Potential to Remove Heavy Metals and Sodium in Contaminated Shrimp Farms by Purple Nonsulfur Phototrophic Bacteria

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ชื่อวิทยานิพนธ์	ศักยภาพในการกำจัดโลหะหนักและโซเดียมที่ปนเปื้อนในบ่อกุ้งโดยแบคทีเรียสังเคราะห์แสงกลุ่มที่ไม่สะสมซัลเฟอร์
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บทคัดย่อ

จากการศึกษาปริมาณการปนเปื้อนของโลหะหนักและโซเดียมอิออนในน้ำที่ใช้สำหรับการเลี้ยงกุ้ง รวมทั้งการคัดแยกเชื้อแบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์เพื่อนำมาศึกษาความสามารถในการบำบัดการปนเปื้อนดังกล่าว จากบ่อเลี้ยงกุ้งในภาคใต้ของประเทศไทยจำนวน 31 บ่อ พบว่าปริมาณของโลหะหนักสูงสุดที่ตรวจสอบในดินตะกอนมี (หน่วยเป็นมิลลิกรัมต่อกิโลกรัมของน้ำหนักแห้ง) แคดเมียม 0.75 ตะกั่ว 62.63 ทองแดง 34.60 และ สังกะสี 58.50 ซึ่งปริมาณที่พบในตัวอย่างดินตะกอนทั้งหมดยังอยู่ในเกณฑ์มาตรฐานดินตะกอนที่ขุดลอกของฮ่องกง ในขณะที่ปริมาณของทองแดง (9-30 µg/L) และสังกะสี (140-530 µg/L) ในตัวอย่างน้ำจำนวน 32 เปอร์เซนต์ และ 61 เปอร์เซนต์ ตามลำดับ มีค่าเกินมาตรฐานสำหรับการเพาะเลี้ยงสัตว์น้ำ ของกรมควบคุมมลพิษ ประเทศไทย (8 µg/L และ 50 µg/L ตามลำดับ) จากแบคทีเรียสังเคราะห์แสงที่แยกได้ทั้งหมด 120 สายพันธุ์ นำมาคัดเลือกสายพันธุ์ที่ทนต่อโลหะหนักได้จำนวน 2 สายพันธุ์ คือ NW16 และ KMS24 จากการเทียบเคียงโดยอาศัยคุณสมบัติทางชีวเคมีและวิธีการทางอาร์ดีเอ็นเอ (rDNA) พบว่าสายพันธุ์ NW16 เป็นเชื้อ *Rhodobium marinum* ในขณะที่สายพันธุ์ KMS24 เป็นเชื้อ *Rhodobacter sphaeroides* ทั้งสองสายพันธุ์สามารถเจริญในอาหารที่มีความเข้มข้นของโลหะหนักปริมาณสูงสุดที่ตรวจพบในดินตะกอนและมีเกลือ 3 เปอร์เซนต์ จากการวัดค่า MIC พบว่ามีค่าความเข้มข้นสูงกว่าในอาหารที่เชื้อเจริญได้มาก โดยลำดับการทนต่อโลหะหนักของเชื้อทั้งสอง คือ ทองแดง > สังกะสี > แคดเมียม ผลจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) ควบคู่กับการวิเคราะห์รังสีเอกซ์โดยใช้ EDX พบว่า ทองแดงและสังกะสีทำให้เกิดการเปลี่ยนแปลงลักษณะทางสัณฐานวิทยาของเซลล์ทั้งสองสายพันธุ์รวมทั้งพบการสะสมของโลหะดังกล่าวที่ตัวเซลล์ด้วย นอกจากนี้ยังพบว่าสาร exopolymeric substances (EPS) ที่สร้างจากทั้งสองสายพันธุ์ ภายใต้สภาวะมีอากาศเล็กน้อย-มีแสง และสภาวะมีอากาศ-ไร้แสง สามารถกำจัดโลหะหนักได้สูงถึง 90.19 - 98.32 เปอร์เซนต์ ในขณะที่การใช้ตัวเซลล์กำจัดได้เพียง 10.71-80.02 เปอร์เซนต์ จากการศึกษากลไกในการกำจัดโลหะหนักแบบยับยั้งกระบวนการเมแทบอลิซึม (metabolic inhibition) และแบบอาศัยกระบวนการเมแทบอลิซึม (metabolic dependent) สรุปว่าได้ว่าทั้ง

สองสายพันธุ์สามารถกำจัดโลหะหนักได้โดยอาศัยทั้งกระบวนการ biosorption และ bioaccumulation โดยสภาวะที่เหมาะสมในการกำจัดโลหะหนักของสายพันธุ์ NW16 คือ ปริมาณชีวมวลของเซลล์ในระยะ log phase ใช้เซลล์สดเทียบเท่า 4.5 มิลลิกรัมน้ำหนักแห้งต่อมิลลิลิตร พีเอช 6.0 อุณหภูมิ 30 องศาเซลเซียส และระยะเวลาในการดูดซับ 30 นาที โดยมีเปอร์เซนต์การกำจัดโลหะหนักเฉลี่ย คือ ตะกั่ว 86.50 เปอร์เซนต์ ทองแดง 64.00 เปอร์เซนต์ สังกะสี 42.50 เปอร์เซนต์ และ แคดเมียม 25.50 เปอร์เซนต์ สำหรับสายพันธุ์ KMS24 พบว่าสภาวะที่เหมาะสมในการกำจัดโลหะหนักคือ ปริมาณชีวมวลของเซลล์ในระยะ log phase ใช้เซลล์สดเทียบเท่า 5.0 มิลลิกรัมน้ำหนักแห้งต่อมิลลิลิตร พีเอช 5.5 และอุณหภูมิ 35 องศาเซลเซียส เป็นเวลา 45 นาที (ค่าเฉลี่ยเปอร์เซนต์การกำจัดตะกั่ว 96 เปอร์เซนต์ ทองแดง 75 เปอร์เซนต์ สังกะสี 46 เปอร์เซนต์ และแคดเมียม 30 เปอร์เซนต์) นอกจากนี้ยังพบว่าเชื้อผสมทั้งสองสายพันธุ์มีประสิทธิภาพในการกำจัดโลหะหนักได้มากกว่าเชื้อบริสุทธิ์ของแต่ละสายพันธุ์ โดยที่แคลเซียมไอออน และแมกนีเซียมไอออน มีผลทำให้ประสิทธิภาพในการกำจัดโลหะหนักของทั้งสองสายพันธุ์ลดลงอย่างมีนัยสำคัญ นอกจากนี้จากการศึกษาศักยภาพในการกำจัดโลหะหนักของทั้งสองสายพันธุ์ในดินตะกอนและน้ำหลังการเพาะเลี้ยงจากบ่อกุ้งที่มีการปนเปื้อนด้วยโลหะหนัก รวมทั้งศึกษาความเป็นพิษต่อพืชหลังการบำบัดโดยทดสอบการงอกของเมล็ด พบว่าในตัวอย่างน้ำชุดทดสอบที่มีเชื้อธรรมชาติร่วมกับเชื้อผสมสามารถกำจัดทองแดงได้ 75 เปอร์เซนต์ สังกะสี 31 เปอร์เซนต์ ซึ่งสูงกว่าชุดปราศจากเชื้อที่เติมเชื้อผสม สำหรับตัวอย่างดินตะกอนชุดเชื้อธรรมชาติร่วมกับเชื้อผสม มีประสิทธิภาพในการกำจัดตะกั่ว ทองแดง สังกะสี และแคดเมียม สูงสุดในสภาวะมีอากาศ-ไร้แสง โดยมีเปอร์เซนต์การกำจัดคือ 84.29 62.52 43.33 และ 40.95 เปอร์เซนต์ตามลำดับ นอกจากนี้ยังพบว่าน้ำที่มีการปนเปื้อนของโลหะหนักภายหลังจากการบำบัดคงมีความเป็นพิษอยู่ โดยมีต่อเมล็ดข้าว (*Oryza sativa*) มากกว่าผักบุ้ง (*Ipomoea aquatic*) และยังมีความเป็นพิษมากกว่าสารละลายดินที่ผ่านการบำบัด โดยชุดบำบัดที่มีเชื้อธรรมชาติร่วมกับเชื้อผสมให้ผลในการบำบัดดีที่สุด โดยเปอร์เซนต์ดัชนีการงอก (%GI) ของเมล็ดข้าวและผักบุ้งในน้ำ คือ 34.50 และ 35.29 และ 115.70 และ 139.33 ของข้าวและผักบุ้งในดินตะกอน จากผลการศึกษาแสดงให้เห็นว่าแบคทีเรียสังเคราะห์แสงที่คัดเลือกได้สามารถนำไปใช้ในการบำบัดโลหะหนักที่ปนเปื้อนในบ่อเลี้ยงกุ้งได้

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ABSTRACT

In order to determine whether water used for shrimp cultivation contained toxic levels of heavy metals (HMs) and sodium (Na), analysis was carried out on 31 shrimp ponds in southern Thailand. Purple nonsulfur bacteria (PNB) were also isolated from the same ponds to investigate if they could be used for bioremediation of the above contaminants. The highest HM concentrations of the sediment samples in mg/kg dry weight were found as follows: 0.75 cadmium (Cd), 62.63 lead (Pb), 34.60 copper (Cu) and 58.50 zinc (Zn). However, all sediment samples met Hong Kong standards for dredged sediment. In contrast, contamination of Cu (9-30 µg/L) and Zn (140-530 µg/L) exceeded the standard guidelines for water used for cultivation of marine aquatic animals set by the Pollution Control Department, Thailand were found in 32 and 61% of water samples. Two metal resistant PNB strains, NW16 and KMS24, were selected from the 120 PNB strains obtained. By using of biochemical and rDNA analytical methods the strain NW16 was identified as *Rhodobium marinum*, while strain KMS24 was *Rhodobacter sphaeroides*. Both strains were tolerant to HMs present at the concentrations in their growth medium containing the higest concentrations detected in shrimp ponds as previously described, with 3% NaCl. The determined MIC values were much higher than those used for their growth with their degree of tolerance being in the order of $Cu^{2+} > Zn^{2+} > Cd^{2+}$. Results of SEM-EDX (a scanning electron microscope equipped with energy dispersive X-ray spectrometer) indicated that Cu^{2+} and Zn^{2+} altered the cellular morphology of both strains and accumulated HMs were found in their cells. Removal of HMs under both incubating conditions (microaerobic-light and aerobic-

dark conditions) by exopolymeric substances (EPS) was between 90.19 and 98.32% but only 10.71-80.02% by the cells (biomass). Based on metabolic inhibition and metabolic-dependent studies, it was concluded that both strains removed HMs using biosorption and also bioaccumulation. The optimal conditions for removal of HMs by strain NW16 were; cells in the log phase with equivalent to 4.5 mg DCW/ml, pH 6.0, and 30ºC for 30 min and the relative averages percent removal of HMs was: Pb, 86.50; Cu, 64.00; Zn, 42.50; Cd, 25.50. Cells in the log phase at 5.0 mg DCW/ml, pH 5.5, and 35ºC for 45 min were optimal conditions for strain KMS24 (averages removal percentages: Pb, 96; Cu, 75; Zn, 46; Cd, 30). The mixed culture of both strains showed a removal efficiency of HMs greater than that found in the pure culture of each strain. The presence of Ca^{2+} and Mg^{2+} significantly decreased the removal capacity of HMs for both strains. In addition, the sediment and water collected from postcultured contaminated shrimp ponds and seed germination was used to assay their plant toxicities after bioremediation. The water from contaminated shrimp ponds was decreased roughly 75% for Cu^{2+} and 31% for Zn^{2+} by a native population with a mixed culture and it was greater than that found in a set of sterile treated water with a mixed culture. For the sediment samples, a set of native with the mixed culture produced the highest efficiency to remove Pb^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} under aerobic-dark conditions with removal percentages of 84.29, 62.52, 43.33, and 40.95, respectively. Water contaminated with HMs after treatment was more toxic to rice seed (*Oryza sativa*) than water spinach (*Ipomoea aquatic*) and more toxic than soil solution from the treated sediment samples. A set with the native population and mixed culture produced the most effective treatment as the % GI index were 34.50 and 35.29 for rice and water spinach in the water and 115.70 and 139.33 for rice and water spinach in the sediment. All results demonstrate that both selected PNB strains could be used for bioremediation of HMs in contaminated shrimp ponds.

Keywords: photosynthetic bacteria, heavy metals, bioremediation, shrimp ponds, seed germination index

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LIST OF ABBREVIATIONS AND SYMBOLS

DCW: Dry cell weight

DW: Dry weight

GM: Glutamate-malate medium

HMs: Heavy metals

ICP-OES: Inductively couple plasma-optical emission spectrometer

LC_{50}: 50% Lethal concentration

MIC: Minimum inhibitory concentration

nm: Nanometer

OD: Optical density

PNB: Purple nonsulfur bacteria

SEM-EDX: Scanning electron microscope equipped with energy dispersive x-ray spectrometer

SLB: Songkhla Lake Basin

TEM: Transmission Electron Microscope

µm: micrometer

CHAPTER 1

INTRODUCTION

Rationale and Background

The increases of population, economic development and use of new technology have lead to environmental pollutions. Waste and pollutants, including toxic metals, such as Cd, Pb, Cu, Ni, and Zn are increasingly released to and are accumulated in the environment by human activities. Other toxic materials such as pesticides and fertilizers used in agriculture, and waste discharged from some industries, i.e. PVC, batteries, and pigments, etc. (Reutergardh and Yen, 1997; Virulhakul and Suntipiriyaporn, 2006).

HMs interact with the soil matrix may persist for a long period of time creating long-term hazards to the environment and human health. Most organic compounds, in contrast, can be biodegraded with time, or can be incinerated. HMs are robust and remain a potential threat to the environment or human health for long time. The presence of HMs in soil leads to serious problems because of their (1) toxicity on biological system and (2) groundwater contamination by leaching process. They can be remobilized to be toxic elements depending on type of elements and condition of environments. Mobility and toxicity of HMs in soil are governed by various parameters including pH and the content of clay minerals and organic matter. Complex formation with naturally occurring or synthetic complexing agents may enhance metal mobility (Neubauer *et al*., 2000).

Thus, HMs in soil will be distributed to accumulate in food chains through absorption by plankton and consequently are transferred to accumulate in animals before being introduced to cause toxic effects in humans (biomagnifications process). Some human diseases occurred through a biomagnification process for example, Minamata disease is caused by consumption of fish, shell fish and crab contaminated with methyl mercury; Itai-Itai disease is caused by consumption of rice contaminated with cadmium (Hodges, 1977). Therefore, plants, animals, and

microorganisms can suffer from a high level of HMs accumulated in food chains (Attewell, 1993).

Thailand is a developing country and shrimp farming is an important business for economic growth of Thailand. Thai shrimp exports have increased to the extent that it has become the world s leader in shrimp e port. The largest export markets for Thai shrimp are the United States and Japan. The demand is increasing yearly. As a result, the increase of areas for shrimp farming is spreading quickly along the coast. Thai farmers have then changed rice field and mangrove forests in the coastal areas to shrimp ponds. An extension of shrimp farming from a coastal area to a freshwater area affected areas used for growing rice paddies, fruit plantations, and fisheries. Rapid growth of shrimp cultivation without a good management also has damaging effects to the environment such as increased soil salinity, reduced water resources, and destruction of mangrove area. Traditionally, most shrimp farms are located near the coast, and seawater is directly introduced to the farms rearing shrimps with no additional treatment processes. However, the coastal portion of seawater is often contaminated by many kinds of pollutants, including HMs (Chua, 1992; Paez-Osuna and Tron-Mayen, 1996). In Thailand, the accumulation of HMs in sea come from industrial wastes and municipal wastes, etc., especially in Gulf of Thailand which receives HMs from 4 major rivers: Chaophraya, Tha Chine, Mae Klong, and Bangpakong. Chaiyakam and Tompolgrung (1995) reported that concentrations of Cd, Cu, Fe, and Pb in the sea exceed the permissible level in water habitat for aquatic cultivation. The HMs contamination in the ocean has affected water quality which is related to poor health, low resistance to diseases, death and finally extinction of some aquatic animals. Accumulation of HMs in shrimp ponds is partly from sea water for use in shrimp farm (Prabnarong, 1993). Other sources of HMs contamination in shrimp farm are from the use of chemical substances and feeding food, including HM leaching from fertilizer used in agriculture. Thus, HMs from those sources are accumulated in shrimp body and consequently affect on their physiological properties and survival rate of shrimp (Tangkerkolan and Cheewaporn, 2001). If concentrations of the HMs in shrimp exceed the standard safety level, they will affect people who consume shrimp and have an impact on shrimp exportation. Moreover, wastewater from shrimp farms, which is discharged to the environment, has caused an

accumulation of HMs and chemical substances to the environment such as soil and water (Manseubchart, 2002).

The conventional processes used for removal of HMs from the environment are chemical and physical processes, e.g. chemical precipitation, ion exchange and reverse osmosis. However, there are some significant disadvantages, such as high cost of treatment and remaining of some toxic substances. Hence, biological approach has been considered as an alternative remediation for HM contamination.

Bioremediation is an attractive alternative to physical and chemical methods. This process uses organisms such as plants or microorganisms to remove HMs. It is very interesting treatment because of its environmental friendly, high efficiency, low cost and easy to operate. Besides, soil, water or sludge which was treated with microorganisms can be reused (Gazso, 2001; Lloyd and Lovley, 2001).

Recently, microorganisms, particularly bacteria have been successfully used as adsorbing agents for removal of HM. Purple nonsulfur photosynthetic bacteria (PNB) that have a variety of physiological properties can grow chemotrophically or phototrophically (Imhoff and Triiper, 1989; Pfenning and Triiper, 1989) have been used for wastewater treatment such as pineapple canning (Noparatnaraporn and Nagai, 1986), seafood (Prasertsan *et al.*, 1993), and rubber latex (Kantachote *et al*., 2005). In addition, PNB s cells can be used as a source of fertili er and single cell protein with high vitamin B12. PNB are normally found in water and sediment that is exposed to sunlight. Previous studies showed that PNB had high efficiency in adsorbing HMs (Seki *et al*., 1998; Watanabe *et al*., 2003). Therefore, the PNB which can be found easily in shrimp ponds should be shown to be usefull on the removed of HMs contaminated in shrimp ponds.

Objectives

1.2.1 To determine the amount of HMs (cadmium, lead, copper, and zinc) and sodium contaminated in shrimp ponds in the lower part of southern Thailand.

1.2.2 To isolate and select purple nonsulfur photosynthetic bacteria (PNB) which are the most tolerant and the most effective strain in removing HMs contaminated in shrimp ponds.

1.2.3 To study the bacterial remediation mechanisms and factors that affect HMs removal.

1.2.4 To investigate the possibility of the selected strains to remove HMs from water and soil samples collected from contaminated shrimp ponds.

1.2.5 To determine the remaining toxicity of HMs and sodium in water and sediment after treatment with PNB selected strains.

Scope of the study

1.3.1 To isolate and characterize the PNB from contaminated shrimp ponds.

1.3.2 To study the HM removal efficiency of the PNB under microaerobic-light and aerobic-dark conditions.

1.3.3 To study optimal conditions e.g. pH, cell mass, cell age and temperature etc. for HM removal by PNB.

1.3.4 To study the HM biosorption properties of PNB strains.

1.3.5 To investigate an application of PNB in removing HMs from contaminated water and soil from post cultured shrimp ponds.

Anticipated Outcomes

1.4.1 This study contributes to a safety measure for consumer s health from HM accumulation in the food chain and reduces a degree of salinity problem in soil and surface water.

1.4.2 Results from this study can be applied to remove HMs contaminated in shrimp farms and to protect and solve the problems or the impacts of HM accumulation in ecosystem or food chain.

1.4.3 Any promising strains may be used for bioremediation in contaminated shrimp ponds.

CHAPTER 2

LITERATURE REVIEW

Heavy metals, An Overview

Heavy metals are metal, with a specific gravity greater than 5.0 g/cm^3, atomic number of 23-92 and 4-7 rows on the periodic table. They are natural constituents of rocks and soils and enter the environment as a consequence of weathering and erosion. HMs enter the soil *via* agricultural additives, such as lime, fertilizers, manure, herbicides, fungicides and irrigation water as well as *via* potentially deleterious material such as sewage sludge, municipal composts, industrial and mine wastes, dredged materials and atmospheric deposits (Giller *et al*., 1999; Berrow, 1986). In aquatic systems, HMs can be found in different forms, whereby influencing their toxicity for fish and other bio-organisms, including free ions, organic and inorganic complexes, precipitates, mineral particles and HMs present in biota. Toxicity of HMs in water bodies depends on the presence of other metals or poisons as well as on water characteristics such as temperature, pH, dissolved oxygen and salinity (Rainbow, 1995). In addition, it depends on the stage of life of the water living organisms and their behavioral response (Fialkowski and Newman, 1998). Based on a biological view, HMs can be classified into 3 groups (Maier *et al*., 2000):

(1) Low toxic elements, this group can easily be found in the nature with large quantity such as Na, K, Mg, Ca, Fe, Li, Rb, and Sr.

(2) High toxic elements such as Co, Ni, Cu, Zn, Sn, As, Se, Au, Ag, Hg, Pt, Pd, Cd, and Be, this group can cause high toxicity at low concentration.

(3) Toxic elements with small quantity are found in nature e.g. Ti, Hf, Zr, W, Nb, Ta, Ga, La, and Ru.

There are some HMs commonly detected in the environment, e.g. cadmium, copper, lead, zinc, manganese, nickel, chromium, iron, and mercury.

Some of these contaminants can remain in the environment for a long time and many of them cannot be degraded. They are ultimately accumulated in the sediments or in organisms. HMs and persistent lipophilic organic compounds are absorbed and accumulated in organisms. This process is known as bioaccumulation (Suess and Erlenkeueser, 1975; Taylor *et al*., 1995). Bioaccumulated substances may be passed up the food chain to predator species, this process, which is known as biomagnification, may become hazardous to humans (Beiras *et al*., 2003).

In shrimp farming, several important HMs such as, Cd, Pb, Cu, and Zn, are intensively found. The physical properties and toxicities of these elements are provided as followed.

Cadmium (Cd)

Cadmium is a soft, malleable, ductile, toxic and bluish-white metal. Naturally cadmium is found with Pb, Cu, Zn, and Hg. It is used in agriculture as a herbicide and fungicide (Friberg *et al*., 1986). About three quarters of cadmium is used in batteries, electroplating, and pigments for coloring of plastics, glass and ceramic glazes. It is commonly used in Ni-Cd battery. Cadmium is well known for its toxicity, bioaccumulation, and biomagnification through the food chain. It is not an essential element for living organism. As a result, cadmium is one of the commonest environmental poisons. Acute poisoning from inhalation of fumes and ingestion of cadmium salts can also occur and at least one death has been reported from self-poisoning with cadmium chloride. In chronic exposure, it also accumulates in the body, particularly in kidney and liver (Goldwater and Clarkson, 1972; Baldwin and Marshall, 1999).

Cadmium is a potential hazard for human who consumes contaminated food and water. Humans can suffer from tracheo-bronchitis, pneumonitis and pulmonary edema if there is a high concentration of cadmium accumulated in the body. The symptoms from cadmium toxicity included cough, irritation of nose and throat, headache, weakness, fever, chest pain, respiratory tract, and kidney problems which can be fatal. Another well-known disease from cadmium poisoning is Itai-Itai occurred in Japanese people who consumed contaminated rice grown in cadmium contaminated water.

An acceptable level of cadmium in human body of ≤ 7.0 μg/kg/person has been defined by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) and it can not be higher than 0.005 mg/L in water for consumption (Lester, 1987).

Cadmium contamination in Thai seafood is a major problem for export. It was found higher than 1.0 mg/kg in 24.8% of 270 samples of octopus, in 24.13% of 472 samples of cuttlefish, and in 18% of 470 samples of loligo (Virulhakul and Suntipiriyaporn, 2006). The reason that higher contamination of cadmium was found in octopus and cuttlefish rather than in loligo may be due to the difference in feeding behavior of octopus and cuttlefish which consume food on the sea bottom at a shallow depth, while the loligo consume food at a deeper depth.

Lead (Pb)

Lead is a dense, ductile, very soft, highly malleable, and bluish-white metal with poor electrical conductivity and very toxic. Lead in nature is mostly found in compound materials. The major source of lead in the environment is in the earth's crust. Lead has been used extensively for both industrial and domestic applications for hundreds of years (Baldwin and Marshall, 1999). Lead has been used for various purposes such as in tetraethylead synthesis process of which the product will be mixed with gasoline for increasing the octane number. In addition, it is used as a component of battery products, herbicide products, fertilizer products, and so on. There is no known biological function of lead. Lead is highly toxic and accumulates in humans. Lead enters food and water supply (Goyer and Chisholm, 1972). Lead is absorbed by foodstuffs (particularly green leafy vegetables) where growing on soil where lead is presented. Lead is also used in paints and some cases of lead poisoning are due to small children eating flakes of this paint (Horner, 1995). Today, lead is still used in batteries, some insecticides, and is found in cigarette smoke where there is between 0.017 and 0.98 μg/cigarette (Horner, 1995). Lead can be distributed and contaminated the environment such as soil, water, and air. Lead from these environments may accumulate in food chains which later can affect human health directly or indirectly from consumption (Tsuchiya, 1986). In human body, more than 90% of lead is absorbed by the stomach, and persist in bone, liver, kidney, and

muscles. Lead reduces formation of red blood cells and reduces cognitive ability of the central nervous system causes anemia, kidney problems, coma and death if it is a long time exposure.

Absorption of lead by the lungs is very efficient, especially when the particle sizes are less than 1 μm in diameter, as may happen for example with fumes from burning lead paint. Gastrointestinal absorption of lead varies with the age of the individual; children absorb around 50% of what they ingest, but adults only absorb 10-20% of what they ingest ((Horner, 1995; Hu *et al*., 1998). Lead also affects sodium, potassium, and calcium ATPase pumps, which maintain the cells' concentration gradients of these ions. Adult and children can be safe from Pb poisoning if they intake Pb less than 7.9 and 3.3 mg/kg/day, respectively (CDC, 1997).

Copper (Cu)

Copper is a reddish-colored metal with a high electrical and thermal conductivity. Copper in nature can be found in an uncompounded mineral which has been mined in several parts of the world. Native copper is found in form of mineral compounds such as chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), malachite ($Cu_2CO_3(OH)_2$), and azurite ($Cu_2(CO_3)_2(OH)$). Copper naturally can also be found in a solid form with Fe and Ag. About 50% of Cu has been used in various products such as electrical switches, electrical busbars, electrical relays, and printed circuit boards, etc. Copper salts are used as a component of pesticide, fungicide, and ceramic glazes (Aaseth and Norseth, 1986). The copper salts are also used to control the growth of algae in the reservoir. This application of copper may result in an increase of copper accumulation in water resources. In the outer Songkhla Lake, the copper concentration of 0.067 mg/L and an average of 7.09 mg/kg in sediments were reported by Chaiyakam and Tompolgrung (1994). Copper in soil is generally found in a range of 2.0 -100.0 mg/kg.

Copper is an essential element for human metabolism which is found in various enzymes and hemoglobin formation. It is absorbed by the intestine and is found in bloodstream (blood cell and serum). Higher level of copper accumulation in liver and tissue organs may inhibit enzyme activities, especially an inherited condition

in the liver called “Wilson’s disease” which causes brain and liver damages. In addition, a high level of copper (II) sulfate may be acutely toxic, causing vomit, low blood pressure, jaundice, coma, and fatal in the final state (Aaseth and Norseth, 1986).

Zinc (Zn)

Zinc is a moderately reactive and bluish-white metal. It is mostly abundant in the earth’s crust and is not found as free metal. In general, it is found in the sulfide ore as sphalerite (ZnS). Zinc is an essential element necessary for sustaining life of plants, animals and microorganisms. Although it is the most abundant element in the nature, zinc and its oxide are only a little soluble in water, while zinc chloride is well soluble in water. The concentration of zinc in water depends on chemical factors of zinc. Zinc ions can be adsorbed by soil sediments. Zinc concentration increases with an increase of acidic condition in water. Zinc has been used in coating steel to prevent rust and corrosion and other products such as building stores, vehicles and houses. Zinc oxide is used in rubber products, as an ingredient of some medicines for malnutrition disease and as a component of pesticides in the carbamate group (Erider, 1986).

Zinc can be absorbed by humans by inhalation of dust or fumes of zinc, by consumption of food and water or by absorption through skin. After ingestion, zinc will be absorbed by the gastrointestinal system, and is distributed in the blood circulation to combine with plasma protein. Zinc is found in red blood cells and may spread into the tissues. Acute toxicity of zinc may occur within 24 hours after the exposure to zinc sulfate. The symptoms include headache, muscle and joint pain, chest pain, cough, sweating and fever.

Heavy metals contamination in aquatic animals and their products

Most information about contamination of HMs in lives of aquatic animals and their products in Thailand are reported by the Quality Control of Food Export Unit, Department of Medical Science and Department of Fisheries. Kitchareonwong, *et al.* (1998) reported that analysis of aquatic animal products (14,818 samples) for exportation from 1986 to 1995 found a mercury content in most of Thai seafood. Its content was lower than the permissible level of mercury content

(0.5 mg/kg) in seafood products for exportation. It was presnt at 0.36% and 0.09% of Mackerel and Tuna samples, respectively, which is higher than the standard level.

In 2004, the Fish Inspection and Quality Control Division, Department of Fisheries reported that mercury contamination in exported products of aquatic animal samples (39,023 samples) between January and December, found mercury in canning fishes, frozen fishes and frozen crabs higher than that in other products with the average contents of 0.136, 0.109 and 0.108 mg/kg, respectively. The 0.2% Hg level in canned fish and the 0.1% frozen fish samples was higher than the standard level (0.5 mg/kg). The cadmium content in frozen squid and canning squid was also higher than the other products with averages of 0.23 and 0.13 mg/kg, respectively. Lead was found in frozen fish and frozen crap with average of 0.109 and 0.108 mg/kg, respectively. These values were higher than those in other products. Lead levels in aquatic products did not pass the standard level (0.5 mg/kg). The average concentrations of lead in frozen fish, frozen crab, canning crab, and canning shrimp with were 0.695, 0.407, 0.685, and 0.615 mg/kg, respectively.

In 2005, cadmium contaminations in aquatic animal samples for exportation (16,169 samples) from January - May reported that the average content of cadmium in frozen and canning squid were 0.35 and 0.12 mg/kg, respectively. Average lead concentration in the frozen shell fish was 0.082 mg/kg, which was higher than those found in frozen shrimp, canning fish, and frozen seafood.

In 2006, Cheung and Wong reported that the concentration of metals in tissues of grey mullet (*Mugil cephalus*) and gei wai shrimp (*Metapenaeus ensis*) were found to be safe for human consumption. Concentration of Cr in tilapia (*Oreochromis mossambicus*xO. nilotica) whole body (0.68-1.10 mg/kg wet weight) were close to or over the guideline value of 1 mg/kg set by the Food Adulteration (metallic contamination) Regulation of Hong Kong. Tilapia fish and small caridean shrimp (*Macrobrachium nipponensis*) collected from gei wais were contaminated by Cr and Pb but were still be safe for human consumption.

Pradit *et al*. (2009) reported that the metal accumulation in fish muscle tissue, and liver and eggs of two cat fish species (*Arius maculatus* and *Osteogeneiosus militaris*) is element-specific, but the concentration of trace elements in fish muscle tissue were well within the limits for human consumption.

These reports show that the contamination of HMs in most of aquatic animals and their products of Thailand did not exceed a standard level and the contaminations depended on the kind of aquatic animals, seasons, and areas. As an accumulation of HMs in environments is changing with time, thus the HMs contents need to be monitored and the current situation of contamination should be continuously updated.

Heavy metals contamination in the coastal areas

HMs contamination of the coastal areas is a serious problem in many part of the world, a study of pollutants in the ocean from 1973 to 1987 (Kaeyuranon, 1998), found that the concentration of the following HMs silver, cadmium, cobalt, copper, mercury, zinc, and ferrous in soil and water had increased with the time of sampling. For example, the mercury content in water samples from the upper gulf of Thailand (close to the coastal of Petchaburi province) collected in May 16, 1982 was 386 µg/L (ppb) and in September 11-16, 1986 the water samples from the central gulf of Thailand showed a the mercury content of 847 µg/L. This result corresponded well to a study of Taemeeyawanich (1984) who found the highest content of HMs in the Thai oceans, directly influences the survival rate of young aquatic animals. Moreover, the HMs have accumulated in some economically aquatic animals.

Suwannarath (1994) studied some HMs in the Klong Wad, Songkhla province and found cadmium, copper, lead, and zinc with concentration ranges from 0.002-0.005 mg/L, 0.002-0.011 mg/L, 0.015-0.089 mg/L and 0.002-0.028 mg/L, respectively. An average content of cadmium was not higher than a standard limit acceptable for surface water (1.0 mg/L), while lead had somewhat higher concentration than a standard limit in surface water (0.05 mg/L). In Songkhla Lake, the contamination of arsenic and HMs were studied by Meesuk (1997), the sediment samples collected in January 1996 from the areas where wastewater was discharged into the lake and from 9 stations close to industrial sites were analyzed. The results showed the concentrations of lead, mercury, zinc, and cadmium were found in a range of 6.55-92.75, 10.45-44.30, 0.28-1.80, 0-1.25, and 0-2.50 µg/L of sediment weight, respectively.

In 2009, Pradit *et al*. reported that the concentrations of Co, Ni, Cu, Zn, Cd, Pb, As, Fe Mn, and Al in sediments of Songkhla Lake, especially the outer section of the lake, in particular the sediments at the mouths of Phawong, U-Taphao and Samrong Canals were significantly enriched with these trace elements due to municipal, agricultural and industrial discharges entering the lake through the canals.

Contamination of heavy metals in the shrimp ponds

In 1995, there was a large increase in low salinity conditions in fresh water areas when the shrimp farm area was 38 km^2 and increased to 320 km^2 in 1998. In 1996, the shrimp product from the fresh water area was 16,041 tons and it was only 6.7% of total shrimp product and its product increased to 23,428 tons or 10.3% of total shrimp products in 1999.

In 2005, shrimp farms in Thailand were surveyed by the Ministry of Agriculture and Cooperatives using aerial photographic map. A total area of about 992 km^2 was reported. It was subdivided into a mangrove forest area of 640 km^2, an estuary area of 320 km^2 and a fresh water area of 192 km^2. However, only half of the total area was used for shrimp farming, while another half of the area was abandoned.

Shrimp cultivation in a low salinity condition or in a fresh water area produces a negative effect on environment such as destroying mangrove forests and the soil has environmental damage. Furthermore, poor management of shrimp farming, use of chemicals, antibiotics, and runoff untreated wastewater has an adverse effect on the environment. Moreover, the water from the ocean which is used for shrimp farming is contaminated with several of HMs from industrial waste and leaching of wastewater to the sea including shrimp feed, chemicals, fertilizers, and antibiotics. Consequently, they were accumulating in the shrimp farms that may be dangerous for shrimp cultivation which, even at reduced concentrations, may negatively affect the health of consumers (Azevedo *et al*., 2009). Some of the HMs that have the greatest risk of contamination are related to mercury, arsenic, cadmium, lead, nickel, copper, and zinc. There are some studies that have been reported about HMs contaminated in shrimp ponds as follows.

