The Use of Purple Non-Sulfur Bacteria as Biofertilizers for Reducing Heavy Metals in Rice Plant and Global Warming

Jakkapan Sakpirom

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

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Author	Mr. Jakkapan Sakpirom
Major Program	Microbiology

Major Advisor

……..
(Prof. Dr. Duangporn Kantachote)

Co-advisor

……...
(Prof. Dr. Eakalak Khan)

Examining Committee :

…….....Chairperson
(Asst. Prof. Dr. Surasak Siripornadulsil)

…….….Committee
(Prof. Dr. Duangporn Kantachote)

…….….Committee
(Prof. Dr. Eakalak Khan)

…….….Committee
(Assoc. Prof. Dr. Sumate Chaiprapat)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Microbiology

..……..……..
(Prof. Dr. Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……Signature
(Prof. Dr. Duangporn Kantachote)
Major Advisor

……Signature
(Mr. Jakkapan Sakpirom)
Candidate

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.

……Signature
(Mr. Jakkapan Sakpirom)
Candidate

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Author Mr. Jakkapan Sakpirom
Major Program Microbiology
Academic Year 2018

ABSTRACT

Purple non-sulfur bacteria (PNSB) are attractive biofertilizers as they can reduce heavy metals in rice plant and greenhouse gases. A total of 235 PNSBs were isolated from various paddy fields contaminated and un-contaminated by cadmium (Cd) and/or zinc (Zn). Only *Rhodopseudomonas palustris* TN110 and *Rubrivivax gelatinosus* TN414 showed great potential as biofertilizers releasing NH_4^+ via N_2 fixationand plant growth promoting substances including 5-aminolevulinic acid (ALA) and indole-3-acetic acid (IAA). In addition, both strains acted as bioremediation agents by reducing heavy metals (Cd and Zn) and greenhouse gases (CH_4 and CO_2). However, only *R. palustris* TN110 bioremediated Cd by biosynthesis of cadmium sulfide (CdS) nanoparticles and simultaneously fixed N_2. This strain produced uniform CdS nanoparticles under its optimal microaerobic-light conditions (pH 7.5, 30 °C and 3,000 lux). The half maximal inhibitory concentration (IC_{50}) of the produced CdS nanoparticles was 1.76 mM. The produced CdS nanoparticles at IC_{50} up-regulated two genes associated with N_2 fixation: Mo-Fe nitrogenase gene (*nifH*) and V-Fe nitrogenase gene (*vnfG*) at 2.83- and 2.27-fold changes, respectively. The results of gene expressions agreed with the amounts of NH_4^+ released. Therefore, only *R. palustris* TN110 was further studied to obtain an optimal mixed carrier to support bacterial cells in solid formulation. Rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA), and spent coffee grounds (SCG) were the carriers studied. With the use of D-optimal experimental design, a mixed carrier of RWA, DCC, RHA and SCG had an optimum ratio of 3: 4: 2: 1. The optimal mixed carrier contained roughly 10 log CFU/g *R. palustris* TN110 and is packed in nylon-LLDPE bags using vacuum sealed at 500 W with a bacterial population decrease of only 3 log cycles after storage for 6 months at room temperature (25±3 °C). PNSB biofertilizer(s) (*R. palustris*

TN110, *Ru. gelatinosus* TN414 and a mixed culture of both strains at 1: 1) were investigated for their phytotoxicity based on rice (*Oryza sativa* L.) seed germination index and found that all PNSB biofertilizers tested either in solid or liquid form showed no phytotoxicity at their optimal dilutions. All PNSB biofertilizer(s) at their optimal dilutions from liquid and solid forms containing 10^8 cells/mL for TN110 and 10^7 cells/mL for TN414 and a mixed culture produced remarkable increases in rice growth on the basis of shoot and root lengths compared with controls (tap water and a mixed carrier with no culture) for both Cd-contaminated and un-contaminated paddy soils. The application of *R. palustris* TN110 in solid form to Cd-contaminated paddy soil significantly improved rice growth based on the maximum shoot dry weight and root length. Additionally, all PNSB biofertilizer(s) in the solid form was better than the liquid form in cutting down Cd and Zn accumulation in rice shoots and roots in Cd-contaminated paddy soil. *R. palustris* TN110 was effective in Cd removal whereas *Ru. gelatinosus* TN414 performed better in decreasing Zn in rice shoot and root. Finally, greenhouse gas emission in rice straw biodegradation model in paddy soil slurry by PNSB biofertilizer(s) was investigated by focusing on light/dark cycles and PNSB biofertilizer(s) formulations. Proliferation between PNSB and methanogens populations under the different light/dark cycles: 0/24, 8/16, 12/12, 16/8 and 24/0 h were evaluated and found that light promoted PNSB growth to compete with methanogens for reducing greenhouse gas emissions. *R. palustris* TN110 was more effective in reducing CH_4 and CO_2 emissions compared with other biofertilizer(s). The carrier control dramatically increased total daily gas volume; all PNSB biofertilizer(s) from liquid form under all light/dark cycles throughout 10 day-incubation showed higher total daily gas volume reduction than a solid form. Strain TN110 in liquid form under natural light cycle (12/12 h) at day 10 was able to reduce 72.27% CH_4 and 34.38% CO_2 emissions. PNSB biofertilizer(s) in the solid form was a suitable form for promoting rice growth and reducing accumulated heavy metals (Cd and Zn) in contaminated soil; however, the liquid form was better for reducing greenhouse gas emissions. Overall discoveries in this research demonstrated that PNSB: *R. palustris* TN110 and *Ru. gelatinosus* TN414 are effective as biofertilizers and bioremediation agents for supporting rice cultivation in Cd-contaminated paddy soil. Moreover, the PNSB could alleviate greenhouse gas emissions by competing

proliferation with methanogens that are responsible for greenhouse gas production. This thesis revealed that PNSB biofertilizer(s) has a great potential to be biofertilizers and also bioremediation agents for producing safe rice. Therefore, application of the PNSB to paddy fields with contamination with heavy metals or un-contamination would be an eco-friendly practice to produce safe rice along with improving soil quality and reducing global warming for sustainable agriculture.

Keywords: Biofertilizer, Bioprecipitation, Cadmium, Cadmium sulfide nanoparticle, Methane, Nitrogenase, Plant growth promoting substances, Purple non-sulfur bacteria, Zinc

ชื่อวิทยานิพนธ์ การใช้แบคทีเรียสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์เป็นปุ๋ยชีวภาพเพื่อลดโลหะหนักในต้นข้าวและภาวะโลกร้อน

ผู้เขียน นายจักรพันธุ์ ศักดิ์ภิรมย์

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2561

บทคัดย่อ

แบคทีเรียสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ (Purple Non-Sulfur Bacteria; PNSB)ได้รับความสนใจในการนำไปใช้เป็นเป็นปุ๋ยชีวภาพ รวมถึงลดการสะสมของโลหะหนักในข้าวและลดการปล่อยก๊าซเรือนกระจก ได้คัดแยกแบคทีเรียกลุ่มนี้จำนวน 235 สายพันธุ์จากนาข้าวในพื้นที่ต่างๆ รวมถึงนาข้าวที่มีการปนเปื้อนด้วยแคดเมียมและสังกะสี จากการศึกษาพบว่าสายพันธุ์ *Rhodopseudomonas palustris* TN110 และ*Rubrivivax gelatinosus* TN414 แสดงให้เห็นถึงความเป็นไปได้สูงที่จะนำไปใช้เป็นปุ๋ยชีวภาพ ด้วยการปลดปล่อยแอมโมเนียมไอออน (NH_4^+) ผ่านการตรึงก๊าซไนโตรเจนและผลิตสารที่ส่งเสริมการเจริญของพืชได้แก่ กรด 5-อะมิโนลีวูลินิก (5-Aminolevulinic Acid; ALA) และกรดอินโดล-3-แอซีติก (Indole-3-Acetic Acid; IAA) นอกจากนี้ทั้ง 2 สายพันธุ์ดังกล่าวยังสามารถเป็นตัวบำบัดโลหะหนัก (แคดเมียมและสังกะสี) และลดการปลดปล่อยก๊าซเรือนกระจก (ก๊าซมีเทนและคาร์บอนไดออกไซด์) แต่มีเพียงเฉพาะสายพันธุ์ TN110 ที่มีความสามารถในการบำบัดแคดเมียมโดยผ่านกระบวนการการสังเคราะห์ให้อยู่ในรูปอนุภาคนาโนแคดเมียมซัลไฟด์พร้อมกับตรึงก๊าซไนโตรเจนไปพร้อมกัน แบคทีเรียสายพันธุ์นี้สังเคราะห์อนุภาคนาโนดังกล่าวได้ลักษณะรูปร่างและขนาดเป็นไปในรูปแบบเดียวกันภายใต้สภาวะมีออกซิเจนเล็กน้อย มีแสง ที่ค่าความเป็นกรดด่าง 7.5 อุณหภูมิ 30 องศาเซลเซียส และความเข้มแสง 3,000 ลักซ์ อนุภาคนาโนที่ผลิตได้นี้ยับยั้งการเจริญของแบคทีเรียสายพันธุ์ TN110 ได้ร้อยละ 50 ที่ค่าความเข้มข้นเท่ากับ 1.76 มิลลิโมลาร์ ค่าความเข้มข้นดังกล่าวสามารถส่งเสริมการทำงานของยีนที่เกี่ยวข้องกับกระบวนการตรึงก๊าซไนโตรเจนของแบคทีเรียเอง โดยส่งเสริมการทำงานของยีนไนโตรจีเนสชนิดโมลิบดีนัม-เหล็ก และวานาเดียม-เหล็ก ได้เพิ่มขึ้น 2.83 และ 2.27 เท่า ตามลำดับ ผลการแสดงออกที่เพิ่มขึ้นของยีนดังกล่าวมีความสัมพันธ์สอดคล้องกับปริมาณ NH_4^+ที่ปลดปล่อยออกมา ดังนั้นสายพันธุ์ TN110 จึงถูกเลือกเพื่อนำไปศึกษาสัดส่วนที่เหมาะสมของการผสมวัสดุพยุงได้แก่ ขี้เถ้าเหลือใช้จากกระบวนการผลิตยางพาราแผ่น กากปาล์มน้ำมัน ขี้เถ้าจากแกลบข้าว และ

กากกาแฟ ที่สามารถช่วยในการเก็บรักษาเซลล์แบคทีเรียเพื่อผลิตปุ๋ยชีวภาพในรูปของแข็ง โดยออกแบบชุดการทดลองด้วยโปรแกรมที่ใช้ตัวเลือกแบบ D-optimal ได้มาซึ่งปริมาณของขี้เถ้าเหลือใช้จากกระบวนการผลิตยางพาราแผ่น กากปาล์มน้ำมัน ขี้เถ้าจากแกลบข้าว และกากกาแฟ ในสัดส่วนที่เหมาะสมคือ 3: 4: 2: 1 สัดส่วนดังกล่าวถูกนำไปผสมกับแบคทีเรียสายพันธุ์ TN110 ความเข้มข้นประมาณ 1หมื่นล้านเซลล์ต่อกรัม บรรจุลงในถุงชนิดไนลอน-พอลิเอทิลีนความหนาแน่นต่ำเชิงเส้น(Nylon-LLDPE) และปิดผนึกโดยใช้แรงดูดสูญญากาศที่กำลังไฟฟ้า 500 วัตต์ พบว่าปริมาณแบคทีเรียลดลงร้อยละ 30 หลังจากเก็บไว้ที่อุณหภูมิห้อง (25±3 องศาเซลเซียส) เป็นเวลา 6 เดือน หลังจากนั้นศึกษาความเป็นพิษของปุ๋ยชีวภาพดังกล่าวต่อดัชนีการงอกของเมล็ดข้าว (*Oryza sativa* L.) ซึ่งปุ๋ยชีวภาพที่ทดสอบได้แก่แบคทีเรียสายพันธุ์ TN110 หรือ TN414 หรือ ผสมทั้ง 2 สายพันธุ์ในอัตราส่วนเท่ากัน ทั้งในรูปแบบของเหลวและของแข็ง ผลการทดสอบพบว่าปุ๋ยชีวภาพทุกสูตรไม่มีความเป็นพิษต่อดัชนีการงอกของเมล็ดข้าวที่ค่าการเจือจางที่เหมาะสมของแต่ละชุดการทดสอบ ปุ๋ยชีวภาพทั้งที่อยู่ในรูปของเหลวและของแข็งที่ผสมแบคทีเรียสายพันธุ์ TN110 ที่ความเข้มข้น 100 ล้านเซลล์ต่อมิลลิลิตร และแบคทีเรียสายพันธุ์ TN110 และแบคทีเรียผสมทั้ง 2 สายพันธุ์ ที่ความเข้มข้น 10 ล้านเซลล์ เพิ่มการเจริญเติบโตอย่างชัดเจนโดยให้ความยาวของลำต้นและของรากข้าวเป็นดัชนีชี้วัดการเจริญของต้นข้าวเปรียบเทียบกับชุดควบคุมทั้งการทดสอบโดยใช้ดินนาข้าวจากพื้นที่ที่มีการปนเปื้อนของแคดเมียมและไม่มีการปนเปื้อน การใช้ปุ๋ยชีวภาพในรูปของแข็งที่ผสมด้วยแบคทีเรียสายพันธุ์ TN110 ในดินนาข้าวจากพื้นที่ที่มีการปนเปื้อนของแคดเมียมให้ค่าน้ำหนักแห้งของลำต้นและรากข้าวสูงสุด อีกทั้งปุ๋ยชีวภาพในรูปของแข็งสามารถลดการสะสมของแคดเมียมและสังกะสีในลำต้นและรากข้าวได้ดีกว่าปุ๋ยชีวภาพในรูปของเหลวในการทดสอบปลูกข้าวในดินนาข้าวจากพื้นที่ที่มีการปนเปื้อนของแคดเมียม สายพันธุ์ TN110 มีประสิทธิภาพในการลดการสะสมของแคดเมียม ในขณะที่สายพันธุ์ TN414 มีประสิทธิภาพในการลดการสะสมของสังกะสีในต้นข้าวทั้งลำต้นและราก สุดท้ายได้มีการศึกษาผลของการใช้ปุ๋ยชีวภาพต่อการปลดปล่อยก๊าซเรือนกระจกในรูปแบบจำลองการย่อยฟางข้าวในนาโดยผสมกับดินนาข้าวที่ให้ช่วงแสงต่อวันแตกต่างกัน ได้แก่จำนวนชั่วโมงของการให้แสงต่อการไม่ให้แสงในรอบเวลา 1 วันที่ 0/24 8/16 12/12 16/8 และ 24/0 เพื่อศึกษาการแข่งขันการเจริญระหว่าง PNSB และจุลินทรีย์กลุ่มที่สร้างก๊าซมีเทน (Methanogens) จากผลการทดลองนี้พบว่า สายพันธุ์ TN110 มีประสิทธิภาพดีที่สุดในการลดการปลดปล่อยก๊าซมีเทนและคาร์บอนไดออกไซด์เมื่อเปรียบเทียบกับชุการทดลองอื่นๆ และชุดควบคุมที่เป็นตัวผยุงมีปริมาณของก๊าซสะสมในแต่ละวันสูงขึ้นอย่างชัดเจนที่เวลาทดสอบ 10 วัน ดังนั้นกล่าวได้ว่าปุ๋ยชีวภาพในรูปของเหลวมีการปลดปล่อยก๊าซสะสมแต่ละวันต่ำกว่าปุ๋ยชีวภาพในรูปของแข็งโดยปุ๋ยชีวภาพในรูปของเหลวสายพันธุ์ TN110 ที่ทดสอบภายใต้การให้แสงต่อการ

ไม่ให้แสงในรอบเวลา 1 วันที่12/12 ชั่วโมง เปรียบเสมือนระบบแสงตามธรรมชาติลดการปลดปล่อยก๊าซมีเทนและคาร์บอนไดออกไซด์ได้ร้อยละ 72.27 และ 34.38 ตามลำดับ ปุ๋ยชีวภาพในรูปของแข็งแสดงให้เห็นผลเด่นชัดในการส่งเสริมการเจริญและลดการสะสมของโลหะหนัก (แคดเมียมและสังกะสี) ของต้นข้าว ส่วนปุ๋ยชีวภาพในรูปของเหลวมีประสิทธิภาพโดดเด่นในการลดการปลดปล่อยก๊าซเรือนกระจก จากผลการศึกษาทั้งหมดครั้งนี้แสดงว่า PNSB สายพันธุ์ TN110 และ TN414 มีประสิทธิภาพในการใช้เป็นปุ๋ยชีวภาพและเป็นตัวบำบัดทางชีวภาพเพื่อส่งเสริมการปลูกข้าวในพื้นที่นาข้าวที่ปนเปื้อนแคดเมียม นอกจากนี้ PNSB ยังมีความสามารถในการลดการปลดปล่อยก๊าซเรือนกระจก โดยการแข่งขันการเจริญกับMethanogens วิทยานิพนธ์นี้แสดงถึงศักยภาพของ PNSB ในการใช้เป็นปุ๋ยชีวภาพเพื่อช่วยให้ได้ผลผลิตข้าวที่ปลอดภัย ดังนั้นการประยุกต์ใช้ PNSB ในนาข้าวทั้งที่ปนเปื้อนหรือไม่มีการปนเปื้อนของโลหะหนักจึงเป็นวิธีการที่เป็นมิตรกับสิ่งแวดล้อม ช่วยปรับปรุงคุณภาพดินและลดภาวะโลกร้อนเพื่อเป็นการทำเกษตรที่ยั่งยืน

คำสำคัญ : การตกตะกอนทางชีวภาพ ก๊าซมีเทน แคดเมียม ไนโตรจีเนส แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ ปุ๋ยชีวภาพ สารส่งเสริมการเจริญเติบโตของพืช สังกะสี อนุภาคนาโนแคดเมียมซัลไฟด์

ACKNOWLEDGEMENTS

I would like to express my special appreciation and sincere grateful thanks to my major advisor, Professor Dr. Duangporn Kantachote for her enthusiasm, helpful suggestions, and precious comments during my study. Heartfelt gratitude is also acknowledged to my co-advisor, Professor Dr. Eakalak Khan for his contribution and valuable advices related to my research.

I also would like to express my gratitude to the Thailand Research Fund through the Royal Golden Jubilee Ph.D. program; grant number PHD/0057/2555 for Ph.D. scholarship, and the Graduate School, Prince of Songkla University (PSU) for supporting a budget to conduct this research.

Sincere appreciation is extended to Assistant Professor Dr. Surasak Siripornadulsil, Department of Microbiology, Faculty of Science, Khon Kaen University for acting as an external committee member and chairperson of my dissertation advisory committee and Associate Professor Dr. Sumate Chaiprapat as an internal committee member for their comments and advices relating this dissertation.

Special thanks go to Professor Dr. John McEvoyfor being as a co-advisor when I did experiments at North Dakota State University, Fargo, USA. I would like to thank Dr. Supachai Pisuchpen from Department of Material Product Technology, Faculty of Agro-Industry, Prince of Songkla University (PSU) for kind supporting PET-nylon-PP and nylon-LLDPE bags used in this study, the walk-in humidity chamber, and suggestions about product packaging.In addition, I would like to thank Associate Professor Dr. Sumana Ratpukdi, Department of Environmental Engineering, Faculty of Engineering, Khon Kaen University for her contribution to my research indirectly through an article publication.

I would like to express my deepest appreciation to my friends at Department of Microbiology, PSU and Department of Civil and Environmental Engineering and Department of Microbiological Science, North Dakota State University for their kindly help throughout my research working. Finally, I wish to thank my beloved family and friends for their support and encouragement.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation/Symbol	Description
ALA	5-aminolevulinic acid
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
CdS	Cadmium sulfide
CFU	Colony forming unit
CH_4	Methane gas
cm	Centimeter(s)
cm^3	Cubic centimeter(s)
CO_2	Carbondioxide gas
DCC	Decanter cake
DNA	Deoxyribonucleic acid
DW	Distilled water
EC	Electrical conductivity
EPS	Exopolymeric substance(s)
Fe	Iron
FTIR	Fourier transform infrared spectrometer
g	Gram(s)
GA	Glutamate-acetate
GC	Gas chromatography
GI	Germination index
h	Hour(s)
HPLC	High-performance liquid chromatography
HR-TEM	High resolution-transmission electron microscope
IC_{50}	Half maximal inhibitory concentration
ICP-OES	Inductively coupled plasma-optical emission spectrometer
IAA	Indole-3-acetic acid
K	Potassium
kg	Kilogram(s)
L	Liter(s)

LA	Levulinic acid
log	Logarithm
mg	Milligram(s)
min	Minnute(s)
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar(s)
Mo	Molybdenum
mS	MilliSiemens
N	Nitrogen
N_2	Nitrogen gas
NH_4^+	Ammonium ion(s)
Nylon-LLDPE	Nylon-linear low density polyethylene
O_2	Oxygen gas
OD_{660}	Optical density at a wavelength of 660 nm
P	Phosphorus
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PET-nylon-PP	Polyethylene terephthalate-nylon-polypropylene
PGPB	Plant growth promoting bacteria
PGPS	Plant growth promoting substances
PNSB	Purple non-sulfur bacteria
PP	Polypropylene
qPCR	Quantitative polymerase chain reaction
RHA	Rice husk ash
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
RWA	Rubber wood ash

SAED	Selected area electron diffraction
SCG	Spent coffee grounds
SEM	Scanning electron microscope
tRNA	Transfer ribonucleic acid
Trp	Tryptophan
U.S.	United State
V	Vanadium
W	Watt(s)
Zn	Zinc
Å	Angstrom
°C	Degree celsius
μ_{max}	Maximum specific growth rate
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
µM	Micromolar(s)
µS	MicroSiemens

CHAPTER 1

Introduction

Background and Rationale

Thailand is an agricultural country and rice is one of the main crops that produce income about ten billion baht (0.3 billion U.S. dollars) per year. The area for rice seedlings of Thailand is roughly 30 million acres and this consists of in-season rice field about 24 million acres, and off-season rice field about 6 million acres (Office of Agricultural Economics, 2017a). The major plant nutrients are nitrogen (N), phosphorus (P), and the potassium (K). Among them, nitrogen is the element that rice needs for the whole life of growing until harvesting; thereby it impacts the production and quality of rice grains (Dobermann and Fairhurst, 2000). In addition, phytohormones are one of plant stimulating factors; however, they are too expensive for rice cultivation. Plants can produce hormones such as auxins, cytokinins and gibberellins, but the amounts do not meet the demand for rice yield increase (Liu et al., 2011).

Chemical fertilizers or artificial fertilizers have been heavily used by rice farmers for many decades because they are simple to use and provide high productivity although they are expensive. Consequently, the cost of rice production increases. These fertilizers have adverse effects on soil by decreasing soil quality such as acidity build up, high soluble salts, and less water available in the soil (Wang and Yang, 2003). Moreover, chemical fertilizers such as phosphate fertilizers can cause environmental impact as they are often contaminated with heavy metals including cadmium (Cd) which originated with rock phosphate (Schipper et al., 2011).

Area for rice cultivation is sometimes limited; therefore, some paddy fields have to be located near mines. This can lead to heavy metals contamination in the fields that caused by inadequately treated mining drainage discharge (Yang et al., 2006). Heavy metals slowly contaminated rice through the roots and then accumulated in the grain. However, most of the accumulation of heavy metals was observed in the roots more

than the grains (Li et al., 2007). Römkens et al. (2009) reported that soils contaminated with Cd in a range of 0.1 to 30 mg Cd/kg of soil resulted in contamination of the grains at an average of 0.2 mg Cd/kg of grain.

The consumption of Cd-contaminated rice at 0.24 µg Cd/g of unpolished rice was likely to contribute a Cd dose of 2.2 µg/kg body weight/day for an adult weighing 60 kg and a dose of 1.5 µg Cd/kg body weight/day for children weighing 40 kg (Yang et al., 2006). In Thailand, the contamination of Cd and zinc (Zn) in rice nearby a Zn mine was observed in Mae Sot District, Tak Province, and caused adverse effects on human health and environment. Jasmine rice was a major export product of the district; therefore, the government paid the farmers to refrain from planting rice in this area during 2005-2007 (Academic and Standard Groups, Office of Management and Environmental Remediation, Department of Primary Industries and Mines, 2004).

Rice cultivation in flood paddy fields in developing countries has been claimed to be a source of greenhouse gases including methane (CH_4) (Zhang et al., 2012). CH_4 is 25 times more potent than carbon dioxide (CO_2) as greenhouse gas (Li et al., 2011). Ahmad et al. (2009) found that rice cultivation without the use of chemical fertilizers released CH_4 at 34.24 g/m^2; whereas rice cultivation with the use of chemical fertilizers emitted more CH_4 at 65.96 g/m^2. CH_4 is produced under anaerobic conditions in paddy fields which is suitable for the growth of methanogens (De Vrieze et al., 2012). Thailand emitted a total of 2.8 million tons of CH_4 in 2000. Of the total, more than 70% came from agriculture, with rice cultivation accounting for a major proportion (51% of CH_4 emissions from agriculture) (Office of Natural Resources and Environmental Policy and Planning, 2011).

Biofertilizers are an alternative to chemical fertilizers. Among biofertilizers that can fix N_2(Harada et al., 2005; Sakpirom et al., 2017 and 2019) and have a high possibility to reduce CH_4 emission by competing the growth with methanogens (Harada et al., 2001 and 2003; Kantha et al., 2015; Sakpirom et al., 2017) are purple nonsulfur bacteria (PNSB). They are a group of bacteria that is found naturally in areas where sunlight can reach such as paddy fields. They can produce certain phytohormones such as auxin (Mujahid et al., 2011; Sakpirom et al., 2017), cytokinin (Serdyuk et al., 2000) including 5-aminolevulinic acid (ALA), a plant growth promoting substance (Sakpirom et al., 2017; Nookongbut et al., 2018). Some

members of PNSB can use hydrogen sulfide (H_2S) surrounding rice roots as an electron donor for photosynthesis thereby resulting in reduction of toxicity (to rice), stronger roots, and consequently higher productivity (Lamer et al., 2013). Moreover, the flooded paddy fields provide anoxic conditions which are suitable for production of H_2S by microbes (Lamers et al., 2013). When H_2S transforms to sulfide (S_2^-), it actively reacts with heavy metals ions in contaminated paddy fields to produce a precipitated form of metal-sulfide nanoparticles (Castillo et al., 2012) that are found in PNSB (Bai et al., 2006 and 2009; Bai and Zhang, 2009). The heavy metals in the precipitated form were less toxic than their soluble form (Reichman, 2002). PNSB has been widely studied for removal of heavy metal ions especially Cd bioaccumulation and biosorption (Smiejan et al., 2003; Watanabe et al., 2003) along with conversion of Cd to more stable forms such as Cd sulfide (Fan et al., 2012; Sakpirom et al, 2019). These bioremediation actions by PNSB are also extended to lead (Pb), copper (Cu), and Zn (Panwichian et al., 2010a). Exopolymeric substances (EPS) of PNSB showed more effective removal of all these heavy metals than their cells (Panwichian et al., 2011).

Biofertilizers are an attractive option for organic agriculture (Kantachote et al., 2016). Formulation of the plant growth promoting bacteria (biofertilizers) requires suitable carriers for supporting growth of one or several bacterial strains, providing a safe environment under stressful conditions during storage, and ensuring survival and establishment after application to soils (Singh et al., 2011; Herrmann and Lesueur, 2013). Decanter cake is a by-product from a palm oil factory that plays an important role in the economy of Thailand (Chavalparit et al., 2006). It is a good candidate for carrier material of biofertilizers because it can improve soil quality and plant growth (Embrandiri et al., 2017). Rubber wood ash is a solid waste from rubber firewood boilers (Saritpongteeraka and Chaiprapat, 2008) that has potential as carrier material for biofertilizers (Demeyer et al., 2001; Park et al., 2005; Gaind and Gaur, 2004; Kumar and Gupta; 2010). Simultaneously, the uses of these materials help in reducing the industrial waste.

According to the above information, it is worth to explore the potential of PNSB as biofertilizers in paddy fields. They can potentially solve heavy metals contamination problems and reduce CH_4 emission from the paddy fields.

Objectives

1. To isolate and select PNSB with abilities to fix N_2, produce ALA and phytohormones, and reduce Cd and Zn.
2. To investigate the efficiencies of Cd and Zn reduction by selected PNSB.
3. To study the efficiency of selected PNSB in synthesizing CdS nanoparticles.
4. To produce a formulation of selected PNSB as biofertilizers in paddy fields.
5. To investigate the possibility of selected PNSB to reduce CH_4 emission.

Scopes of this Study

1. To isolate PNSB and determine amounts of Cd and Zn from soil and water samples collected from contaminated and uncontaminated areas.
2. To investigate the possibility of selected PNSB as biofertilizers.
3. To evaluate the efficiencies of selected PNSB strains for reducing Cd and Zn.
4. To determine the ability of selected PNSB for production of CdS nanoparticles.
5. To assess the efficiency of the formulation of PNSB biofertilizers for rice (*Oryza sativa* L.) growth in laboratory and greenhouse scales.
6. To examine the capacity of selected PNSB strains for mitigating CH_4 emission.

Anticipated Outcomes

1. PNSB as biofertilizers can replace chemical fertilizers in rice cultivation.
2. PNSB as biofertilizers can reduce heavy metals (Cd and Zn) contamination in paddy fields and accumulation in rice shoot and root.
3. Biofertilizer formulations developed in this study can be used to reduce CH_4 emission leading to environmentally friendly and sustainable agriculture.

CHAPTER 2

Review of Literature

Rice

Rice is monocots in the grass family and it is a biennial plant with edible grains. Their leaves are long and thin. Its classification is as follows: (Vaughan, 1989).

Kingdom Plantae
Division Magnoliophyta
Class Liliopsida
Order Poales
Family Poaceae (Gramineae)
Subfamily Oryzoideae
Tq ribe Oryzeae
Genus *Oryza*
Species *Oryza sativa*

Oryza sativa and *Oryza glaberrima* species of rice are planted for food. They are ordinary rice and glutinous rice. *Oryza sativa* is grown widely in many countries. It is classified according to the origin and evolution of the three types of *japonica*, *indica* and *javanica*. In Thailand, the most of them were *indica*(De Datta, 1981).

The status of rice cultivation in Thailand

In 2012, Thailand lost the spot as a top rice exporting country. It was beaten by India and Vietnam. Thailand has the fifth largest amount of land for rice cultivation in the world. Table 2-1 shows information on area, yield, and economics of rice production in Thailand. Average Thai rice yield of 1,217 kg/acre is very low when compared with average rice yield of China (2,568 kg/acre), Japan (2,646 kg/acre), Vietnamese (2,021 kg/acre), Indonesia (1,898 kg/acre), Myanmar (1,609 kg/acre), Bangladesh (1,571 kg/acre), Philippines (1,523 kg/acre) and India (1,298 kg/acre), and the world average rice yield of about 1,688 kg/acre (Phetsuk, 2010).

