Diversity of Purple Nonsulfur Bacteria in Shrimp Ponds and Mercury Resistant Mechanisms of Selected Strains

Kanokwan Mukkata

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

Prince of Songkla University

2016

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ความหลากหลายของแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ในบ่อกุ้งและกลไกการต้านทานปรอทของเชื้อที่คัดเลือกได้

ผู้เขียน นางสาวขนกวรรณ มุขตา

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บทคัดย่อ

เพื่อตรวจสอบปริมาณการปนเปื้อนปรอทและแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria; PNSB) สายพันธุ์ที่ต้านทานปรอทในบ่อกุ้งบริเวณแหลมทองในประเทศไทย จึงเก็บตัวอย่างน้ำและตะกอนดินจำนวน 35 ตัวอย่างจากบ่อกุ้ง ณ สถานที่ต่างๆ เพื่อนำมาตรวจสอบปริมาณปรอททั้งหมด (Hg_T) และเพื่อคัดแยก PNSB สายพันธุ์ที่ต้านทานปรอทเพื่อความเป็นไปได้ในการนำไปใช้บำบัดทางชีวภาพ ปริมาณปรอททั้งหมดในตัวอย่างน้ำมีค่าอยู่ในช่วง <0.0002-0.037 ไมโครกรัมต่อลิตร ขณะที่ในตะกอนดินมีค่าอยู่ระหว่าง 30.73-398.84 ไมโครกรัมต่อกิโลกรัมน้ำหนักแห้ง โดยปริมาณปรอททั้งหมดที่ตรวจวัดได้ในตัวอย่างทั้งสองชนิดมีค่าต่ำกว่าค่ามาตรฐานตามเกณฑ์กำหนดของฮ่องกง ประเทศไทยและแคนาดา จากนั้นได้มีการเก็บตัวอย่างน้ำและตะกอนดินจำนวน 16 ตัวอย่างที่เป็นตัวแทนกลุ่มจากบ่อกุ้งต่างๆ อีกครั้งเพื่อตรวจสอบความหลากหลายของ PNSB และผลของความเข้มข้นของปรอทในบ่อกุ้งต่อความหลากหลายของ PNSB ในบ่อเหล่านั้น โดยความหลากหลายของ PNSB ตรวจสอบจากการตรวจหายีน *pufM* ซึ่งตรวจพบในตัวอย่างน้ำจำนวน

13 บ่อ และในตัวอย่างตะกอนดินจำนวน 10 บ่อ นอกจาก PNSB แล้วยังตรวจพบแบคทีเรียสังเคราะห์แสงที่ไม่ให้ออกซิเจน (anoxygenic phototrophic bacteria; APB) กลุ่มอื่นๆ ด้วยในตัวอย่างทั้งสองชนิด ซึ่งได้แก่แบคทีเรียสังเคราะห์แสงที่ไม่ให้ออกซิเจนกลุ่มใช้ออกซิเจน (aerobic anoxygenic phototrophic bacteria; AAPB) และแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มสะสมซัลเฟอร์ (purple sulfur bacteria; PSB) โดยแบคทีเรียสังเคราะห์แสงกลุ่ม AAPB PSB และ PNSB ที่ตรวจพบในตัวอย่างน้ำมีค่าเท่ากับ 25.71 11.43 และ 8.57 เปอร์เซ็นต์ ขณะที่ในตัวอย่างตะกอนดินมีค่าเท่ากับ 27.78 11.11 และ 22.22 เปอร์เซ็นต์ ผลจากการเทียบเคียงลำดับเบสพบว่า *Roseobacter denitrificans* (AAPB) พบมากที่สุดในตัวอย่างทั้งสองชนิด ตามมาด้วย *Halorhodospira halophila* (PSB) อย่างไรก็ตามไม่พบความสัมพันธ์ระหว่างปริมาณปรอททั้งหมด (ตัวอย่างน้ำอยู่ในช่วง <0.002-0.03 ไมโครกรัมต่อลิตร และตัวอย่างตะกอนดินอยู่ในช่วง 35.40-391.60 ไมโครกรัมต่อกิโลกรัมของน้ำหนักแห้ง) กับความหลากหลายของ APB โดยเฉพาะกับ PNSB ในบ่อกุ้ง จากนั้นได้คัดเลือก PNSB ที่ต้านทานปรอทสามสายพันธุ์คือ *Rhodovulum sulfidophilum* สายพันธุ์ SRW1-5 และ *Afifella marina* สายพันธุ์ SSS2-1 และ SSW15-1 จากความสามารถในการลดความเป็นพิษของปรอทด้วยกลไก volatilization โดยเปลี่ยน Hg^{2+} เป็น Hg^{0} ด้วยเอนไซม์ mercuric reductase และค่ากิจกรรมของเอนไซม์ดังกล่าวในสภาวะมีอากาศมีค่าสูงกว่าในสภาวะมีแสงอย่างมีนัยสำคัญทางสถิติ ซึ่งค่ากิจกรรมของเอนไซม์ของสายพันธุ์ SSW15-1 SRW1-5 และ SSS2-1 มีค่าเท่ากับ 15.75 12.62 และ 12.16 ยูนิตต่อมิลลิกรัมของโปรตีน ตามลำดับ นอกจากนี้ค่าความเข้มข้นต่ำสุดของปรอทที่ยับยั้งการเจริญของแบคทีเรีย (MIC) ของทั้งสามสายพันธุ์มีค่าสูงกว่าความเข้มข้นของปรอทที่พบในบ่อกุ้งมาก จึงยืนยันผลกลไกการต้านทานปรอทของแบคทีเรียทั้งสามสายพันธุ์โดยการตรวจหายีนที่เกี่ยวข้องกับการต้านทานปรอท (*mer* operon) โดยมีการตรวจพบยีนที่ควบคุมการสร้างโปรตีน MerR และโปรตีนเพื่อการส่งผ่านสาร (transporter permease

protein) รวมทั้งพบยีนที่ตำแหน่งระหว่าง *merA* (mercuric reductase enzyme) และ *merD* (secondary regulatory protein) ในแบคทีเรียทั้งสามสายพันธุ์ ในขณะที่การตรวจหายีน *merR* (mercuric resistance operon regulatory protein) และ *merT* (transporter protein) พบเฉพาะใน *A. marina* ทั้งสองสายพันธุ์ ซึ่งยีนดังกล่าวนี้มีความเหมือนกับยีนของแบคทีเรียที่เป็นสมาชิกใน Family *Sphingomonadaceae* นอกจากนี้โปรตีน MerR และ MerT ของแบคทีเรียทั้งสองสายพันธุ์มีความแตกต่างจากโปรตีนของแบคทีเรียอื่นในกลุ่ม *α-Proteobacteria* นอกจากนี้ยังได้ตรวจสอบความเป็นพิษของปรอทต่อเซลล์ของ PNSB พบว่าการสัมผัสปรอท ($HgCl_2$) ที่ความเข้มข้นในช่วง 1-4 มิลลิกรัมต่อลิตรเป็นระยะเวลา 9 ชั่วโมง ทำให้อัตราการขนส่งอิเล็กตรอนสูงสุด (ETR_{max}) ของสายพันธุ์ SRW1-5 และ SSS2-1 ลดลงอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) โดยลดลงประมาณ 37 และ 48 เปอร์เซ็นต์เมื่อเปรียบเทียบกับชุดควบคุม ในขณะที่การทดสอบผลการสัมผัสระยะยาวในสภาวะที่มี $HgCl_2$ (1-3 มิลลิกรัมต่อลิตร) โดยเฉพาะที่ความเข้มข้น 3 มิลลิกรัมต่อลิตรเป็นระยะเวลา 96 ชั่วโมง มีผลลดปริมาณแบคเทอริโอคลอโรฟิลล์เออย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ซึ่งส่งผลต่ออัตราการขนส่งอิเล็คตรอนสูงสุดด้วย อย่างไรก็ตามเซลล์สามารถตอบสนองต่อสภาวะกดดันดังกล่าวโดยการเปลี่ยนแปลงรูปร่างเซลล์ให้ยืดยาวขึ้นเพื่อลดความเป็นพิษ ขณะที่ $HgCl_2$ ที่ความเข้มข้น 4 มิลลิกรัมต่อลิตรสามารถฆ่าแบคทีเรียทั้งสามสายพันธุ์ได้ นอกจากนี้ยังได้มีการศึกษาศักยภาพของเซลล์เป็นและเซลล์ตายของ PNSB ทั้งสามสายพันธุ์ในการกำจัดปรอทและปัจจัยที่เกี่ยวข้องกับการดูดซับปรอท พบว่าเซลล์ตายของแบคทีเรียทั้งสามสายพันธุ์มีประสิทธิภาพในการกำจัด Hg^{2+} มากกว่าเซลล์เป็นอย่างมีนัยสำคัญทางสถิติ แต่สภาวะการเจริญที่แตกต่างกันของสายพันธุ์ SSS2-1 และ SSW15-1 ไม่มีผลต่อการกำจัด Hg^{2+} และผลการทดลองของทั้งสามสายพันธุ์พบว่าสายพันธุ์ SSS2-1 ในการเลี้ยงทั้งสองสภาวะมีประสิทธิภาพในการกำจัด Hg^{2+} สูงสุด โดยในสภาวะที่เหมาะสมในการกำจัด Hg^{2+} โดยใช้เซลล์ปริมาณ 5 มิลลิกรัมของน้ำหนักเซลล์แห้งต่อลิตร และ

ความเข้มข้นเริ่มต้นของ $HgCl_2$ เท่ากับ 4 มิลลิกรัมต่อลิตร สำหรับเซลล์เป็นคือการใช้เซลล์จากช่วง log และ late log phases พีเอชเท่ากับ 7 อุณหภูมิเท่ากับ 35°C และใช้ระยะเวลา 90 นาที ซึ่งสามารถกำจัดปรอทได้ 87 เปอร์เซ็นต์ ส่วนกรณีของเซลล์ตายคือเซลล์จากทุกช่วงของการเจริญ พีเอชเท่ากับ 7 อุณหภูมิช่วง 35-40°C ระยะเวลา 15 นาที ซึ่งสามารถกำจัดปรอทได้ 95 เปอร์เซ็นต์ การดูดซับ Hg^{2+} ของเซลล์เป็นจากการเจริญทั้งสองสภาวะเป็นไปตามแบบจำลอง Freundlich ส่วนการดูดซับของเซลล์ตายเป็นไปตามแบบจำลอง Langmuir ขณะที่แบบจำลองจลนพลศาสตร์ของการดูดซับ Hg^{2+} ของเซลล์ทั้งสองชนิดเป็นไปตามแบบจำลองจลนพลศาสตร์ pseudo-second order จากผลการศึกษาในภาพรวมพบว่า PNSB ทั้งสามสายพันธุ์ที่คัดเลือกได้มีศักยภาพสูงมากในการนำไปบำบัดทางชีวภาพในพื้นที่บ่อกุ้งที่ปนเปื้อนปรอท

คำสำคัญ: การบำบัดทางชีวภาพ ปรอท เมอร์โอเปอรอน แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ บ่อกุ้ง ความเป็นพิษ

Thesis Title Diversity of Purple Nonsulfur Bacteria in Shrimp Ponds and the Mercury Resistance Mechanisms of Selected Strains

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Academic Year 2015

ABSTRACT

To investigate mercury (Hg) contamination and Hg resistant strains of purple nonsulfur bacteria (PNSB) in shrimp ponds along the Thailand peninsular coast, 35 samples of water and sediment from various shrimp pods were collected to detect total Hg (Hg_T) and isolate Hg resistant PNSB strains for the possibility to use in bioremediation. Hg_T levels in samples of water ranged from <0.0002 to 0.037 μg/L while in sediment were between 30.73 to 398.84 μg/kg dry weight. All of these, Hg_T levels were lower than the Thai, Hong Kong, and Canadian standard guidelines. Representative samples of sixteen water and sediment samples were re-collected for investigating the diversity of PNSB and the effect of Hg concentrations on PNSB diversity in shrimp ponds. The diversity of PNSB in shrimp ponds were amplified using *pufM* gene; and the detection was found in 13 and 10 samples of water and sediment. In addition of PNSB, other anoxygenic phototrophic bacteria (APB), aerobic anoxygenic phototrophic bacteria (AAPB) and purple sulfur bacteria (PSB) were detected in both sample types. Among identified groups; AAPB, PSB and PNSB in the samples of water and sediment were 25.71, 11.43 and 8.57%; and 27.78, 11.11 and 22.22%, respectively. Sequencing analysis revealed that *Roseobacter denitrificans* (AAPB) was the most dominant species in both sample types followed by *Halorhodospira halophila* (PSB). However, there was no relationship between Hg_T concentrations in samples (water, <0.002-0.03 μg/L and sediment, 35.40-391.60 μg/kg dry weight) and the APB, particularly the PNSB diversity in the shrimp ponds. Three Hg resistant PNSB strains; *Rhodovulum sulfidophilum* strain SRW1-5 and

Afifella marina strains SSS2-1 and SSW15-1 were selected based on their detoxification mechanism by volatilization of Hg^{2+} to Hg^0 using mercuric reductase enzyme. The activities of this enzyme in dark condition were significantly higher than in light conditions, which were 15.75, 12.62, and 12.16 U/mg protein in strains SSW15-1, SRW1-5, and SSS2-1, respectively. The minimum inhibitory concentration (MIC) values of these selected strains were much higher than those currently contaminated in Thai shrimp ponds. Their Hg resistant mechanism was confirmed by detections of Hg resistant genes in *mer* operon. Genes coding for MerR family transcriptional regulator protein and transporter permease protein including a part of genes between *merA* (mercuric reductase enzyme) and *merD* (secondary regulatory protein) were detected in all selected PNSB strains. Whilst, *merR* (mercuric resistance operon regulatory protein) and *merT* (transporter protein) was detected only in both strains of *A. marina*; and these sequences had high similarity with genes of members in Family *Sphingomonadaceae*. In addition, MerR and MerT proteins in both strains were divergent from other organisms in *α-Proteobacteria*. In addition, the investigation of Hg toxicity on PNSB cells revealed that acute toxic of 4 mg/L $HgCl_2$ after 9 h exposure resulted a significant decrease ($P < 0.05$) of maximal electron transfer rate (ETR_{max}) in strains SRW1-5 and SSS2-1 roughly 37% and 48%, respectively compared with their controls. A longer exposure for 96 h, the Hg^{2+} (1-3 mg/L $HgCl_2$), particularly at 3 mg/L significantly decreased ($P < 0.05$) BChl *a* contents and consequently affected on ETR_{max}; however, exposed cells could respond to stress conditions by elongation of rod shapes to reduce the adverse effect. In contrast, a high concentration of $HgCl_2$ at 4 mg/L could kill all selected PNSB strains. The potential of live and dead PNSB biomass to remove Hg and factors that affect Hg biosorption were also studied in three selected strains. The efficiency to remove Hg^{2+} by the dead cells of all strains was significantly higher than for live cells; but the different growth conditions had no effect with the SSS2-1 and SSW15-1 strains. Among of three strains, strain SSS2-1 showed higher Hg^{2+} removal efficiency under both growth conditions and its optimal conditions under both incubating conditions using biomass (5 mg DCW/L) to remove Hg^{2+} (initial 4 mg/L $HgCl_2$); for live cells were cells from log and late log phases, a pH of 7, 35°C for 90 min with 87% removal while dead cells from all cell ages, a pH of 7, 35-40°C for 15 min with 95% removal.

The isotherms of Hg^{2+} biosoption of live and dead cells under both incubating conditions fitted with the Freundlich and the Langmuir models, respectively. The kinetics of Hg^{2+} biosorption by both types of biomass fitted with the pseudo-second order kinetic model. Overall results concluded that three selected PNSB strains have the great potential to be used for bioremediation of Hg in contaminated shrimp ponds.

Key words: bioremediation, mercury, *mer* operon, purple nonsulfur bacteria, shrimp ponds, toxicity

ACKNOWLEDGMENTS

I would like to express my sincere grateful thanks and sincere appreciation to my advisor, Associate Professor Dr. Duangporn Kantachote, for her helpful suggestion, valuable guidance and comments throughout my study. Sincere appreciation is expended to Associate Professor Dr. Banjong Wittayaweerasak, Professor Dr. Megharai Mallavarapu and Professor Dr. Ravi Naidu, my co-advisor, for their valuable contribution and advice relating to this study.

I would like to thank Associate Professor Dr. Somkiet Techkarnjanaruk and Miss Nimaradee Boonapatcharoen, for their comments and advice relating to this study.

I wish to express my gratitude to The Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. Program, grant number; PHD/0165/2552 and partly was supported by the Graduate School, Prince of Songkla University.

I would like to thank Dr. Brian Hodgson for his suggestions and assistance with the English.

I would like to express my beloved friends at ST 608 Microbiology Laboratory for their kindly and helpful advice during my entire study.

Finally, I wish to thanks my beloved family for their support and encouragement.

Kanokwan Mukkata

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LIST OF ABBREVIATIONS AND SYMBOLS

BAF: Bioaccumulation factor

BCF: Bioconcentration factor

BChl: Bacteriochlorophyll

BrCl: Bromine monochloride

DCW: Dry cell weight

DGGE: Denaturing gel gradient electrophoresis

EF: Emission factors

EPA: U.S. Environmental Protection Agency

FIMS: Flow Injection Mercury System

GA: Glutamate acetate

Hg: Mercury

Hg^{2+}: Divalent mercury

Hg^{0}: Elemental mercury

Hg_T: Total mercury concentrations

IC_{50}: The half maximal inhibitory concentration

MBC: The minimum bactericidal concentration

MIC: The minimum inhibitory concentration

NH_2OH-HCl: Hydroxylamine hydrochloride

PAM: Pulse Amplitude Modulation

PNSB: Purple nonsulfur bacteria

PSB: Purple sulfur bacteria

RC: Reaction center

WHO: World Health Organization

CHAPTER 1

INTRODUCTION

Rationale and Background

Mercury (Hg) is the most toxic of the heavy metals (Zahir et al., 2005) and the sixth most toxic chemical in the list of hazardous compounds that persist in the environment (Nascimento and Chartone-Souza, 2003; Bafana et al., 2010). The most notable examples of environmental contamination with Hg occurred in Japanese Minamata Bay between 1953 and 1961 when fish had been contaminated with methylmercury from an acetaldehyde plant, and people were subsequently poisoned. This incident was called “Minamata disease”. In this case, 121 fishermen and their families were stricken with a mysterious illness characterized by cerebellar ataxia, constriction of visual fields, and dysarthria, resulted a total of 46 deaths (Carrasco et al., 2011). There are two sources of Hg contamination in the environments. Firstly, Hg is released from natural sources including a contribution from primary natural sources such as from dust storms, forest fires, volcanic eruptions and breakdown of the earth's crust by wind and water (Mason, 2009; Pirrone et al., 2010). Secondly, Hg is released from man-made sources or anthropogenic sources, which is a major cause of Hg contamination in the environment. The anthropogenic sources of Hg contamination are from various activities such as burning of coal, oil and biofuel; producing of cement, gold, chlorine and caustic soda; batteries and other electrical apparatus industries (Pirrone et al., 2010).

Shrimp farming is one of the most important aquaculture economic businesses of Thailand; and the worldwide export of shrimp from Thailand i.e. 34,800 t during the first quarter of 2015 (FAO, 2015). In Thailand, most shrimp farms are located near the coastal areas, for seawater is directly introduced to the rearing of shrimp requires no additional treatment process although seawater is often

contaminated with many kinds of pollutants, including heavy metals such as Cd, Cu, Pb, and Zn have been reported (Panwichian et al., 2010). However, there have a few reports of Hg contamination in shrimp ponds. The total Hg (Hg_T) contamination in seawater collected from both coastal areas of Thailand in 1997-1998 was detected and ranged from < 0.01 to 0.54 µg/L in water and 0.05 to 2.14 µg/g dry sediment weight (Thongra-ara and Parkpianb, 2002; Sompongchaiyakul and Sirinawin, 2007). This is due to oil and natural gas production in the Gulf of Thailand and runoff of Hg from the mainland (Thongra-ara and Parkpianb, 2002; Cheevaporn and Menasveta, 2003). Hence, it is possible that the shrimp ponds could become contaminated with Hg from the seawater contaminated with Hg. Hg contamination in environments can consequently accumulate into aquatic animals through the food web. Recently, many studies have reported worldwide on Hg contamination in aquatic animals such as shrimp, fish, shellfish, crab, and mollusks (Brambilla et al., 2013; Li et al., 2013).

It is well recognized that heavy metals including Hg influence on activities of microbes and the biogeochemical processes that they can mediate (Liu et al., 2010). For instance, some studies indicated that Hg affects particular soil functions that microbes mediate, such as soil respiration, enzymatic activities, and nitrification through a combination of physiological and soil microcosm experiments (Giotta et al., 2006; Harris-Hellal et al., 2009; Liu et al., 2010). However, microbes have developed resistant/tolerant mechanisms for living in metal-stressed conditions. It has long been known that microbes have developed resistance mechanisms for converting Hg in both of inorganic and organic toxic forms to less toxic form via detoxification system (Barkay et al., 2003). The *mer* operon is the most widely distributed Hg detoxification genetic systems; and it has been extensively studied in many microorganisms. However, a limitation of *mer* operon has been reported in anoxygenic phototrophic bacteria that are normally found in shrimp ponds (Barkay et al., 2010). The *mer* operon consists of genes; *merR* and *merD* for regulation; *merP*, *merT* and *merC* for transportation and *merA* and *merB* for enzymatic detoxification of inorganic and organic Hg compounds (Nascimento and Chartone-Souza, 2003). Mercuric reductase enzyme as the key detoxification mechanism is governed by *merA* gene coding for flavin oxidoreductase (Mathema et al., 2011). This enzyme reduces

divalent mercury ($Hg^{2+)}$ into the less toxic form of volatile metallic Hg (Hg^0) in a NAD(P)H-dependent reaction. In addition of *merA*, *merB* is one of genes in *mer* operon that codes for an organomercurial lyase to hydrolyze C-Hg bond in organomercurial compound detoxification before Hg^{2+} reduction (Nascimento and Chartone-Souza, 2003).

To deal with a serious problem of Hg contaminated areas, Hg resistant microorganisms have been isolated from contaminated environments to investigate their resistance mechanisms for consideration to use in bioremediation (Glendinning et al., 2005; Zhang et al., 2012; Yu et al., 2014). Bioremediation is an alternative way in recent times to clean up polluted environments as it is efficient, eco-friendly and cost effective (Vijayaraghavan and Yun, 2008). Amongst bioremediation methods, the use of live or dead biomass as biosorbents for bioaccumulation and/or biosorption have been demonstrated to have the great potential to remove metals from an aqueous phase. To clean up Hg contaminated shrimp ponds, normal flora that are distributed in shrimp ponds should be considered; thereby among microbes, purple nonsulfur bacteria (PNSB) are a good candidate for this purpose. This is because these bacteria have a wide range of growth modes to grow under photoheterotrophic, photoautotrophic, and chemoheterotrophic conditions (Imhoff, 1995; Madigan and Jung, 2009), so they have been applied to treat various wastewaters that are high in carbon, nitrogen and sulfur compounds (Kantachote et al., 2005; Antony and Philip, 2006; Zhou et al., 2009b). In addition, PNSB are able to consider as beneficial microbes because their cells have high contents of protein, vitamin B12, ubiquinone and photopigments (Sawatdikul, 2003; Shapawi et al., 2012). Moreover, several research groups reported that PNSB are relatively resistant to heavy metals with the potential to use them as biosorbents for removal of heavy metals (Bai et al., 2008; Panwichian et al., 2010; Panwichian et al., 2012).

However, up to date no work has been done on the diversity of PNSB in shrimp ponds. In order to use PNSB for bioremediation; therefore, it is worth to study their diversity in shrimp ponds to gain knowledge that might be useful for finding a use of them during shrimp cultivation. The diversity and phylogenetic composition of anoxygenic phototrophic bacteria (APB) such as purple sulfur bacteria

(PSB, *γ-Proteobacteria*), PNSB (*α-*, *β-Proteobacteria*) and aerobic anoxygenic phototrophic bacteria (AAPB, *α-Proteobacteria*) have been studied by using *pufM* gene that codes for the M subunit of a pigment-binding protein in the photosynthetic reaction center (Yutin et al., 2007; Asao et al., 2011). Regarding to above information, PNSB are the most useful organism to improve water quality and reduce Hg during shrimp cultivation. Hence, to achieve for obtaining promising PNSB strains and knowledge for applying them in shrimp ponds for producing safe food; the objectives of this thesis are the following provided.

Objectives

1. To study diversity of PNSB in shrimp ponds and the influence of Hg contaminated in shrimp ponds on the PNSB diversity.
2. To isolate and select PNSB strains with abilities to resist Hg and remove Hg.
3. To investigate Hg resistant mechanisms and Hg resistant genes of selected PNSB for clearly understanding of their Hg resistant mechanisms
4. To investigate the toxicity of Hg on selected PNSB.
5. To investigate the possibility of selected PNSB for bioremediation.

Scope of the Study

Water and sediment samples were collected from various shrimp ponds in coastal areas of the Thai Peninsular (Gulf of Thailand and Andaman Sea) to determine the amount of Hg concentration, investigate the diversity of PNSB in shrimp ponds, isolate and select Hg resistant PNSB strains. Selected Hg resistant strains were used to study their Hg resistant mechanisms, Hg resistant genes, toxicity of Hg and the potential to remove Hg with the purpose to use them for bioremediation Hg contaminated shrimp ponds.

Anticipated Outcomes

1. This study explores knowledge of PNSB diversity in shrimp ponds, Hg resistant mechanisms that related to *mer* operon of selected PNSB and their potential for bioremediation.

2. This study contributes the attractive ways to remediate Hg in order to restore the natural environment to create sustainable safe production methods.

3. Results from this study may be applied to remove Hg contaminated shrimp ponds and protect the accumulation of Hg into the human via food chain.

4. Selected PNSB from this study may be used for bioremediation in contaminated shrimp ponds, and also for bioassay candidate of Hg contamination in environments.

5. Selected PNSB from this study may be evaluated for probiotic feed supplement that is one of the strategies for ensuring environmentally friendly safe food.

CHAPTER 2

LITERATURE REVIEW

Mercury

Mercury (Hg) is the only pure metal that is liquid at room temperature and atmosphere pressure (Chang et al., 2009), with an atomic number 80 and atomic weight 200.59 (Wieser and Berglund, 2009). Hg can be frozen (changed into a solid) at a temperature of -38.85°C. It can be changed into a gas (boiled) at 365.6°C. Its density is 13.59 grams per cubic centimeter. Hg is moderately active. It does not react with oxygen in the air very readily. It reacts with some acids when they are hot, but not with most cold (Wieser and Berglund, 2009).

Hg is the most toxic of the heavy metals (Zahir et al., 2005) and is the sixth most toxic chemical in the list of hazardous compounds contamination in the environment. It exists in a large number of physical and chemical forms with a large variety of properties that determine its complex distribution, biological enrichment and toxicity. Hg is a global environmental concern mostly in the form of methylmercury as a potent neurotoxin that affects human and wildlife development and health (Selin, 2009). The most notable examples of environmental contamination with Hg occurred in Japanese Minamata Bay by an acetaldehyde plant. In Minamata bay, between 1953 and 1961, 121 fishermen and their families were stricken with a mysterious illness characterized by cerebellar ataxia, constriction of visual fields, and dysarthria. Of these 121 cases, resulted a total of 46 deaths (Carrasco et al., 2011).

Hg species in the environment

Inorganic Hg

Inorganic Hg may show three states of oxidation: elemental mercury (Hg^0), divalent Hg (Hg^{2+}), and monovalent Hg (Hg^+). Elemental Hg (Hg^0), the

predominant species released from soils, rocks and by anthropogenic activities that is the main source of Hg emitted to the atmosphere (Selin, 2009). Inorganic Hg can bind to form complexes with Cl^- to $HgCl_2$ at a low pH and HgClOH at a neutral pH. Moreover, it might be bound with sulfide ions (S^{2-} and HS^-) and change to insoluble HgS (Navarro, 2008).

Organic Hg

Organic Hg includes two forms: methylmercury (CH_3Hg) and dimethyl mercury (CH_3HgCH_3). Within these forms organic Hg species are more toxic than the other forms. Methylmercury species are presented as chloro and hydroxy complexes (CH_3HgCl and CH_3HgOH) in oxic water while the sulfate complex CH_3HgSO_4 is presented in water with a high sulfate concentration and a low pH (Navarro, 2008). Methylmercury is the most toxic compound and recognized as a major environmental pollution issue and health hazard for humans (Clarkson and Magos, 2006) Methylmercury is biomagnified through the aquatic trophic chain and, as a result, the average proportion of methylmercury over total Hg in fish tissues can be up to 95% (Berzas Nevado et al., 2010)

Sources of Hg contamination and biogeochemical cycle of Hg in the environment

Hg contamination in the environment occurs because of two reasons: firstly, Hg is released from natural sources include the contribution from primary natural sources and emission processes of historically deposited Hg over land and sea surfaces. The Hg emitted from volcanoes, geothermal sources and topsoil enriched in Hg pertains to primary natural sources (Mason, 2009; Pirrone et al., 2010). Secondly, Hg is released from man-made sources or anthropogenic sources (Table 2-1), which is a major cause of Hg contamination in the environment. The anthropogenic sources of Hg contamination results from various activities such as coal, oil and biofuel burning; cement production; gold production; ferrous and non-ferrous metals manufacturing facilities; the using of Hg in chlorine and alkaline plants for electrolytic production of chlorine and caustic soda; cellulose industries for preserving the wet pulp from bacterial and fungal deterioration; plastic industries for catalytic reactions; electrical

industries for production of relays, switches, batteries, rectifiers, lamps, etc.; pharmaceutical industries for production of diuretics, antiseptics, cathartics, some contraceptives and drugs for treatment of congestive heart failure; paint industries, mainly for production of anti-corrosive paints; metal refinement by amalgamation; power plants in special heat engines instead of water vapor; plants treating nuclear wastes for electrolytic purification of wastes; and industries producing industrial and control instruments (e.g. thermometers, barometers, Hg pumps) (Pirrone et al., 2010).

Table 2-1. Global Hg emissions from anthropogenic sources (Pirrone et al., 2010)

Source category	Hg emission (Mg/yr)	Reference
Coal and oil combustion	810	(Pirrone et al., 2010)
Non-ferrous metal production	310	(USGS, 2004)
Pig iron and steel production	43	(Pacyna et al., 2006)
Cement production	236	(Pirrone et al., 2010)
Caustic soda production	163	(Pirrone et al., 2010)
Mercury production	50	(Pirrone et al., 2010)
Artisanal gold mining production	400	(Telmer and Veiga, 2009)
Waste disposal	187	(Pirrone et al., 2010)
Coal bed fires	32	(Pirrone et al., 2010)
VCM production	24	(Pirrone et al., 2010)
Other	65	(Pirrone et al., 2010)
Total	232	

Hg contamination in the environment can be changed by the biochemical processes. In the atmosphere, Hg^0 is the most abundant form of Hg, with a global mean concentration of 1.6 ng/m^3 in surface air. Hg^0 is well mixed in the global atmosphere (Lin and Pehkonen, 1999). Some part is in the form of Hg^{2+} and $Hg_{(P)}$ (Hg associated with particulate matter); Hg^{2+} and $Hg_{(P)}$ are more soluble in

water than Hg^0. Hence, they are the predominant forms of Hg deposited in ecosystems through wet and dry deposition. Hg^{2+} and $Hg(_P)$ have a much shorter lifetime than Hg^0 in the atmosphere (days to weeks), and their surface concentrations are in a range of 1-100 pg/m^3 (Selin, 2009).

Wet and dry deposition brings Hg to the terrestrial surface with a concentration of 20-70 ng/g in soil. The majority of Hg in the terrestrial system (>90%) stays in soil and is associated with organic matter. It can bind strongly with the reduced sulfur groups (Skyllberg et al., 2003). Hg reduction and subsequent emission from soils is considered predominantly a physical (abiotic) process, although some recent research has shown that Hg speciation in and emission from soil can be partially controlled by biotic processes (Gabriel and Williamson, 2004). Hg can be absorbed in soils by organic matter, clays and oxides, such as manganese oxides, iron oxides, aluminium oxides and siliceous oxides. The very strong affinity of Hg for organic matter generally explains the elevated Hg concentrations observed in the organic horizon of temperate and Nordic soils (Berzas Nevado et al., 2010). In some dry ecosystems, increased soil moisture (as a result of precipitation events) promotes volatilization of Hg. In terrestrial food webs, Hg exists predominantly as an inorganic form, and it accumulates in plants. However, the uptake of Hg from soil into plants is usually poor and it does not biomagnify in the organisms that feed on them (Berzas Nevado et al., 2010). Therefore, very few studies have been carried out on terrestrial plants. However, Hg in the soil that becomes methylated form can undergo desorption or run off into a watershed. Factors controlling the adsorption and desorption of Hg in soil systems include the type of soil and other dissolved species, such as S^-, Cl^-, and dissolved organic carbon (Gabriel and Williamson, 2004; Selin, 2009).

Two forms of Hg in the water are inorganic and organic Hg. Inorganic Hg (Hg^{2+}) in the water is converted to the more toxic form of methylmercury by methylation (Figure 2-1). Hg methylation is produced mainly in anoxic sediments and soils, primarily by dissimilatory sulfate-reducing bacteria (SRB) and iron-reducing bacteria (FeRB) (Creswell et al., 2008). In aquatic sediments, microbes convert a small fraction of inorganic Hg (Hg^{2+}) to methylmercury over time. The main sources of methylmercury in the aquatic environment are considered to be atmospheric

deposition and terrestrial runoff, especially from wetlands. However, the relatively high levels of methylmercury found in sediments, biota and water require that other sources of methylmercury should be investigated. Internal processes, i.e., natural methylation of inorganic Hg in the water column and sediments, represent a significant contribution to the overall burden of methylmercury in the aquatic environment (Rudd, 1995). In addition, sulfur, organic carbon, sediment structure, and composition affect methylmercury production by changing the amount of bioavailable inorganic Hg and by stimulating microbial activity. Methylmercury can become bioconcentrated in live organisms and then further biomagnified up the food chain, as predators eat prey contaminated with methylmercury and further concentrate it in their tissues (Selin, 2009).

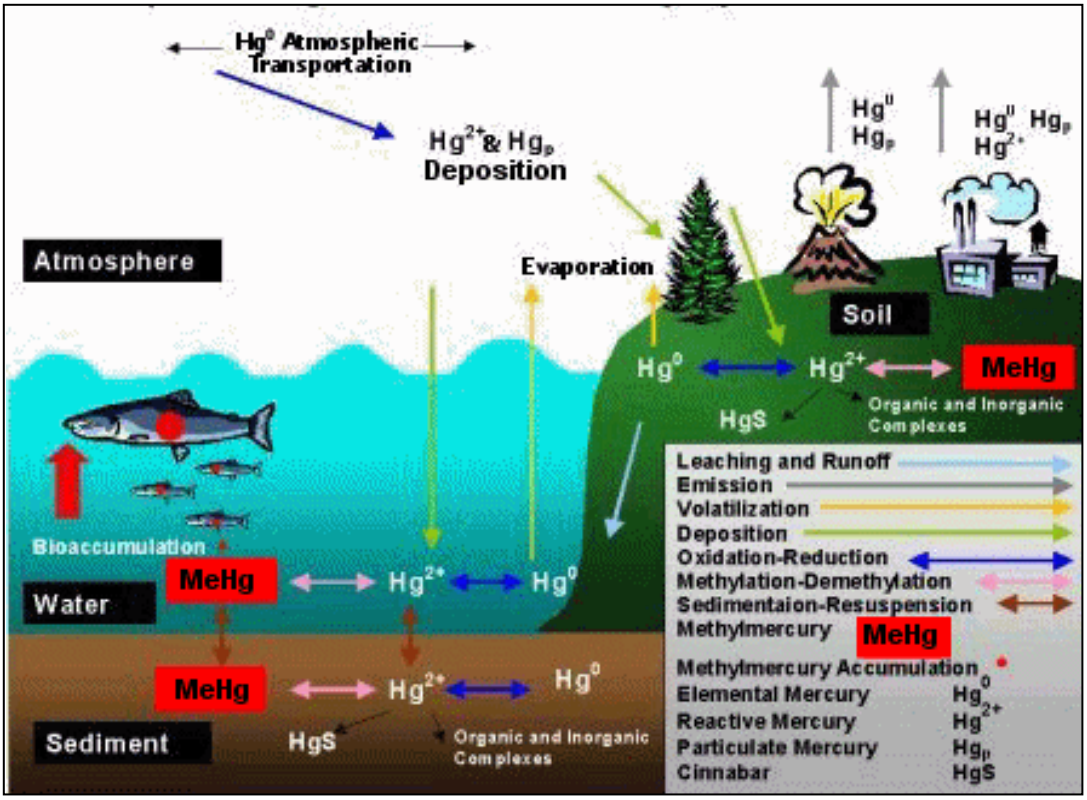

Figure 2-1. Biogeochemical cycle of mercury in the environment (Selin, 2009).

Hg toxicity

Hg occurs in both inorganic and organic mercurial forms in water. Inorganic mercurials include mercuric chloride, mercuric iodide, mercuric oxide, mercuric sulfide, and mercurous chloride. Organic mercurials include ethylmercury, methylmercury, merbromin, merthiolate, and phenylmercuric salts. All forms of Hg can produce systemic toxicity. Hg poisoning can result from ingestion, inhalation, injection, or dermal absorption of mercurials (Graeme Md and Pollack Jr, 1998). Among mercurials, methylmercury is the most toxic of all Hg compounds. The Hg-carbon bond in methylmercury is extremely stable and the attachment of the alkyl radical increases lipid solubility. This facilitates penetration of the blood brain barrier and cell membranes (Clarkson and Magos, 2006). Nervous tissue tends to accumulate the greatest concentrations of methylmercury. Methylmercury rapidly diffuses through the cell membrane and enters the cell where it is rapidly bound with sulfhydryl groups. Inside the cell, methylmercury inhibits protein and RNA syntheses (Zalups and Lash, 1994). The symptoms have been noted specifically with methylmercury exposure include slurred speech, hypersalivation, shouting, dysphagia, scotoma, neurasthenia, loss of libido, depression, hallucinations, focal cramps, chorea, athetosis, myoclonus, paralysis, stupor, coma, and death (Graeme Md and Pollack Jr, 1998). The intake of methylmercury contaminated foods can do extreme damage to human beings.

Accordingly, aiming to safeguard human health, the Joint FAO/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) (FAO/WHO, 2006) sets a Provisional Tolerable Weekly Intake (PTWI) of 1.6 mg/kg body weight or 0.23 mg/kg/day for sensitive groups of the population, such as pregnant women. In the same sense, the U.S. Environmental Protection Agency (EPA) adopted a revised reference dose for methylmercury of 0.1 mg Hg per kg body weight per day (USEPA, 2005). Indeed, the European Commission Regulation (EC) No 466/2001 sets the maximum level of Hg in fish for human consumption at 0.5 mg/kg wet weight; whereas the maximum limit for top fish predators species, such as tuna fish (*Thunnus* spp.), anglerfish (*Lophius* spp.), or swordfish (*Xiphias gladius*), is set at 1 mg/kg wet weight (Carrasco et al., 2011).

Acute exposure to Hg vapor may give rise to lung damage. The aspiration of elemental Hg may result in hemoptysis, cyanosis, pneumonitis, respiratory distress, necrotizing bronchiolitis, pulmonary hemorrhage, pneumothorax, or pulmonary edema. However, the absorption of elemental Hg in the gut is poor and is eliminated in the feces. In contrast, the ingestion of inorganic salts of Hg is very corrosive to the gastrointestinal tract and may result in nausea, vomiting, diarrhea, abdominal pain, and anorexia (Jarup, 2003). Moreover, it can accumulate in the proximal convoluted tubules of the kidneys and may induce proteinuria, hematuria, glycosuria, oliguria, uremia, renal tubular injury, and acute renal failure. Chronic poisoning is characterized by neurological and psychological symptoms, such as tremor, changes in personality, restlessness, anxiety, sleep disturbance, depression (Jarup, 2003) slurred speech, hypersalivation, shouting, dysphagia, scotoma, neurasthenia, loss of libido, hallucinations, focal cramps, chorea, athetosis, myoclonus, paralysis, stupor, coma, and death (Carrasco et al., 2011). The symptoms are reversible after cessation of exposure. Because of the blood-brain barrier there is no central nervous involvement related to inorganic Hg exposure. Metallic Hg may cause kidney damage, which is reversible after exposure had stopped. It has also been possible to detect proteinuria at relatively low levels of occupational exposure.

The Hg contamination in aquatic animals and aquatic products in Thailand

Hg released from anthropogenic sources may be accumulated in aquatic animals through the food web, thereby human health risks may occur through the consumption of food contaminated by Hg. Methylmercury is known as a very important neurotoxin and also the only Hg compound that is bioaccumulated and biomagnified in the food chain (Celo et al., 2006). It is presented as a thiolate complex (likely with cysteine) (Harris et al., 2003), and represents up to 95% of total Hg in top predators. The concentrations of Hg measured in aquatic animals are calculated with a bioconcentration factor (BCF) or bioaccumulation factor (BAF). The BCF is a simple ratio between the concentration of a chemical in an organism's tissues and the concentration in the water to which the organism was exposed (Ivanciuc et al., 2006). The BCF is intended to account only for net uptake directly

from water and thus almost always has to be measured in a laboratory test. On the other hand, the BAF is intended to account for net uptake from both food and water in a real-world situation (USEPA, 1993). Hg concentrations in marine organisms have often been used as a means of assessing the biological impact of Hg on the marine environment and its impact on human health. There has been some research that studied the Hg contamination in the aquatic animal and aquatic products in Thailand as follows.

Thongraa-ar (1987) reported detection of Hg contamination in marine animals that have economic value in the Eastern seaboard in the period from October 1987 to September 1988. The concentrations of Hg in fish, squid, blue swimming crabs, oysters and shrimp were 0.35, 0.25, 0.021, 0.016, 0.017 and 0.010 mg/kg, respectively.

Jintanapan (1994) studied the amount of Hg in the tissues of large blue swimming crabs. The results showed that the liver is the most common accumulator of Hg (11.05 ng/g), while the tissue of steamed crab legs and claws were 7.42 and 5.95 ng/g, respectively.

Ngamprasertwong and Muntrup (1997) studied the amount of Hg in the Asian green mussel in Bangprakong River. The amounts of Hg in the Asian green mussel with a smaller size (<4-7 cm) and a larger size (>7 cm) were 0.17 ± 0.14, 0.14 ± 0.11 and 0.12 ± 0.07 mg/kg, respectively. There was also, a distinctive variation of the Hg content in the tissue of fish vital organs: kidney, stomach, gill, muscle and liver with the average of 0.912, 0.162, 0.186, 0.231 and 0.265 (mg/kg), respectively while with kidney being the major source.

Sawatdikul (2003) studied the Hg content in various tissues of economic marine animal. The result showed that the Hg content exceeded the standard guidelines (0.5 mg/kg) for sea food products at 0.26% of *Portunus pelagius* and 0.53% of *Loligo formasana*. The biological magnification of Hg in food chains has shown that organisms from higher tropical levels had higher Hg residues than those from the lower levels. Furthermore, the results also demonstrated that there was a significant correlation between the concentration of Hg and the weight of the marine animals.

In 2004, the Department of Fisheries reported that the Hg contamination in export aquatic products during January to December of that year. In a total of 39,023 samples found that the tinned fish, frozen fish and frozen crab had higher amounts of Hg than other products. The average Hg concentrations were 0.109, 0.136 and 0.108 mg/kg, respectively. In addition, there were 0.204% and 0.140% of tinned fish and frozen fish that exceeded the standard guidelines (0.500 mg/kg) of sea food.

Hg contamination in shrimp ponds

Intensive shrimp farming has occupied extensive coastal areas worldwide, with its dependence on large inputs of artificial feed, fertilizers, chemical additives and algaecides. It has encouraged many studies to investigate the role of shrimp farms as pollutant sources in coastal environments and the nearby areas in the shrimp ponds (Lacerda et al., 2011). Hg is detected in aquaculture effluents due to its presence as a natural component in aquafeeds, particularly fish meal and as impurities in fertilizers and other chemicals used in shrimp farms (Chou et al., 2002; Berntssen et al., 2004).

In Thailand, there has been only a little research that has studied or detected Hg and other metals in the shrimp ponds. There have been some research about Hg contamination in shrimp ponds in other countries e.g. Brazil. The research estimated Hg emission factors (EFs), the analysis of Hg concentrations in aquafeeds and other chemical additives in shrimp biomass and in inflow and outflow water and suspended particles of the largest shrimp farm, and total Hg loading to the Jaguaribe Estuary, NE Brazil, suffers from intensive shrimp farming in comparison to this and other local anthropogenic activities. The EF reached 83.5 mg/ha/cycle by roughly 175 mg/ha/y. The calculated EF was comparable to Hg EFs from urban wastewaters (200 mg/ha/y) and solid waste disposal (Lacerda et al., 2011).

As most shrimp farming in Thailand is near the sea as well as the water used in shrimp farming comes mostly from seawater it is possible that the shrimp farming has been contaminated with Hg sources such as seawater that was contaminated with Hg. Antony and Philip (2006) reported that total Hg in coastal

water had been monitored along the entire Thailand coastline by the Pollution Control Department since 1997 to 1998, covering 218 sampling sites. Specific areas, such as industrial estates in Map Ta Phut and Laem Chabang, were areas with elevated Hg concentrations as monitored during those years with Hg concentrations that ranged from <0.01 to 0.54 µg/L with an average of 0.032 µg/L; these values are mostly in compliance with the National Coastal Water Quality Standard for mercury of 0.10 µg/L (Thongra-ara and Parkpianb, 2002).

Hg toxicity in shrimp

Some metals are important for the normal metabolic functioning of shrimp such as copper, which is important for the metabolic functioning of hemocyanin, and zinc, a component of many enzymes (Lorenzon et al., 2000); however, in excess these metals become toxic. Other nonessential metals such as Hg may exert a noxious influence as well and are stored in the body. Hg may be accumulated by shrimp either through food or water. The gills function as the major route for uptake of heavy metals including Hg from the medium to the mucous layer (Soundarapandian et al., 2010). The toxicity of Hg to shrimp is due to precipitation of Hg, when it causes a thickening of the epithelial cell of the gill surface that result in reduced gill permeability (Das and Sahu, 2002). In addition, some previous study showed that Hg caused the inhibition of ovarian maturation in shrimp, *Procambarus clarkii* (Reddy et al., 1997).

. Lorenzon et al. (2000) studied the toxicity of Hg, Cd, Cu, Zn, and Pb in the shrimp *Palaemon elegans*. Hg was the most toxic metal in the 96 h assay, (LC_{50} at 96 h = 0.5 mg/L) followed by Cd, Cu, Zn, and Pb. Heavy metals affect the blood glucose levels, yet manipulative stress did not. The intermediate sublethal concentrations of Hg, Cd, and Pb produced significant hyperglycemic responses within 3 h, while the highest concentrations elicited no hyperglycemia in 24 h.

Das and Sahu (2002) studied the toxicity of Hg to the prawns *Penaeues monodon* and *Penaeus indicus* in various salinities. The LC_{50} values with a 95% confidence limit over 96 h were 0.036 (0.032-0.041) and 0.042 (0.036-0.044) mg/L for the large sized groups (55-75 mm) of *P. monodon* and *P. indicus*,

respectively. The small sized group (33-55 mm) was more sensitive to Hg at lower salinities while the large size group was more tolerant at high salinities. *P. monodon* was more sensitive to mercury than *P. indicus*. The threshold limits for *P. monodon* and *P. indicus* were 0.016 and 0.024 mg/L, respectively.

Hg toxicity on microbial activity and community

Metals play an integral role in the life processes of microorganisms. Some metals, such as calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel, and zinc, are required nutrients and are essential although almost any metal at high concentrations is toxic to microorganisms. Other metals such as silver, aluminium, cadmium, gold, lead, and mercury (Bruins et al., 2000) have no biological role and are not essential. Essential metals function as catalysts for biochemical reactions, are stabilizers of protein structures and bacterial cell walls, and can serve in maintaining osmotic balance (Bruins et al., 2000). Toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions. Nonessential metals bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals. Toxicity results from alterations in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Bruins et al., 2000).

Hg as one of the most toxic heavy metals has been widely studied, especially for its effects on soil microbes. Previous study showed that Hg contaminated in the environment affect to microbial community and functional diversity such as soil respiration and enzyme activity (Harris-Hellal et al., 2009).

Rasmussen et al. (2000) studied the effects of Hg on bacterial diversity using denaturing gel gradient electrophoresis (DGGE) on total community DNA. They showed that the number and intensity of DGGE bands decreased rapidly following Hg^{2+} addition, 81 days after the treatment, microbial diversity in treated soils diverged from the initial diversity.

Giotta et al. (2006) studied the influence of heavy metals including Hg^{2+} on the photosynthetic growth of *Rhodobacter sphaeroides*. The results show that

a long term exposure of Hg ions resulted in the prolongation of the lag phase or decrease in the growth rate of *R. sphaeroides*.

Harris-Hellal et al. (2009) studied the effect of Hg^{2+} on soil microbial community, activity, functional and genetic structure by cultural method, measurements of respiration, ECOLOG plates, and DGGE, respectively. The results obtained for the microbial populations enriched with only 1 mg/g $HgCl_2$ being indistinguishable from the controls. Conversely, in the presence of a high Hg^{2+} content (20 mg/g), an immediate effect was found on soil respiration, functional diversity and genetic structure of the microbial communities. However, no significant effect was observed on plate counts or microbial biomass.

Asztalos et al. (2010) studied the effect of Hg on photosynthetic bacteria (*R. sphaeroides*) by changes of the induction of bacteriochlorophyll (BChl) fluorescence. The Hg^{2+} (100 mM) strongly inhibited bacterial growth and damaged of the quinone acceptor pool of the reaction center (RC) including Hg^{2+} resulted the loss of the structural organization and of the pigments.