Towatana and Prabnarong (1996) found zinc at approximately 0.39-2.43 μg/kg in one year use of shrimp ponds, while it was 0.52-2.43 μg/kg in three

years of shrimp ponds use. The amounts of copper found in old and new shrimp ponds were 1.00-2.10 μg/kg and 0.88-1.89 μg/kg, respectively.

Maneepong and Angsupanich (1999) studied lead in the sediments and aquatic animals in the outer Songkhla Lake, the Klong Pawong, and the Khlong U-taphao and Pb content in the sediments were 7.7- 28.2 mg/kg, 2.9- 27.0 mg/kg, and 0.5-21.6 mg/kg, respectively whereas it was found at 8.0 mg/kg in shrimp (*Macrobachium rosenbergii*) from Klhong U-taphao and 6.0 mg/kg for shrimp (*Metapenaeus ensis*) from the outer Songkhla Lake.

Gosavi *et al*. (2004) studied the contamination of HMs in shrimp ponds and found that when the soil type in shrimp ponds was acidic sulfate soil, the caused disease and death of shrimp as a result of the pH drop, thereby an increase in solubility of HMs.

Visuthismajarn *et al.* (2005) studied in an uninhabitated part of a shrimp farm in southern of Thailand. Soil samples were collected from epidemic area of shrimp disease. The result showed that the highest contamination of HM ions is in the Prachuab Khirekhan province followed by Satun and Songkhla, respectively. HMs with highest contamination included Mn, Cd, and Cu (HQ values were 1.9, 4.3, and 1.8, respectively) these values of HQ indicated a risk of causing damage to ecological systems.

Azevedo *et al*. (2009) studied the quantitative assessment of calcium and HMs (iron chromium, copper, zinc, lead, manganese, cadmium and nickel) in water and the sediment of marine shrimp ponds, over three productive cycles and it was found that the metals evaluated in the water of the fisheries, only chromium and zinc were in the safe levels for aquaculture. In contrast, in the sediment the average values of zinc and manganese were classified as high levels for agriculture.

Impacts of heavy metals on tiger shrimps

Based on reports in 1994 and 1995 by Luangthuvapranit who studied toxicities of Hg, Pb, Cu, and Zn in tiger shrimps that showed the LC_{50} of Hg were 0.2148, 0.1637, 0.1495, and 0.1229 mg/kg in 24, 48, 72, and 96 hours, respectively, whereas LC_{50} of Pb was 176.2223, 132.9130, 106.8046, and 99.5230 mg/kg, respectively. The toxicities of Hg and Pb to shrimps were seen at concentrations of

0.1184 and 96.8838 mg/kg, respectively. The safety levels of Hg and Pb for the tiger shrimps were 0.0026-0.0064 mg/kg and 1.9917-4.9794 mg/kg, respectively. The LC_{50} of Cu to tiger shrimp in 24, 48, 72 and 96 hours were 7.3028, 4.8591, 3.0387 and 2.1581 mg/kg, respectively, while LC_{50} of Zn was 8.9925, 42915, 3.5189 and 2.5279 mg/kg, respectively. The initial toxicities of Cu and Zn were seen at 1.6574 and 2.1876 mg/kg, respectively (Luangthuvapranit, 1995). The safe levels of Cu and Zn for the tiger shrimps were 0.0443-0.1106 mg/kg and 0.0527-0.1317 mg/kg, respectively. Tangkerkolan and Cheewaporn (2001) studied changes in physiology, survival rate and acute toxicity of Cd and Pb at different concentrations. They found the LC_{50} of cadmium and lead in 96 hours exposed were 2.42 mg/kg and 0.25 mg/kg respectively. The accumulations of cadmium and lead in the shrimp body did not quickly affect shrimp motility, but more likely cause a physiological changes, including to the feed rate, oxygen consumption, and water balance in the shrimp body.

Impacts of Heavy metals on plant growth

Accumulation of HMs in soil may be leached to groundwater, run off to surface water, and accumulation by plants. They can be transferred and concentrated in the plant tissues, consequently they have caused damaging effects on the plants themselves and may become a health hazard to humans and animals (Athar and Ahmad, 2002). Thus, it is not only the plants that are affected by HMs but also the local ecological system. HMs and plant interact in a specific way, which depends on several factors such as type of soil, growth conditions and the presence of other ions. The sensitivity of plants to those HMs depends on an interrelated network of physiological and molecular mechanisms such as uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents, efflux of HMs from the cytoplasm to extracellular or other compartments including vacuoles and the complexation of HM ions inside the cells by various substances, for example, organic acids, amino acids, phytochelatins, and metallothioneins (Cho *et al*., 2003). Although, some HMs, at the low concentrations, are essential elements for plant such as manganese, zinc, and iron, they are important co-factors of enzymes and critical components of electron transport reaction, but they may cause metabolic disorders and growth inhibition for most of the plants at higher doses (Fernandes and

Henriques, 1991; Claire *et al*., 1991). It can be disturbing to plants by inhibiting metabolism, which results in reduction of growth rate, pigment content and low productivity (John *et al*., 2009). Beside that, it may also influence allocation to sexual reproduction (Saikkonen *et al*., 1998) and delay flowering (Brun *et al*., 2003; Korboulewsky *et al*., 2002). The mechanism of the HMs effect on plants has been reported by many studies e.g., Laetitia *et al*. (2002) studied the effect of Cd^{2+} on *Arabidopsis thaliana* L. which found the cadmium ions released gas exchange and controlled an opening and closing of guard cells. Rana and Masood (2002) showed the study result of HMs affected on the growth and productivity of *Triticum aestivum* L. as protein in the grain was decreased to 19.0-71.4 %. Moreover, they found that a high accumulation of cadmium in soil inhibited growth of *Azotobacter* chromococcum by 84.9%. However, different plants have different tolerances to respond to HMs depending on the kind of plant, size, age and the environment.

Saline soil

Saline soil is that dominated by a high salt content. The saline soil has a negative result on agricultural land. The salinity can be determined by an electrical conductivity (EC) as typical more than 4 dS/m, which is due to several ions composition such as sodium (Na^{+}), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^{-}), and sulfate (SO_4^{2-}). Saline soil may affect on plant growth as the plants can not absorb water and also develop toxic effects to sodium and chlorine. In addition, a high salt condition inhibits potassium and calcium absorption and result in a decrease of potassium, calcium and chlorophyll. Therefore, photosynthesis is decreased while the respiration rate and nitrogen content are increased (Dobermann and Fairhurst, 2000). However, saline soil may be used for growing salt tolerant plant such as *Oryza sativa*, *Brassica* sp., *Ipomoea aquatica*, and *Lactuca satiava* (Towatana *et al.,* 2003).

Conventional methods of heavy metals removal

Many procedures have been applied in order to remove HMs from aqueous streams. Among the most commonly used techniques are chemical precipitation, chemical oxidation and reduction, ion-exchange, filtration, electrochemical treatment, reverse osmosis (membrane technologies), evaporative

recovery and solvent extraction (Xia and Liyuan, 2002). These classical or conventional techniques give rise to several problems such as an unpredictable metal ions removal and generation of a toxic sludge which is often difficult to dewater and requires extreme caution in their disposal (Xia and Liyuan, 2002). Besides that, most of these methods also present some limitations whereby they are only economically viable at high or moderate concentrations of metals but not at low concentrations (Addour *et al*., 1999). Moreover, the classical techniques involve expensive methodologies due to high energy and reagent requirements (Xia and Liyuan, 2002). Some of the conventional methods are explained in brief as following;

Reverse Osmosis

In this process, the HMs are separated by a semi-permeable membrane at a pressure greater than osmotic pressure caused by the dissolved solids in wastewater. The disadvantage of this method is that it is expensive.

Ion-exchange

In this process, metal ions from dilute solutions are exchanged with ions held by electrostatic forces on the exchange resin. The disadvantages include high cost and only partial removal of certain ions.

Ultra filtration

These are pressure driven membrane operations that use porous membranes for the removal of HMs. The main disadvantage of this process is the generation of sludge.

Chemical Precipitation

Precipitation of metals is achieved by the addition of coagulants such as alum, lime, iron salts and other organic polymers. The large amount of sludge containing toxic compounds produced during the process is the main disadvantage (Ahalya *et al*., 2003).

Therefore, the search for efficient, eco-friendly and cost effective remedies for wastewater treatment has been initiated. In recent years, research

attention has been focused on biological methods for the treatment of effluents, some of which are in the process of commercialization (Prasad and Freitas, 2003). Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals (Volesky and May-Philips, 1995; Mallick, 2004).

Bioremediation of heavy metals

Bioremediation is a branch of biotechnology that uses bacteria and other microorganisms to reduce, eliminate, contain and transform toxic agents benign products, contaminants present in soils, sediments, water or air. HM bioremediation involves removal of HMs from wastewater and soil through metabolically mediated or physico-chemical pathways. This is an alternative to traditional remediation technologies and now it can be combined with the physical and chemical methods because of its lower cost than other methods (precipitation, ion exchange, osmosis, and/or extraction) (Rich and Cherry, 1987). In addition, wastewater, sediment and sludge that had been treated with microorganisms could be reused (Gazso, 2001; Lloyd and Lovley, 2001). Microbial processes are now beginning to be used in the clean up of radioactive and HMs contaminants. Microorganisms can interact with metals and radionuclides via many mechanisms some of which may be used as the basis of potential bioremediation strategies. The major types of interaction are summarized in Figure 2-1.

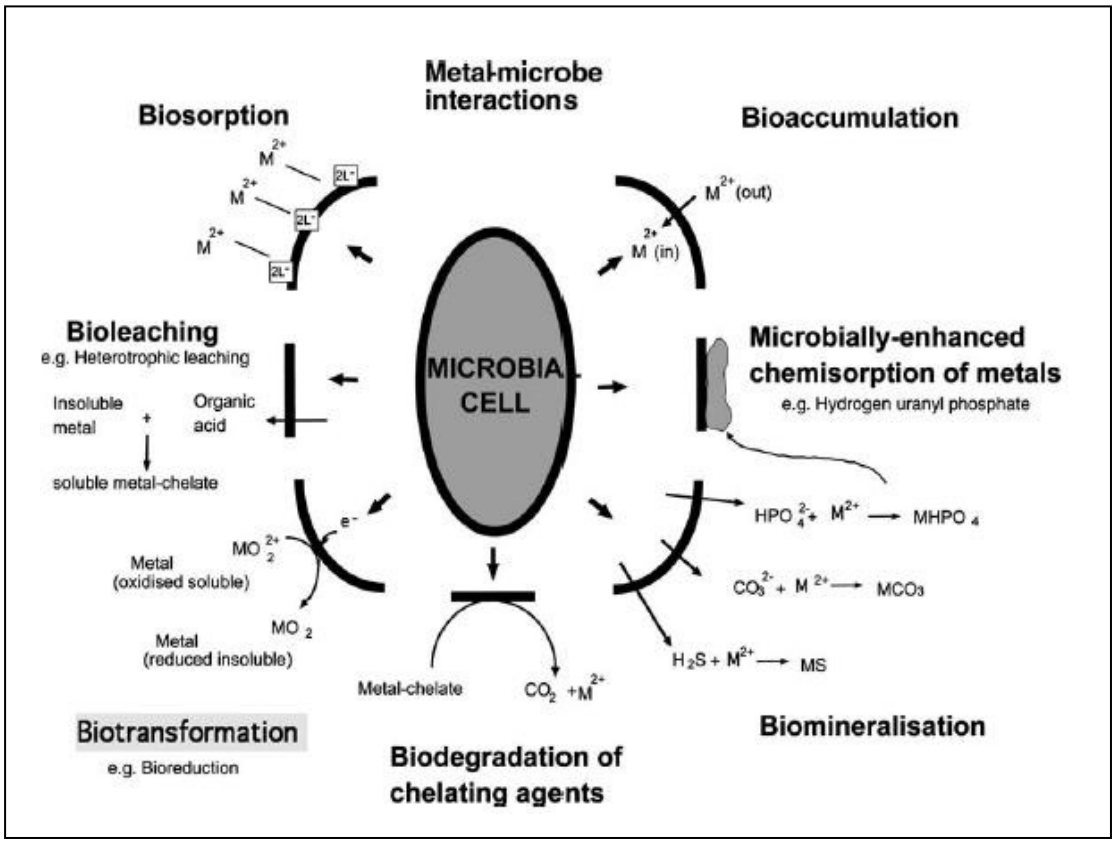

Figure 2-1. Metal-microbe interactions impacting bioremediation. (Tabak *et al*., 2005; p 118)

Mechanisms involve in Bioremediation

The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cells. The bioremediation mechanisms are various and are not fully understood. They may be classified according to various criteria.

- According to the dependence on the cell's metabolism
  - Metabolism dependent and
  - Non -metabolism dependent
- According to the location where the metal removed from solution is found
  - Extra cellular accumulation/ precipitation
  - Cell surface sorption/ precipitation and
  - Intracellular accumulation

Transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of accumulation may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of a toxic metal. During non-metabolism dependent process metal uptake is by a physico-chemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cells' metabolism. Cell walls of microbial biomass and mainly composed of polysaccharides, proteins and lipids have abundant metal binding groups such as carboxyl, sulfate, phosphate and amino groups. This process i.e., a non-metabolism dependent process is relatively rapid and can be reversible (Kuyucak and Volesky, 1988). In the case of precipitation, the metal uptake may take place both in the solution and on the cell surface (Ercole *et al*., 1994). Further, it may be dependent on the cell's metabolism if, in the presence of toxic metals, the microorganism produces compounds that favor the precipitation process. Precipitation may not be dependent on the cells' metabolism, if it occurs after a chemical interaction between the metal and cell surface.

Transportation to cell membrane: HMs could be transported across microbial cell membrane by a two step process. First a physical-chemical method which the metals are bound to the cell wall and then are transported into cells by the same mechanism used to convey metabolical ions such as potassium, magnesium and sodium. The metal ions are transported to cells by the same mechanism charge of essential ions (Costa and Leite, 1991; Gadd, 1988). After that, the HM ions bind with intracellular protein or are precipitation intracellular. The use of mixed bacteria between *Pseudomonas maltophila, Staphylococcus aureus* and Coryneform found that Ag could be accumulated at 300 μg/g dry weight (Brierley, 1990).

Physical-chemical interaction: Microbial cells, especially bacteria at the surface of the cell wall are generally characterized by polyelectrolytes which can be neutralized by interaction with ions in a solution. It may form interactions of vander waals' force, by covalent bonds and an oxidation-reduction process may be found in some bacteria

for instance, *Bacillus* sp. could oxidize manganese to insoluble manganese oxide (Balanco, 2000). Besides that *Bacillus*, *Thiobacillus*, *Micrococcus*, *Rhodopseudomonas* and *Pseudomonas* can reduce ferric ion ($Fe^{3+)}$ to ferrous (Fe^{2+}) as a result of electron transport system in bacterial cells. In *Alterromonas putrefaciens*; it grew by reducing manganese oxide under anaerobic conditions and used manganese ion as a final electron acceptor in metabolism (Brierley, 1990). In addition, other mechanism can occur by addition of an ethyl group to HMs such as Hg as a result to increase the metals toxicity but it is safe to bacterial cells because of its rapidly volatility from the bacterial habitat. In some cases, methyl group is used instead of ethyl group and this can remove HMs from bacterial cells (Roane *et al*., 1998)

Ion Exchange: The bacterial cell is a complex structure especially two main components membrane and cell wall; some bacteria have capsules of polysaccharides. Both capsule and the peptidoglycan layer can have functional groups (phosphate, carboxyl and amino), therefore ion exchange between negative ions and HMs can occur around them as a result and accumulate HMs on the cell surface (Vecchio *et al*., 1998). For example, a marine algae was used to remove HMs in salts solution of K^+ Na^+ Ca^{2+} and Mg^{2+}; these ions can be exchanged with counter ion such as Co^{2+} Cu^{2+} Cd^{2+} and Zn^{2+} (Kuyucak and Volesky, 1988). Muraleedharan and Venkobachar (1990) found that fungi, *Ganoderma lucidium* and *Aspergillus niger* could remove copper by ion exchange mechanism with counter ions of the cell wall.

Complexation of metals with cell surface: The complex formation is a result from the interaction between the metals and the active group on cell surface or some bacterial products as the chelating agent such as siderophores and polymers e.g. polysaccharide, protein and nucleic acid. These polymers have functional groups that can bind with HM ion as a result to form a complex (Brierley, 1990). Aksu *et al*. (1992) found the copper adsorption by cells of *Chlorella vulgaris* and *Zoogloea ramigera* occurr from a coordination bond between metals and amino and carboxyl groups of the cell wall. Complexation is found to be one of the mechanisms responsible for accumulations of calcium, magnesium, cadmium, zinc, copper, and mercury by *Pseudomonas syringae.* Moreover, some bacteria may also produce

organic acids (e.g. citric, oxalic, gluconic, fumaric, lactic and malic acid), that may act with metals to form metallo-organic molecules (Ahalya *el al*., 2003).

Precipitation: HMs may precipitate both in solution and on cell surfaces (Ercole *et al.,* 1994). Some bacteria have outer polysaccharides that can bind with metal ions and then result in precipitation on the outer cell. For example precipitation of cadmium on the capsule found in *Arthrobacteria viscosus* and *Klebsiella aerogenosa* can be observed by electron microscopy (Scott and Palmer, 1988). Besides that, *Klebseilla aerogenosa* when grown in 2-4% of cadmium can form granules of cadmium sulfide that occurr on the outer cell surface (Aiking *et al*., 1984; Gadd, 1990). Formation of phospholic acid (H_3PO_4) by degradation of glycerol 2-phosphate by *Citrobacter* could be used to precipitate cadmium, lead, and uranium. Hence, the bacterium grew in Cd^{2+} and glycerol-2 -phosphate; the cadmium accumulation was found as the $CdHPO_4$ form that is an insoluble compound (Macaskie *et al.,* 1987; Eliora *et al.,* 1992; Brierley, 1990).

Factors affecting heavy metals uptake

There are many factors that an affect bioremediation process as follows:

***Temperature*:** Effect of temperature on biosorption of HMs depends on microbial mechanisms. At the low temperature (0-5 ^{o}C), biosorption of HMs by the metabolism dependent mechanism is low. Aksu *et al*. (1992) found that temperature had no effect on the performance in the range of 20-35 ^{o}C. The optimum temperature which is commonly used to study biosorption of HMs are in the range of 25-35^{o}C. It is also the optimum temperature of bacterial growth (Blackwell *et al*., 1995).

***pH*:** It is the most important parameter in the biosorptive process. It affects the properties of the cell surface and metals (Collins and Stotzky, 1996; Lopez *et al.,* 2000). An increase of pH causes an increase of negative charges on the surface of cells which favor, electrochemical interaction with metal ions and increase of the HMs adsorption (Gourdon *et al.,* 1990). The optimum pH for sorption of lead by *Citrobacter* MCMB-181 was between 4-5, while at a pH lower than 4.5 or higher than 5 a low sorption of HMs may occurr. At pH<3 the lead ion competitively to the cell

surface with H^+. At a high pH the solution solubility of lead is decreased. In addition, adsorption of HMs is very low or does not occur at pH less than 2 due to competitive binding between H^+ and metal ions on the cell surface. In contrast, an increase of pH causes HM precipitation as compounds of hydroxide or oxide. Most bacteria produce optimum adsorption of HMs at the pH range of 4-8 (Blackwell *et al.,* 1995).

***Adsorption time*:** The adsorption time is different for each bacterium. It depends on the mechanism, HM concentration and biomass. The adsorption of cadmium in *Enterobacter aerogenes* which has been fixed on activated carbon had 2 stages. The first step, diffusion occurs at high cadmium concentration as a quick binding of cadmium to the cell surface. It is not energy dependent adsorption. The second step is cadmium adsorption into the cell by active transport. It is energy dependent and a slow reaction. The adsorption time will be proportional to the concentration of cadmium solution. At 25 ppm of cadmium, the adsorption time taken to the equilibrium state was 120 minutes, while at 100 ppm of cadmium the adsorption time to equilibrium state is 30 minute (Scott and Karanjkar, 1992). In *Acinetobacter calca* var. *antratus*, the time taken for cadmium adsorption to equilibrium was only 2-5 minute, but if it needed a longer incubation time, the second equilibrium may occur in 50 hours. The cadmium adsorption was twice 2 times more than the first equilibrium (Hsu and Chiang, 1991).

***Type and concentration of HMs*:** Metal concentrations affect on metal removal by bacteria. Absorption of cadmium, nickel, cobalt and strontium by *Bacillus simplex* ZAN-044 was in the order of Cd > Ni > Co > Sr and its adsorptions increased with increasing metal concentration from 0.01 to 100 μM. However, the adsorptions were decreased at 1,000 μM (Valentine *et al.,* 1996).

In addition, in bacteria the HM concentration not only affects the adsorption but it also affects the inhibition of respiration and protein synthesis. The respiration activity of bacteria from activated sludge was inhibited by 65% when the cadmium concentrations was increased from 0 to 50 mg/L and it produced only 10% respiration as an increase of the cadmium concentration from 50 to 500 μg/L. However, the cadmium adsorption was increased when the concentration of cadmium was increased from 10-100 μg/L (Gourdon *et al.,* 1990).

***Other metals*:** These are not the only HMs that contaminated wastewater but there are more than one of HMs. Therefore other metals are a factor that may affect the removal of HMs, for instance, removal of uranium by the biomass of bacteria, fungi, and yeasts in solution. The metal ions such as manganese, cobalt, copper, cadmium, mercury and lead have no affect on removing uranium (Sakaguchi and Nakajima, 1991; Ahalya *et al.,* 2003). However, cell used of *Rhizopus arrhizus* as a biosorpbent found the Fe^{2+} and Zn^{2+} affected on adsorption of uranium (Tsezos and Volesky, 1982; Ahalya *et al.,* 2003). In addition, the removal of cobalt by other microorganisms may be inhibited by lead mercury and copper in solution (Sakaguchi and Nakajima, 1991; Ahalya *et al.,* 2003).

Photosynthetic bacteria

There are two classes of photosynthetic prokaryote as follows: anoxygenic photosynthetic bacteria and oxygenic photosynthetic bacteria. Purple nonsulfur bacteria (PNB) are a member of the group of the anoxygenic photosynthetic bacteria which are able to grow as phototrophs in anaerobic conditions and availability of light (Pfenning and Truper, 1989; Imhoff, 1992). These bacteria can use organic compounds as the carbon source (photoorganotroph). Some groups of these bacteria can use sulfide or thiosulfate as an electron donor in anaerobic-light conditions but in aerobic-dark conditions these bacteria can grow as chemoorganotroph (heterotroph) which use organic compounds as an electron donor and a sole carbon source (Imhoff, 1992; Staley *et al*., 1994)). The cellular morphology is rod, cocci and spiral and their reproduction is budding or binary fission. Some of these bacteria are motile but some are non-motile. Colors of colonies range among purple, red, brown and orange. The members such as *Rhodobacter, Rhodocyclus, Rhodopseudomonas,* and *Rhodospirillum* are normally found in aquatic environment and wastewater systems (Imhoff, 1992).

Use of purple nonsulfur photosynthetic bacteria to remove heavy metals

Purple nonsulfur bacteria (PNB) have been applied in the field of environmental protection, such as the treatment of sewage and wastewaters (Nagadomi *et al.,* 2000) and the bioremediation of the environment polluted with

organic matters (Takeno *et al*., 2005). They are particularly advantageous for bioremediation processes devoted to the degradation or recovery of pollutants from contaminated environments because the solar radiation is plenty, cheap and a clean energy source. Therefore, they have the potential to use for HMs bioremediation (Sasikala and Ramana, 1995; Seki *et al*., 1998). Several investigations concerning the potentialities of PNB in term of HM accumulation abilities have been reported such as *Rhodobacter sphaeroides, Rhodobacter capsulatus* and *Rhodopseudomonas* sp. The applications of PNB to remove HMs are shown in the following:

Kantachote and Sutunthapareuda (1992) studied the removal of HMs by *Rhodopseudomonas* sp. ST 18 grown with modified Lascelles medium. They found the highest HM adsorption occured by 5 minutes of contact time and temperature did not affect the biosorption. The bacteria could adsorb copper ion, cadmium ion and lead ion as 2.29, 2.74, and 2.48 mg/g dry weight, respectively. Moreover adsorptions of copper and lead in the mixed HMs ions by the bacterium were 2.21 and 2.24 mg/g dry weight, respectively and the HM adsorptions by dead cells were better than that in the living cells.

Seki *et al*. (1998) studied about HMs binding sites in *Rhodobacter sphaeroides* and hydrogen bacteria (*Alcaligenes eutrophus* H 16). The results showed that the biosorption of bivalent metal ions to whole cell bodies of the bacteria was due to monodentate binding to two different types of acidic sites: carboxilic and phosphatic-type sites. The number of metal binding sites of *A. eutrophus* was 2.4-folds larger than that of *R. sphaeroides*.

Watanabe *et al.* (2003) reported that a photosynthetic bacterium, *Rhodobacter sphaeroides* S, and a marine photosynthetic bacterium, *Rhodovulum* sp. PS88, could absorb cadmium ions in a batch culture system. Both of them were able to remove cadmium from culture medium which had 30 g/L sodium chloride and divalent cations (Mg^{2+} and Ca^{2+}). In particular, the strain PS88 showed a high removal ratio and high specific removal rate of cadmium ions from the culture medium under aerobic-dark (heterotrophic) and also anaerobic-light (photoheterotrophic) conditions.

Feng *et al*. (2007) found that the photosynthetic bacterium, *Rhodobacter capsulatus* had a strong ability to adsorb Au (III) and the maximum

specific uptake of living cells was over 92.43 mg HAuCl4/g dry weight of cell in the logarithmic phase and the biosorpion ability would be enhanced by an acidic environment. This study also showed the biosorbtion of Au (III) could be reduced by carotenoid and enzymes embedded and/or excreted by *R. capsulatus*, which might be the mechanism of photosynthetic bacteria metal tolerance.

Bai *et al.* (2008) studied the removal kinetic characteristic and mechanism of cadmium removal by growing *Rhodobacter sphaeroides* and reported that the removal of soluble cadmium was dependent on the cell-biomass, and biosorption played only a minor role compared with cadmium bioremoval by precipitation as cadmium sulfide. Moreover, the content analysis of subcellular fractionation showed that cadmium was mostly removed and transformed by precipitation on the cell wall.

In addition, HMs have differential toxic effects on bacterial growth depending on the kind of metabolism. The *Rhodobacter sphaeroides* was grown in anaerobic conditions acting as a photoheterotroph and also in aerobic conditions. Their aerobic and anaerobic metabolisms may differ in their susceptibility to HMs toxicity. The inhibitory effect exerted by zinc and cadmium on *R. sphaeroides* was stronger in the aerobic conditions than that in the anaerobic conditions (Balsalobre *et al*., 1993). Microbial growth was considerably reduced when HMs concentration was higher than 1 mg/L. Besides, *R. sphaeroides* was quite tolerant to copper as its growth at high concentration of 0.01 to 10 mg/L showed a relatively constant growth under both aerobic and anaerobic conditions (Balsalobre *et al*., 1993).

Plants and their heavy metals toxicity index

HMs contaminated soils may affect the organisms present. Therefore, the presence of organisms can be used as an indicator for a toxicity test. One of these such as plants is very interesting to use because HMs affect plant growth and productivity.

The mechanism of the HMs effect on plant has been reported by many studies e.g., Laetitia *et al*. (2002) studied the effect of Cd^{2+} on *Arabidopsis thaliana* L. and found the cadmium ions released gas exchange and controlled an opening and closing of guard cells. Rana and Masood (2002) studied how HMs affected the growth

and productivity of *Triticum aestivum* L. as protein in the grain was decreased to 19.0-71.4 %. Moreover, they found that a high accumulation of cadmium in soil inhibited growth by 84.9%. The accumulation of toxic elements in plants may be not enough to affect their productivity; however HMs have been concentrated in the food chain. Plants have different tolerances to respond to HMs depending on the kind of plant, size, age and the environment. Hence, it is important to select plants that are appropriate to grow in area of HM contamination. In this study, the plants; rice (*Oryza sativa*), and *Ipomoea aquatic* Forsk, were selected as they normally grow on saline soils in order to examine the toxicity of water and soil after treatment by PNB.

***Ipomoea aquatica* Forsk.**

Ipomoea aquatica Forsk is in the family Convolvulaceae. It is a semi-aquatic tropical plant grown as a leaf vegetable. It can be grown both on terrestrial and water. It can be cultivated on almost kind of soil but the optimum growth for the consumable plant is in friable soils or sand contaminated friable soil. It is found throughout Southeast Asia and is a widely consumed vegetable in this region. The plant growth condition requires high moisture and exposure to sun light and it can grow on the whole year in Thailand.
(http://www.issg.org/database/species/ecology.asp?fr=1&si=477)

Rice: *Oryza sativa*

Oryza sativa is in the family Gramineae. It is often grown in paddies. The trunk is short, slender leaves 50-100 cm long, 2-2.5 cm wide and grows to 1-1.8 m tall. The seed is a grain (caryopsis) 5-12 mm long and 2-3 mm thick. It can be grown practically anywhere. The time to harvest is after 120-130 days and it gives high productivity, strong trunk and is resistant to diseases.
(http://en.wikipedia.org/wiki/*Oryza_sativa*)

CHAPTER 3

Isolation of Purple Nonsulfur Bacteria for the Removal of Heavy Metals and Sodium from Contaminated Shrimp Ponds

Abstract

In order to determine whether waters used for shrimp cultivation contained toxic levels of heavy metals (HMs) and sodium (Na), analysis was carried out on 31 shrimp ponds in areas of southern Thailand. Purple nonsulfur bacteria (PNB) were also isolated from the same ponds to investigate if they could be used for bioremediation of the above contaminants. The highest HMs concentrations of the sediment samples in mg/kg dry weight were found as follows: 0.75 cadmium (Cd), 62.63 lead (Pb), 34.60 copper (Cu) and 58.50 zinc (Zn). However, all sediment samples met Hong Kong standards for dredged sediment. In contrast, contamination of Cu (9-30 µg/L) and Zn (140-530 µg/L) exceeded the standard guidelines for marine aquatic animal set by the Pollution Control Department, Thailand were found in 32 and 61% of water samples, respectively. Two metal resistant PNB strains, NW16 and KMS24, were selected from the 120 PNB strains obtained. Both strains reduced the levels of HMs by up to 39% for Pb, 20% for Cu, 7% for Cd, 5% for Zn and 31% for Na from water that contained the highest levels of HMs found and 3% NaCl when cultured with either microaerobic-light or aerobic-dark conditions. The strain NW16 removed a greater percentage of the HMs than the strain KMS24, but the strain KMS24 was able to survive better under a greater variety of environmental conditions. Both strains were therefore suitable to use for further investigating their abilities to remediate water contaminated with HMs and Na.

Keywords: shrimp farming, heavy metals, salinity, purple nonsulfur bacteria, bioremediatio

บทคัดย่อ

จากการศึกษาปริมาณการปนเปื้อนของโลหะหนักและเกลือโซเดียมในน้ำที่ใช้สำหรับการเลี้ยงกุ้ง รวมทั้งคัดแยกเชื้อแบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ นำมาศึกษาความสามารถในการบำบัดการปนเปื้อนดังกล่าว จากบ่อเลี้ยงกุ้งในภาคใต้ของประเทศไทย จำนวน 31 บ่อ พบว่าปริมาณของโลหะหนักสูงสุดที่ตรวจสอบในดินตะกอนมี (หน่วยเป็นมิลลิกรัมต่อกิโลกรัมของน้ำหนักแห้ง) แคดเมียม 0.75 ตะกั่ว 62.63 ทองแดง 34.60 และสังกะสี 58.50 ซึ่งปริมาณที่พบในตัวอย่างดินตะกอนทั้งหมดยังอยู่ในเกณฑ์มาตรฐานดินตะกอนที่ขุดลอกของฮ่องกง ในขณะที่ปริมาณของทองแดง (9-30 µg/L) และสังกะสี (140-530 µg/L) ในตัวอย่างน้ำจำนวน 32 เปอร์เซนต์ และ 61 เปอร์เซนต์ ตามลำดับ ซึ่งมีค่าเกินมาตรฐานสำหรับการเพาะเลี้ยงสัตว์น้ำ ของกรมควบคุมมลพิษ ประเทศไทย จากแบคทีเรียสังเคราะห์แสงที่คัดแยกได้ทั้งหมด 120 สายพันธุ์ นำมาคัดเลือกสายพันธุ์ที่ทนต่อโลหะหนักได้จำนวน 2 สายพันธุ์ คือ NW16 และ KMS24 จากการศึกษาพบว่าทั้งสองสายพันธุ์สามารถกำจัดโลหะหนักและเกลือโซเดียมที่ปนเปื้อนในน้ำที่มีปริมาณความเข้มข้นของโลหะหนักสูงสุดที่พบในบ่อเลี้ยงกุ้งและมีปริมาณเกลือโซเดียมคลอไรด์ 3 เปอร์เซนต์ ทั้งในสภาวะมีอากาศเล็กน้อย-มีแสง และสภาวะมีอากาศ-ไร้แสง สามารถกำจัดตะกั่วได้ 39 เปอร์เซนต์ ทองแดง 20 เปอร์เซนต์ แคดเมียม 7 เปอร์เซนต์ สังกะสี 5 เปอร์เซนต์ และเกลือโซเดียม 31 เปอร์เซนต์ โดยที่พบว่าสายพันธุ์ NW16 จะมีประสิทธิภาพในการกำจัดโลหะหนักได้ดีกว่าสายพันธุ์ KMS24 ในขณะที่สายพันธุ์ KMS24 สามารถเจริญได้ในสภาวะแวดล้อมที่มีความหลากหลายมากกว่า ดังนั้นทั้งสองสายพันธุ์จึงมีความเหมาะสมที่จะนำมาศึกษาศักยภาพเพื่อใช้ในการกำจัดโลหะหนักและเกลือโซเดียมที่ปนเปื้อนในน้ำของบ่อเลี้ยงกุ้ง

Introduction

Currently, there have been a number of incidents involving food and food safety such as residuals of antibiotics and heavy metals (HMs) occurring in aquatic animals and their products. Fortunately, the contamination by HMs (i.e. mercury: Hg, Cd, Pb and Zn) in all of the aquatic animal products of Thailand did not exceed standard levels (Petroczi and Naughton, 2009). Among aquatic animals, shrimp is one of the prime exports for Thailand although at present there are many problems with shrimp cultivation and export. The rapid expansion in shrimp cultivation over the last 20 years in Thailand has caused several negative environmental and socio-economic impacts. These include destruction of mangrove forests, salination of the soil and the dispersion of toxic chemicals including HMs into the environment (Flaherty *et al.,* 2000; Kautsky *et al.,* 2000).

The Songkhla Lake Basin (SLB) covers some parts of 3 provinces; Nakhon Si Thammarat, Patthalung and Songkhla and is located in southern Thailand along the coastal area of the Gulf of Thailand (Figure 3-1.). SLB is one important area for shrimp farming because this area has many mangroves, so there are few beautiful beaches, and seawater for shrimp cultivation is readily available. Recently, the shrimp business has been on the downturn due to the collapse of the shrimp price and unsuccessful cultivation. There are now many abandoned shrimp ponds in the SLB area, and the soil has been left unfertile and cannot be used to grow other plants due to its increased salinity caused by shrimp farming. However, shrimp farming activities are still being conducted around the SLB.