Table 2-1. Rice Production in Thailand from 2013 to 2015.

Lists	2013	2014	2015
1. Area of cultivation (million acres)	27.60	28.74	28.70
- In-season rice	22.70	23.72	22.54
- Off-season rice	4.90	6.02	5.96
2. Products per acre(kg)			
- In-season rice	1024	1022	984
- Off-season rice	1718	1472	1546
3. Costs of production (baht per ton)			
- In-season rice	8,859	8,448	9,475
- Off-season rice	6,719	8,006	6,904
4. Price which farmers sold (baht per ton)			
- In-season rice (moisture 5 %)	9,612	10,609	9,714
- Off-season rice (moisture 14-15 %)	9,909	8,040	8,040
5. Net income (baht per ton)			
- In-season rice	753	2,162	239
- Off-season rice	3,190	34	1,136

Reference: Office of Agricultural Economics (2017b and c)
Notes:In-season rice planting is from May to October (except for the Southern east coast, June to February) and harvesting period is from August to April. Off-season rice planting takes place from November to April (except for the Southern east coast, March 1 to June 15) and harvesting period is from February to October.

The use of chemical fertilizers in Thai rice cultivation

A long-term use of land for rice cultivation causes adverse effects on the soil; therefore, farmers use chemical fertilizers to increase soil quality (Touraine, 2004). Table 2-2 shows an amount of chemical fertilizers used annually in Thailand that are imported from overseas such as United States, Saudi Arabia and South Korea.

Table 2-2. The amount of chemical fertilizers used in agriculture and value of chemical fertilizer imports in Thailand.

Year	Volume of use (ton)	Import	
		Volume(ton)	Value (million baht)
2013	3,654,797	4,350,516	77,279
2014	3,713,328	3,797,749	68,592
2015	3,775,529	3,833,072	60,721
2016	3,952,356	5,310,037	53,071
2017	3,919,766	5,890,636	58,932

Reference: (Office of Agricultural Economics, 2017a)

The use of chemical fertilizers by rice farmers is one of the factors that help in increasing rice productivity. However, the use of chemical fertilizers causes deterioration of the soil structure and fertility. The use of chemical fertilizers is not nourishing the soil. There is only addition of inorganic nutrients to the plants without addition of organic matter to the soil. The problem of low efficiency of fertilizers increases, particularly when used in a hot arid farmland. The use of chemical fertilizer with more nitrogen changes soil to be more acidic and thus makes unavailable phosphorus available in soil. The continuing use of chemical fertilizer with macronutrients as nitrogen, phosphorus, and potassium leads up to a lack of micronutrients such as zinc, iron, copper, manganese, and magnesium (Naylor, 1996; Sheppard and Wallander, 2004; Craven et al., 2008).

Roles of Nitrogen Element on Rice Growth and Yield

Adding nitrogen to soil could be achieved by three forms of fetilizers: organic fertilizer i.e. manures or compost; biofertilizer such as *Rhizobium* and *Azotobacter* and chemical fertilizers such as ammonium sulfate and urea. Nitrogen is a nutrient that is necessary for the growth of rice and the nutrient availability of nitrogen is approximately 14-16 kg per 1 ton of rice grain (Dobermann and Fairhurst, 2000). Rice plant absorbed nitrogen from soil in the form of ammonium ion and nitrate ion. Concentrations of these nitrogen species are from a mineralization-immobilization process by microbial activity in soil with organic matter as a source of nitrogen. Ammonium ions is transformed into nitrate ions by nitrification, which is easy to leach to the subsoil and resulted in water pollution (Touraine, 2004; Xu-Ri and Prentice, 2008). Therefore, the availability of nitrogen is depended on the concentration of ammonium and nitrate ions around the roots of rice at each stage of growth, especially the periods of the maturation of stem and leaves.

Fixation of atmospheric nitrogen is the role of the nitrogenase enzyme found in nitrogen fixing microbes and this enzyme has molybdenum and iron as its cofactors (Masepohl and Kranz, 2009). These microbes play a critical role in increasing nitrogen and improving soil fertility (Maxwell, 2004). These microbes could be divided into two groups as follows:

Free-living nitrogen fixing microorganisms

Azotobacter, *Azospirillum* and*Burkholderia* require oxygen for nitrogen fixation whereas *Clostridium pasteurianum* does not require. Purple nonsulfur bacteria such as *Rhodobacter* and *Rhodospirillum* are able to fix nitrogen as well (Klipp et al., 2005).

Symbiotic nitrogen fixing microorganisms

In case of *Rhizobium*, this bacterium lives and then gets nutrients and energy from plant host while its fixed nitrogen is given to that plant. *Anabaena* when lives into hollow leaf of *Azolla* it fixes nitrogen better than when lives independently in nature (Pawlowski, 2009).

Phytohormones

Auxins

Indole-3-acetic acid (IAA) is one of auxins plant hormone that can be produced by plants and microbes. The activity of auxins depends on stimuli such as light, temperature, gravitation, and other induction (Kepinski, 2007). There are other compounds that play a role as auxin, such as indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPyA) and indole-3-acetonitrile (IAN) (Mockaitis and Estelle, 2008). The IAA synthesis in bacteria uses various mechanisms in the synthesis depending on the type of bacteria. A precursor for the synthesis of IAA is tryptophan (Trp). The descriptions of the pathways for IAA synthesis are as follows (Spaepen et al., 2007):

1. Indole-3-acetonitrile (IAN) pathway occurs in both plants and bacteria. Trp may be changed to an indolic glucosinolates (glucobrassicin) or indole-3-acetaldoxime before transforming to the IAN. IAN is converted to IAA by nitrilase.

2. Indole-3-acetamide (IAM) pathway is a popular pathway in the synthesis of IAA in bacteria, consists of two main steps. First, Trp is converted to IAM by the try-2-monooxygenase (IaaM) enzyme. In the second step, IAM is transformed IAA by the IAM hydrolase (IaaH) enzyme. This pathway has not been found to occur in plants.

3. Tryptophan side-chain oxidase (TSO) pathway, which is not found in plants, but is found in bacteria. Trp is changed directly to indole-3-acetaldehyde (IAAld) which later became IAA.

4. Indole-3-pyruvate (IPyA) pathway is the most common way for IAA synthesis in plants and is found in bacteria as well. It starts from Trp being converted to IPyA by the aminotransferase enzyme via a transmination reaction, and then IPyA being decarboxylated to IAAld by the indole-3-pyruvate decarboxylase (IPDC) enzyme. Finally, IAAId is transformed to IAA by the IAAld dehydrogenase enzyme.

5. Tryptamine (TAM) pathway in which Trp is decarboxylated to TAM by Trp decarboxylase. It is transformed to IAAld by the amine oxidase enzyme. Finally, it is converted to IAA. In case of plants, they use a flavin monooxygenase-like protein (YUCCA) replacing amine oxidase for transformation of TAM to N-hydroxyl-tryptamine. It is changed to IAAld and IAA respectively.

6. Trp-independent pathway, IAA could be synthesized directly by changing the substance as Indole-3-glycerolphosphate or indole to the IAA.

Cytokinins

In 1950, Skoong and Miller discovered cytokinin and first named it, Kinetin (6-furfuryl aminopurine), the derivatives of adenine (aminopurine), which was extracted from DNA and played a role in cell division, especially cytoplasm. Zeatin (6-(4-hydroxy-3-methylbut-2-enylamino) purine) was discovered in most natural cytokinin species. The synthesis of this compound is found in plants, blue green algae, bacteria and some species of slime mold. There are natural cytokinin, such as N^6-(Δ^2-isopentenyl)-adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ) and dihydrozeatin (DZ) and synthetic cytokinin such as diphenylurea and thidiazuron (Schmülling, 2004). A mechanism of cytokinin biosynthesis in bacteria occurs mostly with an adenosine-5'-monophosphate (AMP) and dimethylallyl pyrophosphate (DMAPP) as precursors and an adenosine phosphate-isopentenyltransferase (IPT). An isopentenyl riboside-5'-monophosphate (iPRMP) is formed as an intermediate which can transformed to different structure of cytokinins (Sakakibara, 2006).

5-Aminolevulinic Acid (ALA)

ALA is a non-protein amino acid consisting of 5 carbon atoms. This chemical is an intermediate in the synthesis of tetrapyrrole such as vitamin B12, chlorophyll, and heme. It could be found in plants, animals, fungi and bacteria. The mechanism of ALA synthesis is classified into two pathways as follows:

1. The C_4 pathway (Shemin pathway) is found mostly in mammals, birds, yeast, some protozoa, and purple nonsulfur bacteria as *Rhodobacter sphaeroides*. The precursor for ALA synthesis is a succinyl-CoA (an intermediate in a citric acid cycle) and glycine. The pyridoxal-5'-phosphate (PLP) as a cofactor binds with the glycine, and then transform to PLP-glycine complex that lead to induce a transaldimination. Afterward, it reacts with succinyl-CoA using the ALA synthase (ALAS) via a decarboxylation, resulting to romoving of CoA, and then become ALA (Sasaki et al., 2002; Kang et al., 2012).

2. The C_5 pathway is found in higher plants, algae, and bacteria. The synthesis of ALA begins from glutamate as a substrate. There are three steps. First, glutamate is bound tRNA by glutamyl-tRNA synthetase (GluS) enzyme with ATP consisting of Zn^{2+} and Mg^{2+} as a cofactor. Next, glutamyl-tRNA is converted to glutamate-1-semialdehyde (GSA) by the NADPH-dependent glutamyl-tRNA reductase (GluTR) enzyme. Finally, GSA is changed to ALA by glutamate-1-semialdehyde aminotransferase (GSA-AM) enzymes (Kang et al., 2012).

The application of ALA in agricultural fields

Plant growth promoting

The use of ALA as a precursor in the synthesis of chlorophyll increased the amount of chlorophyll of *Spirulina platensis* (Sasaki et al., 1995) and ALA at concentrations of 0.06-1.8 mM increased the dry weight of beet (Hotta et al., 1997). Moreover, ALA increased growth in other plants such as red beans, barley, potatoes, and garlic up to 10-60% (Hotta et al., 1997). ALA also increased the synthesis of chlorophyll up to 24.3% at the ALA concentration of 100 ppm (Al-Khateeb, 2006).

Function as pesticides

ALA acts as an herbicide with a photodynamic action which caused the accumulation of tetrapyrrole compounds in plant cells during growing. In the presence of light, it stimulates the oxidation of unsaturated fatty acid at the surface of plant cells leading to cell damage. It affects dicotyledon weeds only; particularly on weed grass, family *Echinochloa crus-galli* L., found in paddy fields. When the concentration of ALA increased, it caused to the destruction of the weed growing and the effects occurred only under light conditions. ALA is induced by a light energy to apply as an insecticide. ALA concentration of 0.06 mM was toxic to grasshopper, *Oxya chinensis*, which is an insect pest for rice, sugarcane, and maize. It made a dead grasshopper up to 71.4% (Hirase et al., 2006).

Heavy Metals

Cadmium

Cadmium (Cd) is classified in the group 12 (IIB), the fifth plane of the periodic table with atomic number 48, atomic weight of 112.4, density of 8.6 g/cm^3, melting point of 320.9 °C, and boiling point of 765 °C. It is a solid at room temperature with silver metal color and is found in zinc, copper or lead ores. Especially zinc ore, the ratio of Cd and Zn was 1:100 - 1:1,000. Cd contaminates the environment via the smelting process. Cd is widely used in various industrial factories; electronics, automotive parts, and batteries, including stabilizers of polyvinyl chloride, plastic painting and glass. Inorganic compounds such as cadmium acetate, cadmium chloride and cadmium sulfate are water soluble whereas cadmium oxide and cadmium sulfide are not soluble. However, cadmium oxide and cadmium carbonate could be soluble in acid solutions (Nordberg et al., 2007).

Cd enters to the body through the respiratory and gastrointestinal tract and it is bound to red blood cells in the bloodstream and spreads to the liver. It could bind to albumin and metallothionein in the body. Cd is eliminated from the body through urine bur could accumulate in kidneys through urine suction, causing nephrotoxicity and Itai-itai. If human uptakes more than 300 mg Cd, it may cause death whereas 10

mg Cd are clearly poisoning. In the atmosphere, the amount of Cd dust or fumes of cadmium oxide must not exceed 0.05 mg/m^3. Humans could be exposed Cd from food, air, water, and tobacco. Food, such as rice, can accumulate high levels of Cd. Plants such as lettuces, broccoli, tomatoes, and beets could absorb Cd better than any other metals and also affected the germination of seeds (Di Salvatore et al., 2008).

Reports of Academic and Standard Groups, Office of Management and Environmental Remediation, Department of Primary Industries and Mines (2004) stated that in 2004 in Thailand, there were contaminations of Cd in water and paddy fields in Mae Sot district, Tak province. The soil in paddy fields is contaminated with Cd in a range of 3.4 to 284 mg/kg soil. This value was higher than the standard of the European Economic Community (EEC) which is 3 mg/kg soil. In addition, it was found in the rice grain about 0.1 to 44 mg/kg rice, which was higher than the standard of Thailand as 0.043 mg/kg grain. However, the maximum permissible by the CODEX Committee on Food Additives and Contaminants (CCFAC) is 0.2 mg/kg grain.

Zinc

Zinc (Zn) is classified in the group 12 (IIB), the fourth plane of the periodic table with atomic number 30, atomic weight of 65.38, density of 7.13 g/cm^3, melting point of 419.5 ℃ and boiling point of 908 ℃. It is in a solid form at room temperature with color of metallic white with slightly blue and it is not found the form of freedom.

Zinc has been utilized in many industrial fields such as battery, building materials, auto parts, and home appliances. Zinc oxide is used in rubber products, bleach solution, basic components of certain drugs for malnourished. Zn is also used as a component of pesticides such as carbamate, and lubricants as well (Sandstead and Au, 2007). Zinc oxide could be toxic to humans through inhalation. The smoke from the plant and mining could cause metal fume fever and breathing of zinc chloride could cause severe pneumonia as well. Zinc in contaminated food or water could cause nausea, vomiting, abdominal pain, and diarrhea, but these symptoms were not severe. Receiving excessive amounts of zinc could affect the intestinal absorption of

copper deficiency. The right consumption amount of zinc is about 10 to 22 mg/day, depending on the need for each span of age (Briefel et al., 2000).

Nanoparticle Biosynthesis

Although nanoparticles are considered a modern discovery, they have a history that dates back to 1857 when Michael Faraday provided the first scientific description of the optical properties of nanometer-scale metals. Nanoparticles have recently begun to gain much attention as they can be useful in many applications. Nanoparticles are defined as materials with at least two dimensions between 1 and 100 nm and thus they possess unique mechanical, catalytic, optical, and electric conductivity properties because of their nano sizes (Klaine et al., 2008). It is well recognized that physical, chemical, and biological methods are available to synthesize nanoparticles. However, physical and chemical methods are more popular, but they are complicated, costly and inefficient, and produce toxic wastes that are harmful to the environment and human health. In contrast, biological methods are attractive ways because of their nontoxic, simple and eco-friendly synthetic technologies. Due to the benefits of biological methods, there has been exponential growth over the past decade in the development of new manufactured or engineered nanomaterials and they can be classified according to their chemical compositions of bionanomaterials. The classification included the metallic nanoparticles, oxide nanoparticles, sulfide nanoparticles, and other typical nanoparticles. The alternative method for their classification was according to morphology which identified as nanoparticles, nanofibers, nanowires and nanosheets (Klaine et al., 2008; Li et al., 2011).

The metal-sulfide nanoparticles such as cadmium sulfide (CdS) and zinc sulfide (ZnS) nanoparticles have been also attracted great attention in both fundamental research and technical applications because of their interesting and novel electronic and optical properties. In the biological synthetic process for sulfide nanoparticles, the soluble sulfate acts as the source of sulfur. The formation mechanism of sulfide nanoparticles by biological transformation reaction could be explained as follows (Edwards and Lefebvre, 2013). First, soluble sulfate enters bacterial cell via diffusion, and later is carried to the interior membrane of bacterial

cell facilitated by sulfate permease. After that, the sulfate is reduced to sulfite by ATP sulfurylase and phosphoadenosine phosphosulfate reductase, and the sulfite was reduced to sulfide by sulfite reductase. The sulfide reacts with *O*-acetylserine to synthesize cysteine via *O*-acetylserine thiolyase (Holmes et al., 1997; Auger et al., 2005), and then the cysteine is converted to sulfide (S^{2-}) by a cysteine desulfhydrase in the presence of metals such as Cd and Zn. After this process, the sulfide reacts with the soluble metal ions and the metal-sulfide nanoparticles are synthesized through disulfide (cystine) bridges. This mechanism may be attributed to cleavage of sulfide-hydrogen (S-H) bond and formation of a new bond, which was a -sulfide-metal (-S-metal) bond of metal-thiolate complex (metal-S-CH_2COOH) on the nanoparticle surface. The carboxylic groups (-COOH) from the metal-thiolate complexes do not react with the amino groups (-NH_2) of protein but interact with hydrogen bond. Therefore, nanoparticles are bonded to amino groups (-NH_2) by hydrogen bond and the particle size is controlled by the culture time of the bacterial cell (Bai et al., 2006; Li et al., 2011).

In recent years, the nanotechnology research is extensively studied. The properties of many conventional materials change when they become the nanoparticles. This is because nanoparticles have a greater surface area per weight than larger particles, which causes them to be more reactive. Therefore, nanotechnology could apply in several fields including medical, optical, electronic, environmental, and agricultural (Li et al., 2011). The application of nanoparticles in agriculture includes the delivery systems for nutrients and plant hormones. Nanoencapsulated fertilizers that slowly release fertilizers have been become a style for application (Chinnamuthu and Boopathi, 2009). In area of environmental application, the nanostructure materials have been developed to control the pollution and improve the remediation technologies to clean up the polluted areas. For example, it is applied to clean up hazardous waste in difficult location to reach such as ground water (Bhawana and Fulekar, 2012).

Methane (CH_4) Production in Paddy Fields

Paddy fields are one of the major sources of the global CH_4 emission (Prinn, 1994; Cicerone and Oremland, 1988). Therefore, the processes involved in CH_4 formation in paddy fields are of great interest (Conrad, 1993; Neue and Sass, 1994). CH_4 is the product of anaerobic degradation of organic matter in the flooded soil. Several studies have shown that the produced CH_4 is derived from the degradation of rice straw, root exudates and soil organic matter. Incorporation of rice straw into the soil as a fertilizer is a common practice in rice cultivation. Therefore, the rice straw is one of the main carbon sources in paddy soil. It has frequently been demonstrated that incorporation of rice straw strongly enhances the emission of CH_4 from paddy fields. Moreover, the addition of organic material to soil is considered as one of the most important factors influencing the source strength of paddy fields for atmospheric CH_4(Holzapfel-Pschorn et al., 1986). The microbes are involved the pathway of CH_4 production which can be summarized the role of microbes as follows (Conrad, 2002):

1. Hydrolytic bacteria

After rice cultivation, there are wastes such as rice straw. Several studies found rice straw as a source of generating CH_4 from paddy soil up to 40% (Wang et al., 1999). Rice straw composes of cellulose (32-37%), hemicelluloses (29-27%), lignin (5-15%) and soluble polysaccharide (8%), and inorganic substances such as silica (Glissmann and Conrad, 2002). The substances as polysaccharides are digested by soil microbes to generate CH_4 and CO_2. The early mechanisms are the polysaccharide catabolism to produce small sugar molecules and bacteria that play a role in this process are Clostridia (Weber et al., 2001). They degrade the polysaccharides by using secreted enzymes, such as β-glucosidase, exo-β-1,4-glucanase and xylosidase. The obtained small sugar molecules will be passed onto the next steps to produce CH_4 (Glissmann and Conrad, 2002).

2. Fermentative acidogenic bacteria

The acidogenic bacteria such as *Clostridium* sp., *Acidobacteria* can convert small sugars into short chain fatty acids such as acetate, propionate and butyrate,

including hydrogen and carbon dioxide as well. The homoacetatogens play a role to produce acetate, which is one of important nutrients for methanogens.

3. Syntrophic bacteria

Propionate and butyrate are fatty acids from the fermentation process, which cannot directly be used as a substrate for methanogens (Müller et al., 2010; Gan et al., 2012). The acetate and H_2-producing bacteria such as *Syntrobacter wolinii* and *Syntrophomonas wolfei* can transform propionate into acetate, carbon dioxide and hydrogen (Krylova and Conrad, 1998). The accumulation of acetate and hydrogen is the major source for producing CH_4 by methanogens (Conrad, 2002).

4. Methanogens

Methanogens or methanogenic bacteria are a large group of bacteria that are very diverse. They are classified into the domain archaea which are closely related to the eubacteria, especially the photosynthetic eubacteria. There are 3 groups of methanogens based on substrates used for producing CH_4 (Whitman et al., 2006).

4.1 Use of H_2, formate, or alcohols as an electron donor and CO_2as an electron acceptor which is reduced to CH_4. The ability to use H_2as an electron donor for reducing CO_2commonly found in almost every type of them as well as the ability to use formate, but there are very few which can use alcohols as an electron donor.

4.2 Use of one carbon atom with a methyl group such as methylamine, dimethylamine, trimethylamine, and dimethylsulfide as an electron donor. Methanogens rarely found in this group.

4.3 Use of acetate as a precursor for production of the CH_4. But the ability to use acetate is limited in species of methanogens. Only *Methanosarcina* and *Methanosaeta* (*Methanothrix*) can use acetate. Acetate is a substance that found commonly in the environment. Methanogens as acetotrophs is found up to 67% for methanogenesis (Conrad, 2002).

Methanogens are very sensitive to oxygen. They can be found everywhere in the environment without oxygen. Most are unable to oxidize sulfur compounds because these compounds could react chemically with oxygen. They are different

from other kinds of archaea because they are normally found in the normal conditions such as mesophile, neutral pH and low salinity. Generally, they can be found in anaerobic digestive system, anaerobic sediments, flooded soil and gastrointestinal tract. In anaerobic conditions with NO_3^-, Fe^{3+} andSO_4^{2-}would inhibit methanogens by competing in the use of nutrients by other microorganisms such as sulfate are found in environments with sulfate-reducing bacteria and then is competed H_2 with them (Whitman et al., 2006).

Purple Non-Sulfur Bacteria (PNSB)

Purple non-sulfur bacteria (PNSB) are anoxygenic phototrophic bacteria by photolithotroph and/or photoorganotroph. However, they can grow under aerobic-dark conditions using fermentation. They are Gram-negative bacteria with various shapes; spiral, rod and ovoid. Most of them are subject to binary cell division and some are divided by budding. They may use polar or peritrichous flagella and some members use gliding movements. PNSB can perform in the environment where the levels of sulfide are either low or undetected. PNSB are usually present in low numbers of bacterial blooms, probably because of their sulfide sensitivity. PNSB are classified by Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005) as follows:

Class Alphaproteobacteria
- Order Rhodospirillales
  - Family Rhodospirillaceae
    - Genus *Rhodospirillum*, *Phaeospirillum*, *Rhodocista*, *Rhodospira*, *Rhodovibrio*, *Roseospira*, *Roseospirillum*
  - Family Acetobacteraceae
    - Genus *Rhodopila*
- Order Rhodobacterales
  - Family Rhodobacteraceae
    - Genus *Rhodobacter*, *Rhodobaca*, *Rhodovulum*, *Rhodothalassium*
- Order Rhizobiales

Family Bradyrhizobiaceae

Genus *Rhodoblastus*, *Rhodopseudomonas*

Family Hyphomicrobiaceae

Genus *Blastochloris*, *Rhodomicrobium*, *Rhodoplanes*

Family Rhodobiaceae

Genus *Rhodobium*

Class Betaproteobacteria

Order Burkholderiales

Family Comamonadaceae

Genus *Rhodoferax*, *Rubrivivax*

Order Rhodocyclales

Family Rhodocyclaceae

Genus *Rhodocyclus*

The applications of PNSB

Free-living nitrogen fixing bacteria

Photosynthethic bacteria are one of the bacterial groups which can fix atmospheric nitrogen, especially PNSB (Hunter et al., 2009). Madigan et al. (1984) found that 18 species of PNSB are able to grow only with nitrogen gas as a nitrogen source, except *Rhodocyclus purpureus*. *Rhodopseudomonas capsulatus* grows quickly in the previous condition and its nitrogenase enzyme is higher rates than other species of *Rhodopseudomonas* (Madigan et al., 1984). In addition, *Rhodospirillum rubrum* has high nitrogenase enzyme expression while *R. fulvum* requires oxygen molecules to fix nitrogen gas under dark condition (Madigan et al., 1984). These bacterial groups commonly found in paddy fields and their cell densities in paddy fields increased rice yield. For example, *R. palustris* KN122 was isolated from a paddy soil and its cell suspension was inoculated into the floodwater once or three times during the cultivation to increase the grain yield (21% for single inoculation and 29% for triple inoculations) (Harada et al., 2005). *Rhodobacter capsulatus* was used with 50% of the recommended chemical nitrogen fertilizer dose; the rice yield was statistically

equivalent to that obtained with 100% of the recommended chemical nitrogen fertilizer dose (Gamal-Eldin and Elbanna, 2011). These studies provided a clear evidence for the potential of *R. capsulatus* to be used as a biofertilizer for paddy fields. In addition, the use of PNSB in rice cultivation also provides a benefit to rice rhizosphere by removing hydrogen sulfide as this gas is normally found in anaerobic conditions of paddy fields. Hydrogen sulfide is a toxic gas to rice roots by inhibiting metabolic processes of rice roots. Hence, the rice roots could grow even more with the use of PNSB leading to stronger rice stems and increases in rice yield (Kobayashi and Kobayashi, 1995).

Phytohormones production

The following PNSB species; *Rubrivivax gelatinosus* ATCC17011, *Rubrivivax benzoatilyticus* JA2, *Rhodopseudomonas fecalis* JCM11668, *R. palustris* DSM123, *Rhodobacter sphaeroides* DSM158 and *Rhodobacter megalophilus* JA194 produced IAA 13.0, 21.6, 9.9, 9.6, 9.1 and 4.5 mg/mL, respectively by using L-tryptophan as a substrate (Mujahid et al. 2011). Many kinds of auxins are produced by *Rb. sphaeroides* OU5 such as sphestrin (Sunayanaet al., 2005a) and rhodestrin (Sunayanaet al., 2005b). The amount of these compounds only 50 nM produced a better result in root germination of tissue-cultured plants than 50 µM of other auxins. The root initiation was observed within 6-8 days with rhodestrin-treated plants, while with other auxins it was observed only after 12-15 days. *Rhodospirillum rubrum* 1R could produce 4-hydroxyphenethyl alcohol (4-HPEA) which is classified in the cytokinin group (Serdyuk et al. 1995). This plant hormone played a role in growth activation of plants and induction of betacyanin synthesis for *Amaranthus caudatus* L. seedlings, reduction of chlorophyll synthesis for radish cotyledons, stimulation of cell division for tobacco, and increasing of fresh weight for radish (Serdyuket al., 2000).

ALA production

ALA has been applied in many fields by acting as herbicide, insecticide, plant growth promoting substance in agriculture, and photodynamic therapy and disease

diagnosis in medicine (Sasaki et al., 2002; Tangprasittipap and Prasertsan, 2002).Sattayasmithstid (2002) studied ALA synthesis of halotolerant *Rhodobacter capsulatus* SS3 that produced 26 µM of ALA under microaerobic-light conditions. The optimal concentration of levulinic acid (LA as ALA dehydratase inhibitor) for ALA production was 15 mM and supplementation of LA two times (15 mM each) increased ALA up to 74 µM although the growth was retarded.

Liu et al. (2005) reported mutant strain *Rhodopseudomonas* sp. strain L-1 produced 22.15 mg/L of ALA under optimal conditions (pH 7.5, 3000 lux and supplemented with 30 mg/L of LA, glycine and succinate). An enhancement of ALA production of *R. palustris* KG31 under aerobic-dark conditions was markedly increased 8-fold yield by supplemented with 10 mM succinate, 4.5 mM glycine, and 15 mM LA (Choorit et al., 2011). Kantha et al. (2010) isolated PNSB from organic saline paddy fields from 14 provinces of the northeast region of Thailand. All of the isolates were identified as *R. palustris*. After incubation in a soil and straw products (SSP) for 4 weeks under microaerobic-dark conditions, the ALA concentration increased with time. The results indicated that SSP containing selected PNSB could produce ALA and could be practically applied to the organic saline paddy fields for increasing growth and yield.

Heavy metal removal

PNSB have been proven to be highly tolerant to heavy metals exposure, especially towards Co^{2+}, Fe^{2+} and MoO_4^{2-}. However, Ni^{2+} and Co^{2+} decreased the cellular content of light harvesting complexes. A characteristic behavior was observed with mercuric ions which produced a significant increase of the lag-phase duration of cell growth (Giotta et al., 2006). Death cells by steam sterilization of *Rb. sphaeroides* were tested for their ability to reduce heavy metals; it was found that biosorption of bivalent metal ions was due to monodentate binding to two different types of acidic sites on surface cells; carboxilic and phosphatic-type sites (Seki et al., 1998). Cd biosorption depended on biomass of *Rb. sphaeroides*; however, biosorption played only a minor role in Cd reduction when compared with sulfide precipitation (Bai et al., 2008). They found that activities of cysteine desulfhydrase in the presence of 10

and 20 mg/L of Cd were higher than the control, while the activities in the presence of 30 and 40 mg/L of Cd were lower than the control. The cysteine desulfhydrase in this organism could produce the sulfide ions under aerobic conditions and precipitate the metal sulfide complexes on the cell wall.

Rhodobium marinum NW16 and *Rb. sphaeroides* KMS24 were used to investigate their potential to reduce heavy metals from contaminated shrimp pond under microaerobic-light and aerobic-dark conditions (Panwichian et al., 2011). The growth of both strains under both incubating conditions was in the order of Pb > Cu > Zn > Cd. Under both incubating conditions, the exopolymeric substances (EPS) produced by both strains showed greater reduction of all heavy metal species (average percentages; 90.52-97.29) than their cells (average percentages; 14.02-75.03). The mechanisms of heavy metals reduction of both strains were dependent on the age of cell, biomass concentration, type of heavy metals, pH of the solution, and other interfering ions (Panwichian et al., 2010a).Fan et al. (2012) studied the ability of *Rb. sphaeroides* for remediation of simulated Cd-contaminated soil; the phytoavailability of Cd was investigated through wheat seedling method to determine the efficiency of remediation. It was found that after remediation, the accumulation of Cd in wheat roots and leaves decreased by 67 and 53%, respectively.

Nanoparticle biosynthesis

Several researchers reported that PNSB have ability to precipitate heavy metals by forming metal-sulfide nanoparticles (Bai and Zhang, 2009; Bai et al., 2009; Sakpirom et al., 2019). Consequently, a novel and clean biological transformation reaction by immobilized *Rb. sphaeroides* has been developed for the synthesis of zinc sulfide (ZnS) nanoparticles with an average diameter of 8 nm and the average diameter of ZnS nanoparticles varied according to the culture time (Baiet al., 2006).