Asztalos et al. (2012) studied the effect of Hg on RC function of *R. sphaeroides*. The results showed that the electron transport (donor/acceptor) sites were sensitive to Hg^{2+}, and the Hg^{2+} (<100 µM) changes the rates of electron transfer kinetics, reduced bound and free cytochrome c_2 and the primary reduced quinone. Moreover, the Hg^{2+} provided the decreasing of the photoactive RCs and the connectivity of the photosynthetic units.

Mechanisms of Hg resistant bacteria

As a response to toxic Hg compounds present in the environment, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity (Barkay et al., 2003; Mathema et al., 2011). However, some bacteria that live in Hg contaminated areas can exchange Hg resistance genes between each other, during their continual exposure to the toxic levels of Hg. An extensively studied resistance system, based on clustered genes in an operon (*mer* operon) as shown in Figure 2-2, allows bacteria to detoxify Hg^{2+} into volatile metallic Hg (Hg^0) by enzymatic reduction. Hg-resistance determinants have been found in a wide range of

Gram-negative and Gram-positive bacteria isolated from different environments. They vary in the number and identity of the genes involved that are encoded by *mer* operons, usually located on plasmids and chromosomes. They are often components of transposons (Nascimento and Chartone-Souza, 2003).

The genetic system that has evolved as *mer* operon is in fact the only well-known bacterial metal resistance system with a high yield transformation of its toxic target. The *mer* operons vary in structure and are constituted by genes that encode the functional proteins for regulation (*merR*), transport (*merT*, *merP* and/or *merC*, *merF*) mercuric reductase (*merA*) and organomercurial-lyase (*merB*) (Barkay et al., 2003; Barkay and Wagner-Döbler, 2005; Mathema et al., 2011). The *merR* is a metalloregulatory DNA binding protein that binds to the promoter-operator region and structural gene transcription in the presence of Hg^{2+}. It induces transcription of the structural genes from the *mer* operon (*merTPCFAD*) (Figure 2-2). The most terminal promoter gene, *merD*, which is co-transcribed with the structural genes, down-regulates the *mer* operon. MerD, a secondary regulatory protein, also binds the same operator-promoter region as MerR. Mercuric ions are transported outside the cell by a series of transporter proteins. This mechanism involves the binding of Hg^{2+} by a pair of cysteine residues on the MerP protein located in the periplasm; Hg^{2+} is transferred to a pair of cysteine residues on merT. In some bacterial operons, *merC* has been attributed for encoding transport function proteins due to its homology to the *merT* gene. MerC is typically a membrane bound protein with a high affinity for Hg ions. When Hg^{2+} is transferred to a pair of cysteine residues on MerT, a cytoplasmic membrane protein, and finally to a cysteine pair at the active site of MerA (mercuric reductase). Mercury reductase, is a flavin oxidoreductase (Barkay et al., 2003; Nascimento and Chartone-Souza, 2003; Mathema et al., 2011) and is fundamentally responsible for the reduction of highly toxic ionic Hg^{2+} into less toxic and volatile Hg^{0} in a NAD(P)H-dependent reaction. Finally, this volatile Hg^{0} is fluxed out from cytosolic region into the outer periplasm. The *merB* gene generally codes for an organomercury lyase which is one of the key enzymes for the detoxification and bioremediation of the organomercurial compound. The processed products by organomercury lyase are finally volatilized by the *merA* gene. MerB catalyzes the

protonolysis of carbon-Hg bound, thereby releasing less toxic and less mobile Hg species which is further acted upon by the *merA* enzyme for complete volatilization of organomercurial compounds (Murtaza et al., 2005; Mathema et al., 2011).

Hg^{2+} ⟶ Hg^{0}

Mercuric reductase

CH_3Hg^{+} ⟶ $Hg^{2+} + CH_4$ ⟶ Hg°

Organomercurial lyase Mercuric reductase

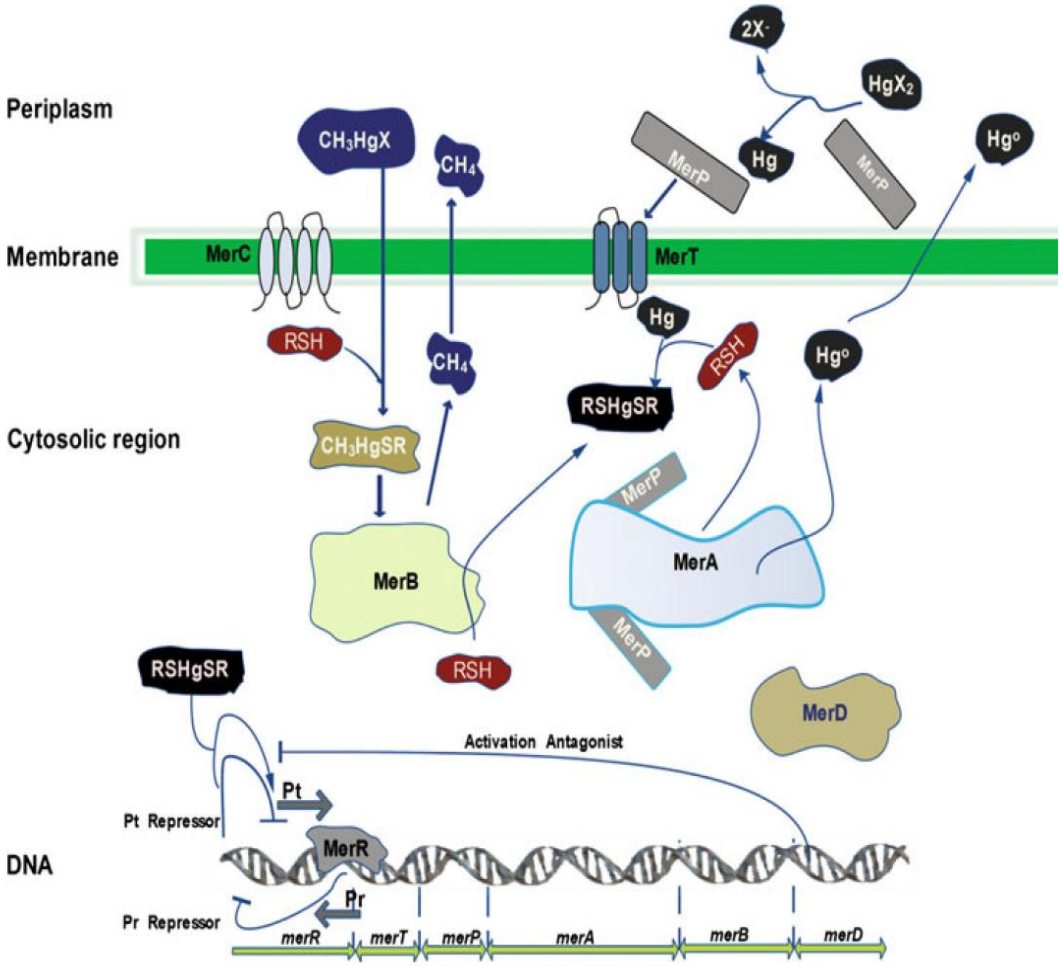

Figure 2-2. Generic *mer* operon in a typical Gram-negative Hg resistant bacterium (Mathema et al., 2011)

Biosorption and bioaccumulation

Various techniques have been employed for the treatment of metals that contaminate the environment. These methods are usually characterized as two broad divisions: abiotic and biotic methods. Abiotic methods include precipitation, adsorption, ion exchange, membrane and electrochemical technologies. Much has been discussed about their downside aspects in recent years (Crini, 2006)**,** as they are all expensive, not environmentally friendly and usually are dependent on the concentration of the waste. Therefore, a search for efficient, eco-friendly and cost effective remedies for remediation is urgently required. In recent years, research attention has been focused on biological methods for the treatments (Vara Prasad and de Oliveira Freitas, 2003). There are three principle advantages of biological technologies for the removal of pollutants; first, biological processes can be carried out in situ at the contaminated site; second, bioprocessing technologies are usually environmentally benign (no secondary pollution) and third, they are cost effective. Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals (Malik et al., 1998; Volesky and Schiewer, 2002).

Biosorption and bioaccumulation differ in that in the first process pollutants are bound to organic materials at the surface of cell wall and in the second, they are accumulated inside the cell. Biosorption is a simple physicochemical process resembling conventional adsorption or ion exchange. The difference lies in the nature of the sorbent which in this case is material of biological origins. Biosorption is a metabolically-passive process, whereas bioaccumulation is the cultivation of an organism in the presence of a pollutant that can be assimilated and removed (Chojnacka, 2010). Since in biosorption an equilibrium is reached; it can be shifted into either side: left in the wastewater treatment system or right if the adsorbate is to be removed and recovered (Figure 2-3). In bioaccumulation, the process goes further. This means that the first stage is biosorption and then subsequent stages occur, related to transport of the pollutant (mainly via energy-consuming active transport systems) into the inside of the cells and eventually the concentration of the pollutant inside the cells increases. Thus, in bioaccumulation more binding sites for the pollutant are

made available and lower residual concentrations can be reached. But in order for the process to occur, some conditions need to be fulfilled as live cells must be used.

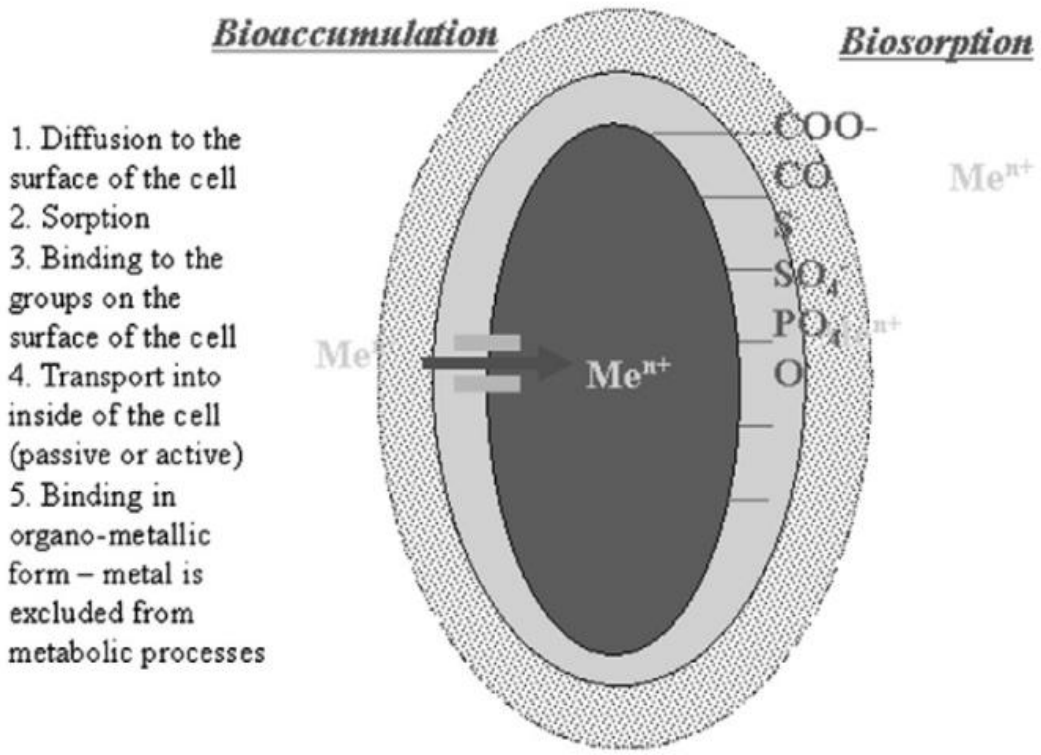

Figure 2-3. The mechanisms of biosorption and bioaccumulation (Chojnacka, 2010).

Factors influence bacterial biosorption

pH

pH plays a major role in biosorption, and seems to affect the solution chemistry of metals and the activity of the functional groups of the biomass. For metals, the pH strongly influences the speciation and biosorption availability of the metal ions (Esposito et al., 2002). At higher solution pH values, the solubility of metal complexes decreases sufficiently and allows for precipitation, which may complicate

the sorption process. The activity of binding sites can also be altered by adjustment of the pH. For instance, during the biosorption of metal ions by bacterial biomass, pH 3 to 6 has been found favorable for biosorption , due to the negatively charged carboxyl groups (p*K*a = 3-5), which are responsible for the binding metal cations via an ion exchange mechanism (Farah et al., 2007).

Temperature

Temperature seems to affect biosorption but to a lesser extent within the range from 20 to 35 °C (Veglio and Beolchini, 1997). Higher temperatures usually enhance sorption due to the increased surface activity and kinetic energy of the solute (Vijayaraghavan and Yun, 2008); however, physical damage to the biosorbent can be expected at higher temperatures. Due to the exothermic nature of some adsorption processes, an increase in temperature has been found to reduce the biosorption capacity of the biomass (Mameri et al., 1999; Aryal and Liakopoulou-Kyriakides, 2014)

Ionic strength

Ionic strength influences the adsorption of solute to the biomass surface (Borrok and Fein, 2005). The effect of ionic strength may be ascribed to the competition between ions, changes in the metal activity, or in the properties of the electrical double layer. When two phases, e.g. biomass surface and solute in aqueous solution are in contact, they are bound to be surrounded by an electrical double layer owing to electrostatic interaction. Thus, adsorption decreases with an increase in ionic strength (Dönmez and Aksu, 2002).

The dosage of a biosorbent

The dosage of a biosorbent strongly influences the extent of biosorption. For instances, lower biosorbent dosages yield higher uptakes and lower percentage removal efficiencies (Mameri et al., 1999; Aksu and Çağatay, 2006; Aryal and Liakopoulou-Kyriakides, 2014). An increase in the biomass concentration generally increases the amount of solute biosorbed, due to the increased surface area of the biosorbent. In contrast, the quantity of biosorbed solute per unit weight of

biosorbent decreases with increasing biosorbent dosage, which may be due to the complex interaction of several factors (Vijayaraghavan and Yun, 2008; Abbas et al., 2014; Aryal and Liakopoulou-Kyriakides, 2014).

The initial solute concentration

The initial solute concentration has an impact on biosorption, with a higher concentration resulting in a high solute uptake (Ho and McKay, 1999; Abbas et al., 2014). This is because at lower initial solute concentrations, the ratio of the initial mole of solute to the available surface area is low; subsequently, the fractional sorption becomes independent of the initial concentration. However, at higher concentrations, the sites available for sorption become fewer compared to the moles of solute present and; hence, the removal of solute is strongly dependent upon the initial solute concentration. It is always necessary to identify the maximum saturation potential of a biosorbent, for which experiments should be conducted, at the highest possible initial solute concentration (Vijayaraghavan and Yun, 2008).

Isotherm of biosorption

The adsorption isotherm indicates how the adsorption molecules distribute between the liquid phase and the solid phase when the adsorption process reaches an equilibrium state. Equilibrium isotherm models are usually classified into empirical equations and the mechanistic models. The mechanistic models are based on the mechanism of metal ion biosorption, which are able not only to represent but also to explain and predict the experimental behavior (Pagnanelli et al., 2002). There are several isothermal equations used in the literature to quantity the biosorptive features of an adsorbent including Langmuir, Freundlich and Sips equations, etc. (Wang et al., 2010).

The Langmuir model

The Langmuir model assumes that the bioadsorption on the surface is a monolayer of metal ion on the surface. The energy of sorption for each ion is the same and independent of surface coverage; the sorption occurs only on localized sites and

involves no interactions between sorbed ions. And the rate of sorption is almost negligible in comparison with the initial rate of sorption. This model is the equation (1) as shown (Abbas et al., 2014).

$$=q_e = \frac{1+K_L C_e}{q_m K_L C_e} \quad (1)$$

q_e is the amount of adsorbate per unit mass of adsorbent (mg/g)

q_m is the monolayer maximal adsorption capacity (mg/L)

K_L is Langmuir equilibrium constants

C_e is the equilibrium concentration of the solute

Freundlich model

The Freundlich model also considers as monomolecular layer coverage of solute by the sorbent. However, it assumes that the sorbent has a heterogeneous valance distribution and then has a different affinity for adsorption. This model takes the following form for a single solute adsorption (Abbas et al., 2014) as shown in the equation (2).

$$q_e = K_F C_e^{1/n} \quad (2)$$

q_e is the amount of adsorbate per unit mass of adsorbent (mg/g)

C_e is the equilibrium concentration of the solute (mg/L)

K_F and n are Freundlich equilibrium constants

Kinetics of adsorption

Kinetics is the study of the rates of chemical processes to understand the factors that influence the rates. Studies of chemical kinetics include careful monitoring of the experimental conditions which influence the speed of a chemical reaction and hence, help attain equilibrium in a reasonable length of time. The study of kinetics can provide information about the possible mechanism of adsorption and

the different transition states on the way to the formation of the final adsorbate-adsorbent complex and help develop appropriate mathematical models to describe the interactions (Sen Gupta and Bhattacharyya, 2011).

Pseudo-first order model

The pseudo-first order rate expression, popularly known as the Lagergren equation has been one of the most used equations particularly for pseudo-first order kinetics and described by the following equation (3).

$$\frac{V(C_0-C_e)}{dt} = K_1(q_e-q_t) \quad (3)$$

q_e is the amount of adsorbate per unit mass of adsorbent (mg/g)

q_t is the values of the amount adsorbed per unit mass at any time (mg/g)

K_1 is the pseudo-first order adsorption rate coefficient (1/min)

Pseudo-second order model

The sorption data has also been analyzed in terms of a pseudo-second order mechanism (Vijayaraghavan and Yun, 2008; Aryal and Liakopoulou-Kyriakides, 2014) given by the following equation (4).

$$\frac{dq}{dt} = K_2(q_e-q_t)^2 \quad (4)$$

q_e is the amount of adsorbate per unit mass of adsorbent (mg/g)

q_t is the value of the amount adsorbed per unit mass at any time (mg/g)

K_2 is the pseudo second-order adsorption rate coefficient (g/mg min)

Purple nonsulfur bacteria (PNSB)

There are two classes of photosynthetic bacteria that include anoxygenic photosynthetic and oxygenic photosynthetic bacteria (Imhoff, 1992). Anoxygenic phototrophic bacteria are diverse and important members of microbial

communities. There are five bacterial phyla of anoxygenic phototrophs: *Proteobacteria* (purple bacteria), *Chlorobi* (green sulfur bacteria), *Chloroflexi* (green nonsulfur bacteria), *Acidobacteria* ("*Candidatus Chloracidobacterium thermophilum*"), and *Firmicutes* (heliobacteria) (Yutin et al., 2007). Purple bacteria are photosynthetic Gram-negative prokaryotes that convert light energy into chemical energy by the process of anoxygenic photosynthesis. Purple bacteria contain photosynthetic pigments-bacteriochlorophyll (BChls) and carotenoids and can grow autotrophically with CO_2 as a sole carbon source. Many genera of purple bacteria are known and the organisms share many basic properties with their nonphototrophic relatives (Madigan and Jung, 2009). Some general characteristics of purple bacteria are listed in Table 2-2.

Table 2-2. General properties of anoxygenic purple phototrophic bacteria (Madigan and Jung, 2009).

Property	Examples
Groups/phylogeny	PSB (*α-Proteobacteria*); PNSB (*α, β-Proteobacteria*)
Major species studied	*Allochromatium vinosum* and *Thiocapsa roseopersicina* (purple sulfur bacteria: PSB); *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, and *Rhodopseudomonas palustris* (purple nonsulfur bacteria)
Pigments/color of dense cell suspensions	BChl *a* or *b*; major carotenoids include spirilloxanthin, spheroidene, lycopene, and rhodopsin, and their derivatives; cell suspensions are purple, purple-red, purpleviolet, red, orange, brown, or yellow brown (BChl *a*-containing species); green or yellow (BChl *b*-containing species)
Location of pigments in cells	Within intracytoplasmic membranes arranged as membrane vesicles, tubes, bundled tubes, or in stacks resembling lamellae
Absorption maxima of living cells	BChl *a*-containing species: near 800 nm, and anywhere from 815-960 nm; BChl *b*-containing species: 835-850 nm and 1010-1040 nm
Electron donors/sulfur globules	H_2S, S^0, $S_2O_3^{2-}$, H_2, Fe^{2+}; if S^0 is produced from the oxidation of sulfide, the S^0 is stored intracellularly only in certain PSB
Photoheterotrophy/dark respiratory growth	PSB limited on both accounts; PNSB typically diverse on both accounts

Purple sulfur bacteria (PSB)

PSB have a color in a range of pink to purple and contain BChl *a* as their major pigment. These phototrophic anaerobes require sulfide, which they oxidize to sulfate for growth. Carbon dioxide is the usual source of cell carbon, but some can also utilize various organic compounds as carbon sources and are usually distributed widely in sulfide-rich reducing environments such as mangroves (Vethanayagam, 1991; Thatoi et al., 2013).

Purple nonsulfur bacteria (PNSB)

PNSB are the most diverse group of the phototrophic purple bacteria. This diversity is reflected in greatly varying morphology, internal membrane structure, carotenoid composition, utilization of carbon sources, and electron donors, among other features. The intracytoplasmic membranes are small, fingerlike intrusions, vesicles, or different types of lamellae. Most species are motile by flagella; gas vesicles are not formed by any of the known species (Imhoff, 1995). PNSB range in color from brown to red and also contain BChl *a* as their major pigment. They have the ability to utilize a remarkably wide spectrum of reducing carbon compounds, like malate or succinate, as electron donors as well as other carbon sources for growth. Sulfur-rich mangrove ecosystems, with their mainly anaerobic soil environment, can also provide favorable conditions for the proliferation of these bacteria (Thatoi et al., 2013).

PNSB can grow as photoautotrophs/photoorganotrophs under anaerobic/microaerobic light conditions or as chemoorganotrophs under aerobic dark conditions (Imhoff, 1995; Madigan and Jung, 2009). Growth of some PNSB, for example, *Rhodobacter capsulatus*, is possible under phototrophic conditions with either CO_2 or organic carbon, or in darkness by respiration, fermentation, or chemolithotrophy. Under phototrophic growth conditions (anaerobic light), many species exhibit a considerable respiratory capacity. The fact that considerable respiratory activity is expressed also under phototrophic growth conditions enables these bacteria (e.g. *R. capsulatus*, *Rhodobacter sphaeroides*, *Rubrivivax* (*Rhodocyclus*) *gelatinosus*, *Rhodospirillum rubrum*) to switch immediately from

phototrophic to respiratory metabolism when environmental conditions change. Some species also may perform a respiratory metabolism anaerobically in the dark with sugars and even nitrate, dimethyl-sulfoxide, or trimethylamine-N-oxide as an electron sink, although poorly with metabolic intermediates as electron acceptors (fermentation) (Imhoff, 1995).

Most representatives can also grow under microaerobic to aerobic conditions in the dark as chemoheterotrophs. Some species are very sensitive to oxygen, but most species are quite tolerant of oxygen and grow well with aerobic conditions in the dark. Under these conditions synthesis of photosynthetic pigments is repressed and the cultures are faintly colored or colorless (Imhoff, 1995). Under anoxic dark conditions, PNSB can grow by either fermentation or anaerobic respiration (Thatoi et al., 2013). For example, pyruvate and certain sugars support fermentative growth of some PNSB. Dark chemolithotrophic growth of certain species PNSB is possible using H_2 or $S_2O_3^{2-}$as electron donors. In *R. capsulatus*, chemolithotrophic growth on H_2 occurs and the organism can be grown in a synthetic medium supplied with the gases; H_2, O_2, and CO_2 as the sources of electron donor, electron acceptor, and carbon, respectively. Oxygen tolerance for respiratory growth varies among species, but some, such as *Rhodobacter* species, can be grown with vigorous aeration (Imhoff, 1995; Madigan and Jung, 2009). Moreover, one or more vitamins are generally required as growth factors, most commonly biotin, thiamine, niacin, and *p*-aminobenzoic acid; these compounds are rarely needed by species of the *Chromatiaceae* and *Ectothiorhodospiraceae*, which may require vitamins as growth factor. Growth of most PNSB is enhanced by small amounts of yeast extract, and some species have complex nutrient requirements (Imhoff, 1995).

The diversity of PNSB in the environment

PNSB have a wide range of growth modes so they are able to grow with photoheterotrophic, photoautotrophic, and chemoheterotrophic conditions (Imhoff, 1995; Madigan and Jung, 2009). They are widely distributed in natural environments such as surficial aquatic sediments, freshwater rivers and lakes including wastewater systems. The diversity and phylogenetic composition of PNSB's

have been studied by microscopy, biomarker profiling, and PCR cloning and sequencing of the *pufM* gene, that codes for the M subunit of the photosynthetic reaction center protein. Analysis of a clone library of the *pufM* gene has been reported as a promising approach to the phylogenetic characterization of phototrophic bacterial communities in aquatic and hot spring environments (Oda et al., 2002). Hence, *pufM* genes have been used to assess the diversity of different aerobic anoxygenic photosynthetic assemblages (Yutin et al., 2007).

Oda et al. (2002) studied the genotypic and phenotypic diversity within species of PNSB found in aquatic sediments. A total of 128 strains were directly isolated from sediment samples by agar plates. All isolates were initially characterized by BOX-PCR genomic DNA fingerprinting, and 60 distinct genotypes were identified. Analyses of 16s rRNA gene sequences of representatives of each genotype showed that there were five and eight different phylotypes of PNSB. One site found 80.5% of the clones were *Rhodopseudomonas palustris*, whereas *Rhodoferax fermentan*s and *R. palustris* were numerically dominant at other sites and constituted 45.9 and 34.4% of the isolates obtained, respectively. BOX-PCR genomic fingerprints showed that there was a high level of genotypic diversity within each of these species.

Karr et al. (2003) studied the diversity of phototrophic purple bacteria in a Permanently Frozen Antarctic Lake that was determined by analysis of the photosynthesis-specific gene, *pufM*. There was an extensive diversity revealed with a highly stratified distribution of PNSB in Lake Fryxell that showed which phylotypes produced *pufM* transcripts in situ. Enrichment cultures for purple bacteria yielded two morphotypes, each with a *pufM* signature identical to signatures detected by environmental screening. Some isolates also contained gas vesicles, buoyancy structures previously unknown in PNSB.

Liang et al. (2010) studied the diversity of PNSB which can accumulate phosphorus in activated sludge. This study used traditional methods; 4', 6-diamidino-2-phenylindole staining as well as chemical analysis including molecular techniques; the PNSB's *pufM* gene demonstrated that most of the isolated PNSB bacteria presented different levels of phosphorus accumulation. The study that used

the traditional method found four of the pure cultures, denoted as *R. palustris*. In contrast, the study of the PNSB's *pufM* gene based on PCR-DGGE found *R. palustris*, *Rhodobacter* sp., *Rhodobacter blasticus*, *R. gelationosus*, *Erythrobacter literalism*, *Rhodocyclus tenuis*, *Rhodomicroblum vannielli*, *R. gelatinosus* and *Allochromatium vinosum*.

The use of PNSB for removal of heavy metals

The conventional processes used to remove heavy metals are chemical and physical methods such as ion exchange, reverse osmosis, carbon adsorption and chemical precipitation. However, the main disadvantage of conventional processes is the expense, the possible accumulation of toxic substances so they are not eco-friendly. Bioremediation with the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment area as it is efficient, eco-friendly and cost effective for remediation (Vijayaraghavan and Yun, 2008). PNSB are an interesting group to use for removal of heavy metals due to the fact that PNSB have been extensively used for wastewater treatment as they are able to utilize a broad range of organic compounds (Kantachote et al., 2005; Kantachote et al., 2010). Some studies have shown that PNSB have the potential to remove heavy metals as follows.

Watanabe et al. (2003) studied the biosorption characteristics of cadmium ions onto a photosynthetic bacterium, *R. sphaeroides* S and a marine photosynthetic bacterium *Rhodovulum* sp. PS 88 in a batch culture system. Both photosynthetic bacteria were capable of cadmium removal in the presence of 30 g/L sodium chloride and divalent cations (Mg^{2+} and Ca^{2+}) in the culture medium. 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 anaerobic light (photoheterotrophic) conditions.

Bai et al. (2008) studied the removal of cadmium by *R. sphaeroides*. They reported that the removal mechanism of cadmium was predominantly governed by bioprecipitation as cadmium sulfide while biosorption contributing only to a minor extent. Also, the results revealed that the activities of cysteine desulfhydrase in strains

grown in the presence of 10 and 20 mg/L of cadmium were higher than in the control, while the activities in the presence of 30 and 40 mg/L of cadmium were lower than in the control. Content analysis of subcellular fractionation showed that cadmium was mostly removed and transformed by precipitation on the cell wall.

Panwichian et al. (2010) studied the ability of PNSB that had been selected from the shrimp ponds in areas of southern Thailand for the removal of heavy metals. Two metal resistant PNSB isolates, NW16 and KMS24 reduced the amounts of Pb, Cd, Cu and Zn at the highest concentrations found in the sediment (dry weight) as follows; 62.63, 0.75, 34.60 and 58.50 mg/kg, respectively. Both isolates reduced the levels of heavy metals by up to 39% for Pb, 20% for Cu, 7% for Cd, 5% for Zn and 31% for Na (initial concentration 3% NaCl) under either microaerobic light or aerobic dark conditions. The isolate NW16 removed a greater percentage of the heavy metals than the isolate KMS24, but the isolate KMS24 was able to survive better under a greater variety of environmental conditions. Two strains removed heavy metals using biosorption and also bioaccumulation. Optimal conditions for removal of heavy metals by strain NW16 were; cells in the log phase at 4.5 mg DCW/mL, pH 6.0, and 30^{o}C for 30 min. With microaerobic light conditions, the relative percentage removal of heavy metals 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). Optimal conditions for strain KMS24 were cells in the log phase at 5.0 mg DCW/mL, pH 5.5, and 35^{o}C for 45 min and there were no significant differences for the removal percentages of heavy metals with either incubating conditions (averages: Pb, 96; Cu, 75; Zn, 46; Cd, 30) (Panwichian et al., 2010).

CHAPTER 3

Diversity of purple nonsulfur bacteria in shrimp ponds with varying mercury levels

Abstract

This research aimed to study the diversity of purple nonsulfur bacteria (PNSB) and to investigate the effect of Hg concentrations in shrimp ponds on PNSB diversity. Amplification of the *pufM* gene was detected in 13 and 10 samples of water and sediment collected from 16 shrimp ponds in Southern Thailand. In addition to PNSB, other anoxygenic phototrophic bacteria (APB) were also observed; purple sulfur bacteria (PSB) and aerobic anoxygenic phototrophic bacteria (AAPB) although most of them could not be identified. Among identified groups; AAPB, PSB and PNSB in the samples of water and sediment were 25.71, 11.43 and 8.57%; and 27.78, 11.11 and 22.22%, respectively. In both sample types, *Roseobacter denitrificans* (AAPB) was the most dominant species followed by *Halorhodospira halophila* (PSB). In addition two genera, observed most frequently in the sediment samples were a group of PNSB (*Rhodovulum kholense, Rhodospirillum centenum* and *Rhodobium marinum*). The UPGMA dendrograms showed 7 and 6 clustered groups in the water and sediment samples, respectively. There was no relationship between the clustered groups and the total Hg (Hg_T) concentrations in the water and sediment samples used (<0.002-0.03 µg/L and 35.40-391.60 µg/kg dry weight) for studying the biodiversity. It can be concluded that there was no effect of the various Hg levels on the diversity of detected APB species; particularly the PNSB in the shrimp ponds.

Keywords: anoxygenic phototrophic bacteria, diversity, purple bacteria, mercury, *pufM* gene, shrimp ponds

บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายของแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria; PNSB) และผลของความเข้มข้นของปรอทในบ่อกุ้งต่อความหลากหลายของ PNSB โดยใช้การตรวจสอบยีน *pufM* ในตัวอย่างน้ำและตะกอนดินซึ่งเก็บมาจากบ่อกุ้งจำนวน 16 บ่อในบริเวณภาคใต้ของประเทศไทย ผลการตรวจพบยีนดังกล่าวในตัวอย่างน้ำจำนวน 13 บ่อ และในตัวอย่างตะกอนดินจำนวน 10 บ่อ นอกจาก PNSB แล้วยังตรวจพบแบคทีเรียสังเคราะห์แสงที่ไม่ให้ออกซิเจน (anoxygenic phototrophic bacteria; APB) กลุ่มอื่นๆด้วย ได้แก่แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มสะสมซัลเฟอร์ (purple sulfur bacteria; PSB) และแบคทีเรียสังเคราะห์แสงที่ไม่ให้ออกซิเจนกลุ่มใช้ออกซิเจน (aerobic anoxygenic phototrophic bacteria; AAPB) แต่ส่วนใหญ่ที่ตรวจพบไม่สามารถบ่งชี้สายพันธุ์ได้ สำหรับแบคทีเรียสังเคราะห์แสงกลุ่ม AAPB PSB และ PNSB ที่ตรวจพบในตัวอย่างน้ำมีค่าเท่ากับ 25.71 11.43 และ 8.57 เปอร์เซ็นต์ ขณะที่ในตัวอย่างตะกอนดินมีค่าเท่ากับ 27.78 11.11 และ 22.22 เปอร์เซ็นต์ จากทั้งตัวอย่างน้ำและตะกอนดินพบแบคทีเรีย *Roseobacter denitrificans* (AAPB) มากที่สุด ตามมาด้วย *Halorhodospira halophila* (PSB) นอกจากแบคทีเรียทั้งสองแล้ว ส่วนใหญ่ที่พบในตะกอนดินเป็นกลุ่ม PNSB ได้แก่ *Rhodovulum kholense Rhodospirillum centenum* และ *Rhodobium marinum* ผลจากการสร้าง UPGMA dendrograms สามารถจัดกลุ่มแบคทีเรียที่ศึกษาได้เป็น 7 กลุ่มในตัวอย่างน้ำ และ 6 กลุ่มในตัวอย่างตะกอนดิน โดยพบว่าผลการจัดกลุ่มดังกล่าวไม่มีความสัมพันธ์กับความเข้มข้นของปรอททั้งหมด (total mercury) ที่ตรวจพบในตัวอย่างน้ำซึ่งมีค่าอยู่ในช่วง <0.002-0.03 ไมโครกรัมต่อลิตร และในตัวอย่างตะกอนดินซึ่งมีค่าอยู่ในช่วง 35.40-391.60 ไมโครกรัมต่อกิโลกรัมของน้ำหนักแห้ง ดังนั้นกล่าวได้ว่าปริมาณปรอทที่ตรวจพบไม่มีผลต่อความหลากหลายของ APB โดยเฉพาะกับ PNSB ในบ่อกุ้ง

คำสำคัญ: แบคทีเรียสังเคราะห์แสงกลุ่มที่ไม่ให้ออกซิเจน ความหลากหลายทางชีวภาพ
แบคทีเรียสังเคราะห์แสงสีม่วง ปรอท ยีน *pufM* บ่อกุ้ง

Introduction

Shrimp farming is one of the most important aquacultural activities in Asia and South America, but farmers face many serious problems like shrimp diseases partly due to problems with the shrimp having to cope with poor water quality during cultivation (Rattanachuay et al., 2011). Only 20-30% of the nitrogen supplied from their feed is converted into shrimp biomass, while the rest accumulates at the bottom of the shrimp ponds (Kutako et al., 2009) and is converted anaerobically by microbes with the production of ammonia and also hydrogen sulfide (H_2S). Both these compounds are toxic for shrimp (Rao et al., 2000; Antony and Philip, 2006); this means that they should be controlled at levels that are safe for shrimp growth. Among the microbes associated with the carbon, nitrogen and sulfur cycles, of shrimp ponds, purple nonsulfur bacteria (PNSB) are probably the most useful to improve the water quality during shrimp cultivation (Antony and Philip, 2006; Zhou et al., 2009b). PNSB are versatile organisms able to grow with photoautotrophic or photoheterotrophic or heterotrophic conditions depending on the availability of light, oxygen and a suitable source of carbon. This means that they can consume organic matter under light and dark conditions and some can also remove H_2S (Antony and Philip, 2006; Kornochalert et al., 2014). Normally, PNSB exist in illuminated anoxic habitats in nature such as in aquatic sediments, of freshwater rivers and lakes, and wastewater treatment systems including shrimp ponds (Panwichian et al., 2010). PNSB are also considered to be beneficial microbes as their cells have a high protein content, they produce essential amino acids, and contain a high content of vitamin B12, ubiquinone and carotenoids (Shapawi et al., 2012; Kornochalert et al., 2014), so they have a great potential for use as probiotics in aquaculture (Shapawi et al., 2012). However, very little work has been published on the diversity of PNSB in shrimp ponds; hence it is worthwhile to study their diversity in shrimp ponds in order to gain knowledge that might be useful for finding a use for them during shrimp cultivation. The sequencing of the *pufM* gene that codes for the M subunit of a pigment-binding protein in the photosynthetic reaction center has been used to study the diversity and

phylogenetic composition of anoxygenic phototrophic bacteria (APB) such as the purple sulfur bacteria (PSB, *γ-Proteobacteria*), PNSB (*α-, β-Proteobacteria*) and aerobic anoxygenic phototrophic bacteria (AAPB, *α-Proteobacteria*) (Yutin et al., 2007; Asao et al., 2011). This is because this gene is part of the unique *puf* operon that codes for the subunits of the light-harvesting complex (*pufB* and *pufA*), and the reaction center complex (*pufL* and *pufM*) (Beja et al., 2002) present in all purple bacteria.

It is well recognized that shrimp cultivation in Thailand has occurred over at least the last 30 years (Lebel et al., 2010), and shrimp ponds are generally located near coastal areas of the Thai peninsular because natural seawater is required for shrimp cultivation. Use of untreated seawater for shrimp cultivation may cause problems if the seawater is contaminated especially with heavy metals including mercury (Hg) and other pollutants (Thongra-ara and Parkpianb, 2002; Cheevaporn and Menasveta, 2003; Panwichian et al., 2010). In some cases the pollutants have accumulated in situations where the shrimp ponds have been used over a long period of time. In addition Hg contamination in the shrimp ponds has been caused by impurities in agricultural fertilizers, lime and chemicals used during shrimp cultivation (Lacerda et al., 2011). The contamination of heavy metals; particularly Hg should be of concern because it is known to have a major effect on the activities of microbes and the biogeochemical processes which they mediate (Harris-Hellal et al., 2009).

Hence, Hg contamination in long term cultivated shrimp ponds could affect the dynamics and the diversity of bacterial communities during shrimp cultivation, in particularly PNSB. As far as we know this is the first report on the effects of Hg concentrations on PNSB in shrimp ponds. With regard to the above information, this work aimed to study the diversity of PNSB in shrimp ponds using the specific *pufM* gene for understanding which species of PNSB were dominant. This might help us to understand their physicochemical properties and roles and propose ways for them to be used optimally to facilitate the cultivation of shrimp in shrimp ponds. This also includes the influence of Hg concentrations on PNSB diversity and their ability to facilitate shrimp growth in the presence of Hg.

Materials and methods

Study areas and samples collecting in shrimp ponds

Water and sediment samples were collected from 16 shrimp ponds used for cultivating white shrimp (*Litopenaeus vannamei*) with different lengths of operation times in the coastal areas of the Thai Peninsular (Gulf of Thailand and Andaman sea). These shrimp ponds were in the districts of Ranot (RN: RN1 and RN2), Tepa (TP: TP1, TP2 and TP3) and Sating Phra (ST: ST1, ST2 and ST3), in Songkhla Province; the districts of Pak Panang (PN: PN1, PN2, PN3 and PN4) in Nakhon Si Thammarat Province; Kantang (KT: KT2), Sikao (SK) and Yan Ta Khao (YT) in Trang Province and Mueang (PT) in Pattani Province. Therefore, the 16 shrimp ponds are in eight districts; and all those in the same district were cultivated by the same owner. Hence, the operation and management systems for shrimp cultivation in the same district were not different.

During intensive shrimp cultivation, nutritionally complete (artificial diet) and mechanical aeration were used to feed shrimp and increase dissolved oxygen in all 16 shrimp ponds; while stocking density, but the feeding regime and supplementary diet feeding including other aquaculture practices depended on the farm owners. Composite subsamples were collected from each pond at various positions to obtain a representative sample of the pond by following two diagonals and a half point from each bank of the pond. Approximately 100 mL of water samples at 50 cm from the surface and 100 g of sediment samples from a depth of 5 cm were collected from each position. All the water and sediment samples were collected during shrimp cultivation near to shrimp harvesting that had shown no signs of shrimp weakness to study the PNSB diversity for evaluating any effects of Hg concentrations on PNSB diversity during the whole process of one crop cycle. As all collected samples were contaminated with Hg at various concentrations; thereby there was no control (without Hg contamination) for comparing PNSB diversity in its absence. Both sample types were kept in sterilized glass bottles and stored in an icebox during transportation, then maintained at 4°C before further studies. In addition a portion of

the water that was separated for determining the amount of total mercury (Hg_T) was preserved by adding 5 ml/L of 0.2 N bromine monochloride (BrCl) before storage.

DNA extraction

The water samples were centrifuged at 8,000 rpm for 20 min to obtain the biomass pellets for DNA extraction following the method of Zhou et al. (1996). Then the biomass pellet and sediment samples were separately washed twice with Tris-EDTA (TE) buffer to eliminate inhibitors that could affect DNA extraction. Genomic DNA was extracted from each sample using 600 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA, phosphate buffer pH 8.0, 1.5 M NaCl, 1% CTAB), 10 µL lysozyme and 10 µL protinase K, followed by mixing and incubating at 37°C for 30 min. After that 100 µL of 20% SDS was added into the mixture and further incubated at 65°C for 30 min. The supernatant was collected after centrifugation at 8,000 rpm for 5 min and mixed with an equal volume of chloroform: isoamyl alchohol (24: 1 v/v) then further centrifuged at 10,000 rpm for 5 min. The aqueous phase was recovered, mixed with 0.6 volumes of isopropanol and kept at -20°C for 1 h to precipitate genomic DNA. The genomic DNA was obtained by centrifugation at 10,000 rpm for 10 min and rinsed with absolute ethanol, air dried, and dissolved in nucleotide-free dH_2O. Genomic DNA was purified with the Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions and left at -20°C for any further steps.

Nested polymerase chain reaction (PCR)

Genomic DNA from each sample was amplified by nested PCR using the *pufL* and *pufM* primers. The first round, *pufLM* genes (1,500 bp) was amplified by *pufL* forward: '5-CTKTTCGACTTCTGGGTSGG-3' (Zeng et al., 2009) and *pufM* reverse 750: 5'-CCATSGTCCAGCGCCAGAA-3' (Achenbach et al., 2001). Each PCR reaction was amplified with 25 µL of PCR reaction mix (QIAGEN PCR Kit) containing 2.5 µL of 10X buffer with 1.5 mM $MgCl_2$, 2.5 µL of Q-Solution, 0.5 µL of 10 mM dNTP mix, 1.0 µL of 10 mM forward and reverse primer, 1.0 µL of DNA template, 0.1 µL of 5U/µL *Taq* DNA polymerase and 16.4 µL RNase-free water. The

reaction cycles were performed in a Thermal Cycle as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 50 s, 60°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min. The PCR product from the first round was used to amplify *pufM* gene (233 bp) in the same way as for the DNA template for the second round using *pufM* forward 557: 5'-CGCACCTGGACTGGAC-3′-GC (Achenbach et al., 2001) and *pufM* reverse 750: 5'-CCATSGTCCAGCGCCAGAA-3'. The PCR mixture and reaction cycles for amplification followed the same procedure as for the first round with the exception of the extension time that was reduced to 30 s. The PCR products were separated by electrophoresis on an 0.8% agarose gel, then stained with ethidium bromide and the bands were observed on a UV transilluminator.

DGGE and Phylogeny

The 233 bp-long *pufM* fragments from the water and sediment samples were separated in a 45-70% and 40-60% (w/v) denaturing gradient, respectively into an 0.8% (w/v) polyacrylamide gel. The electrophoresis was performed at 80 V, and 60°C for 16 h. After electrophoresis, the polyacrylamide gel was stained using the SYBR Gold nucleic acid stain for 20 min. The images were detected and captured on a UV transilluminator. The *pufM* fragments were cut from the gel using Gel Cutting Tips (Cleaver Scientific, England) and re-amplified with the *pufM* forward and *pufM*750-AT-M13 primers. The PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions and then sequenced with *pufM*750-AT-M13 as sequencing primers using the 1st BASE Laboratories Sdn Bhd (Malaysia) and were compared with the GenBank database in the NCBI website. Phylogenetic analysis was constructed by neighbor-joining using the free software MEGA 5.

Hg analysis

Water and sediment samples collected from each shrimp pond were used to determine the amount of Hg_T. All tubes and bottles used for analysis were first cleaned by heating to 65°C in 4 N HCl for 12 h, cooled and rinsed three times

with deionized water, and then dried in a clean oven at 60°C overnight. Method 1631 (2001) and method 1631 (2002) of the U.S. Environmental Protection Agency (USEPA) were used to prepare the sediment and water samples, respectively for detecting Hg_T and then analyzed by the Perkin-Elmer Flow Injection Mercury System (FIMS) 400 using $NaBH_4$ as the reducing reagent and 3% (v/v) HCl for the carrier solution.

Statistical analysis

All experiments in this study were conducted in three replicates. Mean values and the standard deviations are presented. Analysis of the data using one way ANOVA and significant differences among means were analyzed using the Duncan's multiple range test at a *P*-value<0.05. DGGE profiles were compared using the Jaccard coefficient based on the presence or absence of bands. Dendrograms were generated from the Jaccard coefficient using an unweighted paired group analysis of means (UPGMA) and the free trial XLSTAT.

Results

Diversity of purple nonsulfur bacteria in shrimp ponds

As stated in the introduction, the *pufM* gene that we used to study the diversity of PNSB in water and sediment samples collected from the sixteen shrimp pond sites was also present in other groups of APB so it was not specific for PNSB. Using the *pufM* gene primers, bands were detected after DGGE in 13 water samples (Figure 3-1A) and 10 sediment samples (Figure 3-1B). As some of the samples were detected in the different gels, therefore the same numbers of DGGE bands as shown in Figure 3-1 were compared from the positions on the gels and also were confirmed from the results of sequencing analysis. The DGGE profiles from each site differed with many more bands appearing for each of the water samples compared to those from the sediment samples. However, from the water samples, bands 1 and 7 appeared in all while bands 5 and 6 were found from most sampling sites (Table 3-1). Moreover, there were some bands such as bands; 3, 8, 10, 11, 14, 16 and 21 that

appeared frequently among the different sites. Some 35 of the more predominant bands from the water samples were subjected to DNA analysis. Only 3 were identified as known PNSB, 4 were identified as being PSB, 9 were derived from known AAPB and the remaining 19 were not recognized and therefore assigned as uncultured. It is possible that some of these could have been related to PNSB. As for the samples from the sediment only 18 bands were characterized, 4 were PNSB, 2 were PSB, 5 were AAPB while 7 were unrecognized (Table 3-2). Bands 1 and 7 were closely related to *Roseobacter denitrificans* OCh114 (AAPB) and *Roseibacterium elongatum* OCh323 (AAPB), and they were the dominant species in all the water samples while bands 5 and 6 that were closely related to *Halorhodospira halophila* H (PSB) and *Marivita* sp. RCC1921 (AAPB), respectively were found in most water samples.

In contrast, the DGGE profiles in the sediment samples (Figure 3-1B) were quite different and had fewer DGGE profiles than in the water samples. However, lists of bacterial species in the sediment samples belonged to groups of PSB, PNSB and AAPB as well, and again band 1 was closely related to *R. denitrificans* OCh114 that was also the dominant species in the sediment samples followed by band 5, related to *H. halophila* H. For the PNSB group bands; 3, 17 and 18 closely corresponded to *Rhodovulum kholense* type strain JA297T, *Rhodospirillum centenum* SW and *Rhodobium marinum* JA211 (Table 3-2). The following species were detected in both sample types; *R. denitrificans* OCh114, *Marivita* sp. RCC1921, *Loktanella* sp. NP29 (AAPB), *H. halophila* H, *R. marinum* JA211 (PNSB, *α-Proteobacteria*) and *Rubrivivax gelatinosus* (PNSB, *β-Proteobacteria*). However, roughly 54.3% and 38.9% of the DGGE fragments from water and sediment samples respectively had no identified analog in the data base (Tables 3-1 and 3-2).

