	52 (S)	21 (I)	14 (R)	10 (R)
Erythromycin	15	48 (S)	30(S)	52 (S)	45 (S)	NZ (R)	NZ (R)
Steptomycine	10	22 (I)	18 (I)	28 (S)	22 (I)	12 (R)	11 (R)
Neomycin	30	21 (I)	NZ(R)	27 (S)	23 (I)	18 (I)	18 (I)
Colistin sulphate	10	10 (R)	NZ(R)	NZ (R)	NZ (R)	11 (R)	7 (R)
Tetrycycline	30	16 (I)	18 (I)	22 (I)	10 (R)	NZ (R)	7 (R)

NZ = no clear zone, R = resistance, I = intermediate, and S = susceptibility.

4.8 Bacterial capability for heavy metal biosorption

The capabilities of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 to uptake Cd, Pb, and Zn were shown in Table 4.12. The isolates exhibited different biosorption capacity towards the tested metal ions. 65R471 was the most highest efficient in Cd biosorption while 1RER2, 27RER3, and 65R471 exhibited the similar capacity in Zn biosorption. All six tested isolates were capable of uptaking Pb higher than Zn and Cd. 39SHR significantly exhibited the highest biosorption capacity for Pb compared to the other test strains.

Table 4.12 The heavy metal concentration in the selected bacteria.

	Heavy metal concentration (mg/kg) in bacteria		
Bacteria	**Zn**	**Cd**	**Pb**
1RER2	8.74±1.89^{d}	13.25±3.22^{a}	221,592.96±61,723.06^{a}
25R463	5.32±0.77^{ab}	91.47±42.32^{a}	263,317.39±32,636.59^{a}
27RER3	7.00±0.30^{bcd}	20.32±7.37^{a}	166,783.07±53,350.97^{a}
39SHR	3.91±0.24^{a}	19.72±2.67^{a}	578,231.61±27,091.39^{b}
53S462	6.51±0.91^{bc}	85.33±51.65^{a}	150,535.05±30,291.82^{a}
65R471	8.06±0.86^{cd}	236.02±72.95^{b}	298,911.58±11,579.76^{a}

Values in the same column with different superscript are significantly different at $p < 0.05$.

4.9 The effect of PGPR in enhancing heavy metal availability in soil

The concentrations of exchangeable Cd, Pb, and Zn in soil by water extraction were examined to assess the relative efficiency of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 in enhancing metal solubilisation from the soil compared with control treatment (Figure 4.20). 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 inoculation increased the concentrations of soluble Pb in soil as 1.5-, 11-, 9.7-, 1.5-, 4.4-, and 3-folds, respectively. However, the increased Pb solubilisation was only statistically significant with 25R463 and 27RER2 only. All of the strains, except 53S462 showed the trend of increasing Cd soluble concentration. 1RER2 and 65R471 exhibited the highest Zn solubilization efficiency. Moreover, enhancing Zn availability in soil was observed in 65R471 and 53S462 compared to uninoculate condition.

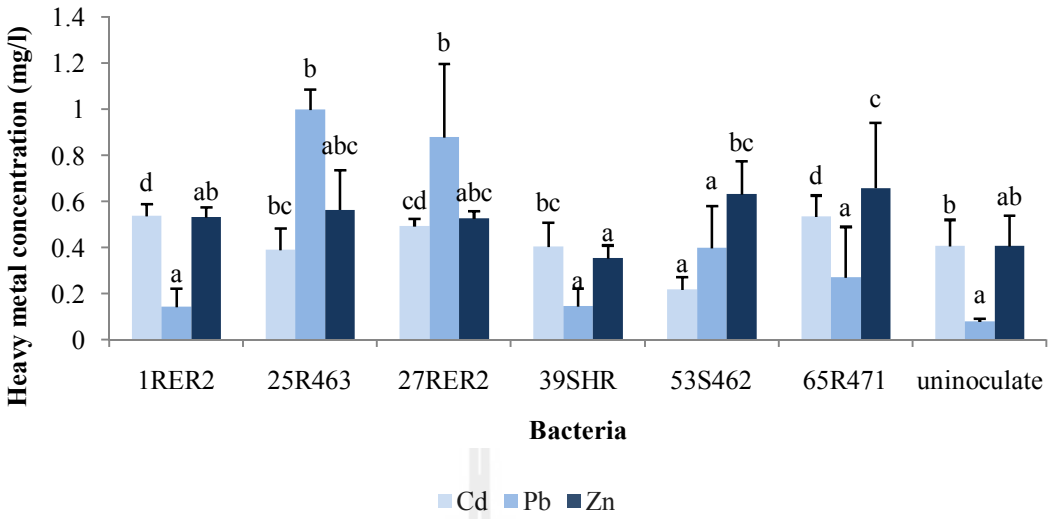
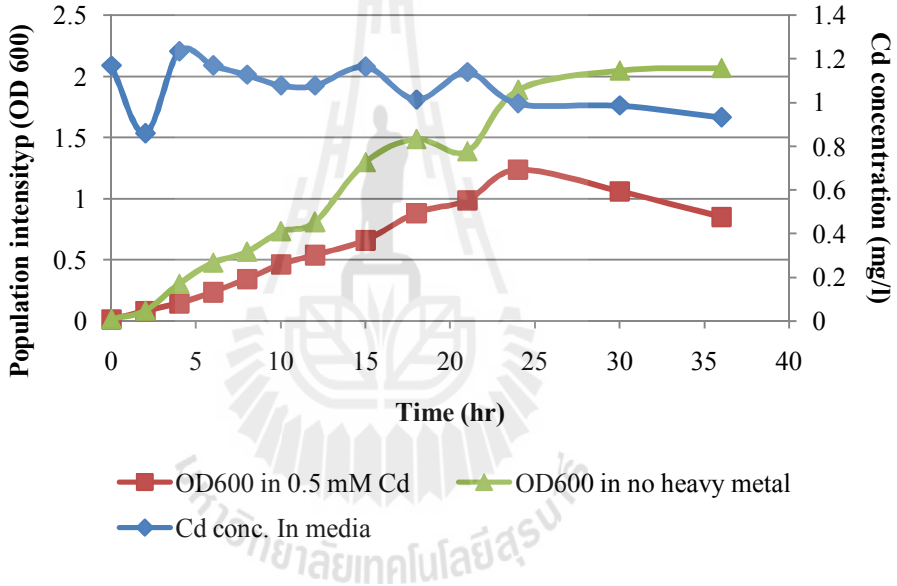


Figure 4.20 Heavy metal availability in soil consisting difference in the bacteria Bars of each heavy metal with different superscript are significantly different at $p < 0.05$.

4.10 Growth curve analysis

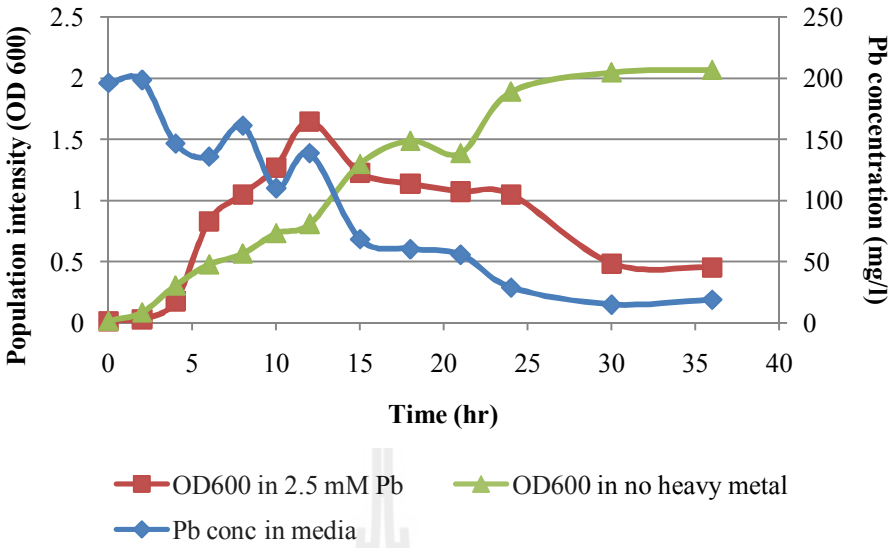
The 53S462 and the 65R471 were selected for the growth curve determination by measuring the optical density at OD600 in different heavy metal stress conditions. The aim of this experiment was to study the relationship of optical density at OD600 of the 53S462 and the 65R471 strains and heavy metal uptake at interval time. Each strain was cultivated in TSB medium supplemented with 0.5 mM Cd, 2.5 mM Pb, and 5.0 mM Zn. The culture was aseptically collected and determined cell density by spectrophotometer for 36 hours, heavy metal concentration in solution, and pH. The results in Figure 4.21 showed that 65R471 grew slightly in the presence of heavy metal. Under Cd and Zn stress condition, the growth pattern of 65R471 displayed exponential phases till 24 hours after incubation and then declined when cells entered the death phase (Figure 4.21). In contrast, Cd and Zn concentration in medium

moderately decreased until the end of observation. For Pb stress condition, the growth curve of 65R471 rapidly reached the exponential phased at 12 hours and the immediately slowed down similar to Pb concentration in the medium. The medium pH, similar to control, slightly increased as alkaline until 30 hours depending on heavy metal conditions. Slightly decreased pH was observed in Pb and Zn containing media after 30 hours, while the pH of media with Zn did not alter mechanism (Figure 4.21).

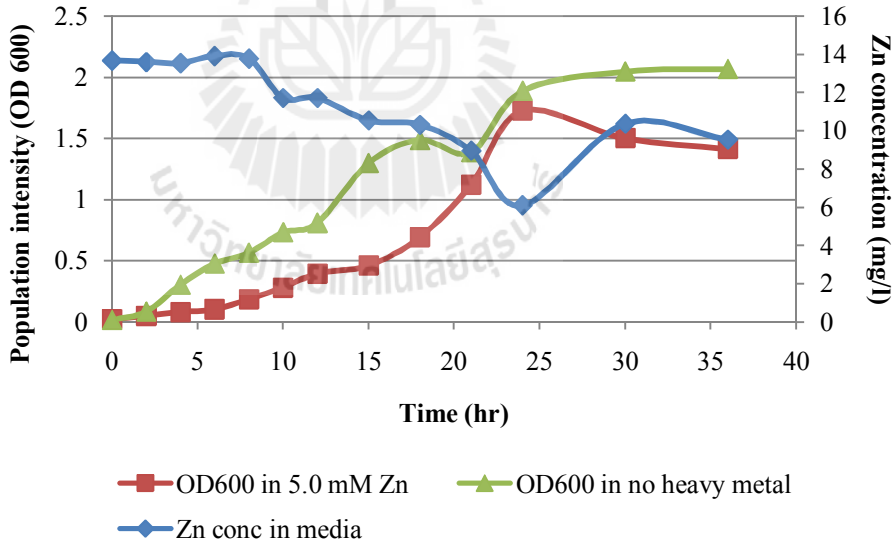


(A)

Figure 4.21 The relationship between 65R471 growth and heavy metal concentrations in the media as (A) Cd, (B) Pb, and (C) Zn.



(B)



(C)

Figure 4.21 (Continued) The relationship between 65R471 growth and heavy metal concentrations in the media as (A) Cd, (B) Pb, and (C) Zn.