R. palustris produced a uniform distribution of CdS nanoparticles, having an average size of approximately 8 nm (Bai et al., 2009). It was observed that cysteine desulfhydrase producing sulfide (S^{2-}) in this organism located in cytoplasm, and the content of cysteine desulfhydrase depending on the growth phase of cells that was responsible for the formation of CdS nanoparticles. Besides, secreted protein by *R.*

palustris stabilized CdS nanoparticles and then transported CdS nanoparticles out of the cell.

CH_4 emission reduction

According to information on CH_4 production and emission from paddy soil in laboratory experiments; there was a positive linear correlation between CH_4 production and incubation temperature from 15 to 37℃, but a negative linear correlation between 37 and 50℃. The application of rice straw, green manure, corncob, xylan, avicel or compost led to stimulate the CH_4 production; but the opposite result was observed for glucose, sucrose and urea supplementation (Yang and Chang, 1998). Anaerobic conditions and water flooding on the surface of soil supported CH_4 emission (Ma and Lu, 2010).

Harada et al. (2001) investigated the interaction between phototrophic dinitrogen fixers and methanogens in soil slurries supplemented with rice straw under light condition. The growth of phototrophic purple bacteria was higher than methanogens in photic zones and thereby CH_4 gas emission was reduced. This agrees with Harada et al. (2003) isolated PNSB from paddy soils, 17 strains were identified as *R. palustris* and one was *Rubrivivax gelatinosus*. The inoculated slurries of all strains showed a positive nitrogenase activity after 10 days incubation under light conditions and CH_4 gas emissions from the soil slurries inoculated with *R. palustris* strains were significantly lower (approximately 44-62%) than the slurries without inoculants. Hence, the growth of PNSB contributed to enhancement of soil nitrogen fixation and mitigation of CH_4 gas emissions from paddy soils.

Biofertilizer

Definition of biofertilizer

The definition of biofertilizer is described in Fertilizer Act (No.2), given on 2007 as follows:

Fertilizer is the organic, synthetic organic, inorganic compounds or microorganisms, whether naturally occurring or synthetic, which is used as a plant nutrient or changed in chemical, physical or biological properties of the soil to nourish the plants for growth.

Biofertilizer is a fertilizer derived from microorganisms that can provide beneficial nutrients to plants. It is used to improve soil in biological, physical or biochemical properties.

Microbial inoculum is microorganism with many cells per unit.

Supporting material or carrier is material used to mix with the microbial inoculum for biofertilizer manufacturing process.

Formulation of biofertilizer

Biofertilizers are being essential component of organic farming as they contain live or latent cells of efficient strains of either nitrogen fixing or phosphate solubilizing microbes and so on with the objectives to increase number of beneficial microorganisms and accelerate microbial processes for providing availability of nutrients that can be easily assimilated by plants (Mahdi et al., 2010). Biofertilizers play a very significant role in improving soil fertility by fixing atmospheric nitrogen, both, in association with plant roots and without or converting insoluble soil phosphates and producing plant growth substances in the soil (Mishra et al., 2013).

The formulation of any effective microorganisms is a multistep process which resulted in one or more strains contained in a particular carrier together with sticking agents or other additives which help in the protection of the cells during storage and transport (Xavier et al., 2004). The perfect formulation does not exist as each type has its own advantages and limitations (Herrmann and Lesueur, 2013) as presented in

Table 2-3. However, there are some critical steps that must be carefully considered during the production of the biofertilizers due to the choices made at these steps can lead to the success or the failure of the inoculation.

Table 2-3. Advantages and limitations of the most common carriers (to be continued).

Carrier	Advantage	Limit
Dry solid (powder)	- Suitable for a wide range of microorganisms - Protective nutritive environment - Moisture content can be adjusted to optimize growth and survival of bacteria during curing, storage, and inoculation - Strong buffering capacity	- Not readily available - Toxic compounds released during drying and sterilization - Highly variable in composition and quality depending on the origin - Seed application: contact with other chemical compounds (toxicity)s
Liquid	- Easy to handle and apply - Easy addition of additives to improve growth or survival of the cells - Composition easily defined and controlled - High cells concentration - Low application rates	- Lack carrier protection: low viability during storage and on seeds - Cool temperatures for storage (4 °C) - Limited shelf life - More sensitive to stressful conditions
Granules	- Easy to store, handle, and apply - Less dusty than dry solid - Application rate easily assessed - Soil application: no direct contact with the other chemical compounds (no toxicity) - Especially efficient under stressful environmental conditions	- Bulky: high transport and storage costs - Higher application rates - Often non-sterile carriers

Table 2-3 Advantages and limitations of the most common carriers.

Carrier	Advantages	Limits
Encapsulated cells	- Suitable for all types of cells (all sizes) - Cells protected in a nutritive shell against mechanical and environmental stresses and against predators - Slow and controlled release of the microorganisms when the shell is degraded - Wide variety of polymers: nontoxic, biodegradable - High concentration of cells/shell - Limited space for storage - Storage at room temperature (dried capsules)	- High production cost - More handling works at the industry level - Specific equipment required - Physiological,morphological, and metabolic changes occurring in the shell - Several applications needed if strains cannot establish in soil - No commercial product available

Rubber wood ash (RWA)

Rubber wood ash is a by-product from rubber firewood boilers (Saritpongteeraka and Chaiprapat, 2008) and it is recognized as a waste (Masae et al., 2013). Generally, wood ash is generally grey in color, abrasive, mostly alkaline and refractory in nature; and it contains different essential elements for plant growth, including both macronutrients P, K, Ca, Mg and micronutrients Zn, Fe, Cu, Mn, B, and Mo (Demeyer et al., 2001). It has been applied in various applications such as soil improvement, plant nutrient supply and adjustment system (Demeyer et al., 2001; Park et al., 2005), and removal of heavy metals (Hasan et al., 2000; Masae et al., 2013). The utilization of ash as a carrier in biofertilizer formulations emerged to be safe and effective alternatives with some evidences that the beneficial bacteria could be survived in this carrier (Gaind and Gaur, 2004; Kumar and Gupta, 2010). Therefore, use of rubber wood ash as the carrier in biofertilizer formulations is an effective way of utilization of problematic this waste in a useful manner (Kumar et al., 2010).

Decanter cake (DCC)

Decanter cake is a solid waste that produced from the third phase separation step of crude palm oil process. The production rate of decanter cake amounts roughly 3-5% (w/w) of fresh fruit bunch processed (Chavalparit et al., 2006). Fresh decanter cake is rather acidic and contains approximately 70% moisture content, while the dry matter contains oils, fiber and inorganic components (Chavalparit et al., 2006). Decanter cake is commonly utilized as fertilizer (Embrandiri et al., 2013) and animal nutrition sources (Gafar et al., 2013) due to the presence of C, N, P, K and Mg (Razak et al., 2012). Embrandiri et al. (2013) reported that the decanter cake supplemented in the planted soil up to 10%, was a probable substitute for inorganic fertilizers with respect to lady's finger (*Abelmoschus esculentus*) plants due to high nutrient content and thus positively affected to yield and biomass as well as morphological characteristics. However, there were observable negative effects over 10% decanter cake amendment ratios. The use of decanter cake will eradicate the use of chemical fertilizers and prevent heavy metal leaching problems through adsorption (Dewayanto, 2010).

Rice husk ash (RHA)

Rice husk ash, by-product of rice milling, presents a potential of properties to be good in an improvement of pollution control and environmental preservation (Foo and Hameed, 2009). The rice husk ash is prepared by burning rice husks at high temperatures (Sekifuji and Tateda, 2019), and it consists of various elements such as silica with high content and other elements (potassium, magnesium and calcium) which are beneficial for plant growth promotion (Habeeb and Mahmud, 2010; Saranya et al., 2018). Kantha et al. (2015) reported that RHA could be a suitable carrier for PNSB biofertilizers as to protect PNSB cells and no phytotoxicity. It acts to promote plant growth by providing of nutrients and also reducing toxicity of heavy metals (Babaso and Sharanagouda, 2017).

Spent coffee grounds (SCG)

Spent coffee grounds (SCG), by-product of coffee brewing, have been applied to support plant growth as SCG by product enriches sources of potassium, magnesium and phosphorus including sugars (Cruz et al., 2012; Mussatto et al., 2011). Previous studies reported that SCG could support growth of plants such as lettuce and mung bean (Batish et al., 2008; Cruz et al., 2012). Moreover, Tokimoto et al. (2005) demonstrated that the SCG could perform as heavy metal remediation agent.

CHAPTER 3

Characterizations of Purple Non-Sulfur Bacteria Isolated from Paddy Fields and Identification of Strains with Potential for Plant Growth Promotion, Greenhouse Gas Mitigation and Heavy Metal Bioremediation

Abstract

This study aimed to select purple non-sulfur bacteria (PNSB) isolated from various paddy fields including Cd and Zn contaminated paddy fields based on their biofertilizer properties. Among 235 PNSB isolates, strain TN110 was the most effective for plant growth promoting substances (PGPS) production, releasing 3.2 mg/L of NH_4^+, 4.11 mg/L of 5-aminolevulinic acid (ALA) and 3.62 mg/L of indole-3-acetic acid (IAA), and reducing methane emission up to 80%. This strain had *nifH*, *vnfG* and *anfG*, which are the Mo, V and Fe nitrogenase genes encoded for the key enzymes for nitrogen fixation under different conditions. This strain provided 84% and 55% removal of Cd and Zn, respectively. Another isolate, TN414, not only produced PGPS (1.30 mg/L of NH_4^+, 0.94 mg/L of ALA and 0.65 mg/L of IAA) but was also efficient in removing both Cd and Zn at 72% and 74%, respectively. Based on 16S rDNA sequencing, strain TN110 was identified as *Rhodopseudomonas palustris*; while strain TN414 was *Rubrivivax gelatinosus*. A combination of TN110 and TN414 would provide potentially a biofertilizer, which is a greener alternative to commercial/chemical fertilizers, and an agent for bioremediation of heavy metals and greenhouse gas mitigation in paddy fields.

Keywords: 5-Aminolevulinic acid, cadmium, indole-3-acetic acid, nitrogenase genes, purple nonsulfur bacteria, zinc

บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อคัดเลือกแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ (Purple Non-Sulfur Bacteria; PNSB) ที่แยกจากนาข้าวในพื้นที่ต่างๆ รวมถึงนาข้าวที่มีการปนเปื้อนด้วยแคดเมียมและสังกะสีโดยพิจารณาจากคุณสมบัติความเป็นปุ๋ยชีวภาพของ PNSB จาก PNSB ทั้งหมด 235 สายพันธุ์ พบว่าสายพันธุ์ TN110 มีประสิทธิภาพในการผลิตสารที่ส่งเสริมการเจริญของพืชได้ดีที่สุด โดยปลดปล่อยแอมโมเนียมไอออน (NH_4^+) กรด 5-อะมิโนลีวูลินิก (5-Aminolevulinic Acid; ALA) และกรดอินโดล-3-แอซีติก (Indole-3-Acetic Acid; IAA) ปริมาณ 3.2 4.11และ 3.62 มิลลิกรัมต่อลิตร ตามลำดับ และลดการปลดปล่อยก๊าซมีเทนได้ถึง 80 เปอร์เซ็นต์ โดยที่ PNSB สายพันธุ์นี้มียีน *nifH vnfG* และ *anfG* ซึ่งเป็นยีนสำหรับไนโตรจีเนสที่มีโมลิบดีนัม-เหล็ก (Molybdenum-Iron; Mo-Fe) วาเนเดียม-เหล็ก (Vanadium-Fe; V-Fe) และเหล็ก-เหล็ก (Fe-Fe) ตามลำดับ ซึ่งเอนไซม์เหล่านี้เป็นเอนไซม์ที่สำคัญสำหรับกระบวนการตรึงก๊าซไนโตรเจนทางชีวภาพภายใต้สภาวะที่แตกต่างกัน นอกจากนี้สายพันธุ์ดังกล่าวยังสามารถกำจัดแคดเมียมและสังกะสีได้ 84 และ 55เปอร์เซ็นต์ ตามลำดับ ส่วนอีกหนึ่งสายพันธุ์คือ TN414 เป็นสายพันธุ์ที่สามารถผลิตสารที่ส่งเสริมการเจริญของพืชได้เช่นกัน โดยปลดปล่อย NH_4^+ ALA และ IAA ได้ 1.30 0.94 และ 0.65 มิลลิกรัมต่อลิตร ตามลำดับ ซึ่งมีประสิทธิภาพในการกำจัดทั้งแคดเมียมและสังกะสีได้ดีที่ 72 และ 74 เปอร์เซ็นต์ ตามลำดับ ผลการเทียบเคียงจากการเรียงลำดับเบสของ 16S rDNA ของเชื้อทั้ง 2 สายพันธุ์ พบว่าสายพันธุ์ TN110 คือ *Rhodopseudomonas palustris* ขณะที่สายพันธุ์ TN414 คือ *Rubrivivax gelatinosus* ดังนั้นการใช้ PNSB ทั้ง 2 สายพันธุ์ร่วมกันซึ่งต่างก็มีศักยภาพในการเป็นปุ๋ยชีวภาพเป็นวิธีทางเลือกที่ดีกว่าการใช้ปุ๋ยเคมี หรือปุ๋ยทางการค้า อีกทั้งยังสามารถทำหน้าที่ในการบำบัดโลหะหนักทางชีวภาพและลดการปลดปล่อยก๊าซเรือนกระจกในนาข้าวที่ก่อให้เกิดภาวะโลกร้อนได้อีกด้วย

คำสำคัญ : กรด5-อะมิโนลีวูลินิก กรดอินโดล-3-แอซีติก แคดเมียม ยีนไนโตรจีเนส แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ สังกะสี

Introduction

The worldwide increase in human population has contributed to shortage of food including rice (*Oryza sativa* L.), which is consumed by half of the world population (Bouman et al., 2007). As a result, chemical fertilizers, particularly nitrogen base, have been heavily used to increase rice yield. In addition to higher cost of production associated with the fertilizers, after their long-term use, loss of soil quality and ground water contamination with nitrate have been reported (Wang and Yang, 2003). In addition, contamination of heavy metals such as Cd and Zn in agricultural land is a serious concern for safe food production. The contamination usually occurs through runoff from mining, industrial drainage discharge and phosphate fertilizer applications (Li et al., 2014; Xu et al., 2014). Zn ore contains Cd naturally at a ratio of 1:25 - 1:500 (Chaney, 2010) while rock phosphate, a source for phosphate fertilizer, is often contaminated with Cd. Cd in a range of 2-100 mg/kg in ten types of rock phosphate was reported (Syers, et al., 1986). This leads to a common problem that agricultural soils are contaminated with Cd through phosphate fertilizers (Schipper et al., 2011). Hence, both heavy metals have been detected in paddy fields in various countries (Akkajit and Tongcumpou, 2010; Sriprachote et al., 2012; Payus and Talip, 2014; Yu et al., 2016). Cd and Zn in rice grains harvested from contaminated paddy fields have been reported (Payus and Talip, 2014). The Cd safety levels in soil and rice are 1.4 mg/kg soil and 0.2 mg/kg grain according to the agricultural soil pollution control standards of the Canadian Council of Ministers of the Environment and the food quality standards of the Codex Committee on Food Additives and Contaminants draft Maximum Permissible Level, respectively (CCME, 1999a and b; Chunhabundit, 2016).

Recently, sustainable agriculture has been promoted and plant growth promoting bacteria or biofertilizers are one of the substitutes for chemical fertilizers and have potential to reduce heavy metals. Nitrogen is one of the major essential elements for plant growth. Biological nitrogen fixation by N_2 fixing microorganisms is an effective mechanism to convert N_2 into ammonium ions (NH_4^+) which can be used by plants (Olivares et al., 2013). A key enzyme for N_2 fixation is called nitrogenase which is found in all N_2 fixing bacteria. There are three nitrogenase

isozymes based on the cofactor: molybdenum (Mo)-iron (Fe), vanadium (V)-Fe and Fe-Fe nitrogenases. The Mo-Fe nitrogenase is a canonical form of the enzymes and is found in all N_2 fixing bacteria. Only some of the N_2 fixing bacteria have the V-Fe and/or the Fe-Fe nitrogenases, which are referred to as alternative nitrogenases (Oda et al., 2005). The alternative nitrogenases are adapted from Mo-Fe nitrogenase under dizotrophic conditions with limited Mo (Bellenger et al., 2014). Purple non-sulfur bacteria (PNSB) are among nitrogen fixing bacteria and are candidates for applications in paddy fields due to their extraordinary metabolic versatility: photoautotrophic, photoorganotrophic, chemoautotrophic and chemoorganotrophic (Larimer et al., 2004; Harada et al., 2005).

PNSB, not only fix N_2 but also produce plant growth promoting substances (PGPS) such as indole-3-acetic acid (IAA) and 5-aminolevulinic acid (ALA). IAA, which is a phytohormone playing a role in growth activation of plants by inducing plant mineral uptake and root cell elongation (Spaepen et al., 2007). ALA is used as a precursor in the synthesis of chlorophyll and induction of antioxidative enzymes under stress conditions such as catalase, glutathione reductase and superoxide dismutase (Nunkaew et al., 2014; Zhang et al., 2015). Studies have found that PNSB strains mitigate methane emission by suppressing the growth of methanogens in paddy soil slurries (Harada et al., 2001). Methane emissions from the soil slurries inoculated with *Rhodopseudomonas palustris* strains were significantly lower than those from the slurries without the inoculant up to 88% (Nunkaew et al., 2015). Other benefits of PNSB are reduction of plant stress from heavy metals by various mechanisms such as adsorption on exopolymeric substances (EPS) on the cell surface, accumulation inside the cells, transformation into less toxic derivatives by redox transformations, and precipitation into carbonate or sulfide compounds (Panwichian et al., 2011; Fan et al., 2012).

Limited work has been conducted on potential use of PNSB as a plant growth promoter in heavy metal contaminated environments including paddy fields. Hence, this study aimed to isolate and select PNSB with capabilities as a plant growth promoter or biofertilizer for NH_4^+, ALA and IAA releases, a bioremediation agent for heavy metals (Cd and Zn) and a greenhouse gas (CH_4) emission reducer.

Materials and methods

Sample collection and heavy metal analysis

Soil or sediment and water samples were collected from Cd and Zn contaminated paddy fields in Thailand. The contaminated paddy fields were close to a zinc mine in Maesot District, Tak Province and a gold mine in Wangsaphung District, Loei Province (referred to as sites C1 and C2, respectively). The soil or sediment samples were collected at a depth of 0-5 cm from the surface of soil while the water samples were collected at 0-50 cm from the surface of water column. Each soil or sediment sample was air dried and passed through a 2 mm sieve. Then, the samples were extracted by acid digestion (HNO_3: HCl = 1: 3) at 70 °C with manual shaking every 15 min for 1 h. After digestion, the extracts were diluted using 6 mL of deionized water and filtered through Whatman filter paper no.1. For water samples, 10 mL of each sample was filtered through Whatman filter paper no.1 before heavy metal analysis (Panwichian et al., 2010b). Cd and Zn in the samples were analyzed by an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Perkin Elmer, Germany).

PNSB isolation and inoculum preparation

A glutamate-acetate (GA) medium consisting of 3.8 g sodium glutamate, 5.44 g sodium acetate monohydrate, 2.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.8 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4$ $7H_2O$, 53 mg $CaCl_2$ $2H_2O$, 1.2 mg $CoCl_2$ $6H_2O$, 1.2 mg $MnSO_4$ $5H_2O$, 0.01 mg biotin, 2.5 mg ferric citrate and 1 mg nicotinic acid in 1,000 mL distilled water (adjusted to pH 7.0) was used to isolate PNSB from soil/sediment and water samples. One gram of each soil sample was transferred to 8 mL of GA medium whereas 4.5 mL from each water sample were transferred to 4.5 mL of double strength GA medium in a screw cap test tube (10 mL volume). Based on the nature of PNSB under the anaerobic photoautotrophic/photoheterotrophic growth, they are stimulated to outcompete other organisms (Kantachote et al., 2005); therefore, the anaerobic-light conditions were used for PNSB isolation. Sterile liquid

paraffin was added on top of the growth medium to achieve anaerobic-light conditions. Light at 3,000 lux was continuously provided using tungsten bulbs for 5-7 days at room temperature. The incubation turned the culture broths from light yellow to pink, red, or brown. The broths were streaked onto GA agar which was then incubated under the same conditions for the liquid cultures to purify the PNSB. The streaking and incubation were repeated until a pure culture was obtained. The pure culture of each isolate was maintained as a stock culture by stabbing it in GA agar and storing at 4 °C or re-suspending the colonies from the agar in 20% glycerol and storing at -80 °C until used.

Each stock culture was sub-cultured twice to obtain an active inoculum which was then inoculated into GA broth under microaerobic-light conditions at a light intensity of 3,000 lux for 48 h at room temperature. Cell pellets were harvested by centrifuging the broth at 8,000 rpm for 15 min. The cell pellets were resuspended in GA medium and diluted by the same medium to an optical density of 0.5 at a wavelength of 660 nm (OD_{660}) to obtain the final inoculums, roughly 1×10^8 cells/mL, used in experiments. The inoculum was prepared under microaerobic-light conditions to acclimate them for further test conditions.

Investigation of the isolated PNSB for biofertilizer properties

Normally, N_2 fixing PNSB fixes atmospheric N_2 to NH_4^+ effectively under obligate anaerobic conditions because N_2 fixation is disrupted by oxygen (Masepohl and Kranz, 2009). Paddy fields are not exactly under obligate anaerobic conditions even when they are flooded, which is a typical practice for rice cultivation. During the day, oxygen is produced by photosynthesis and some of it gets transported from leaves to roots for respiration and may result in oxygen release to the sediments (Larsen et al., 2015). Although some of the released oxygen is used for CH_4 oxidation, some remains in soils and prevents obligate anaerobic conditions (Gutierrez et al., 2014). PNSB such as *Rhodopseudomanas* and *Rhodobacter* are quite tolerant to O_2 and grow well under photosynthesis with limited O_2 (Larimer et al., 2004; Hoffmann et al., 2014). Hence, this study examined N_2 fixing and NH_4^+ release potential by PNSB under microaerobic-light conditions which are more aligned with actual

conditions in the field. Previous studies also investigated their potential production of ALA as PGPS and removal of heavy metals under microaerobic-light conditions (Panwichian et al., 2011; Nunkaew et al., 2015; Kantha et al., 2015).

Selection of the isolated PNSB with N_2 fixing ability

In addition to isolate PNSB from heavy metal contaminated paddy fields in this study, 145 PNSB strains previously isolated from saline paddy fields in southern Thailand (Nunkaew et al., 2015) were also used for selection. Nitrogen free medium (slightly modified from Vatsala et al. (2011)) consisting of 1.0 g sodium acetate, 0.9 g K_2HPO_4, 0.6 g KH_2PO_4, 0.2 g $MgSO_4$, 0.012 g $FeSO_4$, 0.018 g EDTA, 0.075 g $CaCl_2$, 0.01 g Na_2MoO_4 $2H_2O$, 2.8 mg $MnSO_4$ $4H_2O$, 0.75 mg $ZnSO_4$, 0.24 mg $CuSO_4$, 0.016 mg H_3BO_3 and 0.001 mg biotin in 1,000 mL distilled water adjusted pH to 7.0 was used for screening nitrogen fixing PNSB. Ten percent (v/v) of each inoculum was inoculated into the nitrogen free medium in a screw cap tube without headspace, whereas the medium without inoculum served as a control. Nitrogen gas does dissolve in water at approximately 17-20 mg/kg of water under 20-30 °C and 1 bar and therefore there should be enough dissolved nitrogen in the tube for fixation (Kolev, 2007). All culture tubes were incubated under microaerobic conditions, a light intensity of 3,000 lux, and room temperature for 48 h. Then, the cultures were centrifuged at 8,000 rpm for 15 min to obtain cell pellets and 0.5 mL of the supernatant in each sample was tested for NH_4^+ releasing by adding a few drops of the Nessler's reagent. No color change indicates absence of NH_4^+ while soft yellow, yellow, and brown indicate low, medium and high levels of NH_4^+, respectively. The supernatants from any PNSB strains, that showed high or medium levels of NH_4^+ production were selected and measured for the quantity of NH_4^+ release using a Spectroquant® Ammonium kit (Merck, Germany) according to the manufacturer's instructions. To confirm that NH_4^+ release was from nitrogen fixation, an experiment was conducted by culturing some of the selected PNSB in nitrogen free medium without Mo and Fe. It should be noted that nitrogen free medium does not contain V. Without these co-factors (Mo, Fe, and V), nitrogenase genes/enzymes associated with nitrogen fixation cannot function.

Nitrogenase gene investigation of the selected PNSB

Nitrogenase enzymes are the key enzymes for nitrogen fixation. Three nitrogenase isozymes were characterized by the transition metal present at the active site (cofactor): Mo-Fe, V-Fe and Fe-Fe nitrogenases (Oda et al., 2005). The presence of nitrogenase genes encoded for nitrogenase enzymes in PNSB was analyzed by the polymerase chain reaction (PCR) technique. The selected PNSB strains based on NH_4^+ releasing were grown in GA medium and harvested under the same conditions as described above. The cell pellets were extracted for genomic DNA using a Power Soil DNA kit (MO BIO, USA) according to the manufacturer's instructions. Gene description and primers for examining nitrogenase genes are presented in Table 3-1. PCR conditions were according toNavarro-Noyaa et al. (2012) for Mo-Fe nirogenase, and Betancourt et al. (2008) for V-Fe and Fe-Fe nitrogenase. The following *nifH*, *vnfG* and *anfG* genes were amplified corresponding to the Mo-Fe, V-Fe and Fe-Fe nitrogenase, respectively. PCR amplification was performed with 25 µL reaction volume consisting of 10 µM of each primer, DNA template (approx. 10 ng) and iProof™ High-Fidelity Master Mix (BIO-RAD, CA) in a thermal cycler (T100™, BIO-RAD, Singapore). The PCR products were separated in 0.8% agarose gel and observed on a Geldoc/UV transilluminator.

Table 3-1. Nitrogenase genes, primers and target sizes for amplification.

Gene	Primers used	Sequence (5' to 3')	Target size (bp)	References
nifH (Mo-Fe nitrogenase)	nifHRFZ-F	TYGGCAAGTCCACCACC	431	(Navarro-Noyaa et al., 2012)
	nifHRFZ-R	GCGCCATCATCTCRCCGGA		
vnfG (V-Fe nitrogenase)	D6f	CGGGATCCGAAGACTTYGARAAGGTCAT	760	(Betancourt et al., 2008)
	K3r	GCAGTCGTACATCGGGTT		
anfG (Fe-Fe nitrogenase)	D7f	GCTCTAGACGCSATCTAYTCGCCGA	783	(Betancourt et al., 2008)
	K2r	CGGAATTCCGATGCAATCCTTGAT		

ALA production test

The selected PNSB strains based on high levels of NH_4^+ release were prepared for an ALA production test following the same procedure for determining N_2 fixing ability except that the GA medium test was used and the supernatant from the cell suspension was analyzed for ALA by a colorimetric method (Nunkaew et al., 2015) as follows. One mL of each supernatant was added to 2 mL of 1 M acetate buffer (pH 4.7) and 0.05 mL of acetylacetone and then heated at 100 °C for 15 min. After cooling, 3.5 mL of modified Ehrlich's reagent was added, and the mixture was left at room temperature for 15 min prior to measuring optical density at 553 nm by a Microplate Reader (PowerWaveX, BIO-TEK, USA) using uninoculated GA medium as blank. Two-fold serial dilution of the standard stock ALA HCl solution was prepared and the optical density of the diluted standards (a concentration range of 0-16 mg/L) was measured as previously described. A standard curve, which was used to determine the ALA concentration in the sample, was generated by plotting the concentrations of ALA HCl of the diluted standards against optical densities. R^2 value of the standard curve was 0.9958.

The concentration of ALA in the supernatant was confirmed by a high-performance liquid chromatography (HPLC) (Tangprasittipap et al., 2007). Briefly, 50 µL of each supernatant was mixed with 3.5 mL of acetylacetone: ethanol: water (3: 2: 15 v/v) containing 0.4% NaCl and 450 µL of aqueous formalin (8.5% v/v). The mixture was heated at 100 °C for 30 min and then filtered through a membrane with a pore size of 0.22 µm (Syringe filter nylon, 13 mm in diameter, Merck). ALA in the mixture was analyzed using HPLC (Agilent 1200 series, Agilent Technologies, USA) equipped with a fluorescence detector. An Intersil ODS-3 HPLC column (1.5 × 250 mm, 5 µm particle size) (GL Science Inc., Japan) was used. The analytical conditions included 40 °C, methanol and 2.5% (v/v) acetic acid as a mobile phase starting with 60:40 (v/v) at a flow rate of 0.2 mL/min, and excitation and emission wavelengths of 363 and 473 nm, respectively. The samples from any PNSB strains with 1-5 mg/L of ALA production were retained for further selection.

IAA production test

The selected PNSB strains based on high ALA production obtained from the preceding step were grown under microaerobic-light conditions for 48 h in GA medium in which L-sodium glutamate was replaced by 1 mM tryptophan (as a precursor for IAA production) (Mujahid et al., 2011). The cultures were centrifuged at 8,000 rpm for 15 min to obtain supernatant for the determination of IAA release. Each culture supernatant was acidified to pH 2.5 with 5 M HCl and the metabolites were extracted thrice with equal volume of ethyl acetate. The ethyl acetate layer was pooled and evaporated to dryness under vacuum in a rotary flash evaporator (Rotavapor R-210, Buchi, Switzerland) at 45 °C. After drying, the brown residue was dissolved in 1 mL of methanol and filtered through a nylon filter membrane with a pore size of 0.22 µm, and analyzed by HPLC (Agilent 1200 series, Agilent Technologies, USA) equipped with a photodiode array detector and BDS Hypersil C18 column (4.6 × 200 mm, 5 µm particle size) (Thermo Scientific). Acetic acid (1% v/v) and acetonitrile were used as a mobile phase at a flow rate of 1 mL/min.A linear gradient starting with 1% (v/v) acetic acid and acetonitrile at 100:0 %, changing to 50:50% and 0:100% during 0-25 and 25-28 min, and finally returning to 100:0 % was applied. The detection wavelengths were 254 and 280 nm (Modified from Mujahid et al., 2011).

Determination of optimal growth conditions of PNSB

The selected PNSB strains based on the biofertilizer properties were investigated for their optimal growth conditions in GA broth under microaerobic-light conditions for 96 h. Cell growth was monitored by measuring OD_{660} using a spectrophotometer every 6 h for 48 h and after that every 12 h for 48 h. The values of optical density were used to produce a growth curve and calculate the maximum specific growth rate (μ_{max}) and doubling time or generation time. The growth conditions investigated were pH 5, 6, 7, 8, and 9; temperature of 25, 30, 35, and 40 °C; and light intensity of 1,000, 2,000, 3,000, and 4,000 lux.These pH, temperature, and light intensity values were based on conditions observed in tropical agricultural areas (Roger, 1996).