There have been no reports on HMs contamination in the shrimp ponds around the SLB, although some problems have been reported from trace metal contamination i.e. Cd, Cu, Pb, and Zn in the Gulf of Thailand (Cheevaporn and Menasveta, 2003). In addition, the Phawong and U Taphao Canals are major canals that transport wastewater from industries and municipalities to Songkhla Lake (Pradit *et al.,* 2009). Thus, some shrimp farming areas around SLB may be contaminated by HMs from water sources used for shrimp cultivation. The other possible sources of HMs in shrimp ponds are from the use of chemical substances i.e. Cu^{2+}, shrimp food,

and perhaps some leaching from fertilizers used in agriculture (Visuthismajarn *et al.,* 2005). Shrimp farming without good management practices can cause contamination by HMs and increased soil salinity in the vicinity of the shrimp cultivation area. Thus, it is important to remove HMs and treat the salinity in the shrimp pond before water is discharged into the environment.

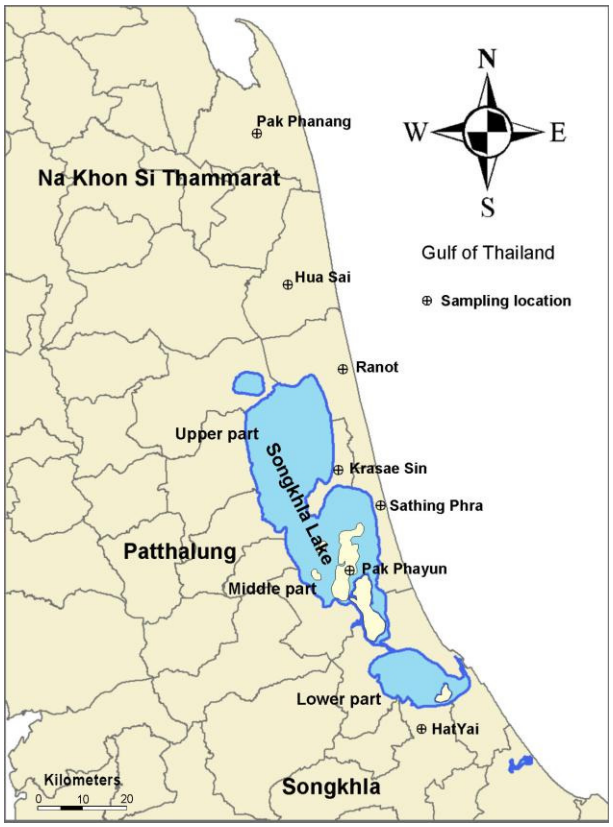

Figure 3-1. Map of Songkhla Lake Basin and Pak Phanang Basin indicating the 7 sampling sites where the samples were collected.

The conventional processes used to remove HMs are chemical and physical methods that are expensive and can accumulate toxic substances. Therefore, bioremediation could be an attractive process to remove HMs and reduce salinity from contaminated areas because it uses organisms like plants or microbes (Vieira and Volesky, 2000; Pietrobelli *et al.,* 2009). Microbes, particularly bacteria, play crucial roles in bioremediation and PNB are an interesting group to use for this purpose as they are normally found in shrimp ponds. PNB have been extensively used for

wastewater treatment because they can grow as photoautotrophs/photoorganotrophs under anaerobic/microaerobic-light conditions or as chemoorganotrophs under aerobic-dark conditions (Imhoff and Triiper, 1989; Cheng *et al.,* 2000) and also they are able to utilize a broad range of organic compounds as carbon and energy sources (Kim *et al.,* 2004; Kantachote *et al.,* 2005). Previous studies have shown that PNB have the potential to remove HMs (Watanabe *et al.,* 2003; Giotta *et al.,* 2006; Feng *et al.,* 2007) but there have been no reports that PNB have been isolated from HMs contaminated shrimp ponds. Therefore, the aims of this study were to explore the concentrations of HMs (Cd, Pb, Cu, and Zn) and Na in shrimp ponds and to isolate and screen for PNB from shrimp ponds based on their resistance to HMs and Na, including an ability to remove metal contaminants.

Material and methods

Study areas

The 31 selected shrimp pond sites are located around the SLB and coastal areas along the Gulf of Thailand in the district of Hua Sai, in Nakhon Si Thammarat Province; districts of Ranot, Krasae Sin, Sathing Phra, and Hat Yai, in Songkhla province and the Pak Payun district, in Patthalung province. Other sites, in the district of Pak Phanang, are not part of the SLB as it is part of the Pak Phanang Basin (Figure 3-1.)

Collecting and analyzing (HMs and Na) the samples

Soil and water samples were collected from 31 shrimp ponds (Table 3-1.). After shrimp harvesting, 13 soil or sediment samples each of about 100 g were collected from the bottom of each pond at a depth of 5 cm in two diagonal and a half point of each bank. For water samples, 100 ml of water at about 50 cm from the surface water level was collected at or close to the time for shrimp harvesting. However, in some farms, only a few samples of water were collected per pond as there was no boat to support the collection of samples following two diagonals of the pond. For collection of water samples in 4 shrimp ponds in the Hua Sai district, 2 ponds were storage ponds for retaining the water before discharge into the environment. All composite samples were kept in an ice box and were

used to isolate PNB as promptly as possible after collection (details will be provided later) and afterwards they were thoroughly combined into a single sample of water or sediment from each pond prior to analysis for HMs (Cd, Pb, Cu, and Zn) and Na using an Inductively couple plasma-optical emission spectrometer (ICP-OES; Perkin Elmer, Germany). Water samples were analyzed directly. Each sediment sample was air dried and passed through a 2 mm sieve. Then 2 g of sample was digested by nitric acid and hydrogen peroxide method (Radojevic and Bashkin, 1999) before analyzed by ICP-OES. The protocol used for the ICP-OES followed the instruction manual for the instrument.

Isolation of PNB from the samples

GM broth consisting of 3.8g sodium L-glutamic acid, 2.7g DL-malic acid, 2.0g yeast extract, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.8g $(NH_4)_2HPO_4$, 0.2g $MgSO_4.7H_2O$, 0.053g $CaCl_2.2H_2O$, 0.001g nicotinic acid, 0.001g thiamine hydrochloride, 0.01g biotin, 0.012g $MnSO_4.5H_2O$, 0.025g ferric citrate, 0.95g $CoCl_2.6H_2O$, and deionized water up to 1000 ml, pH 6.8 was used to isolate PNB from the sediment and water samples. One ml (1 g) from each water and sediment sample, respectively was transferred to 5 ml GM broth in a screw cap test tube and then sterile liquid paraffin was added to the top of the growth medium to achieve anaerobic conditions and finally cultures were incubated under continuous light with incandescent lamps (ca. 3000 lux) for 5-7 days. The anaerobic-light conditions were conducted to isolate PNB although aerobic anoxygenic phototrophs (AAP) may be present in the collected samples. AAP are obligate aerobes and will not grow with anaerobic conditions in continuous light (Yurkov and Beatty, 1998) and thus the conditions were selective for PNB. Afterwards, the culture broths that were pink, red or brown were streaked onto GM agar and then incubated with the same conditions as previously described to purify the organisms. A pure culture of each isolate was maintained by stabing in GM agar and stored at 4^oC until used.

Screening for PNB strains resistant to HMs and Na

The stock culture was subcultured twice to obtain an active inoculum then one loopful of each culture was transferred to a screw cap test tube containing GM broth leaving a little space to provide microaerobic conditions. All culture tubes

were incubated under incandescent lamps (ca. 3,000 lux) for 48 h. The OD_{660} of the culture was adjusted to 0.5 using sterile GM broth as diluent and GM broth as a blank. Four steps were carried out to obtain strains that were resistant to HMs and Na.

1. Primary screening, a 10% inoculum of each culture was added to 10 ml or 18 ml of GM broth in a 15 x 150 mm tube and the cultures were incubated under two growth conditions; aerobic-dark and microaerobic-light for 48 h. For consideration of their possible use in shrimp ponds both growth conditions were investigated. All culture tubes were placed in a shaker at 100 rpm at 30ºC, in darkness for 48 h. for the aerobic-dark conditions while for the microaerobic-light conditions all culture tubes were illuminated at about 3000 lux at the top of the shaker for 48 h. Bacterial growth in GM broth was measured at OD_{660} using a spectrophotometer and any isolates with growth exceeding 0.50 were selected for further screening.

2. In order to screen isolates that were resistant to Na each culture was grown in GM broth plus 1, 3, 5, and 8.5% (w/v) NaCl again under both incubating conditions as described above.

3. Screening for HMs resistant isolates was performed in GM broth containing the highest level of each HM found in the shrimp ponds (Cd 0.75 mg/L, Pb 62.63 mg/L, Cu 34.60 mg/L and Zn 58.50 mg/L). The following inorganic salts; $CdCl_2$, $PbCl_2$, $CuCl_2$ and $ZnCl_2$ were used. Growth in the tubes was measured by the spectrophotometer at OD_{660} and the percent of growth inhibition calculated by comparing with a control set containing no HMs.

4. Selection and characterization of PNB strains that was resistant to mixed HMs and NaCl in shrimp ponds**.** Each PNB isolate was grown with GM broth containing the highest concentration of mixed HMs found in the ponds with varying concentrations of NaCl at 1, 3, and 8.5% under both incubating conditions.

Determining optimum growth conditions for selected PNB resistant strains

Selected strains were used to investigate optimal growth conditions in GM medium under both incubating conditions for 48 h. The growth parameters considered for investigations were as follows: pH, temperature and shaking speed while light intensity was only measured under microaerobic-light conditions. Variations of pH: 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9; temperature: 25, 30, 35, and 40^{o}C;

and shaking speed: 50, 100, 150, and 200 rpm were tested. Light intensities were varied from 2,000, 3,000, and 4,000 lux and were measured by using a Denki light meter model DK-211 and the temperature was maintained at 30ºC.

Counting of viable cells was conducted under each optimal growth condition based on the above results to obtain more information on the effects of various incubating conditions on the 2 selected strains. Conditions included mixed HMs and NaCl concentrations. Each isolate was grown with either GM broth that contained the highest concentration of each of the mixed HMs with 3% NaCl or in the GM broth containing the average determined concentration of the mixed HMs (Cd 0.15 mg/L, Pb 13.25 mg/L, Cu 10.15 mg/L and Zn 23.01 mg/L) and 8.5% NaCl (the highest level detected in shrimp ponds). The cultures were incubated at 30^{o}C for 48 h. under microaerobic-light and aerobic-dark conditions and the growth was then measured using viable plate counts. Plates were incubated under microaerobic-light and aerobic-dark conditions depending on the conditions used for their growth in the broth cultures. For counting growth of PNB under microaerobic-light conditions the plates were placed in a plastic anaerobic jar with Gas-Pak under illumination at 3,000 lux.

Estimation of the efficiency of selected PNB strains to remove HMs and Na

The two selected PNB isolates were grown in GM broth under optimized conditions according to the aerobic-dark and microaerobic-light conditions for 48 h. and bacterial cells were separately harvested by centrifugation at 8,000 rpm for 15 min. The cell pellets were washed twice with 0.1% peptone water and fresh cells equivalent to 31.25 mg DW were suspended in 50 ml of mixed HMs solution at their highest concentrations detected in shrimp ponds and 3% NaCl to obtain 0.625 mg DW/ml. Dry weight was measured following the method in AOAC (2002). The cell suspensions were incubated at 30^{o}C on a shaking incubator with a speed of 100 rpm for 30 min under aerobic-dark conditions and the same condition was set for the microaerobic-light conditions with the light intensity adjusted to 3,000 lux. After that the bacterial cells were centrifuged at 8,000 rpm for 15 min and each supernatant was analyzed for the remaining HMs concentrations and Na to calculate their disappearance.

Data presentation and statistical analysis

All experiments in this study were conducted in triplicate unless otherwise stated. Means and standard deviations are presented. Statistical analysis using one way ANOVA to analyze statistical differences at a p-value < 0.05 and mean comparisons were performed by the Duncan' s multiple range test.

Results

Contamination of HMs and Na in the shrimp ponds

Table 3-1 shows the levels of HMs and Na in shrimp ponds around Songkhla Lake and the Pak Phanang Estuary. Overall, the average concentrations of HMs; Cd, Pb, Cu, and Zn in the sediment samples (mg/kg dry weight: DW) were 0.13 ± 0.16, 13.25 ± 13.48, 10.15 ± 8.76, and 23.01 ± 16.80, respectively, were much higher than those found in the water samples (mg/L) (Cd: 0.002 ± 0.001, Pb: 0.004 ± 0.003, Cu: 0.011 ± 0.017 and Zn: 0.155 ± 0.378). In contrast, the average Na content in the water samples (24.14 ± 21.86 g/L) was higher than that found in the sediment samples (1.44 ± 1.06 g/kg DW). In order to identify areas that might have a serious problem with either HMs and/or Na, the highest concentration of each element was considered. Among the sediments, Pak Phayun was the area that had the highest contaminations in mg/kg DW of Cd (0.75), and Pb (62.63) while Hua Sai was highest for Zn (58.50) and Ranot for Cu (34.60) and Na (3.21 g/kg DW). In contrast, the highest levels (mg/L) of each HM and Na in the water samples were found in different areas such as Pb (0.006) and Cu (0.06) in Sathing Phra, Cd (0.003) in Krasae Sin, Zn (1.70) in Pak Phayun and Na in Hua Sai (84.55g/L).

Table 3-1. The average concentration of heavy metals and sodium detected in shrimp ponds around Songkhla Lake Basin and Pak Phanang Basin.

Area (n) [a]	Sediment (mg/kg)					Water (mg/L)				
	Cd	Pb	Cu	Zn	Na	Cd	Pb	Cu	Zn	Na
Pak Phanang n = 3	0.06 ± 0.00	13.5 ± 0.00	5.00 ± 0.00	25.13 ± 0.00	1004.63 ± 0.04	<0.001	0.002± 0.00	0.004 ± 0.00	0.026 ± 0.00	21016 ± 17479
Hua Sai n = 4	0.10 ± 0.02	9.38 ± 4.47	14.35±12.31	33.49±21.44 (58.5)	2134.96 ± 1496.06 (3208.75)	<0.001	<0.005	0.003 ± 0.00	0.001 ± 0.00	43569 ± 37851 (84550)
Ranot n = 5	0.09 ± 0.06	8.69 ± 5.06	13.16±14.41 (34.60)	17.92±12.54	1164.75 ± 605.15	<0.001	<0.005	0.009 ± 0.00	0.001 ± 0.00	29043 ± 904
Krasae Sin n = 2	0.13 ± 0.00	20.75 ± 0.00	8.50 ± 0.00	32.25± 0.00	364.00± 1.01	0.003 ± 0.00 (0.003 ± 0.0)	<0.005	0.03 ± 0.03	0.22 ± 0.00	5112 ± 1840
Sathing Phra n = 3	0.15 ± 0.00	22.19±10.69	10.19 ± 9.46	35.44±13.70	1589.5 ± 1706.60	0.001± 0.00	0.006±0.00 (0.006±0.0)	0.02 ± 0.02 (0.06 ± 0.03)	0.14 ± 0.11	15524 ± 7751
Hat Yai n = 4	0.11 ± 0.12	4.59 ± 5.33	5.59 ± 7.38	12.34±15.04	810.15 ± 873.79	<0.001	<0.005	0.006 ± 0.00	0.24 ± 0.00	28431 ± 12910
Pak Phayun n = 10	0.19 ± 0.22 (0.75)	19.74±21.59 (62.63)	10.22±11.34	22.26±24.44	1887.50 ± 2033.13	<0.001	<0.005	0.004 ± 0.00	0.53 ± 0.59 (1.70 ± 0.85)	21070 ± 22555
Overall n = 31	0.13 ± 0.16	13.25 ± 13.48	10.15 ± 8.76	23.01 ± 16.80	1437.12 ± 1060.32	0.002 ± 0.00	0.004 ± 0.00	0.01 ± 0.01	0.16 ± 0.38	24144 ± 21863
Sediment [b]	1.5	75	65	200	na	≤ 0.005[c]	≤0.0085[c]	≤0.008[c]	≤0.05[c]	na
Soil [d]	≤ 37	≤ 400	na	na	na					

Data represent mean ± standard deviation. [a] n= Number of ponds, [b] HKGS (1998), [c] Pollution Control Department, (2006), [d] Pollution Control Department (2004), na = not applicable, number in a bracket is the highest concentration of the detected element.

Isolation and selection of PNB resistant strains

A total of 120 PNB strains were isolated from 31 shrimp ponds, 71 strains (59.2%) from water and 49 strains (40.8%) from sediment samples, but only 100 strains were able to grow well (OD_{660} > 0.5) over 48 h. in GM broth under both aerobic-dark and microaerobic-light conditions. Twenty one of these strains (21%) were resistant to 5% NaCl i.e. inhibition < 50%. Most of the strains, that were resistant to 5% salt were inhibited by more than 95% by 8.5% NaCl, only 5 strains survived and some grew well (data not shown). Based on their resistance to Na, 5 strains were classified into being either halotolerant (KTS9, KMS24, and BNW3) or halophilic (NW7 and NW16). Isolates were then further selected for their resistance to the highest levels of each HM (Cd, Pb, Cu, and Zn) found in the shrimp ponds. Each strain had a different tolerance to each HM i.e. NW16, BNW3, and KTS9 were the most tolerant to Zn and Cu under aerobic-dark conditions, and Cd and Pb under both incubating conditions. However, strain KMS24 grown under aerobic-dark conditions was resistant to all HMs tested, particularly with Cd and Pb, whereas NW7 was resistant only to Cd and Cu under aerobic-dark conditions (data not shown).

As none of the 5 strains stood out as the best possible candidate for use in the shrimp ponds they were all retested for their resistance against a mixture of 4 HMs at the highest levels detected in the sediment samples together with varying concentrations of NaCl under both incubating conditions. Strains; KTS9, BNW3, and KMS24 were resistant to the highest levels of mixed HMs at 1% of NaCl with both incubating conditions (< 50% growth inhibition) (Figure 3-2A-2C). In contrast, at 1% NaCl strain NW16 was resistant only with aerobic-dark conditions and the growth of strain NW7 was inhibited (> 50% growth inhibition) with both conditions (see Figure 3-2A). When the NaCl content was adjusted to 3%, only two strains (NW16 and KMS24) were resistant to the highest level of mixed HMs under aerobic-dark and microaerobic-light conditions (Figure 3-2B). Neither of them was resistant under both incubating conditions at the highest levels of mixed HMs at 8.5% NaCl (Figure 3-2C). Based on the above results, strains NW16 and KMS24 were selected for further investigating their ability to remove HMs and Na.

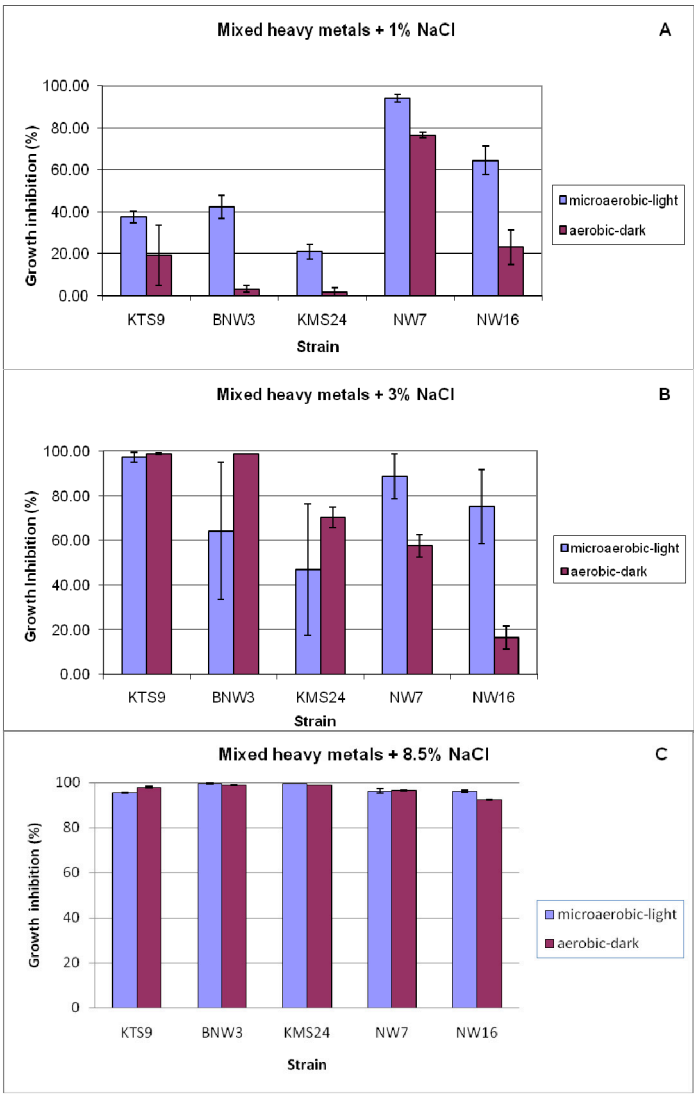

Figure 3-2. Effect of HMs against growth of PNB isolates under microaerobic-light and aerobic-dark conditions in GM medium containing the highest levels of mixed HMs which detected in shrimp ponds with varying level of 1% NaCl (A), 3% NaCl (B), and 8.5% NaCl (C). Each bar represents the mean of three replicates ± standard deviation.

Optimum growth conditions of selected PNB resistant strains

The results of changing the following factors on growth; temperature, pH, shaking speed and light intensity affecting the growth of selected PNB strains, NW16 and KMS24, are shown in Table 3-2. Strain NW16 grew over a range of temperature (25-40^{o}C) with an optimum temperature of between 25-35^{o}C under both

incubating conditions. Under microaerobic-light conditions the optimal temperature for strain KMS24 was between 30-40^{o}C while with aerobic conditions it was 30^{o}C. Both strains could grow in the full range of pH values tested (5.0-9.0). Strain NW16 had a narrow optimum pH of 7.0 with aerobic-dark conditions whereas strain KMS24 had a wide range of optimum pH from 7.5-9.0 and 6.0-9.0 with both microaerobic-light and aerobic-dark conditions. The shaking speed of 150 rpm was an optimum speed for both PNB strains. For the light factor, an intensity of 3,000-4,000 lux produced optimum growth for both PNB strains under microaerobic-light conditions; however, the strain NW16 grew equally in the range of 2,000-4,000 lux.

The effects of the incubating conditions, HMs and Na on bacterial growth (optimal growth conditions) are presented in Figure 3-3. In the control sets without addition of HMs and Na, the growth of both strains reacted in a similar way with the aerobic-dark conditions being less than the growth with microaerobic-light conditions. In contrast, each strain had a different response to the incubating conditions when the medium contained mixed HMs and Na. Strain NW16 grew less with the GM medium containing both the highest and average levels of HMs plus 3.0% NaCl or 8.5% NaCl with microaerobic-light conditions than when grown with aerobic-dark conditions. In contrast, strain KMS24 grew more with the microaerobic-light conditions than with aerobic-dark conditions. In addition, the strain NW16 that was identified as a halophilic *Rhodobium marinum* (data not shown) was more resistant to the highest level of NaCl than with the highest levels of HMs. On the other hand, strain KMS24 that was identified as *Rhodobacter sphaeroides* (data not shown) was more resistant to the highest levels of HMs than to the highest level of NaCl. Overall, the isolate NW16 showed more tolerance to HMs and NaCl than did the strain KMS24.

Table 3-2. Optimum growth[a] conditions in GM medium of selected PNB strains.

Parameter	NW16		KMS24	
	Microaerobic-light	Aerobic-dark	Microaerobic-light	Aerobic-dark
Temperature (^{o}C)				
25	++	++	+++	+++
30	++	++	++++	++++
35	++	++	++++	+++
40	+	+	++++	+++
pH				
5.0	+	+	+	+
5.5	+	+	+	+
6.0	+	+	+	++
6.5	+	+	+	++
7.0	+	++	+	++
7.5	+	+	++	++
8.0	+	+	++	++
8.5	+	+	++	++
9.0	+	+	++	++
Shaking speed (rpm)				
50	++	++	++++	++
100	+++	++	++++	+++
150	+++	+++	++++	++++
200	ND	+++	ND	+++
Light intensity (lux)				
2000	++	ND	+++	ND
2500	++	ND	+++	ND
3000	++	ND	++++	ND
3500	++	ND	++++	ND
4000	++	ND	++++	ND

[a]OD_{660nm}: ≤ 0.5 = +, > 0.5 OD ≤ 1.5 = ++, > 1.5 OD ≤ 3.00 = +++, > 3.00 = ++++

ND = not determined

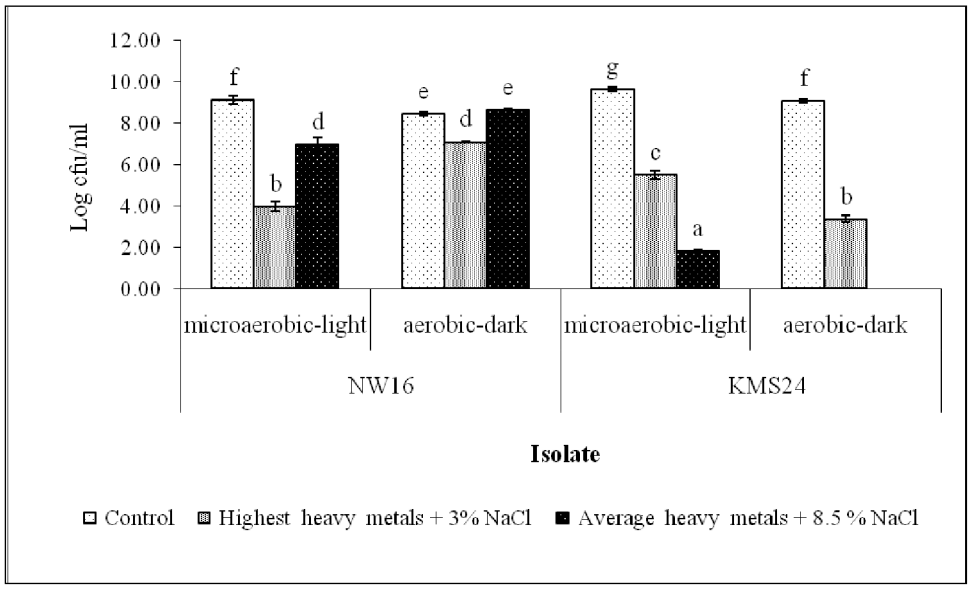

Figure 3-3. The viable cell count of selected PNB strains in GM medium under optimal growth conditions with both incubating conditions in sets of control, the highest levels of mixed HMs with 3% NaCl and the average levels of mixed HMs with 8.5% NaCl. Each bar represents the mean of three replicates ± standard deviation. Lowercase letters above bars indicate significant differences when using a different letter ($p < 0.05$).

Efficiency to remove HMs and Na of selected PNB strains

With optimal growth conditions in GM medium, both strains grew better with microaerobic-light than that with aerobic-dark conditions; strain KMS24 produced a biomass of 0.385 g DW/L and 0.270 g DW/L while strain NW16 produced 0.564 g DW/L and 0.383 g DW/L, respectively. Hence, results (this experiment and Table 3-2.) indicated that comparisons of growth measurements based on turbidity using a spectrophotometer at the same wavelength (OD_{660} nm) may not be accurate, especially if the colors of the cells are different like NW16 is pink while KMS24 is brown and the colors also differ with different growth conditions for the same organism. This is why the removal of the elements was tested with the same weight of bacteria rather than the same OD_{660} nm.

The efficiency of the same dry weight of fresh cells (0.625 mg DW/ml) of each strain to remove the highest levels of a mixed HMs and Na in solution is shown in Table 3-3. Both strains were capable of removing HMs with the relative efficiencies of Pb > Cu > Zn > Cd with both microaerobic-light and aerobic-dark conditions. The highest removal efficiency was found for Pb by the strain NW16 with microaerobic-light and aerobic-dark conditions at 27.70% and 39.49%, respectively. In contrast, the lowest removal efficiency was found for Cd by strains NW16 and KMS24 in a range of 2.33-6.99% with both incubating conditions. The efficiency to remove HMs and Na by strain NW16 in aerobic-dark conditions was more significant than that with microaerobic-light conditions, whereas there was no significant difference found for the strain KMS24. However, the removal percentage of Na by the strain KMS24 was significantly higher with aerobic-dark conditions compared to microaerobic-light conditions. In addition, the strain KMS24 gave a higher efficacy to remove Na (31.12 – 30.07 = 1.05%) than the strain NW16 with aerobic-dark conditions. The overall results clearly showed that strain NW16 had a higher efficiency to remove HMs than the KMS24 with both incubating conditions but there was no significant difference in ability to remove Na found for either strain with microaerobic-light conditions.

As the loss of elements from the solution by fresh cell suspensions with no added substrates is reported as a removal percentage from the solution and the biosorption capacity over the 30 min period could be determined from (biosorption capacity = mg HM removed/gDW biomass). Then, using data in Table 3-3 the biosorption capacity for each element can be calculated e.g. the biosorption of Pb by the strain NW16 with aerobic and microaerobic conditions was 39.68 and 27.78 mg/g DW and equivalent to the removal of 15.20 and 15.67 mg/L culture broth. In the same way, strain KMS24 grown with aerobic and microaerobic conditions remove Pb with a biosorption capacity of 21.38 and 22.88 mg/g DW equivalent to a loss of 5.77 and 8.81 mg/L culture broth. In case of Na, the biosorption capacity of strain NW16 with light and dark conditions was 12.42 and 14.43 g/g DW equivalent to the loss of 7.00 and 5.53 g/L culture broth. The Na biosorption capacity of strain KMS24 with light and dark conditions was 12.74 and 14.94 g/g DW equivalent to a loss of 4.90 and 4.03 g/L culture broth. This means that the removal of HMs and Na depended on both the

biomass capacity and yield of biomass. Based on the above information, it can be concluded that strain NW16 had a higher ability to remove HMs than strain KMS24 with all conditions tested.

Table 3-3. Efficiency of HMs and sodium removal by viable cells of selected PNB strains under microaerobic-light and aerobic-dark conditions for 30 min.

HMs and Na	Initial conc. (mg/L)[A]	Efficiency[B] (% biosorption)			
		Microaerobic-light		Aerobic-dark	
		NW16 (55.38 ml)[C]	KMS24 (81.25 ml)[C]	NW16 (81.63 ml)[C]	KMS24 (114.25 ml)[C]
Cd	0.75	2.33±0.83[a]	3.39±0.19[a]	6.99±1.50[b]	2.50±0.89[a]
Pb	62.63	27.70±2.78[b]	22.82±0.12[a]	39.49±1.44[c]	21.34±1.04[a]
Cu	34.60	12.37±0.89[a]	11.96±0.37[a]	19.92±0.99[b]	11.95±0.70[a]
Zn	58.50	4.63±0.92[ab]	4.57±0.04[ab]	5.46±1.24[b]	3.20±0.29[a]
Na	3.00%	25.84±0.73[a]	26.51±2.04[a]	30.07±1.74[b]	31.12±1.00[c]

[A] Unless stated, [B] Values in same rows followed by a different letter are significantly different ($p < 0.05$) using Duncan's multiple range test. [C] Value in a parenthesis is volume of culture broth that equal to 31.25 mg DW and the fresh cells were suspended in 50 ml of mixed elements solution to obtain 0.625 mg DW/ml cell suspension.

Discussions

Contamination of HMs and Na in the shrimp ponds

This study provides information on the distribution of HMs and Na in some shrimp ponds around the SLB and Pak Phanang Estuary in southern Thailand (Table 3-1). Analysis for HMs showed that sediment samples had significantly higher levels than the water samples. However, based on the standard guidelines issued by the national environmental committee for agricultural soil (Pollution Control Department, 2004) and Hong Kong standards for dredged sediment planning (HKGS,

1998) (Table 3-1) results of sediment samples showed that the HMs concentrations in shrimp ponds of the study areas were lower than the limitation of HMs allowed in agricultural soil and sediment. In contrast, the distribution of Cu and Zn in water samples from most sampling sites was higher than the standard guidelines for marine aquatic animal cultivation (≤ 8 and ≤ 50μg/L for Cu and Zn, respectively) (Pollution Control Department, 2006).

The Cu contents of 9 and 30 μg/L that exceeded the guideline were detected in 32% of water samples collected from shrimp ponds in Sathing Phra, Krasae Sin and Ranot. Whilst contamination by Zn in water samples (140 – 530 μg/L) that also exceeded the guidelines was found in 61% of water samples collected from shrimp ponds in Pak Phayun, Hat Yai, Krasae Sin and Sathing Phra. As there was no correlation between the amounts of each HM and Na in the sediment and water samples from each pond (Table 3-1) this meant that the HMs concentrations in the waters were not caused by the sediments in the shrimp ponds. The results reflect the improper discharge of wastewater from various human activities, e.g. domestic, industry and agriculture in which Cu and Zn were transported into the Songkhla Lake and the gulf of Thailand as previously mentioned (Cheevaporn and Menasveta, 2003; Pradit *et al.,* 2009) and then this water containing HMs was transported to shrimp ponds for shrimp cultivation. This finding was in agreement with findings of Cheung and Wong (2006) who reported that contamination of the shrimp ponds in the Mai Po nature reserve, Hong Kong was caused by municipal and industrial activities in the surrounding areas (northwest Hong Kong and northern part of Deep Bay).

Na levels in the water samples collected from shrimp ponds (Table 3-1) could be classified into 3 groups as follows: high (4.44%), medium (2.11-2.90%) and low (0.51-1.55%). In the area of Hua Sai, farmers used seawater from the gulf of Thailand for shrimp cultivation and in this case a high Na content was found because two storage ponds for waste shrimp water were included in the water samples. In Hat Yai farmers used brackish water from a lower part of the Songkhla Lake which is very close to the gulf of Thailand (Figure 3-1) resulting in a medium level of Na. As expected the shrimp ponds in Krasae Sin and Sathing Phra had a low level of Na in water as these areas are located in the upper and middle parts of Songkhla Lake, respectively where the sea water should have little or no effect on the water in this

area. Therefore, the amount of Na detected in shrimp ponds of the study areas depended on the salinity of the water sources.

From the above information, it seems that contamination by HMs and Na in the water column of shrimp ponds is caused by the sources of water used. In storage ponds there is a high accumulation of Na. Therefore, water after shrimp harvest should be treated before discharge to any environment to prevent HMs and Na into natural areas. If there are no strict regulations by the governmental agencies for shrimp farmers to follow, the situation might get worsen.

Properties of PNB isolates resistant to HMs and Na

Results indicate that the PNB in shrimp ponds are more frequently isolated from the water (59.2%) rather than from the sediment (40.8%). The dominance of PNB in the water column of the shrimp ponds reflects the situation in that the water is directly exposed to sunlight, while the sediment is rather far from the sunlight or the turbidity in water decreases the light penetration.

Among the salt resistant isolates, a halophile group (NW7 and NW16) was isolated from water samples while a halotolerant group (KMS24 and KTS9) was isolated from sediment samples, except BNW3 (Figure 3-2). It can be explained that isolates were familiar with the amounts of Na in their habitats (Table 3-1). However, Xu *et al.* (1998) have reported that PNB isolated from freshwater could adapt themselves to tolerate NaCl in the range of 0% - 4%. In contrast, the halophilic PNB are able to grow well in a medium containing NaCl in the range of 1% to 4% while growth is retarded from 5% to 10% (Xu *et al.,* 2001).