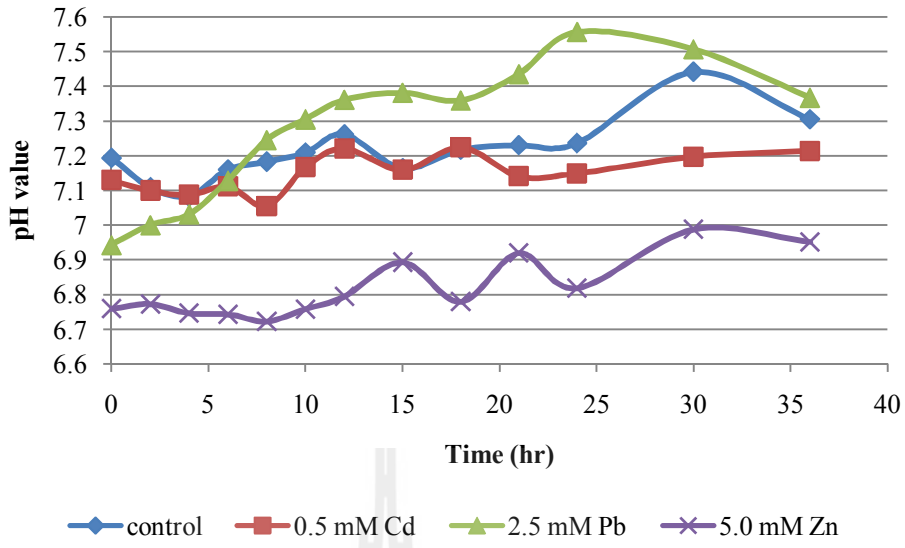


Figure 4.22 The pH of media inoculated with 65R471under heavy metal stress condition.

Similar to 65R471, the growth pattern of 53S462 under Cd and Zn stress condition showed increased cell density until 30 hours (Figure 4.23). The growth curves of 53S462 rapidly reached the exponential phase at 12 hours and immediately slowed down similar to the growth of 65R471in Pb treatment. The pH of the medium with Cd, and Pb were slightly alkaline, while the medium with Zn appeared neutral (pH 6.7-6.9). The medium pH of all treatments slightly increased to alkaline until 30 hours depending on the type of heavy metal condition. Decreased pH was observed after 30 hours as shown in Figure 4.24.

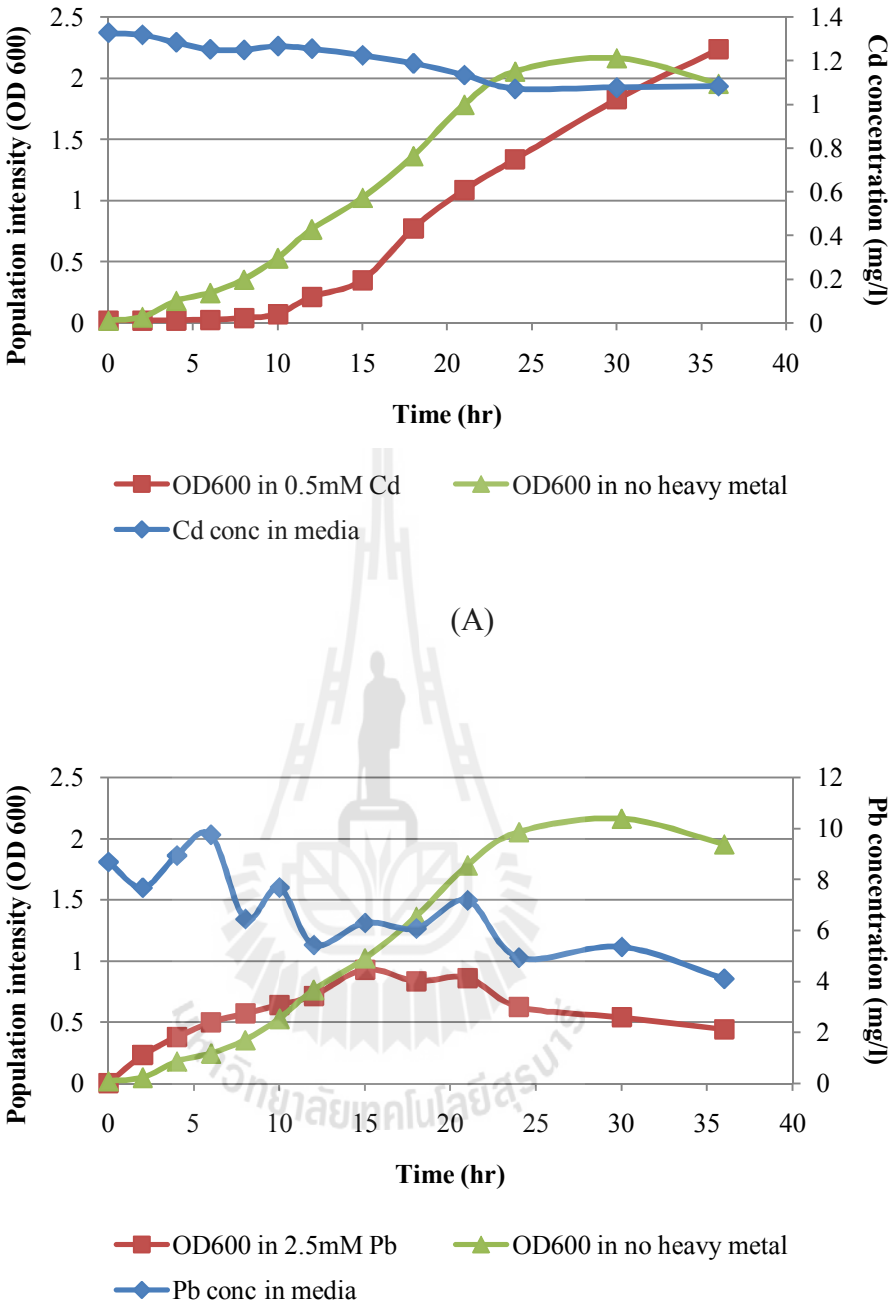
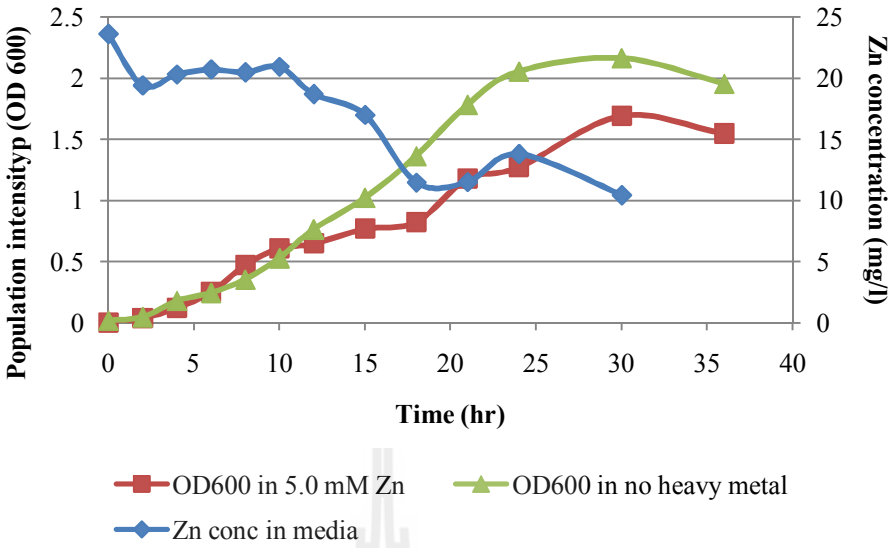


Figure 4.23 The relationship between 53S462 growth and various heavy metal concentrations in media as (A) Cd, (B) Pb, and (C) Zn.



(C)

Figure 4.23 (Continued) The relationship between 53S462 growth and various heavy metal concentrations in media as (A) Cd, (B) Pb, and (C) Zn.

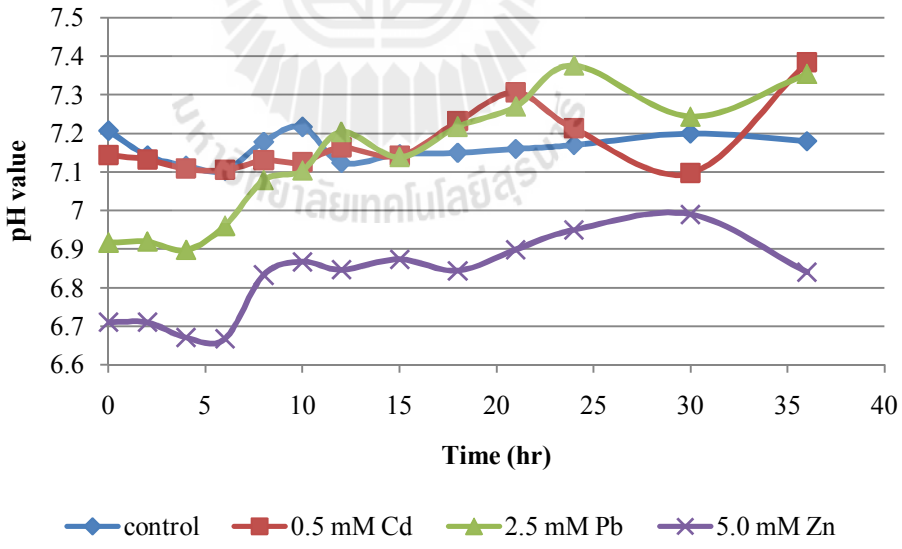


Figure 4.24 pH value of media inoculated with 53S462 in stress condition.

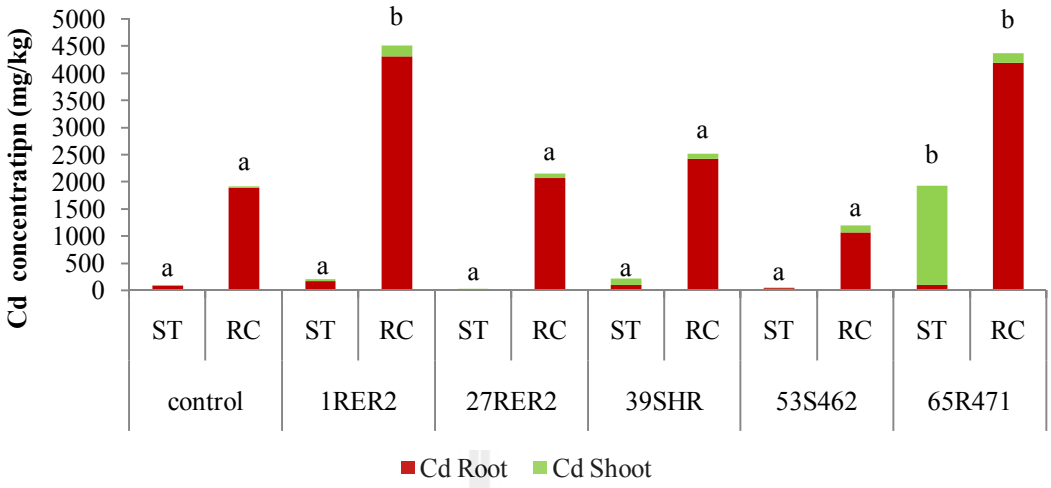
4.11 Effect of the selected PGPR on promoting heavy metal uptake by axenic vetiver

Tissue culture of vetiver grasses ecotype *V. zizanioides* and *V. nemoralis* were grown in axenic condition using Musashige and Skoog agar medium, supplemented with single heavy metal concentration, 0.5 mM Cd, 5.0 mM Zn, or 2.5 mM Pb. Then each culture was inoculated with individual 1RER2, 25R463, 27RER3, 39SHR, 53S462, or 65R471 bacterial cultures. The vetiver grasses were harvested at 14 days after heavy metal application and the concentration of heavy metals were determined in root and shoot parts using the GFAAS.