Abilities of PNSB to resist and remove Cd and Zn

The selected PNSB strains based on the biofertilizer properties were also tested for their abilities to resist and remove Cd and Zn. Ten percent (v/v) of each inoculum was inoculated into GA medium supplemented with Cd or Zn based on the highest concentrations for Cd and Zn in the samples collected from the contaminated paddy fields (both C1 and C2) in this study. However, the selected PNSB strains did not grow well in the GA medium supplemented with the highest concentration for Zn under their optimal growth conditions (Table 3-2 and 3-3). Therefore, a mean value for Zn detected at site C1 and C2 was used. The inoculation medium without Cd and Zn was used as a biotic control while an abiotic control was the medium containing either Cd or Zn without the inoculum. All culture tubes were incubated under microaerobic-light conditions under the optimal growth conditions for 48 h. After incubation, the bacterial growth was measured based on OD_{660}. Each culture was centrifuged at 8,000 rpm for 15 min to obtain the supernatant and then was measured for the residual of heavy metals (Cd and Zn) by ICP-OES. The percentage of heavy metal reduction was calculated by comparing with the abiotic control while the resistance to heavy metals was estimated by comparing the bacterial growth between the treatment and the biotic control. Any PNSB strains providing the highest removal and resistance to both heavy metals were selected for identification.

Ability of PNSB to reduce greenhouse gas emissions

Fresh soil samples collected from a paddy field at NaMom District, Songkhla Province (6°59'22.6"N, 100°34'38.5"E) contained Cd and Zn at concentrations of 0.001 and 19.60 mg/kg soil dry weight. Rice straw was ground and then screened by a sieve with an opening of 0.5 mm. To prepare a microbial rice straw biodegradation system for greenhouse gas (CH_4 and CO_2) emission testing, soil slurry was prepared in a 1,000 mL transparent serum bottle by adding 100 g of fresh paddy soil and 3 g of rice straw to each serum bottle and then adjusting to 900 mL using distilled water. One hundred mL inoculum of each selected PNSB was added to soil slurry and the bottles were sealed with a rubber stopper and then an aluminum cap on top. All

culture bottles were incubated under a light intensity of 3,000 lux at room temperature (25 ± 3 °C) for 10 days. Simultaneously, the incubation of the soil slurry and rice straw without inoculums as a control was maintained under the same condition. Gas samples from the bottles were analyzed for CH_4 and CO_2 using gas chromatography (GC) (7890A GC systems, Agilent Technologies, USA) equipped with a thermal conductivity detector and a HP-PLOT/Q column (length 30 m, diameter 0.53 mm, film thickness 40 µm) (Agilent Technologies, USA). Helium was used as the carrier gas. The inlet and detector temperature, and the oven temperature were 250 °C and 60 °C. The oven temperatures were held for 3 min at 9 psi. A biogas standard consisting of 60% CH_4, 35% CO_2 and 5% N_2 (v/v) was used for GC calibration. The CH_4 and CO_2 percentages were compared with the control and percent reduction was calculated.

Bacterial identification

Identification of the selected PNSB strains based on Bergey's Manual of Systematic Bacteriology vol. 3 (Brenner et al., 2005) was carried out by following the methods described by (Kantachote et al., 2005). In addition, 16S rDNA sequence analysis was also conducted for identification as follows. The selected PNSB strains were grown in GA broth and harvested cells were centrifuged as previously described to obtain cell pellets for the genomic DNA extraction using the Power Soil DNA kit (MO BIO, USA) according to the manufacturer's instruction. PCR amplification was performed with 25 µL reaction volume consisting of 10 µM of each primer (8F; 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R; 5'-GGTTACCTTGTTACGAC TT-3'), 1 U of *Taq* DNA polymerase, DNA template (approx. 10 ng) and iProof™ High-Fidelity Master Mix (BIO-RAD, CA) in a thermal cycler (T100™, BIO-RAD, Singapore). The amplification conditions were 5 min at 95 °C for initial denaturation followed by 30 cycles consisting of 50 sec at 95 °C for denaturation, 30 sec at 55 °C for annealing and 2 min at 72 °C for extension, and 10 min at 72 °C for final extension. The PCR products were separated in 0.8% agarose gel and observed on a Geldoc/UV transilluminator. The collected DNA bands from the gel were purified by a QIAquick gel extraction kit (Qiagen, USA) according to the manufacturer's

instruction. The purified PCR products were sequenced using an automate DNA sequencer at the First Base Laboratories SdnBhd, Malaysia. The partial 16S rDNA sequences were compared with GenBank database on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by BLASTN. CLUSTAL W was used for multiple alignments and a phylogenetic tree was constructed using MEGA6 (Tamura et al., 2013).

Data presentation and statistical analysis

All experiments were conducted in triplicate unless otherwise stated. Means and standard deviations are presented. One way analysis of variance was used to analyze data and statistical differences among means were determined by the Tukey's test (honesty significant difference, HSD, at $P \leq 0.05$).

Results

Contamination of Cd and Zn in paddy fields

The concentrations of Cd and Zn in the contaminated paddy fields are shown in Table 3-4. Both heavy metals in soil/sediment samples collected from site C1 (close to Zn mine, median values of 4.82 and 225.10 mg/kg dry weight for Cd and Zn) were significantly higher than from site C2 (close to gold mine, median values of 0.80 and 169.60 mg/kg dry weight for Cd and Zn). The heavy metal concentrations in water samples collected from both contaminated paddy fields were very low, in a range of 0.003-0.005 mg/L for Cd and 0.008-0.045 mg/L for Zn (Table 3-4).

Selection of PNSB with ability to release NH_4^+ and their nitrogenase genes

A total of 235 PNSB strains consisting of 90 and 145 strains isolated from the heavy metal contaminated and saline paddy fields, respectively, were used to select for their N_2 fixing ability based on NH_4^+ release in nitrogen free medium under microaerobic-light conditions. Out of the 235 isolates, 7 (2.98%) and 29 (12.34%)

strains were classified as medium and low level of NH_4^+ releasers, respectively; whereas NH_4^+ release was not detected for the rest of the strains (199 strains or 84.68%). Seven medium level NH_4^+ releaser strains, four from saline sites (KK415, TN110, TN217 and TN414) and three from the heavy metal contaminated sites (MS072, MS212 and WP051), released 0.90 - 3.20 mg NH_4^+/L with strain TN110 being the highest NH_4^+ releaser (Table 3-5).

Table 3-2. Maximum specific growth rate (μ_{max}) of selected PNSB strains in GA broth under microaerobic-light conditions at different values of pH, temperature, and light intensity. The μ_{max} is given together with its generation time; h (in parentheses).

Parameter		μ_{max} (h^{-1})			
		KK415	TN110	TN217	TN414
pH	5	0.018 (39.15)	0.048(14.35)	0.005(135.88)	0.014 (50.22)
	6	0.085 (8.18)	0.196 (3.54)	0.138 (5.04)	0.148 (4.69)
	7	0.217 (3.20)	0.222 (3.13)	0.209 (3.32)	0.205 (3.38)
	8	0.178 (3.89)	0.212 (3.26)	0.193 (3.59)	0.180 (3.85)
	9	0.170 (4.07)	0.202 (3.44)	0.197 (3.52)	0.168 (4.13)
Temperature	25	0.188 (3.68)	0.191 (3.63)	0.195 (3.55)	0.179 (3.87)
(°C)	30	0.191 (3.63)	0.217 (3.20)	0.209 (3.32)	0.206 (3.36)
	35	0.187 (3.72)	0.200 (3.47)	0.199 (3.49)	0.192 (3.61)
	40	0.173 (4.02)	0.207 (3.35)	0.187 (3.71)	0.184 (3.77)
Light	1000	0.132 (5.26)	0.151 (4.59)	0.155 (4.48)	0.165 (4.20)
intensity	2000	0.187 (3.72)	0.200 (3.47)	0.194 (3.58)	0.177 (3.91)
(lux)	3000	0.201 (3.44)	0.219 (3.16)	0.199 (3.49)	0.192 (3.61)
	4000	0.193 (3.59)	0.192 (3.61)	0.191 (3.63)	0.167 (4.15)

The presence of nitrogenase genes, specifically those associated with three isozymes characterized by the transition metal present at the active site (cofactor) in seven NH_4^+ releaser strains is shown in Figure 3-1. All the NH_4^+ releaser strains had Mo-Fe nitrogenase gene (*nifH*), which is commonly found in N_2 fixing bacteria. Mo-Fe nitrogenase is an outstanding form of the nitrogenase enzyme; while V-Fe and Fe-Fe nitrogenases are normally referred to as alternative nitrogenases (Oda et al., 2005). All strains excluding MS212 and TN414 had Fe-Fe nitrogenase gene (*anfG*) while only TN110 and TN217 contained V-Fe nitrogenase genes (*vnfG*). TN110 and TN217 possessed all three different nitrogenase genes but TN110 produced the most NH_4^+ among all seven strains. All seven strains were further screened for their ability to produce ALA.

Table 3-3. Zn reduction by selected PNSB strains and their resistance to Zn in GA medium supplemented with 784.00 mg/L of Zn (the highest concentration detected in contaminated paddy field C1) under their optimal growth with microaerobic-light conditions for 48 h.

PNSB strain	Zn reduction (%)	Resistance to Zn (%)
KK415	0.94 ± 2.30^{b}	5.42 ± 0.52^{b}
TN110	6.56 ± 2.33^{ab}	6.98 ± 0.95^{b}
TN217	5.47 ± 2.90^{ab}	5.01 ± 0.93^{b}
TN414	8.91 ± 2.07^{a}	11.41 ± 3.28^{a}

Mean and its standard deviation of three determinations are presented. Values with different lowercase letters in the same column are significantly different at $P \leq 0.05$.

Table 3-4. Median and range (in parentheses) of cadmium and zinc concentrations in soil or sediment and water samples from heavy metals contaminated paddy fields (n = number of samples)**.**

Area description		Soil/Sediment (mg/kg dry weight)			Water (mg/L)		
Site	Location	n	Cd	Zn	n	Cd	Zn
C1	16°39'37.7"N, 98°36'29.4"E	22	4.82 (0.60-23.00)	225.10 (35.08-784.00)	9	0.004 (0.003-0.005)	0.019 (0.008-0.045)
C2	17°21'33.1"N, 101°40'31.5"E	10	0.80 (0.48-8.68)	169.60 (49.72-647.60)	4	0.004 (0.003-0.004)	0.014 (0.012-0.031)
Agricultural soil quality guidelines*			1.40	200.00		Not available	Not available

* (CCME, 1999a and b)

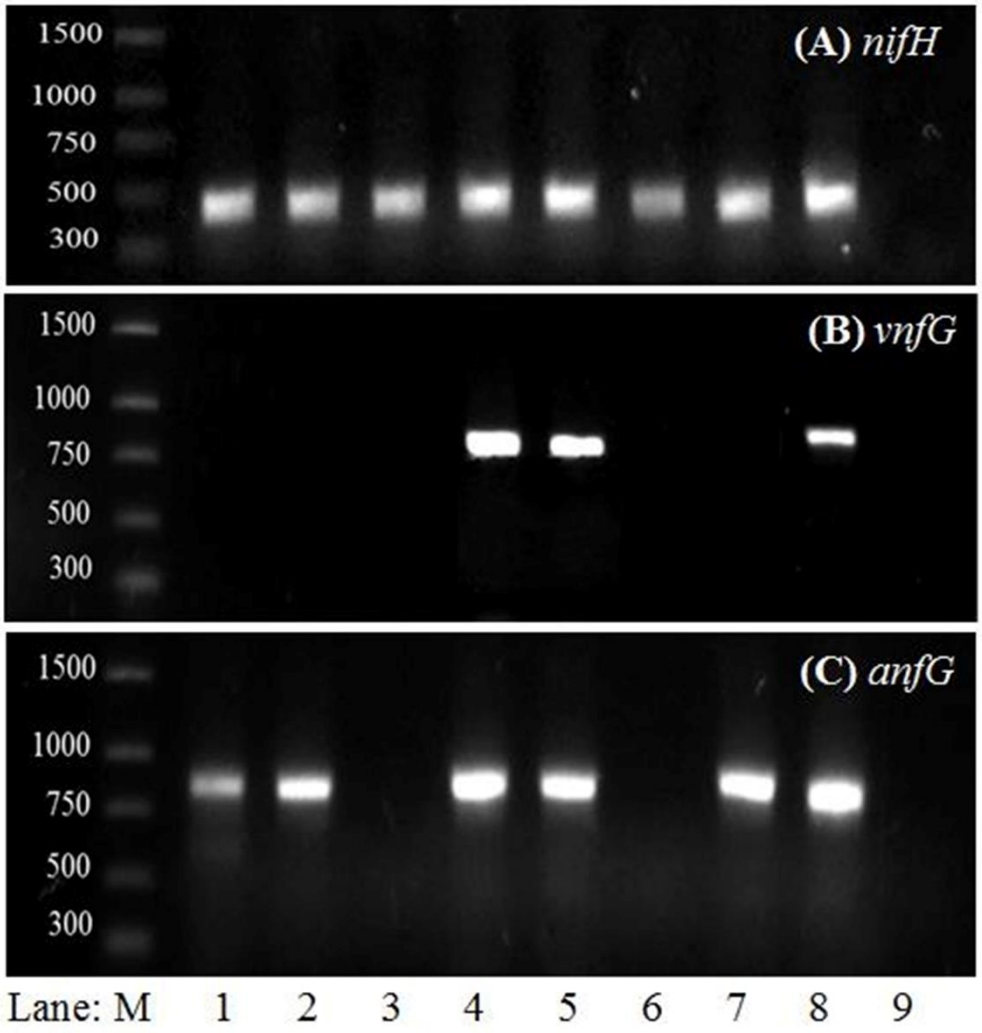

Figure 3-1. DNA bands on 1% agarose gel of *nifH*: Mo-Fe nitrogenase gene (A), *vnfG*: V-Fe nitrogenase gene (B) and *anfG*: Fe-Fe nitrogenase gene (C) amplified using the genomic DNA of isolated PNSB strains (lane nos. 1-7 are KK415, MS072, MS212, TN110, TN217, TN414 and WP051, respectively). Lane no. 8 is positive control (*Azotobacter vinelandii* JCM21475 while negative control is on lane no. 9. Lane M is DNA size marker.

Selection of PNSB with abilities to produce plant growth promoting substances

Only 5 PNSB strains produced extracellular ALA in the GA medium under microaerobic-light conditions (Table 3-5). The released ALA concentration ranged 0.23 - 4.96 mg/L and 0 - 4.24 mg/L based on the colorimetric and HPLC methods, respectively (Table 3-5). For PNSB isolated from the heavy metal contaminated paddy soils, only MS212 released ALA at a very low concentration (0.26 mg/L).

KK415, TN110, TN217 and TN414 were prolific ALA releasers and were selected for further screening although they were isolated from saline paddy soils. All four strains released IAA in a range of 0.65 - 3.62 mg/L in the GA medium supplemented with 1 mM of tryptophan (Table 3-5). Among them, TN110 was the most effective strain in IAA and ALA productions.

Table 3-5. The concentrations of NH_4^+ releasing, extracellular ALA and IAA productions in nitrogen free medium, GA medium and GA medium supplemented with tryptophan, respectively, by the isolated PNSB strains under microaerobic-light conditions for 48 h.

PNSB strain	NH_4^+ (mg/L)	ALA (mg/L)		IAA (mg/L)
		Colorimetry*	HPLC*	
MS072	0.95 ± 0.02^{c}	0.25 ± 0.07^{f}	0.00	nd
MS212	1.10 ± 0.21^{c}	0.76 ± 0.05^{e}	0.26 ± 0.01^{d}	nd
WP051	0.90 ± 0.08^{c}	0.23 ± 0.04^{f}	0.00	nd
KK415	2.05 ± 0.12^{b}	2.90 ± 0.05^{c}	2.54 ± 0.08^{b}	2.39 ± 0.16^{b}
TN110	3.20 ± 0.33^{a}	4.32 ± 0.10^{b}	4.11 ± 0.10^{a}	3.62 ± 0.26^{a}
TN217	1.45 ± 0.32^{c}	4.96 ± 0.07^{a}	4.24 ± 0.07^{a}	0.81 ± 0.46^{c}
TN414	1.30 ± 0.24^{c}	1.57 ± 0.16^{d}	0.94 ± 0.12^{c}	0.65 ± 0.55^{c}

Mean and its standard deviation of three determinations are presented. Different lowercase letters in the same column indicate significant differences ($P \leq 0.05$). Asterisk indicates significant differences in ALA concentrations ($P \leq 0.05$) between two measurement methods.
nd = not determined.

Selection of PNSB with ability to mitigate heavy metals (Cd and Zn) and greenhouse gas emissions

Under microaerobic-light conditions in GA broth, strain TN110 showed higher μ_{max} than the other three strains at pH 5-9, temperature of 30-40 °C and light intensity of 2,000-3,000 lux as shown in Table 3-2.One of the most critical factors for the growth of all four strains was pH as these PNSB preferred neutral and alkaline pH up to 9; lower pH could disrupt plasma membrane and/or inhibit enzyme activities and membrane transport protein (Slonczewski et al., 2009). However, all of them had the

same optimal growth conditions: pH 7, 30 °C and 3,000 lux; and their doubling times were between 3.13 – 3.63 h.

Removal of both heavy metals based on the levels in soils at the contaminated sites (23 mg Cd/kg based on the highest concentration for both sites C1 and C2 as shown in Table 3-4, and 262 mg Zn/kg based on the mean concentration for both sites C1 and C2 (data not shown in Table 3-4) by these PNSB were tested under their optimal growth conditions (Table 3-2). It should be noted that in the heavy metal removal experiments, the mean concentration for both sites C1 and C2 was used for Zn rather the highest concentration of 784 mg/kg (Table 3-3) because all PNSB strains tested were not able to grow well at the highest concentration (Table 3-3). All PNSB tested removed Cd by more than 70% and strain TN110 was the most effective Cd remover at 84% (Table 3-6). However, only strain TN414 could remove Zn efficiently at 74% as the rest of the strains removed 30-55% of Zn. Removal efficiencies of all PNSB strains corresponded to their resistance. For instance, the resistances of strains TN110 and TN414 were 68 and 65% for Cd, and 41 and 51% for Zn, respectively (Table 3-6).

To investigate the possibility of selected PNSB to reduce greenhouse gas emissions, the microbial rice straw degradation experiment was conducted for the competition of the selected PNSB strains with indigenous methanogens that produce CH_4 under microaerobic-light conditions for 10 days. Strain TN110 was the best performer among the four strains, reducing 80% of CH_4 and 33% of CO_2 emissions (Figure 3-2). The red color in the microbial rice straw biodegradation system for the greenhouse gas (CH_4 and CO_2) mitigation test indicates high proliferation of strains TN110 and TN414 compared with the control (Figure 3-3). This observation suggests that the selected PNSB strains grew better than indigenous PNSB (indicated by pale color bloom in the control). Based on overall PNSB screening results, strains TN110 and TN414 were the most promising strains and therefore they were selected for identification.

Table 3-6. Heavy metals (Cd and Zn) reduction by selected PNSB strains and their resistance to heavy metals in GA medium supplemented with 23.00 mg/L of Cd or 262.40 mg/L of Zn under their optimal growth with microaerobic-light conditions for 48 h.

PNSB strain	Heavy metal reduction (%)		Resistance to heavy metal (%)	
	Cd	Zn	Cd	Zn
KK415	77.43 ± 0.45^{c}	45.90 ± 1.51^{c}	54.32 ± 1.07^{b}	36.44 ± 1.57^{bc}
TN110	84.21 ± 0.28^{a}	54.83 ± 0.70^{b}	67.83 ± 1.85^{a}	40.50 ± 0.53^{b}
TN217	82.89 ± 0.19^{b}	29.66 ± 0.59^{d}	58.50 ± 4.23^{b}	33.07 ± 0.89^{c}
TN414	71.52 ± 0.67^{d}	73.60 ± 0.10^{a}	65.30 ± 5.51^{a}	51.44 ± 2.84^{a}

Mean and its standard deviation of three determinations are presented. Values with the different lowercase letters in the same column are significantly different at $P \leq 0.05$.

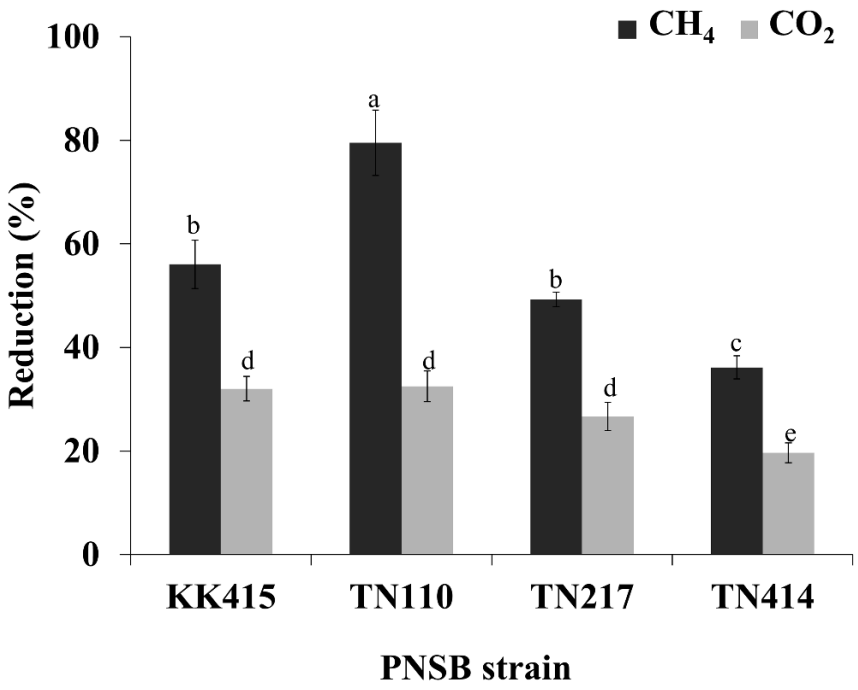

Figure 3-2. Removal efficiency of CH_4 and CO_2 by selected PNSB strains in the paddy soil slurry supplemented with rice straw under microaerobic-light conditions for 10 days. Values with different lowercase letters are significantly different at $P \leq 0.05$.

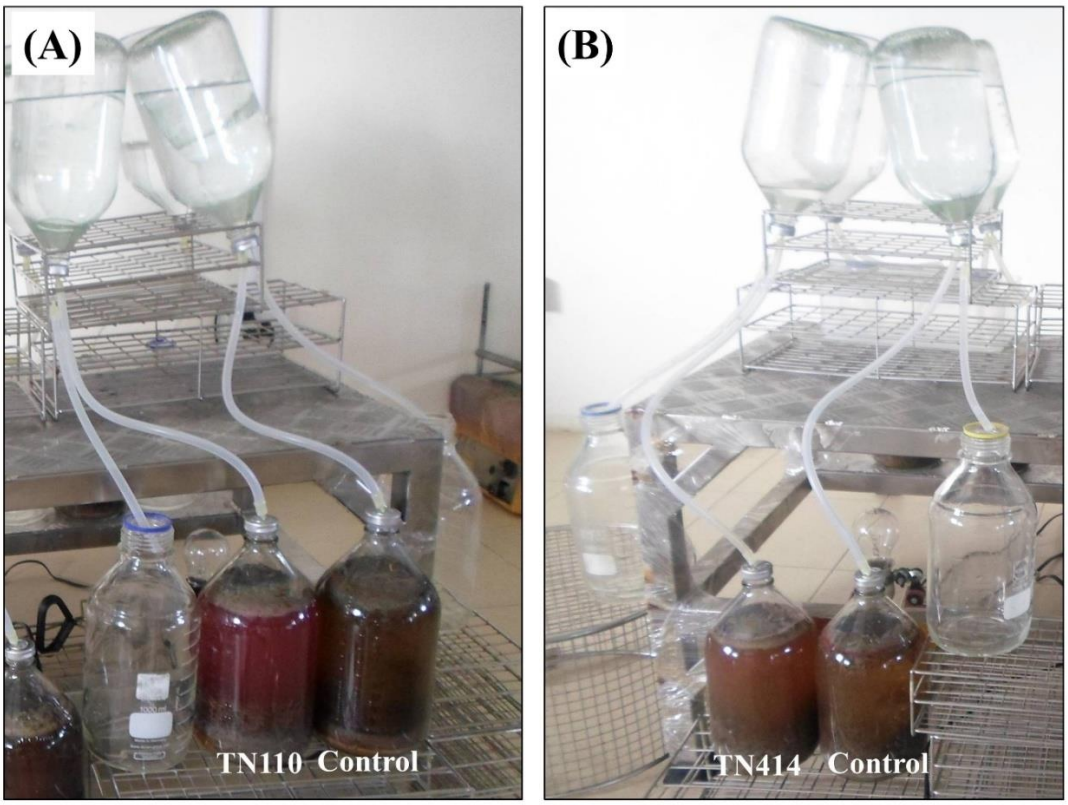

Figure 3-3. The color bloom of strains TN110 (A) and TN414 (B) compared with the control (no PNSB inoculation) in the microbial rice straw biodegradation for the greenhouse gases (CH_4 and CO_2) mitigation test at 25±3 °C for 10 days.

Bacterial identification

The physiological and biochemical properties of strains TN110 and TN414 were very close to *Rhodopseudomonas palustris* and *Rubrivivax gelatinosus*, respectively (Table 3-7). However, strain TN110 could not use benzoate, fumarate, malonate, and propionate. It should be noted that many PNSB bacteria benefit from *p*-aminobenzoate as a growth factor but that is not the case for strains TN110 and TN414. The results of the phenotypic identification corresponded well with the genotypic results. Based on comparison with the Genbank database, strain TN110 was the closest relative to *R. palustris* (HQ647260.1); while strain TN414 was very close to *Ru. gelatinosus* (KC634003.1) (Figure 3-4). The similarities were > 99%. The phylogenetic analysis was conducted using the neighbor-joining method with bootstrapping 1,000 replicates. The strain TN110 was identified to be *R. palustris*

(accession number KU935455); while strain TN414 was highly likely *Ru. gelatinosus* (accession number KU935456).

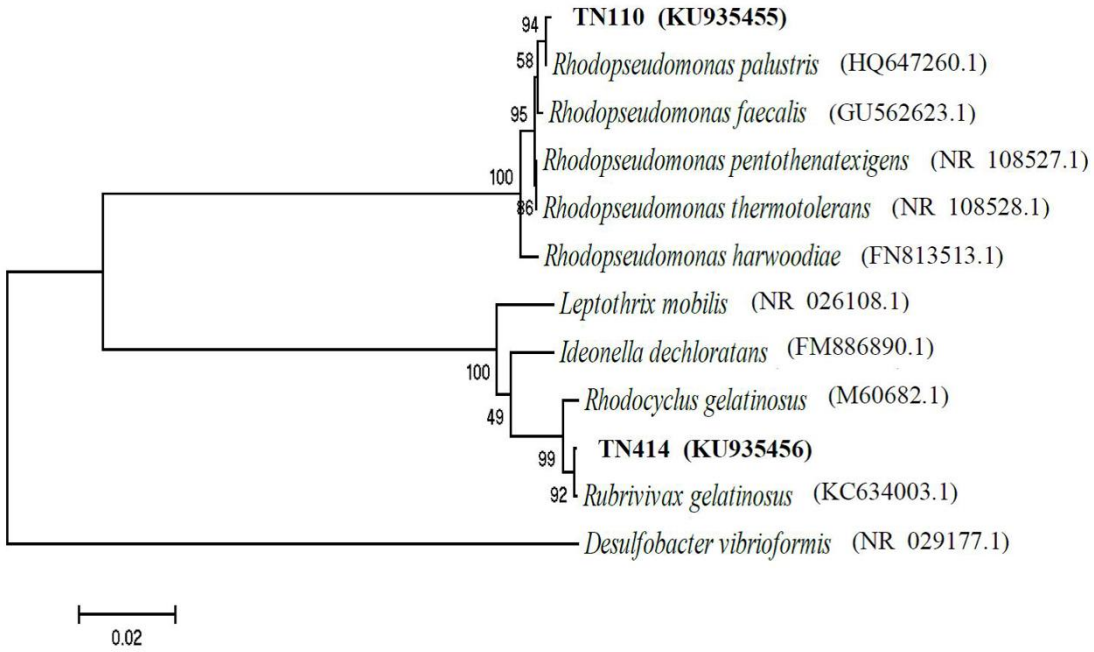

Figure 3-4. Neighbor-joining tree with bootstrapping 1,000 replicates showing the phylogenetic relationship of strains TN110 and TN414 aligned with reference strains from the domain bacteria based on 16S rDNA sequences. The percentage of bootstrap is indicated for each node. The scale bar represents the number of substitutions per site.

Table 3-7. Characteristics of selected PNSB strains (TN110 and TN414) compared to type strains of *Rhodopseudomonas palustris* and *Rubrivivax gelatinosus*.

Characteristic	TN110	*Rhodopseudomonas palustris* [a]	TN414	*Rubrivivax gelatinosus*[b]
Color of culture (anaerobic-light)	Red	Red	Light yellowish	Light yellowish brown
Color of culture (aerobic-dark)	White	White to pink	Pale peach	Pale peach to dirty yellowish brown
Cell shape	Rod	Straight to curved rod or rod	Rod	Straight to curved rod
Gram staining	Negative	Negative	Negative	Negative
Motility	Motile	Motile	Motile	Motile
Biotin	Yes	Yes	Yes	Yes
Nicotinic acid	Yes	Yes	No	No
p-aminobenzoic acid	No[c]	Yes	No	No
Thiamine hydrochloride	No	No	Yes	Yes
Optimal pH	7	6.9	7	6.0-8.5
Optimal temperature (ºC)	30-35	30-37	30	30
Utilization				
Benzoate	-[c]	+	-	-
Butyrate	+	+	+	+/-
Citrate	+	+/-	+	+
Ethanol	+	+/-	+	+
Formate	+	+	+	+/-
Fructose	+	+/-	+	+
Fumarate	-[c]	+	+	+
Glucose	+	+/-	+	+
Glutamate	+	+	+	+
Glycerol	+	+	+[c]	-
Lactate	+	+	+	+
Malate	+	+	+	+
Malonate[c]	-[c]	+	-	o
Mannitol	+	+/-	+[c]	-
Mannose	+	o	+	+
Methanol	+	+/-	+	+/-
Propanol	+	+	+	o
Propionate	-[c]	+	-	+/-
Pyruvate	+	+	+	+
Sorbitol	+	+	+	+
Succinate	+	+	+	+
Sulfide	+	+	+	+
Tartrate	-	-	-	+/-
Thiosulfate	+	+	-	-

+: utilize; -: not utilized; o: not available

[a] and [b] Reference: (Brenner et al., 2005)

[c] Different results between strain TN110 and *R. palustris*; strain TN414 and *Ru. gelatinosus*.