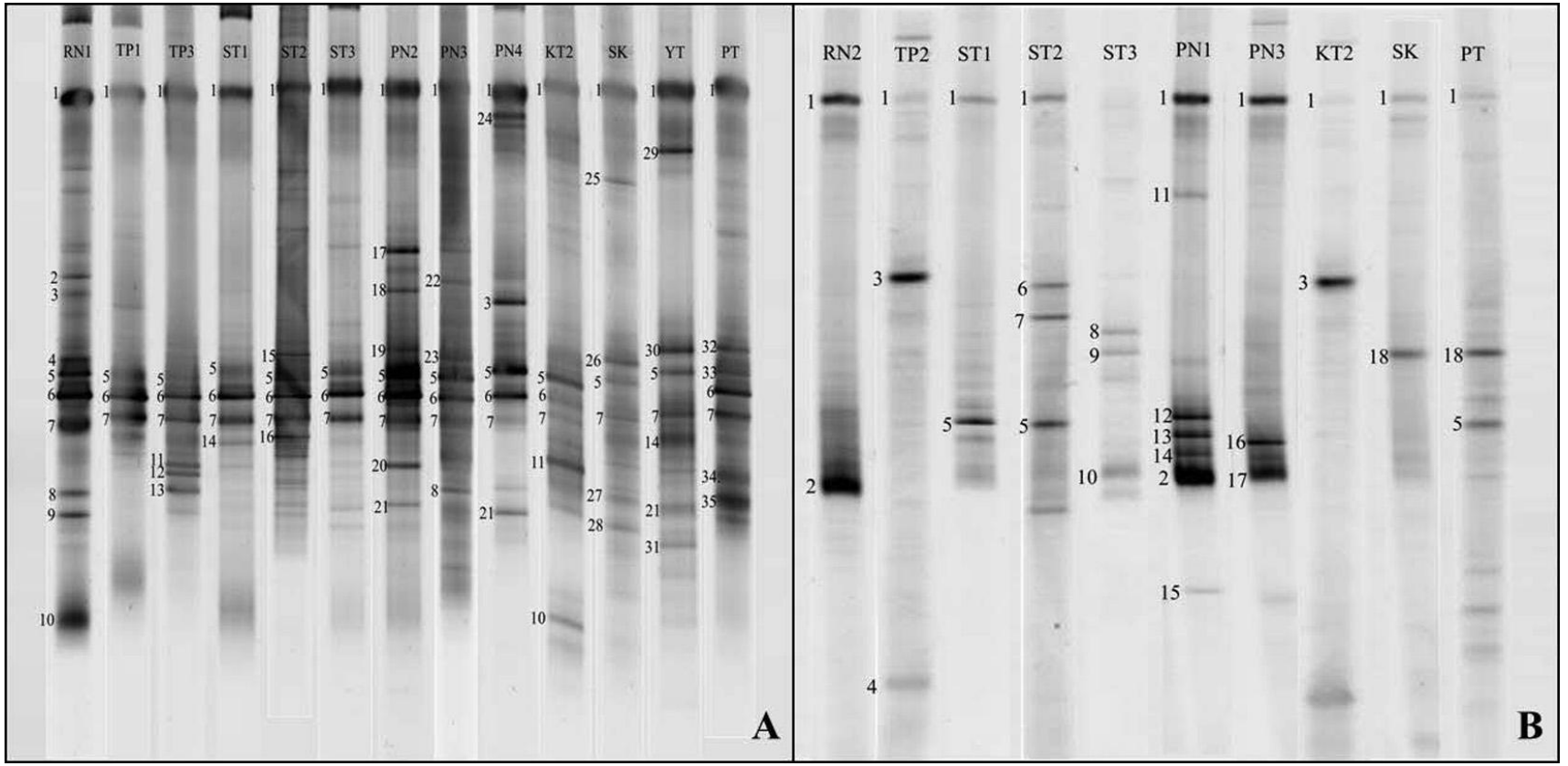

Figure 3-1. DGGE profiles of *pufM* amplified in bacteria from (A) water and (B) sediment samples collected from shrimp ponds located in the districts of Ranot (RN1-RN2), Tepa (TP1-TP3), Sating Phra (ST1-ST3), Pak Panang (PN2-PN4), Kantang (KT2), Sikao (SK), Yan Ta Khao (YT) and Mueang Pattani (PT). Marked numbers on the DGGE profiles represented bands that were excised and sequenced as shown in Tables 3-1 and 3-2.

Table 3-1. Similarity of representative *pufM* sequences in purple bacteria and AAPB from DGGE gels of water samples collecting from shrimp ponds in southern Thailand.

Band	Sampling area	Closet identify relative		Similarity (%)
		Accession number	Bacterial species	
1	All area	CP000362	*Roseobacter denitrificans* OCh 114 (AAPB)	90
2	RN1	EU191249	Uncultured bacterium	95
3	RN1 and PN4	AM944092	*Rhodobium marinum* JA211 (PNSB)	92
4	RN1	EU191257	Uncultured bacterium	96
5	RN1, TP1, TP3, ST1, ST2, ST3, PN2, PN3, PN4, KT2, SK and YT	FN257160	*Halorhodospira halophila* strain H (PSB)	88
6	RN1, TP1, TP3, ST1, ST2, ST3, PN2, PN3, PN4, KT2 and PT	FR852763	*Marivita* sp. RCC 1921 (AAPB)	93
7	All area	JQ694099	*Roseibacterium elongatum* OCh 323 (AAPB)	93
8	RN1 and PN3	AB508087	Uncultured bacterium	88
9	RN1	CP000830	*Dinoroseobacter shibae* DFL 12 (AAPB)	89
10	RN1 and KT2	AB020784	*Rhodovulum sulfidophilum* (PNSB)	98
11	TP3 and KT2	FJ619033	Uncultured bacterium	90
12	TP3	HQ222675	Uncultured bacterium	90
13	TP3	AB486033	Uncultured bacterium	90
14	ST1 and YT	FN869946	Uncultured Rhodobacteraceae bacterium	90
15	ST2	EU009368	*Roseobacter* sp. BS90 (AAPB)	93
16	ST2	AY234384	*Rubrivivax gelatinosus* strain S1 (PNSB)	90
17	PN2	JQ694098	*Roseibacterium* sp. JLT1202r (AAPB)	93
18	PN2	JF523530	uncultured Chromatiaceae bacterium	91
19	PN2	FJ498837	Uncultured bacterium	92
20	PN2	AM944100	*Ectothiorhodospira imhoffii*, type strain JA319T (PSB)	89
21	PN2, PN4 and YT	EU196353	*Loktanella sp.* NP29 (AAPB)	91
22	PN3	KF008154|	Uncultured bacterium	86
23	PN3	KC768183	Uncultured bacterium	86
24	PN4	DQ915720	*Roseovarius tolerans* strain NBRC16695 (AAPB)	90
25	SK	FJ498843	Uncultured bacterium	88
26	SK	HQ222683	Uncultured bacterium	90
27	SK	KC900142	Uncultured bacterium	91
28	SK	JN712795	Uncultured bacterium	91
29	YT	FJ812046	*Allochromatium renukae* strain DSM 18713 (PSB)	90
30	YT	FN257187	*Allochromatium* sp. MTCH3IM086 (PSB)	90
31	YT	FJ589120	Uncultured bacterium	90
32	PT	FR852765	Bacterium RCC 1908	87
33	PT	CP001029	*Methylobacterium populi* BJ001 (AAPB)	85
34	PT	KF008154	Uncultured bacterium	86
35	PT	FJ619028	Uncultured bacterium	90

Table 3-2. Similarity of representative *pufM* sequences in purple bacteria and AAPB from DGGE gels of sediment samples collecting from shrimp ponds in southern Thailand.

Band	Sampling area	Closet identify relative		Similarity (%)
		Accession number	Bacterial species	
1	RN2, TP2, ST1, ST2, PN1, PN3, KT2, SK and PT	CP000362	*Roseobacter denitrificans* OCh 114 (AAPB)	90
2	RN2, PN1	JN712793	Uncultured bacterium	88
3	TP2 and KT2	FM208076	*Rhodovulum kholense*, type strain JA297T (PNSB)	90
4	TP2	AY853584	*Sphingomonas* sp. PB180 (AAPB)	90
5	ST1, ST2, and PT	FN257160	*Halorhodospira halophila* strain H (PSB)	88
6	ST2	DQ017882	*Methylobacterium rhodinum* strain ATCC 14821 (AAPB)	92
7	ST2	AB510456	Uncultured bacterium	89
8	ST3	JN712796	Uncultured bacterium	84
9	ST3	EU191583	Uncultured bacterium	87
10	ST3	AB510450	Uncultured bacterium	89
11	PN1	EU191602	Uncultured bacterium	92
12	PN1	FN257137	*Thiorhodococcus drewsii*, type strain DSM 15006T (PSB)	91
13	PN1	FJ498837	Uncultured bacterium	92
14	PN1	AY234384	*Rubrivivax gelatinosus* strain S1 (PNSB)	89
15	PN1	EU196353	*Loktanella sp.* NP29 (AAPB)	90
16	PN3	FR852763	*Marivita* sp. RCC 1921 (AAPB)	93
17	PN3	CP000613	*Rhodospirillum centenum* SW (PNSB)	89
18	SK and PT	AM944092	*Rhodobium marinum* JA211 (PNSB)	92

When the sequencing analysis was clustered into phylogenetic groups, there were representatives from the *α*-, *β*- and γ-subclass of *Proteobacteria*. The five groups in the water samples were 54.3% of the 'uncultured' group followed by 25.7% of AAPB (*α-Proteobacteria*), 11.4% of PSB (*γ-Proteobacteria*), 5.7% of PNSB (*α-Proteobacteria*) and 2.9% of PNSB (*β-Proteobacteria*) (Figure 3-2A). The five clustered groups in the sediment samples were 38.9% of the 'uncultured' group followed by 27.8% of AAPB (*α-Proteobacteria*), 16.7% of PNSB (*α-Proteobacteria*) followed by 11.0% of PSB (*γ-Proteobacteria*) and 5.6% of PNSB (*β-Proteobacteria*) (Figure 3-2B).

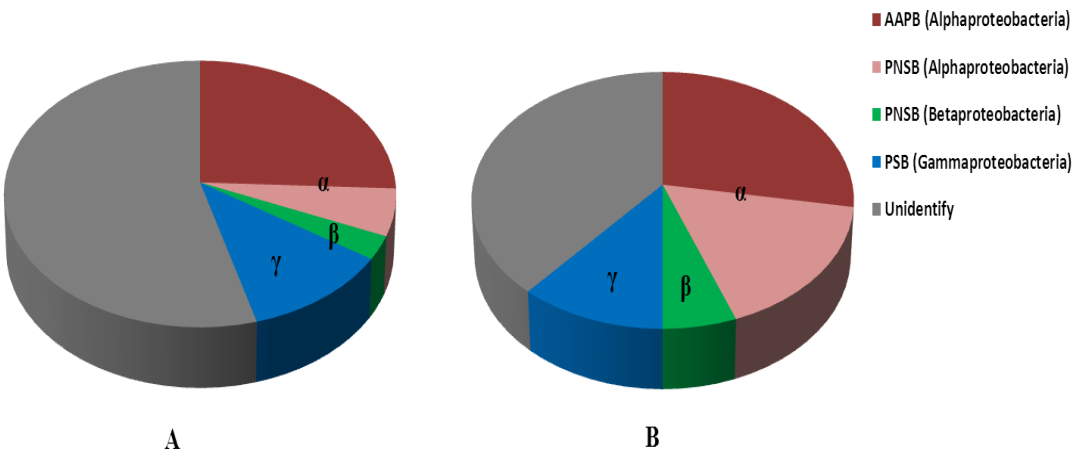

Figure 3-2. Phylum distribution of anoxygenic phototrophic bacteria sequences from water (A) and sediment (B) samples collecting from shrimp ponds in southern Thailand.

The relationship between DGGE profiles and Hg_T concentrations in sampling sites

From the data reported in Table 3-3, do they provide any information on the effects of the Hg_T concentrations in the water and sediment samples on the PNSB diversity? The highest concentration of Hg_T detected in the water was from a sample collected from the KT2 site (0.030 µg/L) followed by sites TP2 and YT (0.004 µg/L) and TP3 (0.003 µg/L) while the Hg_T concentrations in other samples were less than 0.002 µg/L. In contrast, the Hg_T concentrations in the sediment samples were significantly higher than those in the water samples; and their Hg_T concentrations differed from different collecting sites. The Hg_T concentrations in the sediment samples ranged from 35.40 to 391.60 µg/kg dry sediment weight, with a median concentration of 166.01 ± 83.61 µg/kg dry sediment weight. The highest concentration of Hg_T was detected in the PT site while the lowest was from the PN3 site.

The DGGE profiles from the various sites of both sample types were compared by the Jaccard coefficient as shown by the UPGMA dendrograms in Figure 3-3. The DGGE profiles of the water samples were clustered into seven groups (Figure 3-3A). The DGGE profiles from sites; TP1, TP3, ST1-3 and KT2 were clustered in the first group; PN2 and PN4 were in the second group, and the remaining sites (PN3, RN1, SK, YT and PT) were each separated into an individual group. The DGGE profiles of the samples from the sediment were clustered into six groups (Figure 3-3B). The DGGE profiles from sites; KT2 and TP2 were clustered in the first group and the ST1-2, PT and SK were in the second group, and the remaining sites (RN2, PN1, PN3 and ST3) were each separated into an individual group. Comparison of the DGGE profiles in both sample types and their detected Hg_T concentrations in each sample showed that there was no relationship between the bacterial groups.

Comparison of the DGGE profiles in both sample types and their detected Hg_T concentrations in each sample showed that there was no relationship between the bacterial groups and Hg_T concentrations. For example, the sediment samples collected from sites; PT, ST1, ST2 and SK were clustered in the same group (Figure 3-3B and Table 3-3), but the Hg_T concentrations (µg/kg dry sediment weight) were totally different as in PT (391.60), ST1 (367.68), ST2 (183.22) and SK (59.52). Moreover, the Hg_T concentration in RN1 (140.21 µg/kg dry sediment weight) was very close to that of RN2 (135.22 µg/kg dry sediment weight), but the RN1 site had no detectable *pufM* gene. This was similar for the water samples, as the DGGE profiles in KT2 site, with the highest concentrations of Hg_T (0.030 µg/L) was grouped with the TP1 and ST1-3 with the lowest concentration of HgT (<0.002 µg/L). Therefore, the Hg_T concentrations were not in anyways related to the DGGE profiles in the sediment samples and also the water samples with a low Hg_T concentration.

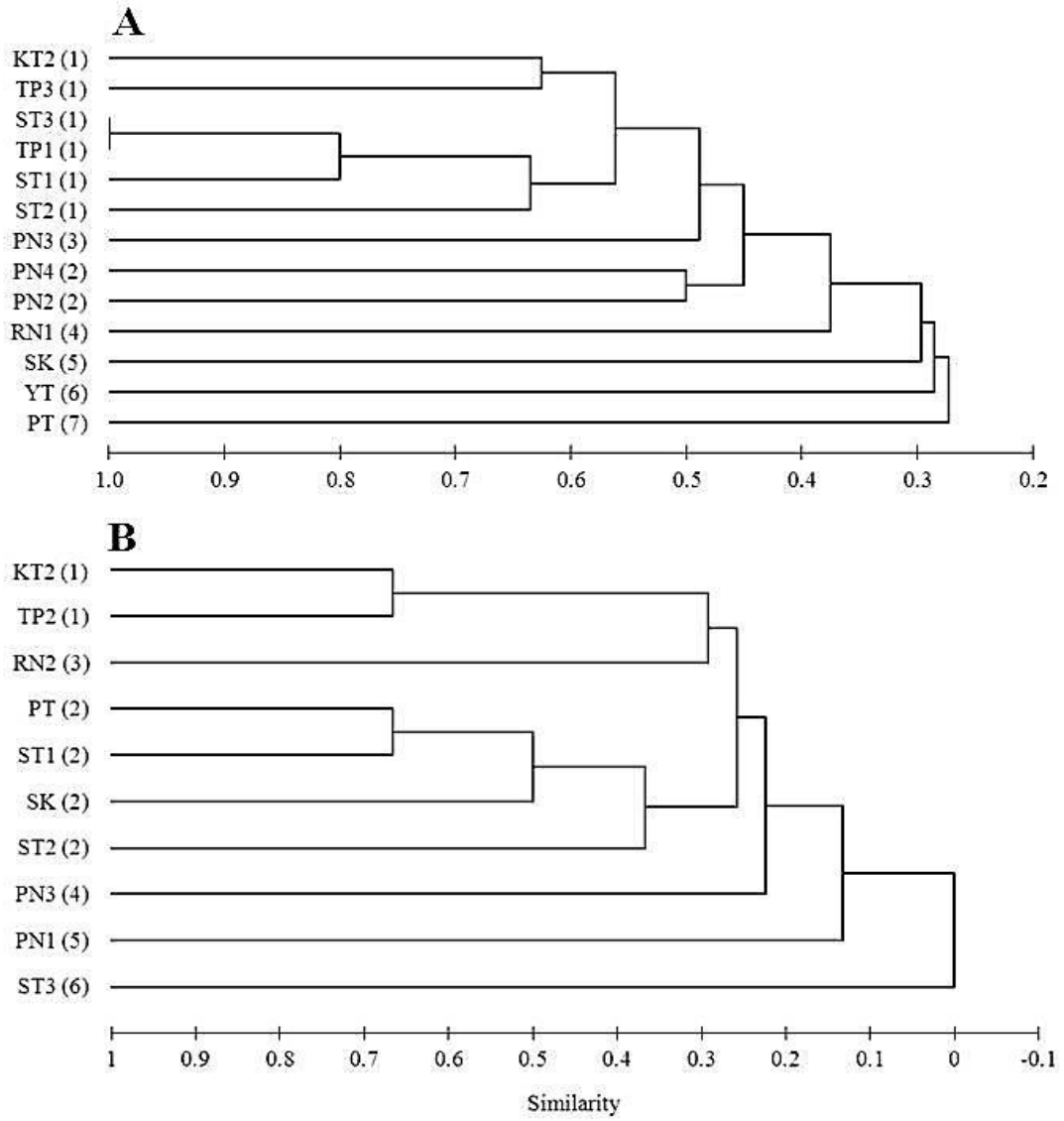

Figure 3-3. UPGMA dendrograms from cluster analysis using the Jaccard coefficient of DGGE profiles from (A) water and (B) sediment samples as shown in Figure 3-1. Number in parentheses is a group of the clustered isolates.

Table 3-3. Group of DGGE profiles in water and sediment samples which were clustered by UPGMA dendrograms using Jaccard coefficient (Figure 3-3) and comparing with Hg_T concentrations in water and sediment samples collected from shrimp ponds in southern Thailand.

Site	Water		Sediment	
	Total mercury (Hg_T) (µg/L)	Group	Total mercury (Hg_T) (µg/kg dry weight)	Group
	<0.002 ± 0.001	4	140.21 ± 3.80	ND
RN2	<0.002 ± 0.000	ND	135.22 ± 7.76	3
TP1	<0.002 ± 0.000	1	169.42 ± 4.07	ND
TP2	0.004 ± 0.000	ND	204.50 ± 2.48	1
TP3	0.003 ± 0.001	1	243.56 ± 8.65	ND
ST1	<0.002 ± 0.000	1	367.68 ± 15.87	2
ST2	<0.002 ± 0.000	1	183.22 ± 1.87	2
ST3	<0.002 ± 0.000	1	221.10 ± 0.75	6
PN1	<0.002 ± 0.000	ND	52.32 ± 0.63	5
PN2	<0.002 ± 0.000	2	63.94 ± 1.16	ND
PN3	<0.002 ± 0.000	3	35.40 ± 2.55	4
PN4	<0.002 ± 0.001	2	85.24 ± 5.51	ND
KT2	0.030 ± 0.004	1	165.92 ± 4.28	1
SK	<0.002 ± 0.000	5	59.62 ± 4.45	2
YT	0.004 ± 0.001	6	87.26 ± 3.08	ND
PT	<0.002 ± 0.001	7	391.60 ± 6.08	2

ND= not detected *pufM* gene.

Data represent mean ± standard deviation of three determinations.

Discussion

Diversity of purple nonsulfur bacteria in shrimp ponds

Although this research was initially focused on the PNSB the results showed that other groups of APB, PSB and AAPB, were also detected based on the amplification of the *pufM* genes (Tables 3-1 and 3-2 and Figure 3-2). Our results are in agreement with the previous work that focused on the diversity of purple bacteria in a permanently frozen Antarctic Lake; however, in addition the purple bacteria, AAPB were also detected due to the use of the *pufM* gene probes (Karr et al., 2003). The AAPB that belong to the *α-Proteobacteria* (Figure 3-2) were the group that showed varied species found in shrimp ponds in both sets of samples. This is not surprising as the *α-Proteobacteria* are the most abundant bacterial group in seawater (Sakami et al., 2008); and seawater is normally used for shrimp cultivation. In addition, aeration is always used in intensive shrimp ponds to improve water quality and increase shrimp yields; and all the 16 shrimp ponds studied were provided with mechanical continuous aeration, particularly at the end of culture. This is the reason why the AAPB such as *R. denitrificans* and *R. elongatum* were detected from all water samples; and *R. denitrificans* was detected from most sediment samples.

The AAPB are aerobic and carry out anoxygenic photosynthesis by capturing energy from light using simple organic compounds as a carbon source for their growth (Yutin et al., 2007; Tang et al., 2009). In contrast, a greater diversity of the PNSB was found in the sediment samples than in the water samples with a higher detection rate of the *pufM* gene; this is because the sediment conditions in shrimp ponds are enriched with organic matter with only a limited amount oxygen and far from the sunlight, but the PNSB also grow in dark conditions using fermentation (Karr et al., 2003). Hence, PNSB could compete better with the PSB in the bottom of the ponds because light is limiting for photosynthesis. The high percentage of *pufM* amplified ‘uncultured’ bacteria in both sample types indicated that there could be many species of purple bacteria and AAPB in the shrimp pond environment that were uncultured species. In addition, it was possible that the extracted DNA template of these species were at low levels, thus resulting in a reduced-specificity for

amplification of the *pufM* gene, so any incomplete sequence was classified as an 'uncultured' bacterium.

One interesting conclusion was that the anthropogenic organic matter that was used in shrimp feed was an important factor that influenced the bacterial community in shrimp ponds (Sakami et al., 2008). The feeding was provided based on the rate of diet feed, rate of shrimp growth and shrimp density that depended on the stocking density in a range of from 35 to 75 postlarvae/m^2 for the 16 shrimp ponds. The daily feeding rate for the shrimp cultivation in this study was in a range of 0.5-2 kg/10^5 shrimps/day for an initial period and 5-6 kg/10^5 shrimps/day just before harvesting. The sampling sites that used similar shrimp feed for artificial diet (CP shrimp feed) although supplementary diet feeding was different produced a similar pattern of the APB community. For example, the DGGE profiles from the water samples collecting from the same district; TP1 and 3 (63 postlarvae/m^2) or different district; ST1–3 (75 postlarvae/m^2) were clustered in the same group (Figure 3-3A), this result demonstrated that because these samples were from ponds with the same artificial diet, the type of shrimp feed in terms of quantity and frequency was similar in the period before harvest (5 kg/10^5 shrimps/day). Furthermore, the DGGE profiles of KT2 (Andaman coast) and TP2 (Gulf of Thailand coast) in the sediment samples (Figure 3-3B) were clustered in the same group that had a different water source.

However, shrimp feeding is not the sole factor that affected the diversity of purple bacteria because the DGGE profiles of the TP1 and TP3 from the same water source were clustered in the same group (Figure 3-3A), but not from sediment samples as the *pufM* gene were not detected from both ponds (Figure 3-3B). Hence, the stocking density of each pond might have an effect on diversity of APB during cultivation because of overfeeding so the uneaten shrimp feed was precipitated to the bottom of the shrimp ponds for stimulating APB growth. This is supported by the DGGE profiles from water and sediment samples of PN1 and 3 (stocking density, 35 postlarvae/m^2; feeding regime, 2 and 6 kg/10^5 shrimps/day for initial and before harvest phases) of detected *pufM* gene were different so they were in different clusters (Figure 3-3A and B). These results pointed to the effects of shrimp feeding and stocking density on the APB found in the water and sediment samples, because the

excess of the shrimp feed was precipitated to the bottom of the shrimp ponds to the more anaerobic conditions; particularly for PN1 and PN3. However, other factors that may affect the growth of APB include: pH, temperature, salinity, sanitizers such as chlorine, iodophors and the community of other bacteria in the shrimp ponds (Rao et al., 2000). Shrimp ponds that differed in these factors also showed a different diversity of APB as observed in the different sampling sites such as from RN1-2, SK, YT and PT (Figure 3-3A) and RN2, PN3 and ST3 (Figure 3-3B).

In this study, we did not determine the physicochemical properties in the samples of water and sediment; however, the dominant species of detected APB could be used to support their roles on some of their physicochemical properties that were related to their characteristics. *R. denitrificans* was the most common species found in both sample types of all studied sites in the water column and almost all sites in the sediment; this organism is a photoheterotroph and also a denitrifying bacterium (Tang et al., 2009). Denitrification occurs in anaerobic conditions; but it is not strictly an anoxic process because nitrogen oxide reductases are expressed when some oxygen is present (Shapleigh, 2009). In addition, the research of Nishimura et al. (1996) found that *R. denitrificans* can grow in aerobic conditions because their photosynthesis genes are highly expressed in aerobic conditions. Hence, this is why this organism was found in both sample types. It is well recognized that the deposit of excess food in the sediment results in an anaerobic digestion process that produces NH_3 and H_2S that diffuse into the water column.

Aeration in the water column helps the bacterial population to convert NH_3 to NO_3^- and H_2S to SO_4^{2-}. However, more PSB were found in the water column; this indicated that the sulfide (S^{2-}) produced in the sediment diffused into the water column. In shrimp ponds the average concentration of SO_4^{2-} ranged from 1.6 to 4.3 g/kg, while the maximum concentration of S^{2-} was 1.5 mg/L (Mirzoyan et al., 2008). Therefore, under illuminated sulfidic conditions PSB grew by consuming S^{2-} as an electron donor for photoautotrophic growth although they are strictly anaerobes. This meant that bacterial communities in the water column including AAPB help to provide anoxic conditions for PSB to grow. In our study a high concentrations of H_2S in the shrimp ponds was assumed by the detection of the PSB species in the water

samples (*E. imhoffii*, *H. halophila*, *A. renkae* and *Allochromatium* sp.) and sediment samples (*H. halophila* and *T. drewsii*) (Tables 3-1 and 3-2); and the sulfide should be completely consumed by them as evidenced by the healthy shrimp in the ponds as previously described. It is well recognized that *T. drewsii* found in the sediment and *R. sulfidophilum* (PNSB) found in the water samples were highly tolerant of the sulfide species, of up to 11 mM (Zaar et al., 2003) and 5-6 mM (Imhoff, 2005), respectively. Hence, this is a feasible biological method for controlling H_2S levels in the shrimp ponds via both groups of purple bacteria.

The relationship between DGGE profiles and Hg_T concentrations in sampling sites

The toxicity of Hg on PNSB has been studied but only by an in vitro test i.e. Hg^{2+} at different concentrations (<100 µM) inhibited the growth of *Rhodobacter sphaeroides* 2.4.1 by affecting the photosynthetic apparatus and the binding with C,O, C–N, C–S, and C–SH groups of the amino acids (Asztalos et al., 2012). In addition, Hg^{2+} at 0.03 mM gave a negative effect on *R. sphaeroides* R26.1 as the growth rate decreased while the lag-phase increased following the increase of the Hg^{2+} concentrations from 0.001 to 0.050 mM (Giotta et al., 2006). However, no work has been done on the effect of Hg on PNSB in shrimp ponds. The Hg_T concentration in the sediment was significantly higher than in the water column (Table 3-3). This was because most of the Hg in the water was attached to particles of suspended sediment, such as organic matter including metal oxides (Domagalski, 2001) and these precipitated onto the sediment. In addition, the Hg in the sediments was strongly bound to S^{2-} to form a highly insoluble, mercury sulfide (HgS) (Gabriel and Williamson, 2004).

The Hg_T concentration at each site was compared with the DGGE profiles (Tables 3-1 and 3-2) that were clustered by the UPGMA dendrograms (Figure 3-3) as shown in Table 3-3. There was no direct relationship between both parameters (levels of Hg contamination and DGGE profiles) found, especially in the water samples, perhaps because the Hg_T concentrations in all water samples were very low. However, the Hg_T concentrations in the sediment samples were much higher than in

the water samples, but there was still no correlation between the Hg_T concentration and the APB diversity. In contrast, use of soil microcosms spiked with inorganic Hg at 20 µg per gram of soil had a significant effect on soil microbial communities (Harris-Hellal et al., 2009). Therefore, it is possible that APB, particularly PNSB have been acclimatized to live in long-term Hg contaminated shrimp ponds. This assumption is supported as more various species of PNSB were detected in the sediment samples with a lower number of *pufM* genes detected when compared with the water samples (Tables 3-1 and 3-2).

Although it was not possible to find any correlation between the APB and Hg levels in the water samples as previously described; the results of the DGGE patterns of the *pufM* bands did change and opened up new avenues for discussion of the bacteria associated with shrimp cultivation. In addition, Hg contamination in shrimp ponds should be of concern because if there was no natural process to remediate and manage these areas, these will definitely increase especially in the sediment and might affect other beneficial microorganisms, and consequently perhaps the bioaccumulation of Hg in the shrimp via food chains. Hence, our future research work will focus on the various PNSB isolates to establish their Hg resistant mechanisms for the possibility of using them for remediation of Hg contaminated shrimp ponds and maintaining the quality of the shrimp cultivation water.

Conclusions

The amplification of APB based on the *pufM* gene found that the most common species found in the shrimp ponds were closely to *Roseobacter denitrificans* OCh 114 and *Halorhodospira halophila* H including some PNSB. These bacteria might play a major role in the nutrient cycles, particularly on the species of nitrogen and sulfur that are related to the water quality for shrimp growth. The Hg contamination in the shrimp ponds tested seemingly had no effect on the APB; in particular the PNSB.

CHAPTER 4

Distribution of mercury in shrimp ponds and volatilization of Hg by isolated resistant purple nonsulfur bacteria

Abstract

The aims of this study were to investigate the presence of mercury (Hg) contamination in shrimp ponds in the south of Thailand and to isolate Hg resistant purple nonsulfur bacteria (PNSB). Contamination by total mercury (Hg_T) in water and sediment samples ranged from <0.0002 to 0.037 μg/L and from 30.73 to 398.84 μg/kg dry weight. In all water and sediment samples, the concentration of Hg_T was less than the Thai, Hong Kong, and Canadian standard guidelines. Of the Hg resistant PNSB, six strains detoxified Hg^{2+} by volatilization to Hg^0 using their mercuric reductase enzyme. The ability of PNSB to resist Hg^{2+} in aerobic dark conditions was better than in microaerobic light, and this corresponded with their Hg reductase activities (dark condition 15.75, 12.62, and 12.16 U/mg protein for strains SSW15-1, SRW1-5, and SSS2-1, respectively). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were the same under both incubating conditions at 2.40 mg/L for SRW1-5 and 1.60 mg/L for SSW15-1. However, both values under light condition of SSS2-1 were 3.20 mg/L while under dark condition MIC and MBC values were 3.20 and 4.00 mg/L. The half maximal inhibitory concentration (IC_{50}) values of Hg^{2+} on strains SSS2-1, SRW1-5, and SSW15-1 under dark and light conditions were 2.16, 1.23, and 0.90; and 1.66, 1.11, and 0.80 mg/L, respectively. They were identified using 16S ribosomal RNA (rRNA) genes establishing that SSS2-1 and SSW15-1 were *Afifella marina*, while SRW1-5 was *Rhodovulum sulfidophilum*. These strains can potentially be used to treat Hg contaminated shrimp ponds.

Keywords: bioremediation, mercury contamination, mercuric reductase, purple nonsulfur bacteria, shrimp farming, volatilization

บทคัดย่อ

การวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อตรวจวัดปริมาณปรอท (Hg) ที่ปนเปื้อนในบ่อกุ้งบริเวณภาคใต้ของประเทศไทย และเพื่อคัดแยกแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria; PNSB) ที่ต้านทานต่อปรอท จากการศึกษาพบว่าปริมาณปรอททั้งหมดในตัวอย่างน้ำมีค่าอยู่ระหว่าง <0.0002-0.037 ไมโครกรัมต่อลิตร และมีค่าอยู่ระหว่าง 30.73-398.84 ไมโครกรัมต่อกิโลกรัมของน้ำหนักแห้งในตัวอย่างตะกอนดิน โดยปริมาณปรอททั้งหมดที่ตรวจวัดได้ทั้งในตัวอย่างน้ำและตะกอนดินมีค่าต่ำกว่าค่ามาตรฐานตามเกณฑ์กำหนดของฮ่องกง ประเทศไทยและแคนนาดา จากการคัดแยก PNSB ที่ต้านทานต่อปรอท พบว่าเชื้อที่คัดเลือกได้จำนวน 6 สายพันธุ์สามารถลดความเป็นพิษของปรอทจาก Hg^{2+} เป็น Hg^{0} ได้ด้วยการสร้างเอนไซม์ mercuric reductase ความต้านทานของเชื้อต่อปรอทในสภาวะไร้แสง มีอากาศดีกว่าสภาวะมีแสง มีอากาศเล็กน้อย ซึ่งสอดคล้องกับกิจกรรมของเอนไซม์ในสภาวะดังกล่าว โดยเชื้อสามสายพันธุ์ที่คัดเลือกได้คือ SSW15-1 SRW1-5 และ SSS2-1 สามารถสร้างเอนไซม์ในสภาวะไร้แสง มีอากาศได้สูงสุดคือ 15.75 12.62 และ 12.16 ยูนิตต่อมิลลิกรัมของโปรตีน ตามลำดับ จากการศึกษาค่าความเข้มข้นต่ำสุดของปรอทที่ยับยั้งการเจริญของแบคทีเรีย (MIC) และความเข้มข้นต่ำสุดของปรอทมีฆ่าแบคทีเรียได้ (MBC) พบว่าเชื้อสายพันธุ์ SRW1-5 และ SSW15-1 มีค่า MIC และ MBC เท่ากันทั้งสองสภาวะการเลี้ยง คือ 2.40 และ 1.60 มิลลิกรัมต่อลิตร ตามลำดับ ในขณะที่เชื้อสายพันธุ์ SSS2-1 จากการเลี้ยงในสภาวะมีแสง มีอากาศเล็กน้อย มีค่า MIC และ MBC เท่ากันคือ 3.20 มิลลิกรัมต่อลิตร ส่วนการเลี้ยงในสภาวะไร้แสง มีอากาศมีค่า MIC และ MBC เท่ากับ 3.2 และ 4.0 มิลลิกรัมต่อลิตร ตามลำดับ ส่วนการตรวจวัดค่าความเข้มข้นของปรอทที่ยับยั้งเชื้อได้ 50 เปอร์เซ็นต์ (IC_{50}) พบว่าเชื้อสายพันธุ์ SSS2-1 SRW1-5 และ SSW15-1 มีค่า IC_{50} เท่ากับ 2.16 1.23 และ 0.90 มิลลิกรัมต่อลิตร ในสภาวะไร้แสง มีอากาศ และมีค่า 1.66 1.11 และ 0.80 มิลลิกรัมต่อลิตร ในสภาวะมีแสง มีอากาศเล็กน้อย ตามลำดับ ผลจากการเทียบเคียงเชื้อทั้งสามสายพันธุ์ด้วยยีน 16 sRNA พบว่าเชื้อสายพันธุ์ SSS2-1 และ SSW15-1 คือ *Afifella marina* ในขณะที่เชื้อสายพันธุ์ SRW1-5 คือ *Rhodovulum sulfidophilum* จากผลการศึกษาดังที่กล่าวมาแล้วข้างต้นแสดงให้เห็นว่าเชื้อดังกล่าวมีศักยภาพในการนำไปใช้เพื่อบำบัดพื้นที่ซึ่งมีปรอทปนเปื้อน

คำสำคัญ: การบำบัดทางชีวภาพ การปนเปื้อนปรอท เอนไซม์ mercuric reductase
แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ การเพาะเลี้ยงกุ้ง
การระเหยของสารแบบเร็ว

Introduction

Mercury (Hg) is the most toxic of the heavy metals and the sixth most toxic element in the list of hazardous compounds found in the environment (Nascimento and Chartone-Souza, 2003; Bafana et al., 2010). The most notable instance of Hg's impact on humans, due to environmental contamination, occurred in the Japan's Minamata Bay between 1953 and 1961 when fish were contaminated with methylmercury, and people were subsequently poisoned. This incident was called "Minamata disease" (Carrasco et al., 2011). Hg contaminates the environment because of two main sources: the release of Hg from natural sources and from anthropogenic sources (Pirrone et al., 2010). Consequently, accumulation of Hg residues may be possible in aquatic animals through the food web, but accumulation depends on the chemical form of Hg such as labile or nonlabile complexed, reduced or oxidized, and inorganic or methylmercury (Baeyens et al., 2003). Recently, many studies have reported worldwide on Hg contamination in aquatic animals such as shrimp, fish, shellfish, crab, and mollusks (Brambilla et al., 2013; Li et al., 2013). Consumption of Hg-contaminated aquatic animals does adversely affect human health.

Shrimp is one of the most important aquatic animals farmed in Thailand. The Department of Fisheries in Thailand (2013) reported that total shrimp production in the first quarter of 2013 was 63,500 t and declined around 30,900 t compared to the same period of 2012. In Thailand, most shrimp farms are located near the coast, to make use of the seawater for rearing shrimp without the need to adjust its composition even though seawater may have many kinds of pollutants, including heavy metals (Panwichian et al., 2010). In Thailand, there have been no reports of Hg contamination in shrimp ponds; however, other contaminating metals such as Cd, Cu, Pb, and Zn have been reported. The Hg_T contamination in seawater collected from both coastal areas of Thailand in 1997-1998 was detected and ranged from < 0.01 to 0.54 µg/L in water and 0.05 to 2.14 µg/g dry sediment weight (Thongra-ara and Parkpianb, 2002; Sompongchaiyakul and Sirinawin, 2007). Hence, it would be possible that the water used for shrimp farming was contaminated with Hg from the

contaminated seawater. In addition, impurities in aqua feeds, fertilizers, lime, and chemicals used in shrimp ponds were a possible source of Hg contamination in shrimp ponds (Lacerda et al., 2011). Currently, organic shrimp farming and integrated aquaculture are becoming attractive ways to reduce the use of chemicals in order to restore the natural environment to create sustainable safe production methods. Using microbes as biological agents for bioremediation or probiotic feed supplements is one of the strategies for ensuring environmentally friendly safe food.

Amongst the microbes, purple nonsulfur bacteria (PNSB) can be employed for this purpose because they normally live in aquatic environment such as ponds and estuaries including shrimp ponds. PNSB have a wide range of growth modes and are able to grow with photoheterotrophic, photoautotrophic, and chemoheterotrophic conditions depending on the light and oxygen conditions (Imhoff, 1995; Panwichian et al., 2010). Several studies have demonstrated that PNSB have the potential to resist heavy metals (Giotta et al., 2006; Panwichian et al., 2010). Due to the response to toxic Hg compounds present in the environments, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity (Barkay et al., 2010). For instance, volatilization is one of the specific resistance mechanisms controlled by the *mer* operon that bacteria use to detoxify inorganic Hg by converting divalent mercury (Hg^{2+}) into the less toxic form of volatile metallic Hg (Hg^{0}), using the mercuric reductase enzyme (Barkay et al., 2003; Zhang et al., 2012). Microorganisms that are resistant to Hg from different environmental regions have been widely investigated (De and Ramaiah, 2007; Zhang et al., 2012; Kurniati et al., 2014); however, no analysis has been reported on Hg resistant PNSB strains from shrimp ponds. Hence, the objectives of this research were first to investigate the distribution of Hg in shrimp ponds in southern Thailand and then to select Hg resistant PNSB strains that could be applied to shrimp cultivation, particularly when shrimp ponds were contaminated by Hg.

Materials and methods

Study areas

The distributions of the shrimp ponds on both the Thai Peninsular coastal areas (Andaman Sea and Gulf of Thailand) where samples were collected are shown in Figure 4-1, and their geographical positions are given in Table 4-1. They were in the districts of Kantang, Sikao, and Yan Ta Khao in the Trang province; the district of Thai Muang in Phang Nga province; the districts of Ranot, Sating Phra, and Tepa in Songkhla province; the districts of Hua Sai and Pak Phanang in Nakhon Si Thammarat province; the district of Pak Phayun in Phatthalung province; and the Mueang district in Pattani province.

Water and sediment sample collecting

Thirty-five samples of water and sediment were collected from various shrimp ponds in southern Thailand after shrimp harvesting (Figure 4-1). Composite subsamples were collected from each pond at various positions following two diagonals and a halfway point to the middle from each bank. The water, roughly 100 mL, was collected at about 50 cm from the surface water while about 100 g of the sediment was collected from the bottom of each pond to a depth of 5 cm. Samples of water and sediment were kept in sterilized glass bottles and stored in an icebox during transportation. All samples were maintained at 4°C before measuring the Hg concentration and to isolate PNSB. Water samples were preserved by adding 5 mL/L of 0.2 N bromine monochloride (BrCl) before mercury analysis.

Hg analysis of samples

All composite subsamples of the water and sediment were combined into a single sample of water or sediment from each pond prior to analysis for Hg. This entailed utilizing the US Environmental Protection Agency Method 1631 for total mercury (Hg_T) analysis in water (USEPA, 2002). The tubes and bottles used for analysis were first cleaned with 4 N HCl at 65°C for 12 h, rinsed three times with deionized water, and were then dried in an oven at 60°C overnight. A 2.5 mL of 0.2 N

BrCl was added into 50-mL portions of the unfiltered water samples to oxidize all forms of Hg to Hg^{2+}. If the yellow color disappeared because of contamination by organic matter or sulfides, more BrCl was added until a permanent yellow color was obtained. Analysis of the Hg_T in the sediments was determined using the USEPA method 1631 (USEPA, 2001). Sediment samples were dried and passed through a 2-mm sieve. Then, 1 g of sample was digested by 9 mL of conc. H_2SO_4/HNO_3 (7:3 v/v) and then placed in a heat blocker at 95 °C for 3 h. To the digest, 40 mL of 0.02 N BrCl was added to destroy the remaining organic matter. The water and sediment samples were maintained at room temperature overnight to accelerate the oxidation reaction. Following oxidation, 0.4 mL of hydroxylamine hydrochloride (NH_2OH-HCl) was added and if necessary more sequentially. Diluted digests of water and sediment were analyzed by the PerkinElmer Flow Injection Mercury System (FIMS) 400 using $NaBH_4$ as the reducing reagent and 3% (v/v) HCl for the carrier solution.

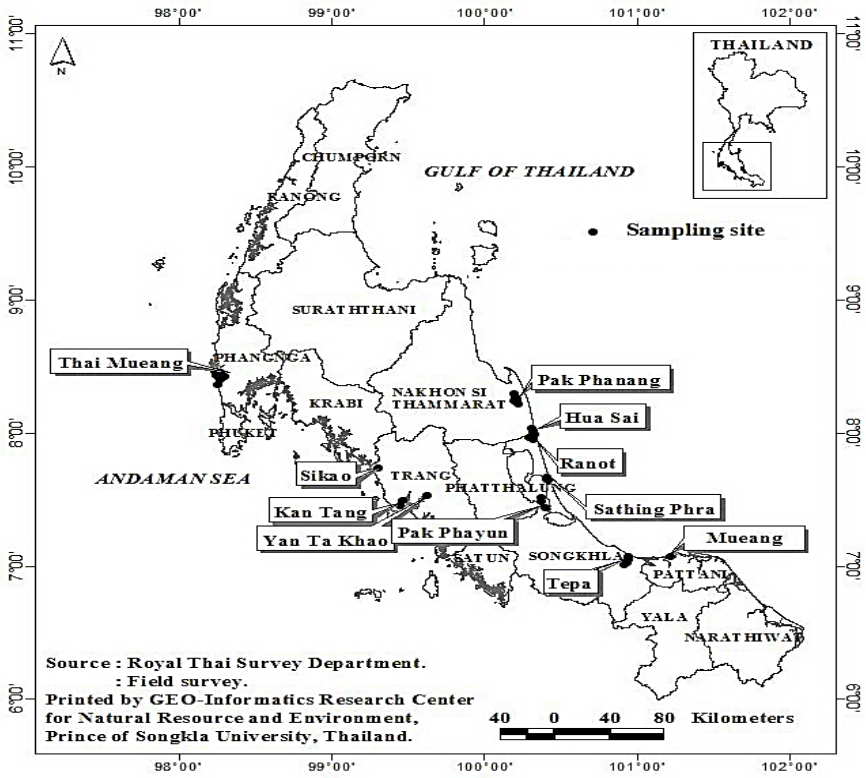

Figure 4-1. Map of sampling sites located on both coastal areas (Andaman Sea and Gulf of Thailand) along the southern coasts of Thailand.

Isolation of PNSB from shrimp ponds

Glutamate acetate (GA) medium was used to isolate PNSB from the sediment and water samples. This medium consisted of 3.8 g sodium glutamate, 5.44 g sodium acetate, 2.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.8 g $(NH4)_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.053 g $CaCl_2 \cdot 2H_2O$, 0.001 g nicotinic acid, 0.001 g thiamine hydrochloride, 0.01 g biotin, 0.012 g $MnSO_4 \cdot 5H_2O$, 0.025 g ferric citrate, 0.95 g $CoCl_2 \cdot 6H_2O$, and deionized water up to 1000 mL, and the pH was adjusted to 7.0. For this study, 2% NaCl was added to the GA broth to provide a simulated medium that was close to that of the shrimp ponds. One milliliter or one gram from each water and sediment subsample was transferred to 17 mL GA broth in a screw cap test tube, and then, sterile liquid paraffin was added to the top of the medium to achieve anaerobic conditions. Finally, cultures were incubated under continuous light with incandescent lamps (light intensity ca. 3000 lux) for 5-7 days. The appearance of a pink, red, or brown color indicated that PNSB had grown; then each culture broth was further utilized to isolate pure colonies by streaking onto GA agar containing 2% NaCl and incubating with the same conditions as previously described. Pure cultures were maintained by stab inoculation of the GA agar containing 2 % NaCl and, after the bacteria grew, were stored at 4°C or in 20% glycerol at -80°C until used.

Inoculum preparation

After two subcultures, inoculation of one loopful of bacterial growth was transferred into a screw cap test tube of GA broth containing 2% NaCl, and all culture tubes were incubated under microaerobic light conditions as described by Panwichian et al. (2010) for 48 h. Each culture broth was adjusted to an optical density at 660 nm (OD_{660}) of 0.5 using sterile GA broth containing 2% NaCl as diluent, and an uninoculated medium was used as the blank. A cell suspension with 0.5 OD_{660} (approximately 1.2×10^8 cell/mL) served as the inoculum, and a 10% inoculum size was applied for all experiments carried out in this study.

Selection of PNSB resistance to salt and Hg

All PNSB isolates were screened for their ability to grow with photoorganotrophic (microaerobic light conditions) and heterotrophic (aerobic dark conditions) to establish if they could be used in ponds that have growing shrimp. A 10% inoculum was added into 18 mL of GA broth containing 2 % NaCl in 50- or 20-mL screw top tubes for incubating under conditions of aerobic dark and microaerobic light, respectively. To set aerobic dark conditions, all cultures were shaken in an incubating shaker at 100 rpm, at 30°C in darkness for 48 h. For the microaerobic light conditions, all cultures were static in tubes exposed to about 3000 lux at room temperature for 48 h. Bacterial growth was measured at an OD_{660} using a spectrophotometer, and any isolates with growth that exceeded 0.50 were selected for salt tolerance studies. The selected isolates were grown in GA broth containing various concentrations (2, 4, 6, 8, and 10%) of NaCl under both incubating conditions. The reason for using the concentrations as described is to follow the salinity conditions in shrimp ponds (Panwichian et al., 2010). The growth inhibition caused by the salt in percentage terms was calculated by comparing growth with a control using no additional NaCl. Salt resistant PNSB strains were selected for their ability to resist Hg in the absence of NaCl and with 3% NaCl under both incubating conditions. For screening the PNSB for resistance to Hg, GA broth containing 398.84 μg/L $HgCl_2$ was used because this was the highest concentration of Hg detected in the shrimp ponds studied. Inhibition of growth by Hg was determined by calculating the percentage difference in growth between those grown with and without Hg. In addition, sets of bacteria grown in GA broth containing $HgCl_2$ and 3% NaCl were conducted to evaluate the impact of both substances on bacterial growth.

Mercury volatilization

Selected PNSB strains from salt and Hg resistance experiments were grown in 10 mL of GA broth containing 3% NaCl under aerobic dark conditions for 48 h. The cell pellet was separated from 2 mL of culture broth by centrifugation at 10,000 rpm for 10 min and washed with phosphate buffer (pH 7), and then, the pellet

was transferred to a microtiter plate followed by adding 200 µL of $HgCl_2$ dissolved in phosphate buffer to obtain a final concentration of 1 mg/L. The method used by Zhang et al. (2012) was modified so that volatilization of $HgCl_2$ was detected by using X-ray film (Kodak), and it was replaced by cutting a Fuji film (Superia 200 CA 135-36), which is the roll film for photography in the dark. Hence, an uninoculated set served as a control. In a dark room, a Fuji film was used to cover the plate that was incubated overnight at a temperature of 30°C. The film was removed from the microtiter plate and then developed. If the PNSB strains could reduce Hg^{2+} to Hg^0 through volatilization, it appeared as a foggy area on the film above a well of the microtiter plate when compared to the control set.

Mercuric reductase activity assay

The method of Glendinning et al. (2005) was used to determine the mercuric reductase activity. Selected strains were grown under aerobic dark and microaerobic light conditions in bottles each with 50 mL of GA broth containing 3% NaCl and 1 mg/L $HgCl_2$ for 96 h. The culture condition was designed to resemble the shrimp pond conditions that normally enrich with nutrients and contain roughly 3% NaCl. Every 12 h, one sampling culture bottle was taken to determine enzyme activity and bacterial growth based on the dry cell weight. Cell pellets were harvested by centrifugation and then washed twice with 10 mL of wash buffer (30 mM Tris/HCl pH 7.6, 20 % sucrose, 1 mM dithiothreitol, 1mM EDTA). The cell pellet was washed again with 10 mL suspension buffer (100 mM sodium phosphate buffer pH 7.0, 1.0 mM β-mercaptoethanol, 0.5 mM EDTA, 0.05 mM phenylmethylsulfonyl fluoride, 0.1 mMbenzamidine) followed by resuspension in 1.0 mL of fresh suspension buffer. Lysis of the cell pellet was achieved using a sonicator, and cell debris was removed by centrifugation. The crude cell extract was stored at 0°C overnight prior to assay. A 100-µL sample of the cell free extract was well mixed with 800 µL of assay buffer (0.1 M sodium phosphate buffer pH 7.0, 1.0 mM β-mercaptoethanol, 0.5 mM EDTA, 0.2 mM NADPH) and preincubated at 30°C for 5 min. This was followed by adding 100 µL $HgCl_2$ solution to obtain a final concentration of 1 mg/L and again was well mixed. A decrease in the NADPH after adding $HgCl_2$ or sterile distilled water (control

set) was measured at A_{340} after varying reaction times: 2, 4, and 6 min, and the amount of NADPH-dependent nonspecific reductase activity was also determined in parallel in the absence of Hg^{2+}. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the Hg^{2+}-dependent oxidation of 1.0 nmol of NADPH per minute (Bafana et al., 2010). The protein content in the crude cell extract was determined using the BCA Protein Assay Kit (Novagen) following the manufacturer's instructions.