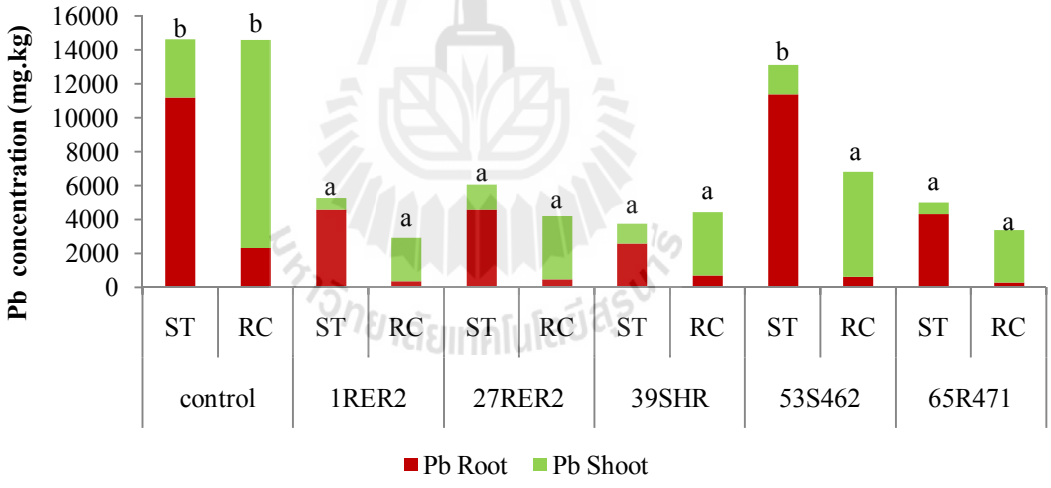
The *V. zizanioides* significantly accumulated higher Cd, Pb, and Zn in roots than shoots (Figure 4.25A-C). In addition, all inoculated PGPR promoted heavy metal uptake by root. The maximum accumulation of Cd in root was higher than shoot of *V. zizanioides* inoculated with all tested strains. The total Cd accumulation by 65R471 and 1RER2 strains was 6,084.03±4,813.78 and 4,247.47±1,706.00 mg/kg, respectively. Based on the bioconcentration of Pb in different compartments of plants, the metal TF at the treatment dose was ranging from 6 to 21 but not statically significant. The data revealed that all tested isolates did not enhance the Pb uptake of *V. zizanioides* compared to the uninoculated control. All inoculums showed bulk Zn contents in shoot which was higher than root. 1RER2 and 39SHR displayed the best potential for Zn accumulation per day (TAR). Compare to the uninoculated control, the total Zn uptake was significantly enhanced ($P < 0.05$) in the *V. zizanioides* with all inoculated test strains.

In *V. nemoralis* (Figure 4.25A), inoculated 65R471 significantly enhanced the uptake of Cd in shoot (133.65±105.38 mg/kg). The total Cd concentration under

inoculation of 1RER2 and 65R471 were higher than the others. The results from the comparison of Pb content between root and shoot (Figure 4.25A) suggested that among tested isolates, 39SHR could stimulate the movement of Pb from root to shoot as observed by the high TF value. Similar to *V. zizanioides*, PGPR inoculated *V. nemoralis* did not enhance Pb uptake compared to the uninoculated control. Similar to Pb, Zn content in root could be observed in all PGPR inoculated vetiver grass grown under axenic condition. However, TF values of vetivers inoculated (Figure 4.25C) with 27RER3, 39SHR, and 65R471 were 90.11±33.95, 92.70±38.26, and 83.53±56.55, respectively. Though not statically significant, the high Zn TAR value of 25.76±0.66 could be observed after 65R471 inoculation. When compared to uninoculated control, the total Zn uptake by *V. nemoralis* was not different in all tested strains, though was a trend of enhancement in 65R471 inoculation was observed (Figure 4.25C).

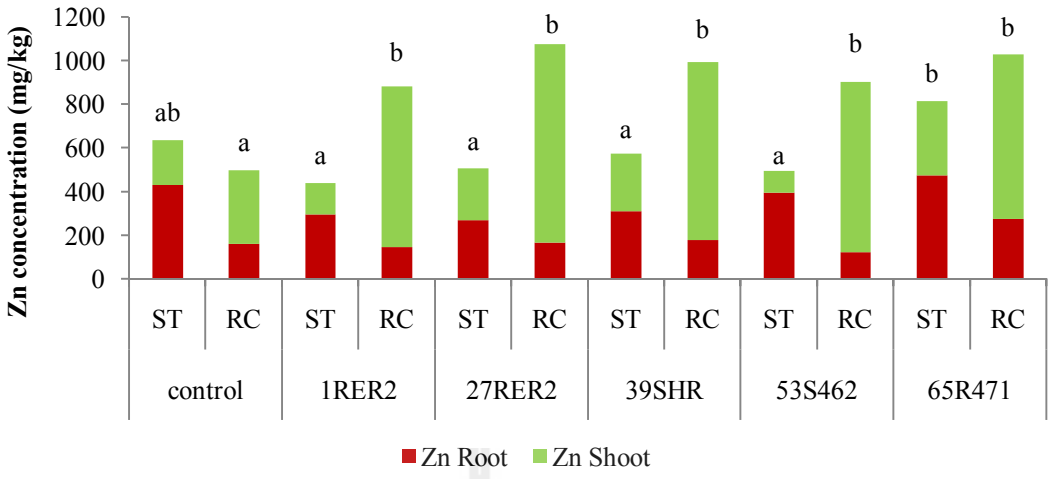


(A)



(B)

Figure 4.25 Total heavy metal uptake of *V. zizanioides* (RC)- and *V. nemoralis* (ST) inoculated with the selected bacteria and grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significantly different at $p < 0.05$.



(C)

Figure 4.25 (Continued) Total heavy metal uptake of *V. zizanioides* (RC)- and *V. nemoralis* (ST) inoculated with the selected bacteria and grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significantly different at $p < 0.05$.

4.12 The colonization of heavy metal tolerated bacteria on vetiver grass

In the presence of 10^8 CFU/ml of each selected PGPR strain, the colonization of bacteria around the *V. zizanioides* and its chlorophyll content was determined after 15 days of inoculation. The 65R471 and 53S462 isolates could colonize around *V. zizanioides* upto 1.4×10^7 CFU/g FW. In addition, they could promote total chlorophyll production of *V. zizanioides* (Table 4.13). The colonization of the selected bacteria on the root of *V. zizanioides* was also photographed under scanning electron microscope as shown in Figure 4.26.

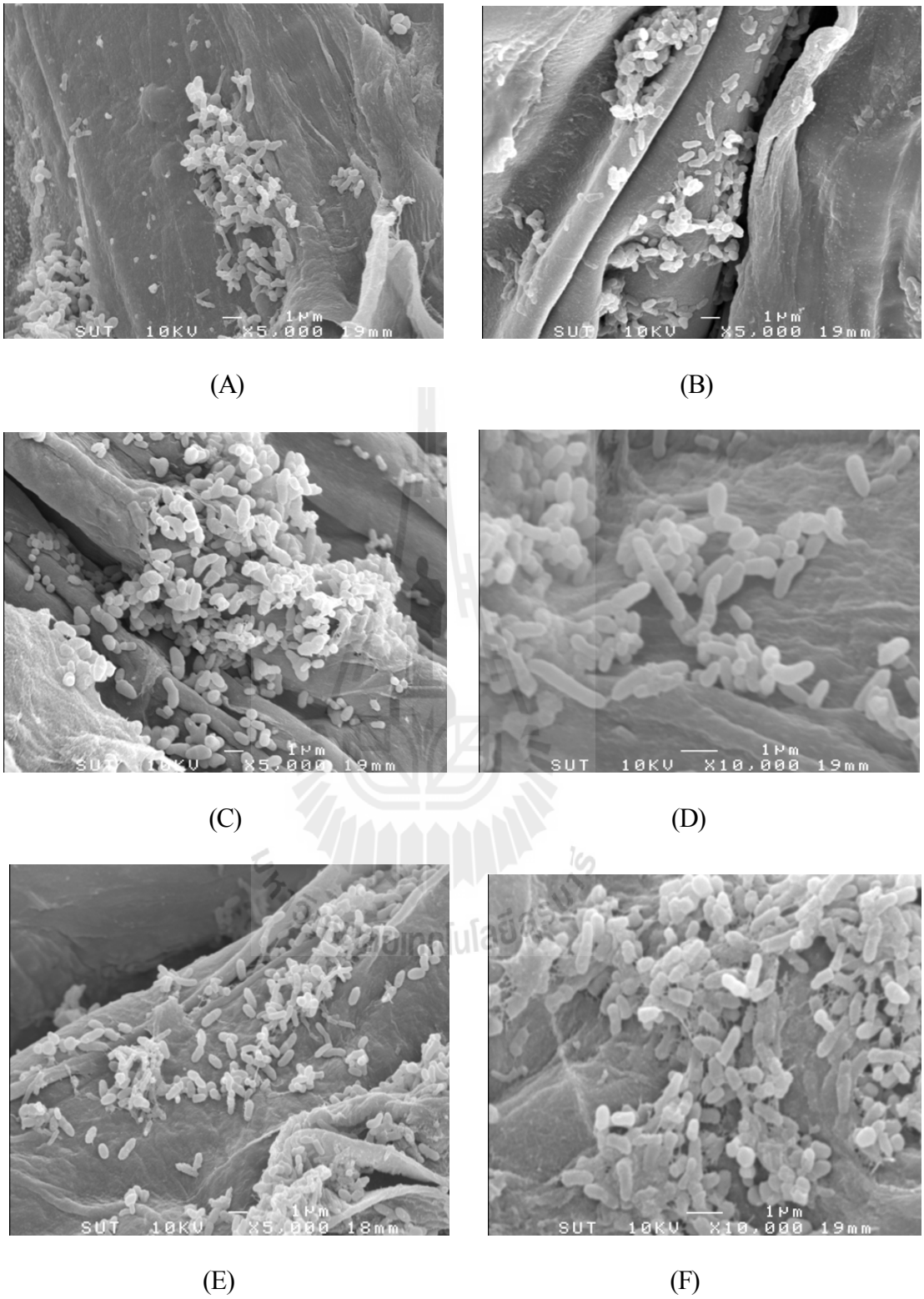


Figure 4.26 Scanning electron microscope depicted the colonization of (A) 1RER2, (B) 25R463, (C) 27RER3, (D) 39SHR, (E) 53S462, and (F) 65R471 at root of *V. zizanioides.*

Table 4.13 The number of heavy metal tolerated PGPR possessing colonizing capability at vetiver root and the plants' chlorophyll content.