Discussion

It is not surprising that median Cd and Zn contents in soil/sediment samples collected from the contaminated paddy fields, particularly site C1 (Table 3-4) were higher than the agricultural soil quality guidelines(CCME, 1999a and b). In addition, the maximum Cd and Zn contents in the heavy metal contaminated paddy fields (23 and 784 mg/kg) were much higher than the standard guidelines (1.4 and 200 mg/kg for Cd and Zn, respectively) (CCME, 1999a and b). The contamination of Cd and Zn occurred through runoff from mining. Cd and Zn detected in the vicinity by previous studies were higher than this study, 0.31 - 172 mg/kg for Cd and 26.12 - 3138 mg/kg for Zn (Akkajit and Tongcumpou, 2010; Sriprachote et al., 2012). Due to the fact that paddy fields around the mine areas are still used for rice cultivation, bioremediation of heavy metals should be considered.

This study initially screened 235 PNSB strains and there were only 7 isolated strains (~ 3%) that are possible biofertilizers based on their NH_4^+ releasing (Table 3-5). After further screening for PGPS production (Table 3-5), only 4 strains remained (KK415, TN110, TN217 and TN414). Finally, after considering CH_4 emission reduction and heavy metal removal abilities (Figure 3-2 and Table 3-6), there were only 2 potential strains (TN110 and TN414). This strongly suggests that to obtain promising strains for field applications both extensive and intensive efforts were required. However, it is worthwhile to obtain them due to their versatility.

For strains KK415, TN110, TN217 and TN414, ammonium release was not observed when the co-factors (Mo, Fe, and V) were not present confirming that ammonium release was from biological nitrogen fixation. Although some of these four strains contain different numbers of nitrogenase type, it is likely that only Mo-Fe nitrogenase was involved in the nitrogen fixation. Fe-Fe and V-Fe nitrogenases are used only when Mo-Fe nitrogenase is not available or depleted. In the study, Mo-Fe nitrogenase should not be limited as an excessive amount of Mo was supplied. That is why strains TN217 and TN414 had comparable ammonium production. The higher ammonium production by strain KK415 could be because it was from a different site that its environmental conditions might promote nitrogen fixation ability. There is no clear explanation for the distinctively higher ability of strain TN110 to release

ammonium than the other three strains (TN217, TN414, and KK415). It could be tied to the finding that the *nifH* gene of TN110 is much different than that of the other three strains (Figure 3-5).

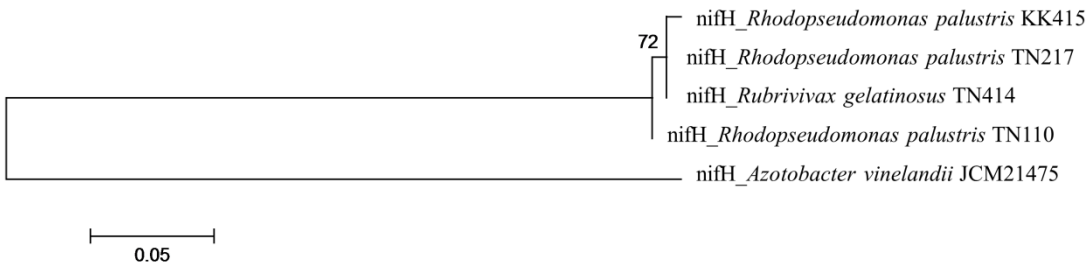

Figure 3-5. Neighbor-joining tree with bootstrapping 1,000 replicates showing an evolutionary relationship of strains *Rhodopseudomonas palustris* KK415, *R. palustris* TN110, *R. palustris* TN217, *Rubrivivax gelatinosus* TN414 and *Azotobacter vinelandii* JCM21475 based on nifHsequences. The percentage of bootstrap is indicated for each node. The scale bar represents the number of substitutions per site.

Only 4 PNSB strains were selected for testing their ability to produce IAA because they were able to release ALA in a range of 1-5 mg/L (Table 3-5). Several studies reported that ALA at low concentrations (1-5 mg/L) can promote plant growth by increasing photosynthesis (Al-Khateeb, 2006; Naeem et al., 2011). Moreover, ALA can reduce reactive oxygen species in plant that are generated from stress conditions such as drought and saline soils (Nunkaew et al., 2014; Kantha et al., 2015). TN110 was the most effective strain for ALA and IAA releases (Table 3-5). It has long been known that IAA, which is one of the phytohormone-like substances, promotes lateral root formation that enhances nutrient uptake ability for plant growth (Spaepen et al., 2007). In addition, IAA can act in a similar manner as ALA by inducing superoxide dismutase that is known as a free radical scavenger to alleviate the toxicity of heavy metals including Cd and Zn (Sun et al., 2014).

Among the 4 PNSB strains, *R. palustris* TN110 was a promising strain for Cd removal (> 80%); however, *Ru. gelatinosus* TN414 was able to remove both Cd and Zn at high levels of nearly 80% (Table 3-6). It was surprising that strain TN414 had high abilities to resist and remove both Cd and Zn while it produced low amounts of ALA and IAA (Table 3-5). It might be that the strain could produce high amounts of EPS to adsorb both heavy metals; this should be further investigated. Panwichian et

al. (2011) showed that EPS produced by *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24 provided greater reduction of heavy metals (Cd and Zn reduction of 90.79 - 91.84%) than the cells by themselves (no EPS) (Cd and Zn reduction from 14.02 to 25.02%) under microaerobic-light and aerobic-dark conditions. Bioremediation of Cd contaminated soil using *Rb. sphaeroides* resulted in a decrease of Cd accumulation in wheat seedling (67% for roots and 53% for leaves) (Fan et al., 2012). It is interesting that both selected PNSB strains were able to use sulfide (Table 3-7) suggesting their potential for reduction of toxic effect of sulfide on rice seedling (Lamers et al., 2013).

The dominance of strains TN110 and TN414 in the microbial rice straw biodegradation for the greenhouse gas (CH_4 and CO_2) mitigation test than indigenous PNSB in the control (Figure 3-3) suggests that the selected PNSB strains could suppress the growth of methanogens to reduce greenhouse gas emissions. Hence, the selected strains had potential to reduce methane emissions as shown in Figure 3-2. This is in agreement with our previous studies (Kantha et al., 2015; Kantachote et al., 2016) that promising strains of *R. palustris* under saline conditions could enhance rice growth and reduce CH_4 emissions in pot and paddy field experiments. In addition, TN110 was the most prolific strain for mitigating both CH_4 and CO_2; while TN414 was the least effective strain. It could be explained that the strain TN110 proliferated better than the strain TN414 in the changes of pH during biodegradation of rice straw based on their μ_{max}(Figure 3-3 and Table 3-2).

Conclusions

Heavy metals (Cd and Zn) in the studied paddy fields around the mine areas are major concerns as they can cause unsafe rice products. Biofertilizers are an attractive approach for decontamination of the heavy metals. PNSB, *R. palustris* TN110 and *Ru. gelatinosus* TN414, showed great potential as biofertilizers as they provide NH_4^+, PGPS (ALA and IAA) and reduce Cd, Zn, and greenhouse gases. TN110 with all nitrogenase genes is the more promising strain for biofertilization while TN414 has more Cd and Zn removal capabilities. The two strains mixed together as biofertilizers should be further investigated. The application of these biofertilizers will reduce or replace the use of chemical compounds/fertilizers and mitigate CH_4 emissions in the paddy fields. Simultaneously, it will lead to heavy metal bioremediation. This study provides promising PNSB with various abilities, particularly biofertilizer production. Their applications in the laboratory settings demonstrated potential direct implications in the fields of environmental and agricultural sciences leading to eco-friendly and sustainable agriculture especially for rice cultivation in heavy metal contaminated areas.

CHAPTER 4

Simultaneous Bioprecipitation of Cadmium to Cadmium Sulfide Nanoparticles and Nitrogen Fixation by *Rhodopseudomonas palustris* TN110

Abstract

This study investigated the abilities of a purple non-sulfur bacterium, *Rhodopseudomonas palustris* TN110 to bioremediate cadmium through the biosynthesis of CdS nanoparticles and to fix nitrogen simultaneously. Under microaerobic-light conditions, *R. palustris* TN110 synthesized CdS nanoparticles. The produced CdS nanoparticles had a spherical shape and an average size of 4.85 nm. The Fourier transform infrared spectrum of the nanoparticles reveals the carbonyl groups, bending vibrations of the amide I and II bands of proteins, and C-N stretching vibrations of aromatic and aliphatic amines. These bands and groups suggest protein capping/binding on the surface of the nanoparticles. *R. palustris* TN110 converted 25.61% of 0.2 mM $CdCl_2$ to CdS nanoparticles under optimal conditions (pH 7.5, 30 °C and 3,000 lux). The half maximal inhibitory concentration (IC_{50}) value of the produced CdS nanoparticles was 1.76 mM. The produced CdS nanoparticles at IC_{50} up-regulated two genes associated with nitrogen fixation: Mo-Fe nitrogenase gene (*nifH*) and V-Fe nitrogenase gene (*vnfG*) at 2.83 and 2.27 fold changes, respectively. On the contrary, the produced CdS nanoparticles slightly down-regulated Fe-Fe nitrogenase gene (*anfG*). The amounts of ammonia released by the strain support the gene expression results. *R. palustris* TN110 has great potential to serve concurrently as a cadmium bioremediation agent and a nitrogen fixer. The strain could be beneficial to paddy fields that are contaminated with Cd through run off from mining and chemical fertilizer applications.

Keywords: bioprecipitation, cadmium sulfide nanoparticle, cysteine desulfhydrase, nitrogenase, purple non-sulfur bacteria

บทคัดย่อ

งานวิจัยนี้เพื่อศึกษาความสามารถของแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ สายพันธุ์ *Rhodopseudomonas palustris* TN110 ในการบำบัดแคดเมียมทางชีวภาพผ่านการสังเคราะห์ในรูปอนุภาคนาโนแคดเมียมซัลไฟด์และตรึงก๊าซไนโตรเจนไปพร้อมกัน แบคทีเรียสายพันธุ์นี้สังเคราะห์อนุภาคนาโนแคดเมียมซัลไฟด์ภายใต้สภาวะมีอากาศเล็กน้อย มีแสง อนุภาคนาโนแคดเมียมซัลไฟด์ที่ผลิตได้มีรูปร่างกลม ขนาดโดยเฉลี่ย 4.85 นาโนเมตร สเปคตรัมของฟลูเรียร์ทรานส์ฟอร์ม อินฟราเรด(Fourier transform infrared) ของอนุภาคนาโนนี้ แสดงถึงหมู่คาร์บอนิลโปรตีนกลุ่มเอมีน 1 และ 2 พันธะที่เกี่ยวข้องกับอะโรมาติกและอะลิฟาติกเอมีน หมู่ฟังก์ชันเหล่านี้แสดงว่ามีหมู่โปรตีนที่ทำหน้าที่ห่อหุ้มอนุภาคนาโน สายพันธุ์ TN110 เปลี่ยน 0.2 มิลลิโมลาร์ของแคดเมียมคลอไรด์เป็นอนุภาคนาโนแคดเมียมซัลไฟด์ได้ร้อยละ 25.61 ภายใต้สภาวะที่เหมาะสมที่ค่าความเป็นกรดด่าง 7.5 อุณหภูมิ 30 องศาเซลซียส และความเข้มแสง 3,000 ลักซ์อนุภาคนาโนที่ผลิตได้นี้ยับยั้งการเจริญของแบคทีเรียสายพันธุ์ TN110 ได้ร้อยละ 50 ที่ค่าความเข้มข้นเท่ากับ 1.76 มิลลิโมลาร์ ค่าความเข้มข้นดังกล่าวสามารถส่งเสริมการทำงานของยีนที่ที่เกี่ยวข้องกับกระบวนการตรึงก๊าซไนโตรเจนของแบคทีเรียเอง โดยส่งเสริมการทำงานของยีนไนโตรจีเนสชนิดโมลิบดีนัม-เหล็ก และวานาเดียม-เหล็ก ได้เพิ่มขึ้น 2.83 และ 2.27 เท่า ตามลำดับแต่ลดการทำงานของยีนไนโตรจีเนสชนิดเหล็ก-เหล็ก ซึ่งสอดคล้องกับปริมาณของแอมโมเนียมไอออนที่ปลดปล่อยออกมาโดยแบคทีเรียสายพันธุ์นี้ ดังนั้นมีความเป็นไปได้ที่จะใช้สายพันธุ์ TN110 เป็นทั้งตัวบำบัดทางชีวภาพสำหรับแคดเมียมและตรึงก๊าซไนโตรเจนไปพร้อมกัน ซึ่งเป็นประโยชน์ในการนำไปใช้ในนาข้าวที่มีการปนเปื้อนของแคดเมียมทั้งจากการชะล้างมาจากการทำเหมืองแร่และการใช้ปุ๋ยเคมี

คำสำคัญ : การตกตะกอนทางชีวภาพ ไนโตรจีเนสแบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ อนุภาคนาโนแคดเมียมซัลไฟด์ เอนไซม์ซิสเทอีนดีซัลไฟด์เดรส

Introduction

Cadmium (Cd) is a toxic heavy metal that usually exists as a divalent cation or a compound with other elements (Bernhoft, 2013; Shi et al., 2019). Cd is naturally present in the environment at low levels; however, human activities have greatly increased those levels through runoff from mining, industrial solid waste and wastewater, and phosphate fertilizers (Li et al., 2014; Xu et al., 2014; Wang et al., 2018). Cd contamination is particularly problematic to paddy fields in Asian countries such as China and Thailand where many people have consumed rice cultivated in Cd-contaminated soils (Khaokaew et al., 2011; Kosolsaksakul et al., 2014; Yu et al., 2016; Wang et al., 2018). Paddy soils in those countries have been reported for Cd contamination higher than a safety level of 1.4 mg/kg soil based on the agricultural soil pollution control standards of the Canadian Council of Ministers of the Environment (CCME, 1999a) and remediation is needed. In-situ bioremediation is an attractive method for the contaminated paddy fields because after treatment by this method the soils remain in place, retain their key characteristics, and can therefore be used for rice production.

To cope with heavy metals including Cd, microorganisms can use many mechanisms such as bioaccumulation inside the cells, biosorption on the cell surface or exopolymeric substances (EPS) and bio-transformation into less toxicity forms (Smiejan et al., 2003; Watanabe et al., 2003; Peng et al., 2018). Bioprecipitation of Cd(II) ions into more stable forms including CdS nanoparticles in bacteria has been studied to reduce the toxicity and bioavailability of Cd to bacteria and plants such as wheat, respectively (Bai et al., 2009; Fan et al., 2012; Yang et al., 2012). A cysteine desulfhydrase enzyme is an intracellular enzyme that is important for bioprecipitation of Cd(II)ions to CdS nanoparticles in bacteria (Bai et al., 2009). This enzyme is light-dependent under a broad range of pH (7-10) and temperature (20-80°C) (Alharbi et al., 2012). These pH and temperature ranges encompass the conditions observed in tropical paddy fields (Roger, 1996). Therefore, it is possible for soil bacteria in tropical paddy fields to produce CdS nanoparticles using cysteine desulfhydrase.

CdS nanoparticles are well known for their use as a semiconductor which has unique optical and electronic properties (Zhu et al., 2018). In addition, it can be used

as a light harvester or photosensitizer to provide an energy source for nitrogenase enzyme (Brown et al., 2016). Yang et al. (2012) reported that CdS nanoparticles up-regulated nitrogenase gene expressions in N_2-fixing bacteria leading to increase in ammonium ion (NH_4^+) release. Nitrogenase enzyme is a key enzyme for biological nitrogen (N_2) fixation, a mechanism for conversion of atmospheric N_2 into NH_4^+ which is readily available nitrogen for terrestrial and aquatic ecosystems (Olivares et al., 2013).

Among of N_2-fixing bacteria, purple non-sulfur bacteria (PNSB) are a promising candidate for use as a biofertilizer in the paddy fields because they naturally distribute around rice rhizosphere where sunlight can reach (Brenner et al., 2005). Rice rhizosphere is under microaerobic conditions due to some oxygen release from photosynthesis during day time (Larsen et al., 2015). Previous studies showed that PNSB can fix N_2 under microaerobic- and anaerobic-light conditions (Harada et al., 2005; Sakpirom et al., 2017). *Rhodopseudomonas palustris* TN110 is a member of PNSB and has 3 types of nitrogenase enzymes, namely molybdenum-iron (Mo-Fe), vanadium-Fe (V-Fe), and Fe-Fe (Sakpirom et al., 2017). These enzymes are based on a requirement of metal cofactors. Mo-Fe nitrogenase is the most superior for N_2 fixation and is found in all of natural N_2 fixing bacteria whereas V-Fe and Fe-Fe nitrogenases are referred to as alternative nitrogenases that are available in only some N_2 fixing bacteria (Oda et al., 2005). The alternative nitrogenases are effective under limited or no Mo environment (Bellenger et al., 2014).

PNSB not only fix N_2 as a nitrogen source for rice but also produce plant growth promoting substances such as indole-3-acetic acid and 5-aminolevulinic acid to support rice growth (Nunkaew et al., 2014; Sakpirom et al., 2017). In addition, PNSB are known for their ability to remediate heavy metals by various mechanisms such as adsorption on the cell surface, accumulation inside the cells, and precipitation into sulfide compounds (Bai et al., 2009; Panwichian et al., 2011; Fan et al., 2012). Bai et al. (2009) reported that PNSB, *R. palustris*, precipitated Cd into CdS nanoparticles. As described above, CdS nanoparticles could up-regulate N_2 fixing genes (Yang et al., 2012). However, limited work has been conducted on the performance of PNSB for CdS nanoparticle bioprecipitation, particularly the effect of the bioprecipitation on N_2 fixation. Hence, it is worthwhile to study PNSB for use as

both biofertilizer and bioremediation agents for Cd-contaminated paddy fields by exploring the role of CdS nanoparticle bioprecipitation by PNSB on N_2 fixation.

This study aimed to examine the bioprecipitation of CdS nanoparticles by a PNSB, *R. palustris* TN110, and the ability of biosynthesized CdS nanoparticles (as a light harvester/photosensitizer) to increase nitrogenase activity. However, the toxicity of CdS nanoparticles has been reported in both prokaryotes and eukaryotes such as *Escherichia coli* and Hela cells (Hossain and Mukherjee, 2013). Up to date, no research work has been conducted on the toxicity of CdS nanoparticles on PNSB. Therefore, the toxicity of CdS nanoparticles produced by *R. palustris* TN110 was also investigated while the biosynthesized CdS nanoparticles were tested for their role on N_2 fixation based on NH_4^+ release and nitrogenase gene expression. An experiment with cadmium chloride ($CdCl_2$) to represent cadmium ion contaminated in the environment was performed for comparative purpose.

Materials and Methods

Preparation bacterial inoculum and CdS nanoparticle bioprecipitation

A glutamate-acetate (GA) medium (Sakpirom et al., 2017) was used for the preparation of a bacterial inoculum. *R. palustris* TN110 was previously isolated from a saline paddy field in southern Thailand (Nunkeaw et al., 2015). The bacterium was sub-cultured twice in GA medium. For each sub-culturing, 1 mL of the culture (10% v/v) was incubated in a 10.2 mL screw-cap test tube containing 9 mL of GA medium and 0.2 mL of headspace (to provide microaerobic conditions) at a light intensity of 3,000 lux for 48 h at room temperature (23±2 °C). After that, a bacterial cell pellet was obtained by centrifugation of the 10.2 mL test tube at 6,000 rpm under 4 °C for 15 min and washed five times with a saline solution (0.85% NaCl). The cell pellet of approximately 0.1 g was used as an inoculum for CdS nanoparticle synthesis.

The inoculum was inoculated in 50 mL of GA broth containing 0.2 mM $CdCl_2$ (an average concentration of Cd contaminated paddy fields in northern Thailand) and 0.2 mM Na_2S as substrates for CdS nanoparticles (Sakpirom et al., 2017) under microaerobic-light conditions and room temperature (23±2 °C) without mixing. The

inoculum in GA broth without the substrates served as a biotic control while the uninoculated broth with the substrates was an abiotic control. The broth was collected at 24, 48 and 60 h of incubation time and used for harvesting CdS nanoparticles.

CdS nanoparticles were harvested according to the method of Bai et al. (2009) which was modified slightly by centrifuging the broth at 4,000 rpm for 20 min. The supernatant and cell pellet were used to obtain extracellular and intracellular nanoparticles, respectively. The supernatant was centrifuged at 4,000 rpm for 5 min to collect an impurified pellet (residual compounds and CdS nanoparticles mixed together) which was later washed with 50% (v/v) ethanol to remove residual compounds. The ethanol washed pellet was centrifuged at 10,000 rpm under 4 °C for 60 min to obtain a purified pellet. The purified pellet was air dried overnight at room temperature to become powder with pale yellow color. This powder is extracellular CdS nanoparticles. Intracellular CdS nanoparticles were obtained in the same manner as the extracellular CdS nanoparticles except that the broth was boiled at 80 °C for 15 min before it was centrifuged at 4,000 rpm for 20 min.

The produced CdS nanoparticles were stable for at least 3 weeks. After centrifugation to separate CdS nanoparticles from the culture supernatant, the nanoparticles were left to air-dry. The air-dried CdS nanoparticles were kept in a microcentrifuge tube and stored at room temperature for 3 weeks or less prior to their experimental use. To ensure the stability of the CdS nanoparticles, the powder was subjected to UV-vis spectrophotoscopy before their use and the result showed that the stored CdS nanoparticles gave the same spectra as the fresh particles (before air-drying).

It is well recognized that CdS nanoparticles are produced intracellularly and then are discharged from the cells with a small amount of them remaining inside the cells (Bai et al., 2009). The intracellular and extracellular CdS nanoparticle concentrations produced in the experiment were 1 and 200 mg/L, respectively. Only extracellular CdS nanoparticles were used for experimentation because a high quantity is needed. Also, the properties of intracellular and extracellular CdS nanoparticles produced by *R. palustris* TN110 were not different based on their UV-vis patterns (Figure 4-1).

For bacterial cell morphological observations before and after the bioprecipitation (at 48 h), *R. palustris* TN110 cells in the broth were collected by centrifugation at 6,000 rpm and 4 °C for 5 min. The cell pellets were washed three times using phosphate-buffered saline (PBS) solution (pH 7.4). The bacterial cells were fixed overnight with 2% glutaraldehyde in PBS solution (pH 7.4), followed by washing for three times with PBS solution. After that, the fixed bacterial cells were dehydrated with ethanol at concentrations of 50, 60, 70 ,80, 90 and 99.9% for 15 min at each concentration. Then, the dehydrated samples were dropped on nuclepore track-etch membrane filters (a pore size of 0.2 µm) and air-dried at 26±2 °C. Finally, the air-dried samples were coated with gold and subjected to scanning electron microscopy (SEM) (SEC, Model SNE-4500M, South Korea).

Characterizations of CdS nanoparticles

UV-vis spectrophotoscopy, high resolution-transmission electron microscope (HR-TEM), energy dispersive X-ray spectroscopy (EDS), selected area electron diffraction (SAED) and Fourier transform infrared spectrometer (FTIR) were applied to characterize the biosynthesized CdS nanoparticles. The CdS nanoparticles (in the powder form prepared as described in the preceding subsection) were processed for the characterizations as follows. All the CdS nanoparticles obtained from one biosysthesis batch (50 mL of GA broth) were dissolved in deionized water by sonication for 1 h and the solution was used to obtain a UV-vis spectrum from 350 to 800 nm using a UV-vis spectrophotometer (Cary 60 UV-vis, Agilent, USA) with deionized water as a blank. The CdS nanoparticle powder of 0.05 g was prepared by drop coating onto a carbon-coated copper grid for HR-TEM and SAED analyses (JEM-2100, JOEL, USA). The nanoparticle size was analyzed from HR-TEM images by using Image J version 1.50i. Purity and elemental analyses of the CdS nanoparticles were examined by EDS attached to the HR-TEM. Approximately 0.3 mg of the CdS nanoparticles were diluted with potassium bromide powder at a ratio of 1:100 and the FTIR spectra were recorded (Nicolet 8700, Thermo Scientific, USA) in a range of 900-2,000 cm^{-1}.

Cysteine desulfhydrase acitivity assay

The culture broths collected at 0, 24, 48 and 60 h from the CdS nanoparticle production procedure described in Subsection "Preparation bacterial inoculum and CdS nanoparticle bioprecipitation" were used to investigate cysteine desulfhydrase acitivity. The preparation of cell lysate for a cysteine desulfhydrase activity assay was slighly adapted from Wang et al. (2000). One mL of the culture broth was centrifuged at 10,000 rpm for 10 min to collect a cell pellet which was then resuspended in 60 µL of 10% sucrose solution in 50 mM Tris solution (pH 7.5). The resuspended cell pellet was lysed using 75 µL of lysis buffer (10% sucrose, 300 mM NaCl, 90 mM EDTA, 3-mg/mL lysozyme, 50 mM Tris-HCl, and pH 7.5). The cell lysate was incubated on ice for 2 h.

Cysteine desulfhydrase activity was measured using a colorimetric assay (adapted from Chu et al. 1997). The assay is based on sulfide production. One µL of the lysate was mixed with 999 µL of 0.1 mM cysteine in PBS solution (pH 7.4). The mixture was incubated for 1 h at 37 °C and then added with 0.1 mL of 0.02 M *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl, and 0.1 mL of 0.3 M $FeCl_3$ in 1.2 M HCl. The sample was mixed by vortexing and incubated for 20 min. Supernatant was collected after centrifugation at 10,000 rpm for 5 min and then diluted at 1:10 ratio in deionized water. The absorbance of the diluted supernatant was measured at a wavelenght of 670 nm by a UV-vis a spectrophotometer (Cary 60 UV-vis, Agilent, USA). A calibration standard was prepared by adding sodium sulfide (Na_2S) to PBS solution at concentrations ranging from 0.0 to 0.2 mM. The cysteine desulfhydrase activity was calculated from produced sulfide (based on the absorbance) divided by g of protein in 1 µL of the cell lysate (used for the cysteine desulfhydrase activity determination). The total protein (in g) of the cell lysate was determined using a Bradford protein assay according Bradford (1976).The unit of cysteine desulfhydrase activity is mM-sulfide/g of cell lysate protein.

Optimization of CdS nanopaticle bioprecipitation

The optimal conditions for producing CdS nanopaticles under microaerobic-light conditions were investigated by considering pH, temperature and light intensity. The values for each parameter were designed based on the conditions observed in tropical paddy fields. The pH was firstly examined by varying the pH of the GA broth at 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The pH that provided the highest yield of CdS nanoparticles was considered as an optimal pH. The optimal pH was used for CdS nanoparticle production by varying the incubation temperature at 25, 30, 35, and 40 °C. Likewise, the temperature that gave the highest CdS nanoparticle yield was an optimal temperature. Finally, the optimal pH and temperature were set for CdS nanoparticle production by varying the light intensity at 1,000, 2,000, 3,000, and 4,000 lux to determine an optimal light intensity.

This experiment included controls: the inoculated medium without substrates ($CdCl_2$ and Na_2S) as a biotic control and the medium containing the substrates without the inoculum as an abiotic control. The CdS nanoparticle bioprecipitation experimental procedure to determine the optimal conditions was the same as that described in Subsection "Preparation bacterial inoculum and CdS nanoparticle bioprecipitation" except that the incubation period was 48 h. The amount of initial cells used was based an optical density at a wavelength of 660 nm (OD_{660}) of 0.07-0.08 (approximately 10^7 cells/mL). After harvesting, the produced CdS nanoparticles were weighed. It was found that the optimal conditions for CdS nanoparticle production were the same as those for the maximal bacterial growth. Percentage of substrate ($CdCl_2$ and Na_2S) tolerance was calculated by comparing the bacterial growth ($OD_{48\ h}$) between the treatment and the biotic control ($OD_{\text{treatment } 48\ h}$ and $OD_{\text{biotic control } 48\ h}$, equation 4-2). The optimal conditions obtained in this part were used for subsequent experiments.

$$\text{Conversion efficiency (\%)} = \frac{\text{Weight of CdS nanoparticle (as Cd)}}{\text{Weight of } CdCl_2 \text{ (as Cd)}} \times 100 \qquad \text{Equation 4-1}$$

$$\text{Substrate tolerance (\%)} = \frac{OD_{\text{treatment } 48\ h}}{OD_{\text{biotic control } 48\ h}} \times 100 \qquad \text{Equation 4-2}$$

Bacterial growth inhibition assay

CdS is a solid compound and insoluble in water; however, it can release Cd(II) ions with changes in physico-chemical properties of the environmental media. Hence, it is imperative to investigate toxicity of CdS nanoparticles produced by *R. palustris* TN110. The toxicity of $CdCl_2$ (representative as Cd(II) ions) and CdS nanoparticles on bacterial growth was determined as follows. *R. palustris* TN110 was grown in the GA broth (no added substrates for CdS nanoparticle production) under microaerobic-light conditions and the optimal conditions for CdS nanoparticle production/bacterial growth idenified by the experiments described in Subsection "Optimization of CdS nanopaticle bioprecipitation". The culture was centrifuged at 4,000 rpm for 20 min, and the cell pellet was resuspended in the GA broth. The re-suspended cell pellet was diluted by the GA broth to reach OD_{660} of 0.5 at which the bacterial density was approximately 10^8 cells/mL. Ten percent of the inoculum (the diluted re-suspended cell pellet) was inoculated into the GA broth supplemented with CdS nanoparticles produced by *R. palustris* TN110. The exposure concentrations for $CdCl_2$ or CdS nanoparticles were 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.25, 2.5, 5.0, 10.0 mM and the exposure time was 48 h under optimal temperaure (30 °C) and pH (7.5). After the incubation, the culture was enumerated for viable cells by a 10 fold serial-diluton using the spread plate technique and incubated under anaerobic-light conditions.

R. palustris TN110 can grow under microaerobic and anaerobic conditions. In this study, microaerobic-light condition was applied based on environmental conditions in paddy fields except for the incubation for enumeration of viable cells for the experiments on the inhibition effect of CdS and Cd(II) ion which were conducted under anaerobic condition to gain bacterial colonies that were more red and visible for counting. The half maximal inhibitory concentration (IC_{50}) was calculated from a relationship between $CdCl_2$ or CdS nanoparticle concentrations and the bacterial count. IC_{50} was the concentration of $CdCl_2$ or CdS nanoparticles that reduced 50% of the bacterial count in comparison with the growth without Cd.