MIC, MBC, and IC_{50} values of Hg on PNSB

Selected PNSB strains were also investigated for the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) including the half maximal inhibitory concentration (IC_{50}) to Hg. All selected strains were grown in GA broth containing 3 % NaCl and $HgCl_2$ concentrations that ranged from 0 to 4.0 mg/L under aerobic dark and microaerobic light conditions for 72 h. Bacterial growth was observed based on turbidity by measuring at an OD_{660} using a spectrophotometer, and viable cells were counted on GA agar containing 3% NaCl. The lowest concentration of Hg at which no turbidity was reported was the MIC. The culture broths that demonstrated no turbidity were used for determining the MBC by streaking onto GA agar containing 3 % NaCl plates, and the lowest concentration with no growth was reported as the MBC. To determine the IC_{50}, the percentages of growth inhibition

Bacterial identification

Identification of the bacterial isolates was made using 16S rDNA sequence analysis by growing selected PNSB strains in GA medium containing 3% NaCl under microaerobic light conditions for 48 h. Then, 1.5 mL of each cell suspension was harvested by centrifugation at 10,000 rpm for 5 min in a microcentrifuge for DNA extraction following the method of Zhou et al. (1996). The cell pellet was mixed with 600 µL of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA, phosphate buffer pH 8.0, 1.5 M NaCl, 1 % CTAB) and 10 µL proteinase K and then incubated at 37°C for 30 min. A 100 µL amount of 20% SDS

was added and further incubated at 65°C for 30 min, after which cell debris was removed by centrifugation at 10,000 rpm for 5 min. The supernatant was collected and mixed with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) and further centrifuged at 13,500 rpm for 5 min. The aqueous phase was recovered, and a 0.6-mL volume of isopropanol was added and then left at -20°C to allow for precipitation of the DNA. The DNA was obtained by centrifugation at 13,500 rpm for 10 min and rinsed with absolute ethanol, air-dried, and dissolved in distilled H_2O. Genomic DNA was amplified by PCR using the primers: 8F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) that target the conserved regions of 16S ribosomal RNA (rRNA) gene in bacteria. The PCR amplification mixture contains 10 μM of each primer, 1.0 U of Taq DNA polymerase, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and DNA template. Reaction cycles in a thermal cycle used the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 50 s, 55°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min. The PCR product was separated by electrophoresis on agarose gels and then stained in ethidium bromide and observed on a UV transilluminator. The purified PCR products were directly cloned using a pGEM-T Easy Vector Cloning Kit (Promega) as described in the manufacturer's instructions. The insertions of the 16S rRNA genes were sequenced with the automated DNA sequencer. The 16S rRNA gene sequencing was compared with the GenBank database and submitted to the NCBI website. Phylogenetic analysis was conducted based on the maximum likelihood method using the free software MEGA 6.

Statistical analysis

All experiments in this study were conducted in three replicates. Medians, mean value, and the standard deviations are presented. Analysis of the data using one-way ANOVA and significant differences among means were analyzed using the Duncan's multiple range test at a P value < 0.05.

Results

Hg concentrations in water and sediment samples

Table 4-1 shows the concentrations of Hg_T in the water and sediment samples from shrimp ponds located around the coastal areas of southern Thailand. The concentrations of Hg_T in water ranged from <0.0002 to 0.037 μg/L. The median concentration of Hg_T in water was 0.006 ± 0.005 μg/L. The highest concentration of Hg_T in water was found in the sample collected from Sikao district in Trang province. In contrast, the lowest Hg_T concentration was observed in water samples collected from the district of Pak Phanang in Nakhon Si Thammarat province and the districts of Kantang and Yan Ta Khao in Trang province.

On the other hand, Hg_T concentrations in the sediment samples were very different and were all higher than the water samples. The Hg_T concentrations in the sediment samples from shrimp ponds ranged from 30.73 to 400 μg/kg dry weights, and the median concentration was 182.19 ± 2.50 μg/kg dry weight. The Hg_T concentration from the district of Sating Phra in Songkla province was the highest while the lowest concentration was detected in the district of Pak Phanang in Nakhon Si Thammarat province.

Table 4-1. Total Hg concentrations in water and sediment samples from shrimp ponds that were located on the coastal areas along the south of Thailand.

Area (n = number of samples)	Location	Mean (range)	
		Water (µg/L)	Sediment (µg/kg dry weight)
Ranot (n = 4)	7° 53.722'N, 100° 19.518'E	0.017 (0.013 - 0.021)	165.79 (138.98 - 185.48)
Sating Phra (n = 3)	7° 32.624'N, 100° 24.880'E	0.006 (0.005 - 0.008)	276.18 (188.75 - 398.84)
Tepa (n = 4)	6° 52.979'N, 100° 56.333'E	0.008 (0.007 - 0.008)	207.81 (183.13 - 251-25)
Hua Sai (n = 4)	7° 57.532'N, 100° 19.568'E	0.005 (0.004 - 0.006)	179.74 (80.65 - 242.77)
Pak Panang (n = 6)	8° 14.973'N, 100° 13.960'E	< 0.002 (<0.0002 - 0.002)	57.77 (30.73 - 78.65)
Kantang (n = 2)	7° 19.910'N, 99° 27.597'E	< 0.002	201.41 (197.19 - 205.63)
Sikao (n = 1)	7° 38.470'N, 99° 19.062'E	0.037	55.21
Yan Ta Khao (n = 1)	7° 22.914'N, 99° 36.743'E	< 0.002	92.19
Pak Phayun (n = 3)	7° 20.516'N, 100° 23.589'E	0.004 (0.003 - 0.007)	186.58 (100.81- 229.57)
Mueang Pattani (n = 1)	6° 52.933'N, 101° 14.052'E	0.006	369.62
Thai Muang (n = 6)	8° 21.921'N, 98° 16.015'E	0.010 (0.008- 0.012)	115.10 (70.21- 200.00)
Median ± median absolute deviation		0.006 ± 0.005	182.19 ± 72.50
Thai standard guidelines		0.10[a]	≤ 23 mg/kg[b]
Hong Kong			0.5 – 1 mg/kg[c]
Canadian quality guidelines		0.10[d]	2.0 mg/kg[d]

[a]Pollution Control Department (2006)

[b]Pollution Control Department (2004)

[c]HKGS (1998)

[d]Gaudet et al. (1995)

Isolation and selection of PNSB resistant to salt and Hg

A total of 131 PNSB strains were isolated from the shrimp ponds. There were 89 strains (67.94%) and 42 strains (32.06 %) from the water and sediment samples, respectively. Of these, only 99 strains (75.57%) produced growth that exceeded an OD_{660} > 0.5 in GA broth containing 2% NaCl in both incubating conditions (aerobic dark and microaerobic light) for 48 h.

There were 99 PNSB strains obtained from the primary screening process that were resistant (growth inhibition < 50%) to 2 and 4% NaCl. The growth of the 99 PNSB strains in GA broth containing 4% NaCl in conditions of aerobic dark and microaerobic light was separated into three levels; 1 strain was for excellent (OD_{660} > 1.5), 16 strains for very good (OD_{660}, 1.1-1.5), and 82 strains for only good (OD_{660}, 0.5-1.0). The numbers of resistant strains quickly decreased at higher concentrations of NaCl; 89 strains were resistant to 6%. Thirty-nine strains were resistant to 8%, and only 31 strains were resistant to 10% NaCl. However, it was found that 19 of the 31 strains exhibited a growth inhibition of less than 20% with 10% NaCl.

The 17 strains with the better growth were selected for testing their ability to resist Hg and a mixture of Hg and high concentrations of NaCl. Of the 17 strains tested in both conditions of aerobic dark and microaerobic light, the presence of 3% NaCl reduced the adverse effect of 398.84 μg/L Hg^{2+} (the highest concentration found in the sediment sample) on the growth, as inhibition was less than 30% (Figure 4-2). In contrast, in the absence of NaCl, a higher growth inhibition was found (data not shown). Under conditions of $HgCl_2$ mixed with 3% NaCl, 12 strains were more highly resistant to $HgCl_2$ (growth inhibition < 10%) than the other 5 strains (Figure 4-2). The growth of all the strains tested in the GA broth with 3% NaCl was better than growth in the GA broth alone (data not shown). This indicated that NaCl stimulated the growth of the PNSB strains tested, and this might be the main reason why the PNSB were more resistant to Hg in the presence of 3% NaCl. In addition, resistance to Hg^{2+} in the presence of 3% NaCl and 398.84 μg/L $HgCl_2$ by the 12 PNSB strains was better when grown in aerobic dark conditions than in microaerobic light

conditions (Figure 4-2). These 12 selected PNSB strains as shown with an asterisk in Figure 4-2 were selected to analyze their ability to catalyze mercury volatilization.

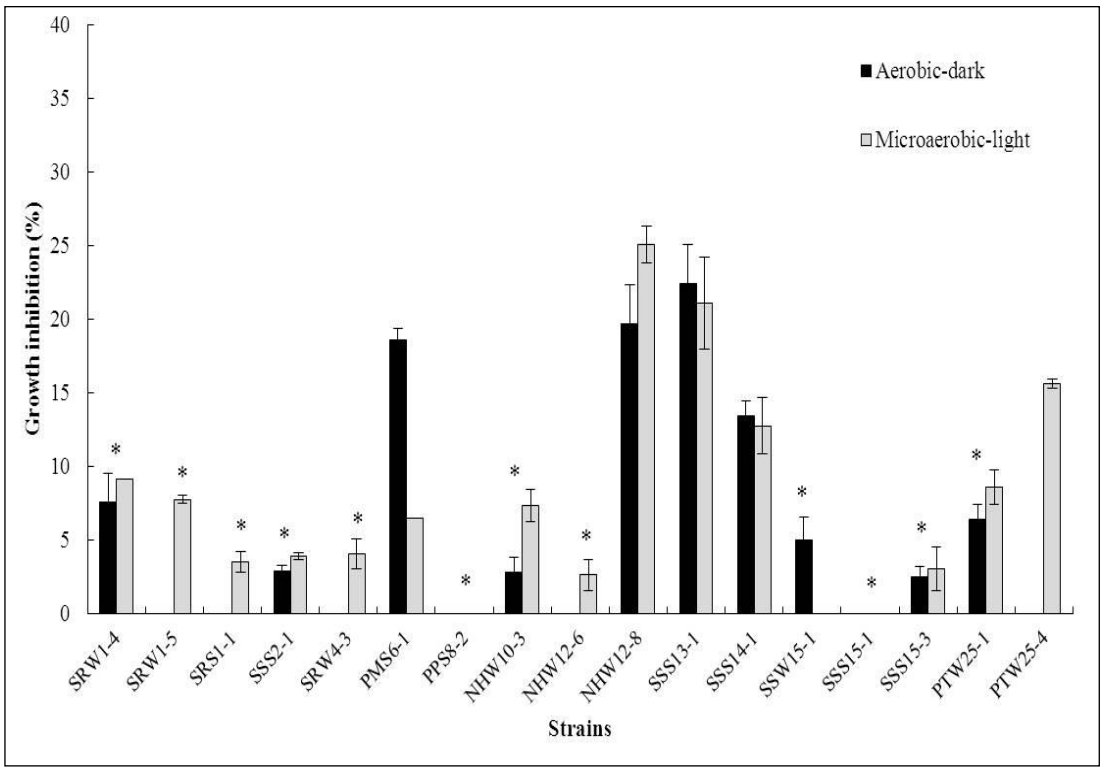

Figure 4-2. The effect of Hg on the growth of the PNSB isolated from contaminated shrimp ponds in GA broth containing 398.84 μg/L $HgCl_2$ and 3 % NaCl under aerobic dark and microaerobic light conditions for 48 h. Each bar represents the mean of three replicates ± standard deviation. The asterisk indicates the strains that were selected for further studies.

Hg volatilization

A cut Fuji film was used to detect Hg volatilization by the 12 Hg resistant strains from 1 mg/L of $HgCl_2$ in the presence of 3% NaCl, and a foggy area was produced by six of the strains: SRW1-5, SRS1-1, SSS2-1, NHW12-6, SSW15-1, and PTW25-1 (Figure 4-3) but not by the others.

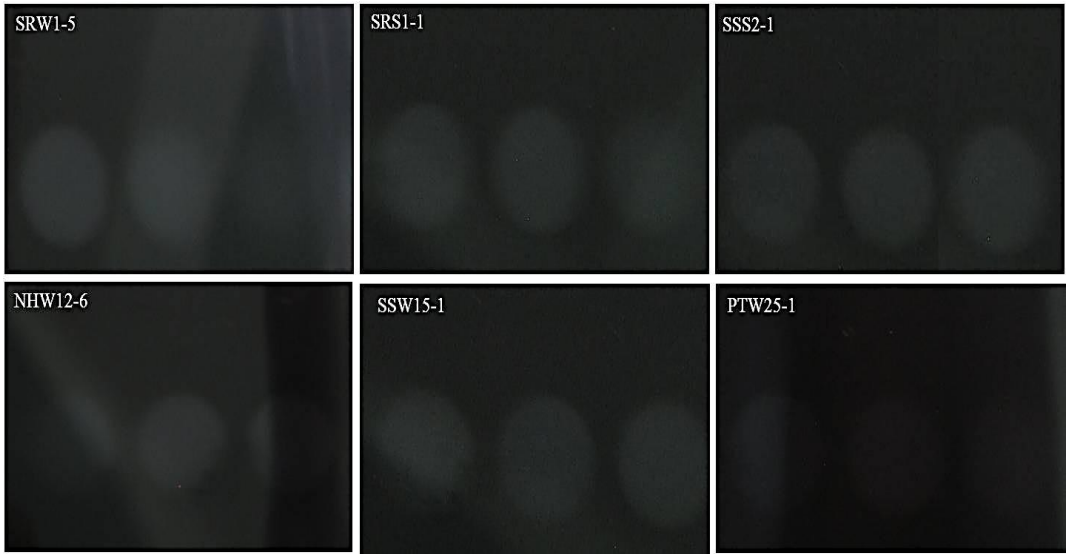

Figure 4-3. Hg volatilization of the selected PNSB strains in a 96-well microtiter plate containing phosphate buffer (pH 7), 1.0 mg/L $HgCl_2$, and 3 % NaCl covered with the Fuji film overnight under aerobic dark conditions.

Mercuric reductase activity

Figure 4-4 shows the results of the mercuric reductase activity of the six selected PNSB strains grown with aerobic dark and microaerobic light conditions. The mercuric reductase activity of all the six selected PNSB, tested under aerobic dark conditions, was significantly higher than that found in cells grown with microaerobic light conditions ($P < 0.05$). Strain SSW15-1 had the highest enzyme activity under both incubating conditions at 48 h (15.75 and 8.50 U/mg protein). Mercuric reductase activity under dark condition was in the order of SSW15-1, SRW1-5, SSS2-1, SRS1-1, PTW25-1, and NHW12-6, i.e., 15.75, 12.62, 12.16, 10.56, 10.26, and 9.58 U/mg protein, respectively. On the other hand, under light condition, strains SSW15-1 showed the highest activity of mercuric reductase followed by SRW1-5, PTW25-1, and the other strains. The mercuric reductase activity under both incubating conditions was highest at 48 h for strain SSW15-1 while it was at 60 h for strains SRW1-5 and SSS2-1. However, strains SRS1-1 and NHW12-6 revealed their highest activity at 48 h after growth with aerobic dark conditions but at 60 h after

growth with microaerobic light conditions. In contrast, strain PTW25-1 produced its highest activity at 48 h when grown with dark condition but at 36 h when grown with light conditions (data not shown). The result proved that volatilization by mercuric reductase is one of the Hg resistance mechanisms in the PNSB tested; and the following strains: SSW15-1 (15.75 U/mg protein), SRW1-5 (12.62 U/mg protein), SSS2-1 (12.16 U/mg protein) with the highest mercuric reductase activity were selected to determine the MIC, MBC, and IC_{50} values for $HgCl_2$.

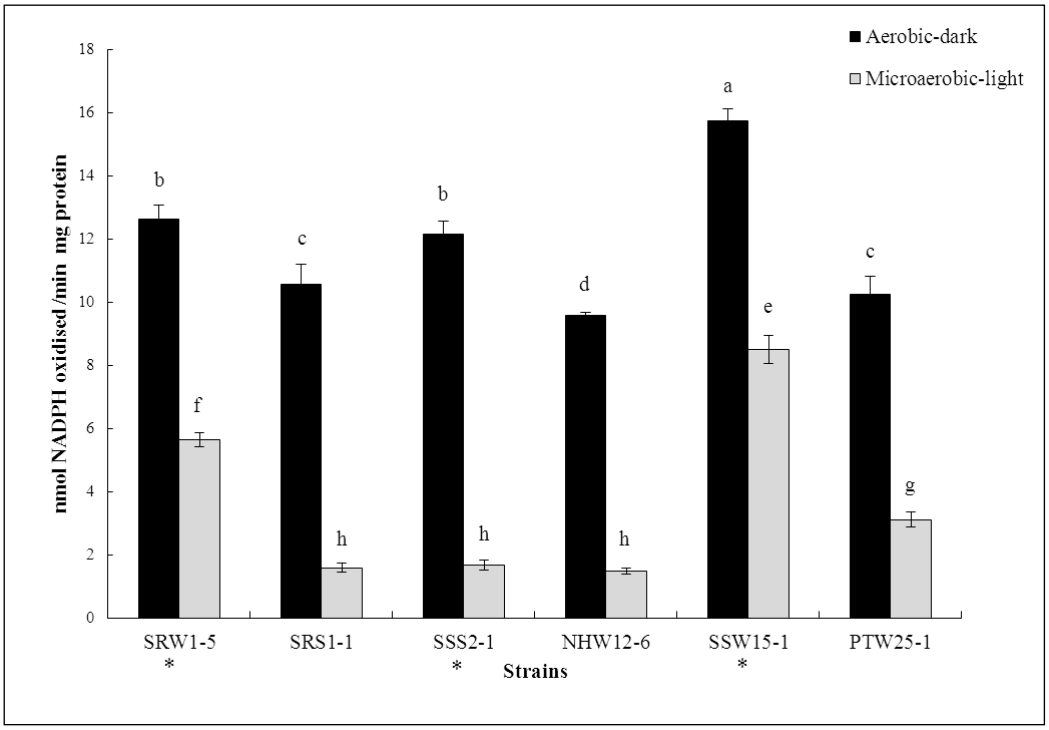

Figure 4-4. Mercuric reductase activity of the selected PNSB strains in GA broth containing 1 mg/L $HgCl_2$ and 3 % NaCl under aerobic dark and microaerobic light conditions. Each bar represents the mean of three replicates ± standard deviation. Different lowercase letters above bars indicate significant differences ($P < 0.05$). The asterisk indicates strains that were selected for further studies

Values of MIC, MBC, and IC_{50}

Strain SSS2-1 was the most resistant to Hg^{2+} when grown with GA broth containing 3% NaCl and various concentrations of $HgCl_2$ under both incubating conditions, with an MIC value of 3.20 mg/L when grown with light conditions, followed by strain SRW1-5 (2.40 mg/L) and strain SSW15-1 (1.60 mg/L) (Table 4-2). Under both incubating conditions, the MIC values for strains SRW1-5 and SSW15-1 were also their MBC values. However, the MBC value of strain SSS2-1 under dark conditions (4.00 mg/L) was higher than that for light conditions (3.20 mg/L) and this corresponded with its mercuric reductase activity under both incubating conditions (Figures 4-2 and 4-4). In addition, the growth inhibitions of various Hg concentrations on three selected PNSB strains were calculated for their IC_{50} values under both incubating conditions as shown Figure 4-5. The IC_{50} values of strains SSS2-1, SRW1-5, and SSW15-1 under dark condition were 2.16, 1.23, and 0.90 mg/L while they were 1.66, 1.11, and 0.80 mg/L under light conditions, respectively (Table 4-2).

Table 4-2. MIC, MBC and IC_{50} values of the selected PNSB strains in GA broth containing various concentrations of $HgCl_2$ (0-4.00 mg/L) and 3% NaCl under conditions of aerobic dark and microaerobic light for 72 h.

Strain	Sample	Area		Aerobic dark			Microaerobic light		
		District	Province	MIC (mg/L)	MBC (mg/L)	IC_{50} (mg/L)	MIC (mg/L)	MBC (mg/L)	IC50 (mg/L)
SRW1-5	Water	Ranot	Songkhla	2.40	2.40	1.63	2.40	2.40	1.37
SSS2-1	Sediment	Sating Phra	Songkhla	3.20	4.00	2.23	3.20	3.20	1.78
SSW15-1	Water	Sating Phra	Songkhla	1.60	1.60	1.22	1.60	1.60	0.67

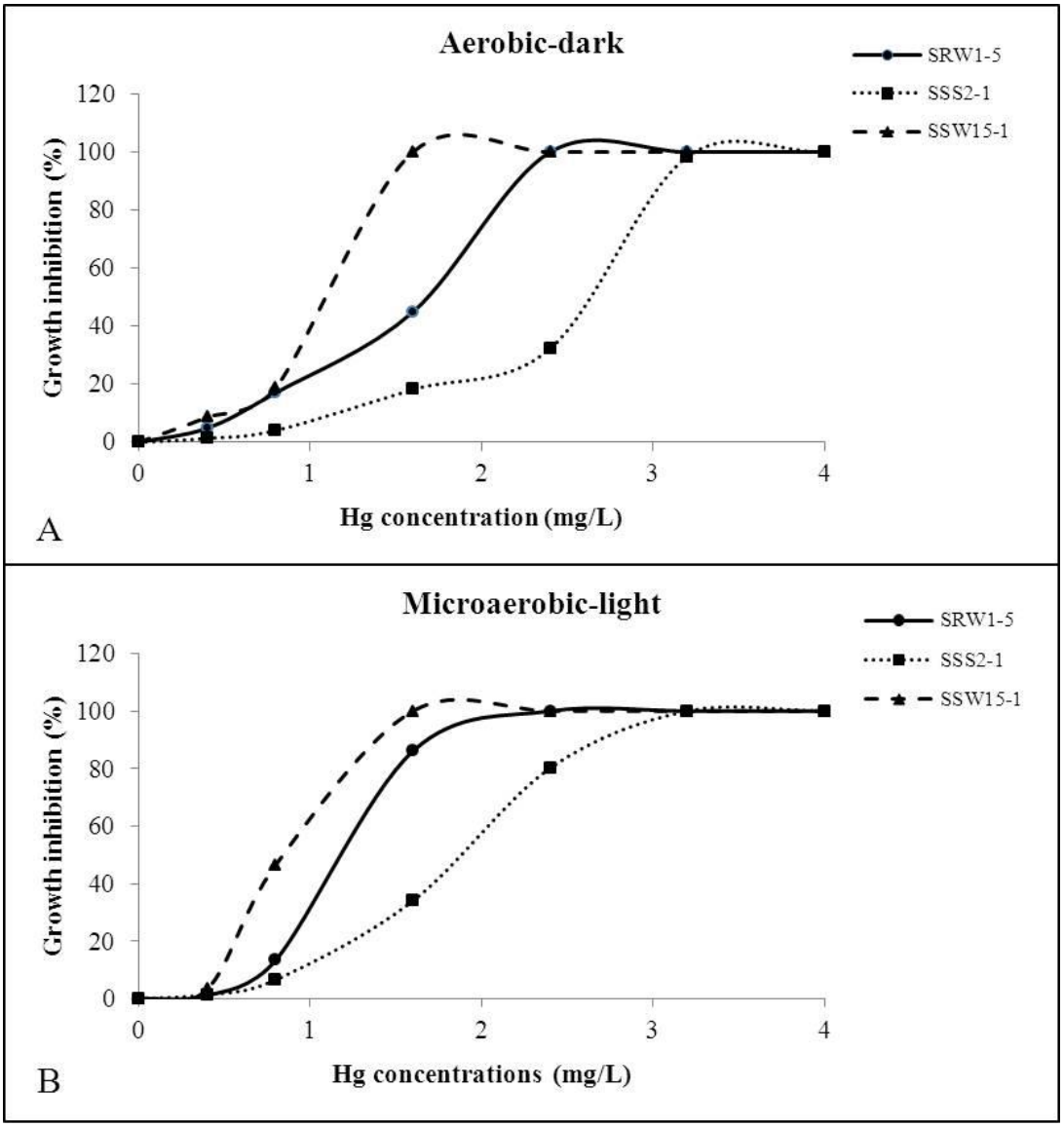

Figure 4-5. The growth inhibition of PNSB strains when growing in GA broth containing various concentrations of $HgCl_2$ ranged from 0-4 mg/L and 3% NaCl under conditions of aerobic dark (A) and microaerobic light (B) for 72 h.

Bacterial identification

The three selected strains, SRW1-5, SSS2-1, and SSW15-1, were identified on the basis of their 16S rRNA gene sequences and submitted to the GenBank database. The submission numbers are KJ508085, KJ508087, and KJ508089, respectively (Figure 4-6). Strains SSS2-1 and SSW15-1 were the closest

relatives to *Afifella marina* (former name, *Rhodobium marinum*) DSM 2698T (HE978266) (Hiraishi et al. 1995; Urdiain et al. 2008) while strain SRW1-5 was very close to *Rhodovulum sulfidophilum* JA198 (AM696694) showing a 99 % homology of the three strains (Figure 4-6).

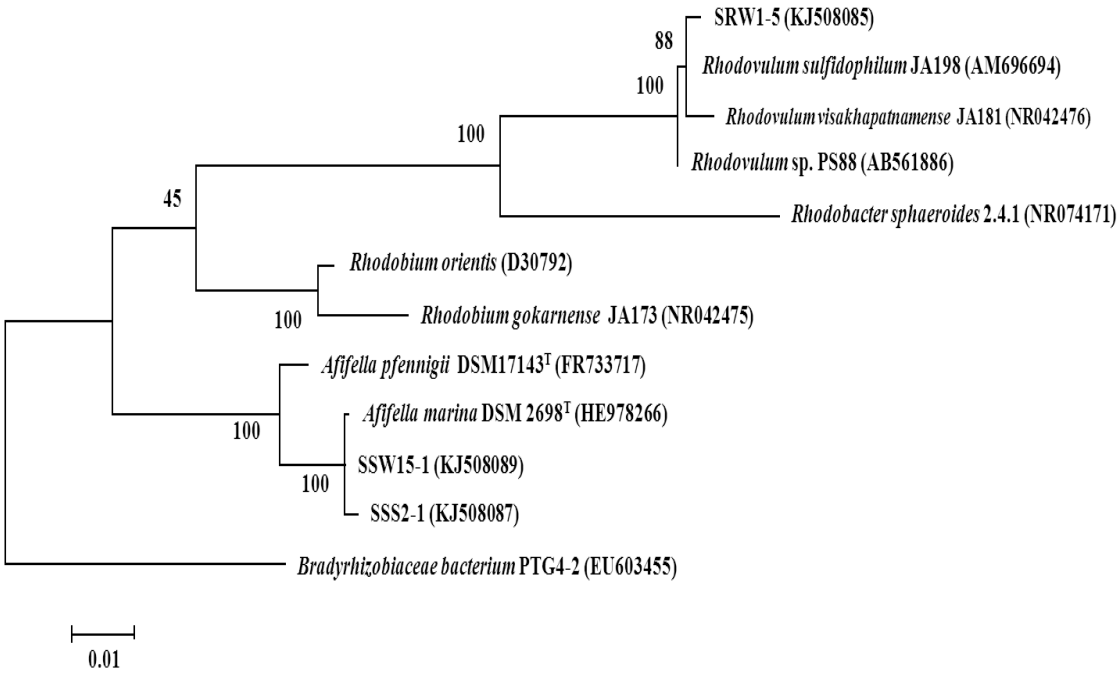

Figure 4-6. Maximum likelihood method with bootstrapping showing the phylogenetic tree of selected PNSB strains based on the 16S rRNA gene sequencing. The percentage of bootstrap is indicated for each node. The scale bar represents the number of substitutions per site.

Discussion

Hg concentration in water and sediment samples

The levels of Hg_T contamination in the water samples of all studied areas were significantly lower than the standard guideline value of 0.10 µg/L as recommended by the standard guidelines of Thailand's Pollution Control Department

(2006) and the Canadian Environmental Quality Guidelines (Gaudet et al., 1995). According to standard guidelines, the values of Thailand's Pollution Control Department (2004) and the Canadian Environmental Quality guidelines (Gaudet et al., 1995) for agricultural and residential land uses are 23 and 2 mg/kg, respectively, while the standard guideline developed by Hong Kong's government for sediment is in the 0.5-1.0 mg/kg range (HKGS, 1998). Hence, Hg_T concentrations in all sediment samples from shrimp ponds were smaller than the standard guidelines. However, if there is no process to remediate and manage the safety of contaminated shrimp ponds, this may result in higher levels of contaminated Hg in the shrimp ponds. Consequently, serious problems could emerge in people's health through the food chain when Hg undergoes bioaccumulation in the shrimp.

The main source of Hg contamination in the water samples studied was the seawater used for shrimp cultivation, and this is in accordance with previous studies that have reported Hg contamination in seawater in the Gulf of Thailand and the Andaman Sea (Thongra-ara and Parkpianb, 2002; Sompongchaiyakul and Sirinawin, 2007). This is due to oil and natural gas production in the Gulf of Thailand and runoff of Hg from the mainland (Thongra-ara and Parkpianb, 2002; Cheevaporn and Menasveta, 2003). The explanation for larger amounts of Hg found in the sediment samples lies in the distribution coefficient or concentration in the particulate phase divided by the concentration in the dissolved phase (Kd) of Hg in water being very high. It means that most of Hg was attached to particles of the suspended sediment (Domagalski, 2001). These particles can be deposited on the bottom, and this process enriches the sediment after repeated shrimp cultivation. Once in the sediment, diagenetic processes can modify that equilibrium: Hg can react with hydrogen sulfide that occurs via the anaerobic respiration of sulfate-reducing bacteria to precipitate as HgS in the sediment (Gabriel and Williamson, 2004). Therefore, the amount of Hg in the sediment was higher than that in the water column. In addition, impurities in the aqua feed, fertilizers, lime, and chemicals used in shrimp cultivation are other possible sources of Hg present in the water samples collected from the shrimp ponds (Lacerda et al., 2011). This agrees with our finding that the largest amount of Hg_T in water samples was found in the shrimp ponds located on the coast

near the Andaman Sea (Figure 4-1 and Table 4-1) since in that area, there was no natural gas production. Moreover, our results established that Hg contamination in the water samples was not very different from sampling sites located in the same district although it was different from other districts. This outcome was due to the water being used in the shrimp ponds in the same district coming from the same canal or sea area.

Isolation and selection of PNSB resistant to salt and Hg

PNSB were more prevalent in the aerobic water column than in the anaerobic sediment. This is due to the effect of exposure to sunlight, as the sediment is some distance from the sunlight, and sunlight penetration is compromised by the turbidity in the water column. Most of the 17 strains were able to grow in GA broth containing 6, 8 and 10 % NaCl, these indicated selected PNSB strains could survive and grow in most shrimp ponds. In addition, all the PNSB strains tested grew in 4% NaCl, and this was expected as the average salt content of shrimp ponds from which they were isolated was approximately 3.5%. However, the highest NaCl level detected in the water samples from shrimp ponds was approximately 8.5% (Panwichian et al., 2010). It is known that some genera of marine anoxygenic phototrophs including PNSB in the group of *β-Proteobacteri*a can grow in from 1 to 12% NaCl (Srinivas et al., 2006). Moreover, it was surprising that all 12 selected Hg resistant strains were isolated from shrimp ponds other than the one that contained the highest level of Hg. Hence, perhaps, resistance to Hg was a normal constitutive characteristic of many PNSB and not necessarily related to the organisms being selected froma site contaminated by Hg. A similar observation had been made by Zeyaullah et al. (2010). However, it is also possible that the water used in the shrimp ponds came from seawater and this was probably the original source of the isolated PNSB that were originally from a marine source and possibly contaminated by Hg. This explanation was later confirmed by the results of the bacterial identification (provided later).

Hg volatilization

These six PNSB strains therefore produced the enzyme mercuric reductase that transformed Hg^{2+} into Hg^{0}, and then, the Hg^{0} diffused out of cell by volatilization to produce the foggy area on the film. The foggy area on the film was the result of reducing Ag^{+} in the film by Hg^{0} that was released from the cells (De and Ramaiah, 2007; Zhang et al., 2012). The other six strains that resisted Hg obviously used alternative mechanisms other than the volatilization detoxification mechanism. One possible example is by using a by-product of normal metabolic processes (Glendinning et al., 2005).

Mercuric reductase activity

Some Hg resistant bacteria use a genetically encoded detoxification mechanism, which is specifically induced in response to Hg (Glendinning et al., 2005). Volatilization is one of the specific resistance mechanisms to detoxify Hg^{2+} to Hg^{0} by mercuric reductase with its coenzyme NADPH (De and Ramaiah, 2007; Bafana et al., 2010; Zhang et al., 2012). Consequently, Hg^{2+} was reduced to the less toxic Hg^{0} (volatile mercury) that diffused from the cells into the gas phase. The higher activity of mercuric reductase when grown with dark condition could be the effect of light condition. In addition mercuric reductase, phototrophs may use some alternative mechanisms to resist Hg as discussed by Barkay et al. (2010) as follows. The intracellular deposit of metacinnabar which is a process that depends on the intracellular thiol pool has been proposed as an Hg resistance mechanism for many strains of cyanobacteria. Reduction of Hg^{2+} to Hg^{0} may be coupled directly to photosynthesis in photosystem II of oxygenic phototrophs. In addition, in the presence of chromophoric dissolved organic matter, photoreduction is the main pathway for reducing Hg^{2+} to Hg^{0} in the photic zone. Our selected PNSB strains are anoxygenic phototrophs that may utilize some of the above Hg resistance mechanisms in light condition; this led to a significant decrease in mercuric reductase compared to the dark condition. The reduction of Hg^{2+} to Hg^{0} is a genetically resistant mechanismthat is controlled via the *mer* operon. In this study, the detection of the mercuric reductase enzyme established that one mechanism of Hg resistance in PNSB was related to the

merA gene, which has not been previously observed in PNSB (Barkay et al., 2010). Hence, the results of this study exposed an interesting area for further studies to clearly understand the Hg resistance mechanism in PNSB, and molecular techniques will be used to investigate the *merA* gene in our selected PNSB soon.

When compared with previous studies of Hg resistant bacteria, the mercuric reductase activity of the PNSB strains here was higher than those of other bacterial strains, i.e., *Bacillus pallidus* and *Ureibacillus thermosphaericus* which had negligible mercuric reductase activity (Glendinning et al., 2005). In contrast, *Methylococcus capsulatus* had a higher activity of this enzyme (352 ± 18 U/mg protein) (Boden and Murrell, 2011). However, in our study, the selected PNSB strains were grown in the medium containing relatively low concentrations of $HgCl_2$ (1 mg/L) when compared to other studies and some activity may be inducible.

Values of MIC, MBC, and IC_{50}

The MIC value in each strain was similar for both incubating conditions; however, the IC_{50} values in the dark condition were higher than those found in the light condition (Table 4-2). This was due to the fact that the three selected strains grew better in dark condition than in light condition; these results could be related to their mercuric reductase activity, as a higher activity was observed under dark conditions. It should be noted that the MIC values of these selected strains were roughly 4 to 10 times higher than the Hg_T content detected in the shrimp ponds. This indicated that our selected PNSB strains could easily survive in Hg contaminated shrimp ponds. As previously mentioned, the MIC values of the selected PNSB strains were similar in both conditions, but this was in contrast to the mercuric reductase activity that showed higher activity in the dark conditions. This indicated that the selected PNSB strains not only were resistant to Hg via volatilization and reduction, but might also have other mechanisms for Hg resistance. It is well recognized that microbes can be resistant to toxic metals using many mechanisms such as efflux of metal ions, the accumulation, and complexation of the metal ions inside the cell, including the structure of the cell envelope that can bind metal ions (Haferburg and

Kothe, 2007). Future studies should consider what kind of Hg resistance or tolerance mechanisms is important for surviving in each set of growing condition for PNSB.

Bacterial identification

The strains SSS2-1 and SSW15-1 were isolated from the sediment and water in shrimp ponds located in the district of Sating Phra in Songkhla province while strain SRW1-5 was isolated from a water sample located in the Ranot district in Songkhla province. Hence, these strains can potentially serve for the bioremediation of Hg contaminated water and sediment in the shrimp ponds. The genotypic and phenotypic strains SSS2-1 and SSW15-1 were similar to *A. marina* DSM 2698T (HE978266), so they are probably originally marine bacteria (Garrity et al., 2005). The strain HE978266 was isolated from coastal seawater in Japan, acting as a facultative aerobic photoheterotroph under anaerobic light or aerobic dark conditions, and achieved maximum growth in the presence of 4 to 5% NaCl (Hiraishi et al., 1995). However, the source for *R. sulfidophilum* JA198 has not been published.

The detection of Hg volatilization and the activity of mercuric reductase enzyme in the three selected PNSB strains revealed the presence of the Hg resistance mechanism that was related to the *merA* gene coding for the mercuric reductase enzyme. However, we have not found any reports that the *merA* gene was previously detected in PNSB. The whole genome sequences of *R. sulfidophilum* DSM 1374 (BASI01000001-01000009) have been studied and deposited at GenBank (Masuda et al., 2013); however, after our checking, there was only mercuric resistance operon regulatory protein (*merR* gene) detected. The *merR* gene is metalloregulatory DNA-binding protein that binds to the promoter/operator region and structural gene transcription (Barkay et al., 2003). Based on the above results, there is a high possibility that our selected PNSB have the *merA* gene including other genes in the *mer* operon; hence, the presence of all the *mer* genes is now being investigated in our current work. If all the *mer* genes are detected, it will be the first report of the *mer* gene operon in PNSB. Most investigations of Hg resistant microorganisms have an aim to develop methods for Hg bioremediation, and the reduction of Hg^{2+} via mercuric reductase enzyme is one such method (Glendinning et al., 2005). Hence, in

the cases described here, strains SRW1-5, SSS2-1, and SSW15-1 would seem to have the potential to remediate Hg contaminated shrimp ponds because they demonstrated excellent growth (OD_{660} = 1.1-1.5) in the presence of Hg. The highest resistance to Hg^{2+} was observed when they were grown with 3% NaCl under both incubating conditions that resembled shrimp pond conditions. Furthermore, other studies have shown that PNSB have the potential to remove other heavy metals (Seki et al., 1998; Bai et al., 2008; Panwichian et al., 2010). Hence, the ability of selected PNSB strains for bioremediation will be studied to confirm their application in metal-contaminated shrimp ponds.

CHAPTER 5

Mercury resistant genes in purple non sulfur bacteria isolated from mercury contaminated shrimp ponds

Abstract

Three mercury (Hg) resistant strains of purple nonsulfur bacteria (PNSB) named *Rhodovulum sulfidophilum* SRW1-5 and *Afifella marina* SSS2-1 and SSW15-1 previously isolated from contaminated shrimp ponds were used to investigate Hg resistant genes for a better understanding of their Hg resistant mechanisms. The gene coding for MerR family transcriptional regulator protein were detected in all strains, while *merR* coding for mercuric resistance operon regulatory protein were detected in strains SSS2-1 and SSW15-1. Both these genes were closely related to the *Sphingomonas* sp. MM-1 showing 99% similarity. In addition, Hg transporter gene (*merT*) was detected in both strains of *A. marina* and showed a 96% similarity with *Novosphingobium pentaromativorans* US6-1. A part of genes analysis between the *merA* and *merD* was also detected in three strains, and they showed 94% similarity with *Halothiobacillus neapolitanus* C2 for the genes in strain SRW1-5, while in strains SSS2-1 and SSW15-1 there was a 100% similarity with *Pseudomonas aeruginosa* IOMTU 133. The genes coding for transporter permease protein were also detected in three strains. The genes in strain SRW1-5 were closely related to those in *Rhodobacter capsulatus* SB 1003 (79% similarity), while strains SSS2-1 and SSW15-1 were closely related to those from *Halomonas elongata* DSM 2581 (78% similarity). Moreover, the multiple alignments of MerR and MerT proteins diverged from other organisms in the *α-Proteobacteria*, and the highest homology was with *Sphingomonas* sp. (AGH51565) and *N. pentaromativorans* (AIT82359), respectively.

Keywords: mercury, *mer* operon, mercury resistant bacteria, purple nonsulfur bacteria

บทคัดย่อ

ได้นำแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์จำนวนสามสายพันธุ์ที่สามารถต้านทานปรอทได้ ได้แก่ *Rhodovulum sulfidophilum* SRW1-5 และ *Afifella marina* SSS2-1 และ SSW15-1 ซึ่งแยกได้จากนากุ้งที่ปนเปื้อนปรอทมาศึกษายีนที่ต้านทานปรอทเพื่อทำความเข้าใจเกี่ยวกับกลไกการต้านทานปรอทของเชื้อ พบว่าแบคทีเรียทั้งสามสายพันธุ์มียีนที่ควบคุมการสร้างโปรตีน MerR ซึ่งยีนที่ควบคุมการสร้างโปรตีน MerR ของกลไกการต้านทานปรอทถูกตรวจพบในแบคทีเรียสายพันธุ์ SSS2-1 และ SSW15-1 เท่านั้นโดยยีนทั้งสองมีความเหมือน 99 เปอร์เซ็นต์กับยีนของ *Sphingomonas* sp. MM-1 นอกจากนี้มีการตรวจพบยีนที่เกี่ยวข้องกับการขนส่งปรอท (*merT*) ใน *A. marina* ทั้งสองสายพันธุ์ โดยยีนดังกล่าวมีความเหมือน 96 เปอร์เซ็นต์กับยีนของ *Novosphingobium pentaromativorans* US6-1 และยังตรวจพบยีนที่ตำแหน่งระหว่าง *merA* และ *merD* ในแบคทีเรียทั้งสามสายพันธุ์ โดยที่ยีนดังกล่าวของสายพันธุ์ SRW1-5 มีความเหมือน 94 เปอร์เซ็นต์กับยีนของ *Halothiobacillus neapolitanus* สายพันธุ์ C2 ส่วนยีนของสายพันธุ์ SSS2-1 และ SSW15-1 มีความเหมือน 100 เปอร์เซ็นต์กับยีนของ *Pseudomonas aeruginosa* สายพันธุ์ IOMTU 133 นอกจากนี้ยังตรวจพบยีนที่เกี่ยวข้องกับการสร้างโปรตีนเพื่อส่งผ่านสาร (transporter permease protein) ของทั้งสามสายพันธุ์ โดยยีนดังกล่าวของสายพันธุ์ SRW1-5 มีความเหมือน 79 เปอร์เซ็นต์กับยีนของ *Rhodobacter capsulatus* SB 1003 ส่วนยีนของสายพันธุ์ SSS2-1 และ SSW15-1 มีความเหมือน 78 เปอร์เซ็นต์กับสายพันธุ์ *Halomonas elongata* DSM 2581 และจากการเทียบเคียง multiple aliments ของ โปรตีน MerR และ MerT มีความแตกต่างจากแบคทีเรียอื่นๆในกลุ่ม *α-Proteobacteria* และมีความคล้ายคลึงสูงสุดกับโปรตีนของ *Sphingomonas* sp. (AGH51565) และ *N. pentaromativorans* (AIT82359) ตามลำดับ

คำสำคัญ: ปรอท เมอร์โอเปอรอน แบคทีเรียที่ต้านทานปรอท แบคทีเรียสังเคราะห์สีม่วงกลุ่มไม่สะสมซัลเฟอร์

Introduction

Mercury (Hg) is a potent neurotoxic chemical, and is listed as the sixth most toxic hazardous compounds (Zahir et al., 2005; Freedman et al., 2012). A major cause of Hg contamination in environments come from anthropogenic sources such as the burning of coal, oil and biofuel; producing of cement, gold, chlorine and caustic soda; batteries and other electrical apparatus industries, and so on (Pirrone et al., 2010). Hg released from anthropogenic and natural sources may bioaccumulate in aquatic animals through the food web and consequently can be toxic to human health due to the consumption of food contaminated by mercurial compounds (Celo et al., 2006).

The presence of heavy metals including Hg compounds in environments also has an effect on microbes in the ecosystems by altering their metabolic pathways such as their respiratory and other enzymatic activities (Rasmussen et al., 2000; Harris-Hellal et al., 2009). However, microorganisms have developed resistant/tolerant mechanisms so they can survive in metal-stressed conditions. These mechanisms might include the presence of efflux mechanisms for metal ions, the accumulation and complexation of the metal ions inside the cell to reduce their toxicity, and the oxidation/reduction of the heavy metal ions to less toxic states (Nies, 1999; Thilakaraj et al., 2007). Microbes have developed Hg resistance mechanisms that can convert inorganic and organic toxic forms to less toxic forms via detoxification system (Barkay et al., 2003). The *mer* operon is the most widely distributed Hg genetic detoxification systems for Hg and it is, usually located on plasmids and chromosomes, and often found attached to components of transposons and integrons which can then facilitate the transfer of the *mer* operon to other bacteria via transformation, transduction or conjugation. Various genes are involved in the *mer* operon including *merR* and *merD* for regulation; *merP*, *merT* and *merC* for transportation or mobilization; and finally *merA* and *merB* for enzymatic detoxification of inorganic and organic Hg compounds (Nascimento and Chartone-Souza, 2003). The *merA* gene coding for the mercuric reductase enzyme is the key detoxification mechanism. It is a flavin oxidoreductase (Mathema et al., 2011), and

reduced Hg^{2+} into Hg^{0} in a NAD(P)H-dependent reaction. Moreover, the broad-spectrum Hg resistant gene, *merB* is one of genes in *mer* operon that codes for an organomercurial lyase which is involved in organomercurial compound detoxification by hydrolyzing the C-Hg bond before Hg^{2+} reduction (Nascimento and Chartone-Souza, 2003). Although, various genes in *mer* operon have been investigated in various microorganisms (Celo et al., 2006; Wang et al., 2010; Boyd and Barkay, 2012; Allen et al., 2013; Yu et al., 2014), at the present there is very little information on any Hg resistant genes present in phototrophic bacteria. The investigation of *merA* from various bacteria in the *α*-, *β*-, *γ*-*Proteobacteria*, the *Firmicutes* and the *Actinobacteria* has shown that *merA* is found in all these groups, but not phototrophic bacteria including cyanobacteria, green sulfur bacteria, purple bacteria, and heliobacteria (Barkay et al., 2010). Hence, it is probably worth to explore the presence of the *mer* operon in phototrophic bacteria, particularly anoxygenic phototrophic bacteria like purple nonsulfur bacteria (PNSB).

This due to the fact that PNSB are versatile organism that have various growth modes including photoautotrophic, photoheterotrophic and chemoheterotrophic that are dependent on the environmental conditions of their habitats (Imhoff, 1995; Madigan and Jung, 2009); so they are widely distributed in various natural habitats and also manmade environments such as wastewater systems and shrimp ponds. It has also been possible to demonstrate that PNSB can be used as bacterial agents not only for agriculture but also for maintaining a clean environment (Panwichian et al., 2011; Kantha et al., 2015; Nunkaew et al., 2015). Three Hg resistant PNSB strains (*Rhodovulum sulfidophilum* SRW1-5 and *Afifella marina* SSS2-1 and SSW15-1 were isolated from Hg contaminated shrimp ponds in southern Thailand. They were selected for the purpose of using them for bioremediation of Hg contaminated shrimp ponds (Mukkata et al., 2015a). They showed resistance to Hg by volatilization converting Hg^{2+} to Hg^{0} using their mercuric reductase enzyme (Mukkata et al., 2015b). This detoxification mechanism is normally associated with *mer* operon; therefore, investigating the *mer* operon in these PNSB was of great interest to study and to gain new knowledge of their Hg resistant genes by comparing with other microorganisms. As above information, this research was focused on the

Hg resistant genes of the selected PNSB resistant strains to help to understand their Hg resistant mechanisms, and to show that the presence of these genes would enable them to support detoxification of Hg for use in bioremediation.

Materials and methods

Culture preparation of selected PNSB

Three Hg resistant PNSB strains (SRW1-5, SSS2-1 and SSW15-1) as previously mentioned were used in this study. To prepare cultures, each culture was subcultured twice by stabbing into GA agar to obtain an active inoculum. Then each activated culture was inoculated into GA broth containing 3% NaCl in screw capped test tubes but leaving a little head space and incubated under microaerobic light conditions (ca. 3,000 lux) for 48 h to prepare inoculum. This inoculum of each PNSB strain was then grown in GA medium containing 3% NaCl and 1 mg/L $HgCl_2$ and incubated under either microaerobic light or aerobic dark conditions for 72 h to prepare cells at their initial stationary phase of growth for investigating their *mer* operon in both conditions. The former condition was set by adding the medium to nearly full volume of the test tubes followed by continuing illumination of roughly 3,000 lux, while the latter condition was achieved by filling the medium to a half volume of the test tubes then the tubes were covered with aluminium foil and shaken at 150 rpm. However, results of this study found that there was no significant difference observed for the *mer* operon using cells from both incubating conditions.

Detection of *mer* operon

DNA was extracted from each PNSB and used to amplify genes using the primers as shown in Table 5-1. To extract DNA, 1.5 mL of each culture broth was harvested by centrifugation at 10,000 rpm for 5 min in a microcentrifuge; and the genomic DNA from the cell pellet was extracted using genomic DNA Isolation Kit (Bioline) by following the manufacturer instructions. The genes in the *mer* operon were detected using primers: merR2, merP1, merT, merA1-5, and merAD for targeting the genes of *merR*, *merP*, *merT*, *merA* and genes between *merA* and *merD*,

respectively. On the other hand, primers merR1 and merP2-3 were also used to detect genes that were associated with MerR family regulators and periplasmic permease proteins. Moreover, *CYC* gene that controlled the cytochrome *c* oxidase that is involved in Fe^{2+}-dependent Hg volatilization reaction, was investigated using primer CYC. The primers; merR1-2, merP1-3, merT, CYC and merA1 primers were designed from the related bacterial genomes of *Sphingobium* sp. and *Sphingomonas* sp. by (Mahbub et al., 2016). The primers merA2 and AD were designed from consensus regions of all known *mer* sequences (Liebert et al., 1997). The primer for merA3 was designed from the consensus regions of *α-Proteobacteria* (Oregaard and Sorensen, 2007), while the primers merA4-5 were designed in this study.