Strain	Log CFU/g DW	Chlorophyll content (mg/g FW)		
		Chl a	Chl b	Total chl
1RER2	7.75±0.1[a]	0.29±0.17[a]	0.09±0.06[a]	0.39±0.23[a]
25R463	7.68±0.78[a]	0.26±0.07[a]	0.09±0.02[a]	0.35±0.10[a]
27RER3	7.97±0.53[a]	0.33±0.13[a]	0.11±0.04[a]	0.45±0.18[ab]
39SHR	8.17±0.11[a]	0.23±0.15[a]	0.08±0.003[a]	0.32±0.01[ab]
53S462	9.26±0.16[b]	0.15±0.10[a]	0.05±0.04[a]	0.21±0.14[ab]
65R471	9.12±0.25[b]	0.40±0.29[b]	0.14±0.09[b]	0.54±0.39[b]

4.13 Effect of the selected PGPR on promoting vetiver growth under heavy metal stress in pots

The experimental soil was sandy loam, pH 7.8, EC 2.79 dS/m, total N 0.07%, total P 200 mg/kg, and total K 2000 mg/kg. PGPR-inoculated and uninoculated *V. zizanioides* was subjected to mixed heavy metal concentration of Cd (33.57 mg/kg), Pb (574.47 mg/kg), and Zn (238.90 mg/kg) in artificial soil for 30 days. The result suggested that inoculation of 65R471 and 53S462 to *V. zizanioides* only enhanced the Zn uptake and had no effect on Cd and Pb uptake, compared to the uninoculated control (Figures 4.27-4.28). The inoculated plants exposed to stress concentration of Cd, Pb, and Zn increased their chlorophyll content compared to uninoculated control.

In the presence of heavy metal, both 65R471 and 53S462 significantly increased total chlorophyll of *V. zizanioides* compared to uninoculated condition (Table 4.14). However, uninoculated showed the higher TF in Pb and Zn. No significant root colonization of 53S462 and 65R471 were observed.

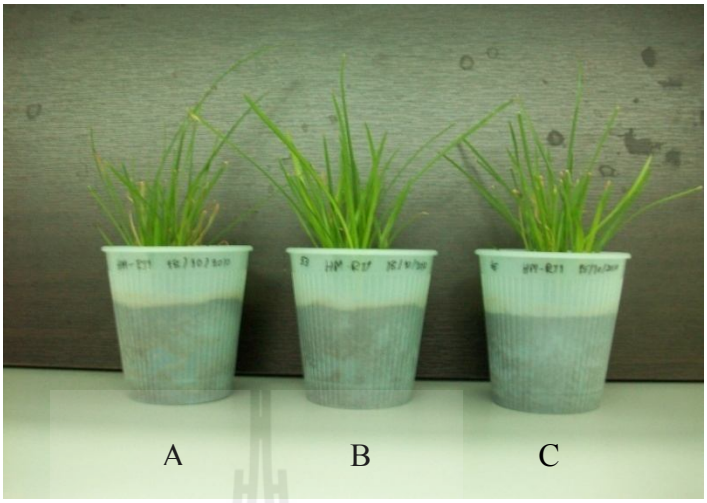


Figure 4.27 An efficiency of heavy metal uptake by *V. zizanioides* (A) uninoculate, (B) inoculated with 53S462, and (C) inoculated with 65R471.

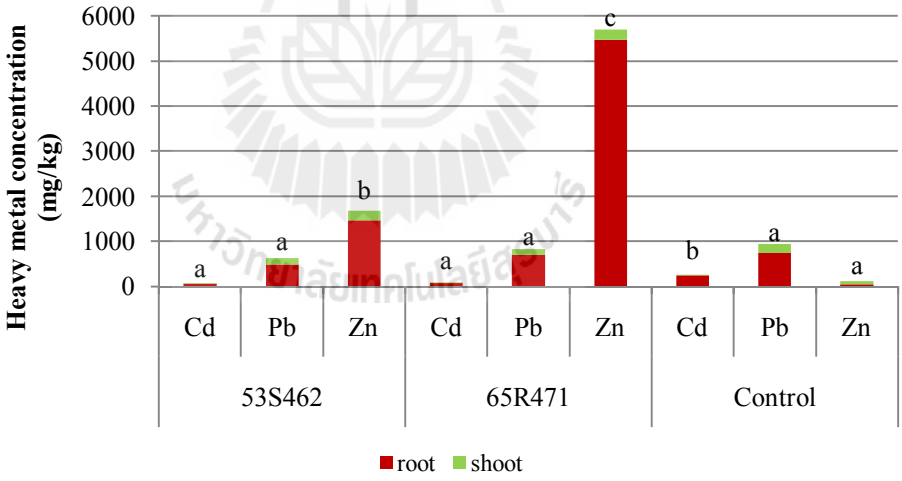


Figure 4.28 Total heavy metal uptake of *V. zizanioides* inoculated with the selected bacteria grown under mixture of heavy metal contaminated soil. Base of each heavy metal with different superscript are significantly different at $p < 0.05$.

Table 4.14 Heavy metal uptake and chlorophyll production.

Strain	Translocation and Accumulation efficiency						Log CFU/g DW	Chlorophyll content (mg/g FW)		
	Cd		Pb		Zn					
	TF*	TAR**	TF	TAR	TF	TAR		Chl a	Chl b	Total chl
Uninoculate	8.29±2.37[a]	0.0014±0.0002[b]	73.28±113.49[a]	0.006±0.001[a]	174.93±6.27[b]	0.004±0.002[a]	-	0.38±0.18[a]	0.17±0.02[a]	0.54±0.26[a]
53S462	22.69±1.58[ab]	0.0007±0.0001[a]	26.91±16.01[a]	0.006±0.003[a]	15.52±0.59[a]	0.012±0.004[a]	8.91±0.17[a]	0.47±0.09[b]	0.22±0.07[b]	0.65±0.12[b]
65R471	46.84±31.78[b]	0.0010±0.0004[ab]	17.53±4.07[a]	0.007±0.004[a]	6.21±2.95[a]	0.034±0.03[a]	8.79±0.29[a]	0.60±0.24[c]	0.15±0.07[c]	0.82±0.31[c]

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF= $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

CHAPTER V

DISCUSSION

Mining activities and their resulting wastes, mine tailings created a complicated problem globally. A raising concern regarding human health risks and environmental consequences associated with these tailings have created a need for efficient and effective remediation strategies. phytostabilization is a term applied to a group of inexpensive and friendly remediation technologies that use plants to reduce, remove, degrade or immobilize xenobiotics which could clean up the heavy metal-contaminated soil (Sekhar *et al*., 2005; Fischerova´ *et al*., 2006). However, slow growth and low biomass of plants in heavy metal-contaminated soil may limit the efficiency of phytoremediation (Kumar *et al*., 1995; Burd *et al*., 2000) due to the inability of plants to survive or perform under undesired conditions including; low nutrient availability, poor soil structure, severely impacted heterotrophic microbial communities, extreme pH values, and high contaminant concentrations. Several scientists revealed that these conditions could be mitigated to some extent by application of plant growth promoting microorganisms to stimulate plant growth under stress condition. This success has inspired research to investigate whether PGPR, screened from heavy metal contaminated sites in Thailand, could be used to overcome limitations to plant growth in heavy metal remediation or restoration applications or otherwise. The selected native PGPRs were collected and their enhancing effect on phytoremediation was studied through inoculation to vetiver

grass. This experiments chose vetiver grass ecotypes *V. zizanioides* and *V. nemoralis* as the tested plants for the bacterial assisted- phytoremediation due to their fast growth and high biomass under highly tolerance to extreme environmental variations including prolonged drought, flood, submergence, extreme fluctuation of temperature (22°C-60°C), soil pH (3.0-10.5) and most importantly high resistance to heavy metal toxicities.

In the experiments, the effects of PGPR traits were determined in the sterile laboratory scale, in which the interaction of PGPR traits on *V. zizanioides* and *V. nemoralis* could be stimulated by Cd, Pb, and Zn application. The different vetiver ecotypes showed the various effects of PGPR traits. AVG, IAA, and pyrocatechol were the proper choices for Cd uptake on *V. zizanioides* while N, IAA, and AVG were suitable for *V. nemoralis* compared to control. Zn uptake by *V. nemoralis* was stimulated by Desferroxamine, GA_3, IAA, AVG, pyrocatechol, and zeatin while phosphate was suitable for *V. zizanioides*. In addition, the different PGPR traits effects on Pb accumulation of both ecotypes suggested that GA_3, and IAA were suitable for Pb uptake of *V. zizanioides* while AVG, DFB, and GA_3 enhanced Pb uptake in *V. nemoralis*. Belimov *et al.* (2001) also reported that AVG, the chemical inhibitors of ethylene biosynthesis, inhibited ACC accumulation in plants which exposed to solutions containing Cd^{2+} brought a significant improvement in the plant growth. Pyrocatechol and desferroxamin as sub-type of siderophore were low molecular mass iron chelators with high association constants for complexing iron and also form stable complex with other heavy metal such as aluminium, Cd, copper, Pb, and Zn. Therefore they promoted the iron uptake by plants in the presence of heavy metal (Dimkpa *et al*., 2009). Wang *et al*. (2007) reported that IAA increased Pb

accumulation in roots, but significantly decreased Pb accumulation in shoots of maize (*Zea mays* L. cv. TY2) seedlings. Similarly, L´opez *et al.* (2004) reported that 0.2 mM Pb plus 10 mM gibberellic acid increased Pb concentration in alfalfa (*Medicago sativa* L.) roots by about 40% compared to treatment with Pb only. The summary could be pronounced that IAA, GA_3, and phosphate demonstrated the indirect effects on Pb accumulation because IAA is a molecule that promotes apical dominance, tropism, stem elongation, and root formation among others, while GA_3 is related to germination and flowering processes. As results, AVG, IAA, GA_3, pyrocatechol, desferroxamine, and phosphate were chosen for PGPRs criteria used in further experiment.

The comparison of heavy metal uptake by two vetiver ecotypes found that *V. zizanioides* and *V. zizanioides* had no significance for heavy metal uptake. Similarly, Roongtanakiat and Chairoj (2003) reported that *V. zizanioides* had significantly higher Mn, Zn, and Cd amounts in shoot and root than *V. nemoralis* and Kamphaeng Phet ecotypes on pot experiment.

The native heavy metal resistant bacteria from Phadaeng Industry Public Company Limited, Tak Province, Thailand have been isolated based on AVG, IAA, GA_3, pyrocatechol, desferroxamine, and phosphate as key selectors. About 521 colonies mostly observed in PSA had capability for producing PGP traits. However, only 6 isolates as 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 were collected to assess the ability for AVG, IAA, GA_3, pyrocatechol, desferroxamine, phosphate solubilization, MIC determination, the impact of heavy metal effects on PGP traits production, soil availability, bioabsorption, and antibiotic resistance. Although a number of studies have demonstrated the importance of bacterial

inoculation for plant growth and heavy metal accumulation in heavy metal-polluted environments (Abou-Shanab *et al*., 2003; Idris *et al*., 2004; Khan, 2005; Sheng and Xia, 2006), to the best of our knowledge, this was the first research report that elucidated the effect of heavy metal on PGP traits production by the rhizobacteeria and the efficiency of heavy metal uptake by vetiver grasses (*V. zizanioides* and *V. nemoralis*) with concurrent promotion of plant growth by the selected PGPR in a pot experiment.