Effect of CdS nanoparticles on N_2 fixation based on ammonium release

An inoculum of *R. palustris* TN110 was prepared under the optimal conditions as previously described in Subsection "Bacterial growth inhibition assay". Ten percent of the bacterial inoculum was inoculated in a nitrogen-free medium (Sakpirom et al., 2017) supplemented with the produced CdS nanoparticles based on the IC_{50} (as determined by the experiments described in Subsection "Bacterial growth inhibition assay"). Three different nitrogenases were studied by supplementing the nitrogen-free medium with the corresponding metal cofactors. The metal cofactors in the nitrogen-free medium were 80 µM of Mo (prepared from Na_2MoO_4 $2H_2O$), 80 µM of V (prepared from $NaVO_3$), and without both Mo and V to examine NH_4^+ release under Mo-Fe, V-Fe, and Fe-Fe conditions, respectively. It should be noted that the nitrogen-free medium contained 80 µM of Fe. Nitrogenase enzymes cannot function when the required cofactor is limited or absent (Oda et al., 2005).

The inoculated nitrogen-free medium without the Mo and V cofactors was used as a biotic control while an abiotic control was the nitrogen-free medium containing the metal cofactors without the inoculum. All of the tests were incubated under microaerobic-light conditions without mixing for 48 h. After the incubation, the culture broths were centrifuged at 8,000 rpm for 15 min to obtain supernatants. The supernatants were measured for NH_4^+ concentration by a salicylate method using an ammonium test kit for a range of 0.00083-0.277 mM (Hach, USA) according to the instruction by the kit manufacturer. The cell pellets were lysed to obtain cell lysate used for the determination of cysteine desulfhydrase activity using the method previously described in Subsection "Cysteine desulfhydrase acitivity assay".

Effect of CdS nanoparticles on gene expressions

To investigate whether CdS nanoparticles increase nitrogenase activity, expressions of Mo-Fe (*nifH*), V-Fe (*vnfG*), and Fe-Fe (*anfG*) nitrogenase genes were probed. Moreover, resistance-nodulation-cell division (RND) divalent metal cation efflux transporter (*czcA*) and Fe-dependent superoxide dismutase (*sodB*) genes which

are associated with bacterial defense mechanisms against heavy metal toxicity, were also examined by following the methods described by Yang et al. (2012).

Bacterial culture

The CdS nanoparticles based on the IC_{50} were used to study their effects on gene expressions. An inoculum of *R. palustris* TN110 was prepared under the optimal conditions as described in Subsection "Bacterial growth inhibition assay" and cultured in the nitrogen-free medium as described previously in Subsection "Effect of CdS nanoparticles on N_2 fixation based on ammonium release" except that the incubation time was only 24 h. Five hundred μL of the bacterial culture was centrifuged at 4,000 rpm at 4 °C for 20 min. Cell pellet was collected and resuspended in 500 μL of PBS at pH 7.4.

RNA extraction and quantitative polymerase chain reaction (qPCR)

RNA was extracted from 500 μL of the resuspended bacterial cell pellets using an RNeasy Plus Mini kit (Qiagen, USA) according to a protocol provided by the manufacturer. cDNA was synthesized using GoScript™ Reverse Transcription System (Promega, USA) according to a protocol provided by the manufacturer. Random primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) were applied. The reverse transcription process was carried out at 25 °C for 5 min followed by 42 °C for 60 min, and at 70 °C for 15 min for enzyme inactivation. Samples without the reverse transcriptase were used as negative controls. The cDNA fragments of *nifH, vnfG, anfG, czcA, sodB* and *16S rRNA* genes were amplified using a iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, USA) in 1 μL of qPCR reaction mixture consisting of 10 ng cDNA SYBR green master mix (5 μL) and 0.3 mM of each primer. 16S rRNA was used as an internal control to normalize gene expression. The fluorescence signal was monitored in an iQ5 thermocycler Real-Time PCR detection system (Bio-Rad, USA). Forward and reverse primers for *nifH, vnfG, anfG, czcA, sodB* and *16S RNA* genes are presented in Table 4-1. Expression data were

analyzed by the comparative C_T method, where C_T is the threshold cycle (Schmittgen and Livak, 2008).

The fold changes in gene expression were calculated based on the method by Schmittgen and Livak (2008) (Equations 4-3 and 4-4). C_T is the threshold PCR cycle presenting the fluorescent signal of the reporter dye detected from complementary DNA amplification. In this study, genes of interests are *nifH*, *vnfG*, *anfG*, *czcA* and *sodB* genes, whereas a *16S rRNA* gene is an internal control. The C_T results of treated (with CdS nanoparticles or Cd(II) ions) and untreated (no Cd) samples were estimated for the fold change.

$$\text{Fold change} = 2^{-\Delta\Delta C_T} \qquad \text{Equation 4-3}$$

$$2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ of treated sample} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ of untreated sample}] \qquad \text{Equation 4-4}$$

Table 4-1. Sequences of quantitative PCR primers.

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
nifH	TYGGCAAGTCCACCACC	GCGCCATCATCTCRCCGGA
vnfG	CGGGATCCGAAGACTTYGARAAGG TCAT	GCAGTCGTACATCGGGTT
anfG	GCTCTAGACGCSATCTAYTCGCCGA	CGGAATTCCGATGCAATCCTTGA T
czcA	GCGTGGTGATGCTGATCTAT	ATGATCGCCTCGGCAATATC
sodB	TGGGAGCACTCCTACTACAT	AGTACAGCTCCTCGACATACT
16S rRNA	GTCTTCGGATTGTAAAGCAC	GCTACACAAGGAAATTCCAC

Note: The primers were designed using a freeware program available at https://sg.idtdna.com/scitools/Applications/RealTimePCR/.

Statistical analyses

All experiments were conducted in triplicate unless otherwise stated. Means and standard deviations are presented. One-way analysis of variance was used to analyze the data and statistical differences among means were determined by the Tukey's test at $P < 0.05$.

Results

Characteristics of CdS nanoparticles, bacterial cells and cysteine desulfhydrase acitivity

The UV-vis spectra for the extracellularly and intracellularly produced CdS nanoparticles, biotic control and abiotic control are shown in Figure 4-1. The spectra for the biotic and abiotic control, which almost completely overlap, do not show any peaks for both extracellular and intracellular cases (Figure 4-1A and B) suggesting no production of CdS nanoparticles. For the case of extracellular production (Figure 4-1A), peaks at the same wavelength of 405 nm corresponding to a UV-vis absorbance characteristic of CdS nanoparticles were observed at all three incubation periods studied (24, 48, and 60 h). The peak increased with incubation time. The UV-vis absorbance spectra for the produced CdS nanoparticles agree well with previous studies (350-450 nm) (Osipyonok et al., 2008; Bai et al., 2009; Prasad and Jha, 2010; Raj et al., 2016).

The characteristic peak was observed only at the incubation period of 48 h for the case of intracellular production (Figure 4-1B). It should be noted that the spectrum for the incubation period of 24 h is difficult to see in Figure 4-1B as it overlaps with that of the biotic control. The absorbance levels of the characteristic peaks for the incubation period of 48 h for the extracellular and intracellular production cases are comparable but much lower than that of the incubation period of 60 h for the extracellular production case.

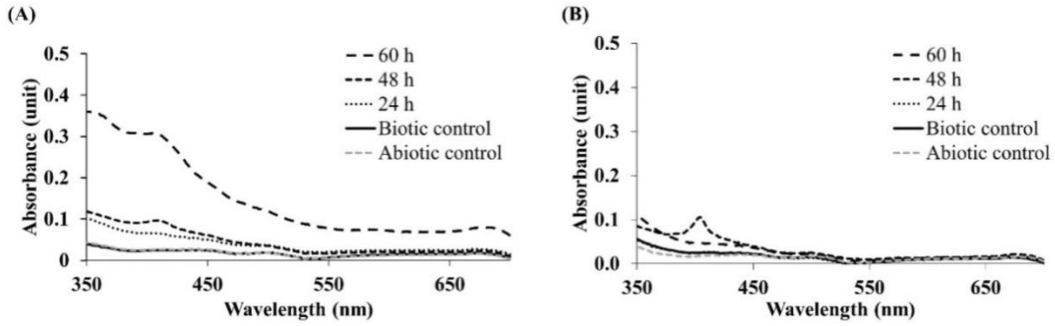

Figure 4-1. UV-vis spectra of (A) extracellular and (B) intracellular biosynthesized CdS nanoparticles by *R. palustris* TN110 under microaerobic-light conditions at room temperature (23±2 °C).

The bacterial cell shapes before and after the bioprecipitation were different (Figure 4-2). Before the bioprecipitation, the cells were in a typical rod shape (Figure 4-2A) whereas after the bioprecipitation, their shape was abnormal (nodular and withered cells) (Figure 4-2B). This is because a transcriptional unit czcABC involving efflux pump system played a role on bacterial cell nodulation and elongation (Yang et al., 2012; Alvarez-Ortega et al., 2013) (Result on *czcA* gene expression is presented in Result Subsection "Effect of CdS nanoparticles on bioligical N_2 fixation based on NH_4^+ release").

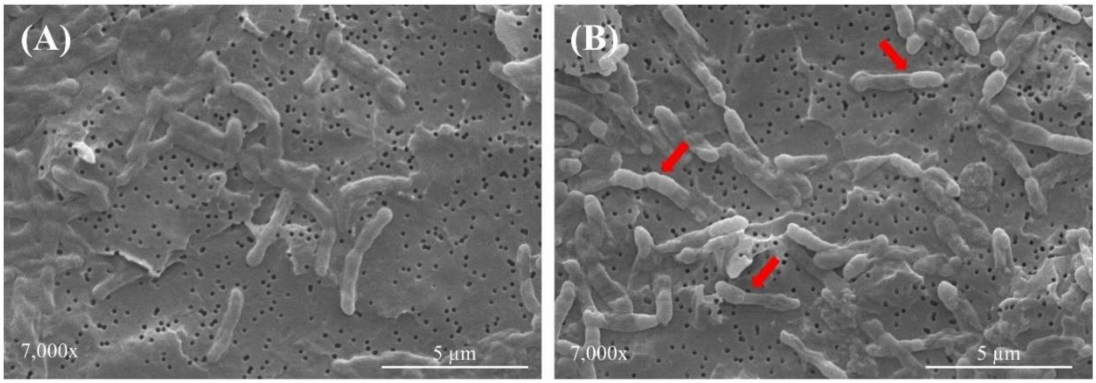

Figure 4-2. SEM images of *R. palustris* TN110 before (A) and after (B) the bioprecipitation of CdS naoparticles. Red arrows indicate abnormal cells based on change of cell shape.

The extracellular CdS nanoparticles were characterized by HR-TEM equipped with SAED and EDS. The representative HR-TEM images of the bioprecipitated CdS nanoparticles are shown in Figure 4-3A. The CdS nanoparticles were essentially spherical in shape and had a uniform size distribution of 3-6 nm with an average size of 4.85 nm (Figure 4-3B). The SAED pattern of these particles suggests a polycrystalline cubic crystal structure (inset of Figure 4-3A). Lattice imaging reveals that the nanocrystals are cubic with a-d spacing of 3.61Å. The EDS pattern shows clearly Cd and S elements in the sample (Figure 4-4). Cl was detected and was likely from $CdCl_2$ that was used as the substrate. Cu is from the Cu grid used for the sample preparation.

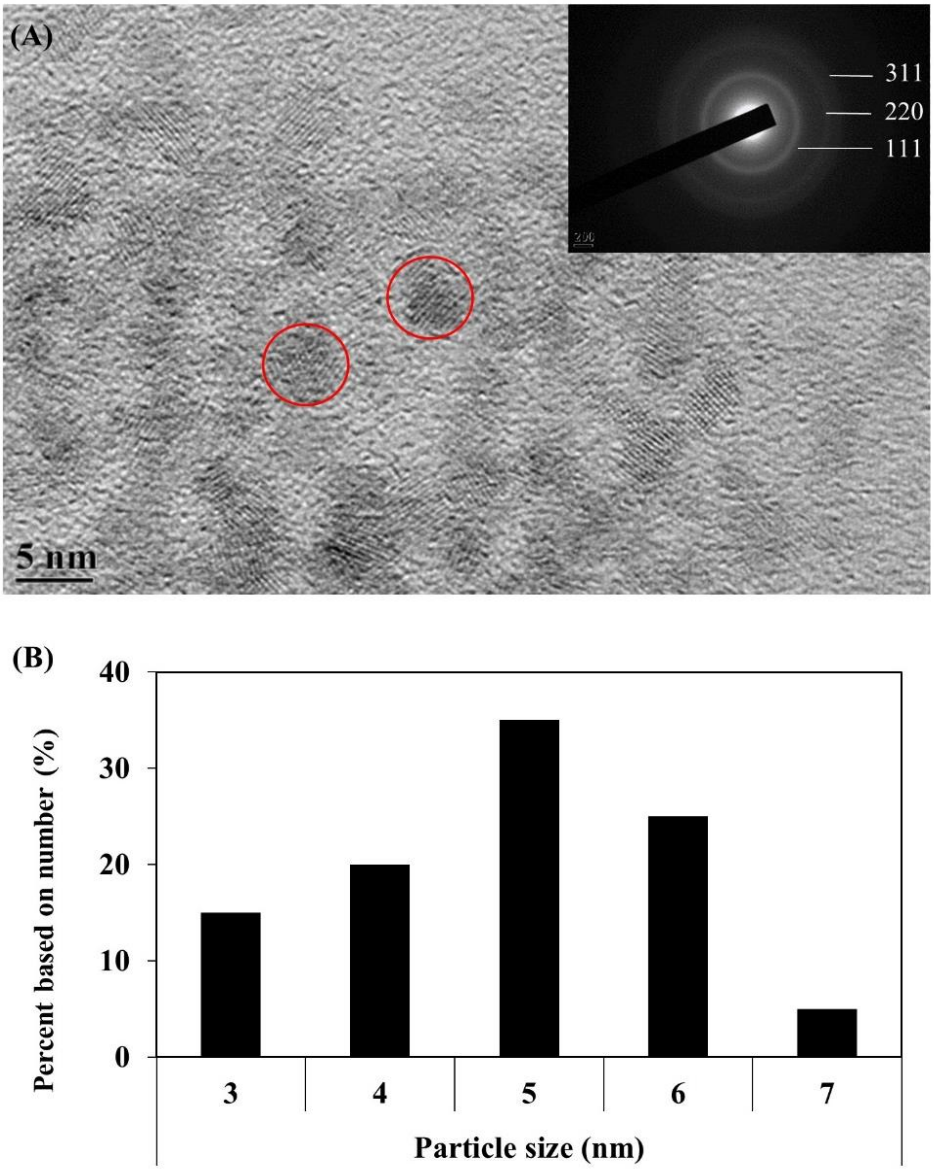

Figure 4-3. (A) HR-TEM micrographs of extracellular CdS nanoparticles (red circles) biosynthesized by *R. palustris* TN110 under microaerobic-light conditions at room temperature (23±2 °C) for 48 h. *Inset*: selected area electron diffraction patterns of the nanocrystals, indicating polycrystalline cubic CdS and (B) Particle size distribution histogram determined graphically from the TEM micrographs.

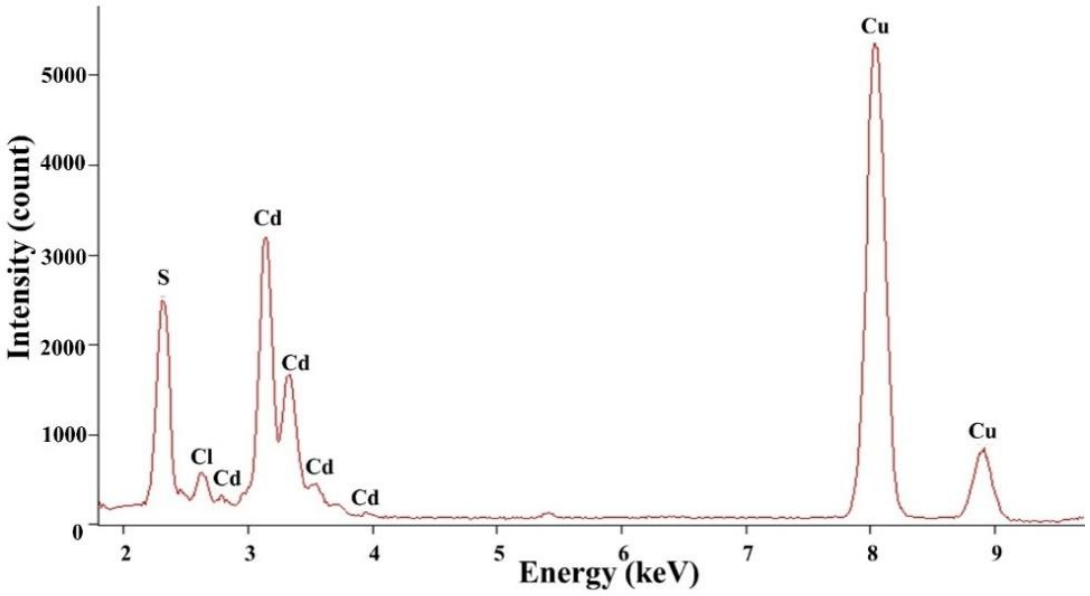

Figure 4-4. EDS spectrum of extracellular CdS nanoparticles biosynthesized by *R. palustris* TN110 under microaerobic-light conditions at room temperature (23±2 °C) for 48 h.

Figure 4-5 presents the FTIR spectrum of the extracellular CdS nanoparticles. The wavenumber at 1,718 cm^{-1} was assigned as a peak of C=O stretching (Pornsuksomboon et al., 2016), whereas the peaks at 1,636 cm^{-1} and 1,540 cm^{-1} are characteristic peaks of the bending vibrations of the amide I and II bands of proteins, respectively (Bai et al., 2009).The wavenumbers at 1,380 cm^{-1} and 1,050 cm^{-1} relate to the C-N stretching vibrations of the aromatic and aliphatic amines, respectively (Bai et al., 2009). The wavenumbers at 1,207, 1,157 and 1,116 cm^{-1} may be due to stretching and vibration between N-H (Prasad et al., 2017). The cysteine desulfhydrase activities of *R. palustris* TN110 under microaerobic-light conditions for Mo-Fe, V-Fe, Fe-Fe cofactors for N_2 fixation were not significantly different: 1.31±0.32, 1.29±0.47 and 1.27±0.24 mM-sulfide/g of cell lysate protein, respectively (Table 4-2).

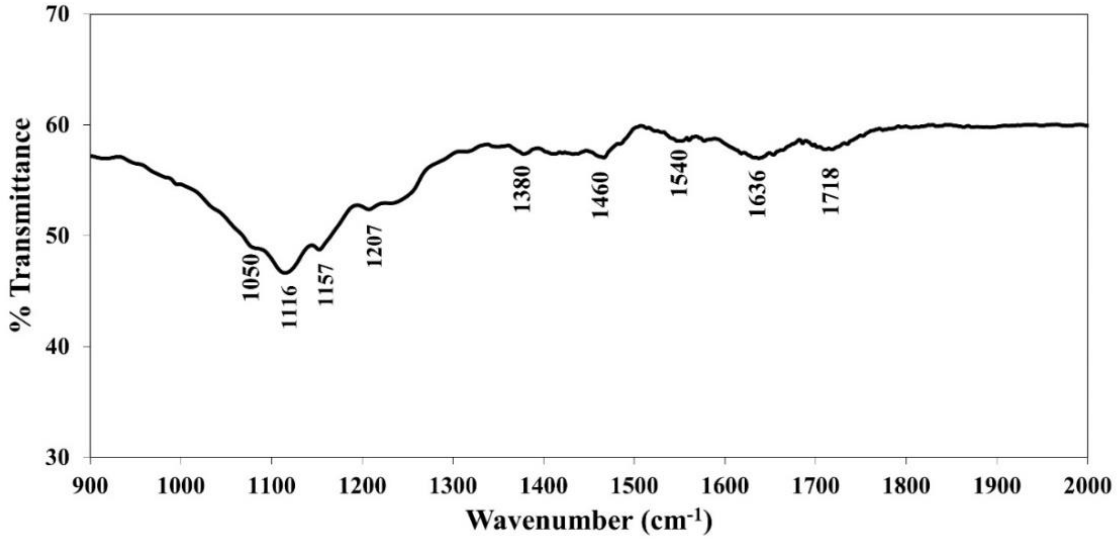

Figure 4-5. FTIR spectrum of extracellular CdS nanoparticles biosynthesized by *R. palustris* TN110 under microaerobic-light conditions at room temperature (23±2 °C) for 48 h.

Table 4-2. Cysteine desulfhydrase activity and NH_4^+ release by *R. palustris* TN110 under miroaerobic-light conditions under different nitrogenases for N_2 fixation.

Tests	Nitrogenases		
	Mo-Fe	V-Fe	Fe-Fe
Cysteine desulfhydrase activity (mM-sulfide/g of cell lysate protein)	1.31 ± 0.32^{a}	1.29 ± 0.47^{a}	1.27 ± 0.24^{ab}
NH_4^+ release (mM)			
• Exposure to CdS nanoparticles	0.51 ± 0.02^{aA}	0.25 ± 0.01^{bA}	0.02 ± 0.01^{cB}
• Exposure to Cd(II) ions	0.20 ± 0.01^{aB}	0.09 ± 0.03^{bC}	nd
• No exposure (biotic control)	0.18 ± 0.01^{aC}	0.11 ± 0.01^{bB}	0.05 ± 0.01^{cA}

Mean and standard deviation of three replicates are presented. Different lowercase letters in the same row and different uppercase letters in the same column indicate significant differences ($P < 0.05$). nd = not detected.

Optimization of CdS nanopaticle bioprecipitation

The optimal conditions for the highest extracellular CdS nanoparticle bioprecipitation yield by *R. palustris* TN110 were initial pH 7.5, 30 °C and light intensity of 3,000 lux (Table 4-3). The optimal conditions provided a CdS nanoparticle conversion efficiency up to 25.61% (calculated by equation 4-1) based on the initial $CdCl_2$ concentration of 0.2 mM. Inhibitory effect of Cd(II) ions on the bacterial growth was mitigated by the bioprecipitation of CdS nanoparticles with the maximal tolerance of 59.74% (calculated by equation 4-2) under the optimal conditions for CdS nanopaticle bioprecipitation.

Bacterial growth inhibition assay

The IC_{50} results indicate growth inhibition of *R. palustris* TN110 exposed to biosynthesized CdS nanoparticles or Cd(II) ions. The IC_{50} values were 1.76 mM for CdS nanoparticles and 0.82 mM for Cd(II) ions. These IC_{50} were used to study their effects on biological N_2 fixation. Higher IC_{50} indicates lower toxicity to bacterial growth. *R. palustris* TN110 exposed to CdS nanoparticles had approximately twice higher IC_{50} than those exposed to Cd(II) ions. It is known that the bioprecipitation of CdS nanoparticles is one of the mechanisms for alleviation of heavy metal toxicity of microorganisms (Bai et al., 2009; Edwards et al., 2013).

Effect of CdS nanoparticles on bioligical N_2 fixation based on NH_4^+ release

NH_4^+ release was the highest under Mo-Fe supplementation (Table 4-2). Exposure to CdS nanoparticles and Cd(II) ions showed increases of NH_4^+ release up to 2.83 and 1.11 times higher, respectively, compared to the non-exposure treatment (biotic control). For V-Fe condition, only exposure to CdS nanoparticles showed increase of NH_4^+ release to 2.27 times more.

The results of NH_4^+ release agree with nitrogenase gene expreesions. *nifH* and *vnfG* encoding for Mo-Fe and V-Fe nitrogenases, respectively were up-regulated by exposure to CdS nanoparticles up to 2.57 and 1.60 fold changes (compared to non-

exposure) while *anfG* encoding for Fe-Fe nitrogenase was down-regulated (Figure 4-6A). For Cd(II) ion exposure, only *nifH* was slightly up-regulated (1.31 fold change) (Fig 4-6B). Moreover, the expressions of *czcA* and *sodB* genes under different N_2 fixation conditions were up-regulated by IC_{50} exposure to the CdS nanoparticles in the ranges of 3.73-5.73 and 2.20-3.64 fold changes, respectively (Figure 4-6A). These trends were also the cases for Cd(II) ion exposure (Figure 4-6B). Positive changes of the expression levels of genes associated with heavy metal efflux pumps (*czcA*) and antioxidant enzyme (*sodB*) were observed and are not substantially different for all the different N_2 fixation conditions (Figure 4-6A and B).

Table 4-3. Optimization of CdS nanoparticle production using *R. palustris* TN110.

Parameters	Conversion efficiency (%)	Bacterial tolerance (%)
pH		
6	1.40	35.25
6.5	14.97	44.07
7	17.19	59.85
7.5	22.81	55.91
8	11.93	55.01
8.5	8.54	53.32
9	3.27	45.27
Temperature (°C) at pH 7.5		
25	16.37	58.23
30	23.51	60.16
35	18.25	55.60
40	2.22	32.21
Light intensity (lux) at pH 7.5 and 30 °C		
1,000	15.91	55.43
2,000	20.58	58.96
3,000	25.61	59.74
4,000	24.21	58.56

Note:
1) The conversion efficiency at 100% refers to the weight of produced CdS nanoparticles (as Cd) being equal to the weight of initial $CdCl_2$ (as Cd). The efficiency at 0% means no CdS nanoparticles produced.
2) The bacterial tolerance at 100% refers to bacterial growth (measured by OD_{660}) from the bioprecipitation ($OD_{\text{treatment 48 h}}$) being equal to the growth of the control (without Cd and S) ($OD_{\text{biotic control 48 h}}$). The bacterial tolerance at 0% means no bacterial growth from the bioprecipitation.

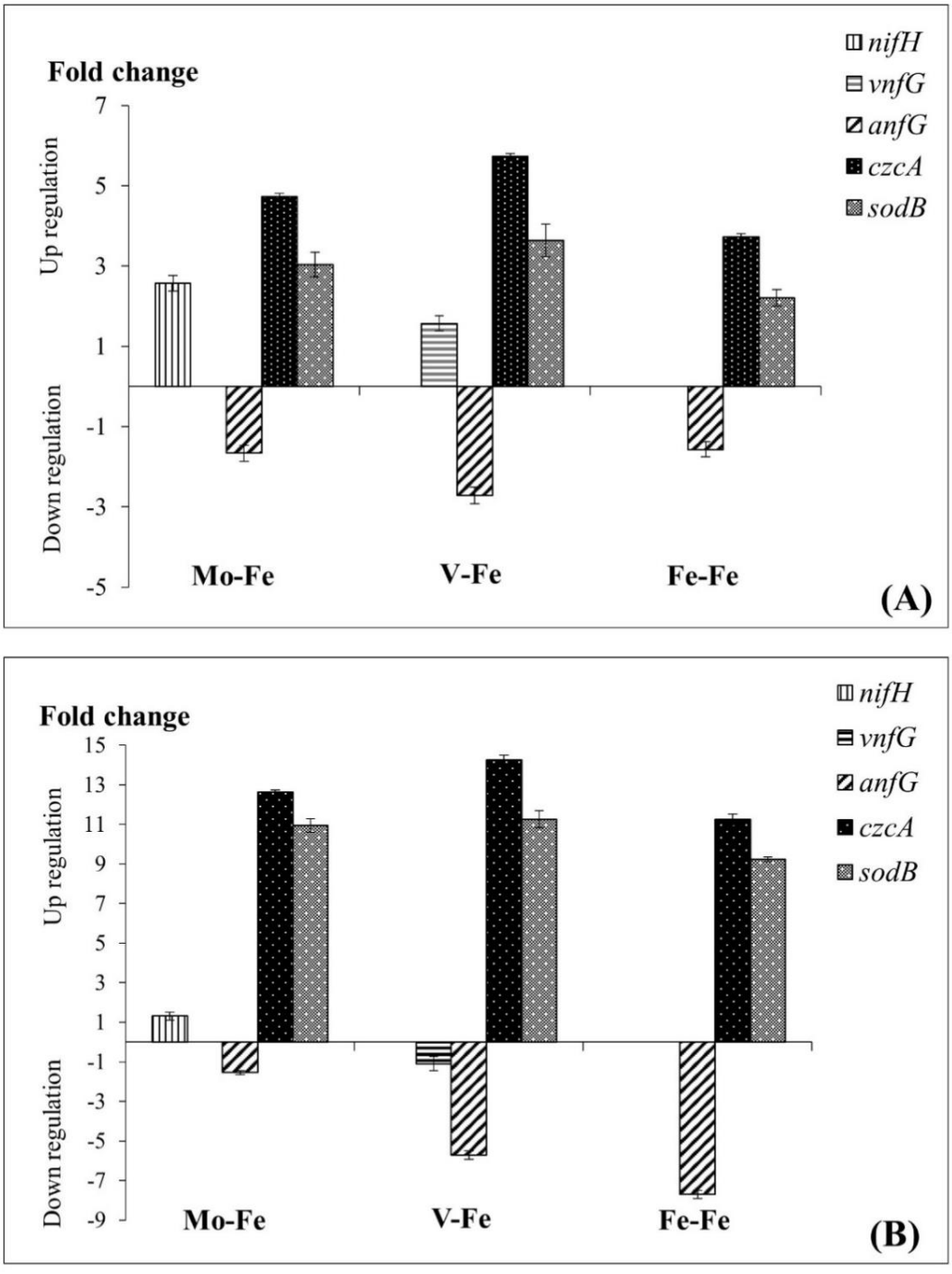

Figure 4-6. *nifH, vnfG, anfG, czcA,* and *sodB* gene expressions of *R. palustris* TN110 exposure to IC_{50} of (A) CdS nanoparticles and (B) Cd(II) ions under miroaerobic-light conditions for different N_2 fixation mechanisms: Mo-Fe, V-Fe, and Fe-Fe.

Discussion

The CdS nanoparticles synthesized in this study were found mostly outside the cells with minimum accumulation of the nanoparticles inside the cells. This result is in agreement with Bai et al. (2009) that reported that CdS nanoparticles were biosynthesized intracellularly and then they were discharged from cells. Previous studies observed a peak in the UV-vis spectrum corresponding to CdS nanoparticles in a wavelength range of 350-450 nm (Osipyonok et al., 2008; Bai et al., 2009; Prasad and Jha, 2010; Raj et al., 2016). The exact wavelength of the peak depends on their size and biosynthesis conditions. The UV-vis spectra of CdS nanoparticles in this study agreed with the spectra reported in these previous studies. High absorbance levels of the peak suggest high quantities of the nanoparticles. In this study, the highest quantities of the extracellular and intracellular CdS nanoparticles were obtained at 60 and 48 h incubation time, respectively while the intracellular CdS nanoparticles were barely or not detected at 24 and 60 h incubation time (Figure 4-1).

Cysteine desulfhydrase has been known as an intracellular enzyme for the production of CdS nanoparticles and then the particles are transported out of the cells (Bai et al., 2009; Qi et al., 2016). Production of CdS nanoparticles increases as the growth progresses (Bai et al., 2009). The extracellular CdS nanoparticles increased with time because the produced CdS nanoparticles were transported via the efflux system and accumulated outside the cells in order to reduce the toxicity (of the produced CdS nanoparticles) particularly during more susceptible growth stages (early exponential growth phase at 24 h and late exponential growth phase at 60 h). That is why the intracellular CdS nanoparticles were detected only at 48 h corresponding to middle exponential growth phase during which the cells are more tolerant (Rolfe et al., 2012).