Each HM and Na had differential toxic effects on the growth of the PNB isolates tested. Additionally, their susceptibility to toxicity of HMs and Na with an aerobic-dark or microaerobic-light conditions differed. It seems that a high amount of Na had a more negative effect on the growth of the 5 PNB isolates than the highest levels of the mixed HMs used (Figure 3-2). However, with a combination of the highest levels of HMs and Na isolates NW7 and NW16 had retarded growth (> 90% inhibition) even though they are halophilic bacteria (Figure 3-2C). Based on the results in Figure 3-2 it is likely that only isolates NW16 and KMS24 could survive in all shrimp ponds after the shrimp were harvested because the pond waters had Na

concentrations in a range of 0.51-4.36% (Table 3-1) and thus their abilities to remove HMs and Na were worth investigating.

Each isolate had a different response to the incubating conditions when the GM medium was supplemented with different concentrations of mixed HMs and Na. The HMs contaminants had a greater negative effect on the isolate NW16 with microaerobic-light conditions while isolate KMS24 was more sensitive with the aerobic-dark conditions. In the control sets with normal media both isolates had the same response to the different incubating conditions as both strains grew better with microaerobic-light than that with aerobic-dark conditions. This indicated that each isolate might have different mechanisms to respond to the contaminants. Some can bind the metal ions present in the medium at the cell surface or transport them into the cells and detoxify them in various ways (Ariskina *et al.,* 2004; Gavrilescu, 2004; Mallick, 2004).

In these experiments, it is possible that the inhibition observed may be due to the combination of HMs used together with the high Na concentrations. However, both isolates did survive the conditions tested. Therefore, it was of interest to know if these isolates could remove HMs with the conditions that exist in the shrimp ponds i.e. the presence of HMs and 3% NaCl.

Efficiency to remove HMs and Na of selected PNB strains

In order to investigate the potential of the 2 isolates to remove HMs and Na, their optimal growth conditions were first investigated. Strain KMS24 grew better at a wide range of pH values (6-9) and temperature (30-40ºC) when compared with the strain NW16 (Table 3-2) but strain NW16 was more resistant to all HMs tested and Na (Figure 3-3). Based on their optimal growth conditions, it was possible to provide one set of conditions for the experiment to determine if suspensions of either strain could remove HMs and Na as follows: pH 7, 30ºC and 150 rpm with a light intensity of 3,000 lux for microaerobic-light conditions.

Both selected strains, NW16 and KMS24, with both incubating conditions did remove HMs but with different efficiencies in the order of Pb > Cu > Zn > Cd that were not directly related to their initial concentrations (Table 3-3). The removal of HMs like Cd, Zn, Cu by halophilic bacteria, PNB and microalgae have

been studied and it has been found that the removal efficiency is higher at higher initial concentrations (Al-Momani *et al.,* 2007; Bai *et al.,* 2008; Monteiro *et al.,* 2009). This could be one reason why the removal of Cd was so low as its initial concentration was very low when compared with other HMs (Table 3-3). However, in case of Zn the removal efficiency was lower than that found in Cu although its initial concentration was higher (Table 3-3). It may be that Zn gave an adverse effect on live cells of organisms tested at a lower concentration than Cu. Balsalobre *et al.* (1993) reported that Zn (10 mg/L) had a negative effect on the growth of *R. sphaeroides* but Cu at 10 mg/L was not toxic to cells. Hence, it could be explained that less toxicity of Cu compared to Zn caused a higher removal of Cu than Zn by both strains tested.

It is possible that the selected isolates may use more than one mechanism to remove metal ions like biosorption (adsorption of metal ions onto the cell surface without requirement of energy) or bioaccumulation (absorption of metal ions into the cells with an energy requirement). However, in order to select for an isolate to use for bioremediation and as the removal efficiency was investigated using living cells suspension only a mixed HMs solution without addition of nutrients and with a contact time of only 30 min was used. It is likely that biosorption would be the main mechanism that the biomass could use in the absence of an energy demanding accumulation inside the cells. The big differences between the removal efficiencies of Pb and Cu and Zn might indicate that their mechanisms of removal are different.

The results in this study have shown that the removal percentage of each HM is not high. However, the biosorption experiment was performed for only 30 min with a viable biomass equivalent to 0.625 mg DW/ml and it might be possible to obtain a higher percentage of HMs removal by both strains by increasing the exposure time and biomass dose including the use of optimal conditions for pH and temperature. In addition, the organisms may use other mechanisms in addition to biosorption to remove HMs and Na as previously mentioned. It was stated earlier that removal of HMs and Na depended on both the biosorption capacity and yield of biomass and it has been proved that the isolate NW16 gave the best result (Table 3-3). However, isolate KMS24 seemed to be a good candidate for use in shrimp ponds (Table 3-2). Therefore, further investigations to achieve the goal of using inoculants

to remove contaminants like Cu and Zn (Table 3-1) from contaminated water will focus on both selected isolates.

Findings from this study indicated that the resistant PNB strains, NW16 and KMS24, have a potential to remove HMs and Na in amounts that were found in shrimp ponds with both aerobic-dark and microaerobic-light conditions. Therefore, it will be possible to use both strains as inoculants for bioremediation of water from shrimp ponds contaminated with toxic HMs and Na.

CHAPTER 4

Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds and their taxonomy

Abstract

Two purple nonsulfur bacteria (PNB) strains NW16 and KMS24, were checked for their potential to bioremediate shrimp pond water contaminated with HMs (HMs). By using biochemical and rDNA analysis the strains NW16 and KMS24 were identified as *Rhodobium marinum* and *Rhodobacter sphaeroides*, respectively. Both strains were tolerant to HMs present at the following concentration in their growth medium : 0.75 Cd^{2+}, 34.60 Cu^{2+}, 62.63 Pb^{2+} and 58.50 Zn^{2+} plus 3% NaCl that were similar to those found in contaminated shrimp ponds, using both microaerobic-light and both were slightly more resistant with aerobic-dark conditions. The determined MIC values were much higher than those used in their growth medium with their degree of tolerance being in the order of $Cu^{2+} > Zn^{2+} > Cd^{2+}$ (Pb was > 65 mg/L but precipitation occurred at 70 mg/L in GM medium). Under aerobic-dark conditions, strain NW16 was the most resistant to Cd^{2+} (48 mg/L). In contrast, this strain was the most sensitive to Cu^{2+} (109 mg/L) and Zn^{2+} (92 mg/L) under microaerobic-light conditions. Results of SEM-EDX (a scanning electron microscope equipped with an energy dispersive X-ray spectrometer) indicated that Cu^{2+} and Zn^{2+} altered the cellular morphology of both strains and accumulated HMs were found in their cells. Their cell walls contained the highest amounts of bound cations followed by the cytoplasm and cell membrane. Removal of HMs at the above concentrations in 3% NaCl under both incubating conditions by exopolymeric substances (EPS) was between 90.19-98.32% but only 10.71-80.02% by the cells (biomass). Strain NW16 removed more metal ions because it produced more EPS. Hence, both strains have the potential for use to remove HMs from contaminated shrimp pond water.

Keywords: accumulation, contamination, heavy metals, exopolymeric substances, photosynthetic bacteria, shrimp farming.

บทคัดย่อ

การทดสอบศักยภาพของแบคทีเรียสังเคราะห์แสง 2 สายพันธุ์ คือ NW16 และ KMS24 เพื่อใช้ในการบำบัดน้ำในบ่อเลี้ยงกุ้งที่ปนเปื้อนโลหะหนัก จากการเทียบเคียงโดยอาศัยคุณสมบัติทางชีวเคมีและวิธีการทางอาร์ดีเอ็นเอ (rDNA) พบว่าสายพันธุ์ NW16 เป็นเชื้อ *Rhodobium marinum* ในขณะที่ไอโซเลท KMS24 เป็นเชื้อ *Rhodobacter sphaeroides* ทั้งสองสายพันธุ์สามารถเจริญในอาหารที่มีความเข้มข้นของโลหะหนัก (แคดเมียม 0.75 ทองแดง 34.60 ตะกั่ว 62.63 และสังกะสี 58.50 มิลลิกรัมต่อลิตร) ในที่มีเกลือ 3 เปอร์เซนต์ ซึ่งเป็นสภาพคล้ายกับความเข้มข้นที่พบปนเปื้อนในบ่อกุ้ง ทั้งในสภาวะมีอากาศเล็กน้อย-มีแสง และสภาวะมีอากาศ-ไร้แสงโดยสภาวะหลังพบว่าเชื้อสามารถทนต่อโลหะหนักได้ดีกว่าเล็กน้อย จากการวัดค่า MIC พบว่ามีค่าความเข้มข้นสูงกว่าในอาหารที่เชื้อเจริญได้มาก โดยลำดับการทนต่อโลหะหนักของเชื้อคือ ทองแดง > สังกะสี > แคดเมียม (ตะกั่วที่ความเข้มข้น > 65 มิลลิกรัมต่อลิตร แต่ที่ 70 มิลลิกรัมต่อลิตรมีการตกตะกอนกับอาหาร GM) โดยที่สายพันธุ์ NW16 เมื่อเจริญในสภาวะมีอากาศ-ไร้แสงทนต่อแคดเมียมมากที่สุด (48 มิลลิกรัมต่อลิตร) ในทางตรงกันข้ามสายพันธุ์ นี้ไวต่อทองแดง (109 มิลลิกรัมต่อลิตร) และสังกะสี (92 มิลลิกรัมต่อลิตร) ที่สุดภายใต้สภาวะมีอากาศเล็กน้อย-มีแสง ผลจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) ควบคู่กับการวิเคราะห์รังสีเอกซ์โดยใช้ EDX พบว่าทองแดงและสังกะสีทำให้เกิดการเปลี่ยนแปลงลักษณะทางสัณฐานวิทยาของเซลล์ทั้งสองสายพันธุ์ รวมทั้งพบการสะสมของโลหะดังกล่าวที่ตัวเซลล์ด้วย โดยที่ผนังเซลล์มีการสะสมของโลหะหนักสูงสุดรองลงมาคือ ไซโตพลาสซึมและ เยื่อหุ้มเซลล์ ตามลำดับ นอกจากนี้ยังพบว่า exopolymeric substances (EPS) ที่สร้างจากทั้งสองสายพันธุ์สามารถกำจัดโลหะหนักซึ่งผสมอยู่ในสารละลายเกลือโซเดียมคลอไรด์ 3 เปอร์เซนต์ ภายใต้สภาวะการบ่มทั้งสองสภาวะ ได้สูงถึง 90.19-98.32 เปอร์เซนต์ ในขณะที่การใช้ตัวเซลล์กำจัดได้เพียง 10.71-80.02 เปอร์เซนต์ และพบว่าสายพันธุ์ NW16 สามารถกำจัดโลหะหนักได้มากกว่าเนื่องจากผลิต EPS ได้มากกว่า ดังนั้นทั้งสองสายพันธุ์จึงมีศักยภาพในการนำมาใช้เพื่อกำจัดโลหะหนักที่ปนเปื้อนในน้ำจากบ่อเลี้ยงกุ้ง

Introduction

The rapid increases in industrialization have not been accompanied by increases in the treatment and disposal of the hazardous wastes they produce and this has resulted in huge environmental problems. The bulk of the hazardous wastes generated have been dumped into the ocean, rivers, canals, any drainage systems and landfills (Bernhard *et al.,* 2004). In addition, many chemicals used as additives to benefit agricultural activities such as lime, fertilizers, manures, herbicides, fungicides and irrigation waters are also sources of HMs (Haq *et al.,* 2003). In Thailand shrimp farming is a major economic aquaculture business that can cause severe ecological problems by introducing toxic substances like HMs to the environment (Cheung and Wong, 2006; Panwichain *et al.,* 2010) and a wide variety of chemicals and biological products (Gräslund and Bengtsson, 2001; Gräslund *et al.,* 2002). Among these contaminants, HMs can cause the most serious environmental problems for wildlife, and in particular, human health. The HMs can accumulate in the shrimp body and consequently affect the physiological properties and survival rates of shrimp (Tangkerkolan and Cheewaporn, 2001) and finally affect humans as the highest trophic level of the food chain (Gorell *et al.,* 1997).

Therefore, standard guidelines for aquaculture products have been established for food safety around the world (Petroczi and Naughton, 2009). This means that if the concentrations of the HMs such as Cd (cadmium), Cu (copper), Pb (lead), Zn (zinc), etc. in shrimp exceed the standard safety levels, they might produce adverse effects on people who consume shrimp and thus have an impact on shrimp exports. Moreover, since there are no strict regulations by governmental agencies for the discharge of effluent from shrimp ponds into the environments, their wastewater is discharged into canals and can flow directly into cultivated areas. This has had a serious polluting effect on soil and agricultural water, especially for the rice-fields and adjacent aquaculture areas. The accumulation of HMs in agricultural soils is therefore of increasing concern due to food safety issues and potential health risk as well as its detrimental effects on the soil ecosystem.

High levels of HMs can affect the qualitative as well as the quantitative composition of microbial communities (Bahig *et al.,* 2008). Several studies have

found that HMs influence microorganisms by harmfully affecting their growth, morphology, and biochemical activities, resulting in a decrease of biomass and diversity (Mohamed *et al.,* 2006; Ahmad *et al.,* 2005). However, microbes living in contaminated environments often become adapted to survive in the presence of existing contaminants using a variety of mechanisms that enables them to tolerate the presence of HMs. These include accumulation and complexation of metal ions and/or metabolic coversions to less toxic compounds (Adarsh *et al.,* 2007; Spain and Alm, 2003). For example, extracellular polymeric substances or exopolymeric substances (EPS) secreted by microbes have been recommended as surface active agents. Because microbes have important roles in the management and sustenance of environments, it is important to characterize bacteria present in areas contaminated with HMs and to explore their roles in resistance to HMs that could include their removal (Affan *et al.,* 2009; Mengoni *et al.,* 2001; Roane and Kellogg, 1996). For example, purple nonsulfur bacteria (PNB) have been reported to resist various HMs (Moore and Kaplan, 1992; Panwichian *et al.,* 2010a)) and they are normally found in water habitats subject to a good light source including shrimp ponds. Hence, PNB also have been extensively studied for their bioremediation potential (Bai *et al.,* 2008; Feng *et al.,* 2007; Smiejan *et al.,* 2003; Watanabe *et al.,* 2003; Seki *et al.,* 1998).

In our previous work, we isolated two PNB strains from HM contaminated shrimp ponds that were resistant to Cd, Cu, Pb and Zn in salt (NaCl) concentrations of up to 8% (Panwichian *et al.,* 2010a). It therefore seemed useful to further explore their characteristics and establish if they could be considered for use in bioremediation of HMs in shrimp ponds. Hence, the aims of this study were to characterize and investigate the degree of resistance to HMs of both selected PNB strains. Moreover, accumulation of HMs and biosorption by EPS and biomass of these PNB were also investigated for consideration of their use for bioremediation in shrimp ponds.

Materials and methods

Heavy metals resistant purple nonsulfur bacteria

PNB strains, NW16 and KMS24, were previously isolated from samples of water and sediment collected from HMs contaminated shrimp ponds in Nakhon Si Thammarat and Songkhla Provinces, Thailand, respectively. Both strains were selected on the basis of their ability to grow well in GM medium (Lascelles, 1956) and were resistant to the HMs tested at the highest concentrations that had been detected in the sediment of shrimp ponds in mg/L; 0.75 Cd^{2+}, 62.63 Pb^{2+}, 34.60 Cu^{2+} and 58.50 Zn^{2+}, and including NaCl up to 5% under both microaerobic-light and aerobic-dark conditions (Panwichian *et al.,* 2010a).

Bacterial identification

Characterization of the selected PNB strains was conducted as follows. Pure cultures of NW16 and KMS24 strains were streaked on GM agar plates at 30º C and incubated for 48 h under anaerobic-light conditions. The colonies were then investigated for their elevation, margin, and other visual features. Gram staining was used to check their cell shapes using a light microscope. Their cell morphology and internal photosynthetic membranes were also investigated with a Scanning Electron Microscope (SEM); model 5800LV, JEOL and Transmission Electron Microscope (TEM); model JEM-2010, JEOL. The bacterial cell fixation and preparation techniques used for SEM and TEM followed the instruction manuals supplied for the instruments. In addition, whole cell pigment scans were carried out using cell pellets suspended in 60% sucrose and scanned by a Hitachi model UV-visible recording spectrophotometer.

The biochemical characteristics (Table 4-1) of both PNB strains were tested under anaerobic-light conditions, unless otherwise stated, according to Bergey's Manual of Systematic Bacteriology, 2nd edition, vol. 2, part C (Garrity *et al.,* 2005). To test their ability to utilize sulfide, thiosulfate and various organic substrates these were added to the basal minimal medium containing 10 mM of ammonium sulfate at a final concentration of 10 mM for each substrate. The requirement for each vitamin present in the basal medium (thiamine, biotin and nicotinic acid) including optimal

growth conditions (pH and temperature) were also examined. Details for characterization using the above mentioned methods were previously described by Kantachote *et al.* (2005).

Phylogenetic analyses were carried out as follows. Genomic DNA was prepared according to Ausubel *et al.* (2002). PNB cells were grown in GM medium until they reached stationary phase. Then 1.5 ml of each cell suspension was harvested by centrifugation for 2 min at 10,000 rpm with a microcentrifuge and resuspended in 567 µL TE buffer. 30 µL of 10% sodiumdodecyl sulfate and 3 µL of 20 mg/ml proteinase K were then added. After incubation at 37ºC for 60 min, 100 µL of 5 M NaCl and 80 µL CTAB/NaCl solution were added, well mixed and incubated at 65ºC for 10 min. An equal volume of chloroform was added, well mixed and then centrifuged at 10,000 rpm for 5 min. After transferring the supernatant to a new tube, 0.6 volumes of isopropanol was added and pellets were washed twice with 1 ml of 70% ethanol (v/v). Washed suspensions were sedimented and the pellets were dried by lyophilization.

Purified DNA was eluted by the addition of 100 µL of TE buffer. The DNA was used as a template in PCR amplifications. The 16S rRNA genes were amplified by PCR using the primers: 27F 5'-AGAGTTTGATCCTGGCTCAG-3', and 1389R: 5'-ACGGGCGGTGTGTACAAG-3'. The amplification conditions were 1 min at 95°C for the initial denaturation followed by 35 cycles consisting of 1 min at 94°C for denaturation, 30 sec 55°C for annealing followed by 2 min at 72°C for elongation. The final extension at 72°C was prolonged to 10 min. Reactions were carried out in a thermocycler and PCR products were separated in 0.8% agarose gels. DNA bands collected from the gel were purified by a Qiagen QIA quick gel extraction kit according to the manufacturer's instructions.

The 16S rDNA amplified PCR product was then used for sequencing. The sequencing reactions were conducted with an automate DNA sequencer (3100-Avant Genetic Analyzer, ABI). The partial 16S rDNA sequence was compared with the GenBank database in the NCBI website (http://www.ncbi.nlm.nih.gov/) by blastn. Alignment and phylogenic analyses were conducted in PAUP* version 4.0 (beta test version). Evolutionary distances and clustering was performed with the neighbor-joining method. The topology of the phylogenetic tree was evaluated by the bootstrap

method with heuristic search. Bootstrap analysis with a maximum of 1,000 trees for 500 bootstrap replicates was indicated in the tree.

Determination of MIC (Minimum Inhibitory Concentration)

The HMs tested for their MIC values were Cd, Pb, Cu, and Zn that were prepared from $CdCl_2$, $PbCl_2$, $CuCl_2$ and $ZnCl_2$, respectively. Stocks of the HMs were prepared in deionized water (DI) and sterilized by a filter membrane with a pore size of 0.22 μm and stored at 4°C until used. Each HM solution was added into GM broth containing 3% NaCl at different concentrations ranging from 0-150 mg/L. Both bacterial strains were separately grown in GM medium with either microaerobic-light (3000 lux) or aerobic-dark conditions at 30°C for 48 h according to the methods previously described by Panwichian *et al.* (2010a). Both incubating conditions were utilized because both PNB strains will be expected to treat water in normal shrimp ponds after harvesting. Each culture broth was adjusted to an optical density of 0.5 at a wavelength of 660 nm (OD_{660nm}) and 10% of each culture was inoculated into GM broth containing each HM with 3% NaCl as previously described. All culture test tubes were incubated at 30ºC with either microaerobic-light or aerobic-dark conditions as mentioned above for 48 h. Bacterial growth was measured as turbidity using a spectrophotometer at a wavelength of 660 nm and the lowest concentration of each HM that prevented growth of each strain is reported as the MIC.

Effect of cations (Cu^{2+} and Zn^{2+}) on the cellular morphology of the selected PNB strains

In order to observe if the uptake of HMs by cells would alter their cell morphology and to confirm their content of HMs in the biomass, SEM-EDX analysis was performed. Among the HMs (Cd, Pb, Cu and Zn) that were present as contaminants in shrimp ponds only Cu^{2+} and Zn^{2+} were considered to study an effect on the cellular morphology of selected PNB strains due to detecting amount of both HMs (Cu^{2+} and Zn^{2+}) in the water of shrimp ponds in amounts that exceeded the standard guidelines for marine aquatic animal cultivation (Panwichian *et al.,* 2010a). The stock cultures of strains NW16 and KMS24 were subcultured twice to obtain active cultures and then 10% of each culture was inoculated into GM broth containing

3% NaCl in the presence and absence (control) of 36.40 mg/L for Cu^{2+} or 58.50 mg/L for Zn^{2+} (the maximum amounts of both ions detected in sediments of shrimp ponds) (Panwichian *et al.* (2010a). All culture tubes were incubated at 30ºC for 60 h with microaerobic-light conditions as in these conditions these bacteria were more sensitive to HMs when compared with aerobic-dark conditions (Table 4-2). After that each culture broth was centrifuged at 8,000 rpm for 15 min and the cell pellet was washed twice with 0.1% peptone water prior to use for studying cell morphology by SEM (JSM-5800LV, JEOL) that was attached to an EDS (Oxford ISIS 300). The bacterial fixation and preparation technique used for SEM and EDS were those described in the instruction manuals for the instruments.

Accumulation and distribution of HMs in cells

The HMs tested for accumulation in cells was Cu and Zn at their previously stated concentrations of 34.60 and 58.50 mg/L, respectively. The HM uptake was tested by culturing each isolate in 1000 ml GM medium containing Cu^{2+} and Zn^{2+} with a shaker speed of 150 rpm at 30 ºC under microaerobic-light and aerobic-dark condition for 60 h (late log phase). Each culture broth was centrifuged at 8,000 rpm for 15 min to obtain the cell pellet and culture supernatant. Each culture supernatant was analyzed for the remaining amount of HMs in the solution using ICP-OES (Inductively coupled plasma-optical emission spectroscopy) and 5 ml of the cell pellet of each organism was analyzed for the distribution of accumulated Cu and Zn in its cells using the method according to Al-Momani *et al.* (2007) as follows:

Cell wall

The accumulation of Cu and Zn at the cell wall surface was determined by washing the cell pellet (5 ml) of each strain with 10 ml of 0.1 M sodium citrate for 10 min, 3 times to release the cations from the cell wall and the wash solution of each organism was analyzed for Cu and Zn using ICP-OES.

Membrane

After washing, the cell pellets from the previous section were separately incubated at 30 ºC for 1 h in a solution of 4 ml lysozyme (1 mg/ml) and 6 ml of 0.01 M sodium phosphate, pH 7.0 to hydrolyze the cell walls under the hypertonic conditions of 10.3% sucrose for protecting the protoplast from rupture.

After incubating, each suspension was centrifuged at 8000 rpm for 10 min and the concentrations of Cu and Zn in the supernatant were analyzed by ICP-OES.

Cytoplasm

Intracellular accumulation (cytoplasm) was determined by using 10 ml of nitric acid (35% HNO_3: DI = 1: 1) for opening the protoplast and then Cu and Zn were measured by ICP-OES.

The percentage accumulation or relative amount (RA) in each cellular part was calculated based on the initial loss of HMs from the culture medium supernatant (accumulated amount in the bacterial cells).

Removal of HMs by exopolymeric substances and cells of PNB

The selected PNB strains; NW16 and KMS24 were grown in GM medium with microaerobic-light conditions until the cells reached stationary phase (72 h). This incubating condition was used because it allowed for a better growth than with aerobic-dark conditions (Panwichian *et al.,* 2010a). Each culture was centrifuged at 8,000 rpm for 15 min to obtain the culture supernatant for detecting EPS while the cell pellets (biomass) were separated to use for measurements for removal of HMs. To obtain EPS, two volumes of cold ethanol (4^{o}C) was added to each culture supernatant and incubated at 4^{o}C, for 24 h to precipitate the EPS. The suspension was then centrifuged at 8,000 rpm for 15 min and some EPS was used to determine the dry weight (DW). For the biosorption experiment, an amount of EPS equivalent to 2.5 mg DW/ml was added to the mixed HM aqueous solution (0.75 mg/L Cd; 62.63 mg/L Pb; 36.40 mg/L Cu and 58.50 mg/L Zn) and the biosorption test conditions were (pH 6, 30°C, 30 min with microaerobic-light or aerobic-dark conditions) as described in our previous paper (Panwichian *et al.,* 2010a). After biosorption any EPS-metal complex was separated by adding one volume of cold ethanol to the solution (slightly modified from Prado Acosta *et al.,* 2005) and centrifuging at 8,000 rpm for 15 min. Residual HM levels in the supernatant were determined by ICP-OES and the loss of HMs was interpreted as the amount that had formed a complex with EPS. In addition, the results of the EPS biosorption experiments were compared with the data from the cell pellets which had produced the EPS for removal of the HMs. Conditions for removal of the HMs by the biomass were the same as those used for the EPS experiment in order to

compare the results directly. The biosorption capacity (mg HM uptake/g DW) of EPS or biomass was also determined as described by Panwichian *et al.,* 201a). Additionally, the yield of EPS per biomass (Yp/b) based on the DW was calculated.

Statistical analysis

All experiments in this work were carried out in triplicate. Data are presented as a mean with a standard deviation of three determinations. One way ANOVA was used to analyze statistical differences at a P-value < 0.05 and mean comparisons were performed by the Duncan's multiple range tests.

Results

Identification of the HM resistant PNB

The preliminary characterization of both PNB strains (NW16 and KMS24) was based on morphological, biochemical and physiological properties (Table 4-1). By streaking each culture on GM agar and incubating for 48 h at 30ºC under anaerobic-light conditions, the colonial morphology of NW16 was similar to that of KMS24, it was smooth and circular in shape. The elevations were convex and the colony margins were entire. The color of the NW16 and KMS24 colonies was red and brown, respectively. Both strains were Gram negative, NW16 was rod shaped whereas KMS24 was ovoid. Based on SEM images (Table 4-1), strains NW16 and KMS24 each had polar flagella. In addition, reproduction by budding was found in strain NW16 while KMS24 reproduced by binary transverse fission (Fig. 4-2A and 2D). TEM images of strains, NW16 and KMS24 both show internal photosynthetic membranes with those of strain NW16 being lamellar, while those of strain KMS24 were vesicular (Table 4-1).

Table 4-1. Characteristics of HM resistant strains, NW16 and KMS24 isolated from contaminated shrimp ponds in southern Thailand.

Characteristics [a]	Purple nonsulfur bacteria			
	NW16	*Rhodobium marinum* [b]	KMS24	*Rhodobacter sphaeroides* [b]
Cell shape	rod	rod	ovoid	ovoid/rod
Cell size (µm)	0.4 x 0.9	0.7-0.9	0.6 x 0.9	2.0-2.5
Gram staining	negative	negative	negative	negative
Absorption maxima (nm)	262, 278, 287, 325, 885	375, 483, 516, 533, 590, 803, 883	317, 375, 447, 477, 511, 589, 801, 852	375, 450, 481, 513, 590, 805, 852, 880
Bacteriochlorophyll	*a*	*a*	*a*	*a*
Internal membrane system	Lamellae	Lamellae	vesicle	vesicle
Motility (flagellum)	+ (polar)	+ (polar)	+ (polar)	+ (polar)
Optimal pH	7.0	6.9-7.1	7.5-9.0	7.0
Optimal temperature (ºC)	25-35	25-30	30-40	30-34
Color of cultures				
Microaerobic-light	pink	pink/red	brown	brown
Aerobic-dark	slightly pink	white/slightly pink	red	red
NaCl requirement	+ (1-8.5%)	+ (1-5%)	-	-
Vitamins requirement				
Biotin	+	+	+	+
Nicotinic acid	+	+	+	+
Thiamine-HCl	+	+	+	+
Slime formation	+	na	+	+
Utilization of				
Acetate	+	+	+	+
Benzoate	-	-	-	-
Butyrate	+	+	+	+
Caproate	-	+	-	+
Citrate	+	±	+	+
Ethanol	+	±	-	-
Formate	-	+	-	-
Fructose	+	+	+	+
Fumarate	+	+	+	+
Glucose	+	+	+	+
Glutamate	+	+	+	+
Glycerol	-	±	-	+
Glycolate	-	nd	+	nd
Gelatin	-	-	-	nd
Lactate	-	±	-	+
Malonate	+	nd	+	nd
Methanol	-	-	-	±
Mannitol	+	+	-	+
Nitrate	-	nd	+	±
Propionate	+	±	+	+
Pyruvate	+	+	+	+
Succinate	+	+	+	+
Sorbitol	-	+	+	+
Tartrate	-	-	-	-
Sulfide	-	±	+	+
Thiosulfate	+	-	+	-

[a] All the tests were conducted under anaerobic-light conditions unless otherwise stated**,** [b] Code from Garrity et al. 2005, nd = not determined, + = growth/requirement, - = no growth, na = not applicable

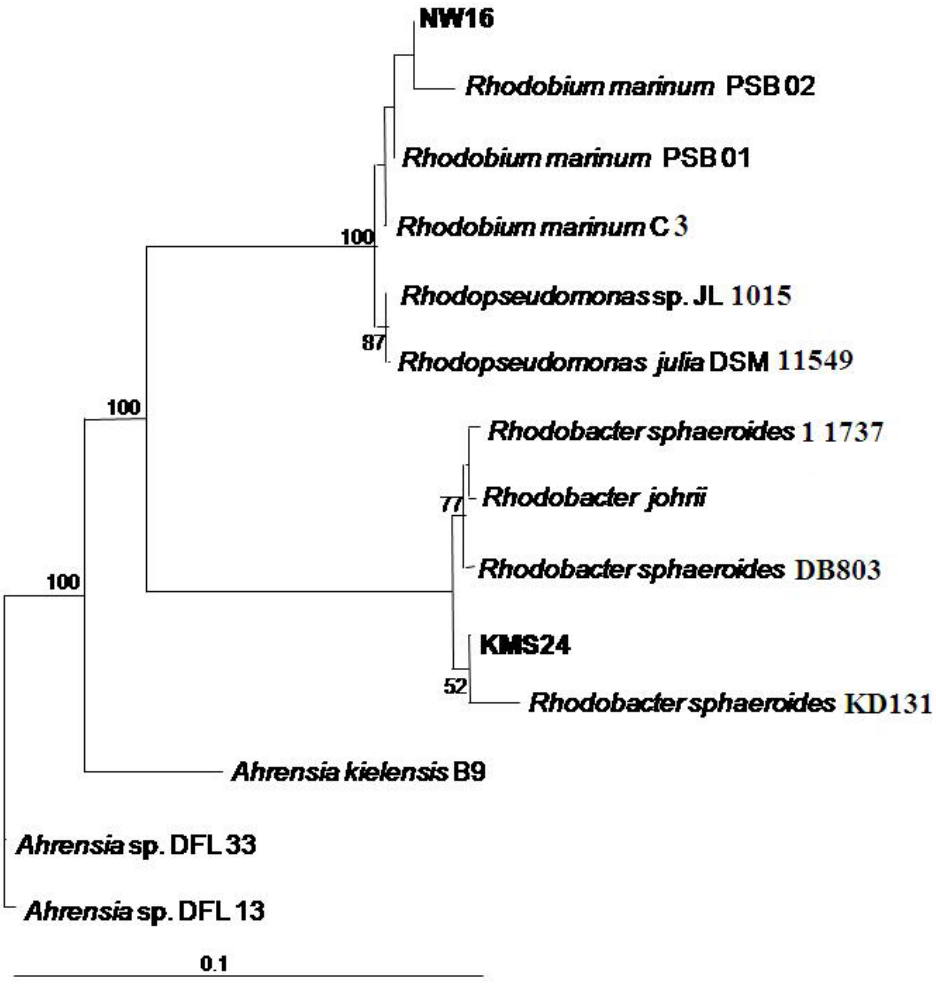

Figure 4-1. Phylogenic relationships between NW16 and KMS24 strains with most closely related species of PNB. The tree was constructed by Neighbor-joining method. bar = 10% substitution.

The biochemical tests results of strains NW16 and KMS 24 are shown in Table 4-1. Both require all the vitamins added to the basal medium for growth with anaerobic-light conditions. The following organic substrates were also utilized by both strains; acetate, butyrate, citrate, fructose, fumarate, glucose, glutamate, malonate, propionate, pyruvate and succinate. Moreover, they also used thiosulfate as an electron donor source. However, neither utilized benzoate, caporate, formate, gelatin, glycerol, lactate, methanol and tartrate. These results indicate that the strains NW16 and KMS24 were most closely related to known species of *Rhodobium marinum* and *Rhodobacter sphaeroide*s, respectively. Some differences were observed. Strain NW16 utilized thiosulfate but not caproate or formate while *R.*

marinum did not utilize thiosulfate but did utilize caproate and formate. Strain KMS24 did not utilize caproate, glycerol, lactate and mannitol but *R. sphaeroides* utilized them all. Using the partial 16S rDNA sequences for a homology search with the BLASTn program from the NCBI database, a neighbor-joining phylogenetic tree was constructed for the strains NW16 and KMS24 and several reference PNB strains (Figure 4-1). This showed that the strain NW16 is included in a cluster of *Rhodobium* strains and it was very closely related to *Rhodobium marinum* PSB02 (accession number EU919184) and PSB01 (EU910275) with a 99.8% sequence identity. Strain KMS24 is grouped in a different cluster and was closely related to *Rhodobacter sphearoides* KD131 with a similarity of 97.7% (CP001151).

Determination of MIC values

The testing of the HM tolerance of NW16 and KMS24 strains in the presence of 3% NaCl was carried out with both incubating conditions; microaerobic-light and aerobic-dark (Table 4-2). With both incubating conditions both strains were resistant to $Cu^{2+} > Zn^{2+} > Cd^{2+}$. The degree of resistance for strain NW16 to Cu^{2+} with aerobic-dark conditions was higher than for microaerobic-light conditions and for strain KMS24's resistance to Zn^{2+}. Moreover, strain NW16 had a significantly higher resistance to Cd^{2+} (48 mg/L) than strain KMS24 (37 mg/L) under aerobic-dark conditions. In contrast, under microaerobic-light conditions strain KMS24 had a significantly higher resistance to Cu^{2+} (127 mg/L) than that found for strain NW16 (109 mg/L). Strain NW16 had the highest resistance against Cu^{2+} with an MIC value of 123 mg/L when grown with aerobic-dark conditions and 127 mg/L by the strain KMS24 when grown with microaerobic-light conditions. These values are not significantly different. The MIC value for Pb^{2+} could not be determined in this study due to the precipitation of lead ions in the GM medium when the concentration of Pb^{2+} was 70 mg/L. However, both selected strains under both incubating conditions did grow in the GM medium containing 65 mg/L Pb^{2+}.