A further quantitative analysis was performed for six of the above PGP activities; ACC deaminase, IAA, GA_3, pyrocatechol, desferroxamine production and phosphate solubilization on heavy metal stress. Although the presence of Pb and Zn except Cd in the growth medium did not affect the ability of the bacterial strains to utilize ACC as the sole nitrogen source, it did influence their growth rate (Figure 4.4). However, the ACC activity was markedly decreased as compared with the control. ACC activity of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 could not be observed in Cd stress. Consistently, Amico, Cavlca, and Andreoni (2005) concluded that the effect of Cd^{2+} on ACCD activity in the ACCD assay reaction mixture depended on the strain. For example, Cd influenced the ACCD activity of *Alcaligenes* sp. ZN4 and Mycobacterium sp. ACC14, but not of *P. fluorescens* ACC9 and *P. tolaasii* ACC23. The acdS gene found in *Pseudomonas* sp. was 6G5 (Klee *et al*., 1991) in spite of having more than one type of ACCD gene (Blaha *et al*., 2006). Moreover, Shah *et al.* (1998) provided a preliminary evidence that some PGPR may contain more than one enzyme that could cleave ACC and some of these enzymes were quite different from the reported ACCD. In control of this experiment, ACC activity of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 were 128.93,

126.42, 854.05, 1472.43, and 1266.62 µmol/hr/mg, respectively. ACC deaminase production of this experiment was quite higher as comparing with Rajkumar and Freitas (2008). They reported that *Pseudomonas* sp and *Pseudomonas jessenii* produced ACC deaminase as 66.32 and 34.23 nmol/hr/mg.

In terms of IAA production, the selected strains have the capability to produce IAA both with or without the supplementation of tryptophan (Figures 4.7-4.9). However, IAA concentration with tryptophan was higher than in the absence of tryptophan. Similarly, Zaidi, Usmani, Singh, and Musarrtat (2006) estimated IAA production in presence and absence of typtophan. They concluded that the bacteria produced 55 µg/ml IAA in the presence of tryptophan, and 21 µg/ml without tryptophan. Thakuria *et al.* (2004) reported the comparable levels of IAA production, ranging from 2.0 to 21.6 mg/l in culture supernatants from soil rhizosphere bacteria. These isolates were supplied with substantially higher amounts of L-tryptophan. Rajkumar and Freitas (2008) reported that *Pseudomonas* sp and *Pseudomonas jessenii* produced 17.74±2.06 and 39.88±3.68 mg/l of IAA amended with 500 µg/ml tryptophan, respectively. In addition, Wani, Khan, and Zaidi (2007) reported that the *Bacillus* strains PSB 1, PSB 7, and PSB 10 produced 19.3, 17.7, and 17.4 µg/ml of IAA, respectively. Although optimal production of IAA by bacteria was achieved under tryptophan induction. The possibility to produce IAA under the absence of added inducing substances (typtophan) was of interest for subsequent application of these bacteria in pot experiment where plant growth and release of auxin-inducing root exudates, was limited by metal stress and attendant poor soil fertility. In heavy metal stress, high amounts of IAA were detected in control treatments in all strains. In contrast, the IAA production in the presence of Cd, Pb, and Zn was reduced and the

detectable levels of IAA which supported by Dimkpa *et al.* (2008). Relatively, Wani, Khan, and Zaidi (2007) reported that Bacillus strains PSB 1, PSB 7, and PSB 10 consistently decreased IAA production with an increase in chromium concentration. These results excluded the possibility that the formation of IAA-metal complexes (Oota and Tsudzuki, 1971) led to decreasing amounts of free IAA.

The results from the study of the effect of Cd, Pb, and Zn on 2 sub-types of siderophores, hydroximate- and catecholate siderophores, showed that the selected strain produced both catecholate- and hydroxymate siderophores which corresponded with the finding of Ma, Rajkumar, and Freitas (2009). They reported that the bacteria produced more catecholate than hydroxymate. Catecholate siderophores were produced more in stressful conditions. Similarly, Cd-induced pyoverdin production in the *Pseudomonas aeruginosa* strain KUCd1, which also showed unusually high resistance to cadmium levels of up to 8 mM (Sinha and Mukherjee, 2008). Siderophores were also produced significantly and more actively under Cd-stress by the isolates. The levels of siderophore production ranging from 56-140 mg/l were comparable to those produced by soil rhizobacteria (Alexander and Zuberer, 1991). In addition, the influence of zinc in stimulating pyoverdine production by *P. aeruginosa* has also been reported (Hoefte *et al*., 1994). Moreover, zinc has also been found to increase the production of the siderophore pyochelin in *Azotobacter vinelandii* (Cornish and Page, 2000). The possible explanation for the stimulating effect of heavy metals on siderophore production has been suggested by Dimpa *et al.* (2008). They reported that the increased production of siderophores by *Streptomyces* in the presence of metals could be explained by the fact that metal ions competed for siderophore binding with the trace amounts of iron present, necessitating increased

siderophore production to obtain equivalent levels of iron to circumvent, or at least alleviate, metal-induced Fe deficiency.

The effect of heavy metal on the potential for phosphate solubilizing in media were determined. The amount of soluble phosphate releasing in liquid medium due to solubilization of tri-calcium phosphate have been detected as shown in Figure 4.10. The bacteria in control released more soluble phosphate than those under heavy metal stress. However, the results showed that less phosphate solubilization stimulated by the selected PGPRs compared to the other investigator. It might be concomitant with pH of media which slightly decreased in the presence of the selected PGPRs. Rajkumar and Freitas (2008) reported that the solubilization of insoluble phosphate by *Pseudomonas* sp. PsM6 and PjM15 were 7.11 ± 3.26 and 88.67 ± 4.46 mg/l, respectively. Wani, Khan, and Zaidi (2007) reported that the *Bacillus* strains solubilized 375 (PSB 1), 340 (PSB 7), and 379 (PSB 10) µg/ml phosphate, respectively, in Pikovskaya broth absence of chromium. At the presence 150 µg/ml chromium, the amount of P solubilized was reduced by 17 (PSB 1), 15 (PSB 7), and 9% (PSB 10) compared to control. The data showed the increase of soluble phosphate which a concomitant with reduction in pH, dues to the release of organic acid. Zaidi, Usmani, Singh, and Musarrat (2006) reported that the bacterial strain PS-1 released 120 µg/ml of soluble phosphate and the extreme drop of pH from 7.5 to 4.8. It means that the phosphorus deficiency in soil could be compensated with the inorganic phosphate-solubilizaing ability of PGPR. Halstead *et al.* (1969) also suggested the solubilization of inorganic phosphates could facilitate the heavy metal uptake from soil.

The study of selected PGPR capability in heavy metal solubilization (Figure 4.12) revealed that bacterial strain 65R471 was the most active strain in the release of Cd, and Zn from the non-soluble phases in the soil. In addition, 39SHR was the most effective strain in Pb solubilization. In spite of the effective phytoextraction that mainly depended on the plant itself and the interaction of plant roots with bacteria and on the bioavailability of heavy metals in the soils. Rajkumar and Freitas (2008) reported that the higher water soluble Zn induced by *Pseudomonas* sp. PsM6 inoculation resulted in a correspondingly higher Zn accumulation in both the shoots and roots of *R. communis*. This suggested that the bioavailability of Zn was increased through bacterial metabolic activities or their interactions with the plants.

The size of inhibition zone which normally indicated the relative Cd, Pb, and Zn resistance ability of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R47 was shown in Table 4.8. The highest Cd, Pb, and Zn MIC could be observed at 3.5 mM, 9.0 mM, and 10 mM, respectively. It means that the selected strains could be high tolerated to Zn as compared with Pb and Cd. The results were supported by Raja, Anbazhagan, and Selvam (2006) who reported the order of resistance to metals by *Psudomonas aeruginosa* BC15 was found that Pb > Ni > Cd > Cr on the agar plate. Also Jiang, Sheng, Qian, and Wang (2008) reported the order of metal toxicity of strain J62 using the plate dilution method as follows chromium > nikel > copper > Zn > Pb > Cd. The different degree of resistance to Cd, Pb, and Zn of the selected stains was quite high when compared with the other experiments. In other studies, He *et al.* (2010) reported that the bacterial strains SWJ11, MT16, GZC24, and YAH27 could be resistant to Cu, Pb, Cd, Ni, and Zn on the plate agar method with the capability of heavy metal resistance bacteria was lower than in this study. He reported a metal

tolerance of 0.09 mM- 0.45 mM for Cd, 0.5 mM - 4.4 mM for Pb and 0.77 mM - 6.15 mM for Zn. On the other hand, the liquid media for heavy metal tolerance was conducted as described by Amico, Cavalca, and Andreoni (2005). They concluded that *P. flurescens* ACC9, *P. tolaasii* ACC23, *Mycobacterium* sp. ACC14, and *Alcaligenes* sp ZN4 had high Cd-resistance and their MIC varied from 0.5 to 2.5 mM. However, the MICs of the heavy metal in solid media were higher than those in liquid media due to the conditions of diffusion, complexation and availability of metals which were different from those observed in solid media. The microbial resistance to heavy metal was attributed to a variety of detoxifying mechanisms developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc. These mechanisms were sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another. In Table 4.11, 53S462 and 65R471 effectively showed the resistance ability in various antibiotic-supplemented media. The result was supported by Verma *et al.* (2001) who concluded that metal tolerance held an association with antibiotic resistance. It clearly indicated a correlation between antibiotic resistance and metal tolerance. The high degree of antibiotic resistance might be associated with higher levels of tolerance of various heavy metals (Rosen, 1996; Hassen *et al.*, 1998). The occurrence of this phenomenon could be attributed to the clustering of tolerant genes in the same plasmid as described by Adarsh *et al.* (2007). Similar to Ghosh *et al.* (2000) reported that plasmids from *Salmonella abortus equi* strains were resistant to ampicillin, arsenic, chromium, cadmium and mercury.

In addition, the selected PGPR exhibited a high degree of metal biosorption potential for Cd and Pb (Table 4.12). Consequently, bacteria have a high surface area

to volume ratio (Beveridge, 1988) and, as a strictly physical cellular interface, should have a high capacity for sorbing metals from solutions (Mullen *et al*., 1989). Several investigations have shown that the relatively large quantities of metallic cations were complexed by bacteria (Samuelson *et al*., 2000). With this intrinsic characteristic, the selected PGPR may also contribute in reducing the phytotoxic effects of the metals by sharing the metal load due to its demonstrated ability of biosorption and bioaccumulation (Zaidi and Musarrat, 2004). Despite of this, the selected strains dramatically showed the highest Zn tolerance but the biosorption was opposed high with the maximum biosorption capacity for Zn ranging from 4.0 to 9.0 mg/kg. Vivas, Biro, Ruiz-Lozano, Barea, and Azcon (2006) reported that *Brevibacillus* cells accumulated only a 5.6% of Zn from a culture medium supplemented with 267 µg/l of Zn^{2+}. The selected strains might be demonstrated for Zn resistance through efflux process which is the most common mechanism of resistance in prokaryotes by the action of P-type ATPases or secondary efflux systems (Paulsen and Saier, 1997). To pump excess zinc ions out of the cell, *E. coli* used a P-type ATPase named ZntA (Beard, Hashim, Membrillo-Hernandez, Hughes, and Poole, 1997; Rensing, Mitra, and Rosen, 1997). The expression of the zntA gene, in turn, was regulated by ZntR, a zinc-responsive regulatory protein (Brocklehurst *et al*., 1999). Proteins with similar functions were identified in the cyanobacterium Synechocystis PCC6803 with ZiaA, a P-type ATPase involved in increased zinc tolerance, and ZiaR, a Zn2- responsive repressor (Thelwell, Robinson, and Turner-Cavet, 1998).