In this study, cysteine desulfhydrase activity was present in all the different conditions for N_2 fixation (Table 4-2) indicating none of the metal cofactors completely inhibited the enzyme. The bioprecipitation by *R. palustris* TN110 was experimented under broard ranges of environmental conditions that are common for tropical agricultural areas (Roger, 1996)and amenable to PNSB growth (Brenner et al., 2005) (Table 4-3). *R. palustris* TN110 has potential as an effective microorganism

for remediation of Cd contaminated areas including paddy fields through bioprecipitation of CdS nanoparticles.

The FTIR spectrum of extracellular CdS nanoparticles (Figure 4-5)suggests that the formation and stabilization of the CdS nanoparticles involved molecular binding with cellular compounds. The carbonyl groups from amino acid residues and peptides have a strong ability to bind metals. They could form a coat covering the metal nanoparticles. The coat promoted agglomeration of the nanoparticles in the medium (Raj et al., 2016). It has been reported that proteins can bind to gold nanoparticles through either free amine groups or cysteine residues (Gole et al., 2001). Moreover, time of incubation is an important parameter to control the size of nanoparticle agglomeration during bioprecipitation (Bai et al., 2006; Raj et al., 2016).

One of the mechanisms for biofertilization is nitrogen fixation to generate nitrogen for plant growth. Nitrogenase enzymes are important for nitrogen fixation and are categorized based on their different cofactors at the active sites. In addition, they have different energy requirements and enzyme specific activities (Bellenger et al., 2014). CdS nanoparticles are known as a photosensitizer that can harvest energy from light for use biological nitrogen fixation (Brown et al., 2016) but are toxic to bacterial cells including *R. palustris* TN110. The CdS nanoparticles (IC_{50} dose) up-regulated Mo-Fe (*nifH*) and V-Fe (*vnfG*) nitrogenase genes (Figure 4-6A)and the gene expression fold changes corresponded with the amounts of NH_4^+ release (Table 4-2). However, this up-regulation was not found in the case of Fe-Fe nitrogenase gene (*anfG*) because the corresponding enzyme (Fe-Fe nitrogenase) is the strongest reductant or demands the most energy and has the lowest enzyme specific activity among the nitrogenases (Oda et al., 2005). Basically, the enzyme is not a promising nitrogenase for N_2 fixation.

The *czcA* and *sodB* genes were up-regulated (Figure 4-6A and B). The *czcA* gene is associated with a divalent metal cation efflux transporter of a transcriptional unit czcABC (encoding a resistance-nodulation-cell division) (Yang et al., 2012). The czcABC proteins comprise a three-component transporter that spans entire cell wall, resulting in efflux of heavy metal ions, including cadmium from both cytoplasm and periplasmic space. The expression of *czcA* gene in this study was well related to the detection of extracellular CdS nanoparticles tied to the efflux system (Figure 4-1A

and B) suggesting that the CdS nanoparticles were released from the bacterial cells via the function of *czcA* gene. Typically, free radicals are produced as organisms are exposed to toxic substances (including CdS nanoparticles). Organisms including bacteria produce antioxidative enzymes to combat with environmental stress (Kashmiri and Mankar, 2014). Among them, *sodB* gene is the gene that governs iron-dependent superoxide dismutase that can destroy free radicals (Yang et al., 2012). The up-regulation of *sodB* during the exposure to CdS nanoparticles suggests that strain TN110 used this enzyme for reducing the toxicity of Cd as well.

CdS nanoparticles might contain and/or release Cd(II) ions and there was no way to decipher the effects of these two chemical species. However, if Cd(II) was present, its amount and effect would be limited as there was an improvement in nitrogen fixation under CdS nanoparticles compared to Cd(II) ions (Figure 4-6).The accumulation of CdS in the natural environment, for example paddy fields, is in a broad range. The level of Cd contamination in paddy fields in Thailand ranged from 0.005 to 12.6 mM (Khaokaew et al., 2011; Sakpirom et al., 2017). Therefore, the CdS level will be sufficient to promote nitrogen fixation of *R. palustris* TN110 for some but not all sites.

The extracellular CdS nanoparticles will be in the vicinity of the cells. A small amount of the produced CdS nanoparticles remains in the cells and promotes nitrogen fixation (Figure 4-1). The extracellular particles around the cells can act as a light harvester and release electrons. Some of the released electrons are transported into the cells and promote the production of cysteine desulfhydrase and in turn CdS nanoparticles and consequently nitrogen fixation (Fleming, 2009).Fate of CdS nanoparticles including half-life in the environment particularly in paddy fields is recommended for future work.

Conclusions

R. palustris TN110 was studied for its performances as a concurrent nitrogen fixation and bioremediation agent. For bioremediation, the strain bio-precipitated Cd(II) ions into uniform CdS nanoparticles. The presence of functional groups of protein coating produced by the strain as a stabilizing agent for CdS nanoparticles was observed. Cysteine desulfhydrase known as a responsible enzyme for nanoparticle formation was found and had similar activity levels under different metal cofactors for N_2 fixation. Furthermore, Mo-Fe and V-Fe nitrogenase genes for N_2 fixation were up-regulated by CdS nanoparticles at IC_{50}. This study demonstrates the potential of *R. palustris* TN110 for the reduction of Cd(II) ion toxicity through bioprecipitation of CdS nanoparticles. Simultaneously, the CdS nanoparticles serve as a photosensitizer to increase biological nitrogen fixation that leads to more NH_4^+ release for plant growth.

CHAPTER 5

Optimizing Combined Agro-Industrial Waste Carrier and Packaging for Purple Non-Sulfur Bacteria Biofertilizer and Obtained Enhancement of Rice (*Oryza sativa* L.) Seedling Growth in Paddy Soil

Abstract

For a biofertilizer to promote plant growth after application, cell survival during storage must be ensured. The carrier formulation and packaging are important factors during storage. This study aimed to optimize the ratio of carriers of rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA), and spent coffee grounds (SCG) to formulate a mixed carrier to support a biofertilizer of purple non-sulfur bacteria (PNSB). The phytotoxicity of the PNSB biofertilizer in both solid and liquid formulations, and its ability to enhance rice (*Oryza sativa* L.) seedling growth in paddy soil were also investigated. D-optimal experimental design based on response surface methodology predicted the most optimal mixed carrier to consist of RWA, DCC, RHA and SCG at a ratio of 3: 4: 2: 1. This mixed carrier contained roughly 10 log CFU/g *Rhodopseudomonas palustris* TN110. Packed in nylon-LLDPE bags and vacuum sealed at 500 W, the PNSB population had decreased by only 3 log CFU cycles after storage for 6 months at room temperature (25±3 °C). All PNSB biofertilizers, strain TN110, *Rubrivivax gelatinosus* TN414 and a mixed culture of both strains at 1: 1, either in solid or liquid form showed no phytotoxicity, whereas the optimal mixed carrier as control was slightly toxic. At the optimal dilution, each biofertilizer formulation from liquid and solid forms in both single culture and mixed cultures increased rice growth compared with controls. PNSB biofertilizers can be produced in both liquid and solid forms, while the solid formulation with an optimal mixed carrier and a suitable packing system prolonged shelf life to 6 months.

Keywords: Biofertilizer, Carrying material, Packaging, Phototrophic bacteria, Phytotoxicity, Vacuum sealing

บทคัดย่อ

การนำปุ๋ยชีวภาพไปใช้เพื่อส่งเสริมการเจริญของพืชจำเป็นต้องแน่ใจว่าเซลล์จุลินทรีย์ที่เป็นปุ๋ยชีวภาพยังมีชีวิตรอดพร้อมที่จะเติบโต ซึ่งกระบวนการผลิตและการบรรจุปุ๋ยชีวภาพเป็นสิ่งสำคัญเพื่อรักษาการอยู่รอดของจุลลินทรีย์ระหว่างการเก็บรักษา ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อหาสัดส่วนที่เหมาะสมของการผสมวัสดุพยุงได้แก่ ขี้เถ้าเหลือใช้จากกระบวนการผลิตยางพาราแผ่น กากปาล์มน้ำมัน ขี้เถ้าจากแกลบข้าว และกากกาแฟ ที่สามารถช่วยในการเก็บรักษาเซลล์แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ สายพันธุ์ *Rhodopseudomonas palustris* TN110 เพื่อผลิตปุ๋ยชีวภาพในรูปของแข็ง จากการศึกษาพบว่า สัดส่วนที่เหมาะสมของปริมาณของขี้เถ้าเหลือใช้จากกระบวนการผลิตยางพาราแผ่น กากปาล์มน้ำมัน ขี้เถ้าจากแกลบข้าว และกากกาแฟ คือ 3: 4: 2: 1โดยใช้โปรแกรมออกแบบชุดการทดลองแบบ D-optimal สัดส่วนดังกล่าวถูกนำไปผสมกับแบคทีเรียสายพันธุ์ TN110 ความเข้มข้นประมาณ 1 หมื่นล้านเซลล์ต่อกรัม บรรจุลงในถุงชนิดไนลอน-พอลิเอทิลีนความหนาแน่นต่ำเชิงเส้น (Nylon-LLDPE) และปิดผนึกโดยใช้แรงดูดสูญญากาศที่กำลังไฟฟ้า 500 วัตต์ พบว่าปริมาณแบคทีเรียสายพันธุ์ดังกล่าวลดลงร้อยละ 30 หลังจากเก็บไว้ที่อุณหภูมิห้อง (25±3 องศาเซลเซียส) เป็นเวลา 6 เดือน หลังจากนั้นศึกษาความเป็นพิษของปุ๋ยชีวภาพดังกล่าวต่อดัชนีการงอกของเมล็ดข้าว (*Oryza sativa* L.)ซึ่งปุ๋ยชีวภาพที่ใช้ได้แก่ แบคทีเรียสายพันธุ์ TN110 หรือ TN414 หรือ ผสมทั้ง 2 สายพันธุ์ในอัตราส่วนเท่ากัน โดยทดสอบทั้งรูปแบบของเหลวและของแข็ง จากการทดสอบพบว่าปุ๋ยชีวภาพทุกสูตรไม่มีความเป็นพิษต่อดัชนีการงอกของเมล็ดข้าวที่อัตราการเจือจางที่เหมาะสมของแต่ละชุดการทดสอบ

คำสำคัญ : การบรรจุภัณฑ์ การปิดผนึกโดยใช้แรงดูดสุญญากาศ ความเป็นพิษต่อพืช วัสดุพยุง แบคทีเรียสังเคราะห์แสงปุ๋ยชีวภาพ

Introduction

Biofertilizers, or plant growth promoting bacteria (PGPB), are an attractive choice for organic agriculture instead of chemical fertilizers (Paul et al., 2013). Purple non-sulfur bacteria (PNSB) are a suitable choice for application in paddy fields as they not only fix N_2 but also release plant growth promoters, such as the phytohormone-like substances indole-3-acetic acid (IAA) and 5-aminolevulinic acid (ALA) (Sakpirom et al., 2017; Nookongbut et al., 2018). They grow in both aquatic and terrestrial environments and can survive in photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic modes (Larimer et al, 2004; Sakpirom et al., 2017).

However, when formulating PGPB, whether from one or several strains of microorganism, an effective carrier is required. Carriers support the survival of PGPB by providing a safe environment that protects them from stressful conditions during storage. They also ensure their survival while they establish themselves in fields (Singh et al., 2011; Herrmann and Lesueur, 2013; Kantha et al., 2015). The formulation of any effective microorganisms are multistep processes which resulted in one or more strains being contained in a carrier together with sticking agents or other additives which help protect the cells during storage and transport (Xavier et al., 2004; Kantha et al., 2015). The perfect formulation does not exist as each type has its advantages and limitations (Herrmann and Lesueur, 2013). Therefore, the formulation of carriers must be carefully investigated along with conditions that support the survival of biofertilizers. Practically, carriers made from dry solid or granular formulations is preferred due to ease of handling and applications as biofertilizer(s)(Herrmann and Lesueur, 2013). The solid waste from rubber wood-fired boilers, known as rubber wood ash (RWA), has potential as a carrier material for biofertilizer (Demeyer et al., 2001; Park et al., 2005; Gaind and Gaur, 2004; Kumar and Gupta; 2010) and rubber production is one of the most important agricultural activities in Southern Thailand (Saritpongteeraka and Chaiprapat, 2008). Decanter cake (DCC) is a by-product from palm oil production, another important activity in the economy of Southern Thailand (Chavalparit et al., 2006). It is a good candidate as carrier material for biofertilizer because it can improve soil quality and plant growth

(Embrandiri et al., 2013). Also, rice husk ash (RHA) and spent coffee grounds (SCG), by-products of rice milling and coffee brewing, respectively, have been applied to support plant growth. SCG, especially, is a rich source of potassium, magnesium and phosphorus (Cruz et al., 2012). New applications for these materials would help in reducing agro-industrial waste and add value to them.

This study aimed to determine an optimal carrier formulation for PNSB using response surface methodology (RSM). RSM is a good approach to predict the optimal proportions of carriers and the D-optimal model is the design normally applied to experiments of optimized conditions (Yin et al., 2009). The shelf life of biofertilizers can also be affected by packing methods and packaging materials. We investigated polypropylene (PP), polyethylene terephthalate-nylon-polypropylene (PET-nylon-PP) and nylon-linear low density polyethylene (nylon-LLDPE) packaging and different sealing methods to obtain the optimal packaging material and sealing method. Finally, the phytotoxicity of PNSB strains was determined based on seed germination and their efficacy at improving rice growth in paddy soil under greenhouse conditions was examnied.

Materials and methods

Preparation of PNSB inoculums

Rhodopseudomonas palustris TN110 and *Rubrivivax gelatinosus* TN414, isolated from saline paddy fields in Southern Thailand (Nunkaew et al., 2015), were selected for this study for their properties as both plant growth promoters and biofertilizers (Sakpirom et al., 2017). To prepare PNSB inoculants, both PNSB strains were separately sub-cultured twice by inoculating in GA broth to obtain active inoculums, and 10% of each culture was transferred to GA broth in a screw cap test tube (Sakpirom et al., 2017). All culture tubes were incubated under their optimal microaerobic-light conditions for 48 h (Sakpirom et al., 2017). Afterwards, the culture broths were centrifuged at 6,000 rpm for 15 min to obtain cell pellets. Each biomass was adjusted to an optical density of 2.0 at a wavelength of 660 nm (OD_{660}) using 0.1% peptone water to provide a cell density of approximately 10^9 CFU/mL. Only *R.*

palustris TN110 based on their high effective performance as biofertilizer and bioremediation agent, was used as biofertilizer in the experiments that optimized ratios of carriers to obtain an optimal mixed carrier.

Optimization of carrier formulations for biofertilizer

Carrier characterization and preparation

RWA, DCC, RHA and SCG were used as carriers to produce biofertilizer in a solid form. The characteristics of these carriers (Table 5-1), individually and in the optimized mixed carrier, were determined as follows. Values of pH and electrical conductivity (EC) were measured by a pH and EC meter (SevenMulti, Mettler Toledo, Switzerland). The Walkley and Black method was used to determine organic matter and organic carbon. The percentage of ash was obtained by igniting a sample at 600 °C. Total nitrogen (N) and total sulfur (S) were determined by combustion and photometric methods, respectively. The methods used are standard methods described in Sparks et al. (1996). Inductively coupled plasma-optical emission spectrometry (ICP-OES) (Perkin Elmer, Germany) was used to determine total P_2O_5, total K_2O, and extractable elements as shown in Table 5-1.

Each carrier was individually well ground manually and then filtered through a sieve with a pore size of 0.5 mm, sterilized at 121 °C for 30 min, dried in an oven at 60 °C until constant weight was reached and then cooled down to room temperature (25±3 °C). Carriers at different proportions for experiments (described later) were prepared and packed in sterile polypropylene bags (size 12 × 18 cm). Each bag had approximately 100 g of a mixed carrier and it was tightly closed using a rubber band.

Ratio optimization of RWA and DCC for maximal PNSB survival

The proportions of RWA and DCC for a mixed carrier were optimized first because they are the most abundant by-products of agro-industry in Southern Thailand (Saritpongteeraka and Chaiprapat, 2008; Chavalparit et al., 2006). Different design values for RWA (X_1) and DCC (X_2) were predicted using RSM. Design Expert

Version 6.0.2 software (Stat-Ease Inc., USA) was used for all experimental designs in this study. To describe the nature of the response surface in the experimental region, D-optimal design was applied. There were 9 experimental runs as shown in Table 5-2A. The proportion of mixed carriers that produced the highest PNSB survival response was determined from the regression equation and analysis of the response surface contour plots using the same software. The quality of fit of the model equations was expressed by the coefficient of determination (R^2). The optimal ratio obtained from the prediction was confirmed by verification sets (Table 5-2B).

Each bag of combined carrier was inoculated aseptically with 60 mL inoculant of PNSB strain TN110 to obtain approximately10^8 cells/g. PNSB biofertilizer and sets of the mixed carrier were adjusted to a final moisture content of 40 % by adding mature coconut water which were suitable for an adjustment of PNSB biofertilizer (Kantha et al. 2015) and mixed well in a Stomacher® blender. After that, they were transferred into sterile polypropylene bags (size 4×6 cm). Each bag had approximately 10 g of the biofertilizer and was machine-sealed without applying vacuum (P-200, Fiji Impulse, Thailand). All sample bags were stored in darkness at room temperature (25±3 ℃) and also at 4 ℃ for comparison. Moisture content loss, pH, EC and PNSB population, were measured every week for 4 weeks. To measure pH and EC, each sample was diluted with distilled water (DW) at 1: 5, while PNSB were counted by a spread plate technique and incubated under anaerobic light condition. The reduction of moisture content of bags containing PNSB biofertilizer was calculated from the weight loss. The optimal proportions of combined carriers that provided the highest PNSB survival was obtained by solving the regression equation and analyzing the response surface contour plots using the software.

Packaging bag permeability test

Polypropylene (PP), polyethylene terephthalate-nylon-polypropylene (PET-nylon-PP) and nylon-linear low density polyethylene (nylon-LLDPE) packaging bags (size 4 × 6 cm) were used in this study. The permeability of the bags was determined from the weight gain of silica gel. Fifteen grams of dried silica gel was packed in PP, PET-nylon-PP and nylon-LLDPE bags and the bags were machine-sealed without

applying vacuum. The bags were kept in a walk-in humidity chamber at 35 °C and 80% relative humidity. The bags containing silica gel were weighed daily for four days. Relationships between weight gains and time were generated.

Since the results of the permeability test for PET-nylon-PP and nylon-LLDPE packaging bags were almost the same, only PP and PET-nylon-PP bags were used in further tests to optimize the ratio of RWA and DCC in the mixed carrier. These tests followed the same packaging protocol as the previous experiment. The parameters of all bags containing PNSB were monitored in the same way as the previous experiment. However, they were kept only at room temperature (25±3 °C) to replicate real-world application.

Adjustment of RWA and DCC ratios with RHA to obtain optimal ratio

The optimized ratio of RWA and DCC was adjusted with RHA to support PNSB survival. This adjustment was necessary because of the increased pH of the optimized two-carrier mix containing PNSB tested in the previous experiment and RHA was used on the basis of its neutral pH (Table 5-1). Following the experimental design, five sets were formulated with various concentrations of RHA (0-10%) (Table 5-3A) mixed with the designed proportions of RWA and DCC and adjusted to pH 7. *R. palustris* TN110 was inoculated into all three-carrier sets to obtain a cell density of approximately 10^8 CFU/g. PNSB biofertilizer sets and controls without inoculation were adjusted with sterile mature coconut water to a moisture content of 40% and mixed well in a Stomacher® blender. Then, they were transferred into sterile PET-nylon-PP bags (size 4 × 6 cm). PET-nylon-PP bags were used for their capacity to prevent moisture content loss and also for the reason previously described (Figure 5-1A). Each bag had approximately 8 g of PNSB biofertilizer and all bags were machine-sealed without applying vacuum. They were stored in darkness at room temperature (25±3 °C) and the parameters were monitored weekly for 4 weeks as previously described. The proportion of three carriers that produced the highest PNSB survival while maintaining a constant pH of roughly 7 for 4 weeks was selected for the next experiment.

Adjustment of RWA, DCC and RHA ratios with SCG to obtain optimal ratio

As SCG had the lowest EC value (0.61 mS/am) and the highest total nitrogen among the carriers (Table 5-1), it was used to minimize the EC value of the optimized combination of RWA, DCC and RHA. Its buffering capacity was expected to prevent pH fluctuation and maintain PNSB survival. Proportions of the four carriers were designed using D-optimal design with low and high limits as shown in Table 5-3B. This involved 20 experimental runs (Table 5-4A). The proportion of mixed carrier that achieved the lowest EC value at a pH of approximately 7 was obtained by solving the regression equation and analyzing the response surface contour plots using the same software. The quality of fit of the model equations was expressed by the coefficient of determination (R^2). The optimal proportions of the mixed carrier with minimal EC value and pH of roughly 7 were selected for a verification test (Table 5-4B).

For the verification test, three 4-carrier mixes at different RWA: DCC: RHA: SCG ratios (Table 5-4B) were inoculated with inoculums of *R. palustris* TN110 to gain a cell density of approximately 10^{10} CFU/g and labeled PNSB biofertilizer. PNSB biofertilizer and carrier controls were adjusted to a moisture content of 40% using sterile mature coconut water, mixed well in a Stomacher® blender, then 8 g of it were transferred into sterile PET-nylon-PP bags (size 4 × 6 cm) and machine-sealed without applying vacuum. All bags were stored in darkness at room temperature (25±3 °C). Moisture content loss, pH, EC and PNSB population were measured weekly for the first 4 weeks and monthly for 6 months thereafter. The PNSB biofertilizer formula that maintained the highest PNSB survival was selected for further experiment.

Influence of vacuum sealing power on biofertilizer packaging

To study the influence of vacuum sealing power on biofertilizer packaging, vacuum sealing machines were used with 150 W (DZ-280A, Smooth Pack, Thailand) and 500 W (DZ-400/2E, Smooth Pack, Thailand) extraction powers. Vacuum sealing

requires a specific bag with vacuum-tolerant and flexible properties (Ranmeechai and Photchanachai, 2017), nylon-LLDPE bags were used in this study as they prevented moisture content loss at the same level as PET-nylon-PP bags (Figure 5-1A). PNSB biofertilizer and carrier controls were prepared to the protocol previously mentioned and packed into sterile nylon-LLDPE bags. All biofertilizer bags were maintained in darkness at room temperature (25±3 °C) to prevent photosynthesis and were monitored in the same manner as previous experiments.

Seed germination index

To check their phytotoxicity, *R. palustris* TN110 and *Rubrivivax gelatinosus* TN414 were used to prepare biofertilizer in solid and liquid forms. Both forms had 6 formulations classified into 3 sets for each form as follows: single strain TN110, single strain TN414 and a mixed culture of both strains (combined equally of each strain). However, there was a control (an optimal mixed carrier without PNSB) only for the solid form. For liquid formulations, a cell density of approximately 10^{10} CFU/mL of each biofertilizer was diluted using sterile DW at different dilutions (1:10, 1:100 and 1:1,000 v/v for biofertilizer: DW). For solid formulations, PNSB biofertilizer in the optimized 4-carrier set and its control was also diluted (w/v) with sterile DW the same as the liquid formulations.

Rice (*Oryza sativa* L.) seeds were soaked in DW overnight, sterilized by soaking in 10% sodium hypochlorite solution for 5 min, and washed gently by shaking in sterile DW for 1 min. Washing was repeated five times. Thirty rice seeds were separately submerged in 10 mL of each PNSB/carrier for each dilution of the liquid and solid formulations, while sterile DW was used as a control. All dilutions were then incubated overnight to ensure the attachment of bacterial cells to the rice seed surface. The submerged seeds were placed on three layers of Whatmanfilter paper no.1 in a 9 cm diameter Petri dish and incubated at room temperature (25±3 °C) in darkness for 5 days. The percentage of rice seed germination and root lengths were measured on the 5^{th} day of incubation. The percentage of relative seed germination (% RSG), relative root growth (% RRG) and the germination index (% GI) were calculated from the equations described by Kantha et al. (2015).

Effectof PNSB-biofertilizer(s) on the growth of rice seedlings in paddy soil

The optimal dilutions among the liquid and solid biofertilizers from the previous experiment were selected to investigate their enhancement of seedling growth in paddy soil. Nine germinated rice seeds were grown in a pot of paddy soil (size 12 × 12 × 25 cm, total soil volume 1,440 mL) containing 30 mL of the optimal dilution of biofertilizer/carrier. Fresh paddy soil collected from KhuanKhanun District, Phatthalung Province (7°45'11.0"N, 100°06'57.0"E) was used in this experiment and its characterization is shown in Table 5-1. Tap water was added to flood the paddy soil 1 cm above the top of the soil for 7 days before planting. Then, the 6 treatments were carried out as follows. TN110, TN414 and a mixed culture in both forms were applied separately into the paddy soils at concentrations of 10^8 and 10^7cells/mL for solid formulations and the optimal dilutions at 1:100 and 1:1,000 for liquid formulations. Sterile tap water and the optimal mixed carrier without PNSB served as the controls for liquid and solid forms of the biofertilizer(s), respectively. The water level was maintained at around 1 cm above the surface soil throughout the experiment. The samples were incubated under greenhouse conditions and experiments were carried out in three replicates (9×3 = 27 seedling/set). After 21 days of applying either liquid or solid biofertilizer(s) with their controls, seedling growth was measured based on the length of shoots and roots and their dry weight. To measure dry weight, rice roots and shoots were dried in an oven at 60 ℃ overnight or until they reached a constant weight.

Data presentation and statistical analysis

All experimental data were conducted in triplicate unless otherwise stated. Means and standard deviations are presented. Statistical differences among means were performed by Duncan's multiple tests at $P \leq 0.05$.

Results

Optimization of carrier formulation and packing for biofertilizer

RWA, DCC, RHA and SCG were used as carriers in this study.All carriers contained primary macronutrients, secondary macronutrients and micronutrients (Table 5-1). Nitrogen (N), phosphorus (including P_2O_5) and potassium (including K_2O) are primary macronutrients for plant growth (Fageria, 2009). Among the carriers tested, RWA contained the highest total P_2O_5 (2.51%) and K_2O (10.92%), whereas SCG presented the highest total N content (2.25%). Secondary macronutrients comprised calcium (Ca), magnesium (Mg) and sulfur (S) (Fageria, 2009) and the highest contents of Ca (15.45%), Mg (1.98%) and S (0.26%) were found in RWA. Plant growth also requires micronutrients such as manganese (Mn), copper (Cu), Iron (Fe), boron (B), zinc (Zn) and molybdenum (Mo) (Fageria, 2009). The greatest concentrations of micronutrients (Mn, Cu, B and Zn) were observed in RWA, while DCC presented the highest Fe content. However, no Mo (molybdenum) was detected in any of the tested carriers. Moreover, high levels of organic matter were found in SCG (92.38%) and DCC (83.82%) and RWA: DCC: RHA: SCG carrier optimized at 3:4:2:1 was also characterized (Table 5-1).

Before obtaining PNSB biofertilizer, the carriers used were optimized step-by-step. The RWA and DCC were considered first to make biofertilizer as both are abundant raw materials. In this study, D-optimal design was used to determine the optimal ratio of the mixed carriers for PNSB survival. Experimental and predicted values of PNSB survival after mixing with the combined RWA and DCC are shown in Table 5-2A. The experimental data obtained from this experiment were analyzed using multiple linear regression analysis to obtain predicted values. Design Expert software was used to analyze the relationship of RWA and DCC to the response of PNSB survival using the regression model. The cubic model showed significant F-values which indicates that the model was good. According to the ANOVA results, the cubic model showed R^2 values of 88.64% and 87.46% for the experiments at room temperature (25±3 °C) and 4 °C, respectively. These values imply that this model could explain PNSB survival in the combination of RWA and DCC. A higher R^2value

indicates a higher representing capability of the full cubic equation for the model under the given experimental domain.

Table 5-1. Characteristics of individual carriers; rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA) and spent coffee grounds (SCG); the optimal mixed carrier at 3: 4: 2: 1 ratio of RWA: DCC: RHA: SCG and paddy soil collected from Khuan Khanun District, Phatthalung Province, Thailand.

Parameter	Type of carrier				Optimal mixed carrier	Paddy soil
	RWA	DCC	RHA	SCG		
pH	11.08	5.55	7.00	5.43	7.09	5.29
EC (mS/cm)	16.40	1.28	0.64	0.61	2.62	0.073
Organic matter (%)	1.40	83.82	22.14	92.38	47.61	9.09
Organic carbon (%)	0.81	48.62	12.84	53.58	27.62	5.27
Ash (%)	74.57	6.78	71.04	1.34	39.43	76.91
Total N (%)	0.05	0.96	0.43	2.25	0.71	0.46
Total P_2O_5 (%)	2.51	0.24	0.28	0.21	0.93	0.10
Total K_2O (%)	10.92	0.62	0.37	0.48	3.65	0.04
Total S (%)	0.26	0.12	0.10	0.10	0.16	0.01
Extractable elements (%)						
Ca	15.45	0.63	0.32	0.14	4.97	1000 (mg/kg)
Mg	1.98	0.20	0.11	0.11	0.71	700 (mg/kg)
Co	0.32	0.17	0.34	0.12	0.24	2.48 (mg/kg)
V	0.78	0.38	0.57	0.00	0.50	19.98 (mg/kg)
Extractable elements (mg/kg)						
Mn	220,000	50.80	302.87	76.12	66,000	50.53
Cu	50.99	18.20	5.75	12.18	24.95	12.17
Fe	498.28	714.00	481.17	37.23	535.04	3300.00
B	116.37	5.45	2.76	6.96	38.34	not determined
Zn	142.05	16.80	23.69	11.21	55.19	20.86
Mo	0.00	0.00	0.00	0.00	0.00	0.00
Si	114.24	192.50	339.45	119.4	191.10	44.87
Na	223.51	227.50	393.95	168.1	253.66	181.15
Ni	30.48	0.98	1.89	0.81	10.00	6.43
Cd	0.20	0.10	0.10	0.10	0.11	0.10

Regression analysis of the experimental results based on contour plots (data not shown) found that the results were suitably explained by the following cubic equations:

$$Y = 4.98138X_1 - 2.68317X_2 + 8.97363X_1X_2 - 21.01681X_1X_2(X_1-X_2) \quad \text{Equation 5-1}$$

and

$$Y = 5.63348X_1 + 4.10658X_2 + 4.43139X_1X_2 - 5.15302X_1X_2(X_1-X_2) \quad \text{Equation 5-2}$$

in which Y represents PNSB survival (log CFU/g) and X_1 and X_2 refer to RWA and DCC ratios, respectively.

Equations 5-1 and 5-2 were used to predict PNSB survival in combined carriers at varying ratios of RWA and DCC kept at room temperature (25±3 ℃) and 4 ℃, respectively.