The PCR amplification mixture contained 2 µL of 10 µM of each primer, 25 µL of PCR mixture (Bioline), 19 µL of dH_2O and 2 µL of the optimal amount of the DNA template. Reactions were carried out in a Thermal Cycler using the following parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C between 30 s to 2 min, with a final extension step of 72°C for 10 min. The resulting amplification products were subjected to electrophoresis in 0.8% agarose gels. The gels were stained in ethidium bromide and observed on a Geldoc/UV transilluminator, photographed and compared with marker bands. The DNA fragment of each gene was cut and purified with the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. The purified PCR products were directly cloned using a pGEM-T Easy Vector Cloning Kit (Promega). Plasmid was extracted using the Plasmid Isolation Kit (Bioline) as described in the manufacturer's instructions. The insertion of each gene was checked by digestion with the restriction enzyme (*EcoR1*) followed by agarose gel electrophoresis. The insert was sequenced at Flinders Sequencing Facility, Flinders Medical Centre, Adelaide, Australia. All gene sequences were subjected to BLAST with the GenBank database in the NCBI website. The multiple alignments of amino acid sequences of MerR and MerT proteins were obtained using ClustalW.

Table 5-1. The primer sequences used for PCR analysis of *mer* genes in selected purple nonsulfur bacteria (PNSB) resistant strains.

Primer	Sequence (5'-3')	Size (bp)	Reference
merR1F	ATGAAGCCGGTGATGATTGG	417	Mahbub, K.R.*
merR1R	CGGTACCTTATTGATCGGCTTGAGGGT	417	Mahbub, K.R.*
merR2F	ATGGAGCAACAGGTCGGC	423	Mahbub, K.R.*
merR2R	CGGGGTACCTCAATTGCCTTGCCTTGATGGGT	423	Mahbub, K.R.*
merP1F	ATGAAAAAGACAGTATGTATC	330	Mahbub, K.R.*
merP1R	CGGGGTACCTCAGTTTTGAATAGCGCGCG	330	Mahbub, K.R.*
merP2F	ATGGATTATTCTCGGCGTCG	1,359	Mahbub, K.R.*
merP2R	CGGGGTACCTTCATCGGGTAGCGGCCAGGG	1,359	Mahbub, K.R.*
merP3F	ATGGCCAGCCATGCCATGCC	1,056	Mahbub, K.R.*
merP3R	CGGGGTACCTTCAGCTAAGCCGTGATCCGCGTG	1,056	Mahbub, K.R.*
merTF	ATGGTTTCGAGATTGCAGCC	540	Mahbub, K.R.*
merTR	CGGGGTACCTCAATAGAACCAGGGTGCCC	540	Mahbub, K.R.*
CYCF	ATGCTCTCGTTGCGGCTGCTC	1,209	Mahbub, K.R.*
CYCR	CGGGGTACCTTCAGCTAAGCCCGTGATCCGCGTG	1,209	Mahbub, K.R.*
merA1F	ATGAACGACTGTTGCAACCG	1,419	Mahbub et al. (2016)
merA1R	CGGGGTACCTCACCCGGCGCAGCAAGAC	1,419	Mahbub et al. (2016)
merA2F	ACCATCGGCGGCACCTGCGT	1,238	Liebert et al. (1997)
merA2R	ACCATCGTCAGGTAGGGGAACAA	1,238	Liebert et al. (1997)
merA3F	TCCAAGGCGMTGATCCGCGC	800	Oregaard and Sorensen (2007)
merA3R	TAGGCGGCCATGTAGACGAACTGGTC	800	Oregaard and Sorensen (2007)
merA4F	TCCAAGGCGMTGATCCGCGC	1,449	This study
merA4R	AG GCGGCCATGTAGACGAACTGGTC	1,449	This study
merA5F	GGCTGCGTTCCTTCCAAGRCGC	1,000	This study
merA5R	GCGCCATAGGCKGCCATGTARACG	1,000	This study
merADF	GCCGACCAGTTGTTCCCCTACCTGACG	928, 181	Liebert et al. (1997)
merADR	CGCACGATATGCACGCTCACCC	928, 181	Liebert et al. (1997)

*Primer sequences were desired by Khandaker Rayhan Mahbub, and data have not been published.

Results

Hg resistant genes in selected PNSB

The use of specific primers (Table 5-1) and the PCR amplification of the Hg resistant genes from three PNSB strains, were resolved on a 0.8% agarose gel (Figure 5-1), for detected the genes in *R. sulfidophilum* SRW1-5 and *A. marina* SSS2-1 and SSW15-1. All sequences were analyzed and compared with the sequences in the database in the NCBI as shown in Table 5-2.

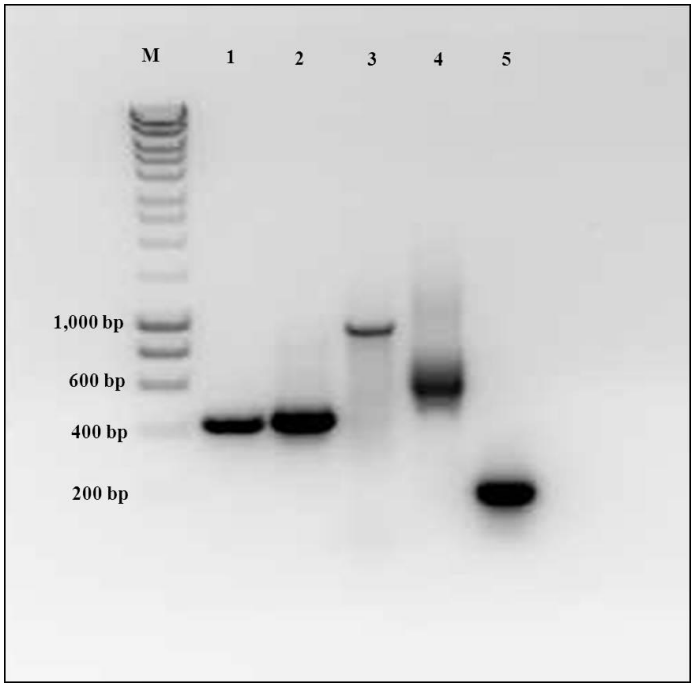

Figure 5-1. Agarose gel of PCR profiles from PNSB strains. Lanes M, DNA molecular weight markers; Lane 1, DNA fragment amplified with primers merR1; Lane 2, merR2; Lane 3, merP3; Lane 4, merT and Lane 5, merAD.

It was found that the target genes using primer merR1, merP3 and merAD were detected in all three strains. In contrast, the detection of the target gene using primer merR2 and merT were only detected in members of *A. marina* (strains SSS2-1 and SSW15-1). The sequence analysis amplified from primer merR1 in all strains and from primer merR2 in strains SSS2-1 and SSW15-1 was closely related to the gene coding for MerR family transcriptional regulator and mercuric resistance operon regulatory protein of *Sphingomonas* sp. MM-1 by showing 99% similarity for both genes. While the *merT* gene in strains SSS2-1 and SSW15-1 showed a 96% similarity with gene coded for the mercuric transport protein in *Novosphingobium pentaromativorans* US6-1. In contrast, the sequence amplified from the primer merP3 in strain SRW1-5 was closely related to the gene from *Rhodobacter capsulatus* SB 1003 with a 79% similarity, while strains SSS2-1 and SSW15-1 were closely related to *Halomonas elongata* DSM 2581 with a 78% similarity to both strains. The function of this gene in strain SRW1-5 was different from strains SSS2-1 and SSW15-1, because the functional gene in strain SRW1-5 was associated with an ATP-binding-cassette (ABC) transporter, an ATP-binding/permease protein, while a major facilitator superfamily (MFS) transporter was found in strains SSS2-1 and SSW15-1. In addition, a part of gene analysis between *merA* and *merD* genes in strain SRW1-5 was closely related to *Halothiobacillus neapolitanus* C2 by showing 94% similarity, whereas in strains SSS2-1 and SSW15-1 it showed 100% similarity with genes of *Pseudomonas aeruginosa* IOMTU 133. Moreover, the detection and analysis of the *mer* genes in the three strains grown under both conditions were not different. On the other hand, the target genes using the primer merP1, merA1-5 and CYC were not detected in any strains used of this study.

Table 5-2. Similarity of representative gene sequences in selected mercury resistant PNSB strains

Strain	Primer	Closet identify relative		Similarity (%)	Proteins
		Accession number	Bacterial species		
SRW1-5	merR1	CP011450	*Sphingomonas* sp. MM-1	99	MerR family transcriptional regulator
	merP3	CP001312	*Rhodobacter capsulatus* SB 1003	79	ABC transporter, ATP-binding/permease protein
	merAD	NG036549	*Halothiobacillus neapolitanus* C2	94	Mrrcuric reduactase enzyme (MerA) and regulatory function in expression of mercury (MerD)
SSS2-1	merR1	CP011450	*Sphingomonas* sp. MM-1	99	MerR family transcriptional regulator
	merR2	CP004037	*Sphingomonas* sp. MM-1	99	Mercuric resistance operon regulatory protein
	merT	CP009293	*Novosphingobium pentaromativorans* US6-1	96	Mercuric transport protein
	merP3	FN869568	*Halomonas elongata* DSM 2581	78	MFS transporter permease proteins
	merAD	AB852526	*Pseudomonas aeruginosa* IOMTU 133	100	Mercury reductase enzyme (MerA) and regulatory function in expression of mercury (MerD)
SSW15-1	merR1	CP011450	*Sphingomonas* sp. MM-1	99	MerR family transcriptional regulator
	merR2	CP004037	*Sphingomonas* sp. MM-1	99	Mercuric resistance operon regulatory protein
	merT	CP009293	*Novosphingobium pentaromativorans* US6-1	96	Mercuric transport protein
	merP3	FN869568	*Halomonas elongata* DSM 2581	78	MFS transporter permease proteins
	merAD	AB852526	*Pseudomonas aeruginosa* IOMTU 133	100	Mercury reductase enzyme (MerA) and regulatory function in expression of mercury (MerD)

Multiple alignments of the MerR and MerT proteins

The Blastx was used to obtain putative conserved domains of the MerR and MerT proteins; and then both amino acid sequences were blasted using BlastP for finding any homology with MerR and MerT proteins from other organisms in the group of *α-Proteobacteria*. The amino acid sequences between strains SSS2-1 and SSW15-1 were 100% homology in both proteins after aliment with ClustalW (Figure 5-2 and 5-3). The multiple alignments of the 140 amino acids (aa) of the MerR protein in strains SSS2-1 and SSW15-1 showed the highest homology with *Sphingomonas* sp. (AGH51565) which was 98% identical. Other multiple alignments, the percentages of identity were 81, 58, 59, 56, 51 and 44 with MerR proteins of *N. pentaromativorans* (WP007015440), *Thalassobaculum salexigens* (WP 028793073), *Rhizobium* sp. (WP 052642499), *Roseovarius tolerans* (WP 050664246), *Loktanella hongkongensis* (WP 026147360) and *Lutibaculum baratangense* (WP 023432424), respectively (Figure 5-2). Whilst the multiple alignments of the 128 amino acids of the MerT proteins in strains SSS2-1 and SSW15-1 showed the highest homology with *N. pentaromativorans* (AIT82359) that was 95% identical. In addition, the percentage identities were 90, 54, 54, 54, 38 and 23 with the MerT proteins of *Sphingobium herbicidovorans* (KFG90066), *R. tolerans* (WP 050664247), *Phaeobacte*r sp. (WP 040181274), *Rhizobium* sp. (WP 052642501), *Oceanibaculum indicum* (WP 008946214), *Sinorhizobium fredii* (WP 014332374) and *L. baratangense* (WP 023432425), respectively (Figure 5-3). Moreover, the 151 alignment length of the MerR amino acid in all selected bacterial strains showed that: identity was 24.50% (37/151 aa); strongly similar was 19.21% (29/151 aa); weakly similar was 5.96% (9/151 aa); and the difference was 50.33% (75/151 aa) (Figure 5-2). Besides, they showed that: identity was 18.44% (26/141 aa); strongly similar was 12.06% (17/141 aa); weakly similar was 9.93% (14/141 aa); and the difference was 59.57% (84/151 aa) in the 141 alignment length of the MerT amino acids (Figure 5-3).

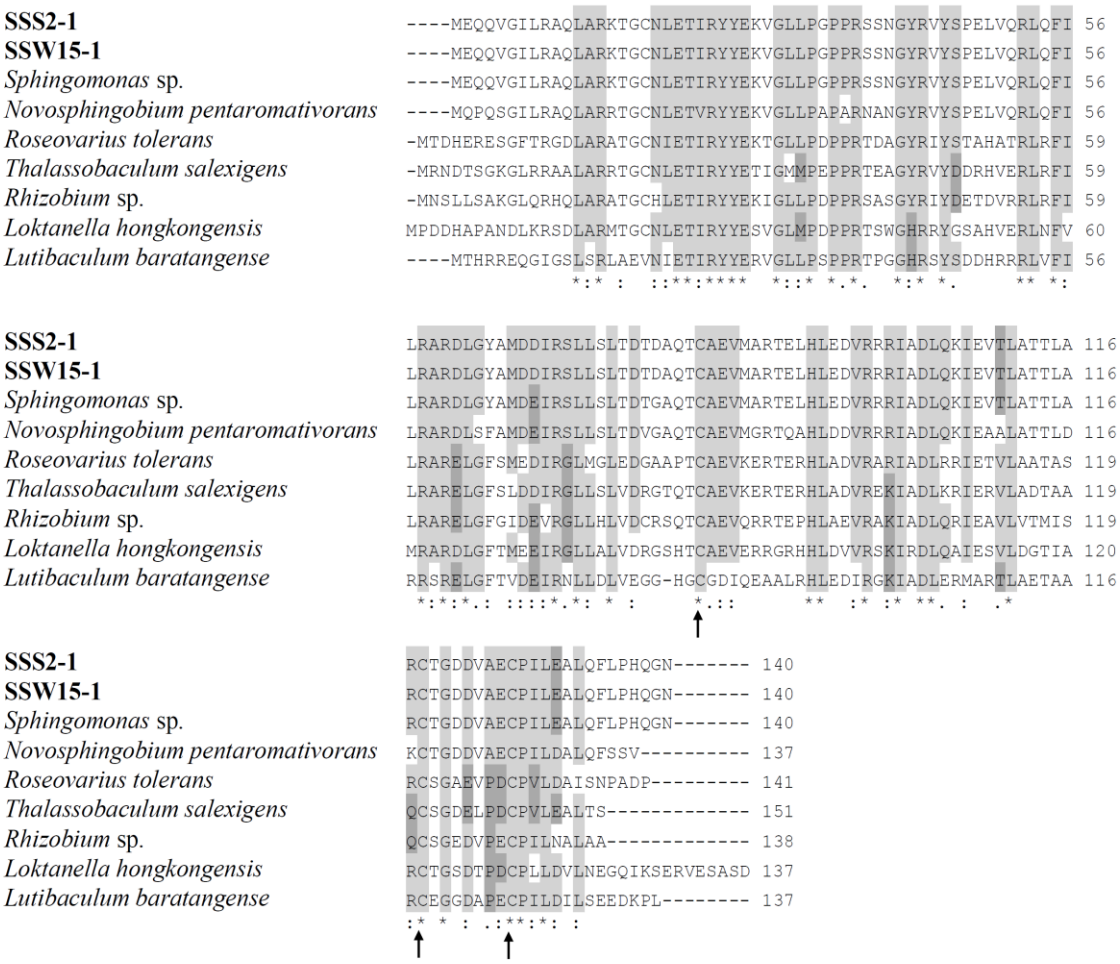

Figure 5-2. Multiple sequence alignment of the mercuric resistance operon regulatory protein of *Afifella marina* strains SSS2-1 and SSW15-1, *Sphingomonas* sp. (AGH51565), *Novosphingobium pentaromativorans* (WP007015440), *Roseovarius tolerans* (WP 050664246), *Thalassobaculum salexigens* (WP 028793073), *Rhizobium* sp. (WP 052642499), *Loktanella hongkongensis* (WP 026147360) and *Lutibaculum baratangense* (WP 023432424). Highlights are marked at conserved regions of identity (*), strongly similar (:) and weakly similar (.). The Hg^{2+} binding cysteine residues are marked with an arrow.

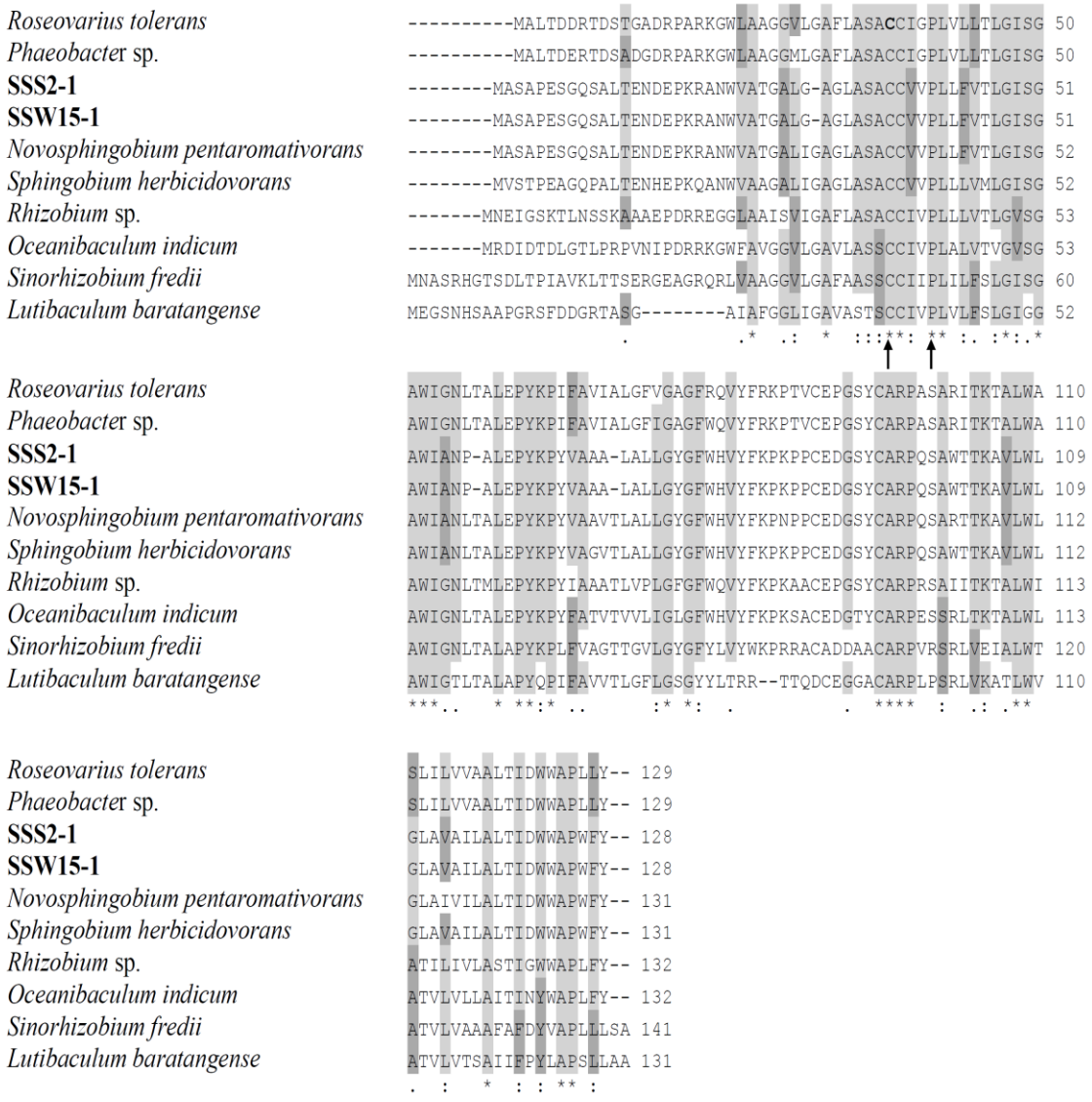

Figure 5-3. Multiple sequence alignment of the mercuric transporter protein of *Afifella marina* strains SSS2-1 and SSW15-1, *Roseovarius tolerans* (WP 050664247), *Phaeobacter* sp. (WP 040181274), *Novosphingobium pentaromativorans* (AIT82359), *Sphingobium herbicidovorans* (KFG90066), *Rhizobium* sp. (WP 052642501), *Oceanibaculum indicum* (WP 008946214), *Sinorhizobium fredii* (WP 014332374) and *Lutibaculum baratangense* (WP 023432425). Highlights are marked at conserved regions of identity (*), strongly similar (:) and weakly similar (.). The Hg^{2+} binding cysteine residues are marked with an arrow.

Discussion

Hg resistant genes in selected PNSB

The *merR* is usually the first gene in *mer* operon. It is a metalloregulatory DNA binding protein (MerR) that binds to the promoter/operator region and is the structural transcription gene for protecting the cell against toxic mercurial compounds (Mathema et al., 2011). MerR family regulator are classified into several groups that sense for Hg^{2+} (MerR), Cu^{+} (CueR/SctR), Cd^{2+} (CadR), Pb^{2+} (PbrR), Zn^{2+} (ZntR), as well as some with unknown specificity (Permina et al., 2006; Hobman, 2007); and some of these different MerR family regulators can respond to different metals that have similar physical and electrochemical properties because the promoter is recognized and binds to a specific regulator. The detection of MerR family regulator in a group of CadR-PbrR might confirm the ability of the three PNSB strains to have resistant mechanisms for toxic metals such as cadmium (Cd) and lead (Pb) in addition to Hg. However, the resistance mechanisms to Cd and Pb in the three PNSB strains have not been studied, it is possible that these three PNSB can resist both these heavy metals due to the isolation of the heavy metal resistant PNSB strains (*Rhodobacter sphaeroides* KMS24 and *Rhodobium marinum* NW16, which is now *Afifella marinum*) isolated from contaminated shrimp ponds (Panwichian et al., 2010). This is associated to the contamination of both heavy metals in various shrimp ponds (Cheung and Wong, 2006; Nemati et al., 2009; Panwichian et al., 2010) that have resulted in the selection for microbes able to resist these heavy metals. Hence, if those PNSB strains can resist many heavy metals contaminating in shrimp ponds; it would be helpful to use them as bacterial agents to remediate any heavy metals contaminated shrimp ponds including other aquatic animal ponds used for producing safe foods.

Mercuric ions are transported into the cells by a series of transporter proteins. This mechanism involves the binding of Hg^{2+} by a pair of cysteine residues on the MerP protein that is located in the periplasm (Mathema et al., 2011), and is then transferred to the residues on MerT for transportation of Hg^{2+} into the cytoplasm for enzymatic detoxification via the mercuric reductase enzyme (MerA) (Barkay et al., 2003). In this study, the *merP* gene was not detected in all PNSB strain used;

however, the *merT* gene was detected in strains SSS2-1 and SSW15-1. As the *merT* gene was not detected in strain SRW1-5; it could be that the primer used for its merT was not sufficiently specific for the *merT* gene in *R. sulfidophilum* SRW1-5, which is from a different genus than strains SSS2-1 and SSW15-1 of *A. marina*.

It should be noted that comparison of the sequences of *merR2* and *merT* from the three PNSB strains with the databases in NCBI found that strains SSS2-1 and SSW15-1 had a high similarity with the *merR* gene in *Sphingomonas* sp. MM-1 and the *merT* gene in *N. pentaromativorans* US6-1, all being member of the *a-Proteobacteria* in the Family *Sphingomonadaceae*. This is because both strains as *A. marina* are also members of the *a-Proteobacteria*, but they are in the Family *Rhodobacteraceae*. These results were similar to the multiple alignments of the MerR and MerT proteins that have putative conserved domains for MerR and MerT with a higher homology to the genera in members of the Family *Sphingomonadaceae* (Figure 5-2 and 5-3) more than for *R. tolerans* and *L. hongkongensis* which are the same familty with *A. marina* SSS2-1 and SSW15-1 in the Family *Rhodobacteraceae*. These results are in agreement with the research of Yamaguchi et al. (2007) who constructed a phylogenetic tree to provide the correlation between the Hg transport proteins and 16S rRNA genes. The results showed that there was no correlation between the phylogenies of Hg transport proteins and their 16S rRNA genes. Hence, from these results can be concluded that *mer* operon of PNSB is not correlate to their source organism (16S rRNA genes) because the *mer* operon has been obtained by a lateral gene transfer (LGT) (Boyd and Barkay, 2012).

Although *merP* gene in the *mer* operon was not detected in all three PNSB strains, other transport proteins were detected (Table 5-2). The sequencing analysis of strain SRW1-5 using primer P3 was similar to the gene coding for the ABC transporter, ATP-binding/permease protein of *Rhodobacter capsulatus* SB 1003, which is a member in group of PNSB. In contrast, the sequencing analysis of strains SSS2-1 and SSW15-1 were similar to the gene coding for the MFS transporter permease of *Halomonas elongata* DSM 2581. The ABC transporter permease proteins and MFS transporter are families of periplasmic protein can mediate transport of natural toxic compounds through biological membranes. The ABC transporter

permease proteins hydrolyze ATP and use the energy generated to transport solutes across the cell membranes, while the MFS transporters do not hydrolyze ATP, and compounds are driven through the membrane by the proton-motive force (Del Sorbo et al., 2000).

The study of interactions between the metals and ABC transporter permease proteins showed their important for metal homeostasis and resistance mechanism that involved co-working with the ATP-dependent efflux pumps for cellular extrusion to prevent the accumulation of metals in the cell (Achard-Joris and Bourdineaud, 2006; Reis et al., 2010). Whilst the MFS transporter permeases are divided into 76 subfamilies related to transport with many carbohydrates, lipids, amino acids and peptides, nucleosides, and other molecules including the efflux systems for antibiotics (Yan, 2013). Moreover, one part of the amino acid sequences of strains SSS2-1 and SSW15-1 when compared with the protein databases in the NCBI were related to a sensor histidine kinase associated with heavy metal resistance with efflux systems for copper, silver, cadmium, and/or zinc (data not shown). Therefore, the detection of periplasmic permease proteins in the three PNSB strains might be related to the first step of mercuric ions transportation into the cells by the periplasmic permease proteins before they are transported via specific transport proteins due to the promoter of *mer* operon is activated by Hg^{2+}.

The full length of the *merA* gene has not been detected in all three PNSB strains; however, detection of the short length of *merA* gene amplified from the primer AD confirmed the presence of *merA* genes in these three PNSB isolates. The primer merAD was designed from the consensus regions of all know sequences in Gram-negative bacteria between the end of the *merA* gene and the start of *merD* gene. However, the PCR amplification in this study between *merA* and *merD* gene was only a 181 bp (Figure 5-1); and this result indicated the absence of that particular *merB* gene in all three PNSB strains. This is because the sequences produce a 928 bp when *merB* was present or a 181 bp when *merB* gene was absent (Liebert et al., 1997) as the *merB* gene was found to be downstream from the *merA* gene in the *mer* operon. It is well recognized that the broad spectrum Hg resistant genes in bacteria contain both *merA* and *merB* genes for enzymatic detoxification of both inorganic and organic

mercurial compounds, respectively (Mathema et al., 2011). Hence, the detection of MerD confirmed the downstream regulation protein of operon, a secondary regulatory protein, that binds to the same operator-promoter region as the MerR (Nascimento and Chartone-Souza, 2003).

With regard to the above information the study of *merA* gene in the three PNSB strains should be continued to try to obtain the full length of the *merA* gene sequences. Although the *merR* and *merT* genes in strain SRW1-5 and *merP* in all three PNSB strains were not detected, the detections of *merR* and *merT* in strains SSS2-1 and SSW15-1, including *merAD* in all selected PNSB strains was indicative that there were similar genes in the *mer* operon of PNSB. Nevertheless, those that have not been detected it might be because the primers used for detection of each gene were not specific for their sequences. Also the mercuric reductase activity was previously detected in all three PNSB strains in our previous study (Mukkata et al., 2015b). Hence, studying of the *mer* genes in three strains should be continued until the *mer* operon of PNSB is completed in future studies.

Multiple alignments of the MerR and MerT proteins

The *merR* genes of the transposons Tn*501* from *Pseudomonas aeruginosa* (Stanisich et al., 1977) and Tn*21* from the *Shigellafexneri* R100 plasmid (Barrineau et al., 1984) were the first to be studied. The research on both transposons has confirmed that the three conserved cysteine (Cys) residues including Cys82, Cys117 and Cys126 (Wang et al., 2009) are the Hg^{2+} binding site, and all conserved cysteine residues also contains the MerR amino acids from both PNSB strains (SSS2-1 and SSW15-1); however, they also contain the conserved amino acids Cys83, Cys118 and Cys126 (Figure 5-2). Moreover, in the transport process of Hg^{2+} into the cell, the cysteine residues are very important because they are the first binding site for Hg^{2+} before they are transferred to the MerA mercuric reductases for detoxification (Mathema et al., 2011). The multiple alignments of the MerTs in microbes studied in previous research revealed that the best-conserved motif for Hg^{2+} binding was CC(LIV)GP (Yamaguchi et al., 2007). However, in this study the conserved motif in both strains was CCVVP and this was similar to *N. pentaromativorans* (AIT82359)

and *S. herbicidovorans* (KFG90066) (Figure 5-3). In addition, the MerT proteins in both strains was 128 amino acids which are similar with most of the other MerT proteins in bacteria (73-136 aa) (Yamaguchi et al., 2007). The protein sequences shown in the Hg resistant systems of PNSB including their multiple aliments with other bacteria indicate that the conserved regions of MerR and MerT are different from bacteria in the *α-Proteobacteria*, except for some members of the Family *Sphingomonadaceae*. This indicated that some Hg resistant genes in PNSB might have developed more recently than the others associated with the known mercury resistant plasmids.

Conclusions

At present, this is the first report on Hg resistant genes in Hg resistant PNSB on the basis of using strains; *R. sulfidophilum* SRW1-5 and *A. marina* SSS2-1 and SSW15-1 to confirm that their detoxification mechanisms were related to the *mer* operon. Comparisons of the Hg resistant genes and proteins in these PNSB with other genera in the *α-Proteobacteria* revealed that the Hg resistant genes in PNSB had most likely been a lateral gene transfer (LGT) and might have been achieved only recently. This study has explored the *mer* operon of PNSB although continuing studies should be investigated for obtaining their complete *mer* operon.

CHAPTER 6

Toxic effects of mercury on photosynthesis process and cell morphology of Hg resistant purple nonsulfur bacteria

Abstract

This study aimed to investigate the toxic effects of Hg^{2+} on photosynthetic apparatus, electron transport rate (ETR) and cell morphology of three Hg resistant purple nonsulfur bacteria (PNSB), *Rhodovulum sulfidophilum* SRW1-5 and *Afifella marina* SSS2-1 and SSW15-1. No significant difference of bacteriochlorophyll *a* (Bchl *a*) contents was found in strains SRW 1-5 and SSS2-1 grown in medium containing $HgCl_2$ from 0 to 2 mg/L for 96 h, but their Bchl *a* contents significantly decreased at 3 mg/L $HgCl_2$. In contrast, the Bchl *a* contents of strain SSW15-1 significantly dropped following the higher concentrations of Hg^{2+}. No detection of Bchl *a* in three PNSB strains at 4 mg/L $HgCl_2$ because they could not grow in this condition. In short-term exposure showed that the maximal electron transfer rate (ETR_{max}, measurement by Pulse Amplitude Modulation fluorometer) of strains SRW1-5 and SSS2-1 significantly decreased ($P < 0.05$) following the increasing of Hg^{2+} concentrations to 4 mg/L $HgCl_2$ and 9 h exposure times; and at 9 h exposure the ETR_{max} decreased 37% for the former and 48% for the latter when compared with their controls. The ETR_{max} levels of both strains in long-term incubation for 96 h in all sets including controls significantly increased ($P < 0.05$) following incubation times between 48-60 h; however, over 60 h significantly decreased was observed only in controls while the opposite results were found in treatment sets. Results of cell morphology using scanning electron microscope (SEM) showed that three PNSB strains exposed to $HgCl_2$ for 96 h had elongated rod shaped than their controls. Overall results indicated that a remarkable decrease of ETRmax was the acute toxic of Hg^{2+} while a longer exposure to Hg^{2+}, Hg resistant PNSB responded to stress condition by changing of their cell shapes for survive.

Keywords: bacteriochlorophyll, electron transport rate, mercury, Pulse Amplitude Modulation, purple nonsulfur bacteria, toxicity

บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของปรอท (Hg^{2+}) ต่อรงควัตถุที่ใช้ในการสังเคราะห์แสง อัตราการขนส่งอิเล็กตรอน และสัณฐานวิทยาของแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria: PNSB) สายพันธุ์ที่ต้านทานปรอทสามสายพันธุ์ได้แก่ *Rhodovulum sulfidophilum* สายพันธุ์ SRW1-5 และ *Afifella marina* สายพันธุ์ SSS2-1 และ SSW15-1 จากการศึกษาไม่พบความแตกต่างของปริมาณแบคเทอริโอคลอโรฟิลล์เอของสายพันธุ์ SRW1-5 และ SSS2-1 เมื่อเลี้ยงในอาหารที่ผสม $HgCl_2$ ความเข้มข้น 0 ถึง 2 มิลลิกรัมต่อลิตร เป็นเวลา 96 ชั่วโมง แต่ปริมาณแบคเทอริโอคลอโรฟิลล์เอลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเลี้ยงในอาหารที่ผสม $HgCl_2$ 3 มิลลิกรัมต่อลิตร ในทางตรงกันข้ามปริมาณแบคเทอริโอคลอโรฟิลล์เอของสายพันธุ์ SSW15-1 ลดลงอย่างมีนัยสำคัญทางสถิติตามความเข้มข้นที่สูงขึ้นของ Hg^{2+} และที่ความเข้มข้น 4 มิลลิกรัมต่อลิตรของ $HgCl_2$ ตรวจไม่พบ แบคเทอริโอคลอโรฟิลล์เอในทุกสายพันธุ์เนื่องจากเชื้อไม่เจริญ ขณะที่อัตราการขนส่งอิเล็กตรอนสูงสุด ซึ่งวัดโดยเครื่อง Pulse Amplitude Modulation fluorometer ลดลงอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ตามความเข้มข้นของปรอทและระยะเวลาการสัมผัส $HgCl_2$ ของเซลล์ที่เพิ่มขึ้นถึง 4 มิลลิกรัมต่อลิตร เป็นระยะเวลา 9 ชั่วโมง โดยอัตราการขนส่งอิเล็คตรอนสูงสุดของสายพันธุ์ SRW1-5 และ SSS2-1 ที่เวลา 9 ชั่วโมง ลดลง 37 และ 48 เปอร์เซ็นต์เมื่อเปรียบเทียบกับชุดควบคุม อัตราการขนส่งอิเล็คตรอนสูงสุดของเชื้อทั้งสองสายพันธุ์ในทุกชุดการทดสอบรวมทั้งชุดควบคุมที่เลี้ยงในสภาวะที่มีปรอทเป็นระยะเวลา 96 ชั่วโมง มีค่าสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) ตามระยะเวลาของการบ่มในช่วงชั่วโมงที่ 48 ถึง 60 แต่หลังจากชั่วโมงที่ 60 ค่าดังกล่าวของชุดควบคุมลดลงอย่างมีนัยสำคัญทางสถิติซึ่งตรงข้ามกับชุดทดสอบที่เพิ่มขึ้น ส่วนลักษณะสัณฐานวิทยาของเซลล์ที่ตรวจสอบด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด (SEM) พบว่ารูปร่างของเซลล์ของทั้งสามสายพันธุ์ ที่เลี้ยงในสภาวะที่มีปรอทเป็นเวลา 96 ชั่วโมงมีลักษณะแท่งที่ยืดยาวขึ้นเมื่อเทียบกับชุดควบคุม จากที่กล่าวมาสรุปได้ว่าผลเฉียบพลันของปรอทต่อเซลล์ PNSB คืออัตราการขนส่งอิเล็กตรอนสูงสุดลดลงอย่างเด่นชัด ในขณะที่เซลล์ของทุกสายพันธุ์ที่ต้านทานปรอทเมื่อสัมผัสกับปรอทเป็นระยะเวลานานขึ้นมีการตอบสนองต่อสภาวะกดดันโดยการเปลี่ยนแปลงรูปร่างเซลล์เพื่อการอยู่รอด

คำสำคัญ: แบคเทอริโอคลอโรฟิลล์ อัตราการขนส่งอิเล็กตรอน ปรอท Pulse Amplitude Modulation
แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ ความเป็นพิษ

Introduction

Heavy metals are known to have a considerable effect on the activities of microbes and the biogeochemical processes that they can mediate (Liu et al., 2010). Mercury (Hg) is one of the most toxic heavy metals, and it has been intensively studied for its effects on microbes (Šeršeň et al., 1998; Antal et al., 2009; Asztalos et al., 2010; Liu et al., 2010; Asztalos et al., 2012; Deng et al., 2013). Generally, Hg produces an adverse effect on microbes; particularly Hg in a form of Hg^{2+} due to its high solubility (Giotta et al., 2006; Harris-Hellal et al., 2009; Liu et al., 2010). Among microbes that have high opportunity to expose Hg contamination in environments are anoxygenic phototrophic bacteria; particularly purple nonsulfur bacteria (PNSB) as they have versatile growth modes that can grow in various environments including aquatic and terrestrial environments (Madigan and Jung, 2009). For instance, these PNSB under anaerobic light conditions can harvest the photons of light by pigments of a reaction center (RC) including several varieties of bacteriochlorophyll (BChl) and various carotenoids, and then convert light energy into chemical energy by photosynthesis process (Madigan and Jung, 2009). On the other hand, without light PNSB also grow under aerobic dark or anaerobic dark conditions using fermentation or anaerobic respiration (Imhoff, 1995). Regarding to above information, this leads to their resistance to heavy metals; so they have the potential to be applied for bioremediation in heavy metals contaminated environments (Bai et al., 2008; Panwichian et al., 2010; Mukkata et al., 2015b).

The effect of Hg on PNSB, *Rhodobacter sphaeroides*, was previously studied, and it showed that Hg influence to increase the lag-phase and decrease the growth rate of *R. sphaeroides* (Giotta et al., 2006). Due to the fact that photosynthesis is very important process of photosynthetic organisms; hence, the toxicity of Hg on photopigments such as chlorophyll, bacteriochlophylls (BChl) and carotenoids have been investigated. Previous studies showed that Hg^{2+} strongly interacted with the following groups C=O, C-N, C-S, C-SH of amino acids in photopigments (Bernier and Carpentier, 1995; Šeršeň et al., 1998). In addition, the study of Hg affecting on *R. sphaeroides* strain 2.4.1 using fluorescence induction technique revealed that Hg^{2+}

destroyed the BChl pigments and decreased the connectivity within the antenna; consequently, the antenna organization and photosynthetic electron transfer reaction of the cells were inhibited (Asztalos et al., 2012). Although, the interaction between Hg and PNSB were reported, the researches of Hg toxicity on photosynthesis process of Hg resistant PNSB strains have not much be focused. From our previous study, Hg resistant PNSB strains were isolated from contaminated shrimp ponds and the selected strains showed high abilities of biovolatilization (Mukkata et al., 2015b). Hence, the toxicity of Hg on Hg resistant PNSB strains is worth to investigate for better understanding how Hg effects on their photosynthesis and morphology, and finally effecting their growth.

Pulse Amplitude Modulation (PAM) fluorometer has been used to measure light reaction of photosynthesis in photosynthetic organisms such as vascular plants and algae by measuring the viable fluorescence arising from Photosystem II (PSII) (Franklin and Badger, 2001; Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). Whilst PAM using blue light has been recently used to measure the light reaction of photosynthetic *Afifella marina* and *Rhodopseudomonas palustris* , which are members in PNSB (Ritchie, 2013; Ritchie and Runcie, 2013). Moreover, PAM is also used to study the effect of heavy metals on photosynthetic process of algae (Ahmed and Häder, 2010; Deng et al., 2013). Hence, it is valuable that fast technique could be used to monitor the damage of photosynthetic apparatus exposed to Hg. Therefore, the aims of this research were to investigate the effects of Hg on photosynthetic apparatus, cell morphology of selected Hg resistant PNSB and their photosynthetic process using the PAM fluorometer.

Materials and methods

Bacterial strains and inoculum preparation

Three PNSB strains, *R. sulfidophilum* strain SRW1-5 and *A. marina* strains SSS2-1 and SSW15-1 isolated from Hg contaminated shrimp ponds were used in this study. The stock culture of each PNSB strain was grown in glutamate acetate (GA) broth containing 3% NaCl and incubated under microaerobic light conditions

provided by the continuous illumination of tungsten lamps (ca. 3,000 lux) for 48 h. The cell cultures were subcultured twice and adjusted to an optical density of 0.5 at OD_{660} using a spectrophotometer for using as inoculum.

The effect of Hg^{2+} on amount of photosynthetic pigments

A 10% of each inoculum was inoculated into GA broth containing 3% NaCl by varying concentrations of $HgCl_2$; 1, 2, 3, and 4 mg/L; and all culture tubes were incubated under optimum growth of microaerobic light conditions for 96 h based on our preliminary study. Cell cultures were taken every 12 h for determination of extracted photosynthetic pigments by the method of Biebl et al. (2005). A 10 mL of each culture broth was centrifuged at 8,000 rpm for 15 min at 4 °C and washed twice with deionized water. The cell pellets were extracted in 1 mL of acetone/methanol (7: 2 v/v) and left for 1 h at room temperature in the dark; and then supernatant were obtained by centrifugation as the same condition. After extraction Bchl *a* was determined by a UV-visible spectrophotometer at 771 nm. The amount of Bchl *a* (mg/L) was calculated according to the following formula (Chen et al., 2006).

$$TC = (ADV_1)/(kV_2)$$

Where TC denotes the amount of BChl *a* yield (mg/L, based on culture broth), A is the absorbance of BChl *a* in diluted extract solution, D is the dilution ratio, V_1 the volume of acetone/methanol, k is extinction coefficient of Bchl *a*, and V_2 is the volume of culture broth. The total Bchl *a* is compared with a control set as PNSB grown in GA broth containing 3% NaCl without addition of $HgCl_2$.

The effect of Hg^{2+} on bacteriochlorophyll fluorescence

Cell cultures preparation

The effect of Hg^{2+} on photosynthesis process was done in only two strains; SRW1-5 and SSS2-1 while the cells of strain SSW15-1 were not studied due to it was the same species with strain SSS2-1. The experiments were designed in two sets, short and long-term exposures. In short-term exposure, 10% of each inoculum was grown in GA broth containing 3% NaCl, and then incubated under microaerobic

light until cell cultures reached to their log phase. Then cell cultures were added with $HgCl_2$ at the final concentrations of 1, 2, 3 and 4 mg/L. Cell cultures after mixing with $HgCl_2$ were incubated under microaerobic light conditions, and taken the samples at 0, 3, 6 and 9 h after incubation for further studies. While, in long term exposure, 10% of each inoculum was grown in GA broth containing 3% NaCl with various concentrations of $HgCl_2$; 1, 2 and 3 mg/L, and then incubated under microaerobic light for 96 h. Cell cultures were taken every 12 h for further studies.

Preparation of cells for absorbance measurement

The preparation of cell cultures and absorbance measurement were done according to the protocol described by Ritchie (2013). Cell cultures were filtered onto Whatman GF/C glass fiber disks, which had 206.12 x 10^{-6} m^2 of surface area for cell pellet adhesion on the filtered disks. The filtered disks were placed on a Petri dish with a layer of filter paper moistened with GA broth and kept in the dark condition, and not allowed to dry out prior to absorbance measurement with the absorbance meter, Blue-reflectance absorbance transmission (Blue-RAT) designed by Aquation Pty Ltd, Australia and the Junior-PAM machine. The Blue-RAT uses a blue diode light source with a maximum at 465 ± 40 nm similar to that used in the Junior-PAM machine and a diode set at 45° to the light beam in an arrangement based on Schultz (1996). The transmission (%T) and reflectance (%R) of light through specimen on filtered disks were measured and then the absorbance (%A) was calculated as %A = 100 - %T - %R.

Pulse amplitude modulation fluorometry

The Junior-PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Würzburg, Germany) was used to detect light situation curve from both PNSB cells on filtered disks. The Junior-PAM fitted with a 1.5 mm diameter optic fiber and a blue diode (465 ± 40 nm) light source. The filtered disks were kept in the dark for 10 min and not more than 30 min and then measured with Junior-PAM set to measure light curve about 88 s to complete with 10 s between saturating flashes of light source using methods previously described (Ritchie, 2013;

Ritchie and Runcie, 2013; Ritchie and Mekjinda, 2015). Minimal fluorescence (F_0) was measured after kept in the darkness, while maximal fluorescence (F_m) was measured after the filtered disk received a flash of actinic saturating light using standard Walz Software (Walz, Wurzburg, Germany). The Junior-PAM parameters were calculated using WINCONTROL software (v2.08 & v2.13; Heinz Walz GmbH, Effeltrich, Germany) (Ritchie, 2013; Ritchie and Mekjinda, 2015).

The effect of Hg^{2+} on cell morphology

A 10% of each inoculum was cultured in GA broth containing 3% NaCl and 3 mg/L $HgCl_2$ for 96 h under microaerobic light; and their cell morphology were observed using scanning electron microscope (SEM) by comparing with a control set as PNSB grown in GA broth containing 3% NaCl with no added $HgCl_2$. Cell pellets were obtained by centrifugation at 8,000 rpm for 15 min and then washed with 0.1 M sodium phosphate buffer (pH 7.0) for three times. The bacterial cells were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 2 h and then washed with the same buffer for 3 times. Cells were dehydrated in a series of ethanol (50-90%) for 5 min in each step for 2 times and 100% ethanol for 30 min. The samples were dried by the critical-point method (CPD), coated with gold, and observed with SEM Quanta 400.

Results

The effect of Hg^{2+} on amount of photosynthetic pigments

Figure 6-1 shows a complete difference of the BChl *a* contents in three PNSB strains *(R. sulfidophilum* SRW1-5 and *A. marima* SSS2-1 and SSW15-1) after 96 h exposure with Hg^{2+} in a range of 0-4 mg/L $HgCl_2$, especially at the higher concentrations of Hg^{2+}. The highest Bchl *a* contents (4.06 and 0.72 mg/L) in strains SRW1-5 and SSS2-1 in sets of control and treatment (1 mg/L $HgCl_2$) were detected at 60 and 72 h after incubation, respectively; however, a higher $HgCl_2$ at 2 and 3 mg/L it took a longer time at 84 h to reach the maximum contents (4.03 and 2.94 mg/L for SRW1-5; 0.66 and 0.37 mg/L for SSS2-1) (Figure 6-1A, B). On the other hand, the

BChl *a* content of strain SSW15-1 was highest (0.52 mg/L) at 60 h in a control set, but it was highest in treatments at 1-3 mg/L $HgCl_2$ at 96 h; 0.41, 0.29 and 0.10 mg/L, respectively (Figure 6-1C). The data of maximal Bchl *a* contents as shown in Figure 6-2 revealed the significant differences of maximal Bchl *a* contents in three strains; and also the effect of Hg^{2+} to cause a remarkable decrease the maximal contents of Bchl *a* of strains SRW1-5 and SSS2-1 found at 3 mg/L $HgCl_2$ while strain SSW15-1 started at 1 mg/L $HgCl_2$. The highest Bchl *a* contents in strain SRW1-5 exposed to 0, 1 and 2 mg/L $HgCl_2$ were roughly 4.00 mg/L, while they were between 0.65 and 0.72 mg/L in strain SSS2-1. On the other hand, the highest Bchl *a* contents at 3 mg/L $HgCl_2$ in strains SRW1-5 (2.94 mg/L) and SSS2-1 (0.37 mg/L) significantly decreased. In contrast, the highest Bchl *a* content of strain SSW15-1 significantly dropped from control (0.52 mg/L) following the concentrations of Hg^{2+} in a range of 1-3 mg/L $HgCl_2$ that were between 0.10 and 0.41, mg/L (Figure 6-2). However, no detection of Bchl *a* contents in three strains at 4 mg/L $HgCl_2$ because they could not grow in this condition.