For the effects of the selected strains on promoting heavy metal uptake by both vetiver ecotypes, the experiment was done in laboratory scale as similar to the other experiments. The utilization of sterilized substrates provided a valuable data regarding

accurate effects of an individual strain, so it would not contaminate the other strains. The stimulation of individual Cd, Pb, and Zn plant uptake by1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 inoculation to different ecotypes of vetiver showed the different effect of heavy metal plant uptakes. Similarly, Peeiulyte, Repeekiene, Levinskaite, and Lugouskas (2006) proposed the different strategies for heavy metal uptake by maize plants and vetch plants.

Besides, the ability of 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 to produce PGPR traits in enhancing heavy metal uptake by vetiver, the others mechanism for enhancing the efficiency of phytoremediation was an ability of root colonization and an ability to immobilize heavy metals in nutrient media and soils. Similarly, the successful plant growth promoting inoculants, bacteria must be able to rapidly colonize the root system during plant growth (Defreitas and Germida, 1992). Screening studies for determining root colonization and chlorophyll production was performed under laboratory conditions using surface-sterilized vetiver grass under sterile conditions. Consequently, these isolates undoubtingly enhanced one aspect or another of plant growth under controlled conditions. In the study, all selected strains were able to colonize, especially 53S462 and 65R471 have very high capacity for root colonizing. In addition, vetiver grass inoculated with strain 65R471produced higher total of chlorophyll ($p < 0.05$) in axenic experiment. Defreitas and Germida (1992) demonstrated that strain J62 facilitated maize and tomato growth, but could not increase the growth of the Indian mustard in pot experiment. Their result showed that strain J62 was able to colonize and develop in the rhizosphere soil of maize and tomato after root inoculation and was able to promote the growth of maize and tomato. Being a successful plant growth promoting inoculants the bacteria must be

able to rapidly colonize the root system during the growing season. Root colonization was a prerequisite for successful inoculation of PGPR was also suggested by Bashan and de-Bashan (2005) and Lucy *et al.* (2004). Accordingly, some PGPR may display desirable growth promotion mechanisms but remain ineffective due to their inability to colonize and/or compete in the rhizosphere of host plants (Benizri *et al*., 2001).

In pot experiment, strain 53S462 as *Pseudomonas putida* and 65R471were chosen to inoculate with *V. nemoralis*. The selected strains had the characteristics of producing IAA, siderophores, ACC deaminase and inorganic phosphate solubilization under stressed condition. In addition both strains also showed very high degree of resistance to heavy metals such as Cd, Pb, and Zn and exhibited antibiotic resistance characteristics to neomycin (30 μg), tetracycline (30 μg), chloramphenical (30 μg), colistin sulphate (10 μg), streptomycin (10 μg) and erythromycin (15 μg). Moreover, strain 53S462 and 65R471 was able to colonize the root of vetiver grass in sterile condition as shown in Table 4.13. Therefore the ability of 65R471 for root colonization was higher than 53S462 in axenic experiment. Contrary to the laboratory experiment, 53S462 showed slightly higher root colonization than 65R471 in pot experiments. This difference might involve soil properties which were attributed to adverse colonization of 65R471. Soil moisture content following inoculation may also play a role in root colonization (Burr *et al*., 1978; Oliveira *et al*., 2004). The different results between under axenic cultures and in pots were also observed when prospective PGPB were used to enhance arsenic accumulation (Jankong *et al*., 2007). Although 53S462 showed better capabilities in root colonizing than 65R471, 65R471 may also be used for heavy metal stabilization because all the heavy metal contained was concentrated in the bulk of root part. The effectiveness of 65R471 for heavy

metal soil availability may promote heavy metal uptake. As discussed by Dell'Amico *et al.* (2008), the application of strain J62 effectively increased the bioavailability of Pb and Cd in the rhizosphere soils and promoted the growth of maize and tomato plants and subsequently resulted in increasing the total Pb and Cd uptakes of the plants even under non-sterile conditions. Thus, with the innate capability of expressing multiple traits, the strain of 65R471 might serve as an effective metal sequestering and growth promoting bioinoculant for plants in Pb-, Zn-, and Cd-stressed soil. Compared to the uninoculated *V. zizanioides*, significant decreased in the shoot Pb-, Zn-, and Cd concentration of the inoculated *V. zizanioides* was observed, indicating that Pb-, Zn-, and Cd was mainly accumulated in the root of the inoculated *V. zizanioides*.

CHAPTER VI

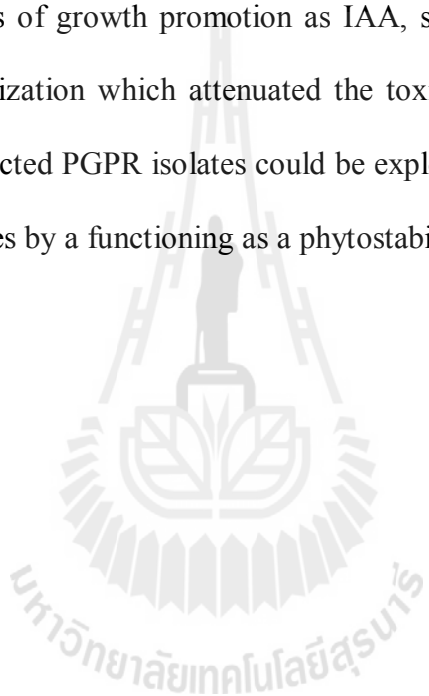
CONCLUSIONS

In the present study, the heavy metal resistant bacteria from Phadaeng Industry Public Company Limited, Tak Province, Thailand was isolated and characterized for their appropriate PGPR traits particularly increased plant biomass and improved plant efficiency for Cd, Zn, and Pb removal. Vetiver ecotypes were chosen as tested plant in this experiment because of high biomass production and fast growth. The ability of Cd-resistant bacteria to promote growth and Cd uptake of vetiver in highly heavy metal contaminated soil may indicate the potential role of the bacteria in enhancing phytoremediation. The representative of upland and lowland vetiver was similar phytoremedial activity in heavy metal removal.

The 521 isolates of 50 mg/l Cd-resistant bacteria were obtained from Phadaeng Industry Public Company Limited, Tak Province, Thailand and were screened and selected based on PGPR traits, including, ACC deaminase, siderophore, IAA, GA_3, zeatin, phosphate and nitrogen production. Six isolates as 1RER2, 25R463, 27RER3, 39SHR, 53S462, and 65R471 which displayed high PGPR traits activity for AVG, IAA, GA_3, pyrocatechol, desferroxamine and P production in Cd, Pb, and Zn stress condition. In addition the 6 isolates were characterized by determining their MIC, soil availability, bioabsorption, antibiotic resistance, and root colonization on the vetiver grass. Among tested isolates 53S462 and 65R471 are found to be the best PGPR traits

production, high bioavailability and root colonizing capability and were chosen for the subsequent pot experiment.

Pot experiment demonstrated that the application of the bacterial strain 65R471 could significantly enhance *V. zizanioides* ecotype biomass, chlorophyll content and heavy metal uptake. However, the *V. zizanioides* ecotype root was the bulk site for heavy metal accumulation. This experiment suggested that the bioinoculant due to its multifarious properties of growth promotion as IAA, siderophores, ACC deaminase and phosphate solubilization which attenuated the toxic effects of Cd, Pb, and Zn. Consequently, the selected PGPR isolates could be exploited for remediation of metal from contaminated sites by a functioning as a phytostabilization for plant.



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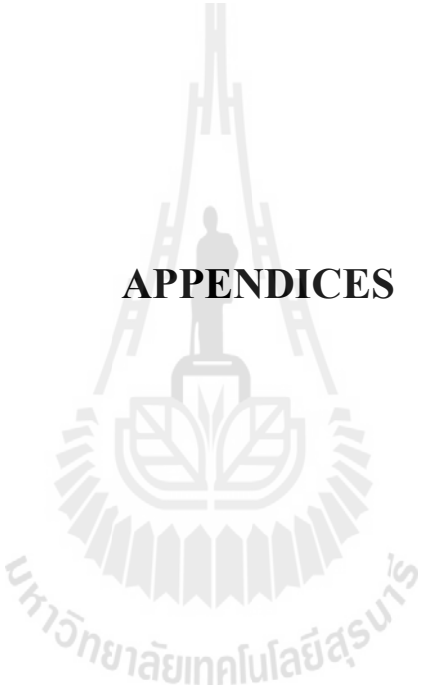
APPENDICES



APPENDIX A

CULTURE MEDIA AND REAGENT PREPARATION

A.1 Culture media for PGPR traits

A.1.1 Fe-free Hoagland's Nutrient

Component	Stock Solution (g/l)	ml Stock Solution/l
Majors:		
2M KNO_3	202	2.5
$Ca(NO_3)_2$. $4H_2O$	472	0.5
2M $MgSO_4.7H_2O$	493	1.0
1M NH_4NO_3	80	1.0
Minors:		1.0
H_3BO_3	2.86	
$MnCl_2.4H_2O$	1.81	
$ZnSO_4$.$7H_2O$	0.22	
$CuSO_4$	0.051	
$H_3MoO_4.H_2O$ or Na_2MoO_4 .$2H_2O$	0.09	
1M KH_2PO_4	0.12	
pH = 6.0 with 3M KOH	136	0.5

Make up stock solutions and store in separate bottles with appropriate label. After that, add each component to 800mL deionized water then fill to 1 l and completely mixed for seed growth germination

A.1.2 DF salts minimal medium utilized for hormone production (Dworkin and Foster, 1958)

Major:

KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
$MgSO_4.7H_2O$	0.2 g
$FeSO_4.7H_2O$ 0.001 (Stock solution of 100 mg/10 ml)	
Glucose	2.0 g
Gluconic acid (Ksalt)	2.0 g
Citric acid (Tri-Na salt)	2.0 g
$(NH_4)_2SO_4$	2.0 g

Dissolved in 1000 ml of distilled H_2O

Minor: (Stock solution: 0.1ml l^{-1} was added to above DF salts minimal medium)

H_3BO_3	10 mg
$MnSO_4$	11.2 mg
$ZnSO_4$	124.6 mg
$CuSO_4$	78.2 mg
MoO_3	78.2 mg

Dissolved in 1000 ml of distilled H_2O

A.1.3 MM9 meadium for siderophore production

Na_2HPO_4	6.8 g/l
KH_2PO_4	0.3 g/l
NaCl	0.5 g/l
NH_4Cl	1.0 g/l

0.4% glucose

Dissolved in 1000 ml of distilled H_2O

A.1.4 Modified DF salts minimal medium utilized for ACC assay (Dworkin and Foster, 1958)

Major:

KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
$MgSO_4.7H_2O$	0.2 g

$FeSO_4.7H_2O$ 0.001 g (Stock solution of 100 mg/10 ml)

Glucose	2.0 g
Gluconic acid (Ksalt)	2.0 g
Citric acid (Tri-Na salt)	2.0 g

All of the above were dissolved in 1000 ml of distilled H_2O

Minor: (Stock solution: 0.1ml/l was added to above DF salts minimal medium)

H_3BO_3	10.0 mg
$MnSO_4$	11.2 mg
$ZnSO_4$	124.6 mg
$CuSO_4$	78.2 mg
MoO_3	78.2 mg

Dissolved in 1000 ml of distilled H_2O

ACC, $(NH_4)_2SO_4$ and 0.1M $MgSO_4$ (stock solution)

ACC 30.33 mg in 10 ml of distilled H_2O

$(NH_4)_2SO_4$	13.21 g/l
$MgSO_4$	24.64 g/l

A.1.5 National Botanical Research Institute's phosphate growth medium (NBRIP) for phosphate solubilization

Glucose	10.0 g
$Ca_3(PO_4)_2$	5.0 g
$MgCl_2.6H_2O$	5.0 g
$MgSO_4.7H_2O$	0.25 g
KCl	0.2 g
$(NH_4)_2SO_4$	0.1 g

Dissolved in 1000 ml of distilled H_2O

pH = 7.0±0.2 at 25°C

A.2 Reagents preparation for PGPR traits

A.2.1 Salkowski's Reagent

Concentrated Sulphuric acid	150 ml
Distilled water	250 ml
0.5M $FeCl_3.6H_2O$	7.5 ml

A.2.2 CAS (Chrome Azurol S) Solution (Schwyn and Neilands, 1987)

A. Chrome Azurol S	12.2 mg
Deionized water	10.0 ml
B. HCl (concentrated)	84.0 µl
Deionized water	100.0 ml
$FeCl_3$ $6H_2O$	27.0 mg
C. HDTMA	21.9 mg
Deionized water (warm)	25.0 ml

Seven and half millilitres of (A) were mixed with 1.5 mL of (B), then added slowly to (C) while stirring and then placed in a 100-ml volumetric flask and autoclaved.