Table 4-2. Minimum inhibitory concentrations (MICs) of HMs against growth of purple nonsulfur bacteria (NW16 and KMS24) isolated from contaminated shrimp ponds.

Strain/Growth conditions	MICs (mg/L)			
	Cd^{2+}	Cu^{2+}	Zn^{2+}	Pb^{2+}
NW16				
Microaerobic-light	45.33±4.62^{bc}	108.67±12.05^{a}	91.66±2.89^{a}	na
Aerobic-dark	48.00±5.77^{c}	123.33±5.77^{bc}	98.67±3.21^{ab}	na
KMS24				
Microaerobic-light	33.33±5.77^{a}	126.67±5.77^{c}	98.67±3.21^{ab}	na
Aerobic-dark	36.67±5.77^{ab}	123.33±5.77^{bc}	100.00±5.00^{c}	na

Different lowercase letters in the same column indicate significant differences ($P < 0.05$), na: not applicable due to the precipitation of Pb^{2+} in GM medium at 70 mg/L

Data are a mean and a standard deviation from three determinations.

Effects of cations (Cu^{2+} and Zn^{2+}) on the cellular morphology of selected PNB

SEM was used to examine the morphology of the PNB cells after growing in GM medium containing 3% NaCl with and without 36.40 mg/L Cu^{2+} or 58.50 mg/L Zn^{2+} for 48 h and microaerobic-light conditions (Figure 4-2). Comparisons between the cell morphology in the controls (no HM) (Figure 4- 2A and 2D) and cells grown with either Cu or Zn, clearly showed marked differences. In Figure 4-2B and Figure 4-2C strain NW16 was elongated or even filamentous in the presence of Cu or Zn. Strain KMS24 also showed filamentous cells in the presence of Cu (Figure 4-2E), but with Zn they were dumbbell shaped (Figure 4-2F). Moreover, analysis for EDS confirmed that it was present after growth with Cu or Zn but not in the control set. From the ten areas of treatment sets that were selected for EDS analysis, the amounts of Cu and Zn in the NW16 cells grown in their presence were 0.44% and 0.38% of the total element components found in the cells, respectively. In contrast, strain KMS24 had even higher levels of Cu and Zn with 1.23% and 2.82% of the total element components, respectively (Table 4-3).

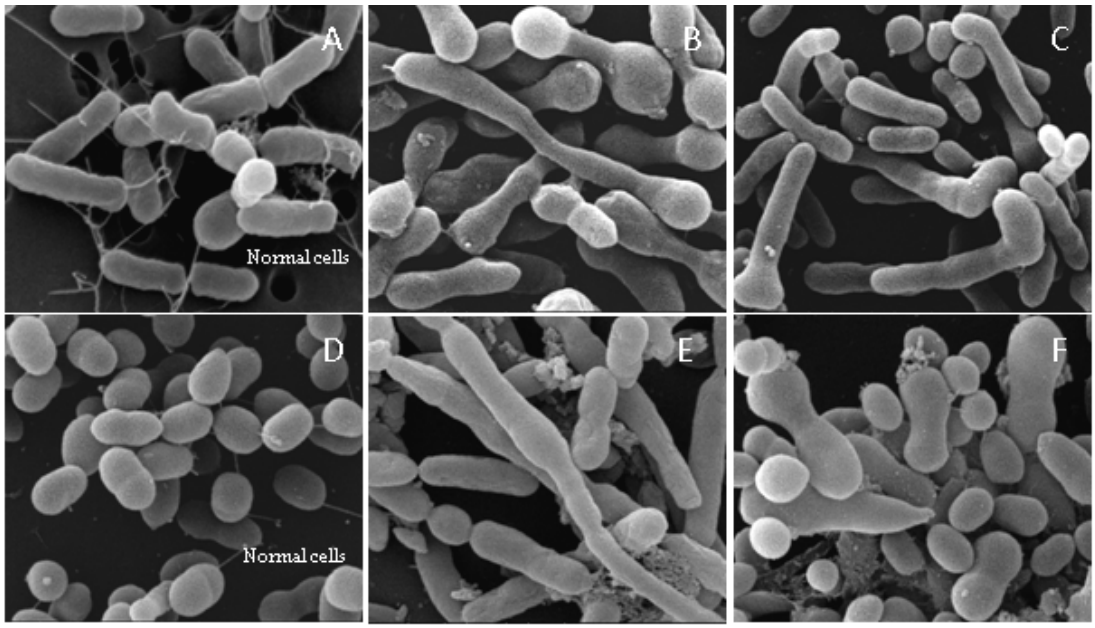

Figure 4-2. Scanning electron micrographs (20,000X) of PNB cells when grown with GM medium; NW16 control (A), NW16 treated with Cu (B), NW16 treated with Zn (C), KMS24 control (D) KMS24 treated with Cu (E) and KMS24 treated with Zn (F).

Table 4-3. EDS (energy dispersive X-ray spectrometry) analysis for Cu and Zn in cells of PNB strains.

Treated HM	Minimum (%)	Maximum (%)	Mean (%)[a]
NW16			
Cu	0.20	0.67	0.438±0.123
Zn	0.18	0.74	0.383±0.172
KMS24			
Cu	0.95	1.41	1.227±0.150
Zn	2.68	3.07	2.821±0.134

[a] Data are a mean of ten independent determinations ± a standard deviation, and Cu and Zn were not detected in the control set of each strain.

Accummulation and distribution of HMs in cells

Table 4-4 shows that Zn and Cu were accumulated by strains NW16 and KMS24 in three cellular fractions; cell wall, plasmamembrane (cell membrane) and cytoplasm.

Table 4-4. Accumulation and distribution of Cu and Zn in bacterial cells (NW16 and KMS24) under microaerobic-light and aerobic-dark conditions.

Conditions/ Cell fractions	Amount of heavy metals in each fraction							
	Rhodomium marinum NW16				*Rhodopseudomonas sphaeroides* KMS24			
	Cu		Zn		Cu		Zn	
	mg	RA* (%)	mg	RA (%)	mg	RA (%)	mg	RA (%)
Microaerobic-light								
Whole cell	13.56±0.77 (36.73±0.69)	100.00	7.39±0.22 (24.15±1.25)	100.00	17.00±0.77 (38.47±0.95)	100.00	11.00±1.34 (28.62±0.56)	100.00
Cell wall	1.18±0.02	8.72	0.20±0.00	2.69	1.68±0.00	9.86	0.34±0.01	3.09
Cell membrane	0.26±0.00	1.93	0.07±0.00	0.99	0.34±0.01	1.98	0.13±0.00	1.19
Cytoplasm	1.12±0.00	6.59	0.13±0.00	1.75	1.30±0.00	7.66	0.24±0.00	2.14
Undetected	11.03±0.04	82.76	6.99±0.00	94.57	13.68±0.00	80.50	10.29±0.00	93.58
Aerobic-dark								
Whole cell	16.94±0.52 (45.89±1.20)	100.00	8.12±0.81 (26.54±1.50)	100.00	17.58±0.72 (44.18±0.85)	100.00	9.96±1.28 (25.92±1.15)	100.00
Cell wall	1.66±0.02	9.82	0.16±0.00	2.00	2.07±0.02	11.77	0.21±0.01	2.16
Cell membrane	0.28±0.00	1.69	0.07±0.00	0.91	0.48±0.00	2.74	0.11±0.00	1.11
Cytoplasm	1.16±0.00	8.53	0.12±0.00	1.51	1.21±0.02	6.88	0.14±0.00	1.41
Undetected	13.84±0.00	79.96	7.77±0.00	95.58	13.82±0.00	78.61	9.50±0.00	95.32

*RA = relative amount, numbers in brackets are the removal percentages of heavy metals from the media. Data are presented as a mean and a standard deviation of three determinations.

Over the 48 h of cultivation in GM medium, the removal percentages of HMs in 3% NaCl under microaerobic-light and aerobic-dark conditions by the strain NW16 were 36.73 and 45.89% for Cu and 24.15% and 26.54% for Zn respectively. The amount of each HM removed, calculated as a percentage of the initial HM available, was assumed to have been accumulated within the cells. The amount of HM detected in the various cell fractions was then calculated as a percentage of the amount thought to be associated with the cell (RA). For both strains the relative amounts associated with each of the three cell fractions was similar cell wall > cytoplasm > cell

membrane. For instance, uptake of Cu by strain NW16 as a percent RA in the fractions of cell wall, cytoplasm and cell membrane was 8.72, 6.59 and 1.93% (see details for Zn and strain KMS24 in Table 4-4). Furthermore, the amount of HM not detected in any fraction of the biomass was extremely high and similar for both strains: undetected Cu (78.61-82.76%) and undetected Zn (93.58-95.58%).

Removal of HMs by exopolymeric substances and cells of PNB

Comparisons between the ability of the biomass and EPS of the two PNB strains (NW16 and KMS24) for removal of each HM from the mixed HMs solution (Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+}) are shown in Figure 4-3. The EPS from both strains exhibited a significantly higher ability to removal HMs ($P < 0.05$) when compared to their biomass. Amongst the HMs, a high uptake of Pb was observed both by the biomass and EPS. The efficiency to remove HMs by the EPS produced by two strains under both conditions had the following order of removal of average percentages: Pb > Zn ≈ Cd ≈ Cu (97.29, 91.83, 90.78 and 90.52%, respectively), while the biomass had an order of removal average percentage of: Pb > Cu > Zn > Cd (75.03, 40.81, 25.02 and 14.02%). Results show that the removal average percentages of Cd, Zn, Cu and Pb by EPS were increased with about 76.76, 66.81, 49.71 and 22.26%, respectively. In addition, the results indicated that the incubating conditions, either microaerobic-light or aerobic-dark conditions, had no affect on the removal efficiency of EPS produced by NW16 and KMS24 strains (Table 4-5A). In contrast, the efficiency to remove HMs by the biomass of both strains in microaerobic-light conditions was significantly higher than with aerobic-dark conditions for the case of Cu by the strain NW16 and for both Cu and Pb by strain KMS24 (Table 4-5B). There were no significant differences found for the removal efficiency of HMs by EPS collected from either strain NW16 or strain KMS24 under both incubating conditions (Figure 4- 3). However, when consideration on biosorption capacity of the amount of EPS produced by the strain NW16 was higher than for strain KMS24 (Table 4-5A) so the biosorptive ability overall is higher for NW16 than for KM24. Yield (Yp/b) of EPS produced by the strains NW16 and KMS24 was 0.859 (1.630/1.898) and 0.512 (0.922/1.7996), respectively.

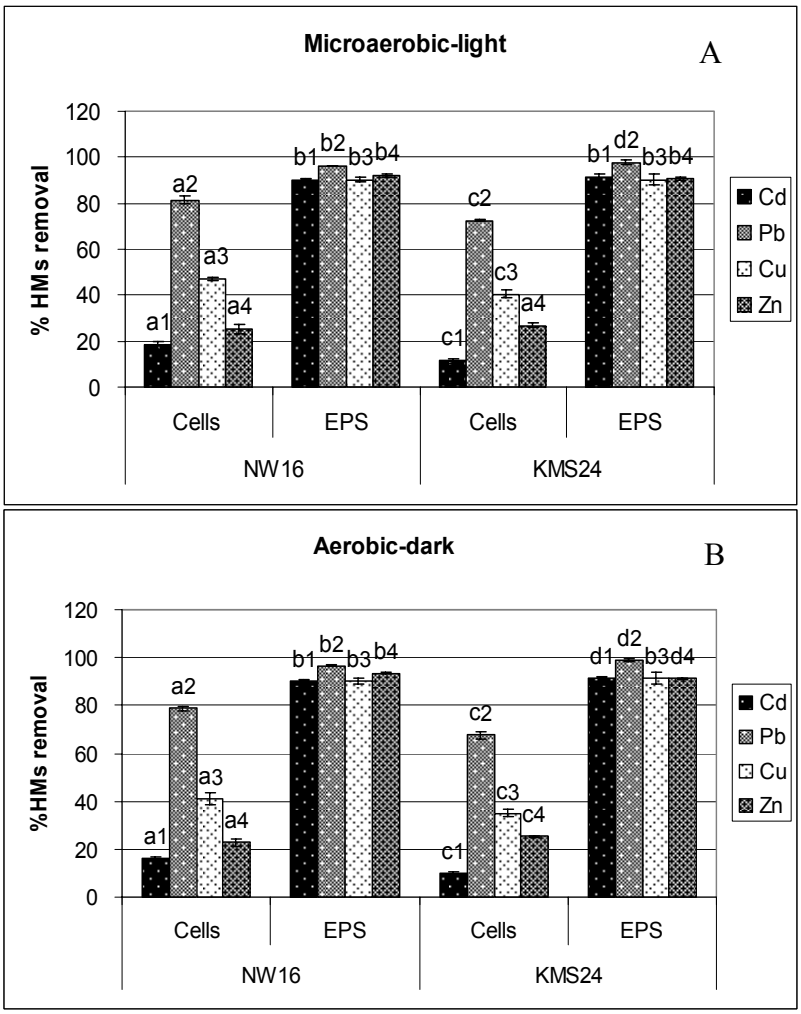

Figure 4-3. Heavy metals~~HMs~~ removal capacity by EPS and cell biomass of PNB strains; NW16 and KMS24, microaerobic-light (A) and aerobic-dark (B).

Table 4-5. Efficiency to remove heavy metals and biosorption capacity by (A) exopolymeric substances (EPS) and (B) biomass of strains NW16 and KMS24 under microaerobic-light and aerobic-dark conditions.

(A) EPS

Heavy metal	Initial conc. (mg/L)	NW16			KMS24		
		Remove (%)[1]	Biosorption capacity (mg/gDW)[2]	Biosorption (mg/L)[3]	Remove (%)[1]	Biosorption capacity (mg/g DW)[2]	Biosorption (mg/L)[3]
Cd	0.75	90.19^{a1}	0.29^{b1}	0.47^{c2}	91.38^{a1}	0.29^{b1}	0.27^{c1}
Cu	34.60	90.22^{a1}	13.28^{b2}	21.65^{c4}	90.81^{a1}	13.37^{b2}	12.33^{c3}
Pb	62.63	96.26^{a3}	25.62^{b4}	41.82^{c6}	98.32^{a4}	26.20^{b4}	24.16^{c5}
Zn	58.50	92.69^{a2}	23.07^{b3}	37.61^{c5}	90.98^{a1}	22.65^{b3}	20.88^{c4}

[1] average percentage under both incubating conditions, [2] mg/g dry weight EPS, [3] mg/L culture supernatant. Lowercase letters a, b and c for % removal, biosorption capacity as mg/g DW and biosorption as mg/L with different numbers in each column indicating significant differences ($P < 0.05$).

(B) Biomass

Heavy metal	Initial conc. (mg/L)		NW16			KMS24		
			Remove (%)[1]	Biosorption capacity (mg/gDW)[2]	Biosorption capacity (mg/L)[3]	Remove (%)[1]	Biosorption capacity (mg/gDW)[2]	Biosorption capacity (mg/L)[3]
Cd	0.75		17.33^{a2}	0.06^{b1}	0.10^{c2}	10.71^{a1}	0.06^{b1}	0.07^{c1}
		light	46.99^{a6}	6.92^{b3}	13.13^{c5}	40.42^{a5}	5.95^{b2}	10.71^{c3}
Cu	34.60	dark	40.78^{a5}	6.00^{b2}	11.40^{c4}	35.07^{a4}	5.16^{b2}	9.29^{c3}
			80.02^{a9}	21.33^{b5}	40.48^{c8}			
		light				72.52^{a8}	19.33^{b4}	34.78^{c7}
Pb	62.63	dark				67.57^{a7}	18.01^{b4}	32.41^{c6}
Zn	58.50		24.05^{a3}	5.99^{b2}	11.36^{c4}	25.99^{a3}	6.47^{b3}	11.64^{c4}

[1] No significant difference was found under both incubating conditions; thereby an average percentage is shown unless otherwise stated, [2] mg/g dry weight biomass, [3] mg/L culture broth. Lowercase letters a, b and c for % removal, biosorption capacity as mg/g DW and biosorption as mg/L with different numbers in each column indicate significant differences ($P < 0.05$).

Discussions

Bacterial identification

Results (Table 4-1 and Figure 4-2A and 2D) indicate that NW16 seemed to be a known species, *R. marinum* of the genus *Rhodobium* although this isolate had some different characteristics than the previously recorded strains of *R. marinum* for the utilization of caproate, formate and thiosulfate. However, phylogenic analysis of the partial 16S rDNA sequence showed that this isolate was very closely related to *Rhodobium marinum* PSB02, PSB01 and C3 with a similarity of 99.8%. It is well recognized that, members of the *Rhodobium* genus have been isolated from a wide range of saline environments such as brackish water and sea water. Strain PSB01 was isolated from a marine sediment of the Bohai Sea and had an ability to produce bioactive compounds; however, this strain was different from the strain NW16 as it had no flagella (Zhao *et al*., 2010). Strain C3 was isolated from a saline microbial mat (Urdiain *et al.,* 2008) and our strain was isolated from the water sample collected from a HMs contaminated shrimp pond. Therefore, it is not surprising that the strain NW16 was resistant to HMs in 8.5% NaCl (Table 4-1 and Panwichian *et al.,* 2010a).

Strain KMS24 closely resembled known species of the genus *Rhodobacter sphaeroides* although some of its biochemical properties were different such as the utilization of caproate, glycerol, lactate and mannitol (Table 4-1). However, the 16S rDNA sequence data showed that it had 97.7% identity with *Rb. sphaeroides* KD131 and 11737 (Figure 4-1). The strain KD131 has been reported to be one of the best strains for producing H_2 from a variety of organic substrates and at different light intensities (Kim *et al.,* 2006) while the isolate KMS24 is able to resist HMs in high concentrations of NaCl.

Resistance to and accumulation of HMs by PNB strains

The aim of this study was quite different from other previous studies on PNB strains as the MIC values for HMs of isolates NW16 and KMS24 were investigated in a medium containing 3%NaCl due to the expectation that they might be used for bioremediation of contaminated shrimp ponds. It was of interest that the

MIC value for each HM under both the incubating conditions tested was significantly higher than the maximum concentrations of the HM levels found in the shrimp ponds (Table 4-2). Hence, these organisms could be used as inoculants for removal of HMs in shrimp farm ponds. It was not surprising that the selected strains were the most sensitive to Cd, followed by Zn and Cu (Table 4-2) and these results were similar to those reported by Panwichian *et al.,* 2010a. Cu is an active component of enzymes such as cytochrome c oxidase including other oxygenases and thus bacteria can tolerate a higher concentration than for the other HMs tested. Zn is also an essential trace element for organisms and is a co-factor of some enzymes and forms complexes with enzymes like RNA and DNA polymerases (Nies, 1999). Therefore, both HMs at higher concentrations were less toxic than Cd. Cd is a toxic element and there have been no reports of its possible biological function. Consequently it may not be surprising that Cd was the most toxic element to both organisms. Results indicate that Cd was more toxic than Pb for the organisms tested because they could grow at 65 mg/L Pb while the MIC values for Cd for both organisms were between 33-48 mg/L under both incubating conditions. Based on results in Table 4-2, the strain KMS24 seemed to have a higher resistance to Cu and Zn than strain NW16 and this was supported by finding higher levels of metal ions either Cu or Zn in cells of the former organism (Table 4-4). Hence, the strain KMS24 is perhaps the best candidate for removal of HMs in shrimp ponds.

For the reasons previously described we have studied only the accumulation of Cu^{2+} and Zn^{2+} and their distribution in cellular fractions. At least 2 steps are required for metal uptake first their absorption onto the cell surface (passive) using metal-binding groups such as carboxyl, phosphate hydroxyl, etc. followed by active uptake using energy (Wang and Chen, 2006). In the latter step metal ions penetrate the cell membranes and enter into the cells. The present study reveals that the uptake of both metal ions by the selected strains consisted of both passive and active transport as the accumulated metal ions bound on the surface cell were eluted by 0.1 M sodium citrate but were also detected in the cell membrane and cytoplasm (Table 4-4). Under both the incubating conditions tested most of the metal ions accumulated by the selected strains were observed in their cell wall followed by their cytoplasm and the least was found in the cell membrane (Table 4-4).

It is known that both Cu and Zn are essential trace elements for bacterial growth and can be detected and accumulated in various subcellular compartments of cells (Table 4-4) However, when grown at high concentrations of HMs such as those found in contaminated shrimp ponds (34.60 mg/L Cu and 58.5 mg/L Zn) they produced adverse effects on bacterial cells as filamentous or dumbbell shapes were observed (Figure 4-2B, 2C, 2E and 2F). A similar result was previously reported by (Mohamed *et al.,* 2006) who showed that Cd altered the cellular morphology of *Rhodobacter capsulatus* B10 from rods to filamentous cells. As the excess metal ions in cells, results in alterations to cell morphology, selected strains might become resistant by trying to expel metal ions using efflux mechanisms. This may be one reason why the distribution of metal ions was in order of cell wall > cytoplasm > cell membrane. Biosorption of metal ions on the cell surface might reflect intracellular uptake and/or their efflux mechanism required for their HM resistance mechanisms. In the present study results indicated that the main resistance mechanism of the 2 selected isolates involves biosorption of HMs via EPS (Table 4-5A).

Removal of HMs by exopolymeric substances and PNB cells

It is well recognized that one of the important roles of EPS produced by microbes is to bind with HMs to allow for growth at high concentrations of HMs (Roane *et al.,* 1998) and thus EPS has been studied for use as a biosorbent for removing HMs (Watanabe *et al.,* 2003; Iyer *et al.,* 2005). There are two types of EPS: those attached to cells and those present in their culture fluid or non attached fractions (Xu *et al.,* 2009). EPS is often only loosely bound to cells in the form of slimes and can be easily shed into the medium. In this study the EPS used was in the form of a slime as it was collected from the culture supernatant using cold ethanol for precipitation. In general, most EPS consist of polysaccharides, protein, RNA and inorganic moieties such as sulfate or phosphate (Iyer *et al.,* 2005; Xu *et al.,* 2009). Based on the compositions of EPS, biosorption of HMs by EPS is non-metabolic, and no energy is required, as they bind with HMs using their negative charges (Watanabe *et al.,* 2003). Hence, the different incubating conditions used, either microaerobic-light or aerobic-dark had no affect on the HMs removal efficiency (Table 4-5A). It is

interesting that the EPS produced by both strains without optimizing their production conditions had a very good efficiencies to remove all the HMs tested with no significant differences being observed for their efficiencies to remove HMs; however, the strain NW16 gave a better biosorption capacity for HMs in mg/L of culture supernatant (Table 4-5A) because it produced a higher yield than the strain KMS24 of roughly 1.68 times.

EPS produced by both organisms bound Pb more efficiently than Zn (only strain KMS24), Cd and Cu (Table 5A). One possible reason to explain may be concerned with the different initial concentration of each HM ion and to the possibility that the uptake rate of the metal ion increases along with its increasing initial concentration when the amount of biosorbent is kept unchanged (Wang and Chen, 2006). Therefore, the higher uptake of Pb by EPS was due to the higher initial concentration. However, there were no significant differences found for Cd, Cu and Zn although their initial concentrations were widely different. Another factor that could have an influence on the amounts of different HMs bound is the possibility that the EPS had different affinities for different HMs. This means that Cd had a higher affinity for EPS than did Cu and Zn.

In contrast the efficiency of cells from both strains (biomass) to remove HMs under both incubating conditions was in the order of Pb > Cu > Zn > Cd (Table 4-5B). This indicates that some functional groups (i.e. carboxyl, phosphate, hydroxyl, amino, etc.) present on the cell surface may have different binding abilities to those of the EPS. These different affinities might also explain why Zn had a more toxic effect on the structural organization of the organism than did Cu. (Panwichain *et al.,* 2010a.) In the present study, the removal efficiency of HMs by both strains was significantly higher than found in our previous work (Table 4-5B and Panwichian *et al.,* 2010a) probably because a higher biomass was used with an increase from 0.625 mg DW/ml to 2.5 mg DW/ml. However, the biomass of these organisms was less efficient than the EPS in removing HMs. Factors that can affect the efficiency of the biomass to remove HMs will be further investigated and optimized.

Conclusions

Two selected strains, NW16 and KMS24, isolated from shrimp ponds contaminated with HMs were identified as *Rhodobium marinum* and *Rhodobacter sphaeroides*, respectively. Both strains could resist HMs at concentrations that were much higher than those found in the contaminated shrimp ponds and their HMs resistant mechanisms might include biosorption, metal uptake and efflux. One benefit of using these organisms is that they produced HM binding EPS in addition to the cells themselves accumulating HMs. Hence, the performance of both PNB strains indicated that they would be potential candidates for bioremediation processes in shrimp ponds.

CHAPTER 5

Factors affecting immobilization of heavy metals by purple nonsulfur bacteria isolated from contaminated shrimp ponds

Abstract

In order to remove heavy metals (HMs) from contaminated shrimp pond at the highest concentrations found of; 0.75 mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}, two strains of purple nonsulfur bacteria isolated from shrimp ponds (NW16 and KMS24) were investigated for their ability to immobilize HMs in 3% NaCl in both microaerobic-light and aerobic-dark conditions. Based on metabolic inhibition and metabolic-dependent studies, it was concluded that both strains removed HMs using biosorption and also bioaccumulation. The efficiency of removal by both strains with both incubating conditions tested was in the order of lead (Pb) > copper (Cu) > zinc (Zn) > cadmium (Cd). Optimal conditions for removal of HMs by strain NW16 were; cells in the log phase at 4.5 mg DCW/ml, pH 6.0, and 30ºC for 30 min. With microaerobic-light conditions, the relative percent removal of HMs was: Pb, 83; Cu, 59; Zn, 39; Cd, 23 and slightly more with the aerobic-dark conditions (Pb, 90; Cu, 69; Zn, 46; Cd, 28). Cells in the log phase at 5.0 mg DCW/ml, pH 5.5, and 35ºC for 45 min were optimal conditions for strain KMS24 and there were no significant differences for the removal percentages of HMs with either incubating conditions (averages: Pb, 96; Cu, 75; Zn, 46; Cd, 30). The presence of Ca^{2+} and Mg^{2+} significantly decreased the removal capacity of HMs for both strains.

Keywords: immobilization, heavy metals, photosynthetic bacteria, shrimp ponds

บทคัดย่อ

เพื่อที่จะใช้แบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ ที่แยกได้จากบ่อเลี้ยงกุ้ง จำนวน 2 สายพันธุ์ (NW16 และ KMS24) ในการกำจัดโลหะหนักที่ปริมาณความเข้มข้นสูงสุดที่พบปนเปื้อนในบ่อเลี้ยงกุ้งคือ แคดเมียม 0.75 มิลลิกรัมต่อลิตร ตะกั่ว 62.63 มิลลิลกรัมต่อลิตร ทองแดง 34.60 มิลลิกรัมต่อลิตร และสังกะสี 58.50 มิลลิกรัมต่อลิตร ในสภาวะที่มีความเข้มข้นเกลือโซเดียมคลอไรด์ 3 เปอร์เซนต์ ทั้งในสภาวะมีอากาศเล็กน้อย-มีแสงและสภาวะมีอากาศ-ไร้แสง จากการศึกษากลไกในการกำจัดโลหะหนักแบบยับยั้งกระบวนการเมแทบอลิซึม (metabolic inhibition) และอาศัยกระบวนการเมแทบอลิซึม (metabolic dependent) สรุปได้ว่าทั้งสองสายพันธุ์สามารถกำจัดโลหะหนักได้โดยอาศัยทั้งกระบวนการ biosorption และ bioaccumulation โดยมีประสิทธิภาพในการกำจัดโลหะหนักในทั้งสองสภาวะตามลำดับดังนี้คือ ตะกั่ว > ทองแดง > สังกะสี > แคดเมียม และพบว่าสภาวะที่เหมาะสมในการกำจัดโลหะหนักของสายพันธุ์ NW16 คือ ปริมาณชีวมวลของเซลล์ในระยะ log phase ใช้เซลล์สดเทียบเท่า 4.5 มิลลิกรัมน้ำหนักแห้งต่อมิลลิลิตร พีเอช 6.0 อุณหภูมิ 30 องศาเซลเซียส และระยะเวลาในการดูดซับ 30 นาที โดยในสภาวะมีอากาศเล็กน้อย-มีแสง สามารถกำจัดตะกั่วได้ 83 เปอร์เซนต์ ทองแดง 59 เปอร์เซนต์ สังกะสี 39 เปอร์เซนต์ และ แคดเมียม 23 เปอร์เซนต์ ส่วนสภาวะมีอากาศ-ไร้แสงพบว่ามีเปอร์เซนต์การกำจัดโลหะหนักได้สูงกว่าเล็กน้อย (ตะกั่ว 90 เปอร์เซนต์ ทองแดง 69 เปอร์เซนต์ สังกะสี 46 เปอร์เซนต์ และแคดเมียม 28 เปอร์เซนต์) สำหรับสายพันธุ์ KMS24 พบว่าสภาวะที่เหมาะสมในการกำจัดโลหะหนักคือ ปริมาณชีวมวลของเซลล์ในระยะ log phase ใช้เซลล์สดเทียบเท่า 5.0 มิลลิกรัมน้ำหนักแห้งต่อมิลลิลิตร พีเอช 5.5 และอุณหภูมิ 35 องศาเซลเซียส เป็นเวลา 45 นาที โดยที่พบว่าเปอร์เซนต์การกำจัดโลหะหนักในแต่ละสภาวะไม่มีความแตกต่างกันอย่างมีนัยสำคัญ (ค่าเฉลี่ยเปอร์เซนต์การกำจัดตะกั่ว 96 เปอร์เซนต์ ทองแดง 75 เปอร์เซนต์ สังกะสี 46 เปอร์เซนต์ และแคดเมียม 30 เปอร์เซนต์) นอกจากนี้ยังพบว่าแคลเซียมไอออน และแมกนีเซียมไอออน มีผลทำให้ประสิทธิภาพในการกำจัดโลหะหนักของทั้งสองสายพันธุ์ลดลงอย่างมีนัยสำคัญ

Introduction

Thailand is recognized as one of top ten aquaculture producers in the world (FAO, 2007). Contamination of seafood by heavy metals (HMs) such as lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn) has become a barrier for export due to the well documented health hazards associated with ingestion of HMs (Petroczi and Naughton, 2009). For example, chronic exposure to HMs like Cu, Pb and Zn has been reported to be associated with Parkinson's disease (Gorell *et al.,* 1997) and these are present in shrimp farm cultivation water that is normally derived from the coastal environment (Cheevaporn and Menasveta, 2003; Pradit *et al.,* 2009). In order to ensure that cultivated shrimp are safe, it has been recognized for a long time that removal of HMs from shrimp ponds particularly Cd and Pb that have been accumulating within the aquatic food chain is essential (Mdegela *et al.,* 2009; Mokhtar *et al.,* 2009; Yap *et al.,* 2004).

Traditional methods for removal of HMs e.g. chemical precipitation, ion exchange, and reverse osmosis have some significant disadvantages e.g. a high cost of treatment, low efficiency at low concentrations of HMs and the remnants of some toxic substances etc. (Lloyd and Lovley, 2001). An alternative method, like bioremediation, that uses microbes has many advantages such as high efficiency, low cost, easy to operate, and environmentally friendly. Furthermore, perhaps the most important benefit is that the residual soils, sediments, water, or sludge after removal by microbial actions can be reused (Gazso, 2001; Lloyd and Lovley, 2001). Immobilization of HMs by microbes occurs by precipitation, biosorption and bioaccumulation (Gazso, 2001). These processes have been considered to remove HMs from water as part of the microbial biomass as not only living cells but also dead cells can bind HMs either actively, or passively or by a combination of both processes (Al-Momani *et al.,* 2007; Bai *et al.,* 2008; Gavrilescu, 2004). Biosorption of metal ions on the cell surface is based on non-enzymatic processes such as adsorption while bioaccumulation involves the intracellular uptake of metal ions and requires an energy dependent transport system (Gazso, 2001; Pardo *et al.,* 2003). The capacity of any biosorbent is mainly influenced by the biomass characteristics, physicochemical properties of the target metals, and the micro-environmental factors of the contact

solution including pH, temperature, and interaction with other ions (Chan and Wang, 2007).

Microorganisms living in a contaminated environment are often well adapted themselves to survive in the presence of existing contaminants by evolving mechanisms to tolerate the presence of HMs such as by using an efflux of metal ions, or by accumulating and complexing metal ions inside the cell (Gazso, 2001; Nies, 1999). Thus bacteria that can stay alive in the environment of HMs contamination should be isolated and used as inoculants for bioremediation. Many research workers have studied removal of HMs by purple nonsulfur bacteria (PNB) (Giotta *et al.,* 2006; Seki *et al.,* 1998; Smiejan *et al.,* 2003; Watanabe *et al.,* 2003) mainly because PNB are normally used to treat many kinds of wastewater (Kantachote *et al.,* 2005; Nagadomi *et al.,* 2000). The unique advantages of PNB are that they can use solar radiation as an energy source under anaerobic-light conditions and organic matter as sources of energy and carbon under aerobic-dark conditions (Imhoff and Truper, 1989). Therefore, the PNB isolated from various HMs contaminated shrimp ponds may be good candidates to remove HMs by immobilization. Hence, the aims of this work were to investigate the potential of PNB strains isolated from contaminated shrimp ponds to immobilize HMs and also the ability of other factors to have an effect on their immobilization.

Materials and methods

Preparation of cell pellets of PNB for immobilization of HMs

Two PNB strains, *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24, used in this study were isolated in our laboratory from water and sediment samples collected from HMs contaminated shrimp ponds. Our previous work showed that both strains grew well in GM medium (Lascelles, 1956) and were resistant to HMs at the highest concentrations that were found in the sediments from the shrimp ponds (0.75 mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}). A ten percent inoculum from a growing culture (NW16 and KMS24) was added to GM medium and incubated with their optimal growth conditions under microaerobic-light and also aerobic-dark conditions. Conditions included an initial

medium pH of 7; shaking speed, 150 rpm; incubating temperature, 30ºC and their optimal light intensity was 3000 lux. Cells grown with microaerobic-light and aerobic-dark conditions were used to test for immobilization of HMs when incubated with microaerobic-light and aerobic-dark conditions, respectively. These experiments were designed because both PNB strains will be expected to treat water in shrimp ponds, after harvesting, where both sets of conditions can prevail. Cells in the log phase (48 h) unless otherwise stated were harvested by centrifugation at 8,000 rpm for 15 min and were washed twice with sterile 0.1% peptone water to obtain cell pellets for testing the immobilization of HMs.

Preparation and analysis of HMs ions

The following HMs tested in this study; Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} were prepared from $CuCl_2$, $ZnCl_2$, $CdCl_2$, and $PbCl_2$, respectively. Stocks of each HM (HM) ion were prepared in deionized water and then sterilized by a (0.22 μm) filter membrane. They were stored at 4°C until used. The HM concentrations were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (PerkinElmer, Germany).