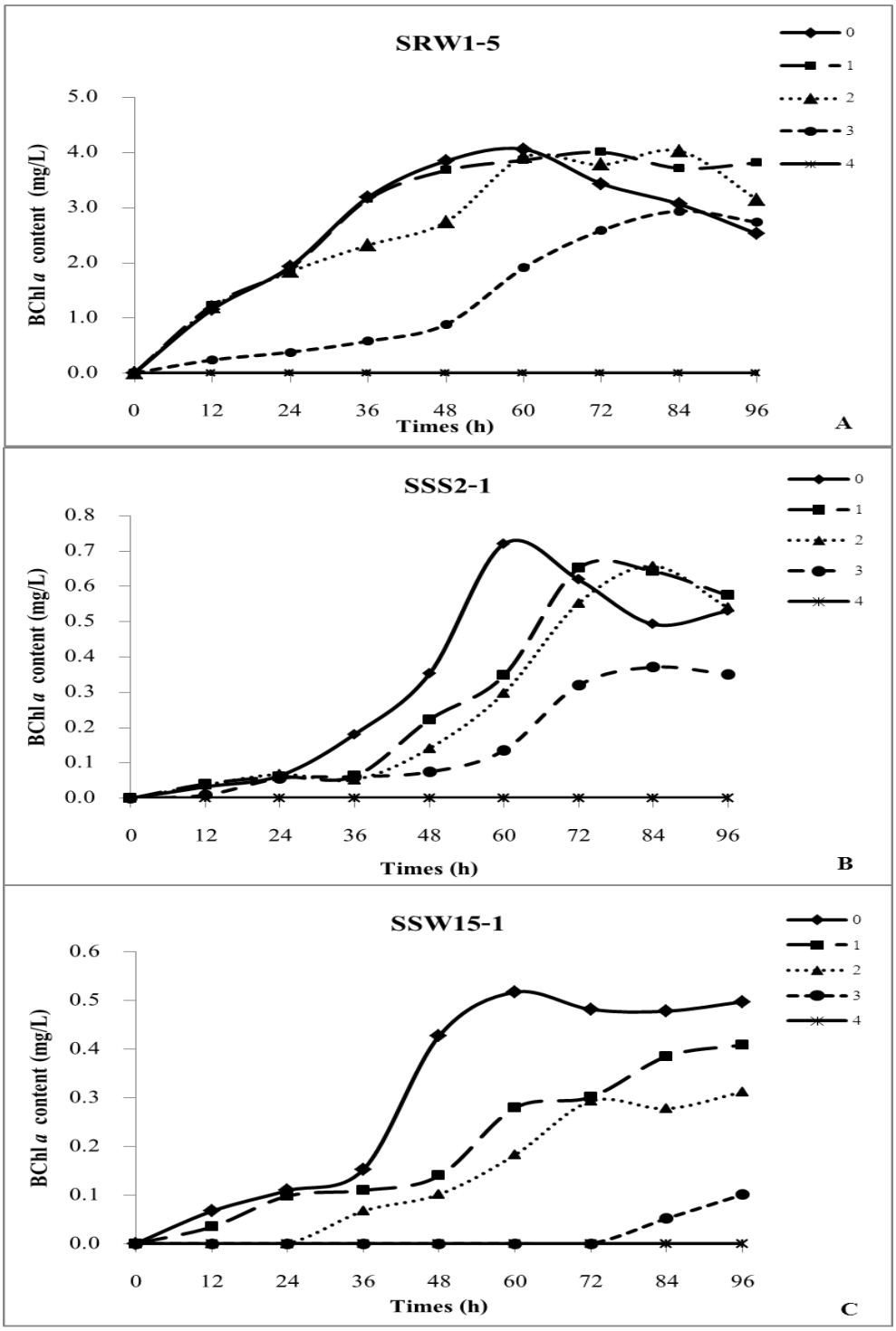

Figure 6-1. Effect of Hg^{2+} on bacteriochlorophyll *a* (BChl *a*) contents of PNSB strains; SRW1-5 (A), SSS2-1 (B) and SSW15-1 (C) grown in GA broth containing 3% NaCl with varying concentrations of 0-4 mg/L $HgCl_2$ under microaerobic light conditions during 96 h incubation.

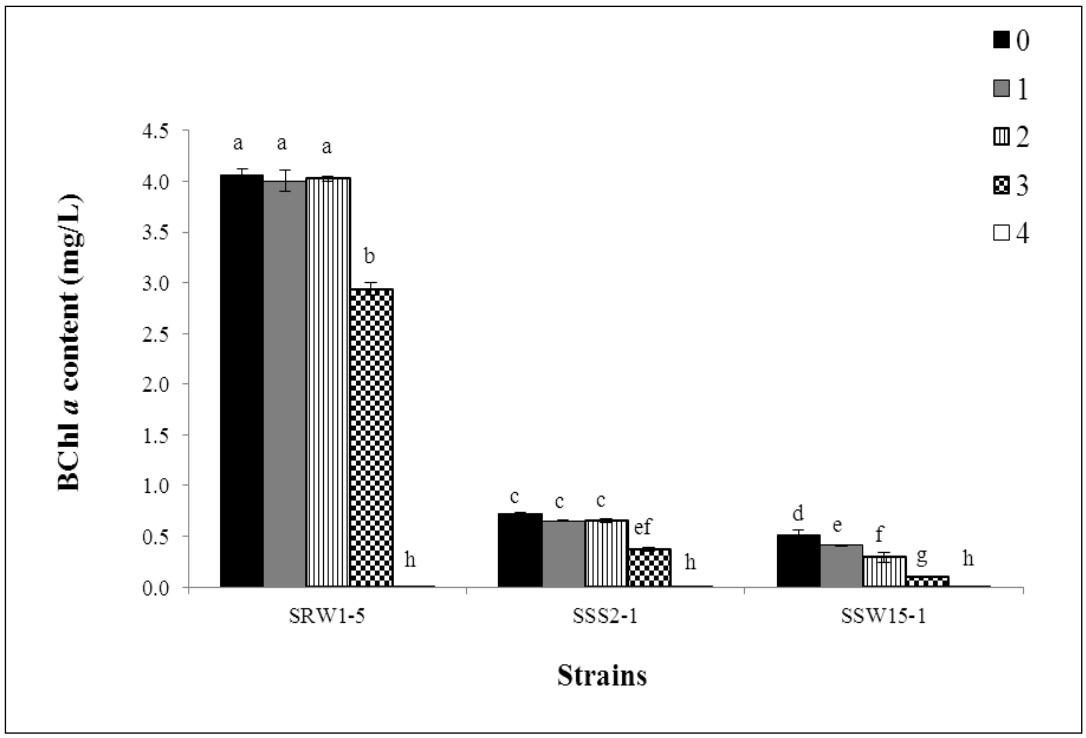

Figure 6-2. The maximal amount of BChl *a* of PNSB strains (SRW1-5, SSS2-1 and SSW15-1) grown in GA broth containing 3% NaCl with various concentrations of $HgCl_2$ (0, 1, 2, 3 and 4 mg/L) under microaerobic light conditions for 96 h. Different lowercase letters above bars indicate significant differences among means ($P < 0.05$). Error bars indicate standard deviations of three determinations.

The effect of Hg^{2+} on electron transported rate (ETR)

The effect of Hg^{2+} concentrations on ETR after acute and long-term toxicity was done in strains SRW1-5 and SSS2-1. In short-term exposure, both cell cultures at their log phase were mixed with $HgCl_2$ at the final concentrations of 1, 2 and 3 mg/L, and cell cultures at 0, 3, 6 and 9 h after exposure were taken to detect ETR using the Junior-PAM machine. The maximal ETR (ETR_{max}) values significantly decreased ($P < 0.05$) following the increasing Hg^{2+} concentrations and exposure times (Figure 6-3). Compared with the control after 9 h exposure, the ETR_{max} values of strain SRW1-5 decreased by 9, 13, 26 and 37% at 1, 2, 3 and 4 mg/L $HgCl_2$, respectively (Figure 6-3A), while the ETR_{max} values of strain SSS2-1 decreased by 11, 10, 33 and 48% at 1, 2, 3 and 4 mg/L $HgCl_2$, respectively (Figure 6-3B).

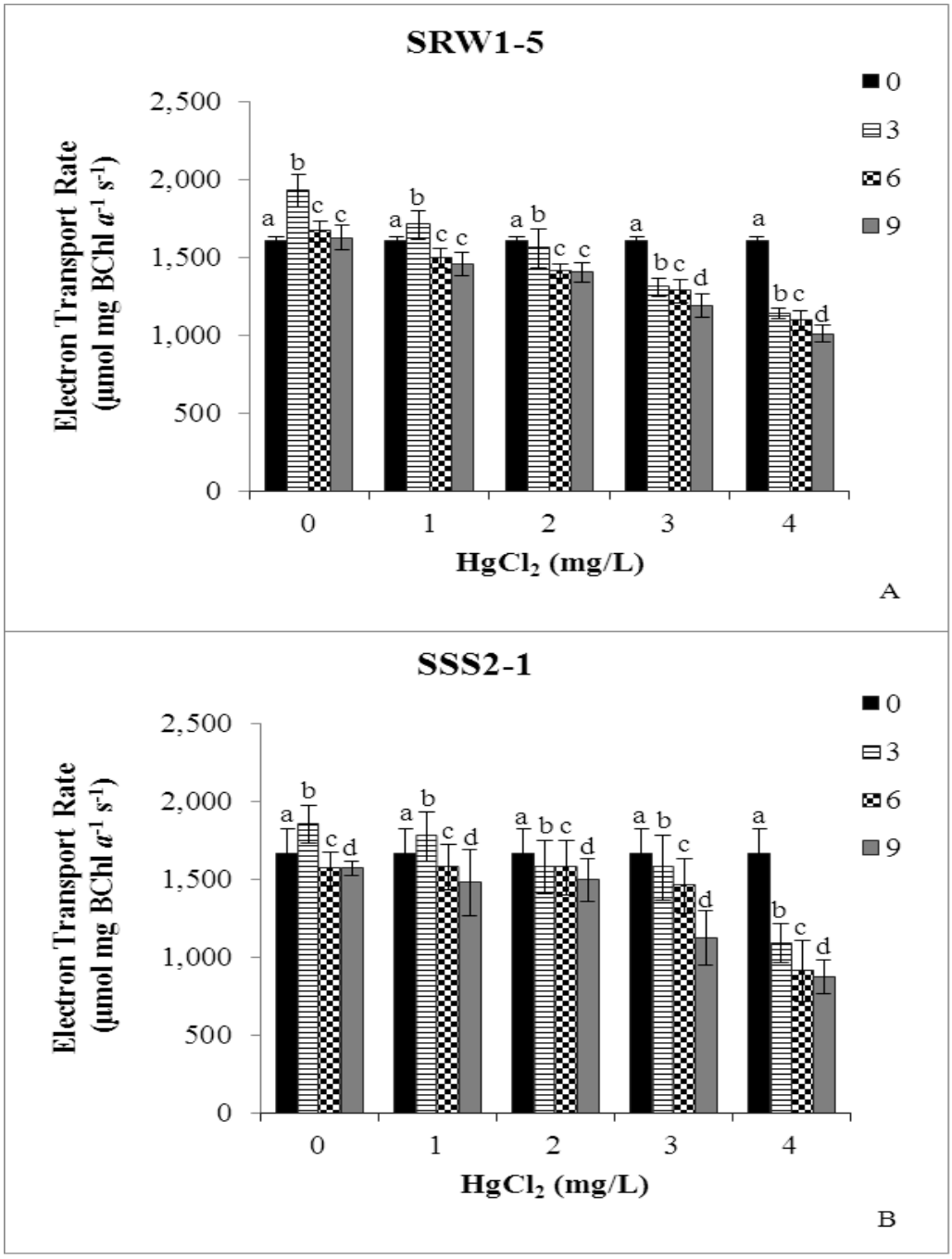

Figure 6-3. Acute effect of $HgCl_2$ (0-4 mg/L) on the ETR_{max} of PNSB strains; SRW1-5 (A) and SSS2-1(B) after 9 h exposure. As $HgCl_2$ concentrations and exposure times significantly decreased on ETR_{max} so only significant differences between means of control and treatment sets in each time interval are indicated by different lowercase letters above bars ($P < 0.05$). Error bars indicate standard deviations of three determinations.

In long-term incubation, PNSB cells (SRW1-5 and SSS2-1) were grown in GA medium containing 1, 2 and 3 mg/L $HgCl_2$, then incubated under microaerobic light conditions for 96 h, and cell cultures were taken every 12 h to detect ETR as well. As shown in Figure 6-4, significant differences of ETR_{max} values of both PNSB strains were observed when compared with controls at different concentration of Hg^{2+} at 12 h interval of sampling for 96 h. The ETR_{max} values in control sets of both strains increased following incubation time until 48 h (1,610 and 1,650 µmol mg BChl a^{-1} s^{-1}), and then they dramatically decreased at 60 h, and finally sharply decreased to nearly zero at 96 h (Figure 6-4). In treated cells after exposure with 1 mg/L $HgCl_2$ for 48 and 60 h, the highest ETR_{max} values in strains SRW1-5 and SSS2-1 were roughly 1,360 µmol mg BChl $a^{-1}s^{-1}$. Whilst at 2 mg/L $HgCl_2$, the highest ETR_{max} values in strains SRW1-5 and SSS2-1 were between 1,047 and 1,148 µmol mg BChl $a^{-1}s^{-1}$ at 72 and 84 h, but at 96 h when cells exposed with 3 mg/L $HgCl_2$ they were 563 and 800 µmol mg BChl $a^{-1}s^{-1}$, respectively.

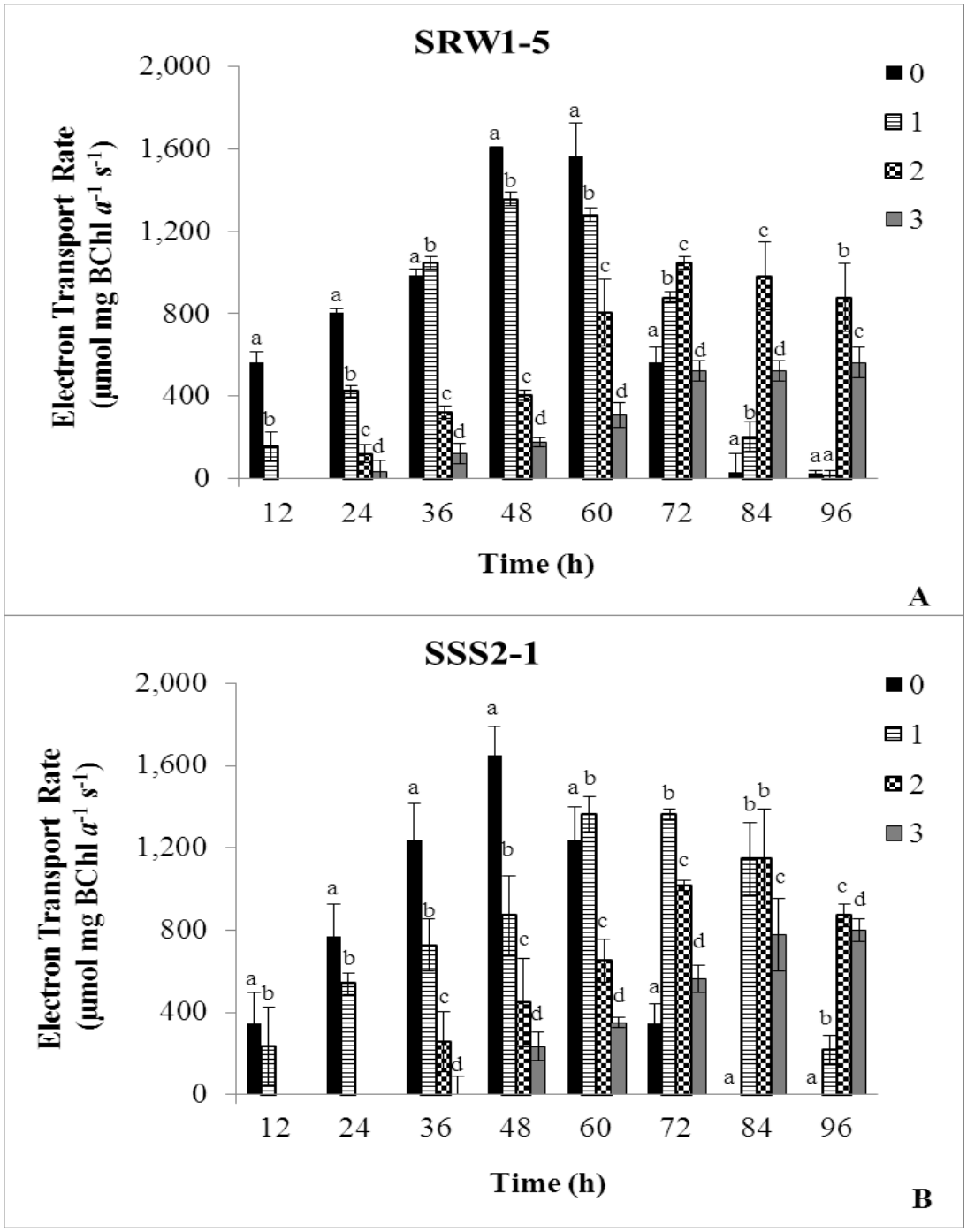

Figure 6-4. The ETR_{max} of PNSB strains SRW1-5 (A) and SSS2-1(B) grown in GA broth containing 3% NaCl with various concentrations of $HgCl_2$ from 0-3 mg/L under microaerobic light conditions. As $HgCl_2$ concentrations and exposure times significantly affected on ETR_{max} so only significant differences between means of control and treatment sets in each time interval are indicated by different lowercase letters above bars ($P < 0.05$). Error bars indicate standard deviations of three determinations.

The effect of Hg^{2+} on cell morphology

The effect of Hg^{2+} on cell morphology of PNSB strains; SRW1-5, SSS2-1 and SSW15-1 was observed by SEM after growing in GA broth containing 3% NaCl and 3 mg/L $HgCl_2$ for 96 h under microaerobic light conditions (Figure 6-5). Cell morphology of strain SRW1-5 as ovoid shape in the control (no Hg^{2+}) (Figure 6-5A) changed to elongated rod in the treated cells (Figure 6-5D). A similar result was observed for both strains, SSS2-1 and SSW15-1, in control sets (Figure 6-5B and C); however, after exposure to Hg^{2+} their shapes were quite differences as a little longer and abnormally like finger for strain SSS2-1 (Figure 6-5E) while not much change in strain SSW15-1 although elongated cells were found (Figure 6-5F).

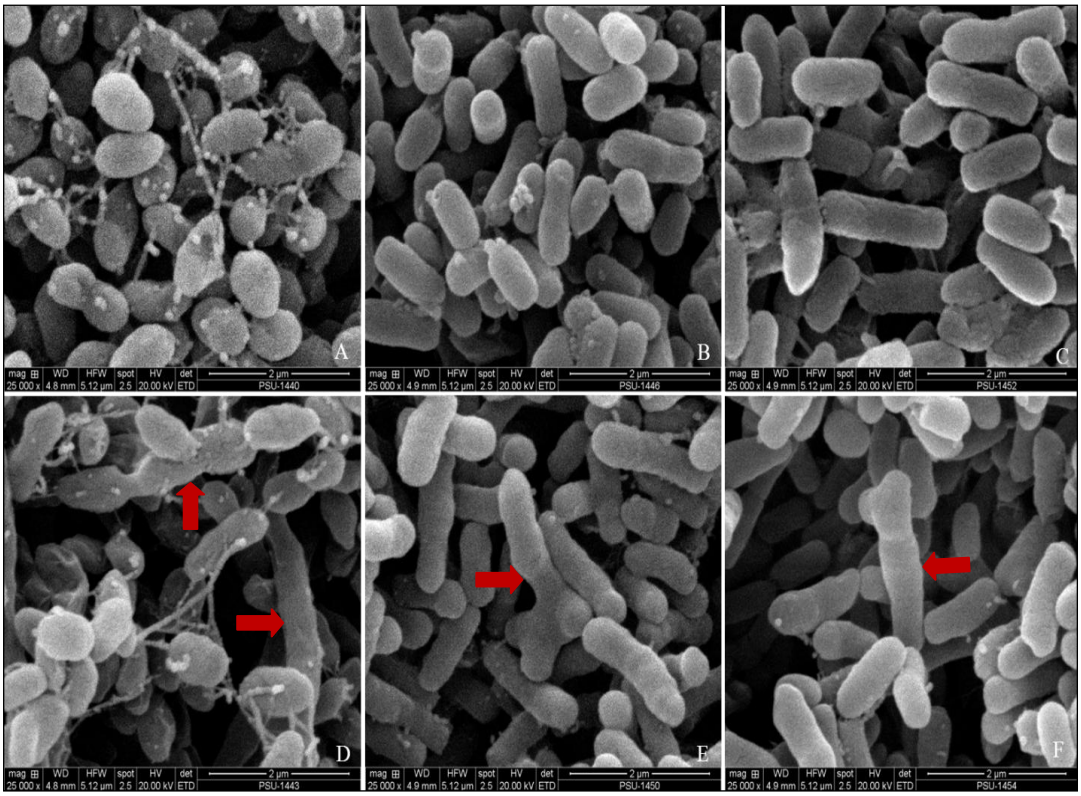

Figure 6-5. Scanning electron micrographs (25,000X) of PNSB strains grown in GA broth containing 3% NaCl in the absence and present of 1 mg/L $HgCl_2$ in microaerobic light conditions for 96 h: control sets; SRW1-5 (A), SSS2-1 (B), SSW15-1 (C) and treatment sets, SRW1-5 (D), SSS2-1 (E) and SSW15-1 (F). The arrow indicates the cell changes when compared with their controls.

Discussion

The effect of Hg^{2+} on amount of photosynthetic pigments

BChl *a* is the most important photopigment for photosynthesis of PNSB (Koblízek et al., 2005), so the toxicity of Hg^{2+} to BChl *a* content and photosynthesis have been studied. Many previous researches showed that Hg has toxicity to photosynthetic apparatus of phototrophic bacteria such as cyanobacteria and algae (Murthy and Mohanty, 1993; Šeršeň et al., 1998; Asztalos et al., 2010; Asztalos et al., 2012; Deng et al., 2013). In this study, a similar pattern was observed for the toxicity of Hg^{2+} on BChl *a* content as a remarkable decreased was found at 3 mg/L $HgCl_2$ (Figure 6-1). This might be that three PNSB strains used in this study are Hg resistant strains (Mukkata et al., 2015b). This result was similar with research of Giotta et al. (2006) that reported about the toxicity of heavy metal ions including Hg^{2+}, Ni^{2+} and Co^{2+} on *R. sphaeroides*, and results showed that these heavy metal ions produced a drop of the BChl molecules when compared to unexposed cells.

As no significant difference for BChl *a* contents was found between controls and treatment sets containing 1-2 mg/L $HgCl_2$ of *R. sulfidophilum* SRW1-5 and *A. marina* SSS2-1 although they are different genera (Figure 6-2). In contrast, significant differences were found for strain *A. marina* SSW15-1 for control and all treatment sets (Figure 6-2). This suggested that resistance to Hg^{2+} depends on strains of PNSB. This imply that our PNSB may detoxify toxic form of Hg^{2+} into less toxic form of Hg^0 via their mercuric reductase enzyme (Mukkata et al., 2015b). However, at the high concentration of $HgCl_2$ up to 4 mg/L, Bchl *a* contents sharply decreased to zero when compared with their control sets (Figure 6-2), so their growth were completely inhibited. At the high concentration of Hg^{2+}, the production of the Bchl *a* contents were dropped because Hg^{2+} can bind strongly to the groups; C=O, C-N, C-S, C-SH of amino acids in photosynthetic apparatus (Bernier and Carpentier, 1995; Šeršeň et al., 1998; Asztalos et al., 2012); consequently damage of photosynthetic apparatus like Bchl *a* and inhibition of their photosynthesis. The investigation of Hg^{2+} toxicity on PNSB cells showed that at the low concentrations was not much negative effect to their Bchl *a* contents in Hg-resistant PNSB strains; on the other words, the

high concentrations of Hg^{2+} had significant toxicity to Bchl *a* contents. These results are in agreement with previous studies (Giotta et al., 2006; Antal et al., 2009; Asztalos et al., 2010; Asztalos et al., 2012).

The effect of Hg^{2+} on electron transported rate (ETR)

The pattern of Hg^{2+} toxicity on amount of BChl *a* of both strains in both short-term and long-term exposures were in accordance with ETR_{max} detected by the Junior-PAM (Figure 6-1, 3 and 4). The ETR_{max} levels of both strains in their controls kept consistency for short-term exposure while they were significantly decreased in treatment sets as increasing of exposure for 9 h and concentrations of $HgCl_2$ up to 4 mg/L (Figure 6-3). This means that Hg^{2+} produced acute toxic to altered BChl *a*, and leading to decrease ETR_{max} levels. In the research of Asztalos et al. (2010), the effect of Hg^{2+} on photosynthetic apparatus of *R. sphaeroides* strain 2.4.1 by the fluorescence induction of whole cells, explained that firstly negative effect of Hg^{2+} became from the attack of Hg^{2+} to the acceptor and/or donor sites of reaction center. Secondly, the Hg^{2+} destroys the light harvesting BChl pigments and weakens the connectivity within the antenna, hence the antenna organization and electron transfer of cells were inhibited. Furthermore, Hg^{2+} affected both light and dark reactions of photosynthesis and strongly inhibited the photosynthetic electron transport chain. The inhibition of ETR by Hg^{2+} was proposed by damage of the electron transfer from Q_A and Q_B and/or by increase of the fraction of closed (Q_{A^-}) RC (Kukarskikh et al., 2003)

For long-term exposure, a remarkable increase of ETR_{max} levels of both strains was found in their controls following incubation time until at 48 h; and then they decreased to nearly zero at 96 h after incubation (Figure 6-4). These seemed to be the same pattern of time course of their BChl contents (Figure 6-1); and the ETR_{max} levels significantly decreased at longer incubation because cells in control sets reached to their death phase (data not shown). The ETR_{max} levels of both strains that exposed to Hg^{2+} were lower than that found in their controls for 60 h incubation; however, the opposite results were found from 72 until 96 h incubation (Figure 6-4). This slightly prolonged the ETR_{max} levels in treatments sets of both strains due to

increasing of the lag-phase duration was observed in treatment sets compared to their controls (Figure 6-1 and 4). The investigation of Hg influence on PNSB, *Rhodobacter sphaeroides* strain R26.1 found that its growth lag-phase duration appeared strongly increased upon increasing of Hg^{2+}; and this indicated a detoxification process of bacteria under Hg stress condition (Giotta et al., 2006).

Hence, the results in this study pointed that decreasing of the ETR_{max} levels of both PNSB strains after growing in $HgCl_2$ might resulted from loss of BChl *a* or damage of reaction center molecules, consequently reduced their photosynthesis efficiency and finally their growth (Chen et al., 2008; Ahmed and Häder, 2010). In addition, it might be that although BChl *a* can be synthesized at the low concentrations of Hg^{2+}, it is highly sensitive to Hg^{2+} and its function may not be complete. The evidence to support as there was no significantly different of the highest Bchl *a* contents of both strains grown in medium containing 1 and 2 mg/L $HgCl_2$ when compared with their controls (Figure 6-2), but the ETR_{max} levels at the same concentrations of Hg^{2+} were significantly less than in control sets (Figure 6-4). However, both PNSB strains could survive at high concentrations of Hg^{2+} resulted from their toxification by mercuric reductase as previously described (Mukkata et al., 2015b) and recovery of cells for withstand in long-term stress condition by changing cell morphology may be possible.

The effect of Hg^{2+} on cell morphology

The effect of Hg^{2+} on cell morphology was observed by SEM confirmed that Hg resistant PNSB cells responded to Hg^{2+} by changing their morphological cells for protective themselves in stress conditions (Figure 6-5). Cell morphology in strains SRW1-5 and SSS2-1 was elongated and abnormally that was similar with the research of Nithya et al. (2011). The increasing of cell surface from cell elongation resulted from the reduction of surface area-volume ratio, and the reduction of surface area-volume ratio decreased the attachment/uptake sites on the cell surface for the heavy metals (Neumann et al., 2005). Hence, cell elongation was the one of protective mechanism of bacteria in stress condition. In addition, the results in this study is in agreement with (Panwichian et al., 2011) who reported that *R.*

sphaeroides KMS24 and *Rhodobium marinum* NW16 (now *Afifella marina*) had elongated cells when exposed with other heavy metals (Cu^{2+}, Zn^{2+}). In addition, the application of the Junior-PAM in this study showed that it would be useful as one of tools for monitoring the effect of Hg^{2+} on photosynthesis process of PNSB in addition of eucaryote. Overall results in the present study concluded that the acute and long-term toxicity of Hg^{2+} affected on photosynthesis process; and the response of Hg resistant PNSB strains to Hg^{2+} were dependent on strains. Long-term exposed cells with Hg^{2+} had adapt themselves for surviving in stress conditions, in contrast, for short-term Hg^{2+} produced an adverse effect to photosynthesis process by decreasing ETR_{max}.

Conclusions

The toxic effect of Hg^{2+} on BChl *a* content, ETR_{max} and cell morphology of Hg resistant PNSB, *R. sulfidophilum* SRW1-5 and *A. marima* SSS2-1 and SSW15-1 was investigated and found that 4 mg/L $HgCl_2$ caused all strains dead. In addition, a significant decrease of ETR_{max} was the acute toxic of Hg^{2+} on PNSB cells. However, lower concentrations of $HgCl_2$ with a longer exposure, exposed cells responded to stress conditions by changing their cell shape to reduce the adverse effect although Hg^{2+} significantly decreased BChl content and consequently affected on ETR_{max} to retard the growth of PNSB. Hence, the information from this study for the effects of Hg^{2+} on PNSB could be used for finding the appropriate ways to use them for bioremediation and also as bioassay candidate of Hg contamination in environments.

CHAPTER 7

Mercury removal by live and dead cells of Hg resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds

Abstract

The aim of this study was to investigate the possibility to remove mercury (Hg) using the live or dead biomass of Hg resistant purple nonsulfur bacteria (PNSB) strains isolated from contaminated shrimp ponds for their potential application during shrimp cultivation. The efficiency of Hg^{2+} removal by three selected PNSB strains; *Rhodovulum sulfidophilum* strain SRW1-5, and *Afifella marina* strains SSS2-1 and SSW15-1, grown with 2 different growth conditions (microaerobic light and aerobic dark conditions) were tested with a 2 mg/L $HgCl_2$ solution using 4.5 mg dry cell weight (DCW)/mL biomass for 30 min. The efficiency to remove Hg^{2+} by the dead cells of all strains was significantly higher than for live cells but the different growth conditions had no effect with the SSS2-1 and SSW15-1 strains. However, both cell types (dead or live) of strain SRW1-5 had a higher efficiency to remove Hg^{2+} when grown with microaerobic light conditions than with aerobic dark conditions. Strain SSS2-1 showed higher Hg removal efficiency under both growth conditions, therefore strain SSS2-1 was selected for further investigation. The highest Hg removal percentage under the optimum conditions using a 4 mg/L initial $HgCl_2$ concentration by live and dead cells grown with both incubating conditions were approximately 87 and 95%, respectively. Under both incubating conditions, Hg biosoption by strain SSS2-1 fitted the Freundlich model for live cells and the Langmuir model for dead cells. The kinetics of Hg biosorption by both cell types of strain SSS2-1 suggests the process as a pseudo-second order kinetic model rather than a pseudo-first order kinetic model. This study demonstrates that the SSS2-1 live or dead biomass has great potential for its application to remove Hg from contaminated shrimp ponds by biosorption.

Keywords: biosorption, mercury, purple nonsulfur bacteria, shrimp cultivation

บทคัดย่อ

การวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ของการใช้เซลล์เป็นและเซลล์ตายของแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria; PNSB) สายพันธุ์ที่ต้านทานต่อปรอทซึ่งคัดแยกได้จากนากุ้งที่ปนเปื้อนปรอทเพื่อพิจารณาศักยภาพการนำมาประยุกต์ใช้ในระหว่างการเพาะเลี้ยงกุ้ง การทดสอบประสิทธิภาพการกำจัดปรอทของ PNSB สามสายพันธุ์ที่ถูกคัดเลือกได้แก่ *Rhodovulum sulfidophilum* สายพันธุ์ SRW1-5 และ *Afifella marina* สายพันธุ์ SSS2-1 และ SSW15-1 ซึ่งเจริญในสองสภาวะ (สภาวะมีแสง มีอากาศเล็กน้อย และไร้แสง มีอากาศ) ซึ่งสภาวะการทดสอบมีสารละลายปรอทเท่ากับ 2 มิลลิกรัมต่อลิตร ปริมาณเซลล์เทียบเท่ากับ 4.5 มิลลิกรัมของน้ำหนักเซลล์แห้งต่อมิลลิลิตร และใช้ระยะเวลา 30 นาที ประสิทธิภาพการกำจัดปรอทของเซลล์ตายของทั้งสามสายพันธุ์มีค่าสูงกว่าเซลล์เป็นอย่างมีนัยสำคัญทางสถิติ แต่เซลล์ที่เจริญในสภาวะที่ต่างกันของสายพันธุ์ SSS2-1 และ SSW15-1 ไม่มีผลต่อการกำจัดปรอท แต่อย่างไรก็ตามเซลล์ทั้งสองชนิด (เซลล์เป็นและเซลล์ตาย) ของสายพันธุ์ SRW1-5 ที่เลี้ยงในสภาวะมีแสง มีอากาศเล็กน้อย มีประสิทธิภาพต่อการกำจัดปรอทสูงกว่าเซลล์จากการเลี้ยงในสภาวะไร้แสง มีอากาศ โดยที่สายพันธุ์ SSS2-1 จากการเลี้ยงทั้งสองสภาวะมีค่าการกำจัดปรอทสูงสุด ดังนั้นสายพันธุ์ SSS2-1 จึงถูกคัดเลือกเพื่อใช้ในการศึกษาในขั้นตอนต่อไป การกำจัดปรอทสูงสุดภายใต้สภาวะที่เหมาะสมที่ความเข้มข้นของสารละลายปรอทเท่ากับ 4 มิลลิกรัมต่อลิตรของเซลล์เป็นและเซลล์ตายจากการเลี้ยงในสองสภาวะมีค่าประมาณ 87 และ 95 เปอร์เซ็นต์ ตามลำดับ การดูดซับปรอทภายใต้สภาวะที่เหมาะสมของสายพันธุ์ SSS2-1 พบว่าการดูดซับของเซลล์เป็นเป็นไปตามแบบจำลอง Freundlich ส่วนการดูดซับของเซลล์ตายเป็นไปแบบจำลอง Langmuir แบบจำลองจลนพลศาสตร์ของการดูดซับปรอทของเซลล์ทั้งสองชนิดของสายพันธุ์ SSS2-1 เป็นไปตามแบบจำลองจลนพลศาสตร์ pseudo-second order มากกว่าแบบจำลอง pseudo-first order จากการศึกษาในครั้งนี้แสดงให้เห็นว่าทั้งเซลล์เป็นและเซลล์ตายของสายพันธุ์ SSS2-1 มีประสิทธิภาพที่ดีต่อการนำไปใช้ในการกำจัดปรอทด้วยวิธีการดูดซับทางชีวภาพในพื้นที่นากุ้งที่ปนเปื้อนปรอท

คำสำคัญ: การดูดซับ การปนเปื้อน แบบจำลองจลนพลศาสตร์ ปรอท แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์ การเพาะเลี้ยงกุ้ง

Introduction

Shrimp farming is one of the important aquaculture businesses in Thailand; and the worldwide export of shrimp from Thailand was about 34, 800 tonnes during the first quarter of 2015 (FAO, 2015). However, the residues of chemicals and antibiotics used during shrimp cultivation has become a major concern due to their persistence and toxicity in shrimp ponds (Gräslund et al., 2003; Visuthismajarn et al., 2005; Lacerda et al., 2011). Mercury (Hg) is one of the toxic elements that have been recently reported to be present in aquatic animals such as shrimp, fish, shellfish, crab, and mollusks (Brambilla et al., 2013; Li et al., 2013). During our previous studies we have found Hg contamination in water and sediment samples from various shrimp ponds located in the south of Thailand (Mukkata et al., 2015b). Hence, Hg contamination can be one critical problem for shrimp cultivation that should be dealt with, because it might accumulate in shrimp through the food web and through contaminated shrimp consumption pose risks to human health.

As a response to toxic Hg in either the inorganic or organic forms present in the contaminated environment, microorganisms have developed some resistance mechanisms for detoxification (Barkay et al., 2003). To deal the problem of Hg contaminated areas, Hg resistant microorganisms have been isolated from contaminated environments to investigate their resistance mechanisms to assist in bioremediation (Glendinning et al., 2005; Zhang et al., 2012; Yu et al., 2014). Bioremediation has received increasing attention in recent times to help to clean up polluted environments, because it is efficient, eco-friendly and cost effective (Vijayaraghavan and Yun, 2008). Of the many bioremediation methods, the use of live or dead biomass as biosorbents for bioaccumulation and/or biosorption have been demonstrated to possess good potential to remove metals from an aqueous phase. Biosorption is a non-metabolic mediated passive process of removing toxicants by adsorption on dead or inactive biomass, while bioaccumulation is the metabolic driven uptake of toxicants by living cells (Vijayaraghavan and Yun, 2008; Chojnacka, 2010). The capacity to remove metals by microbes depends on the biomass types and their properties that are related to the functional groups of the

biding sites on cells wall and also the parameters associated with the sorption systems such as the maximum concentrations of the biosorbent and the contaminant metals ions, the pH and temperature (Abbas et al., 2014). Moreover, the isotherm and kinetic sorption parameters are also investigated to determine optimum conditions (Lesmana et al., 2009; Chojnacka, 2010) for the highest potential of the biosorption systems.

Purple non sulfur bacteria (PNSB) are the most versatile group of photosynthetic bacteria that gain their energy from light by anoxygenic photosynthesis process while using CO_2 or organic compounds as a carbon source. In addition, they can grow by using organic compounds for energy and carbon sources using aerobic dark conditions via fermentation processes that include anaerobic respiration in the dark (Imhoff, 1995; Madigan and Jung, 2009). Normally, these bacteria are widely distributed in various natural habitats that are enriched with nutrients and exposed to sunlight including manmade environments such as wastewater systems and shrimp ponds (Mukkata et al., 2015b). Several research groups have reported that PNSB are relatively resistant to heavy metals and have the potential to remove them (Watanabe et al., 2003; Bai et al., 2008; Panwichian et al., 2010; Panwichian et al., 2012).

It should be noted that PNSB are so versatile that they can be potential probiotics and are also used to clean up water in shrimp cultivation (Qi et al., 2009; Shapawi et al., 2012). Hence, they have the potential to be used for many purposes in shrimp cultivation. Our previous studies demonstrated that PNSB are part of the normal flora in shrimp ponds. A few PNSB strains can volatilize Hg and are therefore Hg resistant (Mukkata et al., 2015b). In addition to removing Hg from contaminated shrimp ponds by biovolatilization, the selected PNSB should be investigated for their potential to decontaminate Hg in shrimp ponds through bioaccumulation and biosorption. There have been no previous reports on the removal of Hg from shrimp ponds by PNSB. The aims of this work were to determine the potential of live and dead PNSB biomass to remove Hg including factors that affect Hg biosorption prior to their application in removing Hg and bioremediation in shrimp ponds during shrimp cultivation.

Materials and methods

Inoculum and biomass preparations

The Hg resistant PNSB ; *Rhodovulum sulfidophilum* strain SRW1-5, and *Afifella marina* strains SSS2-1 and SSW15-1 isolated from our previous study (Mukkata et al., 2015b) were used in this work. To obtain an active inoculum, each strain was subcultured twice by inoculating into glutamate acetate medium (GA) containing 3% NaCl and incubated under microaerobic light conditions (ca. 3,000 lux) for 48 h prior to their use in further studies. To obtain biomass, all selected PNSB strains were grown with GA broth containing 3% NaCl with a half volume of the test tubes covered with aluminium foil to provide aerobic dark conditions in a shaker at 150 rpm, at 35 ºC for 48 h. At the same time microaerobic light conditions were set by filling the same medium with a full volume of the test tubes and providing an illumination of roughly 3,000 lux for 48 h. Culture broths were centrifuged at 8,000 rpm for 15 min and washed three times with 0.1% peptone water (pH 7.0) for preparing live cells, whereas dead biomass was prepared from similarly grown live cells but dried at 60^{o}C in an oven for 10 h (Wang et al., 2010).

Biosorption experiments

Each wet pellet and dead biomass that was equivalent to 4.5 mg dry cell weight (DCW)/mL (Panwichian et al., 2010) was added into 10 mL of a 2 mg/L $HgCl_2$ solution, at a pH 7.0 and incubated with both conditions of aerobic dark and microaerobic light as previously described for 30 min depending on the growth conditions i.e. cells harvested from aerobic dark used aerobic dark conditions. Cell suspensions were centrifuged at 8,000 rpm for 15 min and the supernatant was mixed with 5% HNO_3 and the remaining Hg concentration was then determined by a Flow Injection Mercury Systems (FIMS). The potential of live and dead cells of three PNSB strains to remove Hg^{2+} was calculated and compared with control sets without biomass under both sets of incubating conditions.

Factors affecting the removal of Hg^{2+} by a selected PNSB strain

For growth studies, a ten percent inoculum of a selected strain was grown with GA broth containing 3% NaCl under both optimal growth conditions as previously described. Cell growth was observed every 6 h. Bacterial cells were harvested from their various phases (log, late log, and stationary). The cell pellets for biosorption were prepared as previously described to study the optimal growth phases and the following factors; biomass doses by collecting cells from the optimum growth phase for Hg^{2+} removal and a varying cell density of 3.5, 4.0, 4.5, 5.0 and 5.5 mg DCW/mL; initial Hg concentrations by using $HgCl_2$ solution at 1-8 mg/L; varying pH values of 5.0, 6.0, 7.0, 8.0 and 9.0; varying temperatures of 25, 30, 35 and 40°C; and varying contact times of 0-120 min. In addition, the presence of other cationic ions; 3.5% NaCl (Na^+), 85 mg/L $CaCl_2$ (Ca^{2+}), 160 mg/L $MgSO_4$ (Mg^{2+}) and 0.75 mg/L $CdCl_2$ (Cd^{2+}) were also investigated by following the methods as described by Panwichian et al. (2010). Values for the pH and temperature were designed according to the conditions of the shrimp cultivation. The Hg concentrations were set to match the contamination levels of Hg found in the shrimp ponds (Mukkata et al., 2015b).

Biosorption isotherm models

The biosorption isotherms for removing Hg by a selected PNSB strain SSS2-1 was obtained with varying Hg concentrations of 1 and 8 mg/L $HgCl_2$ solutions, a pH of 7.0 and 5.0 mg DCW/mL of live or dead cells that had been grown with both incubating conditions of microaerobic light and aerobic dark for a 60 min exposure. Each biomass was separated from the solutions containing $HgCl_2$ by centrifugation at 8,000 rpm for 15 min, and then the remaining Hg concentration was determined by FIMS. The remaining Hg concentrations were used to determine the Hg sorption isotherms. The amount of metal ions (Hg^{2+}) sorbed by the biomass (q_e, mg/g) was calculated as follows:

$$\frac{}{m} \quad (1)$$

where C_0 and C_e are the initial and equilibrium solution concentrations (mg/L), respectively, V is the volume of the solution (L) and m is the amount of adsorbent or biomass (g).

The results were fitted to the Langmuir and Freundlich models that were described by formulae (2) and (3), respectively (Abbas et al., 2014).

$$q_e = \frac{1+K_L C_e}{q_m K_L C_e} \quad (2)$$

$$q_e = K_F C_e^{1/n} \quad (3)$$

Where q_e and q_m are the final amount and the maximum of Hg^{2+} per unit mass of adsorbent (mg/g), C_e is the equilibrium Hg concentrations (mg/L), K_L is the Langmuir equilibrium constant, and K_F and n are Freundlich equilibrium constants.

A plot of C_e/q_e versus C_e yielded a straight-line with a slope and intercept that corresponded to $(1/q_m)$ and $(1/K_L.q_m)$, respectively, from which q_m and K_L were calculated from equation (2) of the Langmuir models. Meanwhile, a plot of $\log q_e$ versus $\log C_e$ yielded a straight line with a slope and intercept that correspond to $(1/n)$ and $(\log K_F)$, respectively, from which n and K_F were calculated assuming the adsorption process follows equation (3) of the Freundlich model. Then, a regression co-efficient (R^2) was calculated and compared to the linear regression equations of the Langmuir and Freundlich model graphs. The model that fitted better was considered for Hg sorption by PNSB (SSS2-1) biomass.

Biosorption kinetic models

In the biosorption kinetic models for Hg by a selected PNSB strain, a 5.0 mg DCW/mL was suspended in 4.0 mg/L $HgCl_2$ solution, pH 7.0. Samples were taken from the solutions at contact times of 0, 5, 15, 30, 45, 60, 75, 90 and 120 min, centrifuged at 8,000 rpm for 15 min and the supernatants were collected. The

remaining Hg concentrations were determined by FIMS and then q_e (mg/g) was calculated using equation (1). The results were adjusted to the pseudo-first and pseudo-second order models. The pseudo-first and pseudo-second order models were calculated by the equations (4) and (5), respectively (Abbas et al., 2014):

$$\frac{dq}{dt} = K_1(q_e - q_t) \quad (4)$$

$$\frac{dq}{dt} = K_2(q_e - q_t)^2 \quad (5)$$

Where q_e and q_t are the values of the amount adsorbed per unit mass at equilibrium and any times, respectively (mg/g) and K_1 (1/min) and K_2 (g/mg min) are the pseudo-first and pseudo-second order adsorption rate coefficient, respectively. The plot of log ($q_e - q_t$) versus t gives a straight-line graph with a slope and intercept that were used to calculate the K_1 and q_e for the pseudo-first order kinetics. Meanwhile, the plot of (t/q_t) versus t provided a straight-line graph with a slope and intercept that were used to calculate q_e and K_2 for the pseudo-second order kinetics. Then, R^2 was calculated and compared to the linear regression equations of the graphs of the pseudo-first and second order models. If the R^2 values of any of the models is higher than another model, the kinetics of Hg sorption by the PNSB biomass is based on that model.

Statistics

All experiments in this study were conducted in triplicate and data presented as mean with standard deviation. For analysis of the data one way ANOVA was performed and significant differences among means were analyzed using the Duncan's multiple range tests at a P-value < 0.05.

Results

The potential of selected PNSB strains to remove Hg^{2+} by biosorption

The potential of the three selected PNSB strains following their growth under microaerobic light and aerobic dark conditions were tested for their ability to remove Hg^{2+} from a 2 mg/L $HgCl_2$ solution using 4.5 mg DCW/mL of live or dead cells of each strain for 30 min using the same conditions as for the growth. The efficiency of Hg^{+2} removal by live cells of strains SRW1-5, SSS2-1 and SSW15-1 under conditions of microaerobic light were 81.11, 76.31 and 79.29% while from aerobic dark conditions they were 71.67, 78.93 and 77.14%, respectively (Figure 7-1A). In contrast, the removal percentage of Hg^{2+} by dead cells was 92.94, 89.90 and 92.92% for SRW1-5, SSS2-1 and SSW15-1 under microaerobic light and 87.50, 89.45 and 91.61% under aerobic dark conditions (Figure 7-1B). A higher Hg^{2+} biosorption by all selected PNSB strains was observed using dead cells, particularly strain SRW1-5 when compared to the live cells (Figure 7-1). Strains SSS2-1 and SSW15-1 showed no significant difference for their efficiency to remove Hg^{2+} under both incubating conditions suggesting their potential for bioremediation in shrimp ponds. However, the strain SSS2-1 was selected for further studies due to its higher resistance to Hg^{2+} compared to strain SSW15-1 (Mukkata et al., 2015b).

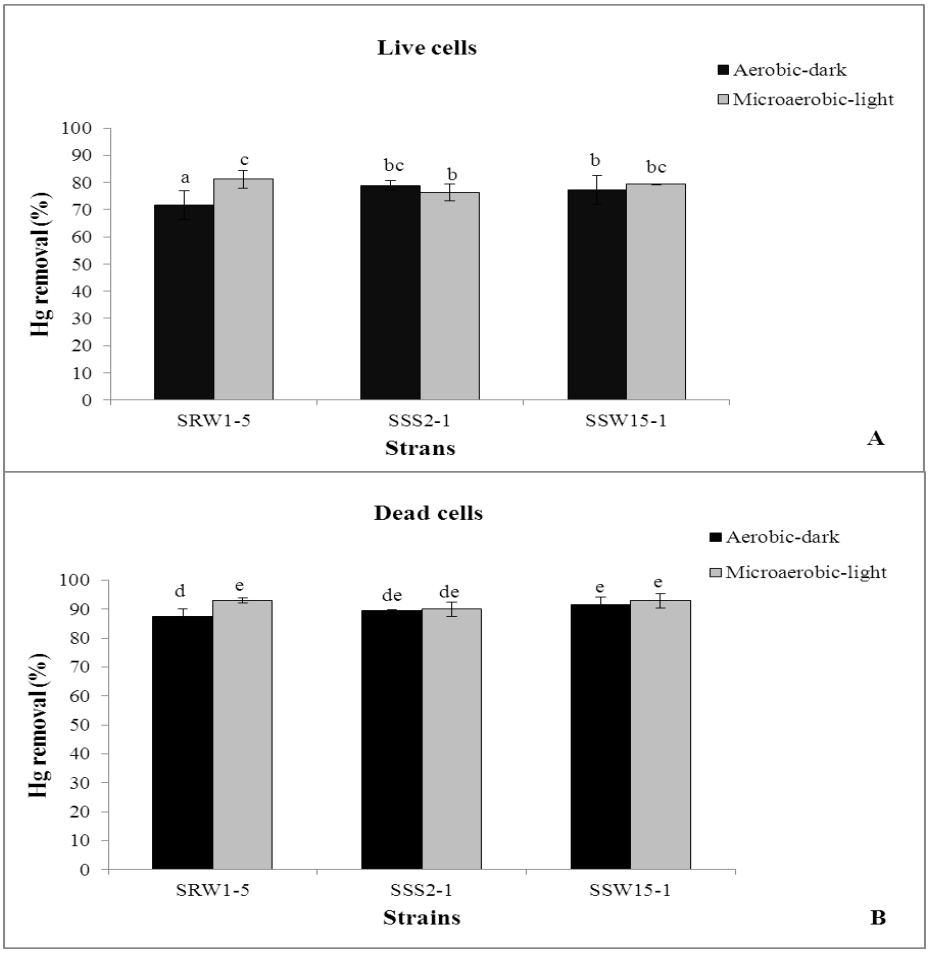

Figure 7-1. The potential of three PNSB strains to remove Hg^{2+}at 2 mg/L $HgCl_2$, pH 7.0 for 30 min by 4.5 mg DCW/mL of live (A) and dead cells (B) under microaerobic light and aerobic dark conditions. Different lowercase letters above bars indicate significant differences among the means of both cell types ($P < 0.05$). Error bars indicate standard deviations of three determinations.