D. MES

The buffer solution prepared by dissolving 9.76 g MES in 50 ml water and adjusted pH to 5.6 with 50% KOH. Solution (D) was rinsed into the volumetric flask and made to a volume of 100 ml with sterile distilled water. This coloured solution was stored at 5°C and covered to keep the solution from breaking down.

A.3 Culture media for biochemical tests.

A.3.1 TSI (Triple sugar iron)

Peptic digest of animal tissue	10.0 g
Casein enzyme hydrolysate	10.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Lactose	10.0 g

Sucrose	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Ferric sulfate	0.2 g
Sodium thiosulfate	0.3 g
Phenol red	0.024 g
Agar	12.0 g

pH = 7.4± 0.2 at 2°C

All ingredients except carbohydrates were added to distilled water and gently heated until dissolved. Then, the media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches. The media was allowed to cool down to 45-50°C and added the sterile carbohydrates into medium.

A.3.2 LIM (Lysine indole motile medium)

Peptone	10.0 g
Tryptone	10.0 g
Yeast extract	3.0 g
L-lysine hydrochloride	10.0 g
Dextrose	1.0 g
Ferric ammonium citrate	0.5 g
Brom cresol purple	0.02 g
Agar	2.0 g

pH = 6.6±0.2 at 25°C

All components were added to distilled water and brought volume up to 1.0 l. The medium was mixed thoroughly and gently heated until dissolved. The media

were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

A.3.3 MR-VP (Methyl Red Voges-Proskauer medium)

Peptone 5.0 g	5.0 g
Glucose 5.0 g	5.0 g
K_2PO_4	5.0 g

pH = 7.5±0.2 at 25°C

All components were added to distilled water and brought volume up to 1.0 l. The medium was mixed thoroughly and gently heated until dissolved. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

A.3.4 Simmons citrate agar

$MgSO_4$	0.2 g
$(NH4)_2PO_4$	1.0 g
K_2PO_4	1.0 g
Sodium citrate	2.0 g
NaCl	5.0 g
Agar	15.0 g
Bromthymol blue	0.05 g

pH = 6.8±0.2 at 25°C

All components were added to distilled water and brought volume up to 1.0 l. The medium was mixed thoroughly and gently heated until dissolved. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

A.3.5 Nitrate reduction broth

Peptone	5.0 g
Beef extract	3.0 g
KNO_3	1.0 g

pH 6.9±0.2 at 25°C.

A.3.6 Oxidation-Fermentation medium

Sodium chloride	5.0 g
Pancreatic digest of casein	2.0 g
di-Potassium hydrogen phosphate	0.3 g
Bromthymol Blue	0.03 g
Agar	2.5 ml
Glucose solution	100.0 ml

Preparation of glucose solution was added 10 g of glucose pallet to 100 ml distilled water and filtrated using sterile technique with sterile filter paper. The solution did not autoclaving. Media preparation was added all gradients to distilled water and brought volume up to 900 ml. The medium was mixed thoroughly and gently heated until dissolved. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation. The media was allowed to cool down to 45-50°C and filled 100 ml of glucose solution.

A.3.7 LST

Tryptose	20.0 g
Lactose	5.0 g
NaCl	5.0 g
Lauryl sulfate sodium salt	0.1 g

K_2HPO_4	2.75 g
KH_2PO_4	2.75 g

pH 6.8±0.2 at 25°C

All components were added to distilled water and brought volume up to 1.0 l. The medium was mixed thoroughly and gently heated until dissolved. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

A.3.8 Motility test medium

Tryptone	10.0 g
Agar	5.0 g
NaCl	5.0 g

pH 7.2±0.2 at 25°C

Tryptone and agar were added to distilled water and brought volume up to 1.0 l. The medium was mixed thoroughly and gently heated until dissolved. The media were sterilized by autoclaving for 15 min at 121°C, 15 lb/square inches after preparation.

A.4 Reagents for nucleic acid analysis

A.4.1 STE buffer

NaCl	2.92 g
Tris base	1.21 g
EDTA $2H_2O$-Na_2	1.86 g

Final concentration of solution per litter was 100 mM NaCl, 10 mM Tris/HCl, and 1 mM EDTA. The solution was adjusted pH to 8.0 with NaOH and autoclaving at 121°C, 1, 5 lb/square inches for 15 min.

A.4.2 TE buffer

Tris Base	1.21 g
EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	0.37 g

The ingredients were dissolved and adjusted the volume to 1.0 l with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

A.4.3 Tris-saturated phenol (pH 8)

Dissolved phenol was transferred to 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA pH 8.0.

A.4.4 Loading buffer

Bromophenol blue	25.0 g

The dye was dissolved and adjusted the volume to 10.0 ml with 40% sucrose in water.

A.4.5 Ethidium bromide (10 mg/ml)

Ethidium bromide (Sigma)	1.0 g

The chemical was dissolved and adjusted the volume to 10 ml with sterilized deionized water.

APPENDIX B

PHYSICAL AND CHEMICAL SOIL ANALYSIS

B.1 Determination of soil moisture content

Soil moisture content has been expressed as the ratio of the mass of water in a sample to the mass of the sample when dried to constant weight. The procedures of the determination of soil moisture content are as follows:

1. The empty sample container was dried at 105°C for 24 hours or until weight becomes constant and allowed to cool in the desiccator. Then, the containers were weighed and recorded in the unit of grams.

2. 50.0 g of sample were placed in the container. The container was weighed, and recorded as the weight of the wet soil plus container.

3. After that the container was dried in 105°C oven for 24 hours. The container was removed from the oven and allowed to cool in the desiccator.

4. The container was weighed and recorded as the weight of the dry soil plus container.

The soil moisture content was calculated by using the following equation. Soil moisture content (%) = (wet weight soil + tin) - (dry weight soil + tin) x 100 / (dry weight soil + tin) – tin weight

B.2 Determination of soil pH

Soil pH is a measure of the activity of ionized H (H+) in the soil solution. Soil pH was determined as follows:

1. 20 g air-dry soil was weighed into a 100 ml beaker. Then 20 ml of distilled water was added to each beaker and then stirred periodically with a glass rod for a period of 30 minutes.
2. Soil suspension was stood for 10 minutes. After that, an electrode was inserted into the container and swirled slightly. The pH was read and recorded.

B. 3 Soil texture determination

A 2-mm sieve soil were dried for 24 hours at 105°C and put them in the desiccator. Each 50 g soil was transferred to 600 ml beakers (already weighted) and labled as beaker one and two, respectively. Added 100 g soil instead of 50 g if it was sandy soil After that 100 ml distilled water and the rest of H_2O_2 were added and then left the soil for 1 hour. After that, the first beaker was dried in the oven at 105°C until the soil was dried. The soil was weighted in order to find the weight of the soil without organic matter (for calculation). The second beaker was added with 100 ml Calgon into and left for 10 minutes before transfer the soil from the beaker to the dispersing cup. The soil was thoroughly washed with water and then stirred for 5 minutes. The soil was removed from the cup, put in cylinder and added water until it reaches the specified level. (If the soil is 50 g, water was added until the volume reaches 1,130 ml. If the soil is 100 g, water was added until the volume reaches 1,205 ml). After that 100 ml of Calgon was put into the prepared cylinder of soil

suspension solution (blank) and shaked the cylinder. After 20 seconds, the hydrometer was put in the cylinder, read, record the result on the hydrometer after 40 seconds passed thoroughly and measured temperature of the soil suspension. After 2 hours, the system was read, record the hydrometer and the temperature again. The percentages of sand, silt, and clay was calculated as formular given below. Soil textural class was determined by using the "Textural Triangle".

% Silt + clay = Rs at 40 sec x 100/weight of the soil

% clay = Rs at 2 hr x 100/ weight of the soil

% sand = 100-% (silt + clay)

% silk = % (slit + clay) - % clay

APPENDIX C

BIOCHEMICAL CHARACTERIZATION

Table 1C The Biochemical characteristics of the selected PGPRs.

Characteristics	**1RER2**	**25R463**	**27RER3**	**39SHR**	**53S462**	**65R471**
Catalase	+	+	+	+	+	+
Oxidase	+	+	-	-	+	+
TSI	NC/K, H_2S-,g-	A/K, H_2S-,g+	A/A, H_2S-,g-	NC/K, H_2S-,g-	K/K, H_2S-,g-	K/K, H_2S-,g-
Lysine	P+	Y-	P+	P+	P+	P+
Simmon's Citrate	G	B	G	G	B	B
Motile in liquid media	-	-	-	-	-	-
Indole	Y-	P+	Y-	Y-	Y-	Y-
MR-VP	-/-	-/+	+/+	-/-	-/-	-/-
O-F test	Oxi	Fer	Fer	Fer	Oxi	NGF
LST (gas)	-	-	-	-	-	-
Nitrate reduction	Pink	Pink	Clear/ Pink	Clear/ Clear	Pink	Pink

APPENDIX D

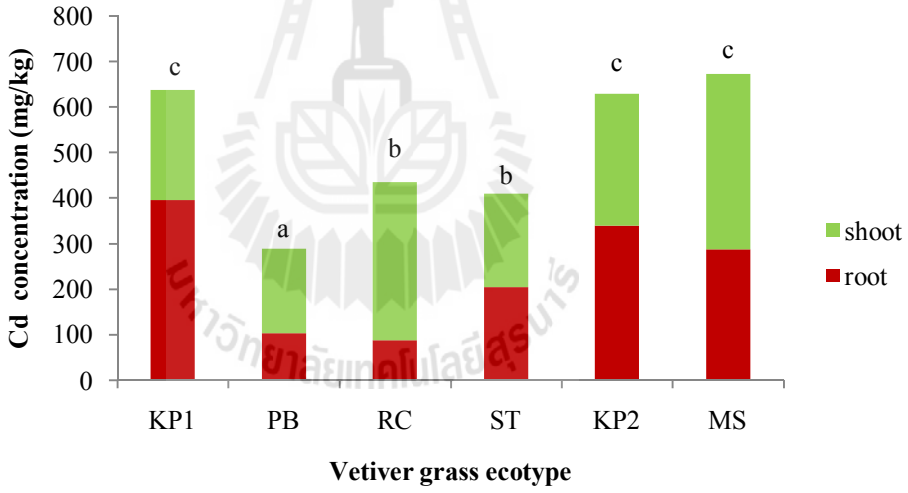
THE EFFICIENCY OF VETIVER GRASS FOR HEAVY METAL UPTAKE

Tissue culture of vetiver grasses both *V. zizanioides* (Kumphang Phet1, Prajuab Kirikhun, and Ratchaburi ecotype) and *V. nemoralis* (Kumphang Phet2, Mae Hongson, and Suratthani ecotype) were grown in Hoagland's solution supplemented with single heavy metal concentration, 20 mg/l Cd, 500 mg/l Zn, or 500 mg/l Pb for 5 days. The vetiver grasses were harvested after heavy metal application and the concentration of heavy metals were determined in root and shoot parts using the GFAAS.