Immobilization of HMs ions by PNB

Metabolism-independent

In this experiment cell pellets of each culture harvested from a late log phase culture (60 h) was suspended in 1 M sodium azide (NaN_3) solution for 45 min to inhibit metabolic activity (Gourdon *et al.,* 1990). Cell suspensions were centrifuged then washed with the same processes as previously mentioned. The wet cell pellet equivalent to 0.625 mg dry cell weight (DCW)/ml was resuspended in the mixed HMs solution (Highest concentrations detected in shrimp sediments as previously mentioned) containing 3% NaCl, at a pH of 5.8. The 3% NaCl was added to equate with the average concentration that was detected in the water from shrimp ponds. The cell suspensions were incubated at 30^{o}C on a shaking incubator with a speed of 100 rpm for 30 min with aerobic-dark conditions and the same condition was set for the microaerobic-light conditions but with the light intensity adjusted to 3,000 lux. After

centrifugation, the remaining HMs in the supernatant was analyzed using ICP-OES. Two control sets were also prepared; negative control (without addition of cell suspension) and positive control (without treatment by 1 M sodium azide). The percentage of each HM removed was finally calculated based on their initial concentrations.

Metabolism-dependent

Wet cell suspensions of NW16 and KMS24 were prepared as previously described except that peptone, yeast extract, Na_2SO_4, and KH_2PO_4 were added into the mixed HMs solution at concentrations of 5.0 g/L, 5.0 g/L, 0.20 g/L, and 0.02 g/L, respectively (Gourdon *et al.,* 1990) and a control set had no added supplement of nutrients and produced a clear solution. As some turbidity appeared after adding a set of nutrients, this precipitate was removed by centrifugation and the supernatant was used to measure the initial concentration of each HM before inoculation. In addition, the initial concentration of each HM in a control set was adjusted by using the measured concentrations found in the supernatant of the set with added nutrients. Immobilization of HMs by both bacterial strains was conducted as previously described.

Factors affecting immobilization of HMs

The effects of the biomass or cell properties (cell growth phase, biomass dose) and environmental factors (pH, temperature, contact time and presence of other cationic ions) were determined consecutively on the immobilization capacity of HMs by both PNB strains with both standard microaerobic-light and aerobic-dark conditions as previously mentioned. After each experiment, optimum conditions were adjusted before carrying out the next series of tests and measurements of HMs by ICP-OES.

Effect of the cell growth phase

A ten percent inoculum size of each culture was grown in GM medium under the optimal conditions as previously mentioned and their growth were observed every 6 h. The cells were harvested at log phase (48 h), late log phase (60 h), and

stationary phase (72 h). The cell pellets were prepared as previously described and then HMs immobilization studies were carried out as previously described. However, to achieve a higher immobilization of HMs in this experiment the wet cells equivalent to 2.5 mg DCW/ml was used instead of 0.625 mg DCW/ml.

Effect of biomass dose

Cell pellets of each PNB isolate that were harvested at the log phase (optimum growth phase for removing of HMs) were prepared by varying wet cell concentrations of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mg DCW/ml for HMs immobilization tests.

Effect of pH

The pH of the mixed HMs solution was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0.

Effect of temperature

The temperature was adjusted to 20, 25, 30, 35, and 40 ºC.

Effect of contact time

The time of incubation with the mixed HMs solution varied from 0, 5, 10, 15, 20, 30, 45, and 60 min.

Effect of other cationic ions

The effect of the presence of other cationic ions, 85 mg/L Ca^{2+} ($CaCl_2$) and 160 mg/L Mg^{2+} ($MgSO_4$) on immobilization from the mixed HMs solution was investigated. This included the presence of a control set (HMs solution in 3% NaCl) without the addition of both cationic ions. The amounts of cationic ions used were determined by reference to the concentrations that were found in the water from shrimp ponds. Cell pellets were prepared and incubated based on the optimal conditions of each factor obtained from the previous experiments.

Data presentation and statistical analysis

All experiments in this study were conducted in three replicates. Means and standard deviations are presented. Statistical analysis using one way ANOVA to analyze statistical differences at a p-value < 0.05 and mean comparisons were performed by the Duncan' s multiple range test.

Results

Immobilization of HMs by PNB

Metabolism- independent

Results of the effect of the metabolic inhibitor sodium azide, with both incubating conditions (microaerobic-light and aerobic-dark conditions) show that it strongly inhibited the immobilization of standard HMs concentrations (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by both PNB strains (NW16 and KMS24) (Figure 5-1). The percentage reduction of immobilization by strains NW16 and KMS24 under both incubating conditions was between 45% - 58% and 38% - 57%, respectively when compared to a set of untreated cells (Figure 5-1). The relative efficiency of removal of HMs by both PNB strains was in the order of Pb > Cu > Zn > Cd with both of the incubating conditions. HMs removed by strain NW16 with microaerobic-light (% ions removal: Pb, 33.21; Cu, 16.15; Zn, 13.36; Cd, 9.89) was only slightly different from that with the aerobic-dark conditions, except for Pb^{2+} (Pb, 37.80; Cu, 16.15; Zn, 13.04; Cd, 9.33). A similar result was also found with strain KMS24, except that in this case the removal of Pb^{2+} was not different.

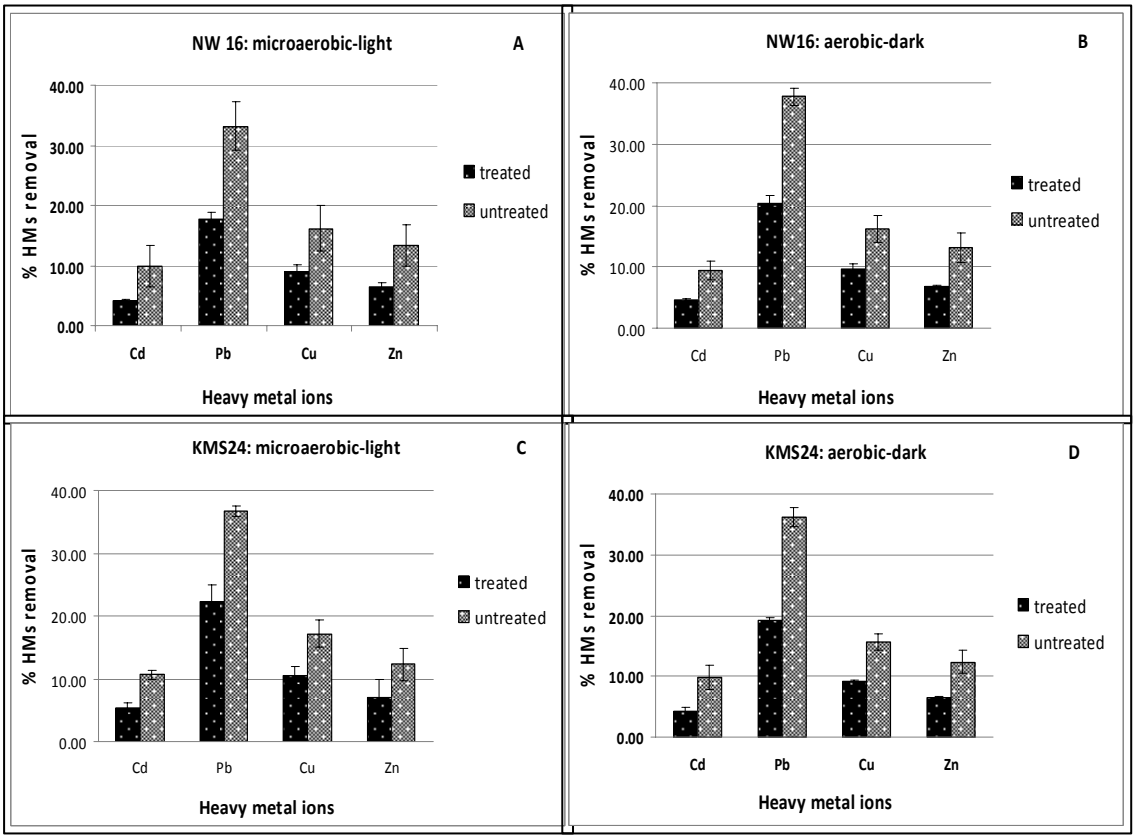

Figure 5-1. Effect of metabolic inhibition using 1M sodium azide on the immobilization of mixed HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 0.625 mg DCW/ml from late log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (A) and aerobic-dark (B), KMS24 strain with microaerobic-light (C) and aerobic-dark (D).

Metabolism-dependent

Results of immobilization of HMs by both PNB strains under all conditions tested (microaerobic-light and aerobic-dark conditions) show that both strains did remove HMs in the presence of nutrients in g/L (5 peptone, 5 yeast extract, 0.2 Na_2SO_4, and 0.02 KH_2PO_4) when compared with those of no added nutrients. HMs did interact with the nutrients as the turbidity increased slightly when the HMs were added to the medium containing nutrients before inoculation. However, this precipitation had been removed by centrifugation. Therefore, the loss of HMs from the medium with added nutrients was due to the presence of bacterial cells. Both PNB

strains removed ≥ 83% of Pb^{2+}, when the medium was supplemented with nutrients under the conditions tested, 31-34% for Cu^{2+}, 22-26% for Zn^{2+} and 17-20% for Cd^{2+} (Figure 5-2). In the absence of nutrients the removal capacity of; Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} by the strain NW16 was decreased by about 43%, 59% ,47% and 43%, respectively under microaerobic-light and about 53%, 56%, 49% and 51% under aerobic-dark conditions. A similar result was also found for the strain KMS24.

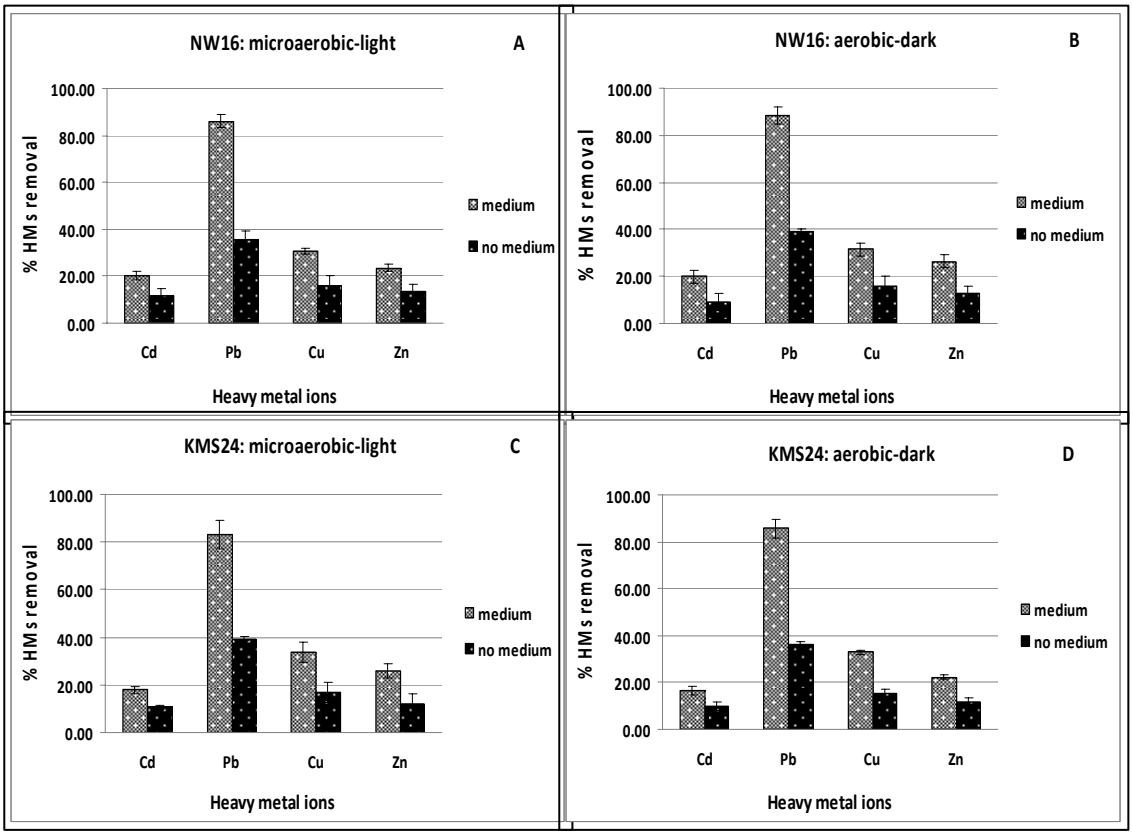

Figure 5-2. Effect of adding nutrients on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 0.625 mg DCW/ml from late log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (A) and aerobic-dark (B), KMS24 strain with microaerobic-light (C) and aerobic-dark (D).

Factors affecting HMs immobilization

Cell age or cell growth phase

In all conditions tested both strains were more effective in immobilizing HMs in the log phase of growth (Figure 5-3). Differences were not significant when comparing cells in the late log and stationary phases. The biggest removal of Pb^{2+} detected was about 80% by KMS24 with both conditions tested and also by the strain NW16 but in this case only with aerobic-dark conditions. In contrast, the removal percentage of Pb^{2+} was only 67% by strain NW16 under microaerobic-light conditions. Both strains under all conditions tested showed removal percentage of Cu^{2+}, Zn^{2+} and Cd^{2+} in a range of 30-50, 10-20 and $\leq$ 10, respectively.

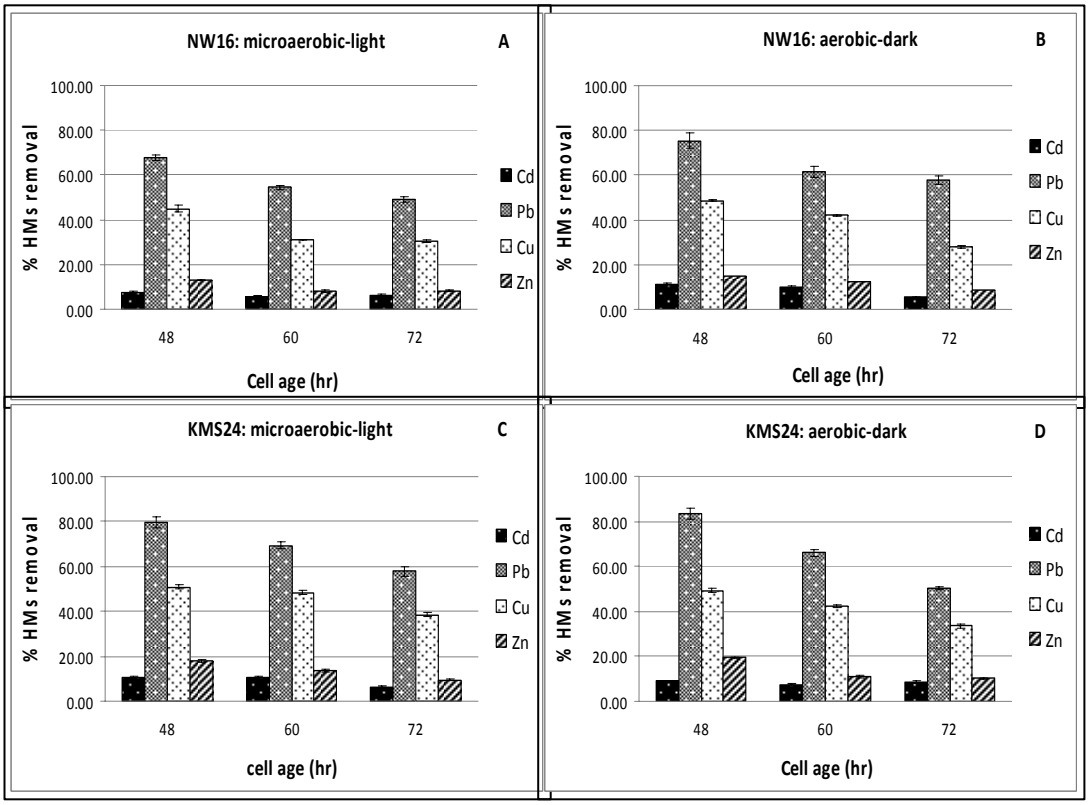

Figure 5-3. Effect of cells age on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 2.5 mg DCW/ml, pH 5.8, 30 min; NW16 strain with microaerobic-light (A) and aerobic-dark (B), KMS24 strain with microaerobic-light (C) and aerobic-dark (D).

Biomass dose

The removal capacity of HMs by both strains, harvested in the log phase, increased with increasing biomass from 2.0 to 5.0 mg DCW/ml (Figure 5-4). However, a wet biomass dose equivalent to 4.5 mg DCW/ml of the strain NW16 under both incubating conditions provided the best removal percentage of all HMs. With strain KMS24 under both incubating conditions there was no difference when the dose of biomass increased from 4.5 to 5.0 mg DCW/ml for Cu^{2+} and Pb^{2+} but the removal capacity of Cd^{2+} and Zn^{2+} was highest at 5.0 mg DCW/ml. Therefore, the optimum amount of biomass for NW16 and KMS24 was 4.5 and 5.0 mg DCW/ml, respectively. These concentrations were selected for further studies.

pH

The removal capacity of HMs by strain NW16 in the pH range from 5.0–9.0, with all conditions tested, significantly increased with increasing pH, in the range of 5.0-6.0, with an optimum pH of pH 6.0 for removal of all HMs by log phase cells at biomass doses of 4.5 and 5.0 mg DCW/ml for NW16 and KMS24, respectively (Figure 5-5). However, for the strain KMS24 under all conditions tested the optimum pH was 5.5. Therefore, the selected optimal pH for further studies with strain NW16 was 6.0 while for strain KMS24 a pH of 5.5 was chosen.

Temperature

The highest removal ability of each HM by both strains under all incubating conditions was between 30 and 35ºC (Figure 5-6). However, removal of Zn^{2+} by strain NW16 under both incubating conditions decreased significantly at 35ºC. Hence, the optimal temperature for HMs removal by strain NW16 was 30ºC. In the case of strain KMS24 removal of Pb^{2+} significantly increased at 35ºC under both incubating conditions hence 35ºC was chosen as the optimal temperature for the strain KMS24. In addition, the removal ability of HMs particularly Zn^{2+} and Cd^{2+} by the strain KMS24 was higher than the strain NW16 under both incubating conditions (Figure 5-6).

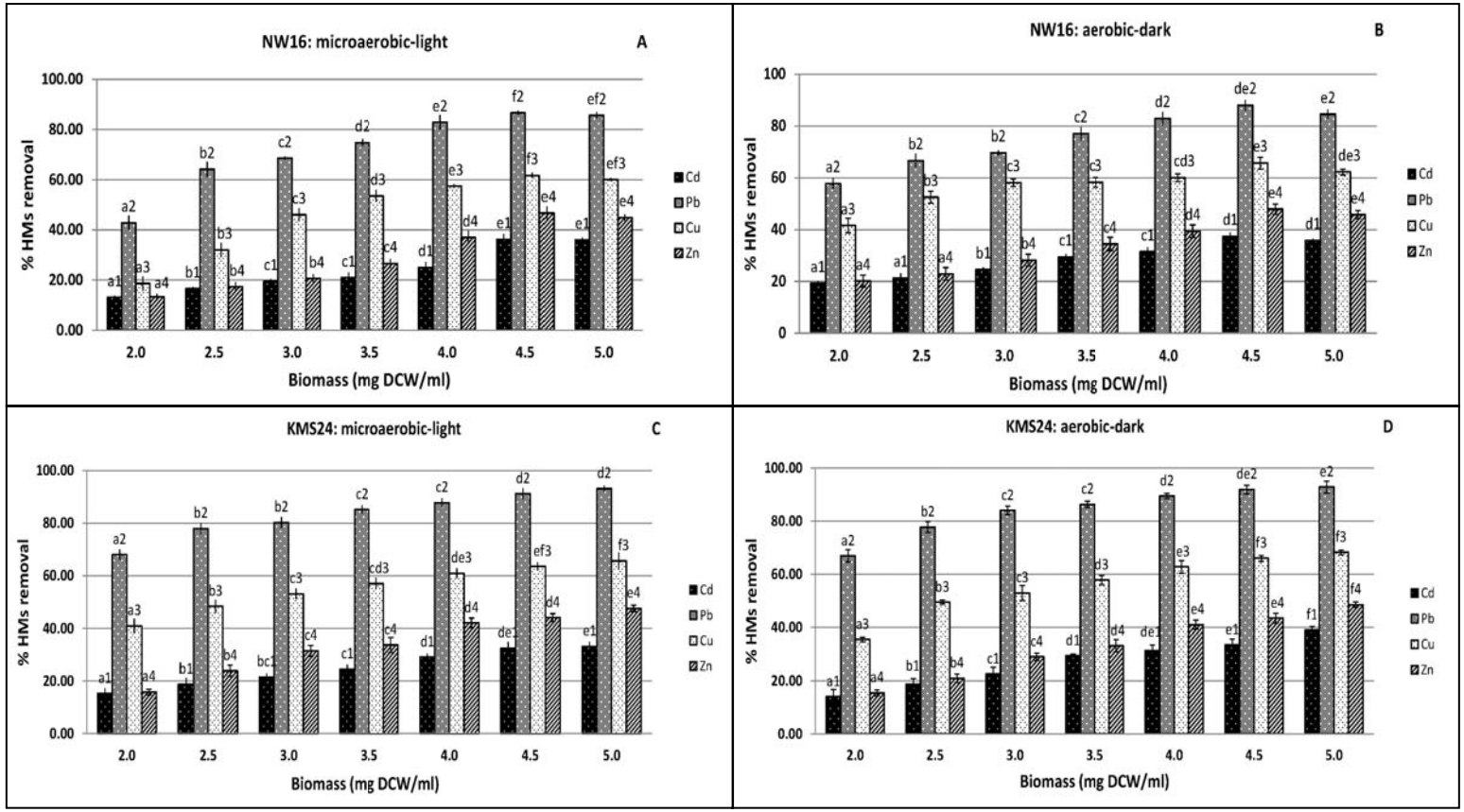

Figure 5-4. Effect of biomass dose on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 2.5 mg DCW/ml from log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (A) and aerobic-dark (B), KMS24 strain with microaerobic-light (C) and aerobic-dark (D) Lowercase letters with numbers above bars with different letters indicate significant differences ($p < 0.05$).

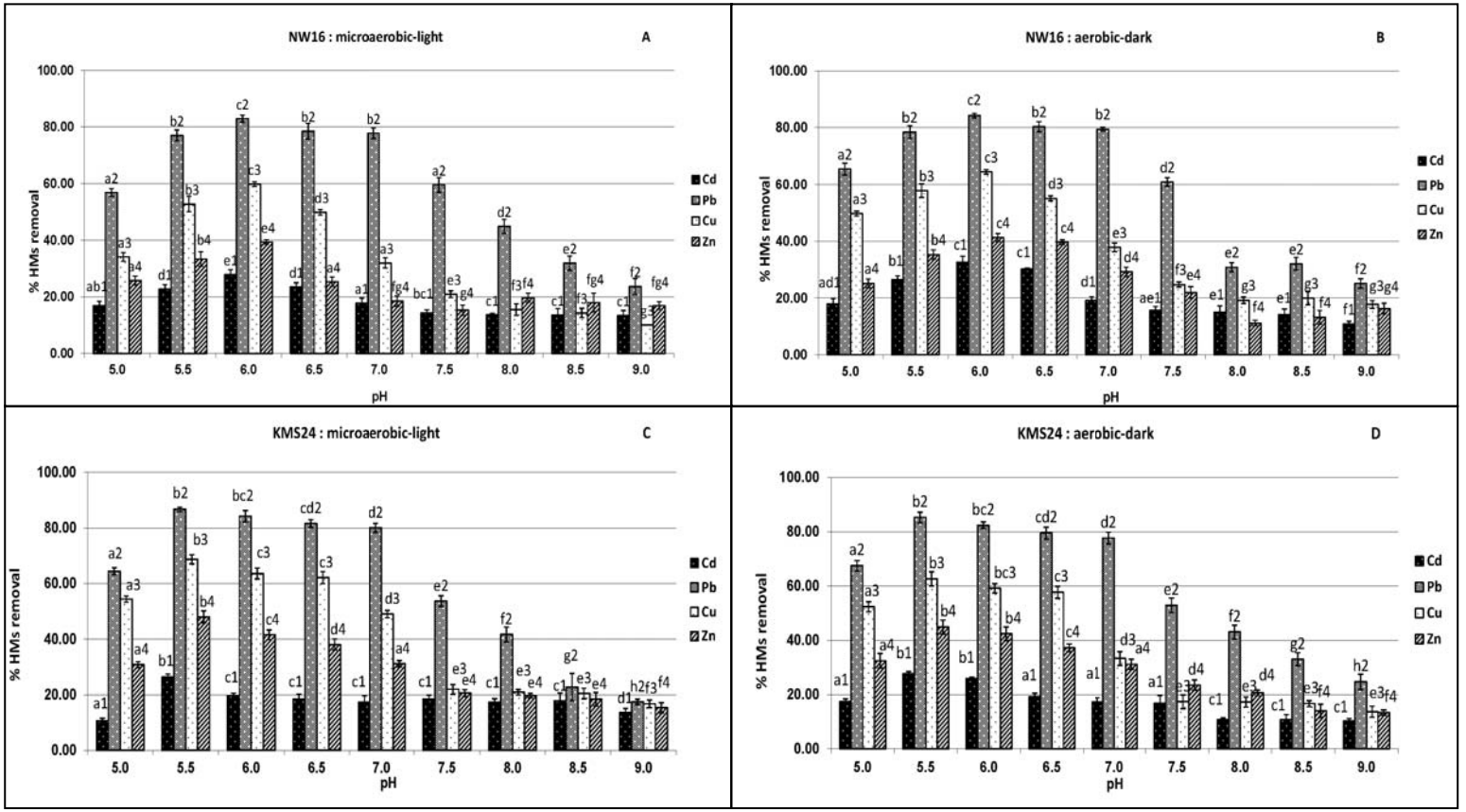

Figure 5-5. Effect of pH on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase, 30 min; NW16 strain (4.5 mg DCW/ml) with microaerobic-light (A) and aerobic-dark (B), KMS24 strain (5.0 mg DCW/ml) with microaerobic-light (C) and aerobic-dark (D) Lowercase letters with numbers above bars using a different letter indicate significant differences ($p < 0.05$).

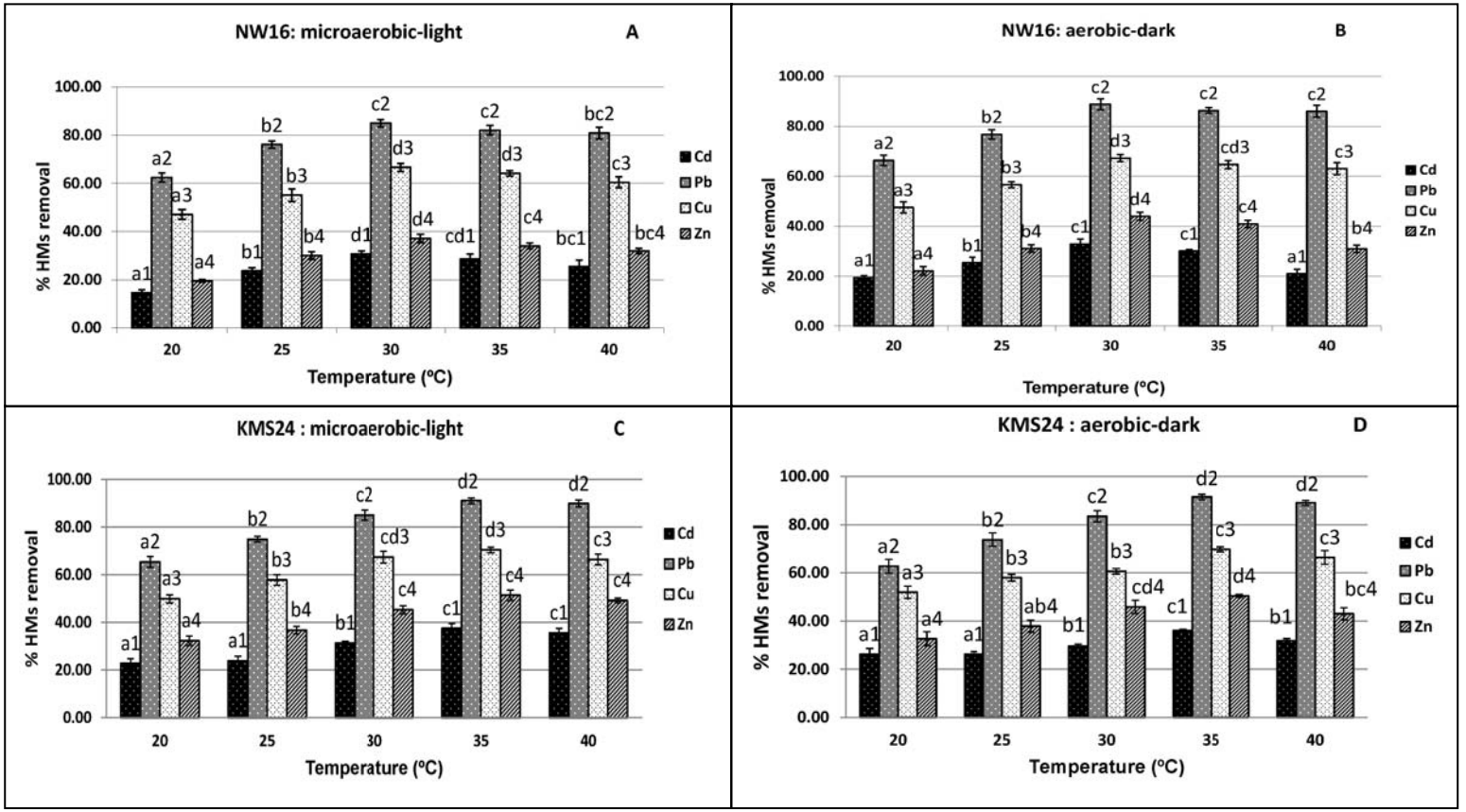

Figure 5-6. Effect of temperature on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNSB isolates. Conditions used: cells from log phase, 30 min); NW16 strain (4.5 mg DCW/ml, pH 6.0) with microaerobic-light (A) and aerobic-dark (B), KMS24 strain (5.0 mg DCW/ml, pH 5.5) with microaerobic-light (C) and aerobic-dark (D) Lowercase letters with numbers above bars using a different letter indicate significant differences ($p < 0.05$).

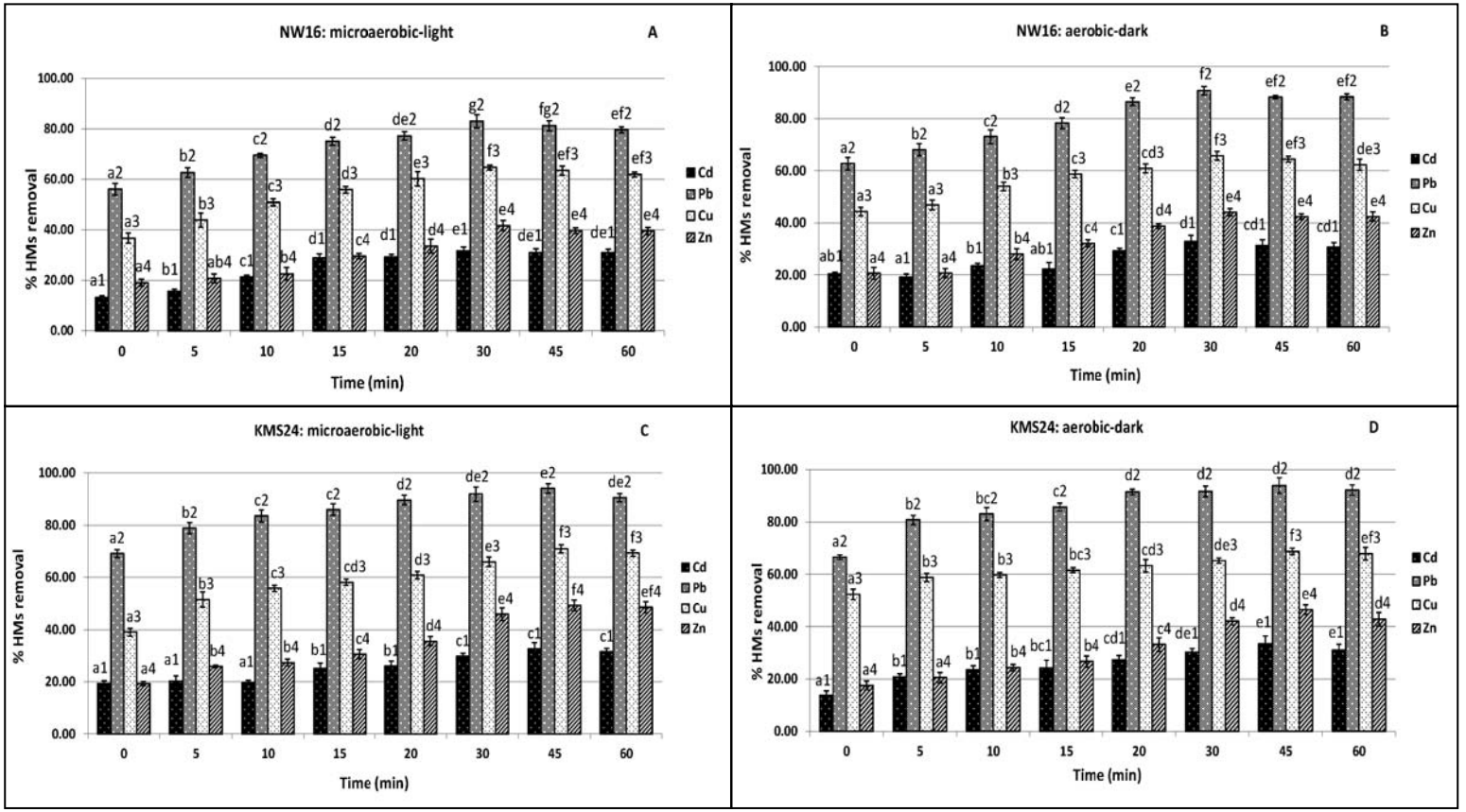

Figure 5-7. Effect of contact time on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase; NW16 strain (4.5 mg DCW/ml, pH 6.0, 30ºC) with microaerobic-light (A) and aerobic-dark (B), KMS24 strain (5.0 mg DCW/ml, pH 5.5, 35ºC) with microaerobic-light (C) and aerobic-dark (D) Lowercase letters with numbers above bars using a different letter indicate significant differences ($p < 0.05$).

Contact time

The removal of all HMs by cells of both strains harvested in the log phase under both new standard incubating conditions at a biomass dose of 4.5 mg DCW/ml, a pH of 6.0 and 30ºC for strain NW16 and at a biomass dose of 5.0 mg DCW/ml, a pH of 5.5 and 35ºC for strain KMS24 increased at a significant rate from t = 0 but only until 30 min for the strain NW16 and 45 min for the strain KMS24 (Figure 5-7). Therefore, the optimal contact times to remove HMs under both incubating conditions for the strains NW16 and KMS24 were 30 and 45 min, respectively.

Other cationic ions

In the presence of calcium and magnesium ions at 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+}, respectively, the immobilization of the mixed HMs in 3% NaCl by both PNB strains under both incubating conditions was significantly decreased (Figure 5-8). For example, removal of HMs by strain NW16 with optimal conditions (control set) with aerobic-dark conditions was Pb, 90% ; Cu, 69%; Zn, 46%; Cd, 28% whereas with microaerobic-light conditions it was Pb, 83%; Cu, 59%; Zn, 39%; Cd, 23%. The average percentage reduction of HMs by the strain NW16 under both incubating conditions was roughly 32, 40, 44 and 52 for Pb, Cu, Zn and Cd, respectively. A similar trend for a reduced removal of HMs was observed in the presence of both cations by the strain KMS24 under both incubating conditions (Pb, 26%; Cu, 36%; Zn, 50%; Cd, 55%). In contrast, no such differences for removal of HMs was found between microaerobic-light and aerobic-dark conditions when the average removal percentages in the control set were 96, 75, 46 and 30 for Pb, Cu, Zn and Cd, respectively.