Factors affecting Hg removal by a selected PNSB strain

Based on previous experiments, *A. marina* SSS2-1 was selected for further studies as its removal of Hg^{2+} by both live or dead cells from both incubating conditions was very high (Figure 7-1). Results of the cell growth phases from log, late log and stationary phases on Hg^{2+} removal by live or dead cells of strain SSS2-1 are shown in Figure 7-2A, B. The percentage Hg^{2+} removal of live cells from their log

and late log phases from both incubating conditions were similar of about 80%, while biosorption of cells from their stationary phase decreased to 61% and 69% for the microaerobic light and aerobic dark conditions, respectively (Figure 7-2A). In contrast, dead cells obtained from both incubating conditions showed no significant difference in their ability to remove Hg^{2+} as both were in the range of 92-96% removal (Figure 7-2B).

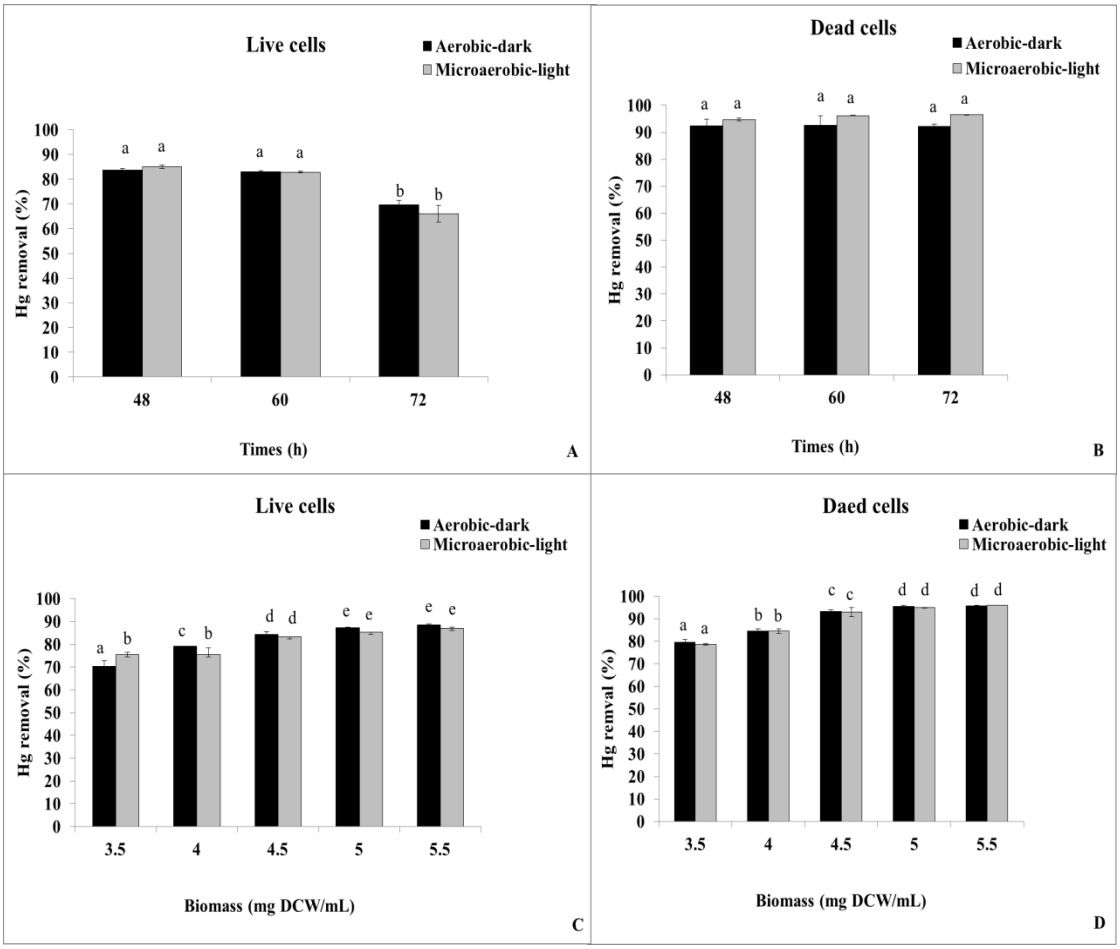

Figure 7-2. Effects of cell ages (48 , 60 and 72 h for log, late log and stationary phases) of live (A) and dead cells (B), and biomass doses of live (C) and dead cells (D) of *A. marina* SSS2-1 on Hg^{2+} removal at 4 mg/L $HgCl_2$, pH 7.0 for 30 min under microaerobic light and aerobic dark conditions. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bars indicate standard deviations of three determinations.

The effects of increasing the biomass dose from 3.5 to 5.5 mg DCW/mL of live or dead cells grown under both incubating conditions was tested using a 4 mg/L $HgCl_2$, a pH of 7.0 solution for 30 min incubated with the same conditions as for growth. The removal percentage of Hg^{2+} by both cell types significantly increased with increasing biomass from 3.0 to 5.0 mg DCW/mL. Therefore, the optimum biomass dose for Hg^{2+} removal was 5.0 mg DCW/mL but there was no significant difference for the 5.0 and 5.5 mg DCW/mL. This biomass dose was therefore selected for further studies. Under both incubating conditions of microaerobic light and aerobic dark at a dosage of 5.0-5.5 mg DCW/mL, the maximum Hg^{+2} removal of live cells were 87 and 88 %, respectively, but it was 95 % using dead cells (Figure 7-2C, D).

Increasing the initial $HgCl_2$ concentrations from 1 to 8 mg/L produced different effects on the percentage Hg^{2+} removal by live or dead cells; however, no significant differences were found between both incubating conditions (Figure 7-3A, B). Live cells showed a dose-dependent decrease in their ability to remove Hg (Fig 3A). In contrast, the dead cells did not show any significant difference in their Hg removal ability at 1-3 mg/L $HgCl_2$ concentrations although a marginal decrease (up to 20%) in their Hg^{2+} removal efficiency was observed between 4-8 mg/L $HgCl_2$ concentrations (Figure 7-3B).

Removal of Hg^{2+} under both incubating conditions with the use of 5.0 mg DCW/mL of live or dead biomass significantly increased over a range of pH from 5-7 and thereafter significantly decreased at pH values between 8-9 (Figure 7-3C, D). The optimum pH to remove Hg^{2+} for both cell types under both incubation conditions was 7.0 followed by 6 and 8; and thus the optimum pH of 7.0 was selected for further studies.

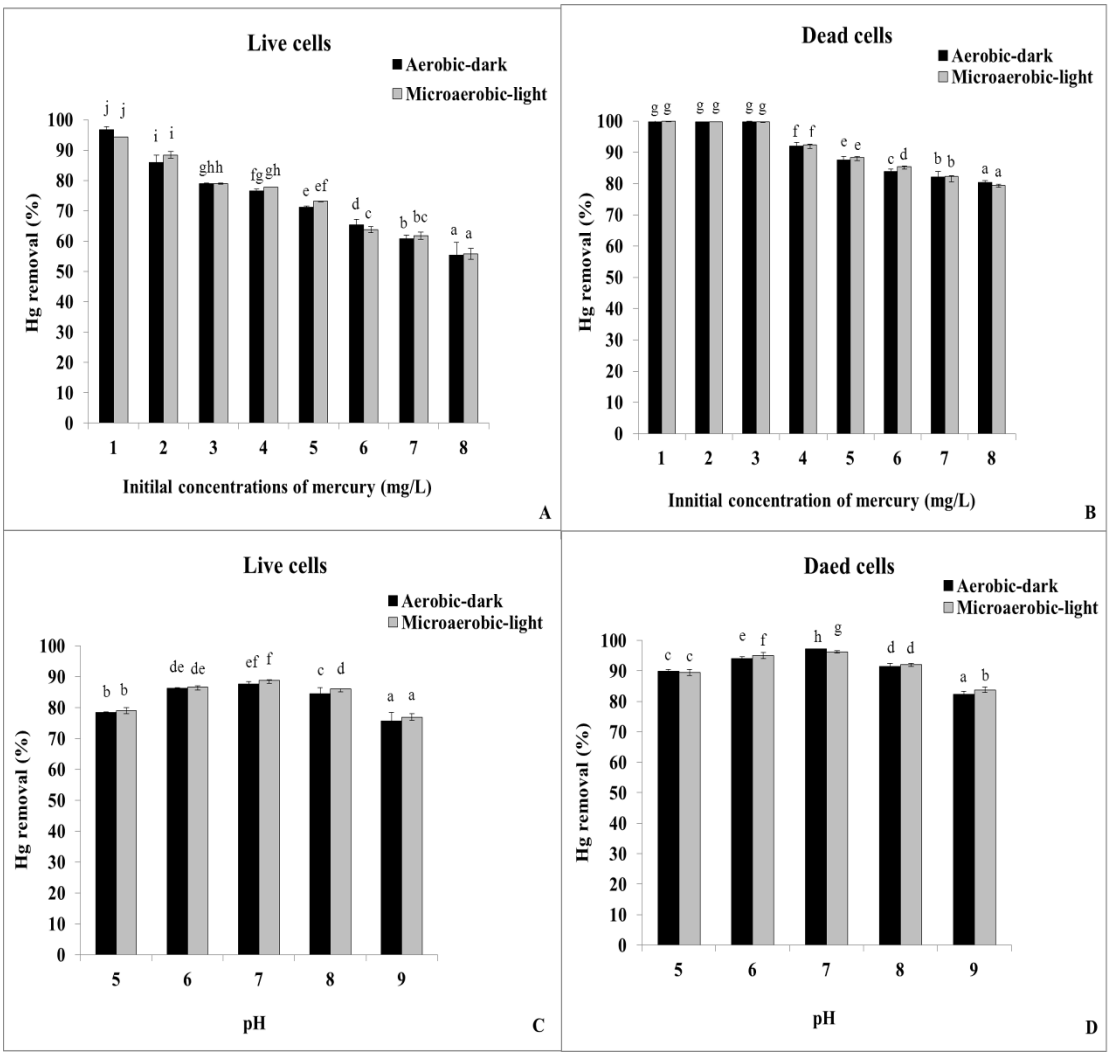

Figure 7-3. Effect of initial concentrations of Hg^{2+}on its removal at pH 7.0 by live (A) and dead cells (B), and the effect of pH at 4 mg/L $HgCl_2$, pH 5.0-9.0 by live (C) and dead cells (D) from the biomass of *A. marina* SSS2-1 (5.0 mg DCW/mL) under microaerobic light and aerobic dark conditions for 30 min. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bars indicate standard deviations of three determinations.

Increasing contact times from 5 to 120 min increased Hg^{2+} removal by live or dead cells (5 mg DCW/mL, pH 7.0) with a marked effect on live cells than dead cells under both incubating conditions (Figure 7-4A, B). For live cells, the removal percentage of Hg^{2+}significantly increased from about 60% to 89% with

increasing contact times from 5-90 min (Figure 7-4A). However, the biosorption of dead cells increased suddenly after 5 min of contact time (≈82-88%), but there was little further increase up to 60 min. Furthermore, the removal percentage of Hg^{2+} significantly decreased at higher contact times (75, 90 and 120 min) (Figure 7-4B). According to these results 60 min of contact time was selected for further studies of both cell types under both incubating conditions because the aim was to investigate Hg^{2+} removal by biosorption although over the longer time of 90 min the efficiency of live cells was similar to the dead cells at 15-60 min.

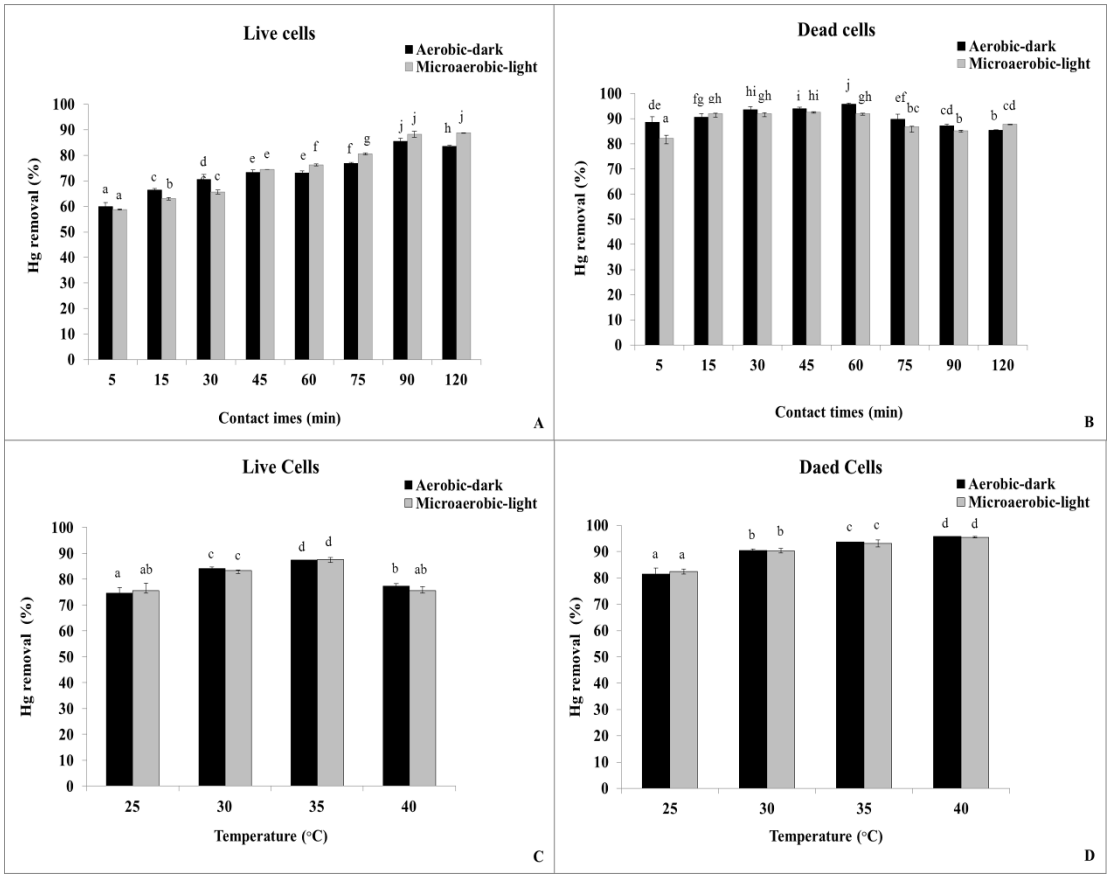

Figure 7-4. Effects of contact time and temperature on Hg^{2+} removal by live (A, C) and dead cells (B, D) from biomass (5.0 mg DCW/mL) of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions at 4 mg/L $HgCl_2$ pH 7.0 for 30 min. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bars indicate standard deviations of three determinations.

The effect of temperature (25 to 35°C) on Hg^{2+} removal by live or dead cells under both incubating conditions was investigated using conditions of 5.0 mg DCW/mL of live or dead cells, pH 7.0 for 60 min (Figure 7-4C, D). Under both incubating conditions, the removal percentage of Hg^{+2} by live cells significantly increased from 75 to 87% with an increase of the temperature up to 35°C, but at a higher temperature of 40°C there was a significant reduction of 76% (Figure 7-4C). However, the removal percentages of Hg^{2+} using dead cells increased from 82 to 95% with an increase of the temperatures from 25 to 40°C with a significant increase at 30°C (Figure 7-4D). Hence, 35^{o}C was selected as the optimum temperature for further studies for both cell types.

The effect of the presence of other cationic ions; 3.5% NaCl (Na^{+}), 85 mg/L $CaCl_2$ (Ca^{2+}), 160 mg/L $MgSO_4$ (Mg^{2+}) and 0.75 mg/L cadmium ($CdCl_2$) (Panwichian et al., 2010) on the Hg removal by live or dead cells (5.0 mg DCW/mL) was evaluated at a pH of 7.0 and temperature of 35°C for 60 min. The presence of other ions significantly decreased the removal percentage of Hg^{+2} when compared with the control sets (Figure 7-5). There was a significant reduction of the percentage of Hg^{+2} removed under both incubating conditions by both cell types in the order of Cd^{2+} and Mg^{2+} (28-31%) > Ca^{2+} (24-25%) > Na^{+} (18-20%); however, the presence of these other light and heavy metal ions had more effect on live cells than on dead cells.

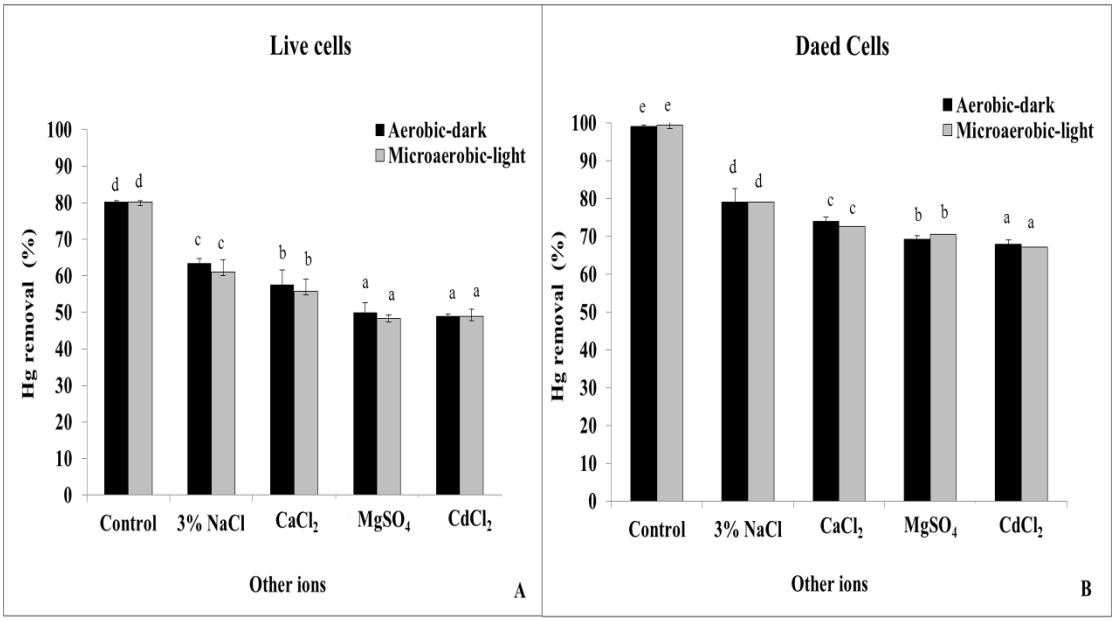

Figure 7-5. Effect of other ions on Hg^{2+} removal at 4 mg/L $HgCl_2$, pH 7.0 for 60 min by 5.0 mg DCW/mL by live (A) and dead cells (B) of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bar indicates standard deviations of three determinations.

Isotherms of Hg sorption by a selected PNSB biomass

The adsorption isotherms of Hg biosoption by live or dead cells of *A. marina* SSS2-1 grown with either microaerobic light or aerobic dark conditions were analyzed using the Langmuir and Freundlich models. Both models have been commonly used for interpretation of biosorption processes. The linear regressions of the data of the Langmuir and Freundlich models are presented in Figure 7-6. The values of constants from both models, and the linear correlation coefficients (R^2) are displayed in Table 7-1. The prediction of Hg biosorption by the Langmuir model of live cells was less precise with a lower R^2 values of 0.954 and 0.970 when growth with microaerobic light and aerobic dark conditions, respectively, while the prediction by the Freundlich model gave R^2 values of 0.994 and 0.991 under microaerobic light and aerobic dark conditions, respectively. In contrast, the values of R^2 from the dead

cells (0.986 and 0.993) that were predicted by the Langmuir model were higher than the predicted from the Freundlich model (0.824 and 0.712). Therefore, the Hg^{2+} sorption of live cells under both conditions was in agreement with the Freundlich model. In contrast, the biosoption of dead cells collected from both incubating conditions was in agreement with the Langmuir model.

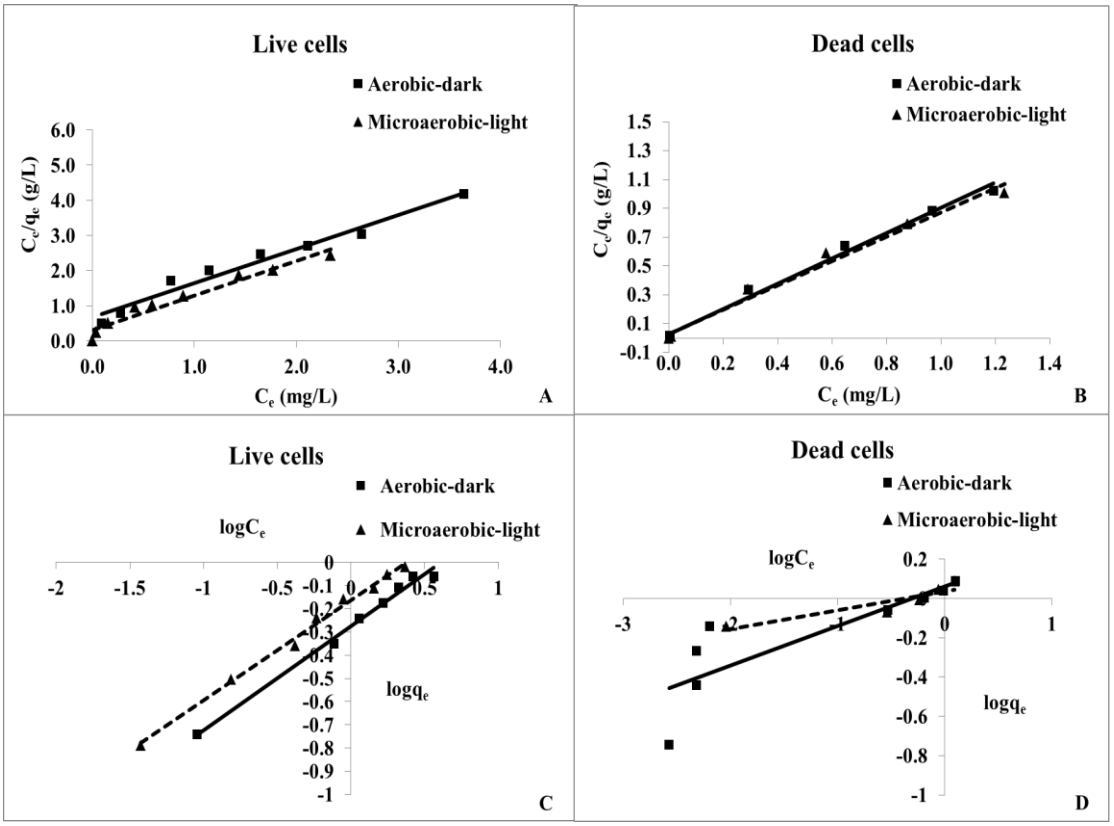

Figure 7-6. Langmuir model of Hg^{2+} biosorption by live (A) and dead cells (B) and Freundlich model by live (C) and dead cells (D) of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions.

From the prediction with the Freundlich model, the K_F value of live cells were 0.686 and 0.531, and n was 2.312 and 2.237 with microaerobic light and aerobic dark conditions, respectively. While the predicted maximum adsorption capacities (q_m) by the Langmuir model for the dead cells were 1.184 and 1.139 mg/L, and their K_L values were 29.323 and 36.596 with growth under microaerobic light and aerobic dark conditions, respectively (Table 1).

Table 7-1. Langmuir and Freundlich isotherm constants and values of linear correlation co-efficient (R^2) for Hg^{2+} biosorption by live and dead cells of *Afifella marina* SSS2-1 under microaerobic light and aerobic dark conditions.

Biomass	Conditions	Langmuir			Freundlich		
		q_m (mg/L)	K_L (L/mg)	R^2	K_F (L/g)	n	R^2
Live	Light	1.253	3.224	0.954	0.686	2.312	0.994
Live	Dark	0.982	13.483	0.970	0.531	2.237	0.991
Dead	Light	1.184	29.323	0.986	1.087	10.460	0.824
Dead	Dark	1.139	36.596	0.993	1.153	4.987	0.712

A study of the kinetic modeling of Hg^{2+} removal by a selected PNSB biomass

The adsorption kinetics of Hg^{2+} biosoption by living or dead cells of *A. marina* SSS2-1 with microaerobic light and aerobic dark conditions were analyzed using the pseudo-first and pseudo-second order models. The linear regressions of the data of the pseudo-first and pseudo-second order models by the live and dead cells are shown in Figure 7-7. The kinetic constants and correlation coefficient values of the pseudo-first and pseudo-second order models are given in Table 7-2. The correlation coefficient of the first-order kinetics of live and dead biomass under both incubating conditions was lower than in the case of the second-order kinetic model. The lower of the R^2 values from the regression lines of the pseudo-first order kinetic model in living cells and dead cells were calculated to be 0.930 and 0.295 for the microaerobic light condition, and 0.892 and 0.165 for the aerobic dark condition. In contrast, the R^2 values from the regression lines of the pseudo-second kinetic models in the living and dead cells were 0.988 and 0.999 with microaerobic light conditions, and 0.987 and 0.996 with aerobic dark conditions (Table 7-2). This shows that the kinetics of Hg^{2+}

biosorption by the strain SSS2-1 biomass was better described by pseudo-second order kinetic model rather than by a pseudo-first order kinetic model. From the pseudo-second order kinetic model, the q_e value (mg/g) for the live cells were 0.678 and 0.672, and 0.691 and 0.707 for the dead cells grown with microaerobic light and aerobic dark conditions, respectively. While the K_2 for the live cells were 0.220 and 0.195, and 1.182 and 1.030 for the dead cells grown with microaerobic light and aerobic dark conditions, respectively (Table 7-2).

Figure 7-7. Pseudo-first order model of Hg^{2+} biosorption by live (A) and dead cells (B) and pseudo-second order model by live (C) and dead cells (D) of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions.

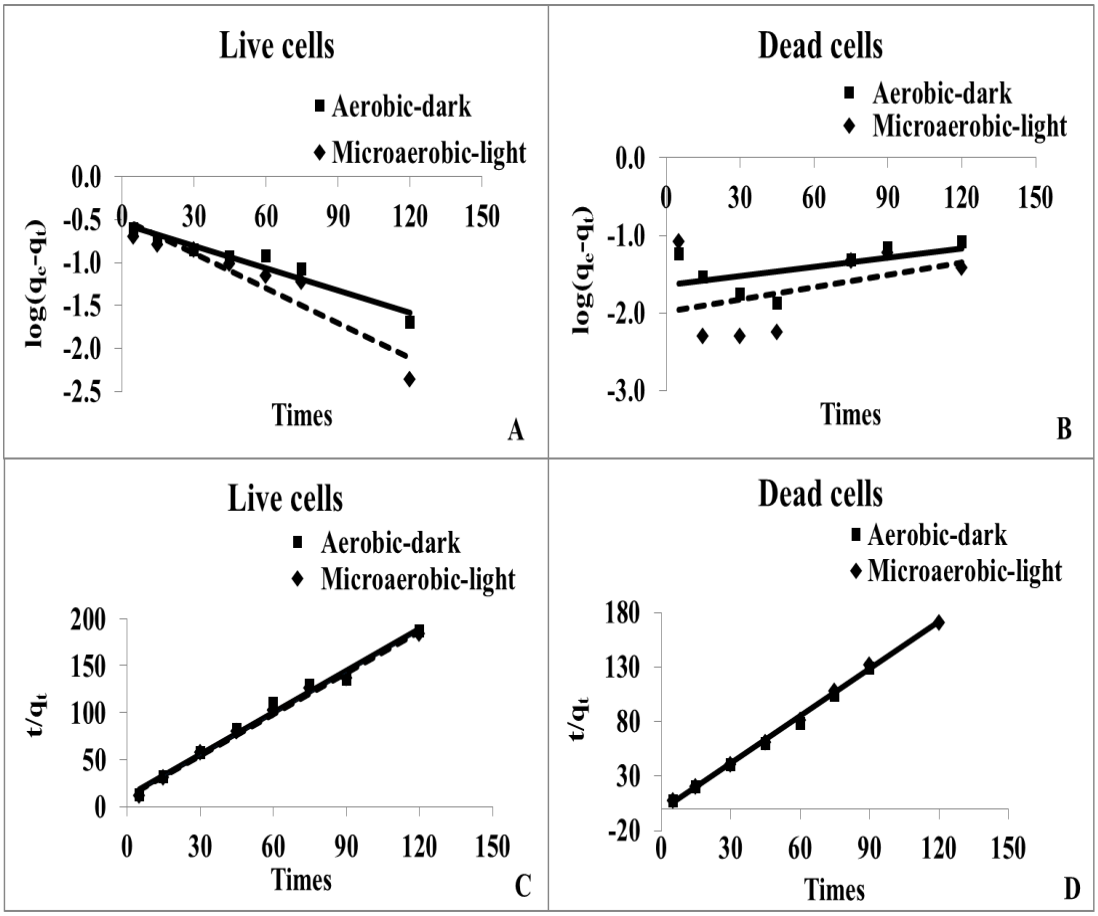

Table 7-2. Rate constants for kinetic models of the Hg^{2+} biosorption by live and dead PNSB cells of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions.

Biomass	Conditions	Pseudo-first order			Pseudo-second order		
		q_e (mg/g)	K_1 (min^{-1})	R^2	q_e (mg/g)	K_2 (g/mg min)	R^2
Live	Light	0.568	$4x10^{-5}$	0.930	0.678	0.220	0.988
Live	Dark	0.567	$6x10^{-5}$	0.892	0.672	0.195	0.987
Dead	Light	0.595	$3x10^{-5}$	0.295	0.691	1.182	0.999
Dead	Dark	0.548	$3x10^{-5}$	0.165	0.707	1.030	0.996

Discussion

The potential of selected PNSB strains to remove Hg^{2+} by biosorption

This study explored the potential use of either live or dead selected Hg resistant PNSB strains as a biological approach to remove contaminating heavy metals, especially Hg in shrimp ponds. Such a bioremediation process would provide many advantages such as being cost effective, eco-friendly and provide opportunities for metal recovery, including the advantage that the removal of the metals can be carried out *in situ* at a contaminated site (Jafari and Cheraghi, 2014). Some previous studies have indicated that the biosorption capacity by dead cells can be higher than for live cells (Kaçar et al., 2002; Bayramoğlu and Arıca, 2008; Velásquez and Dussan, 2009), because biosorption by dead cells occurs passively in the absence of metabolism so binding of metals occurs rapidly and mainly at the cell surface. In contrast, bioaccumulation by live cells is a metabolically active process and accumulation may be limited by toxicity (Chojnacka, 2010). The results obtained in this study support this possibility as the percentage removal of Hg^{+2} from a solution

containing 2 mg/L $HgCl_2$ was significantly higher using dead cells of the three PNSB strains tested, in both incubating conditions when compared with their live cells (Figure 7-1A, B). There were no significant differences for the percentage removal of Hg^{2+} by cells obtained from the 2 sets of incubating conditions, except for strain SRW1-5 for which Hg binding by cells from the aerobic dark conditions was lower for both live and dead cells (Figure 7-1A, B). It was also clear that the growth of the strain SRW1-5 (*R. sulfidophilum*) with microaerobic light conditions provided more suitable binding conditions compared to those provided by the other strains SSS2 and SSW15-1 (*A. marina*).

Normally, the biomass consists of many functional groups on the cell surfaces such as carboxyl, sulfonate, phosphoryl, amido, amino and imidazole (Volesky and Schiewer, 2002; Chojnacka, 2010). These functional groups play an important role in metal binding to the cell biomass. The Hg^{2+} removal capacity by strains SSS2-1 and SSW15-1 were similar because their surface biomass functional groups are similar as they belonged to the same species. On the other hand, the percentage of Hg^{2+} removal by both cell types of strain SRW1-5 when growth with microaerobic light conditions was significantly higher than with aerobic dark conditions. This could be explained by this strain's capacity to produce slime or other extracellular polymeric substances (EPS) when grown under microaerobic light conditions (data not shown). It is well recognized that EPS is secreted by many microbes in response to some physiological stresses such as heavy metals and osmotic salts such as Na^+ (Panwichian et al., 2011; Qurashi and Sabri, 2011; Nunkaew et al., 2015); and EPS can contain rich functional groups for binding metal ions such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups (Liu and Fang, 2002; Joshi and Juwarkar, 2009). Hence, the production of EPS by strain SRW1-5 when growing under microaerobic light conditions enhanced the percentage of Hg^{2+} that could bind to the cell surface. There is no significant difference in the Hg^{2+} removal by dead cells of all three PNSB strains (Figure 7-1); the efficiency of live cells under both incubating growth conditions was considered for shrimp cultivation and/or bioremediation. Therefore, strain SSS2-1 was chosen because it had a higher

efficiency to remove Hg^{2+} using both incubating conditions in addition to its Hg^{2+} resistance as previously described (Mukkata et al. 2015).

Factors affecting of Hg^{2+} removal by a selected PNSB strain

The efficient removal of Hg^{2+} by bacterial biomass depends on many factors such as the growth phase of the cells, biomass dose, environmental conditions such as the initial Hg concentration, pH, contact time, temperature and the presence of other ions (Abbas et al., 2014). With both sets of incubating conditions, the percentage of Hg^{2+} removal by live cells from the log and late log phases was significantly higher than by cells from the stationary phase; however, the amounts of Hg^{2+} bound by dead cells was not significantly different from all three growth phases (Figure 7-2A, B). Bioaccumulation involved with removal of Hg^{2+} by live cells is a metabolically driven detoxification processes (Chojnacka, 2010). Hence, cells in the log and late log phases had a higher capacity for bioaccumulation of Hg^{2+} than cells from the stationary phase. During the stationary phase the composition and flexibility of the cell wall changes and binding sites may not be so accessible (Abbas et al., 2014). All three growth phases of dead cells produced a similar result for removal of Hg^{2+}; this is because only the sorption of Hg^{2+} on preformed binding sites on the cells surface occurs with dead cells. This is also why the binding of Hg^{2+} by dead cells under both sets of growth conditions was always similar and slightly greater than by live cells.

The dosages of the biomass strongly influenced Hg^{2+} removal because an increase in biomass dose increased the surface area and numbers of binding sites for Hg ions (Vijayaraghavan and Yun, 2008) (Figure 7-2C, D). The initial Hg^{2+} concentration also influenced the percentage removal of Hg. At the low concentration of Hg^{2+}, the removal percentage of Hg^{2+} was extremely high, especially at 1-3 mg/L by dead cells (Figure 7-3A, B). In this case the biosorption sites on cell surface were sufficient for binding most of the Hg^{2+}; hence all of Hg^{2+} could bind to the cell surface (5 mg DCW/L) of dead cells. In contrast, an increase of the initial concentration of Hg^{2+} significantly reduced the removal percentage of Hg^{2+} because of a decrease in binding sites. These results were similar to those of a previous study

(Mo and Lian, 2010). For live cells, the initial Hg^{2+} concentration was not only affect the sorption sites on the cell surface but were toxic to the cells; therefore the percentage removal of Hg^{2+} was significantly decreased with an increase of the initial Hg^{2+} dose (Figure 7-3A, B).

The pH plays an important role in metal removal because its influence on the metal ion solubility and the total charges of the functional binding groups such as carboxylate, phosphate and amino groups on the biomass (Abdel-Ghani and El-Chaghaby, 2014). The percentage Hg^{2+} biosorption capacity of both cell types increased with increasing pH up to a pH of 5 to 7 (Figure 7-3C, D). This could be due to an increase in pH values providing more negative charges on the biomass surface for binding with positive charges like Hg^{2+} (Aksu and Gülen, 2002; Aryal and Liakopoulou-Kyriakides, 2014). However, at pH values higher than 7, hydroxo species of the metals can be formed and these will not bind to sorption sites on the biomass surface (Kaçar et al., 2002; Green-Ruiz, 2006). This causes the reduction of metal ion binding such as Hg^{2+} so the Hg removal capacity by both types of biomass at a pH of 9 was significantly decreased (Figure 7-3C, D). Many previous studies have reported that the optimum pH for Hg^{2+} sorption by live and dead cells of bacteria was pH 6 (Kaçar et al., 2002; Green-Ruiz, 2006; Jafari and Cheraghi, 2014). For strain SSS2-1 it was between 6-7 with a small decrease at pH 8; this provided the opportunity to use this bacterium in shrimp farm either for cultivation or bioremediation as the pH of shrimp ponds is in the range of 6-8 (Cheung and Wong, 2006).

The results of this study are in agreement with many previous studies in that the percentage of metals removed is most rapid at the beginning of the sorption time because of the larger surface area of biomass for the sorption of metals until it reached the equilibrium (Zhou et al., 2009a; Mo and Lian, 2010). Hence, the percentage of Hg^{2+} removal by dead cells was very high within the first 5 min and reached a maximum at 15 min and then was stable until 60 min after contact; but the Hg^{2+} removal declined afterwards for up to 75 min of contact time (Figure 7-4A, B), as the Hg^{+2} desorbed (Fowle and Fein, 2000). However, the percentage removal of Hg^{+2} by live cells gradually increased until it reached equilibrium at 90 min (Figure 7-

4A). The results indicate that in addition to biosorption, live cells also used bioaccumulation for detoxification as they continually increased Hg^{2+} uptake into the cells.

The capacity for the biosorpton of heavy metals also depended on the temperature as increases of temperature enhanced surface activity and kinetic energy leading to an increase in the biosorption capacity (Aryal and Liakopoulou-Kyriakides, 2014). This is the reason why the percentage of Hg^{2+} removal increased following an increase of the temperature from 25-40°C for dead cells (Figure 7-4C, D). However, higher temperatures can destroy biomass, and reduce the biosorption capacity (Aryal and Liakopoulou-Kyriakides, 2014), so this might be one reason for the reduction of Hg^{2+} removal by live cells as the temperature increased up to 40°C (Figure 7-4A). The optimum temperature of 35°C for the binding of Hg by *A. marina* SSS2-1 was in agreement with Jafari and Cheraghi (2014) who studied the Hg sorption by *Vibrio parahaemolyticus*; both these organisms are aquatic Gram negative bacteria.

The effect of other ions (Na^{+}, Ca^{2+}, Mg^{2+} and Cd^{+2}) normally present in shrimp ponds on Hg^{2+} removal was associated with an increase of positive charges; and the positive charges in an aqueous environment will compete with the positive charges of the Hg ions for binding with negative charges on the surface of the cells (Vijayaraghavan and Yun, 2008). Hence, the Hg^{2+} removal was reduced in the presence of other ions with Mg^{2+} and Cd^{+2} > Ca^{2+} > Na^{+} (Figure 7-5). The amount of reduction was dependent on the numbers and types of available binding sites and their relative binding affinities for the different metal ions.

The investigation of the several parameters that affected Hg^{2+} removal based on biosorption showed that dead cells had a higher efficiency than live cells (Figures 7-2, 7-5). However, the highest removal percentage of Hg^{2+} under the optimum condition by live cells under both incubating conditions was quite high (87%) and could become as high as the dead cells by increasing the contact time from 30 min to up to 90 min (Figure 7-4A). Also, live cells of strain SSS2-1 could remove Hg via different mechanisms during shrimp cultivation such as by biovolatilization, biosorption and bioaccumulation (Mukkata et al. 2015; this study) whereas biosorption was the only method used by dead cells. On the other hand, dead cells

would be an alternative way when the focus was to remediate Hg from contaminated shrimp ponds before/after cultivation. In addition, the versatile growth modes of PNSB allow the possibility of their application in remediation of various Hg contaminated areas.

Isotherms for Hg^{2+} sorption by a selected PNSB biomass

There are several isotherm equations that have been used to provide information on sorption processes such as cell surface properties and biosorption mechanisms. However, two models have been frequently used; the Langmuir and Freundlich models because both models are simple, well-established and have physical meaning and are easily interpretable (Vijayaraghavan and Yun, 2008). The Langmuir model assumes that the sorption process is based on a monolayer of a homogeneous surface with constant sorption energy and with no interactions between adsorbed molecules. The Freundlich model assumes that the sorption process is based on a monolayer with a heterogeneous surface and molecules that attach to a surface site will have an effect on the neighboring sites (Plaza et al., 2011; Jafari and Cheraghi, 2014).

The Hg^{2+} biosorption by dead cells corresponded to the Langmuir model (Figure 7-6B) while live cells corresponded to the Freundlich model (Figure 7-6C). These results indicated that the biosorption process of live cells occurred on a monolayer with a heterogeneous surface that means the biosorption of Hg^{2+} was not only to the cell surface, but also Hg^{2+}accumulated intracellularly (Kaduková and Virčíková, 2005; Li et al., 2010). In contrast, the biosorption process of dead cells occurred only on a monolayer with a constant sorption energy as previously described. These results are in agreement with previous studies for the appropriate isotherms of metal sorption by live and dead biomass (Li et al., 2010; Mo and Lian, 2010; Jafari and Cheraghi, 2014)

In the case of the Freundlich model of live cells, the *n* value indicated formation of strong bonds between the biosorbent and heavy metal with a value of between 1 and 10 (Li et al., 2010; Jafari and Cheraghi, 2014). In this study, the *n* values of Hg sorption were 2.312 and 2.237 for microaerobic light and aerobic dark

conditions (Table 7-1); this means that the SSS2-1 biomass is a good biosorbent. The n and K_F values for the SSS2-1 live biomass were not high; however, the n values were a little higher than found in previous studies for Hg biosorption by *Bacillus* sp., and *Vibrio parahaemolyticus* that were 1.51 and 1.53-1.68 (Jafari and Cheraghi, 2014). The values of q_m and K_L from the Langmuir model were calculated for description and the maximum monolayer biosorption capacity of the biosorbent (mg/g) and the affinity of the binding sites, respectively. Although the high value of q_m and K_L indicated that biomass provided good biosorption, the low values of q_m and high K_L were found, when biosorption was carried out at low metal concentrations (Li et al., 2010). This could be used to explain the results of the dead cells SSS2-1 that were tested on low concentration of Hg (1-8 mg/L) and showed a low q_m (1.184 and 1.139 mg/L) and a high K_L (29.323 and 36.596) under microaerobic light and aerobic dark conditions, respectively (Table 7-1). Moreover, R_L values that are normally used to predict if an adsorption system is favorable or unfavorable (Rasmussen et al., 2000) were calculated from the equation (7):

$$R_L = \frac{1}{1+K_L C_0} \tag{7}$$

where C_0 is the highest metal concentration (mg/L). The R_L value can indicate unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$) and irreversible ($R_L = 0$) adsorption (Abdel-Ghani and El-Chaghaby, 2014). From our results, the R_L values of the dead cells grown with microaerobic light and aerobic dark conditions were 0.004 and 0.003 (data not shown), and indicated that the Hg^{2+} biosorption by dead biomass SSS2-1 was favorable.

A study of the kinetic models of Hg^{2+} removal by a selected PNSB biomass

The two kinetic models namely, pseudo-first and pseudo-second order models, were investigated for their ability to describe the transport mechanisms of the Hg ion in the adsorbent (Lesmana et al., 2009). It was very clear that the kinetic

models for Hg^{2+} removal by both the live and dead PNSB biomass fitted to a pseudo-second order model rather than to a pseudo-first order model (Figure 7-7). Our results agreed with several literature studies that have reported the prevalent kinetic models of metals biosorption by microbes is more often associated with the pseudo-second order model rather than the pseudo-first order model (Imhoff, 1995; Bayramoğlu and Arıca, 2008; Khambhaty et al., 2008a; Jafari and Cheraghi, 2014; Kőnig-Péter et al., 2014). The pseudo-second order model was used to demonstrate that the rate-limiting step was by chemisorption, not limitation by mass transport (Lesmana et al., 2009). It also assumed that the reaction of biosorption included two reactions; the first one was fast and reached equilibrium quickly and the second was a slower reaction that continued for long time periods (Khambhaty et al., 2008b). This assumption correlated well with the results in this study that showed that the Hg^{2+} removal of live and dead cells was high within the first 5 min and then the biosorption rates were slow until they reached equilibrium at about 90 min and 15 min, respectively (Figure 7-4A, B). Furthermore, the calculated q_e values for both types of biomass and incubating conditions agreed with the experimental data (data not shown) and these supported the view that the biosorption of Hg with SSS2-1 biomass fitted to the pseudo-second order kinetic model. In comparison the rate parameter k_2 (g/mg min) as shown in Table 7-2 indicated that the biosorption with microaerobic light and aerobic dark conditions by the dead biomass (1.182 and 1.030) was faster than that of the live cells (0.220 and 0.195).

Conclusions

Overall these results have proven that the live or dead biomass of *A. marina* SSS2-1 grown with either microaerobic light or aerobic dark conditions have the potential to remove Hg^{2+} by biosorption. The optimal conditions under both incubating conditions for live or dead cells (5 mg DCW/L) to remove Hg^{2+} (initial 4 mg/L $HgCl_2$); for live cells were using cells from log and late log phases, a pH of 7, at 35°C for 90 min while dead cells from all cell ages, required a pH of 7, 35-40°C for 15 min. The Hg^{2+} biosoption of live and dead cells under both incubating conditions

fitted with the Freundlich and the Langmuir models, respectively. The kinetics of Hg^{2+} biosorption by live and dead SSS2-1 biomass fitted with the pseudo-second order kinetic model. Dead cells showed higher efficiency for Hg^{2+} removal through biosoption; however, live cells also should be considered as they may have alternative mechanisms to remove Hg from contaminated areas.

CHAPTER 8

CONCLUSIONS

The findings of the research work from each chapter of this thesis are discussed in this final chapter in order to attempt for integration the conclusion drawn and also to identify future research needs. The general conclusions from the thesis are the following provided.

Thirty-five samples of water and sediment collected from various shrimp ponds along the Thailand peninsular coast after shrimp harvesting were contaminated with Hg_T <0.0002 to 0.037 μg/L in water and 30.73 to 398.84 μg/kg dry weight in sediment. Of these Hg_T concentrations in shrimp ponds were lower than the Thai, Hong Kong, and Canadian standard guideline values. However, bioremediation of Hg contaminated shrimp ponds would prevent shrimp being contaminated with Hg and subsequently enter the human food chain. The results of this part lead to investigate the effect of Hg distribution on diversity of PNSB in shrimp ponds.

Representative samples of water and sediment from 16 Hg contaminated shrimp ponds were re-collected for investigating the diversity of PNSB in shrimp ponds using the specific *pufM* gene to know which species of PNSB were dominant in shrimp ponds. Amplification of the *pufM* gene was detected in 13 and 10 samples of water and sediment found that in addition of PNSB, APB like PSB and AAPB were also observed although most of them could not be identified. Among identified groups; AAPB, PSB and PNSB in the samples of water and sediment were 25.71, 11.43 and 8.57%; and 27.78, 11.11 and 22.22%, respectively. The most dominant species found in both sample types were closely to *R. denitrificans* OCh 114 and *H. halophila* H including some PNSB such as *R. kholense, R. centenum* and *R. marinum*. In addition, the UPGMA dendrograms revealed that no relationship between the clustered groups and the Hg_T concentrations in the water and sediment samples used (<0.002-0.03 μg/L and 35.40-391.60 μg/kg dry weight). Therefore, it can be concluded that there was no effect of the various Hg levels on the diversity of

detected APB species; particularly the PNSB in the shrimp ponds. This suggests that PNSB might have Hg resistant mechanisms for live in Hg contaminated shrimp ponds.

Therefore, 131 PNSB strains isolated from Hg contaminated shrimp ponds were selected for their resistance to Hg; and obtained three promising Hg resistant PNSB strains (*R. sulfidophilum* strain SRW1-5 and *A. marina* strains SSS2-1 and SSW15-1). They could detoxify Hg^{2+} by volatilization to Hg^{0} using their mercuric reductase enzyme which significantly higher in aerobic dark than microaerobic light conditions. The mercuric reductase activities in dark condition of strains SSW15-1, SRW1-5, and SSS2-1 were 15.75, 12.62, and 12.16 U/mg protein, respectively. It is interesting to note that the MIC values of these selected strains were much higher than those currently contaminated in Thai shrimp ponds. This means that three selected PNSB strains could survive in Hg contaminated shrimp ponds. To know more on Hg resistant mechanisms related to *mer* operon; Hg resistant genes of the selected PNSB had been investigated.

It was found that the gene coding for MerR family transcriptional regulator protein and a part of genes analysis between *merA* (mercuric reductase enzyme) and *merD* (secondary regulatory protein) including the genes coding for transporter permease protein were detected in all selected PNSB strains. While, *merR* coding for mercuric resistance operon regulatory protein and *merT* coding for transporter gene was detected only in both strains of *A. marina*. The comparison the sequences of *merR* and *merT* from both PNSB strains with the databases in NCBI found they had high similarity with *merR* gene of members in Family *Sphingomonadaceae*. In addition, the comparing of MerR and MerT proteins in both PNSB with other genera in *α-Proteobacteria* revealed that the Hg resistant genes in PNSB were divergent from other organisms in *α-Proteobacteria*; and they are a lateral gene transfer (LGT) and might recently develop during exposure to Hg contaminated shrimp ponds. This study achieved to explore the *mer* operon of PNSB although continuing studies should be investigated for obtaining their complete *mer* operon.

The toxicity of Hg on PNSB, particularly on their photosynthesis might have an adverse effect on ecosystem or their application. Hence, acute and long-term effects of Hg^{2+} on BChl, ETR and cell morphology of PNSB were investigated. A high concentration of $HgCl_2$ at 4 mg/L could kill all selected PNSB strains. After 9 h exposure, a significant decrease of ETR_{max} levels in strains SRW1-5 and SSS2-1 roughly 37% and 48%, respectively compared with their control was the acute toxic of Hg^{2+} on PNSB cells. However, lower concentrations of $HgCl_2$ (1-3 mg/L $HgCl_2$) with a longer exposure for 96 h, exposed cells could respond to stress conditions by changing their cell shapes to reduce the adverse effect although Hg^{2+}, particularly at 3 mg/L $HgCl_2$ significantly decreased BChl content and consequently affected on ETR_{max} to retard the growth of PNSB. Results of this study it might be useful for finding the appropriate ways to apply the selected PNSB for bioremediation, and for investigating bioassay candidate of Hg contamination in environments. To achieve the goal of using the selected PNSB for bioremediation in Hg contaminated shrimp ponds, their biomass was investigated to use as biosorpbent agents.