The total Cd accumulation of *V. zizanioides* was in the order of Kumphang Phet1 > Ratchaburi > Prajuab Kirikhun ecotype ($p < 0.05$). For *V. nemoralis*, Mea Hongson and Kumphang Phet2 showed higher Cd accumulation than Surat Thani ecotype. For over all comparison, Kumphang Phet1, Mea Hongson and Kumphang Phet2 exhibited the highest efficiency of Cd uptake (Figure 1D). The maximum translocated ability (TF value) was observed in Ratchaburi ecotype (Table 1D). No significantly different of TAR values were observed. Based on the bioconcentration of Pb in different plant compartments, the highest metal TF was observed in Surat Thani ecotype but not statically significant. The data revealed that all tested vetiver grass did not significantly enhance the Pb uptake per day. All vetiver grass ecotypes except Kumphang Phet1 and Mea Hongson showed bulk Zn contents in shoot rather

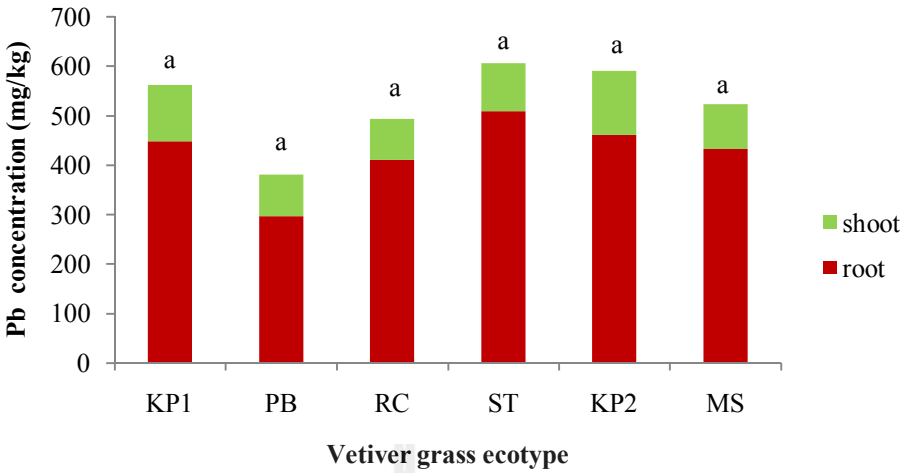
than root suggesting their high potential of Zn translocation (TF). The highest average total Zn uptake was seemed to be Ratchaburi ecotype, but not significantly different.

In conclusion, Kumphang Phet1, Mea Hongson, and Kumphang Phet2 exhibited the highest total Cd uptake. There was no significantly different of total Pb and Zn uptake in all the vetiver grass tested. Ratchaburi ecotype exhibited the Cd and Zn translocation capability (Table 1D). The high average TF values of Pb and Zn were exhibited by Surat Thani ecotype (Table 1D). Consequently, Ratchaburi and Surat Thani ecotypes, the representative for *V. zizanioides* and *V. nemoralis*, respectively, were chosen for further experiment because they possessed high shoot uptake capability.

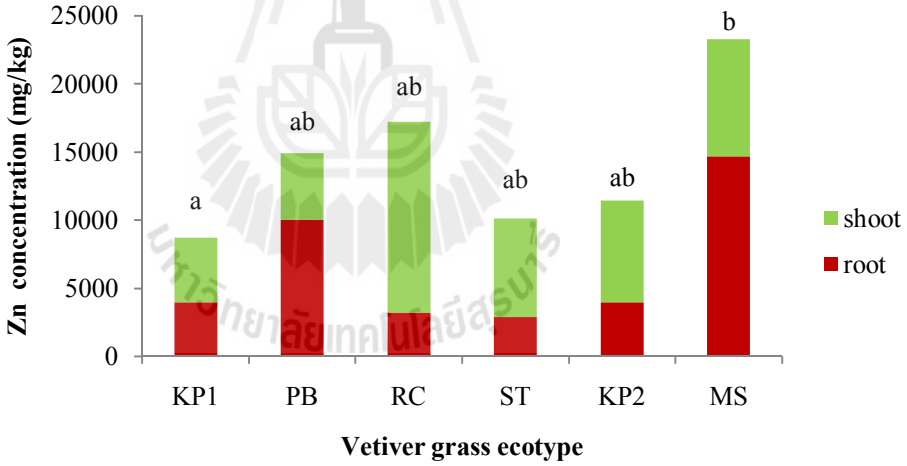


(A)

Figure 1D Total heavy metal uptake of *V. zizanioides* (Kumphang Phet1 (KP1), Prajuab Kirikhun (PB), and Ratchaburi (RC)) and *V. nemoralis* (Kumphang Phet2 (KP2), Mae Hongson (MS), and Surat Thani (ST) ecotypes grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significantly different at $p < 0.05$.



(B)



(C)

Figure 1D (Continued) Total heavy metal uptake of *V. zizanioides* (Kumphang Phet1 (KP1), Prajuab Kirikhun (PB), and Ratchaburi (RC)) and *V. nemoralis* (Kumphang Phet2 (KP2), Mae Hongson (MS), and Surat Thani (ST)) ecotypes grown under Cd (A), Pb (B), or Zn (C). Bars of each ecotype with different superscript are significantly different at $p < 0.05$.

Table 1D Heavy metal concentration in different vetiver grass ecotypes grown under heavy metal stress.

Vetiver	20 mg/l Cd		500 mg/l Pb		500 mg/l Zn	
	TF*	TAR**	TF	TAR	TF	TAR
Kum Phangphet1	61.08±12.68^{a}	2.547±0.255^{a}	26.49±6.73^{ab}	0.03±0.013^{a}	41.88±8.13^{a}	1.081±0.552^{a}
Kum Phangphet2	86.78±24.89^{a}	2.296±0.559^{a}	28.91±8.23^{ab}	0.034±0.001^{a}	247.24±83.98^{ab}	1.087±0.045^{a}
Mae Hongson	141.39±82.96^{a}	2.293±0.622^{a}	20.27±6.39^{a}	0.033±0.007^{a}	70.19±33.76^{a}	1.557±0.458^{ab}
Prajuab Kirikun	180.39±43.06^{a}	1.702±0.603^{a}	29.48±13.93^{ab}	0.021±0.007^{a}	116.66±148.1^{a}	0.847±0.474^{a}
Ratchaburi	410.13±184.66^{b}	1.992±0.591^{a}	20.99±16.68^{a}	0.028±0.001^{a}	441.14±27.91^{c}	1.884±0.596^{b}
Surat Thani	77.02±12.65^{a}	2.612±1.059^{a}	45.48±7.39^{b}	0.028±0.011^{a}	379.28±139.11^{bc}	1.197±0.428^{ab}

Value in the same column with different superscript are significantly different at $p < 0.05$.

*TF = $\left[\frac{\text{Shoot heavy metal conc (µg/g)}}{\text{Root heavy metal conc (µg/g)}}\right] x\ 100$

** TAR = $\frac{\text{(Shoot dry weight (g)x Shoot HM conc (µg/g))+(Root dry weight (g)x Root HM conc (µg/g))}}{\text{((Shoot dry weight (g)+Root dry weight (g))x days of harvest)}}$

APPENDIX E

LIST OF PRESENTATIONS

E.1 Poster Presentation

Aksorn, E. and Chitsombun, B. (2010). **Isolation of cadmium-resistant plant growth promoting rhizobacteria from heavy metal contaminated soil.** The 3nd SUT Graduate Conference, November 21-23, 2010, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

E.2 Oral Presentation

Aksorn, E. **The effect of heavy metal on production of plant growth promoting factors by cadmium-resistant plant growth promoting bacteria.** International Conference on the Environment and Natural Resources 2010 (ICENR 2010) *"The Changing Environment: Challenges for Society"* November 10-12, 2010. Faculty of Environment and Resource Studies, Mahidol University, Salaya, Thailand.

Isolation of cadmium-resistant plant growth promoting rhizobacteria from heavy metal contaminated soil

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Abstracts

Several plant growth promoting rhizobacteria (PGPR) are known to promote plant growth through several direct and indirect mechanisms. In search of PGPR that might enhance the efficiency of phytoremediation for heavy metal removal, the rhizobacteria were isolated from highly cadmium (Cd)-contaminated soils in Tak Province, Thailand. The 380 isolates, tolerating to 290 mg Cd/L onto media, were screened for their plant growth promoting traits including the production of indoleacetic acid (IAA), gibberellins, ammonia, siderophore, 1-aminocyclopropane-1-carboxylic acid utilization and phosphate solubilization. To explore the effects of Cd on the production of plant growth promoting factors by PGPR, five Cd-resistant isolates with the highest ability to synthesize most of the factors were selected and cultured in the absence or presence of 56 mgCd/L –containing media. The results suggested that isolates No. 53 and No. 65 showed higher capacity for IAA and siderophore production ($p < 0.05$) under Cd-stressful condition. Therefore, the selected PGPR isolates might be useful for further investigation of exploitation in Cd-contaminated soil phytoremediation.

Keyword: Plant growth promoting rhizobacteria, cadmium

International Conference on the Environment and Natural Resources 2010 (ICENR 2010) "The Changing Environment: Challenges for Society" 10-12 November, 2010 No.O4_11

The effect of heavy metal on production of plant growth promoting factors by cadmium-resistant plant growth promoting bacteria

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Abstract

The application of plant growth promoting bacteria (PGPB) to overcome survival or limitation of plant growth in the phytoremediation of pollutants is an emerging area of interest. The present study aims to explore the effects of heavy metals; cadmium (Cd), lead (Pb) and zinc (Zn), on the production of plant growth promoting factors by selected PGPB. Five Cd-resistant bacteria, with plant growth promoting characteristics; namely, the production of indoleacetic acid (IAA), gibberellins, siderophore, 1-aminocyclopropane-1-carboxylic acid utilization and phosphate solubilization, were isolated from soils of highly heavy metal-contaminated sites in Tak province, Thailand. The selected PGPB were cultured in the absence or presence of either heavy metal containing media (0.5 mM CdCl2, 2.5 mM Pb(NO3)2 or 5.0 mM ZnSO4). The results suggested that isolates of PGPB No. 25, No.39, No.53 and No. 65, exposed to highly heavy metal-stressful conditions, could still maintain plant growth promoting properties as suggested by the higher capacity for IAA and siderophore production ($p < 0.05$) compared to the unexposed control culture. Therefore, the selected PGPB isolates should be useful for further investigation of exploitation in heavy metal contaminated soil phytoremediation.

Key Words: Plant Growth Promoting Bacteria, Cadmium, Lead, Zinc, Phytoremediation

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