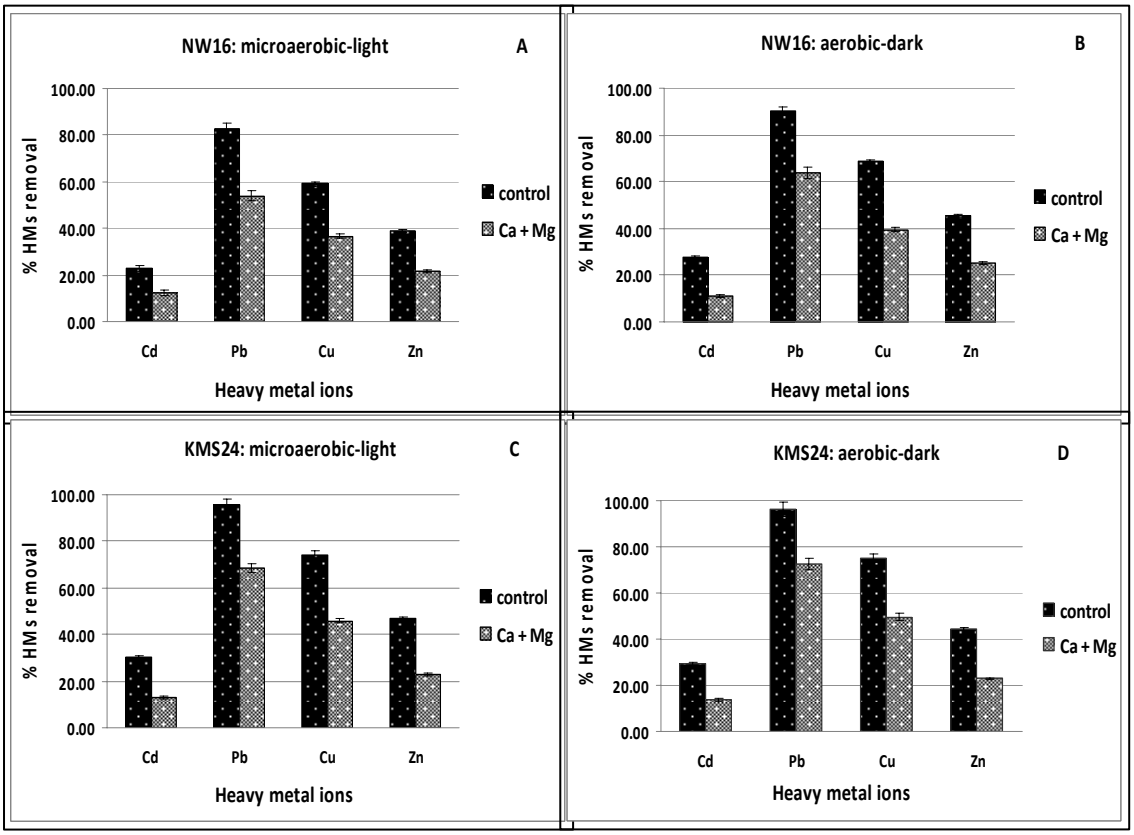

Figure 5-8. Effect of other cationic ions on the immobilization of HMs (0.75mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase; NW16 strain (4.5 mg DCW/ml, pH 6.0, 30ºC, 30 min) with microaerobic-light (A) and aerobic-dark (B), KMS24 strain (5.0 mg DCW/ml, pH 5.5, 35ºC,45 min) with microaerobic-light (C) and aerobic-dark (D).

Discussion

Immobilization of HMs by PNB

The present study demonstrates that the PNB cells (NW16 and KMS24) with microaerobic-light and aerobic-dark conditions can effectively remove HMs from an aqueous solution (mixed solution containing 3% NaCl, 0.75 mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}, 34.60 mg/L Cu^{2+} and 58.50 mg/L Zn^{2+}). The metabolic inhibition and metabolic-dependent studies (Figure 5-1 and Figure 5-2) reveal that the processes of HMs immobilization by PNB cells may involve intracellular uptake (bioaccumulation) and surface binding (biosorption). It is clear that 1 M sodium azide

strongly decreased HMs removal by both strains with both incubating conditions (Figure 5-1) and as live cells were used that means bioaccumulation is also involved with removal of HMs. The intracellular accumulation of HMs is governed by energy dependent transport systems. This is also supported by the increase of the HMs removal capacity after adding nutrients into the mixed HMs solution (Figure 5-2). This indicates that there were some metabolic processes that facilitated the uptake of HMs into the cells. These mechanism-dependent processes are also energy dependent, requiring an active energy generating system by the cells and probably through specific transport systems (Prado Acosta *et al.,* 2005).

The problem caused by the precipitation after adding nutrients was eliminated by first centrifuging the medium as described in the methods section. This indicates that the metabolically dependent accumulation led to the remarkable removable of ≥ 83% of the Pb^{2+}. Metal precipitation through the formation of Pb-phosphates may be possible as one of the added nutrients was 20 mg/L KH_2PO_4 although the pH of the system tested was 5.8 and precipitation is normally accelerated at a more alkaline pH. It has long been recognized that degradation of organophosphates to ortho-phosphate by microbes can lead to metal precipitation as metal-phosphates, especially above pH 7 do precipitate (Gazso, 2001). Therefore, it could be concluded that a higher removal of metal ions (Pb^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}) in the solution with added nutrients was mainly caused by both biosorption and bioaccumulation. As no precipitation occurred when azide inhibited metabolism and in the control with no nutrient supplements in the metabolic- dependent experiment and also because the testing time was for only 30 min we suggest that immobilization of HMs by both PNB strains is governed by bioaccumulation and biosorption but not by precipitation. To date the accumulation of HMs by living PNB cells such as *Rhodobacter sphaeroides* has been restricted to the case of tellurite, selenite and rare earth metal oxides (Bebien *et al.,* 2001; Moore and Kaplan 1992) and this study is the first to report the accumulation of HMs from a mixed solution of Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} in 3%NaCl by *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24.

Factors affecting HMs immobilization

It is well recognized that the removal capacity of HMs is mainly influenced by micro-environmental factors of the contact solution including pH, temperature, and interaction with other ions. Additionally, biomass itself, like cell age and the biomass dose, also affect the efficiency of removal of HMs. Cells in their log phase gave the best removal capacity but there were no significant differences found for the cells in their late log and stationary phases (Figure 5-3). These results are similar to reports from previous studies (Goyal *et al.,* 2003; Simmons and Singleton, 1996) that cells at an early stage of growth have a higher biosorptive capacity for HMs than do those of stationary phase cultures.

As removal of HMs by both PNB strains was catalyzed by both biosorption and bioaccumulation (Figure 5-1 and Figure 5-2) therefore removal of HMs from aqueous solution by both PNB strains was increased as the cell concentration increased (Figure 5-4). Increasing biomass provides an increase of surface area and of functional groups on the cell wall for binding HMs. An increase of the biomass dose in the biosorption system resulted in increasing the sorption site interactions and thus an increased rate of immobilizing of the HMs occurred. In addition, the efficiency of removing HMs increased with an increasing initial concentration of HMs when the amount of biomass was constant. This agrees with the findings of Monteiro *et al.* (2009). Hence, the biosorptive capacity of HMs is related to the ratio of the concentration of initial HMs to the concentration of biomass and this is the main reason why Pb^{2+} was the most efficiently removed HM by both strains of PNB as its initial dose was 62.63 mg/L whereas the initial dose of Cd^{2+} was only 0.75 mg/L and this was the HM that was least efficiently used (Figure 5-4). However in case of Zn^{2+} although its initial concentration was 58.50 mg/L, its removal efficiency was lower than that found in Cu^{2+} with an initial concentration of 34.60 mg/L. One possible reason for this may be that Zn^{2+} has more adverse effects than Cu^{2+} on living cells (Balsalobre *et al.,* 1993) and therefore has reduced bioaccumulation. The order of the biosorption capacity found in yeast cells was Pb > Cu > Cd (Goksungur *et al.,* 2005).

In addition, more viable biomass accounts for more bioaccumulation of HMs ions. The largest percentage of HMs removal was with the viable biomass

equivalent to ≤ 5 mg DCW/ml and this indicates the effectiveness of PNB to remove HMs from solution. For biosorption of HMs, pH is one of the more important environmental factors, as this parameter affects the protonation state of the functional groups on the cell wall of the biomass (Bayramoglu and Arica, 2008). The surface charge of the cell wall at a low pH level is more positive and when the pH is raised, there is more affinity for metal ions as more ligands bearing negative charges increase (Gupta and Rastogi, 2008). This phenomenon was also found in this study (Figure 5-5). The most suitable pH values for removal of all HMs by both PNB strains were 5.5-7.0. The results of this study agree with a study of Blackwell *et al.* (1995) that the optimal pH ranged from 4.0-8.0 for metal uptake for almost all types of biomass. The reason for this can be explained by the extra protons at the low pH value tends to compete with the metal ions for the binding sites as previously mentioned. On the other hand, at a high pH value metal complexes will precipitate. Hence, the optimal pH for strains NW16 and KMS were 6.0 and 5.5 as depicted in Figure 5-5.

The removal capacity of HMs with both incubating conditions significantly increased when the temperature increased from 20ºC to 30ºC for the strain NW16 and up to 35ºC for the strain KMS24 (Figure 5-6). This can be explained as living cells were used and thus in addition to biosorption, bioaccumulation is also involved with the removal efficiency of HMs. Both strains grew well in a range of temperature from 20ºC to 30 or 35ºC. Therefore, it will be possible to use both strains for removing HMs in shrimp ponds water without any temperature control. The optimum biomass dose of the strain KMS24 (5.0 mg DCW/ml) was higher than for strain NW16 (4.5 mg DCW/ml); however, strain KMS24 gave higher efficiencies for the removal of Zn^{2+} and Cd^{2+} than the strain NW16 in all conditions tested. Additionally, the incubating conditions had no additional adverse effects for the strain KMS24. Hence, in order to achieve the optimum removing HMs contaminated in shrimp ponds, mixed cells of both strains might be even more effective.

The removing of HMs by both PNB strains with both incubating conditions occurred very quickly at the start of the incubation and this amount significantly increased until 30 min and 45 min for strains NW16 and KMS24, respectively (Figure 5-7). The fast binding of HMs at the starting time of contact was most likely associated with adsorption of HMs onto the cell surface. This process is

usually completed rapidly at around 5 min (Gaber *et al.,* 2008; Kadukova and Vircikova, 2005). However, the additional rapid uptake of HMs absorption over the next 30 or 45 min indicates that this process was due to a continuous metabolic uptake of HMs after the initial physical adsorption (Figure. 1, 2 and 7). Hence, the advantage of using living cells is due to their ability to remove HMs continuously through bioaccumulation.

As shrimp water contains many additional ionic components, that includes metal cations like Na^{+}, Ca^{2+} and Mg^{2+} and anions like Cl^{-} and SO_4^{2-} normally the biosorption of any HM might be modified by their presence. The observed decrease of HMs uptake in competitive conditions is believed to be a response to increased competition between similar charged species for binding onto the cell surface (Goksungur *et al.,* 2005). This explains the reduced biosorption capacity of PNB cells in the presence of the bivalent cations, Ca^{2+} and Mg^{2+} (Figure 5-8) as both calcium and magnesium ions will increase competition for binding sites on the cell surface.

Conclusions

These results have proved that both PNB strains (NW16 and KMS24) can effectively remove HMs in the 3% NaCl aqueous system with either microaerobic-light or aerobic-dark conditions and thus have a good potential for use to remove HMs present in contaminated shrimp ponds. Removal efficiency is governed by both energy independent (biosorption) and dependent (bioaccumulation) processes and the removal efficiency is mainly influenced by cell age, biomass dose, properties of the target metals, pH and competition with other bivalent cations. Consequently, removal of HMs from shrimp pond water using both these promising strains is now being economically assessed in our laboratory.

CHAPTER 6

Toxicity assessment of sediment and water from shrimp ponds contaminated with heavy metals after treatment by selected purple nonsulfur bacteria

Abstract

The potential to remove heavy metals (HMs) by the purple nonsulfur bacteria (PNB) isolates, NW16 and KMS24, were investigated in a synthetic solution (62.63 Pb^{2+}, 34.60 Cu^{2+}, 58.5 Zn^{2+}, 0.75 Cd^{2} mg/L) containing 3% NaCl, and from sediment and water collected from post cultured contaminated shrimp ponds. After seed germination was used to assay their plant toxicities after bioremediation. With or without addition of 85mg/L Ca^{2+} and 160 mg/L $Mg^{2+}$to the synthetic HMs solution containing 3% NaCl, the removal efficiency of HMs in the mixed culture of both strains was greater than that found in the pure culture of each strain and all treatments removed Pb^{2+}> Cu^{2+}> Zn^{2+}> Cd^{2+}. The presence of both light metal ions significantly decreased the efficiency of removal of HMs and using the optimal condition for removal of HMs by KMS24 there was a higher efficiency to remove HMs, particularly with the mixed culture (removal percentages; 85 Pb^{2+}, 74 Cu^{2+}, 47 Zn^{2+}, and 28 Cd^{2+}). The water from shrimp ponds contaminated with 0.043 mg/L Cu^{2+} and 0.057 mg/L Zn^{2+} was decreased by roughly 75% for Cu^{2+} and 31% for Zn^{2+} by a set of the native population plus the mixed culture and it was greater than that found in a set of sterile treated water with a mixed culture although no significant differences was found for the abiotic and native control sets. For the sediment samples , a set with a native population together with the mixed culture produced the highest efficiency to remove Pb^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} (initial concentrations; 23.15, 15.05, 22.16 and 0.29 mg/kg) under aerobic-dark conditions with removal percentages of 84.29, 62.52, 43.33, and 40.95 respectively. Water contaminated with HMs after bioremediation was more toxic to rice seed (*Oryza sativa*) than water spinach (*Ipomoea aquatic*) and

more toxic than soil solution from the treated sediment samples. The native population with the mixed culture produced the most effective treatment to remove toxicity as the % GI index was 34.50 and 35.29 for rice and water spinach with the treated water and 115.70 and 139.33 for rice and water spinach from the sediment respectively.

Keywords: bioremediation, contaminated shrimp ponds, HMs, photosynthetic bacteria, rice, seed germination index

บทคัดย่อ

ทดสอบศักยภาพในการกำจัดโลหะหนักของแบคทีเรียสังเคราะห์แสงสายพันธุ์ NW16 และ KMS24 ในสารละลายสังเคราะห์ (ตะกั่ว 62.63 ทองแดง 34.60 สังกะสี 58.50 และแคดเมียม 0.75 มิลลิกรัมต่อลิตร) ที่ผสมด้วยเกลือโซเดียมคลอไรด์ 3 เปอร์เซนต์ กับตัวอย่างดินและน้ำหลังการเพาะเลี้ยงจากบ่อกุ้งที่มีการปนเปื้อน รวมทั้งความเป็นพิษต่อพืชหลังการบำบัดโดยทดสอบการงอกของเมล็ด พบว่าเชื้อผสมทั้งสองมีประสิทธิภาพในการกำจัดโลหะหนักมากกว่าเชื้อบริสุทธิ์ของแต่ละสายพันธุ์ทั้งในสภาวะที่มีการเติมและไม่เติม แคลเซียมไอออน 0.85 มิลลิกรัมต่อลิตร และแมกนีเซียมไอออน 160 มิลลิกรัมต่อลิตร ในสารละลายโลหะหนักสังเคราะห์ที่มีส่วนผสมของเกลือโซเดียมคลอไรด์ 3 เปอร์เซนต์ โดยทุกชุดทดสอบให้ผลในการกำจัดตามลำดับคือ ตะกั่ว > ทองแดง > สังกะสี > แคดเมียม ซึ่งโลหะเบาทั้งสองมีผลทำให้ประสิทธิภาพในการกำจัดโลหะหนักลดลงอย่างมีนัยสำคัญทางสถิติ และพบว่าในสภาวะที่เหมาะสมต่อการกำจัดโลหะหนักของสายพันธุ์ KMS24 มีประสิทธิภาพในการกำจัดโลหะหนักได้สูงกว่า โดยเฉพาะในเชื้อผสม (เปอร์เซนต์การกำจัด คือ ตะกั่ว 85 ทองแดง 74 สังกะสี 47 และแคดเมียม 28) ในน้ำจากบ่อกุ้งที่มีการปนเปื้อนทองแดง 0.43 มิลลิกรัมต่อลิตร และสังกะสี 0.057 มิลลิกรัมต่อลิตร พบว่าชุดทดสอบที่มีเชื้อธรรมชาติร่วมกับเชื้อผสมสามารถกำจัดทองแดงได้ 75 เปอร์เซนต์ สังกะสี 31 เปอร์เซนต์ ซึ่งพบว่าสูงกว่าชุดของน้ำที่มีการฆ่าเชื้อที่เติมเชื้อผสม อย่างไรก็ตามไม่มีความแตกต่างทางสถิติระหว่างชุดควบคุมที่ไม่มีเชื้อและที่มีเชื้อธรรมชาติ สำหรับตัวอย่างดินตะกอนชุดเชื้อธรรมชาติร่วมกับเชื้อผสมมีประสิทธิภาพในการกำจัดตะกั่ว ทองแดง สังกะสี และแคดเมียม (ความเข้มข้นเริ่มต้น 23.15 15.05 22.16 และ 0.29 มิลลิกรัมต่อกิโลกรัม) ได้สูงสุดในสภาวะมีอากาศ-ไร้แสง โดยมีเปอร์เซนต์การกำจัดคือ 84.29 62.52 43.33 และ 40.95 เปอร์เซนต์ ตามลำดับ นอกจากนี้ยังพบว่าภายหลังการบำบัดน้ำที่มีการปนเปื้อนของโลหะหนักคงมีความเป็นพิษต่อเมล็ดข้าว (*Oryza sativa*) มากกว่าผักบุ้ง (*Ipomoea aquatic*) และยังมีความเป็นพิษมากกว่าสารละลายดินจากตัวอย่างดินตะกอนที่ผ่านการบำบัด โดยชุดธรรมชาติที่มีเชื้อผสมให้ผลในการบำบัดเป็นเปอร์เซนต์ของดัชนีการงอก (%GI) ของเมล็ดข้าวและผักบุ้งในน้ำ คือ 34.50 และ 35.29 และ 115.70 และ 139.33 ของข้าวและผักบุ้งในดินตะกอน

Introduction

The increased demand for shrimp in world markets has encouraged many developing countries to enter into shrimp farming but this can have damaging effects on the local environment (Chua, 1992). The extension of shrimp farming from coastal areas to freshwater areas has affected those areas, some previously used for growing rice, fruit plantations and fisheries. Traditionally, seawater from coastal waters is directly used to rear the shrimp with no additional processes and often these coastal waters are contaminated by many kinds of pollutants including heavy metals (HMs) (Cheevaporn and Menasveta, 2003; Cheung and Wong, 2006). Contaminants other than HMs from seawater, that can become concentrated in shrimp farms include chemical substances from shrimp food, antimicrobial compounds to prevent shrimp infections and , HMs, pesticides, fertilizers etc that leach from agricultural practices (Visuthismajarn *et al*., 2005). In addition, water removed during shrimp pond drainage during harvesting is often directly discharged into canals and flows into other cultivated areas, together with the illegal disposal of shrimp pond sediments (Dierberg and Kiattisimkul, 1996). Consequently, this can cause serious pollution to soil in agricultural areas, especially rice-fields and vegetable crops, resulting in an accumulation of HMs and chemical substances (Manseubchat, 2002). Therefore, it is very likely that the paddy soil and rice grown in these areas display raised levels of HMs.

The accumulation of HMs in agricultural soil is of increasing concern due to food safety issues and potential health risks because they cannot be biodegraded and they may be leached to surface water run-off, groundwater storages, plant absorption etc. HMs are frequently accumulated by agriculturally important crops and become concentrated in the plant tissues to produce damaging effects on the plants themselves and may also pose a health hazard to animals and humans (Athar and Ahmad, 2002; Yap *et al*., 2004; Mokhtar *et al*., 2009). The sensitivity of plants to HMs depends on an interrelated network of physiological and molecular mechanisms such as uptake and accumulation of metals by binding to extracellular exudates and cell wall constituents, efflux of HMs from the cytoplasm to extranuclear

compartments including vacuoles and the complexation of metal ions inside the cell by various substances; for example, organic acids, amino acids, phytochelatins, and metallothioneins (Cho *et al*., 2003). Although, some HMs, at low concentrations, are essential elements for plants such as manganese, zinc, and iron, where they are important as co-factors of enzymes and critical components of electron transport reactions, but at higher doses they may cause metabolic disorders and growth inhibition for most plants (Fernandes and Henriques, 1991; Claire *et al*., 1991). Stress from HMs can have a negative impact on processes associated with biomass production and grain yield in almost all major field grown crops and this can result in reduction of growth rate, pigment content and low productivity (John *et al*., 2009). Moreover, HMs may also influence plant sexual reproduction (Saikkonen *et al*., 1998) and delay flowering (Brun *et al*., 2003; Korboulewsky *et al*., 2002).

Thailand is the biggest rice exporting country; however, the development of shrimp farming in Thailand has opened the door for shrimp farming away from the coast into the paddy land, particularly in this region of southern Thailand. Hence, low rice yields and the contamination of ground-water aquifers has rendered large areas of land unsuitable for cultivation (Flaherty *et al*., 1999). In general rice is tolerant to salt; however, almost all rice varieties are sensitive to salinity (Greenland, 1997) and HMs in rice may cause some illness such as Itai Itai by Cd (Shimbo *et al*., 2001). Water spinach (*Ipomoea aquatica*) is an herbaceous aquatic or semi-aquatic perennial plant of the tropics and subtropics. It is a fast growing plant and can be cultivated on most kinds of soils. Contamination of HMs in the water where *I. aquatica* was grown may cause the risk of poisoning to consumers (Gothberg *et al*., 2002).

There are many advantages for using bioremediation instead of physical and/or chemical processes as it is a natural process, produces harmless end products and any bioremediated soil/water can be re-used (Burmeier, 1995; Barker and Bryson, 2002). Bioremediation of HMs from contaminated water and soil would provide decontaminated soil/water that could be used for agriculture (Barker and Bryson, 2002). In our previous studies, two bacterial isolates , NW16 and KMS24, purple nonsulfur bacteria (PNB) have proven their abilities to effectively remove HMs that are present in contaminated shrimp pond water (Cd, Cu, Pb and Zn)

containing 3% NaCl (Panwichian *et al*., 2010a, Panwichian *et al*., 2010b). Therefore, our aims in the present study were to investigate the potential of these PNB strains to remove HMs from the sediment and water collected from contaminated shrimp ponds after harvesting and to assay the water using a seed germination index of economic plants; rice (*Oryza sativa*) and water spinach (*Ipomoea aquatica*) for investigating the toxicity of the sediment and water after treatment.

Materials and methods

Collection of sediment and water from contaminated shrimp ponds

Post cultured contaminated shrimp ponds in the following areas: Ranot, Songkhla province; HuaSai, Nakhon Si Thammarat province and Pak Phayun, Pattalung province were chosen for collecting sediment because they were contaminated with Cu, Zn and Pb (Panwichian *et al*., 2010a). Contaminated shrimp pond water was collected from post cultured contaminated shrimp ponds in the Pak Phayun district, Pattalung (Panwichian *et al*., 2010a). After shrimp harvesting, sediment sub-samples, each of about 100 g were collected from the bottom of a pond at a depth of 5 cm in two diagonal and a half points from each bank. Water sub-samples were collected at the time for shrimp harvesting, roughly 100 ml of water at about 50 cm below the surface water level. All sub samples of sediment and water were kept in a big ice box during transport and then at our laboratory all sub-samples were promptly mixed well to obtain one representative sample each for sediment and water. Concentrations of HMs (Cd, Pb, Cu, and Zn) were analyzed using the inductively coupled plasma optical emission spectroscopy (ICP-OES) (PerkinElmer, Germany). In addition, samples of sediment and water were also measured for pH, EC and salinity as described by Panwichian *et al*. (2010a).

Preparation of heavy metal solutions

The following inorganic salts; $CdCl_2$, $PbCl_2$, $CuCl_2$ and $ZnCl_2$ were used for preparing stock solutions of each HM ion whereas $CaCl_2$ and $MgCl_2$ were used for preparing light metal ions. Each metal was dissolved in deionized water to obtain the concentration as designated and then the stock solution was sterilized using

a 0.22 μm filter membrane. They were stored at 4°C until used. The concentration of HMs was analyzed using ICP-OES (Perkin Elmer, Germany).

Preparation of PNB for uptake of HMs

Two PNB strains, NW16 and KMS24, used in this study were isolated from water and soil samples collected from shrimp ponds contaminated with HMs (Panwichian *et al*., 2010a)**.** A ten percent inoculum of each active isolate was grown in GM medium under microaerobic-light conditions (3000 lux). Culture broths were harvested in the log phase of growth because previously it had been established that this was the most effective time for them to remove HMs (Panwichian *et al*., 2010b). After centrifugation at 8000 rpm for 15 min, the cell pellets were washed twice with 0.1% peptone water. The cell pellets were later prepared for uptake of HMs with the optimum biomass equivalent to 4.5 and 5.0 mg DCW/ml for NW16 and KMS24, respectively. In this study, mixed culture of 2.5 mg DCW/ml of NW16 and 2.5 mg DCW/ml of KMS24 was also prepared for testing the uptake of HMs.

Effect of Ca^{2+} and Mg^{2+} on removal of HMs by PNB

Experiments in this study was designed based on the concentrations of the highest concentration of HMs and the average concentrations of Na^{+}, Ca^{2+} and Mg^{2+} in shrimp ponds (Panwichian *et al*., 2010a). The mixed solution of HMs containing 0.75 mg/L Cd^{2+}, 62.63 mg/L Pb^{2+}; 34.60 mg/L Cu^{2+}; 58.50 mg/L Zn^{2+} in 3% NaCl solution and with or without added 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} were prepared. These were used for the treatment and control sets for investigating the effects of both Ca^{2+} and Mg^{2+} on the HM removal efficiency of both pure cultures and a mixed culture of PNB. The optimum conditions for removing HMs by each culture was adopted from one of our previous studies (Panwichian *et al*., 2010b) as follows; 4.5 mg DCW/ml, pH 6.0, 30 ºC, 30 min for strain NW16 and 5.0 mg DCW/ml, pH 5.5, 35ºC, 45 min for strain KMS24. The mixed culture consisted of 2.5 mg DCW/ml of each culture and it was tested at both the optimal conditions for removal of HMs by strains NW16 and KMS24. Cell suspensions were shaken in an incubator at a speed of 150 rpm for 30/45 min under aerobic-dark conditions. Aerobic-dark conditions were designated because they provided a higher efficiency for removal of HMs when

compared to microaerobic-light conditions (Panwichian *et al*., 2010b). Cell suspensions were centrifuged, and the remaining HMs in each supernatant was analyzed using ICP-OES.

Removal of HMs in the water collected from post cultured shrimp ponds

The uptakes of HMs by biomass of both PNB strains were conducted under both microaerobic-light and aerobic-dark conditions; the cells of NW16 and KMS24 as either a pure or mixed culture were added into the collected water samples that had been sterilized (autoclaving at 121ºC, 15 min) and not sterilized (native set). A sterile water set without inoculation of PNBs served as an abiotic control while a single culture or mixed culture was inoculated into a sterile set namely a pure culture (NW16 or KMS24) or mixed PNB set. In contrast, to the non sterile water sets namely a native set was prepared together with NW16 or KMS24 or a mixed PNB set with the two strains inoculated together. The uptake of HMs was investigated under optimum conditions as previously described in the previous section as follows: a pH of 6.0, 30ºC, 30 min for strain NW16 and a pH of 5.5, 35ºC, 45 min for strain KMS24. The study of uptake of HMs by the mixed culture of NW16 and KMS24 cells was investigated at the optimum condition of pH 5.5 and 35ºC for 45 min based on the result of the previous experiment. In these studies, removal of HMs was focused on only Cu^{2+} and Zn^{2+} as these are 2 cations in the water column of the collected shrimp ponds that exceeded the standard guidelines for aquaculture (the present study and Panwichian *et al*., 2010a).

Removal of HMs in sediment collected from post cultured shrimp ponds

The sediment was made into a soil slurry using sterile DI water with a ratio of 1:1. The uptake of HMs was also investigated using the same protocol as used for the water samples. After incubation the soil slurry samples were centrifuged at 8,000 rpm for 20 min and the loss of each HM was calculated based on the amounts of HMs in the supernatant together with the amounts determined in the pellet (sediment) at zero time and at the end of the experiment.

Toxicity assessment by seed germination for the sediment and water after treatment

Toxicity of the water and sediment samples from post cultured contaminated shrimp ponds after treatment with PNB; NW16, KMS24, a mixed culture of NW16 and KMS24 in the presence or absence of native flora from the previous experiments was tested for their effects on seed germination. To follow the real situation in shrimp ponds, samples of treated water in each set from both incubating conditions were mixed for obtaining one sample of each set and it was used for testing toxicity. Again, treated sediment samples were prepared in a similar way with the treated water samples for testing toxicity. One of the most common techniques used to assess phytotoxicity is the seed germination test (Kapanen and Itavaara, 2001). The plants used in this study were rice (*Oryza sativa*) and water spinach (*Ipomoea aquatica*). Results were evaluated by comparing the results among sets of treatments and control sets (abiotic control and native control). Briefly for the seed germination test, water samples from each set were filtered with a 0.45 μm of filter membrane to remove the organisms. 5 ml of filtered water sample without dilution was added to a 9 cm sterile petri dish, using Whatman # 1 as a bed, then 10 grains were placed on the bed. All petri dishes were incubated in dark conditions at room temperature for 72 h. The percentage of seed germination (RSG), relative to root growth (RRG) and the germination index (GI) were calculated and they were compared with the distilled water as the control set (Hoekstra *et al.,* 2002). The sedimented soil samples were prepared to obtain soil solution by adding 25 ml of sterile DI water into 5 g wet weight of sediment and shaking overnight. After that it was centrifuged at 12,000 rpm for 10 min. The supernatant as the soil solution was filtered with a 0.45 μm of filter membrane and the toxicity was tested by the same method as previously described for the water samples.

Statistical analysis

All experiments in this work were conducted in triplicate. Data are presented as a mean with a standard deviation. One way ANOVA was used to analyze statistical differences at a P-value < 0.05 and mean comparisons were performed by the Duncan's multiple range test.

Results

Effect of Ca^{2+} and Mg^{2+} on removal of HMs by PNB

Results of the removal of HMs in 3% NaCl in the presence and absence of 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} by the pure cultures or a mixed culture of PNB strains (NW16 and KMS24) under aerobic-dark conditions are shown in Figure 6-1. The mixed culture produced a significantly higher HM removal efficiency for all HMs than each of the pure cultures separately both with and without the added cations (Ca^{2+} and Mg^{2+}). In addition, the light metal ions significantly decreased the HMs removal efficiency by all cultures (see details in Figure 6-1A and 1B). In the presence of Ca^{2+} and Mg^{2+} in the HM solution containing 3% NaCl using optimal conditions for the strain NW16, the pure culture NW16 removed Pb^{2+} Cu^{2+} Zn^{2+} and Cd^{2+} by about 65, 56, 21 and 11% respectively, but the removal percentages of the mixed culture was roughly 78, 65, 36 and 21% respectively (Figure 6-1 A). Ca^{2+} and Mg^{2+} had less effect on the efficiency of HM removal by the pure culture of strain KMS24 and a mixed culture under the optimum conditions for removing HMs by this strain. The removal percentages of Pb^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} with the condition containing both light metal ions by the pure culture KMS24 were 75, 62, 27 and 15, respectively but the mixed culture performed with a higher efficiency of 85, 74, 47 and 28%, respectively (Figure 6-1B). Therefore, the optimal condition for removal HMs of the strain KMS24 was applied for use in the further experiments in the case of a mixed culture.

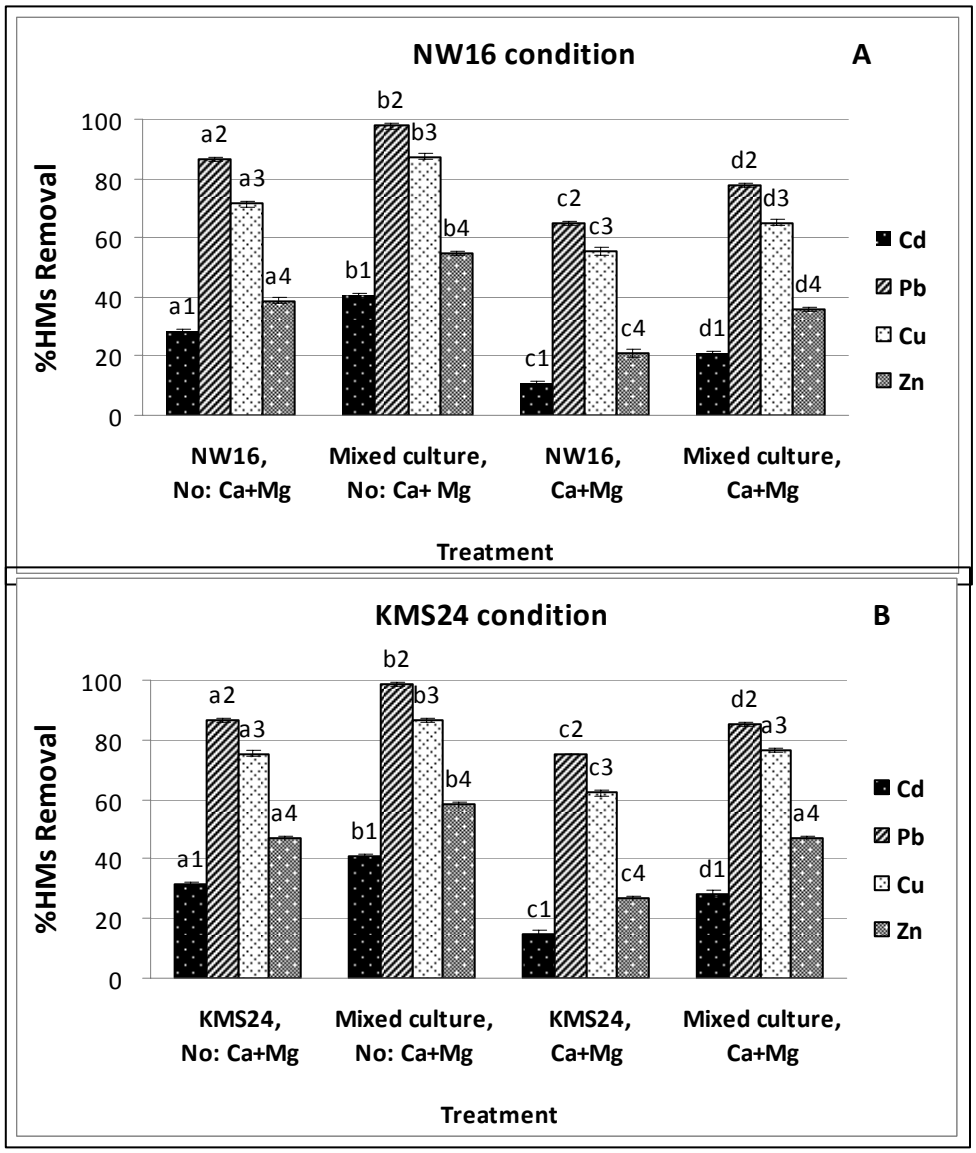

Figure 6-1. The HMs ions removal efficiency in the synthetic solution (Cd, 0.75 mg/L; Pb, 62.63 mg; Cu 34.60 mg/L; Zn, 58.50 mg/L) containing 3% NaCl in the presence or absence of 85 mg/L Ca^{2+} and 160 mg/L Mg^{2+} under aerobic-dark conditions by (A) pure culture of 4.5 mg DCW/ml of NW16 and the mixed of NW16 and KMS24 (2.5+2.5 mg DCW/ml) under optimum conditions for removal of HMs of NW16; pH 6.0, 30ºC, 30 min and (B) pure culture of 5.0 mg DCW/ml of KMS24 and the mixed culture (2.5+2.5 mg DCW/ml) under optimum conditions for removal of HMs of KMS24; pH 5.5, 35ºC, 45 min.