The potential of live and dead PNSB biomass to remove Hg including factors that affect Hg biosorption prior to their application in removing Hg in shrimp ponds during shrimp cultivation was studied. The results proved that live or dead biomass of all selected PNSB strains grown with either microaerobic light or aerobic dark conditions has the potential to remove Hg^{2+} by biosorption. The strain SSS2-1 showed higher Hg^{2+} removal efficiency under both growth conditions and its optimal conditions under both incubating conditions using biomass (5 mg DCW/L) to remove Hg^{2+} (initial 4 mg/L $HgCl_2$); for live cells were using cells from log and late log phases, a pH of 7, at 35°C for 90 min with 87% removal while dead cells from all cell ages, required a pH of 7, 35-40°C for 15 min with 95% removal. The Hg^{2+} biosoption of live and dead cells under both incubating conditions fitted with the Freundlich and the Langmuir models, respectively. The kinetics of Hg^{2+} biosorption by live and dead biomass fitted with the pseudo-second order kinetic model. Therefore, overall results demonstrate that live or dead biomass of PNSB has the great potential for applying to remove Hg from contaminated shrimp ponds by biosorption. A higher efficiency for

remove of Hg^{2+} by biosoption was found in dead cells; however, live cells should be considered as they have alternative mechanisms to remove Hg from contaminated areas by biovolatilization and also bioaccumulation.

Future research needs

1. Although, Hg contamination in shrimp ponds was lower than the standard guidelines, Hg contamination in shrimp ponds should be monitored to prevent of shrimp contaminated with Hg that can subsequently enter the human food chain.

2. To better understanding, the dynamics of the PNSB community in shrimp ponds, monitoring the changes of PNSB community during shrimp cultivation should be studied. Moreover, the abiotic parameters in shrimp ponds such as pH, temperature and nutrient should be determined to provide their influences on PNSB community in shrimp ponds.

3. Due to some of gene in *mer* opern did not be detected in three selected PNSB strains; therefore, *mer* genes should be continued until the *mer* operon of PNSB is completed.

4. The effect of Hg on photosynthesis process of PNSB, especially its effect on photosynthetic pigments should be continued for investigating bioassay candidate to monitor Hg contamination in environments.

5. Hg biosorption by the selected PNSB biomass should be done in water from Hg contaminated shrimp ponds to confirm the possibility to use PNSB biomass for bioremediation in shrimp ponds cultivation.

6. Biovolatilization to remove Hg from sediment collected from Hg contaminated shrimp ponds by the selected PNSB should be investigated to assess their potential to remove Hg from contaminated shrimp ponds.

BIBLIOGRAPHY

Abbas, S.H., Ismail, I.M., Mostafa, T.M., and Sulaymon, A.H. 2014. Biosorption of Heavy Metals: A Review. *Journal of Chemical Science and Technology* 3(4): 74-102.

Abdel-Ghani, N.T., and El-Chaghaby, G.A. 2014. Biosorption for metal ions removal from aqueous solutions: a review of recent studies. *International Journal of Latest Research in Science and Technology* 3(1): 24-42.

Achard-Joris, M., and Bourdineaud, J.-P. 2006. Heterologous expression of bacterial and human multidrug resistance proteins protect *Escherichia coli* against mercury and zinc contamination. *Biometals* 19(6): 695-704.

Achenbach, L.A., Carey, J., and Madigan, M.T. 2001. Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Applied and Environmental Microbiology* 67(7): 2922-6.

Ahmed, H., and Häder, D.-P. 2010. Rapid ecotoxicological bioassay of nickel and cadmium using motility and photosynthetic parameters of *Euglena gracilis*. *Environmental and Experimental Botany* 69(1): 68-75.

Aksu, Z., and Çağatay, Ş.Ş. 2006. Investigation of biosorption of Gemazol Turquise Blue-G reactive dye by dried *Rhizopus arrhizus* in batch and continuous systems. *Separation and Purification Technology* 48(1): 24-35.

Aksu, Z., and Gülen, H. 2002. Binary biosorption of iron(III) and iron(III)-cyanide complex ions on *Rhizopus arrhizus*: modelling of synergistic interaction. *Process Biochemistry* 38(2): 161-173.

Allen, R.C., Tu, Y.K., Nevarez, M.J., Bobbs, A.S., Friesen, J.W., Lorsch, J.R., McCauley, J.A., Voet, J.G., and Hamlett, N.V. 2013. The mercury resistance (*mer*) operon in a marine gliding flavobacterium, *Tenacibaculum discolor* 9A5. *FEMS Microbiology Ecology* 83(1): 135-48.

Antal, T.K., Graevskaia, E., Matorin, D.N., Volgusheva, A.A., Osipov, V.A., Krendeleeva, T.E., and Rubin, A.B. 2009. Study of the effect of methylmercury and copper ions on primary photosynthesis processes in the green algae *Chlamydomonas moevusii* using the parameters of kinetic curves of the variable of chlorophyll fluorescence. *Biofizika* 54(4): 681-687.

Antony, S.P., and Philip, R. 2006. Bioremediation in Shrimp Culture Systems. *NAGA, WorldFish Center Quarterly Vol. 29 No. 3 & 4 Jul-Dec 2006* 29(3-4): 62-66.

Aryal, M., and Liakopoulou-Kyriakides, M. 2014. Bioremoval of heavy metals by bacterial biomass. *Environmental Monitoring and Assessment* 187(1): 1-26.

Asao, M., Pinkart, H.C., and Madigan, M.T. 2011. Diversity of extremophilic purple phototrophic bacteria in Soap Lake, a Central Washington (USA) Soda Lake. *Environmental Microbiology* 13(8): 2146-2157.

Asztalos, E., Italiano, F., Milano, F., Maroti, P., and Trotta, M. 2010. Early detection of mercury contamination by fluorescence induction of photosynthetic bacteria. *Photochemical & Photobiological Sciences* 9(9): 1218-23.

Asztalos, E., Sipka, G., Kis, M., Trotta, M., and Maróti, P. 2012. The reaction center is the sensitive target of the mercury(II) ion in intact cells of photosynthetic bacteria. *Photosynthesis Research* 112(2): 129-140.

Baeyens, W., Leermakers, M., Papina, T., Saprykin, A., Brion, N., Noyen, J., De Gieter, M., Elskens, M., and Goeyens, L. 2003. Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. *Archives of Environmental Contamination and Toxicology* 45(4): 498-508.

Bafana, A., Krishnamurthi, K., Patil, M., and Chakrabarti, T. 2010. Heavy metal resistance in *Arthrobacter ramosus* strain G2 isolated from mercuric salt-contaminated soil. *Journal of Hazardous Materials* 177(1–3): 481-486.

Bai, H.-J., Zhang, Z.-M., Yang, G.-E., and Li, B.-Z. 2008. Bioremediation of cadmium by growing Rhodobacter sphaeroides: Kinetic characteristic and mechanism studies. *Bioresource Technology* 99(16): 7716-7722.

Barkay, T., Kritee, K., Boyd, E., and Geesey, G. 2010. A thermophilic bacterial origin and subsequent constraints by redox, light and salinity on the evolution of the microbial mercuric reductase. *Applied and Environmental Microbiology* 12(11): 2904-17.

Barkay, T., Miller, S.M., and Summers, A.O. 2003. Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews* 27(2-3): 355-384.

Barkay, T., and Wagner-Döbler, I. 2005. Microbial Transformations of Mercury: Potentials, Challenges, and Achievements in Controlling Mercury Toxicity in the Environment. In *Advances in Applied Microbiology*, 1-52. Academic Press.

Barrineau, P., Gilbert, P., Jackson, W.J., Jones, C.S., Summers, A.O., and Wisdom, S. 1984. The DNA sequence of the mercury resistance operon of the IncFII plasmid NR1. *Journal of molecular and applied genetics* 2(6): 601-619.

Bayramoğlu, G., and Arıca, M.Y. 2008. Removal of heavy mercury(II), cadmium(II) and zinc(II) metal ions by live and heat inactivated *Lentinus edodes* pellets. *Chemical Engineering Journal* 143(1–3): 133-140.

Beja, O., Suzuki, M.T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., Eisen, J.A., Fraser, C.M., and DeLong, E.F. 2002. Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* 415(6872): 630-633.

Bernier, M., and Carpentier, R. 1995. The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II. *FEBS Letters* 360(3): 251-254.

Berntssen, M.H.G., Hylland, K., Julshamn, K., Lundebye, A.K., and Waagbø, R. 2004. Maximum limits of organic and inorganic mercury in fish feed. *Aquaculture Nutrition* 10(2): 83-97.

Berzas Nevado, J.J., Rodríguez Martín-Doimeadios, R.C., Guzmán Bernardo, F.J., Jiménez Moreno, M., Herculano, A.M., do Nascimento, J.L.M., and Crespo-López, M.E. 2010. Mercury in the Tapajós River basin, Brazilian Amazon: A review. *Environment International* 36(6): 593-608.

Biebl, H., Allgaier, M., Tindall, B.J., Koblizek, M., Lunsdorf, H., Pukall, R., and Wagner-Dobler, I. 2005. *Dinoroseobacter shibae* gen. nov., sp. nov., a new aerobic phototrophic bacterium isolated from dinoflagellates. *International Journal of Systematic and Evolutionary Microbiology* 55(Pt 3): 1089-96.

Boden, R., and Murrell, J.C. 2011. Response to mercury (II) ions in *Methylococcus capsulatus* (Bath). *FEMS Microbiology Letters* 324(2): 106-110.

Borrok, D.M., and Fein, J.B. 2005. The impact of ionic strength on the adsorption of protons, Pb, Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic, diffuse, and triple-layer models. *Journal of Colloid and Interface Science* 286(1): 110-126.

Boyd, E.S., and Barkay, T. 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Frontiers in Microbiology* 3: 349.

Brambilla, G., Abete, M.C., Binato, G., Chiaravalle, E., Cossu, M., Dellatte, E., Miniero, R., Orletti, R., Piras, P., Roncarati, A., Ubaldi, A., and Chessa, G. 2013. Mercury occurrence in Italian seafood from the Mediterranean Sea and possible intake scenarios of the Italian coastal population. *Regulatory Toxicology and Pharmacology* 65(2): 269-277.

Bruins, M.R., Kapil, S., and Oehme, F.W. 2000. Microbial Resistance to Metals in the Environment. *Ecotoxicology and Environmental Safety* 45(3): 198-207.

Carrasco, L., Bayona, J., and Díez, S. 2011. Mercury in Aquatic Organisms of the Ebro River Basin. In *The Ebro River Basin*, edited by D. Barceló and M. Petrovic, 239-258. Springer Berlin Heidelberg.

Celo, V., Lean, D.R.S., and Scott, S.L. 2006. Abiotic methylation of mercury in the aquatic environment. *Science of The Total Environment* 368(1): 126-137.

Chang, T.C., You, S.J., Yu, B.S., Chen, C.M., and Chiu, Y.C. 2009. Treating high-mercury-containing lamps using full-scale thermal desorption technology. *Journal of hazardous materials* 162(2-3): 967-972.

Cheevaporn, V., and Menasveta, P. 2003. Water pollution and habitat degradation in the Gulf of Thailand. *Marine Pollution Bulletin* 47(1-6): 43-51.

Chen, D., Han, Y., and Gu, Z. 2006. Application of statistical methodology to the optimization of fermentative medium for carotenoids production by Rhodobacter sphaeroides. *Process Biochemistry* 41(8): 1773-1778.

Chen, W., Yang, X., He, Z., Feng, Y., and Hu, F. 2008. Differential changes in photosynthetic capacity, 77 K chlorophyll fluorescence and chloroplast ultrastructure between Zn-efficient and Zn-inefficient rice genotypes (*Oryza sativa*) under low zinc stress. *Physiologia Plantarum* 132(1): 89-101.

Cheung, K.C., and Wong, M.H. 2006. Risk assessment of heavy metal contamination in shrimp farming in Mai Po Nature Reserve, Hong Kong. *Environmental Geochemistry and Health* 28(1-2): 27-36.

Chojnacka, K. 2010. Biosorption and bioaccumulation – the prospects for practical applications. *Environment International* 36(3): 299-307.

Chou, C.L., Haya, K., Paon, L.A., Burridge, L., and Moffatt, J.D. 2002. Aquaculture-related trace metals in sediments and lobsters and relevance to environmental monitoring program ratings for near-field effects. *Marine Pollution Bulletin* 44(11): 1259-1268.

Clarkson, T.W., and Magos, L. 2006. The Toxicology of Mercury and Its Chemical Compounds. *Critical Reviews in Toxicology* 36(8): 609-662.

Creswell, J.E., Kerr, S.C., Meyer, M.H., Babiarz, C.L., Shafer, M.M., Armstrong, D.E., and Roden, E.E. 2008. Factors controlling temporal and spatial distribution of total mercury and methylmercury in hyporheic sediments of the

Allequash Creek wetland, northern Wisconsin. *Journal of Geophysical Research* 113.

Crini, G. 2006. Non-conventional low-cost adsorbents for dye removal: A review. *Bioresource Technology* 97(9): 1061-1085.

Das, S., and Sahu, B.K. 2002. Toxicity of Hg (II) to prawns *Penaeus monodon* and *Penaeus indicus* (Crustacea: Penaeidae) from Rushikulya estuary, Bay of Bengal. *Indian Journal of Marine Sciences* 31(4): 337-339.

De, J., and Ramaiah, N. 2007. Characterization of marine bacteria highly resistant to mercury exhibiting multiple resistances to toxic chemicals. *Ecological Indicators* 7(3): 511-520.

Del Sorbo, G., Schoonbeek, H.-j., and De Waard, M.A. 2000. Fungal Transporters Involved in Efflux of Natural Toxic Compounds and Fungicides. *Fungal Genetics and Biology* 30(1): 1-15.

Deng, C., Zhang, D., Pan, X., Chang, F., and Wang, S. 2013. Toxic effects of mercury on PSI and PSII activities, membrane potential and transthylakoid proton gradient in *Microsorium pteropus.* *Journal of Photochemistry and Photobiology B: Biology* 127: 1-7.

Domagalski, J. 2001. Mercury and methylmercury in water and sediment of the Sacramento River Basin, California. *Applied Geochemistry* 16(15): 1677-1691.

Dönmez, G., and Aksu, Z. 2002. Removal of chromium(VI) from saline wastewaters by Dunaliella species. *Process Biochemistry* 38(5): 751-762.

Esposito, A., Pagnanelli, F., and Vegliò, F. 2002. pH-related equilibria models for biosorption in single metal systems. *Chemical Engineering Science* 57(3): 307-313.

FAO. 2015. First quarter farmed shrimp production moderate with export prices weakening, generating further demand in international trade. (Accessed (October 24).

Farah, J.Y., El-Gendy, N.S., and Farahat, L.A. 2007. Biosorption of Astrazone Blue basic dye from an aqueous solution using dried biomass of Baker's yeast. *Journal of Hazardous Materials* 148(1–2): 402-408.

Fowle, D.A., and Fein, J.B. 2000. Experimental measurements of the reversibility of metal–bacteria adsorption reactions. *Chemical Geology* 168(1–2): 27-36.

Franklin, L.A., and Badger, M.R. 2001. A comparison of photosynthetic electron transport rates in macroalgae measured by pulse amplitude modulated chlorophyll fluorometry and mass spectrometry. *Journal of Phycology* 37(5): 756-767.

Freedman, Z., Zhu, C., and Barkay, T. 2012. Mercury resistance and mercuric reductase activities and expression among chemotrophic thermophilic Aquificae. *Applied and Environmental Microbiology* 78(18): 6568-75.

Gabriel, M., and Williamson, D. 2004. Principal Biogeochemical Factors Affecting the Speciation And Transport of Mercury through the terrestrial environment. *Environmental Geochemistry and Health* 26(3-4): 421-434.

Gaudet, C., Lingard, S., Cureton, P., Keenleyside, K., Smith, S., and Raju, G. 1995. Canadian Environmental Quality Guidelines for mercury. *Water, Air, and Soil Pollution* 80(1-4): 1149-1159.

Giotta, L., Agostiano, A., Italiano, F., Milano, F., and Trotta, M. 2006. Heavy metal ion influence on the photosynthetic growth of *Rhodobacter sphaeroides*. *Chemosphere* 62(9): 1490-9.

Glendinning, K.J., Macaskie, L.E., and Brown, N.L. 2005. Mercury Tolerance of Thermophilic *Bacillus* sp. and *Ureibacillus* sp. *Biotechnology Letters* 27(21): 1657-1662.

Graeme Md, K.A., and Pollack Jr, M.D.F.C.V. 1998. Heavy Metal Toxicity, Part I: Arsenic and Mercury. *The Journal of Emergency Medicine* 16(1): 45-56.

Gräslund, S., Holmström, K., and Wahlström, A. 2003. A field survey of chemicals and biological products used in shrimp farming. *Marine Pollution Bulletin* 46(1): 81-90.

Green-Ruiz, C. 2006. Mercury(II) removal from aqueous solutions by nonviable *Bacillus* sp. from a tropical estuary. *Bioresource Technology* 97(15): 1907-1911.

Haferburg, G., and Kothe, E. 2007. Microbes and metals: interactions in the environment. *Journal of Basic Microbiology* 47(6): 453-467.

Harris-Hellal, J., Vallaeys, T., Garnier-Zarli, E., and Bousserrhine, N. 2009. Effects of mercury on soil microbial communities in tropical soils of French Guyana. *Applied Soil Ecology* 41(1): 59-68.

Harris, H.H., Pickering, I.J., and George, G.N. 2003. The Chemical Form of Mercury in Fish. *Science* 301(5637): 1203.

Hiraishi, A., Urata, K., and Satoh, T. 1995. A new genus of marine budding phototrophic bacteria, *Rhodobium* gen. nov., which includes *Rhodobium orientis* sp. nov. and *Rhodobium marinum* comb. nov. *International Journal of Systematic Bacteriology* 45(2): 226-34.

Ho, Y.S., and McKay, G. 1999. The sorption of lead(II) ions on peat. *Water Research* 33(2): 578-584.

Hobman, J.L. 2007. MerR family transcription activators: similar designs, different specificities. *Molecular Microbiology* 63(5): 1275-8.

Imhoff, J. 1992. Taxonomy, Phylogeny, and General Ecology of Anoxygenic Phototrophic Bacteria. In *Photosynthetic Prokaryotes*, edited by N. Mann and N. Carr, 53-92. Springer US.

Imhoff, J. 1995. Taxonomy and Physiology of Phototrophic Purple Bacteria and Green Sulfur Bacteria. In *Anoxygenic Photosynthetic Bacteria*, edited by R. Blankenship, M. Madigan and C. Bauer, 1-15. Springer Netherlands.

Ivanciuc, T., Ivanciuc, O., and Klein, D. 2006. Modeling the bioconcentration factors and bioaccumulation factors of polychlorinated biphenyls with posetic quantitative super-structure/activity relationships (QSSAR). *Molecular Diversity* 10(2): 133-145.

Jafari, S.A., and Cheraghi, S. 2014. Mercury removal from aqueous solution by dried biomass of indigenous *Vibrio parahaemolyticus* PG02: Kinetic, equilibrium, and thermodynamic studies. *International Biodeterioration & Biodegradation* 92: 12-19.

Jarup, L. 2003. Hazards of heavy metal contamination. *British Medical Bulletin* 68(1): 167-182.

Jintanapan, T. 1994. The concentration of cadmium, lead and mercury in the tissues of large blue swimming crabs. Senior project of Science, Department of Marine Science, Faculty of Science, Chulalongkorn University.

Joshi, P.M., and Juwarkar, A.A. 2009. In Vivo Studies to Elucidate the Role of Extracellular Polymeric Substances from *Azotobacter* in Immobilization of Heavy Metals. *Environmental Science & Technology* 43(15): 5884-5889.

Kaçar, Y., Arpa, Ç., Tan, S., Denizli, A., Genç, Ö., and Arıca, M.Y. 2002. Biosorption of Hg(II) and Cd(II) from aqueous solutions: comparison of biosorptive capacity of alginate and immobilized live and heat inactivated *Phanerochaete chrysosporium*. *Process Biochemistry* 37(6): 601-610.

Kaduková, J., and Virčíková, E. 2005. Comparison of differences between copper bioaccumulation and biosorption. *Environment International* 31(2): 227-232.

Kantachote, D., Kornochalert, N., and Chaiprapat, S. 2010. The use of the purple non sulfur bacterium isolate P1 and fermented pineapple extract to treat latex rubber sheet wastewater for possible use as irrigation water. *African Journal of Microbiology Research* 4(21): 2296-2308.

Kantachote, D., Torpee, S., and Umsakul, K. 2005. The potential use of anoxygenic phototrophic bacteria for treating latex rubber sheet wastewater. *Electronic Journal of Biotechnology* 8(3): 314-323.

Kantha, T., Kantachote, D., and Klongdee, N. 2015. Potential of biofertilizers from selected *Rhodopseudomonas palustris* strains to assist rice *(Oryza sativa* L. subsp. indica) growth under salt stress and to reduce greenhouse gas emissions. *Annals of Microbiology* 65(4): 2109-2118.

Karr, E.A., Sattley, W.M., Jung, D.O., Madigan, M.T., and Achenbach, L.A. 2003. Remarkable Diversity of Phototrophic Purple Bacteria in a Permanently Frozen Antarctic Lake. *Applied and Environmental Microbiology* 69(8): 4910-4914.

Khambhaty, Y., Mody, K., Basha, S., and Jha, B. 2008a. Hg(II) Removal from Aqueous Solution by Dead Fungal Biomass of Marine Aspergillus niger: Kinetic Studies. *Separation Science and Technology* 43(5): 1221–1238.

Khambhaty, Y., Mody, K., Basha, S., and Jha, B. 2008b. Pseudo-second-order kinetic models for the sorption of Hg(II) onto dead biomass of marine *Aspergillus niger*: Comparison of linear and non-linear methods. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 328(1–3): 40-43.

Koblízek, M., Shih, J.D., Breitbart, S.I., Ratcliffe, E.C., Kolber, Z.S., Hunter, C.N., and Niederman, R.A. 2005. Sequential assembly of photosynthetic units in Rhodobacter sphaeroides as revealed by fast repetition rate analysis of variable bacteriochlorophyll a fluorescence. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1706(3): 220-231.

Kőnig-Péter, A., Kocsis, B., Kilár, F., and Pernyeszi, T. 2014. Bioadsorption characteristics of *Pseudomonas aeruginosa* PAOI. *Journal of the Serbian Chemical Society* 79(4): 495-508.

Kornochalert, N., Kantachote, D., Chaiprapat, S., and Techkarnjanaruk, S. 2014. Use of *Rhodopseudomonas palustris* P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. *Annals of Microbiology* 64(3): 1021-1032.

Kukarskikh, G.L., Graevskaia, E.E., Krendeleva, T.E., Timofeedv, K.N., and Rubin, A.B. 2003. Effect of methylmercury on primary photosynthesis processes in green microalgae *Chlamydomonas reinhardtii*. *Biofizika* 48(5): 853-859.

Kurniati, E., Arfarita, N., Imai, T., Higuchi, T., Kanno, A., Yamamoto, K., and Sekine, M. 2014. Potential bioremediation of mercury-contaminated substrate using filamentous fungi isolated from forest soil. *Journal of Environmental Sciences* 26(6): 1223-1231.

Lacerda, L.D., Soares, T.M., Costa, B.G.B., and Godoy, M.D.P. 2011. Mercury Emission Factors from Intensive Shrimp Aquaculture and Their Relative Importance to the Jaguaribe River Estuary, NE Brazil. *Bulletin of Environmental Contamination and Toxicology* 87(6): 657-661.

Lebel, L., Mungkung, R., Gheewala, S.H., and Lebel, P. 2010. Innovation cycles, niches and sustainability in the shrimp aquaculture industry in Thailand. *Environmental Science & Policy* 13(4): 291-302.

Lesmana, S.O., Febriana, N., Soetaredjo, F.E., Sunarso, J., and Ismadji, S. 2009. Studies on potential applications of biomass for the separation of heavy metals from water and wastewater. *Biochemical Engineering Journal* 44(1): 19-41.

Li, H., Lin, Y., Guan, W., Chang, J., Xu, L., Guo, J., and Wei, G. 2010. Biosorption of Zn(II) by live and dead cells of *Streptomyces ciscaucasicus* strain CCNWHX 72-14. *Journal of Hazardous Materials* 179(1–3): 151-159.

Li, P., Feng, X., Liang, P., Man Chan, H., Yan, H., and Chen, L. 2013. Mercury in the seafood and human exposure in coastal area of Guangdong province, South China. *Environmental Toxicology and Chemistry* 32(3): 541-547.

Liang, C.-M., Hung, C.-H., Hsu, S.-C., and Yeh, I.-C. 2010. Purple nonsulfur bacteria diversity in activated sludge and its potential phosphorus-accumulating ability under different cultivation conditions. *Applied Microbiology and Biotechnology* 86(2): 709-719.

Liebert, C.A., Wireman, J., Smith, T., and Summers, A.O. 1997. Phylogeny of Mercury Resistance (mer) Operons of Gram-Negative Bacteria Isolated from the Fecal Flora of Primates. *Applied and Environmental Microbiology* 63(3): 1066–1076

Lin, C.-J., and Pehkonen, S.O. 1999. The chemistry of atmospheric mercury: a review. *Atmospheric Environment* 33(13): 2067-2079.

Liu, H., and Fang, H.H. 2002. Characterization of electrostatic binding sites of extracellular polymers by linear programming analysis of titration data. *Biotechnology and Bioengineering* 80(7): 806-11.

Liu, Y.-R., Zheng, Y.-M., Shen, J.-P., Zhang, L.-M., and He, J.-Z. 2010. Effects of mercury on the activity and community composition of soil ammonia oxidizers. *Environmental Science and Pollution Research* 17(6): 1237-1244.

Lorenzon, S., Francese, M., and Ferrero, E.A. 2000. Heavy Metal Toxicity and Differential Effects on the Hyperglycemic Stress Response in the Shrimp *Palaemon elegans*. *Archives of Environmental Contamination and Toxicology* 39(2): 167-176.

Madigan, M., and Jung, D. 2009. An Overview of Purple Bacteria: Systematics, Physiology, and Habitats. In *The Purple Phototrophic Bacteria*, edited by C.N. Hunter, F. Daldal, M. Thurnauer and J.T. Beatty, 1-15. Springer Netherlands.

Mahbub, K.R., Krishnan, K., Megharaj, M., and Naidu, R. 2016. Bioremediation potential of a highly mercury resistant bacterial strain *Sphingobium* SA2 isolated from contaminated soil. *Chemosphere* 144: 330-337.

Malik, P., Terry, T.D., Bellintani, F., and Perham, R.N. 1998. Factors limiting display of foreign peptides on the major coat protein of filamentous bacteriophage capsids and a potential role for leader peptidase. *FEBS Letters* 436(2): 263-266.

Mameri, N., Boudries, N., Addour, L., Belhocine, D., Lounici, H., Grib, H., and Pauss, A. 1999. Batch zinc biosorption by a bacterial nonliving *Streptomyces rimosus* biomass. *Water Research* 33(6): 1347-1354.

Mason, R. 2009. Mercury emissions from natural processes and their importance in the global mercury cycle. In *Mercury Fate and Transport in the Global Atmosphere*, edited by R. Mason and N. Pirrone, 173-191. Springer US.

Masuda, S., Hori, K., Maruyama, F., Ren, S., Sugimoto, S., Yamamoto, N., Mori, H., Yamada, T., Sato, S., Tabata, S., Ohta, H., and Kurokawa, K. 2013. Whole-Genome Sequence of the Purple Photosynthetic Bacterium *Rhodovulum sulfidophilum* Strain W4. *Genome Announc* 1(4).

Mathema, V., Thakuri, B., and Sillanpää, M. 2011. Bacterial *mer* operon-mediated detoxification of mercurial compounds: a short review. *Archives of Microbiology* 193(12): 837-844.

Mirzoyan, N., Parnes, S., Singer, A., Tal, Y., Sowers, K., and Gross, A. 2008. Quality of brackish aquaculture sludge and its suitability for anaerobic digestion and methane production in an upflow anaerobic sludge blanket (UASB) reactor. *Aquaculture* 279(1–4): 35-41.

Mo, B.-B., and Lian, B. 2010. Hg(II) adsorption by Bacillus mucilaginosus: mechanism and equilibrium parameters. *World Journal of Microbiology and Biotechnology* 27(5): 1063-1070.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., and Boonapatcharoen, N. 2015a. Diversity of purple nonsulfur bacteria in shrimp ponds with varying mercury levels. *Saudi Journal of Biological Sciences*, 10.1016/j.sjbs.2015.05.014.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M., and Naidu, R. 2015b. Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria. *Water, Air, & Soil Pollution* 226(5): 1-14.

Murtaza, I., Dutt, A., Mushtaq, D., and Ali, A. 2005. Molecular Cloning and Genetic Analysis of Functional *mer*B Gene from Indian Isolates of *Escherichia coli*. *Current Microbiology* 51(5): 297-302.

Murthy, S.D.S., and Mohanty, P. 1993. Mercury ions inhibit photosynthetic electron transport at multiple sites in the cyanobacterium *Synechococcus* 6301. *Journal of Biosciences* 18(3): 355-360.

Nascimento, A.M.A., and Chartone-Souza, E. 2003. Operon *mer*: Bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genetics and Molecular Research* 2(2): 92-101.

Navarro, A. 2008. Review of characteristics of mercury speciation and mobility from areas of mercury mining in semi-arid environments. *Reviews in Environmental Science and Bio/Technology* 7(4): 287-306.

Nemati, K., Bakar, N.K.A., and Abas, M.R. 2009. Investigation of heavy metals mobility in shrimp aquaculture sludge-Comparison of two sequential extraction procedures. *Microchemical Journal* 91(2): 227-231.

Neumann, G., Veeranagouda, Y., Karegoudar, T.B., Sahin, Ö., Mäusezahl, I., Kabelitz, N., Kappelmeyer, U., and Heipieper, H. 2005. Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. *Extremophiles* 9(2): 163-168.

Ngamprasertwong, T., and Muntrup, S. 1997. The analysis of heavy metals: cadmium, lead and mercury in the asian green mussel in Bangprakong river. Department of General Science, Faculty of Science, Chulalongkorn University.

Nies, D.H. 1999. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* 51(6): 730-750.

Nishimura, K., Shimada, H., Ohta, H., Masuda, T., Shioi, Y., and K., T. 1996. Expression of the *puf* operon in an aerobic photosynthetic bacterium, *Roseobacter denitrificans*. *Plant & Cell Physiology* 37: 153–159.

Nithya, C., Gnanalakshmi, B., and Pandian, S.K. 2011. Assessment and characterization of heavy metal resistance in Palk Bay sediment bacteria. *Marine Environmental Research* 71(4): 283-294.

Nunkaew, T., Kantachote, D., Nitoda, T., Kanzaki, H., and Ritchie, R.J. 2015. Characterization of exopolymeric substances from selected *Rhodopseudomonas palustris* strains and their ability to adsorb sodium ions. *Carbohydrate Polymers* 115: 334-341.

Oda, Y., Wanders, W., Huisman, L.A., Meijer, W.G., Gottschal, J.C., and Forney, L.J. 2002. Genotypic and Phenotypic Diversity within Species of Purple Nonsulfur Bacteria Isolated from Aquatic Sediments. *Applied and Environmental Microbiology* 68(7): 3467-3477.

Oregaard, G., and Sorensen, S.J. 2007. High diversity of bacterial mercuric reductase genes from surface and sub-surface floodplain soil (Oak Ridge, USA). *ISME J* 1(5): 453-467.

Pacyna, E.G., Pacyna, J.M., Steenhuisen, F., and Wilson, S. 2006. Global anthropogenic mercury emission inventory for 2000. *Atmospheric Environment* 40(22): 4048-4063.

Pagnanelli, F., Esposito, A., and Vegliò, F. 2002. Multi-metallic modelling for biosorption of binary systems. *Water Research* 36(16): 4095-4105.

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

Panwichian, S., Kantachote, D., Wittayaweerasak, B., and Mallavarapu, M. 2011. Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds. *Electronic of Journal of Biotechnology* 14(4).

Panwichian, S., Kantachote, D., Wittayaweerasak, B., and Mallavarapu, M. 2012. The use of selected purple nonsulfur bacteria to remove heavy metals and salts from sediment and water collected from contaminated areas to decrease their phytotoxicity. *African Journal of Biotechnology* 11(29).

Permina, E.A., Kazakov, A.E., Kalinina, O.V., and Gelfand, M.S. 2006. Comparative genomics of regulation of heavy metal resistance in Eubacteria. *BMC Microbiology* 6: 49.

Pirrone, N., Cinnirella, S., Feng, X., Finkelman, R.B., Friedli, H.R., Leaner, J., Mason, R., Mukherjee, A.B., Stracher, G.B., Streets, D.G., and Telmer, K. 2010. Global mercury emissions to the atmosphere from anthropogenic and natural sources. *Atmospheric Chemistry and Physics* 10(13): 5951-5964.

Plaza, J., Viera, M., Donati, E., and Guibal, E. 2011. Biosorption of mercury by *Macrocystis pyrifera* and *Undaria pinnatifida*: Influence of zinc, cadmium and nickel. *Journal of Environmental Sciences* 23(11): 1778-1786.

Qi, Z., Zhang, X.-H., Boon, N., and Bossier, P. 2009. Probiotics in aquaculture of China — Current state, problems and prospect. *Aquaculture* 290(1–2): 15-21.

Qurashi, A.W., and Sabri, A.N.S. 2011. Osmoadaptation and plant growth promotion by salt tolerant bacteria under salt stress. *African Journal of Microbiology Research* 5(21).

Rao, P.S.S., Karunasagar, I., Otta, S.K., and Karunasagar, I. 2000. Incidence of bacteria involved in nitrogen and sulphur cycles in tropical shrimp culture ponds. *Aquaculture International* 8(5): 463-472.

Rasmussen, L.D., Sørensen, S.J., Turner, R.R., and Barkay, T. 2000. Application of a mer-lux biosensor for estimating bioavailable mercury in soil. *Soil Biology and Biochemistry* 32(5): 639-646.

Rattanachuay, P., Kantachote, D., Tantirungkij, M., Nitoda, T., and Kanzaki, H. 2011. Antivibrio compounds produced by *Pseudomonas* sp. W3: characterisation and assessment of their safety to shrimps. *World Journal of Microbiology and Biotechnology* 27(4): 869-880.

Reddy, P.S., Tuberty, S.R., and Fingerman, M. 1997. Effects of Cadmium and Mercury on Ovarian Maturation in the Red Swamp Crayfish, *Procambarus clarkii*. *Ecotoxicology and Environmental Safety* 37(1): 62-65.

Reis, F.C., Madureira, D.J., Vicentini, R., Carlos, C., Ferraz, L.F.C., Garcia, O., and Ottoboni, L.M.M. 2010. Transporter protein genes are differentially expressed in Acidithiobacillus ferrooxidans LR maintained in contact with covellite. *World Journal of Microbiology and Biotechnology* 26(11): 2061-2068.

Ritchie, R.J. 2013. The Use of Solar Radiation by the Photosynthetic Bacterium, *Rhodopseudomonas palustris*: Model Simulation of Conditions Found in a Shallow Pond or a Flatbed Reactor. *Photochemistry and Photobiology* 89(5): 1143-1162.

Ritchie, R.J., and Mekjinda, N. 2015. Measurement of Photosynthesis Using PAM Technology in a Purple Sulfur Bacterium *Thermochromatium tepidum* (Chromatiaceae). *Photochemistry and Photobiology* 91(2): 350-358.

Ritchie, R.J., and Runcie, J.W. 2013. Photosynthetic Electron Transport in an Anoxygenic Photosynthetic Bacterium *Afifella* (*Rhodopseudomonas*) *marina* Measured Using PAM Fluorometry. *Photochemistry and Photobiology* 89(2): 370-383.

Rudd, J.M. 1995. Sources of methyl mercury to freshwater ecosystems: A review. *Water, Air, and Soil Pollution* 80(1-4): 697-713.

Sakami, T., Fujioka, Y., and Shimoda, T. 2008. Comparison of microbial community structures in intensive and extensive shrimp culture ponds and a mangrove area in Thailand. *Fisheries Science* 74(4): 889-898.

Sawatdikul, S. 2003. Mercury contents in various tissues of economic marine anmimals from the eastern coast Thailand. Master degree, Environmental Science, Burapa University.

Schultz, H.R. 1996. Leaf absorptance of visible radiation in Vitis vinifera L.: estimates of age and shade effects with a simple field method. *Scientia Horticulturae* 66(1–2): 93-102.

Seki, H., Suzuki, A., and Mitsueda, S.-I. 1998. Biosorption of Heavy Metal Ions on *Rhodobacter sphaeroides* and *Alcaligenes eutrophus* H16. *Journal of Colloid and Interface Science* 197(2): 185-190.

Selin, N.E. 2009. Global Biogeochemical Cycling of Mercury: A Review. *Annual Review of Environment and Resources* 34: 43-63.

Sen Gupta, S., and Bhattacharyya, K.G. 2011. Kinetics of adsorption of metal ions on inorganic materials: A review. *Advances in Colloid and Interface Science* 162(1–2): 39-58.

Šeršeň, F., Král'ová, K., and Bumbálová, A. 1998. Action of Mercury on the Photosynthetic Apparatus of Spinach Chloroplasts. *Photosynthetica* 35(4): 551-559.

Shapawi, R., Ting, T.E., and Al-Azad, S. 2012. Inclusion of Purple Non-sulfur Bacterial Biomass in Formulated Feed to Promote Growth, Feed Conversion Ratio and Survival of Asian Seabass *Lates calcarife*r Juveniles. *Journal of Fisheries and Aquatic Science* 7(6): 475-480.

Shapleigh, J. 2009. Dissimilatory and Assimilatory Nitrate Reduction in the Purple Photosynthetic Bacteria. In *The Purple Phototrophic Bacteria*, edited by C.N. Hunter, F. Daldal, M. Thurnauer and J.T. Beatty, 623-642. Springer Netherlands.

Skyllberg, U., Qian, J., Frech, W., Xia, K., and Bleam, W. 2003. Distribution of mercury, methyl mercury and organic sulphur species in soil, soil solution and stream of a boreal forest catchment. *Biogeochemistry* 64(1): 53-76.

Sompongchaiyakul, P., and Sirinawin, W. 2007. Arsenic, Chromium and Mercury in Surface Sediment of Songkhla Lake System, Thailand. *Asian Journal of Water, Environment and Pollution* 4(1): 17-24.

Soundarapandian, P., Premkumar, T., and Dinakaran, G.K. 2010. Impact of Bioaccumulation of Mercury in Certain Tissues of the Marine Shrimp, *Penaeus monodon* (Fabricius) from the Uppanar Estuary, Cuddalore, Tamilnadu, India. *Current Research Journal of Biological Sciences* 2(2): 114-117.

Srinivas, T.N.R., Kumar, P.A., Sasikala, C., Ramana, C.V., Süling, J., and Imhoff, J.F. 2006. *Rhodovulum marinum* sp. nov., a novel phototrophic purple non-sulfur alphaproteobacterium from marine tides of Visakhapatnam, India. *International Journal of Systematic and Evolutionary Microbiology* 56(7): 1651-1656.

Stanisich, V., A., Bennet, P.M., and Richmond, M.H. 1977. Characterization of a Translocation Unit Encoding Resistance to Mercuric Ions That Occurs on a

Nonconjugative Plasmid in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 129(3): 1227-1233.

Tang, K.-H., Feng, X., Tang, Y.J., and Blankenship, R.E. 2009. Carbohydrate Metabolism and Carbon Fixation in *Roseobacter denitrificans* OCh114. *PLoS ONE* 4(10).

Telmer, K., and Veiga, M. 2009. World emissions of mercury from artisanal and small scale gold mining. In *Mercury Fate and Transport in the Global Atmosphere*, edited by R. Mason and N. Pirrone, 131-172. Springer US.

Thatoi, H., Behera, B., Mishra, R., and Dutta, S. 2013. Biodiversity and biotechnological potential of microorganisms from mangrove ecosystems: a review. *Annals of Microbiology* 63(1): 1-19.

Thilakaraj, R., Raghunathan, K., Anishetty, S., and Pennathur, G. 2007. In silico identification of putative metal binding motifs. *Bioinformatics* 23(3): 267-71.

Thongra-ara, W., and Parkpianb, P. 2002. Total Mercury Concentrations in Coastal Areas of Thailand: A Review. *ScienceAsia* 28(301-312).

Thongraa-ar, V. 1987. The contamination of mercury in marine animals that have economic value of the Eastern seaboard. Institute of Marine Science, Burapha University.

USEPA. 1993. Quality criteria for water. Washington, D.C.

USEPA. 2001. Appendix to Method 1631 total mercury in tissue, sludge, sediment, and soil by acid digestion and BrCl oxidation.

USEPA. 2002. Method 1631, Revision E: Mercury in water by oxidation, purge and trap, and cold vapor atomic fluorescence spectrometry. United States Environmental Protection Agency, Office of Water 4303.

USEPA. 2005. Water quality criterion for the protection of human health. Washington, DC: Office of Science and Technology, Office of Water.

USGS. 2004. Minerals yearbook, Tech. rep., US Geological Survey. http://minerals.usgs.gov/minerals /pubs/commodity/mercury/index.html#myb.

Vara Prasad, M.N., and de Oliveira Freitas, H.M. 2003. Metal hyperaccumulation in plants: Biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology* 6: 285-321.

Veglio, F., and Beolchini, F. 1997. Removal of metals by biosorption: a review. *Hydrometallurgy* 44(3): 301-316.

Velásquez, L., and Dussan, J. 2009. Biosorption and bioaccumulation of heavy metals on dead and living biomass of Bacillus sphaericus. *Journal of Hazardous Materials* 167(1–3): 713-716.

Vethanayagam, R.R. 1991. Purple photosynthetic bacteria from a tropical mangrove environment. *Marine Biology* 110(1): 161-163.

Vijayaraghavan, K., and Yun, Y.S. 2008. Bacterial biosorbents and biosorption. *Biotechnology Advances* 26(3): 266-91.

Visuthismajarn, P., Vitayavirasuk, B., Leeraphante, N., and Kietpawpan, M. 2005. Ecological Risk Assessment of Abandoned Shrimp Ponds in Southern Thailand. *Environmental Monitoring and Assessment* 104(1-3): 409-418.

Volesky, B., and Schiewer, S. 2002. Biosorption, Metals. In *Encyclopedia of Bioprocess Technology*. John Wiley & Sons, Inc.

Wang, X.S., Li, F.Y., He, W., and Miao, H.H. 2010. Hg(II) Removal from Aqueous Solutions by *Bacillus subtilis* Biomass. *CLEAN - Soil, Air, Water*, 10.1002/clen.200900201: NA-NA.

Wang, Y., Freedman, Z., Lu-Irving, P., Kaletsky, R., and Barkay, T. 2009. An initial characterization of the mercury resistance (*mer*) system of the thermophilic bacterium *Thermus thermophilus* HB27. *FEMS Microbiology Ecology* 67(1): 118-29.

Watanabe, M., Kawahara, K., Sasaki, K., and Noparatnaraporn, N. 2003. Biosorption of cadmium ions using a photosynthetic bacterium, *Rhodobacter sphaeroides* S and a marine photosynthetic bacterium, *Rhodovulum* sp. and their biosorption kinetics. *Journal of Bioscience and Bioengineering* 95(4): 374-378.

Wieser, M.E., and Berglund, M. 2009. Atomic weights of the elements 2007 (IUPAC Technical Report). *Pure and Applied Chemistry* 81 (11): 2131–2156.

Yamaguchi, A., Tamang, D.G., and Saier, M.H. 2007. Mercury Transport in Bacteria. *Water, Air, and Soil Pollution* 182(1-4): 219-234.

Yan, N. 2013. Structural advances for the major facilitator superfamily (MFS) transporters. *Trends in Biochemical Sciences* 38(3): 151-159.

Yu, Z., Li, J., Li, Y., Wang, Q., Zhai, X., Wu, G., Liu, P., and Li, X. 2014. A *mer* operon confers mercury reduction in a *Staphylococcus epidermidis* strain isolated from Lanzhou reach of the Yellow River. *International Biodeterioration & Biodegradation* 90: 57-63.

Yutin, N., Suzuki, M.T., Teeling, H., Weber, M., Venter, J.C., Rusch, D.B., and Béjà, O. 2007. Assessing diversity and biogeography of aerobic anoxygenic phototrophic bacteria in surface waters of the Atlantic and Pacific Oceans using the Global Ocean Sampling expedition metagenomes. *Environmental Microbiology* 9(6): 1464-1475.

Zaar, A., Fuchs, G., Golecki, J., and Overmann, J. 2003. A new purple sulfur bacterium isolated from a littoral microbial mat, *Thiorhodococcus drewsii* sp. nov. *Archives of Microbiology* 179(3): 174-183.

Zahir, F., Rizwi, S.J., Haq, S.K., and Khan, R.H. 2005. Low dose mercury toxicity and human health. *Environmental Toxicology and Pharmacology* 20(2): 351-360.

Zalups, R.K., and Lash, L.H. 1994. Advances in understanding the renal transport and toxicity of mercury. *Journal of Toxicology and Environmental Health* 42(1): 1-44.

Zeng, Y., Shen, W., and Jiao, N. 2009. Genetic diversity of aerobic anoxygenic photosynthetic bacteria in open ocean surface waters and upper twilight zones. *Marine Biology* 156(3): 425-437.

Zeyaullah, M., Islam, B., and Ali, A. 2010. Isolation, identification and PCR amplification of merA gene from highly mercury polluted Yamuna river. *African Journal of Biotechnology* 9(24): 3510–3514.

Zhang, W., Chen, L., and Liu, D. 2012. Characterization of a marine-isolated mercury-resistant *Pseudomonas putida* strain SP1 and its potential application in marine mercury reduction. *Applied Microbiology and Biotechnology* 93(3): 1305-1314.

Zhou, J., Bruns, M.A., and Tiedje, J.M. 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* 62(2): 316–322.

Zhou, L.-C., Li, Y.-F., Bai, X., and Zhao, G.-H. 2009a. Use of microorganisms immobilized on composite polyurethane foam to remove Cu(II) from aqueous solution. *Journal of Hazardous Materials* 167(1–3): 1106-1113.

Zhou, Q., Li, K., Jun, X., and Bo, L. 2009b. Role and functions of beneficial microorganisms in sustainable aquaculture. *Bioresource Technology* 100(16): 3780-3786.

APPENDIX

A. Medium

Glutamate-Acetate medium

Sodium L-glutamic acid	3.8 g
Sodium acetate	5.4 g
Yeast extract	2.0 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$(NH_4)_2HPO_4$	0.8 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.053 g
Nicotinic acid	0.001 g
Thiamine hydrochloride	0.001 g
Biotin	0.010 g
$MnSO_4.5H_2O$	0.012 g
Ferric citrate	0.025 g
$CoCl_2.6H_2O$	0.950 g
Deionized water	1,000 mL
pH	6.8

B. Report of microbial identification by 16s rDNA sequence analysis

LOCUS	KJ508085 1428 bp DNA linear BCT 19-MAY-2014
DEFINITION	*Rhodovulum sulfidophilum* strain SRW1-5 16S ribosomal RNA gene, partial sequence.
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ORGANISM	*Rhodovulum sulfidophilum* Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Rhodovulum*.
REFERENCE	1 (bases 1 to 1428)
AUTHORS	Mukkata,K.
TITLE	Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria
JOURNAL	Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M., and Naidu, R. 2015. Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria. Water, Air, & Soil Pollution 226(5): 1-14.
REFERENCE	2 (bases 1 to 1428)
AUTHORS	Mukkata,K.
TITLE	Direct Submission
JOURNAL	Submitted (27-FEB-2014) Microbiology, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
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TITLE Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria
JOURNAL Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M., and Naidu, R. 2015. Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria. Water, Air, & Soil Pollution 226(5): 1-14.
REFERENCE 2 (bases 1 to 1428)
AUTHORS Mukkata,K.
TITLE Direct Submission
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Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
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AUTHORS Mukkata,K.
TITLE Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria
JOURNAL Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M., and Naidu, R. 2015. Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria. Water, Air, & Soil Pollution 226(5): 1-14.
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Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
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1381 ccggccacgg tagggtcagc gactggggtg aagtcgtaac aaggtaacc

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List of Publication and Proceeding

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S.,Mallavarapu, M., and Naidu, R. 2015. Distribution of Mercury in Shrimp Ponds and Volatilization of Hg by Isolated Resistant Purple Nonsulfur Bacteria. *Water, Air, & Soil Pollution* 226(5): 1-14.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., and Boonapatcharoen, N. 2015. Diversity of purple nonsulfur bacteria in shrimp ponds with varying mercury levels. *Saudi Journal of Biological Sciences*. DOI: 10.1016/j.sjbs.2015.05.014.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M., and Naidu, R. 2015. Distribution of mercury in shrimp ponds and volatilization of Hg by isolated resistant purple nonsulfur bacteria. RGJ- Ph.D. Congress XVI "ASEAN: Emerging Research Opportunities". June 11-13. Jomtien Palm Beach, Pattaya, Thailand. (Poster Presentation).

Mukkata, K., Kantachote, D., Krishnan, K., Mallavarapu, M., and Naidu, R. Mercury resistant genes in purple non sulfur bacteria isolated from mercury contaminated shrimp ponds, *Manuscript,* will be submitted.

Mukkata, K., Kantachote, D., Ritchie, R.J., and Nunkaew, T. Toxic effects of mercury on photosynthesis process and cell morphology of Hg-resistant purple nonsulfur bacteria, *Manuscript,* will be submitted.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Mallavarapu, M., and Naidu, R. Mercury removal by live and dead cells of Hg resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds, *Manuscript*, will be submitted.