Removal of HMs in the water collected from post cultured shrimp ponds

The water sample used in this study was a composite sample collected from various shrimp ponds contaminated with HMs as previously described and its physicochemical properties were as follows: salinity, 10.23±2.53 ppt; pH 8.07±0.57 and EC, 4.68±1.06 ms/cm. The composite sample consisted of the following HMs in mg/L; <0.001 Cd^{2+}, <0.005 Pb^{2+}, 0.043 ± Cu^{2+}, and 0.057 ± Zn^{2+}. As previously stated only the efficiency of removal of Cu^{2+} and Zn^{2+} was studied. The removal of HMs and control sets is presented in Table 6-1. No significant difference was found for the removal percentage for Cu^{2+} and Zn^{2+} under the two incubating conditions (microaerobic- light and aerobic-dark) between the abiotic and native control sets.The pure culture of KMS24 performed with a significantly higher efficiency to remove both HM ions than strain NW16 both in the presence and absence of native flora and with both incubating conditions. The presence of the native flora in all cases produced a significant increase in removal of HMs. However, the most effective treatment for Cu^{2+} was observed with a mixed culture in the presence of the native flora that removed 73.78 and 75.92% under conditions of microaerobic-light and aerobic-dark respectively. The mixed culture had a significant increased ability to remove Cu^{2+} from 48.40 % for the pure KMS24 culture plus native flora to 75.92 %, while the comparable increase for Zn^{2+} removal was from 29.93 to 31.67, both with dark conditions. In most treatment sets more HMs were removed in the aerobic-dark than the microaerobic-light conditions although the differences may be not significant for some.

Table 6-1. The removal percentage of Cu^{2+} and Zn^{2+} in the contaminated water from post cultured shrimp ponds by the selected purple nonsulfur bacteria under microaerobic-light and aerobic-dark conditions.

Treatment	% Removal			
	Cu^{2+} (initial conc., 0.043 mg/L)		Zn^{2+} (initial conc., 0.057 mg/L)	
	Light	Dark	Light	Dark
Control				
Abiotic	11.87±6.05[a]	13.42±1.02[a]	7.95±0.89[a]	7.78±2.09[a]
Native	10.40±4.16[a]	11.14±1.72[b]	6.44±0.82[a]	6.41±1.02[a]
NW16				
Sterile + NW16	35.29±1.03[b]	40.62±0.97[c]	18.60±0.83[b]	22.52±1.06[b]
Native + NW16	41.18±1.26[c]	45.84±1.26[d]	22.58±1.11[c]	25.73±0.82[c]
KMS24				
Sterile + KMS24	39.41±1.41[c]	43.23±0.91[e]	25.93±1.04[d]	25.47±0.74[c]
Native + KMS24	45.12±0.96[d]	48.40±0.86[f]	30.52±1.77[e]	29.93±1.38[d]
Mixed culture (NW16 + KMS24)				
Sterile + Mixed culture	65.91±1.50[e]	71.97±1.65[g]	29.24±1.77[e]	26.35±0.72[c]
Native + Mixed culture	73.78±1.34[f]	75.92±0.52[h]	30.40±2.35[e]	31.67±1.03[e]

Values in the same columns with different lowercase letters indicate significant differences ($p < 0.05$)

Removal of HMs in the sediment collected from post cultured contaminated shrimp ponds

A composite sediment sample collected from shrimp ponds contaminated with HMs as previously described had the following physicochemical properties: 1269 µs/cm EC, 0.84 ppt salinity, and a pH 6.93. The composite sediment sample was contaminated with the following HMs in mg/kg; 0.29 Cd^{2+}, 23.15 Pb^{2+}, 15.05 Cu^{2+}, and 22.16 Zn^{2+}. The removal of HMs by the pure culture of NW16 or KMS24 or their mixed culture with sterile sediment or non sterile sediment (native) under conditions of microaerobic-light and aerobic-dark is shown in Table 6-2.

Comparing the removal percentage between the abiotic and native control sets under both incubating conditions showed no significant difference for Cd^{2+} and Cu^{2+} but a slight significant increase with the native flora was found for Pb^{2+} and Zn^{2+}. In all controls the percentage removal from the sediment samples was higher than for the water samples especially for Zn^{2+} removal and this was reflected in the values for Zn^{2+} removal with the other pure and mixed cultures. The presence of the native flora with the pure or the mixed culture with both culture conditions caused a significant increase in the removal of all HMs. As was observed from the results with the contaminated water, strain KMS24 was mostly slightly better than strain NW16 at removing HMs. Also the best removal rate was for Pb^{2+} with 84.29% removed by the mixed culture plus native flora in aerobic-dark conditions while the corresponding figure with the same conditions for Cu^{2+} was 62.52% for Cd (43.33%) and for Zn (40.95%).

Table 6-2. The removal percentage of Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+} in the contaminated sediment from shrimp ponds after harvesting by the selected purple nonsulfur bacteria under microaerobic-light and aerobic-dark conditions

Treatment	% Removal							
	Cd^{2+} (0.29 mg/kg)		Pb^{2+} (23.15 mg/kg)		Cu^{2+}(15.05 mg/kg)		Zn^{2+}(22.16 mg/kg)	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Control								
Abiotic	20.2±2.54^{a}	18.92±3.87^{a}	21.49±3.41^{a}	19.64±1.99^{a}	20.67±2.15^{a}	20.52±2.36^{a}	17.48±1.50^{a}	19.06±2.91^{a}
Native	19.96±3.19^{a}	17.76±2.47^{a}	24.31±5.22^{b}	24.31±4.22^{b}	21.09±1.79^{a}	19.53±2.70^{a}	20.57±1.40^{b}	21.67±2.16^{b}
NW16								
Sterile + NW16	30.28±0.77^{b}	32.54±0.54^{b}	60.20±1.99^{c}	63.24±0.99^{c}	31.83±0.64^{b}	34.25±0.65^{b}	33.76±0.80^{cd}	35.63±0.54^{d}
Native + NW16	36.83±1.08^{c}	40.32±1.07^{c}	66.95±1.41^{c}	69.35±0.72^{d}	41.24±0.68^{c}	39.63±1.09^{c}	39.15±0.98^{e}	40.12±0.90^{e}
KMS24								
Sterile + KMS24	29.96±1.08^{b}	32.26±0.49^{b}	67.66±1.27^{c}	65.27±1.08^{cd}	35.28±0.43^{d}	39.74±0.92^{c}	30.48±0.86c	34.82±1.04^{d}
Native + KMS24	39.34±0.95^{d}	40.19±1.01^{c}	71.01±1.23^{d}	70.97±1.38^{d}	41.72±0.87^{e}	45.89±0.94^{d}	35.40±0.61^{d}	37.75±0.69^{de}
Mixed of PNB cells								
Sterile + Mixed PNB	36.65±1.56^{c}	39.23±1.22^{c}	76.20±1.81^{e}	81.35±2.91^{e}	57.57±1.02^{f}	59.16±1.62^{e}	32.83±2.60^{cd}	30.02±2.39^{c}
Native + Mixed PNB	39.80±0.81^{d}	43.33±0.92^{d}	78.48±1.12^{e}	84.29±3.41^{f}	60.30±2.04^{g}	62.52±1.07^{f}	35.48±1.93^{d}	40.95±1.05^{e}

The number in each bracket is the initial concentration of heavy metal ion; values in the same columns with different lowercase letters indicate significant differences ($p < 0.05$)

Toxicity assessment by seed germination for the sediment and water after treatment

The toxicity of water and samples from the sediment after treatment by pure cultures of NW16 or KMS24 or their mixed culture was assayed by seed germination and results are presented as a germination index in percent (% GI) with rice (*Oryza sativa*) and water spinach (*Ipomoea aquatic*) (Table 6-3). All samples from contaminated water inhibited germination but after any treatment the inhibition was considerably reduced with the most significant reduction occurring after treatment with the mixed culture plus the native population. Samples from the sediment before treatment also inhibited germination and again all treatments reduced this inhibition. Treatment with the mixed culture with and without native flora resulted in an increased % GI. There were no significant differences found between the sets of pure cultures (NW16 or KMS24) but together they did have a synergistic effect. In all cases the presence of the native population significantly increased the germination index up to 139.3% after treatment of the sediment samples with the mixed culture plus the native population. Rice was always more susceptible to inhibition than water spinach.

Table 6-3. Germination index of rice and water spinach in sediment and water samples after treatment by the selected purple nonsulfur bacteria under both incubating conditions*.

Treatment	Germination index (% GI)			
	Rice (*Oryza sativa)*		Water spinach: *(Ipomoea aquatica)*	
	Water	Sediment	Water	Sediment
Control				
Abiotic	10.50±0.59^{a}	59.38±1.32^{a}	10.59±0.59^{a}	78.30±1.62^{a}
Native	15.44±0.29^{b}	70.04±0.30^{b}	13.41±0.07^{b}	92.38±2.09^{b}
NW16				
Sterile+NW16	20.47±4.44^{c}	80.57±7.44^{c}	21.75±2.82^{c}	114.83±6.86^{c}
Native+NW16	23.86±2.26^{c}	85.39±6.29^{c}	24.41±1.06^{c}	120.71±4.13^{cd}
KMS24				
Sterile+KMS24	21.46±0.85^{c}	79.41±0.45^{c}	22.16±2.54^{c}	115.34±3.86^{c}
Native+KMS24	24.03±0.66^{c}	83.35±2.59^{c}	26.91±2.91^{d}	126.90±4.61^{d}
Mixed of NW16 and KMS24 cells				
Sterile + PNB cells	28.33±1.28^{d}	106.14±6.56^{d}	30.11±3.17^{e}	121.89±2.83^{cd}
Native + PNB cells	34.50±2.23^{e}	115.70±3.22^{e}	35.29±1.03^{f}	139.33±9.51^{e}

Values in the same columns with different lowercase letters indicate significant differences ($p < 0.05$). *Samples from both incubating conditions were mixed.

Discussion

Effect of Ca^{2+} and Mg^{2+} on removal of HMs by PNB

In this study, biosorption by biomass of the selected PNS strains (NW16 and KMS24) was used to remove HMs in 3% NaCl from a synthetic solution (with and without light metal ions; Ca^{2+} and Mg^{2+}), and samples of water and sediment were collected from shrimp ponds contaminated with HMs. The exposure time for binding between HMs and cells was short between 30 and 45 min, so

biosorption was likely to be the main mechanism for removing HMs (no energy requirement). However, some bioaccumulation (requiring energy) of HMs by PNB cells was possible (Panwichian *et al*., 2010b) and this might be one explanation for the mixed culture being more efficient than a pure culture. The biosorption process itself requires a combination of a series of passive and active transport mechanisms, starting with the diffusion of the metallic ions into the biomaterial. This accumulation requires many passive processes such as adsorption, covalent bond formation, complexation, chelation, ion exchange and micro-precipitation (Ahluwalia and Goyal, 2007; Panwichian *et al*., 2010b). Different organisms might use different processes resulting in a significant increase in the removal of HMs when incubated together. The native population may also use alternative processes and might even facilitate some bioaccumulation because of their previous adaptation to the culture medium.

In general, co-ions interfere and reduce the biosorption capacity of another metal ion such as by competition to use the same processes such as competition for binding sites on the PNB cells. In the present study, light metal ions, such as Na^{+}, Ca^{2+} and Mg^{+2}, were present in the synthetic solution and the experimental data showed that Ca^{2+} and Mg^{2+} light bivalent cations but not Na^{+} had a big effect on the biosorption of HMs (Fig. 6-1). This indicates that the cell surface binding exhibits a low degree of specificity and similarly charged cations will also increase competition for binding sites on the cell surface. However, the results in the present study indicate that the use of a mixed culture of PNB cells does have the potential to remove HMs in the conditions of the shrimp ponds.

HMs removal of water and sediment samples from post culturing contaminated shrimp ponds

The physicochemical properties of the composite post cultured water from contaminated shrimp ponds had a pH of 8.07 and this may have an effect on the solubility of HMs as a high pH normally accelerates their precipitation (Gazso, 2001), particularly for Pb and Cd (< 1.00 for Cd^{2+} and < 5.00 μg/L Pb^{2+}). However, the amount of Cu and Zn ions were present at 43 and 57 μg/L, respectively and they exceeded the standard guidelines for marine aquatic animal cultivation (≤ 8 and ≤ 50μg/L for Cu and Zn) (Pollution Control Department, 2006). In addition, any HM

accumulation in the food webs might have an adverse effect on human beings as previously described. Therefore, the HMs contaminated water was treated by the selected PNB strains under of microaerobic-light and aerobic-dark conditions. Fortunately removal of HMs by PNB cells did occur under both incubating conditions and in general the conditions of aerobic-dark produced a better removal efficiency than microaerobic-light conditions. In general, actively growing PNB cells have been used to treat various wastewaters including that in shrimp ponds (Watanabe *et al*., 2003; Kantachote *et al*., 2005). These results show that the application of PNB cells to clean up water will rapidly remove HMs from both the sediment which has microaerobic-light conditions and in the water column with aerobic-dark conditions at night time. However, biosorption using the biomass of PNB should be investigated under the conditions of aerobic-light as well although the biosorption process did occur without requiring energy from the cells.

The results in Table 6-1 show that removal of Cu^{2+} and Zn^{2+} in an abiotic control occurred (7.78-13.42%) in the water tested under both incubating conditions tested. This indicates that adsorption of HMs either Cu^{2+} or Zn^{2+} to non-living organism or inorganic and organic matters in the water. It is well recognized that adsorption can remove metals over a wider range of pH values at lower concentrations, as in the present study, than can be removed by alkaline precipitation (Baker and Khalili, 2004). In addition, biopolymers produced by microorganisms also bind metals strongly (Watanabe *et al*., 2003; Iyer *et al*., 2005). However, in the present study there was no significant difference for the removal percentage of HMs by native and abiotic controls. It might be that in this case biopolymers derived from native flora were not present. Results in this study indicate that the biomass of KMS24 might have a greater affinity for both HMs ions than the biomass of NW16 as it provided higher efficiencies under either a set of pure culture or with native flora. In addition to biosorption of HMs to cells, bioaccumulation of HMs into cells can occur as previously described. This was supported by the removal of Zn^{2+} being significantly higher using a mixed culture than found for NW16 but there was no significant difference with KMS24. This is due to the toxicity of Zn^{2+} on the strain NW16 being higher than the strain KMS24 and this result was in agreement with Panwichian *et al*. (2010a). As each culture may have some different properties for

binding or uptake HMs including HMs tolerance; thereby synergistic removal of HMs was significantly increased with their mixed culture and again with higher efficiencies being obtained in the set with native flora.

Results in Table 6-2 demonstrate that contamination of HMs in the sediment collected from shrimp ponds was at acceptable levels for use as agricultural soil (≤ 1.5, ≤75, ≤65 and ≤ 200μg/kg for Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+}, respectively) (Pollution Control Department, 2004 ; HKGS 1998) although the sediment initially contained higher concentrations of HMs than that found in the water. The abiotic control in the sediment samples removed more HMs than found for the water samples (Tables 6-1 and 6-2) due perhaps to the sediment having more organic and inorganic matter including clay particles to bind HMs. Moreover, a higher concentration of HMs in the sediment could increase its removal efficiency as the uptake rate of the metals ions will increase along with its increasing initial concentration when the amount of adsorbent is constant (Wang and Chen, 2006). There was no significant difference found for removal of Cd^{2+} and Cu^{2+} in both control sets (abiotic and native) but there was a higher significant removal of Pb^{2+} and Zn^{2+} found in the native control. In addition results obtained from the pure culture sets and the culture of NW16 or KMS24 with native flora confirmed that native flora did increase the efficiency to remove HMs.

As previously described the strain KMS24 was more resistant to Zn^{2+} in the water tested than strain NW16 and the experiments with the sediment samples also showed that the former strain was more resistant to Cu^{2+} (Tables 6-1 and 6-2). This can be explained using the same reasoning that was previously given in the water experiment as bioaccumulation could be involved and thus a pure culture of KMS24 produced a higher efficiency to remove Cu^{2+} in the sediment samples tested. Again, it is not surprising that the mixed culture of both PNB strains had the highest efficiency to remove HMs from the sediment by a synergistic action as previously explained. Some difference was found for the removal efficiency of HMs from the synthetic solution ($Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$) and the sediment samples ($Pb^{2+} > Cu^{2+} > Cd^{2+} \approx Zn^{2+}$) (Figure 6-1 and Table 6-2). There are many factors such as organic matter, soil particles like clay in the sediment samples that can affect removal efficiency when compared with the synthetic solution and that are why a higher efficiency was

observed in the sediment samples because removal was by both biosorbtion and also adsorbtion. Furthermore, those factors also had an impact on the removal of Zn or Cd from the sediment samples.

Toxicity assessment by seed germination for the sediment and water after treatment

The seed germination bioassay has been documented as one of the popular techniques for investigating the toxicity of HMs (Ye *et al*., 2002) and the germination index (% GI) is regarded as the most sensitive parameter that is able to detect low toxicity that affects root growth and seed germination (Zucconi *et al*., 1981). Results of the germination of rice seed (*Oryza sativa*) and water spinach seed (*Ipomoea aquatic*) in treated of water and sediment samples by all treatments was significantly higher than that in both the control sets (native > abiotic) and the % GI of both plants, rice and water spinach, in the treated sediment were remarkably higher than that found in the treated water samples (Table 6-3). Biosorption of HMs by PNB strains as a pure culture or mixed culture either with native flora or not produced a significant decrease of HM toxicity to plants and this corresponded with the HMs removal percentage. In order to explain why treated sediment had less toxicity to plants, there could be two main reasons; first, contamination of HMs in the sediment was within the acceptance values for each HMs to allow plants to grow. Secondly, the salinity of the sediment samples (0.84 ppt) was much less than in the water (10.23 ppt). Moreover, the presence of higher amounts of nutrients in the sediment may enhance plant growth and help to reduce the toxicity of HMs.

Conclusions

The presence of light metal ions, Ca^{2+} and Mg^{2+}, had a negative effect on the removal of HMs by biosorption. However, the use of a mixed culture of PNB (NW16 and KMS24) demonstrated the potential for successful application. The results clearly indicate that the biosorption of HMs by the selected PNBs in the water or sediment samples collected from contaminated shrimp ponds after harvesting; particularly with the mixed culture alone or with native flora significantly decreased

the toxicity of HMs to plants as demonstrated by an increased % GI value. However, toxicity was still found in the treated water but that could be caused by salinity. It is therefore suggested that the wastewater from post culturing shrimp ponds could be treated by bioremediation such as biosorption prior to discharge into the environment.

CHAPTER 7

CONCLUSIONS

The findings of the research study are discussed in this final chapter in order to attempt to integration the conclusions drawn from each section and also to identify future research needs. The general conclusions from the work are as follows.

Chapter 3 reported on the isolation of purple nonsulfur bacteria for the removal of HMs and sodium (Na) from contaminated shrimp ponds. There was very less information available about the levels and distribution of HMs in areas of the Songkhla Lake Basin and the Pakphanang Estuary in southern Thailand. Thus, the distribution of HMs and Na in shrimp ponds was determined, and it was found that the concentration of HMs and Na were different depending on the areas of study. For sediment samples (the highest concentrations in mg/kg DW; 0.75 Cd, 62.63 Pb, 34.60 Cu, and 58.50 Zn, and 3.21 g/kg DW Na) had significantly higher levels of HMs than the water samples (the highest concentrations in mg/L; 0.003 Cd, 0.006 Pb, 0.06 Cu, and 1.70 Zn, and 84.55 g/L Na). However, the concentrations of HMs in the sediment samples were with in acceptancable levels for agricultural soil and sediment (Pollution Control Department, 2004; HKGS, 1998). In contrast, water samples had levels of Cu and Zn that are higher than the standard guidelines for marine aquatic animal cultivation (Pollution Control Department, 2006). This indicates that strict regulations by government agencies for shrimp farmers must be enforced. In order to remediate HMs and Na from contaminated shrimp ponds, two PNB strains, NW16 and KMS24, were isolated and selected for investigating their abilities to remediate contaminated shrimp ponds with high HMs and Na.

Chapter 4 focused on the identification of two strains with good abilities to remove HMs and their tolerance to HMs. The distribution of HMs in cells including the removal efficiency of HMs occurred by binding to EPS and biomass. Both strains were identified, one was *Rhodobium marinium* NW16 and the other was *Rhodobacter sphearoides* KMS24. Both grew better with conditions of microaerobic-light than that with aerobic-dark. The results showed that the most toxic metal with

the lowest MIC was Cd, whereas the least toxic metal tested was Cu. For Pb, it was precipitated with the phosphate in GM medium and it has been argued that the HMs and media components may interact in various ways and this makes it difficult to interpret the exact value for HMs tolerance to its (Gadd, 1983; Trevors *et al*., 1985). One of the mechanisms for HMs resistance is their capacity to accumulation the HMs. The greater amounts of Cu and Zn taken up by PNB cells were found on the cell wall, with a minor part being taken up inside the cells. The presence of HMs in the cytoplasm and cell membrane was confirmed by SEM and EDX analysis. This indicate that both PNB used more than one mechanism to accumulate Cu and Zn in addition to biosorption (metabolism- independent) and PNB cells responded to HMs by changing their cellular morphology. This study also proved that EPS produced by PNB had the greatest potential for removing HMs higher than did their biomass. Removal of HMs with the highest concentrations detected in shrimp farms in 3% NaCl under conditions of microaerobic-light and aerobic-dark by EPS was significantly higher (97.2% Pb, 91.83% Zn, 90.78 % Cd and 90.52 %Cu) when compared with their biomass (75% Pb, 40% Cu, 25% Zn and 14% Cd. This indicates that the EPS were very effective and played an important role in accumulating HMs and the toxicity of HMs affected on active cells. This kind of property makes PNB useful for bioremediation and in order to prove what factors affected the immobilization of HMs were further investigated in the next chapter.

Chapter 5 deals with the mechanisms of HMs immobilization by PNB cells and the results supported the above finding that some HMs immobilization occurred by intracellular uptake (bioaccumulation) and surface binding (biosorption). The removal efficiency of HMs in 3% NaCl (initial concentration of each HM as previously mentioned in chapter 4) in both incubating conditions was in the order of Pb > Cu > Zn > Cd. The HMs uptake capacity of the PNB was affected by the different environmental growth conditions; cell age, biomass dose, pH, temperature, and contact time and the optimal conditions for removal of HMs by the NW16 strain were found as cells in the log phase at 4.5 mg DCW/ml, pH 6.0, and 30ºC for 30 min. Under microaerobic-light conditions, the relative percent removal of HMs was: Pb, 83; Cu, 59; Zn, 39; Cd, 23 and slightly more under the aerobic-dark conditions (Pb, 90; Cu, 69; Zn, 46; Cd, 28). For KMS24, cells in the log phase at 5.0 mg DCW/ml,

pH 5.5, and 35ºC for 45 min were found to be the optimal conditions, and there were no significant differences for the removal percentages of HMs with either incubating conditions (averages: Pb, 96; Cu, 75; Zn, 46; Cd, 30). Additionally, the biosorption capacity of the HMs by each PNB strain was found to be significantly decreased in the presence of the other competing metal ions such as Ca^{2+} and Mg^{2+} and this may be a serious problem for application. Therefore, the use of mixed culture would be a better solution to the problem as the results show that the strain NW16 had a better efficiency to remove HMs but the strains KMS24 had more resistance to HMs.

Chapter 6 focused on the effects of light metal ions on HMs removal in the synthetic HMs solution containing 3% NaCl using pure cultures of each strain or their mixed cultures. Moreover, the possibility to remove HMs from contaminated in water and sediment samples collected from shrimp ponds after harvesting and the toxicity of HMs in both samples after treatment were investigated by seed germination. The combination of both strains produced a big increase for removal of HMs although the light metals ions caused some reduction on removal and the average removal percentages were 81.5 Pb, 69.5 Cu, 41.5 Zn, and 24.5 Cd. Both strains present either as pure culture or their mixed cultures performed with a significantly higher efficiency to remove HMs than found in the control sets (abiotic and native). The highest removal percentage of Cu and Zn from the water was found in the set of mixed PNB cells together with the native population under aerobic-dark conditions with 75.92% and 31.67% respectively. A similar result was found in the sediment that showed that a mixed culture of PNB cells with native flora produced the highest potential to remove Pb, Cu, Zn, and Cd under aerobic-dark condition with the removal percentages; 84.29, 62.52, 43.33, and 40.95 respectively. Tests for the toxicity in the water and sediment were conducted with rice (*Oryza sativa*) and water spinach (*Ipomoea aquatic*) seeds and the results confirmed that the high to remove of HMs did produce a significantly higher GI for both plants tested. However, some toxicity did remain in the water as the % GI for rice and water spinach was 34.50 and 35.29 and this was due to water being saline as it is known that both plants are sensitive to saline condition (10.23 ppt). In contrast, no toxicity in the sediment remained as 115.70 and 139.33 % GI for rice and water spinach were found and this

may be contamination of HMs was in the acceptance levels for plant growth and only 0.84 ppt for its salinity.

The results presented in this thesis can be used to answer some questions posed in the introduction. Contamination of HMs and Na in the shrimp ponds does have an adverse effect on the environment, particularly the water after shrimp harvest that should be treated before discharge into the surrounding area. HMs resistant PNB strains were found in shrimp ponds but only two strains, *Rhodomium marinum* NW16 and *Rhodobacter sphaeroides* KMS24, were selected for further study. Both strains have been proved to efficienciency remove HMs in 3% NaCl both in the synthetic solution and the real conditions of water and sediment from contaminated shrimp ponds. Immobilization of HMs by PNB is governed by biosorption and bioaccumulation with their high content of potential active site, mainly in the cell wall and secretion of high amount of EPS that have a more extensive in biosorption capacity. Hence, the NW16 and KMS24 strains together with the resident population have a great potential to remove HMs from the contaminated shrimp ponds.

Future research needs

There are many questions that remain with no answers and some specific suggestions that arising from the current studies are given below.

1. Only the chapter 3 that focused on removal of Na by PNB and thus the removal of Na should be investigated further to obtain optimal conditions due to salinity being the most serious problem in the water of shrimp ponds.

2. As immobilization of HMs involves both biosorption and bioaccumulation, it is thereby the conditions of aerobic-light for removal of HMs and Na should be studied to follow the conditions of shrimp cultivation.

3. One of the outstanding tasks in evaluating the role of PNB in metal binding is in ability to produce EPS. These have been no attempts at present to optimize the parameters for biosorption process of EPS, its purification and chemical analysis by both PNB strains. This is an essential future area for research.

4. Induction, production, and properties of the EPS that brought both PNB strains for removing of HMs in the conditions of water from shrimp ponds should be vigorously investigated.

5. To further remove toxic agents from contaminated water, the next step of phytoremediation should be done with the water bioremediation by both PNB strains.

6. Due to both PNB strains being able to use H_2S and many organic compounds found in shrimp ponds; thereby they should be tried for clean up water and removing of HMs including Na in shrimp ponds as concomitant activity.

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APPENDIX

A. Medium

GM medium

Sodium L- glutamate	3.8	g
DL-malic acid	2.7	g
Yeast extract	2.0	g
KH2PO4	0.5	g
KHPO4	0.5	g
(NH4)2HPO4	0.8	g
MgSO4.7H2O	0.2	g
CaCl2. 2H2O	0.053	g
MnSO4.5H2O	0.0012	g
CoCl2.6H2O	0.00095	g
Ferric Citrate	0.0025	g
Nicotinic acid	0.001	g
Thiamine hydrochloride	0.001	g
Biotin	0.00001	g
Deionized water	1000	ml
pH	6.8	

B. Report of Microbial Identification by partial 16S rDNA sequence analysis

B1. NW16

546 bp Identification

Homology Search with BLASTn program from NCBI database

Sequences producing significant alignments:		SCORE	E VALUE
EU919184	*Rhodobium marinum* strain PSB-02	980	0.0
EU910275	*Rhodobium marinum* strain PSB-01	980	0.0
EU445270	*Rhodobium marinum* strain C3	980	0.0
DQ985044	*Rhodopseudomonas* sp. JL1015	980	0.0
AY428572	*Rhodopseudomonas julia* strain DSM 11549	980	0.0

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Description	NW16
Molecule type	nucleic acid
Query Length	546
Database Name	nr
Description	All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
Program	BLASTN 2.2.20+

Reference

Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

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gene, partial
sequence
Length=1249

Score =  980 bits (1086),  Expect = 0.0
Identities = 545/546 (99%), Gaps = 0/546 (0%)
Strand=Plus/Plus

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LOCUS EU919184 1249 bp DNA linear BCT 30-AUG-2008

DEFINITION *Rhodobium marinum strain* PSB-02 16S ribosomal RNA gene, partial sequence.

ACCESSION EU919184

VERSION EU919184.1 GI:197253677

KEYWORDS .

SOURCE *Rhodobium marinum*

ORGANISM *Rhodobium marinum*

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;

Rhodobiaceae; Rhodobium.

REFERENCE 1 (bases 1 to 1249)

AUTHORS Zhao,Y. and Mu,J.

TITLE Isolation and characterization of marine photosynthetic bacteria from abyssal deposits of the Bohai Sea

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1249)

AUTHORS Zhao,Y. and Mu,J.

TITLE Direct Submission

JOURNAL Submitted (23-JUL-2008) School of Environmental and Chemical Engineering, Dalian Jiaotong University, No.794, Huanghe Road, Dalian, Liaoning 116028, China

FEATURES Location/Qualifiers

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/product="16S ribosomal RNA"

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121 tagcttgttg gtggggtaac ggcctaccaa ggcaacgatc cgtagctggt ctgagaggat

181 gatcagccac actgggactg agacacggcc cagactccta cgggaggcag cagtggggaa

241 tcttggacaa tgggggaaac cctgatccag ccatgccgcg tgagtgaaga aggccctagg

301 gttgtaaagc tctttcagcg gggaagataa tgacggtacc cgcagaagaa gccccggcta

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B2. KMS24

533 bp Identification

Homology Search with BLASTn program from NCBI database

Sequences producing significant alignments:	SCORE	E VALUE
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Molecule type	nucleic acid
Query Length	533

Database Name nr

Description All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)

Program BLASTN 2.2.20+

Reference

Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query Length 533

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Length=1297647

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rRNA-16S Ribosomal RNA

Score = 957 bits (1060), Expect = 0.0
Identities = 532/533 (99%), Gaps = 0/533 (0%)
Strand=Plus/Plus

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Features in this part of subject sequence:
rRNA-16S Ribosomal RNA

Score = 957 bits (1060), Expect = 0.0
Identities = 532/533 (99%), Gaps = 0/533 (0%)
Strand=Plus/Minus

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Features in this part of subject sequence:
rRNA-16S Ribosomal RNA

Score = 957 bits (1060), Expect = 0.0
Identities = 532/533 (99%), Gaps = 0/533 (0%)
Strand=Plus/Plus

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LOCUS CP001151 1110 bp DNA linear BCT 22-JAN-2009

DEFINITION *Rhodobacter sphaeroides* KD131 chromosome 2, complete sequence.

ACCESSION CP001151 REGION: 105..1214

VERSION CP001151.1 GI:221161990

PROJECT GenomeProject:31111

DBLINK Project:31111

KEYWORDS .

SOURCE *Rhodobacter sphaeroides* KD131
ORGANISM *Rhodobacter sphaeroides* KD131
Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter.
REFERENCE 1 (bases 1 to 1110)
AUTHORS Lim,S.K., Kim,S.J., Cha,S.H., Oh,Y.K., Rhee,H.J., Kim,M.S. and Lee,J.K.
TITLE Complete Genome Sequence of Rhodobacter sphaeroides KD131
JOURNAL J. Bacteriol. 191 (3), 1118-1119 (2009)
PUBMED 19028901
REFERENCE 2 (bases 1 to 1110)
AUTHORS Lim,S.K., Cha,S.H., Kim,S.J., Oh,Y.K., Kim,M.S. and Lee,J.K.
TITLE Direct Submission
JOURNAL Submitted (04-SEP-2008) R&D Center, GenoTech Corp. 59-5 Jang-dong, Yuseong-gu, Daejeon 305-343, South Korea
COMMENT Bacteria available from KCTC (http://www.brc.re.kr/English/_SearchView.aspx?sn=12085).
FEATURES Location/Qualifiers
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ORIGIN

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```

VITAE

Name Miss Saijai Panwichian

Student ID 4910230017

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. General Science	Prince of Songkla University	1993
M.Sc. Microbiology	Prince of Songkla University	2000

Scholarship Awards during Enrolment

2007-2009 Scholarship, the Commission on Higher Education Congress: University Staff Development Consortium CHE-USDC Congress.

Thesis Research Grant, Project number SCI520001S, Faculty of Science, Prince of Songkla University.

List of Publications and Proceedings

Panwichian, S., Kantachote, D., Wittayaweerasak, B., Mallavarapu, M. 2010. Isolation of purple nonsulfur bacteria for the removal of HMs and sodium from contaminated shrimp ponds. Electronic Journal of Biotechnology, Vol. 13(4), 1-12.

Panwichian, S., Kantachote, D., Wittayaweerasak, B., Mallavarapu, M., Factors affecting immobilization of HMs by purple nonsulfur bacteria isolated from contaminated shrimp ponds. World Journal of Microbiology and Biotechnology, Published online 18 April 2010.

Panwichian, S., Mallavarapu, M., Wittayaweerasak, B., Kantachote, D., Influences of HMs and Sodium Ions on Purple Non sulfur Photosynthetic Bacteria Growth under Aerobic Dark Conditions. Proceedings of the 12th National Graduate Research Conference, February 12-13, 2009, Khon Kaen University.

Panwichian, S., Mallavarapu, M., Wittayaweerasak, B., Kantachote, D., Effect of HMs and Sodium on Growth of Purple Nonsulfur Photosynthetic Bacteria Isolated from Contaminated Shrimp Farms. Proceedings of the conference of the Commission on Higher Education Congress I: University Staff Development Consortium CHE-USDC Congress I, September 5-7, 2008, the Ambassador City Jomtien Hotel, Chonburi.

Panwichian, S., Kantachote, D., Wittayaweerasak, B., Mallavarapu, M., Removal of HMs by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds and their taxonomy, *Manuscript*, to be submitted.

Panwichian, S., Kantachote, D., Wittayaweerasak, B., Mallavarapu, M., Toxicity assessment of sediment and water from shrimp ponds contaminated with HMs after treatment by selected purple nonsulfur bacteria, *Manuscript*, to be submitted.