The results of the cubic model of optimal proportions of RWA and DCC at room temperature (25±3 ℃) and 4 ℃ (Table 5-2B) were verified. At room temperature (25±3 ℃), the optimal ratio of RWA: DCC was 40.50:59.50 and at 4 ℃it was 40.99:59.01. The desirability of the optimal proportion of RWA and DCC at room temperature (25±3 ℃) was 0.920 and at 4 ℃ it was 0.854. The optimal ratio of RWA: DCC was simplified to 41:59 for practical application. However, after 4 weeks storage in the optimal ratio of a mixed carrier at room temperature (25±3 ℃) PNSB survival was almost 2 times lower than it was at 4 ℃ (Table 5-2B). Since high moisture content loss was observed (Figure 5-1B); the type of packaging bag might influence moisture content in PNSB biofertilizers.

Regarding the above results, the permeability of the packing bags (PP, PET-nylon-PP and nylon-LLDPE) was investigated. A significant increase in weight gain was observed only in PP bags. A small increase occurred in the rest of the bags (Figure 5-1A). As previously described; thereby, only PPPET-nylon-PP and PP bags were later tested for the survival of PNSB in an optimal mixed carrier of RWA and DCC. PET-nylon-PP bags could maintain moisture content 10 times higher than PP bags (Figure 5-1B). A higher pH was observed in samples in PP bags than in PET-nylon-PP bags, although the pH increased with longer incubation in both packaging bags (Figure 5-1C). The difference in the evolution of moisture content and pH in both packaging bags led to a considerable difference in the maintenance of PNSB survival. In PET-nylon-PP bags, survival of PNSB was1.88 times higher than in PP

bags (Figure 5-1D). The results indicate that the type of packaging bag used strongly affected PNSB survival and the PET-nylon-PP bag was selected for next study. However, the undesirable pH previously described required the adjustment of the two-carrier mixture by adding pH-neutral RHA to aid the maintenance of PNSB survival.

Table 5-2. Experimental design for optimizing the proportions of RWA and DCC in a mixed carrier to support PNSB (*R. palustris* TN110) at room temperature (25±3 °C) and at 4 °C for 4 weeks.

A: D-optimal design using RSM.

Run number	Carrier (%)		PNSB survival (log CFU/g)			
	RWA	DCC	25±3 °C		4 °C	
			Actual	Predicted	Actual	Predicted
1	25.00	75.00	2.93	2.89	5.82	5.80
2	75.00	25.00	2.81	2.78	5.62	5.60
3	50.00	50.00	3.61	3.39	6.04	5.98
4	37.50	62.50	3.51	3.53	5.90	6.02
5	62.50	37.50	2.86	2.98	5.74	5.80
6	41.67	58.33	3.41	3.55	6.10	6.03
7	25.00	75.00	2.97	2.89	5.77	5.80
8	75.00	25.00	2.77	2.78	5.59	5.60
9	25.00	75.00	2.77	2.89	5.84	5.80

B: Verification test.

Verification set	Carrier ratio (RWA: DCC)	Desirability	PNSB survival (log CFU/g)	
			Actual value	Predicted value
At 25±3 °C	40.50: 59.50	0.920	3.41	3.55
At 4 °C	40.99: 59.01	0.854	6.10	6.03

The results showed that increasing the proportion of RHA in a range from 0 to 10% (Table 5-3A) increased PNSB density from 6.28 to 6.68 log CFU/g. The optimal RWA: DCC: RHA ratio of 41:49:10 maintained PNSB density at 6.68 log CFU/g for 4 weeks under storage at room temperature (25±3 °C). The optimal RWA: DCC ratio of 41:59 from the previous experiment shows almost the same PNSB density at 6.40 log CFU/g under storage at 4 °C (Figure 5-1D). The results revealed that the added

RHA maintained PNSB survival without requiring storage at 4 °C. Nevertheless, the pH of the optimal ratio of RWA: DCC: RHA still increased during storage and also exhibited a high EC value of approximately 10 to 20 mS/cm. Therefore, to increase the possibility of maintaining the PNSB population in the RWA: DCC: RHA carrier, initial pH should be maintained at roughly 7 and EC value of the mixed carrier required a small adjustment. SCG was selected to solve this problem as it might have a high buffer capacity (maximum organic matter) to prevent pH change and also the lowest EC value of all the carriers (Table 5-1).

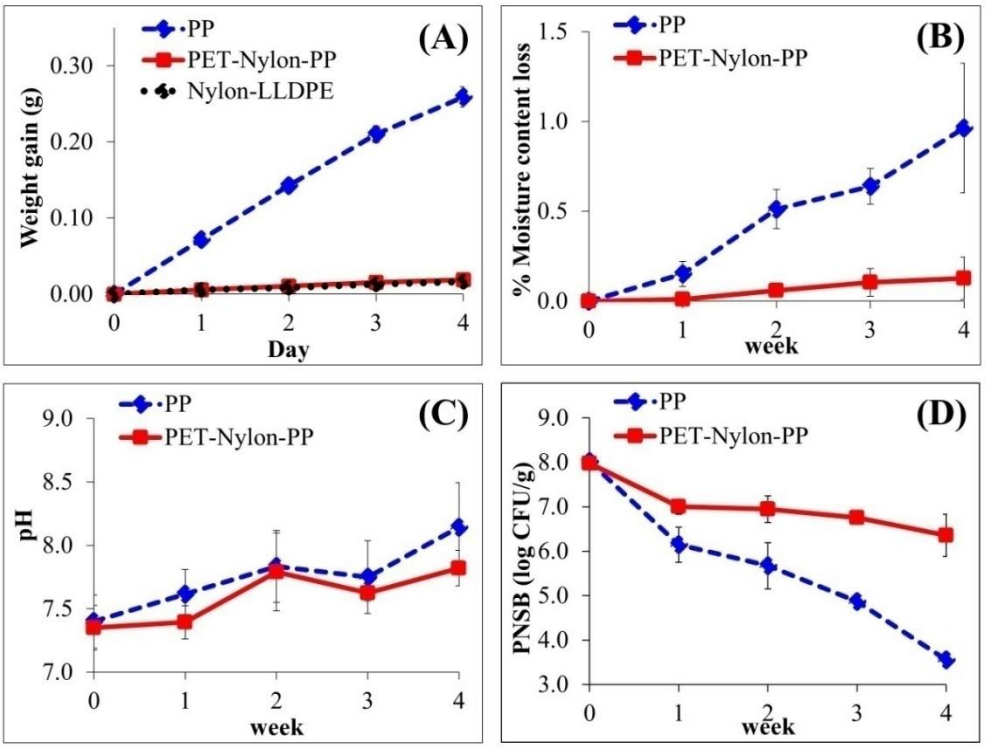

Figure 5-1. Bag permeability test (A) weight gain according to silica gel method in walk-in humidity chamber at 35 °C and 80% relative humidity; and effect of packaging bags (PP and PET-nylon-PP) containing an optimal mixed carrier of rubber wood ash (RWA) and decanter cake (DCC) and PNSB on (B) moisture content, (C) pH, and (D) PNSB survival at room temperature (25±3 °C) for 4 weeks.

Limitation and design of experiments based on the D-optimal design for a carrier of RWA, DCC, RHA and SCG that gave constant pH of approximately 7 and minimal EC were set up as defined in Table 5-3B and Table 5-4A. The experimental data obtained from the experimental runs were analyzed by multiple linear regression

analysis to provide predicted values. The special cubic model showed a significant F-value which indicates that the regression model was good. The experimental results were analyzed by regression analysis based on contour plots (data not shown), which consisted of the effect of variables as can be found in the following equation:

$$Y = -0.277(X_1) - 0.068(X_2) - 0.115(X_3) - 0.246(X_4) + 0.010(X_1X_2) + 0.012(X_1X_3) + 0.018(X_1X_4) + 0.005(X_2X_1) + 0.008(X_2X_4) + 0.010(X_1X_4) - 0.0001(X_1X_2X_3) - 0.0003(X_1X_2X_4) - 0.0003(X_1X_3X_4) - 0.0001(X_2X_3X_4) \quad \text{Equation 5-3,}$$

in which Y represents pH, X_1, X_2, X_3 and X_4 refer to RWA, DCC, RHA and SCG ratios, respectively. Equation 5-3 was used to predict the pH of each mixed carrier. According to the ANOVA results, the cubic model showed a R^2 value of 99.99% for the experimental runs at room temperature (25±3 °C), which implied that the effect of the ratio of RWA, DCC, RHA and SCG on pH could be explained by this model. A higher R^2 value indicated a higher representing capability of the full special cubic equation for pH value under the given experimental domain.

Table 5-3. Experimental design to investigate an optimal ratio of a mixed carrier from various carriers.

A: Combination of RWA, DCC and RHA.

Run number	Carrier level (%)		
	RWA	DCC	RHA
1	41	59	0
2	41	56.5	2.5
3	41	54	5
4	41	51.5	7.5
5	41	49	10

B: Limits of proportions of RWA, DCC, RHA and SCG based on D-optimal design.

Type of carrier	Limitation (%)	
	Low	High
RWA	10	30
DCC	20	50
RHA	10	40
SCG	10	30

Table 5-4. D-optimal experimental design to investigate optimal proportions of a mixed carrier among RWA, DCC, RHA and SCG to provide a neutral pH with minimal EC value.

A: Experimental design showing carrier content, actual and predicted pH in each run.

Run number	Carrier content (%)				pH	
	RWA	DCC	RHA	SCG	Actual	Predicted
1	17	27	40	17	6.68	6.68
2	30	20	30	20	7.61	7.62
3	30	50	10	10	6.74	6.73
4	10	20	40	30	6.52	6.52
5	30	20	40	10	7.88	7.88
6	23	20	27	30	7.35	7.33
7	30	30	10	30	7.21	7.21
8	10	35	25	30	6.07	6.08
9	10	50	10	30	5.91	5.91
10	10	50	30	10	5.93	5.93
11	30	35	25	10	7.03	7.02
12	20	43	18	20	6.31	6.30
13	10	40	40	10	6.09	6.09
14	20	50	20	10	6.36	6.37
15	10	50	20	20	5.90	5.88
16	30	30	10	30	7.21	7.21
17	10	50	10	30	5.91	5.91
18	10	20	40	30	6.52	6.52
19	30	50	10	10	6.74	6.73
20	30	20	40	10	7.88	7.88

B: Verification test to select the most optimal mixed carrier.

Treatment (Verified set)	Carrier ratio (RWA: DCC: RHA: SCG)	Desirability	pH	
			Actual	Predicted
1	30: 28: 19: 23	1.000	7.05	7.00
2	29: 32: 11: 29	1.000	6.97	7.00
3	30: 40: 20: 10	0.987	7.09	7.03

The verification test of the optimal proportions of RWA, DCC, RHA and SCG to obtain a constant pH of approximately 7 and minimal EC under storage at room temperature (25±3 °C) (Table 5-4B) found that, after 6 months storage, treatment 3 with ratios of RWA: DCC: RHA: SCG at 30:40:20:10 provided the lowest moisture content loss among sets containing biofertilizer (Figure 5-2A). It should be noted that moisture content loss in all treatments was significantly higher than in the control (a mixed carrier without PNSB). However, pH in treatment 3 at 4 months' storage was higher than treatments 1 and 2 including the control, and then no difference was found at 5-6 month-storage (Figure 5-2B). Among all treatments, the lowest EC value (2.0-2.6 mS/cm) with the highest PNSB population was observed in treatment 3 throughout storage (Figure 5-2C and D). At longer storage time, PNSB population for treatment 3 decreased from initial 10 log CFU/g to 8, 6 and 3.8 log CFU/g at months 2, 4 and 6.

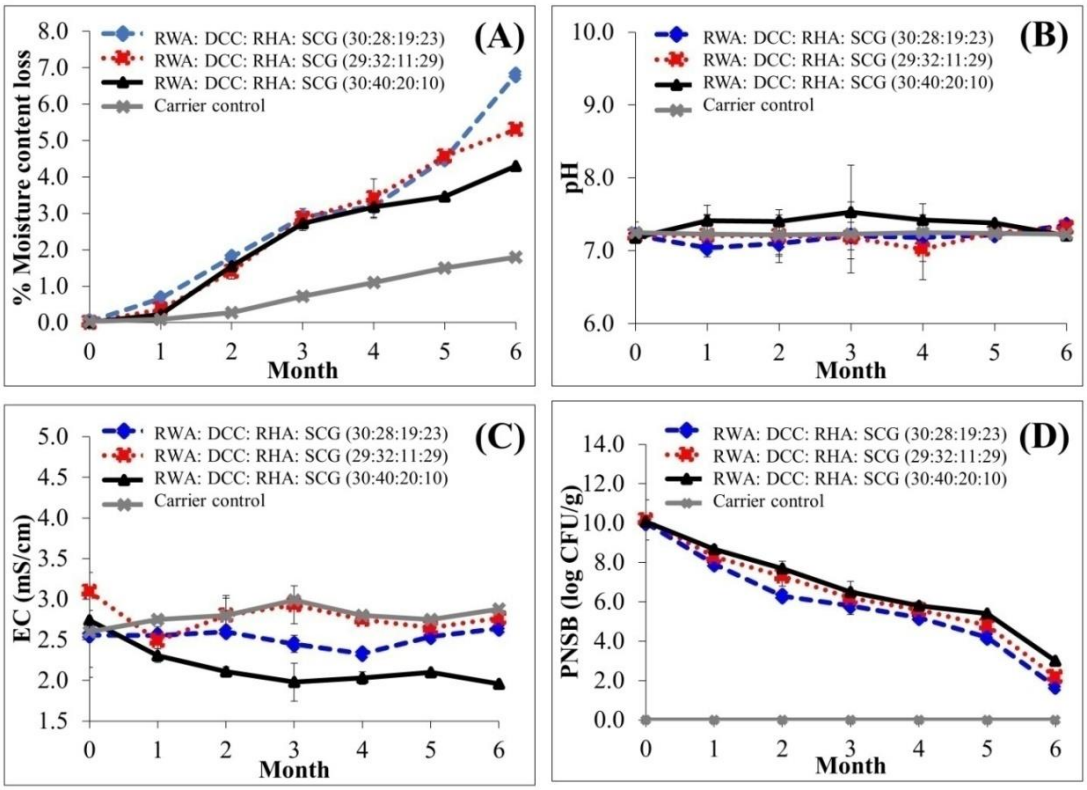

Figure 5-2. Effects of different ratios of RWA, DCC, RHA and SCG in a mixed carrier for PNSB packed in PET-nylon-PP bags and stored at room temperature (25±3 °C) for 6 months on (A) moisture content, (B) pH, (C) EC and (D) PNSB survival: Treatments 1, 2 and 3 are RWA: DCC: RHA: SCG carrier ratios at 30:28:19:23, 29:32:11:29 and 30:40:20:10, respectively.

With the expectation of prolonging the shelf life of the PNSB biofertilizer, packing methods were investigated by vacuum sealing bags at low and high power. Normal sealing without vacuum served as the control. The use of vacuum sealing preserved moisture content, constant pH, and minimal EC, better than sealing without vacuum (Figure 5-3A to C). Moisture content loss was significantly higher at low sealing power compared with high sealing power (Figure 5-3A); however, similar results were found for pH and EC values in treatments sealed at both powers and in the control. The results showed that after 6 months of storage, the use of high vacuum sealing power (500 W) maintained significantly higher PNSB populations in the RWA: DCC: RHA: SCG carrier at a ratio of 30:40:20:10 than were maintained in carrier sealed with the use of low vacuum sealing power (150 W) (Figure 5-3D). PNSB-biofertilizer(s) using the normal sealing decreased PNSB population remaining approximately 40% for 6 month-storagewhereas PNSB-biofertilizer(s) using the high vacuum sealing preserved PNSB population up to 70% (Figure 5-3D). PNSB-biofertilizer(s) using the normal sealing lost PNSB population to approximately 40% of the original population after 1 month-storage (Figure 5-1D) whereas PNSB-biofertilizer(s) using the high vacuum sealing retained PNSB population up to 90% for 1 month-storage (Figure 5-3D). Populations of PNSB in the biofertilizer decreased in bags sealed at both vacuum sealing powers. The reduction of PNSB population density was found to be roughly 1 log cycle after storage for 3 months and 3 to 4 log cycles after storage for 6 months. On the other hand, after storage for 6 months, the PNSB population density in the control was roughly 3 log CFU/gcorresponding to a decrease of 7 log cycles.

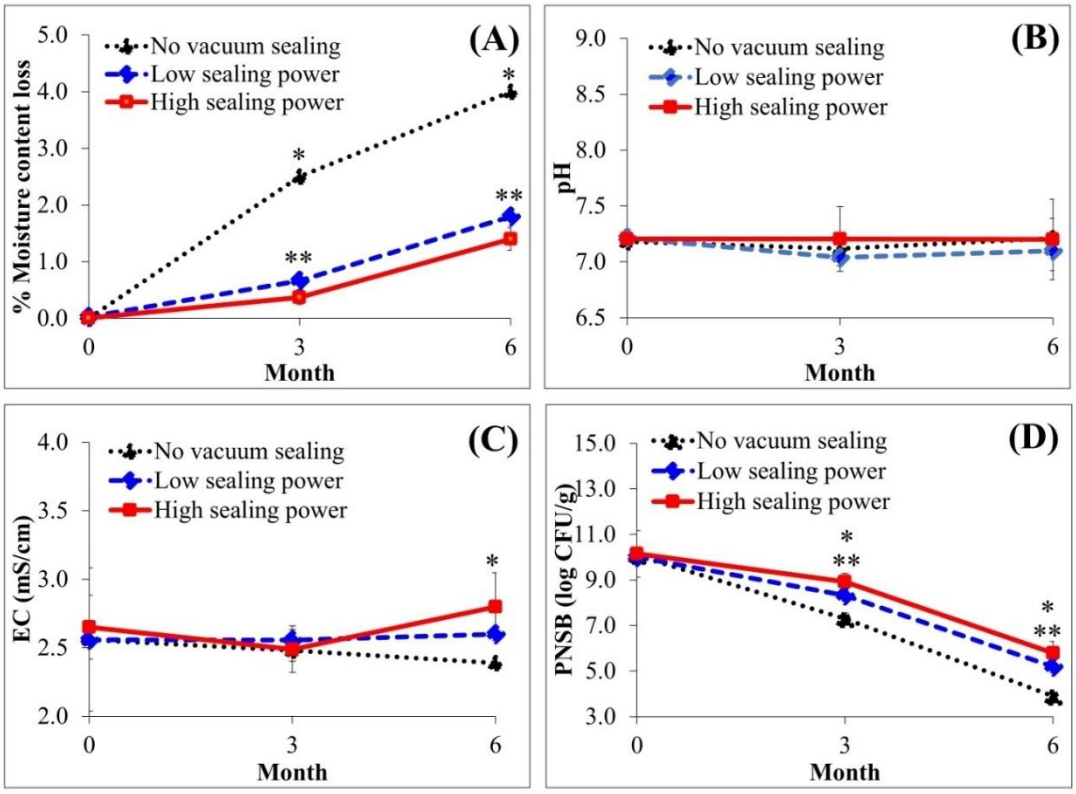

Figure 5-3. Effects of different sealing methods for nylon-LLDPE bags containing the optimal ratio of RWA, DCC, RHA and SCG on (A) moisture content, (B) pH, (C) EC and (D) PNSB survival at room temperature (25±3 °C) for 6 months: Asterisk indicates significant differences among control and treatments, and ** among treatments.

Seed germination index and effect of the PNSB-biofertilizers on the growth of rice seedlings

PNSB biofertilizers (strains TN110, TN414 and a mixed culture) in both solid and liquid forms were separately serially diluted in sterile DW and tested for their phytotoxicity on rice seeds using seed germination based on GI value (Figure 5-4). Each concentration of all biofertilizers and an optimal mixed carrier without PNSB as control produced significant differences in the percentage of GI (P<0.05). The optimal concentrations of PNSB biofertilizer in solid form were obtained from 1 g suspended in 100 mL DW for TN110, and 1 g in 100 mL for TN414 and 1,000 mL of DW for the mixed culture. These concentrations produced the best GIs of 120.70, 107.16 and 107.23%, respectively. In liquid preparations, the optimal concentrations

were obtained at 1: 100 for TN110 and at 1:1000 for both TN414 and the mixed culture sets. These concentrations produced the best GIs of 115.53, 109.22 and 111.61%, respectively. The best GI of an optimal mixed carrier without PNSB supplementation was 99.71 % obtained with 1 g suspended in 1,000 mL of DW. Strain TN110 delivered greater GI than strain TN414 at the same dilution at 1:1000 in solid form and at all dilutions tested in liquid form. A similar result was found for strain TN414 and the mixed culture in both forms at all dilutions tested. The optimal concentrations were selected for study of their stimulating effect on rice seedling growth. No phytotoxicity was observed in any of the PNSB biofertilizers tested at their optimal concentrations based on GI value (Figure 5-4); however, the optimal mixed carrier without PNSB supplementation was slightly toxic.

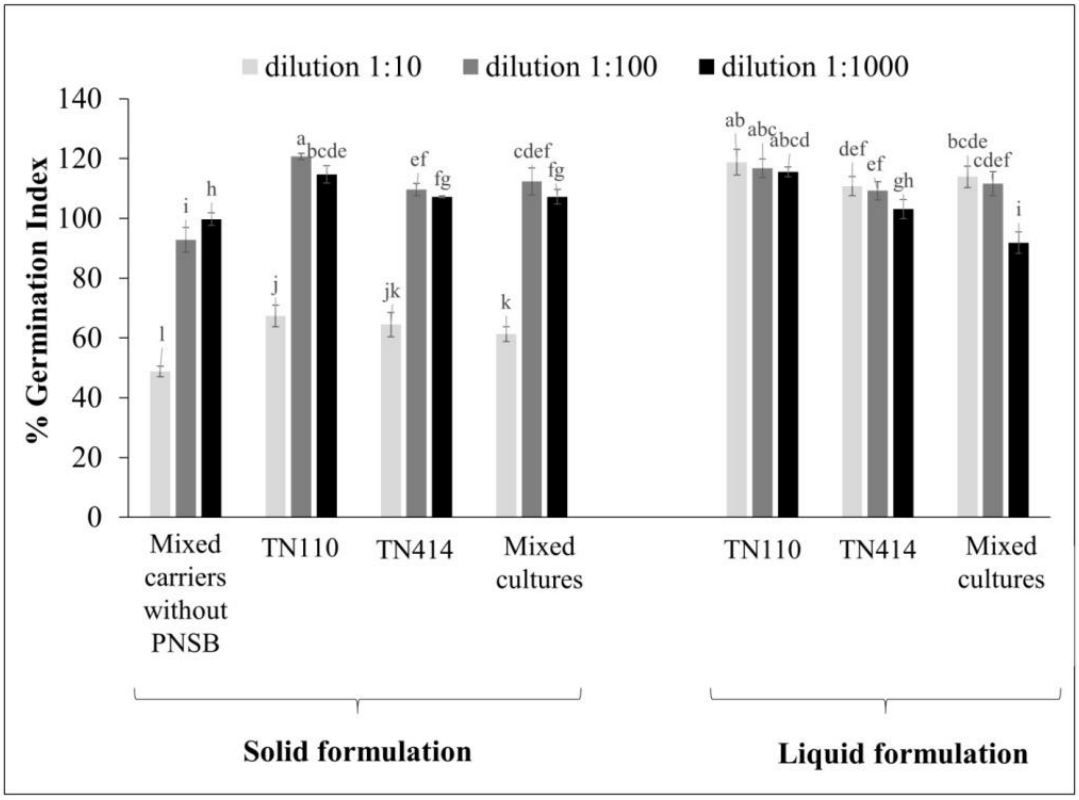

Figure 5-4. Effects on seed germination index of rice (*Oryza sativa* L.) of biofertilizer formulations in solid and liquid forms at different concentrations of the suitable mixed carrier, PNSB-biofertilizer in the suitable mixed carrier and PNSB in liquid form. Mean values with the different lowercase letters are significantly different at $P \leq 0.05$.

The effect of the PNSB biofertilizers in solid and liquid forms on rice seedling growth was studied in paddy soil under greenhouse condition for 28 days. Plant growth parameters including dry weight and length of shoot and root. Results were compared between solid and liquid forms of different biofertilizers. All measured plant growth parameters were significantly higher ($P < 0.05$) after application of all PNSB biofertilizers than in the controls (Figure 5-5). No significant difference was observed among controls in both forms on the basis of all measured parameters. Based on dry weight of root and shoot, the solid form of all biofertilizers performed much better than the liquid form (Figure. 5-5A and B). The difference in length of shoots was insignificant between solid and liquid forms but root length was significantly increased by the liquid formulation of PNSB biofertilizer strain TN110 (Figure. 5-5C and D). Among the optimized biofertilizers, strain TN110 in both solid and liquid forms was the most effective at promoting rice growth in paddy soil (Figure. 5-5A to D).

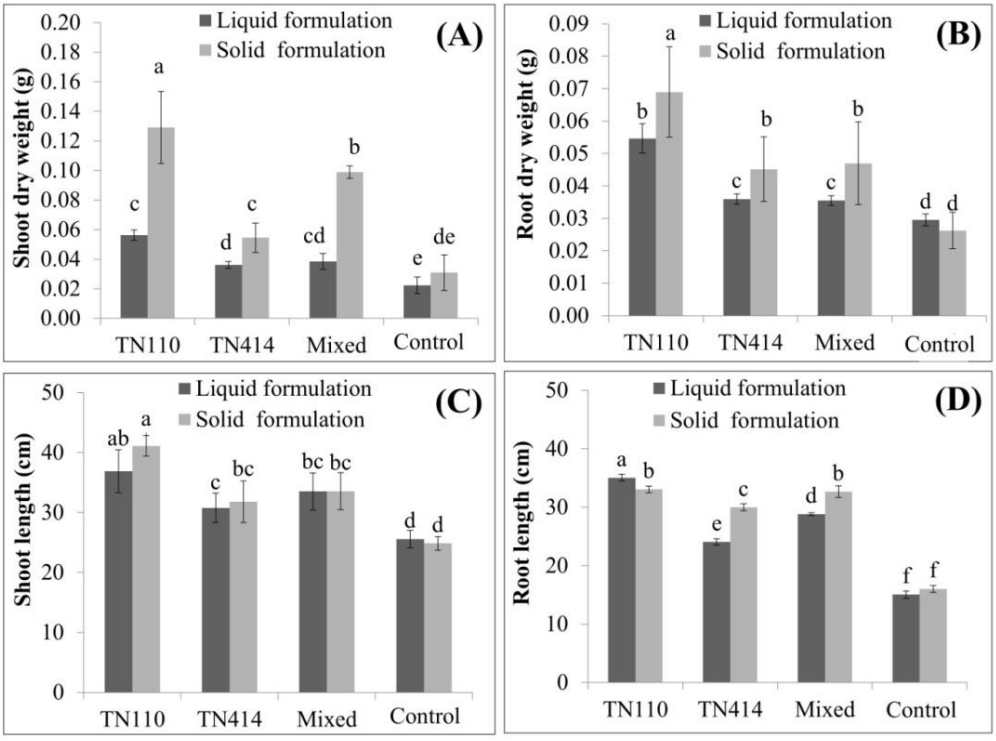

Figure 5-5. Effects of different PNSB-biofertilizer in both formulations as liquid and solid forms on (A) shoot dry weight, (B) root dry weight, (C) shoot length and (D) root length of rice (*Oryza sativa* L.) seedlings grown in paddy soil under greenhouse condition. Mean values with the different lowercase letters are significantly different at $P \leq 0.05$.

Discussion

Optimization of mixed carrier ratios for biofertilizer formulation

The first considerations were the choice of carrier and packaging that would best support the growth of PNSB as biofertilizer which their formulation produced in solid form (Herrmann and Lesueur, 2013). All carriers used in this study contained nutrients essential to plants (Kumar and Gupta; 2010; Cruz et al., 2012; Embrandiri et al., 2013). However, none of the carriers contained Mo (molybdenum) that is necessary for N_2 fixation (Oda et al., 2005). A combination of RWA with DCC at the optimal ratio was not an optimal mixed carrier to preserve PNSB in a biofertilizer (Table 5-2B). This was due to the high pH and EC of RWA (Table 5-1). The high pH and EC might threaten bacterial survival during storage. High pH refers to low concentration of proton ions that affect bacterial metabolism and alter bacterial growth (Kim et al., 2016). Likewise, the EC value defines the potential of electrical ions to be transported through water. The high electrical ions which affect bacterial metabolism from the carrier of RWA with DCC indicate PNSB strain was not in a state of hibernation for good preservation of PNSB strain. Therefore, we investigated the reduction of pH and EC values to maintain optimal conditions or the stability of both values.

RHA with a neutral pH (7.00) and low EC (0.64 mS/cm) was selected to improve conditions by decreasing pH to support PNSB survival in a mixed carrier of RWA and DCC. The addition of RHA enabled higher PNSB survival although it did not maintain a constant pH of approximately 7 and minimal EC. However, it did reduce pH to a level that better supported PNSB survival. The pH of the mixed carrier of RWA and DCC was initially 7.40 and rose to 7.94 after 4 weeks of storage. The pH of the mixed carrier of RWA, DCC and RHA was initially only 6.99 (pH reduction of 0.41 from initial pH) but reached 7.87 after 4 weeks of storage. On the other hand, the EC values of mixed carriers of RWA and DCC fluctuated between 15.80 and 17.95 mS/cm. This fluctuation was also found in the mixed carrier of RWA, DCC and RHA but at lower levels between 12.81 and 16.43 mS/cm. In addition to pH and EC of the mixed carrier, another factor might involve in the survival of PNSB, was such as a

storage temperature and carrier surfuce. Siddique et al. (2016) reported that bacteria could survive by attaching themselves inside the surface pores of RHA. According to the results, the protection of PNSB cells still required better maintenance of the pH level and minimal EC values. Therefore, SCG, a by-product from the coffee brewing process (Cruz et al., 2012), was added to in the mixed carrier of RWA, DCC and RHA. An optimized ratio of the 4-carrier mix obtained an optimal mixed carrier with pH 7.09 and EC of 0.002 mS/cm (Table 5-1).

The characterization of the optimal mixed carrier (Table 5-1) indicates that it not only supports PNSB biofertilizer but also provides plant nutrients. The considerable reduction in the value of EC in the 4-carriermixmay be due to the high level of organic matter in SCG. Moreover, total N and other plant nutrients found in SCG are good for soil quality and support plant growth (Cruz et al., 2015; Embrandiri et al., 2017).

While packaging methods are also important for survival of biofertilizer, due to the effect of carrier type on biofertilizer formulations, moisture content is considered essential to ensure bacterial survival (Lim and Matu, 2015). Hence, this study maintained a 40% moisture content (Kantha et al., 2015) to support the PNSB biofertilizer. Interestingly, this study found that PET-nylon-PP or nylon-LLDPE bags were the most suitable packaging based on their ability to prevent moisture content loss (Figure 5-1A). It should be noted that with vacuum sealing at either low or high power the shelf life of high PNSB populations could be extended from 3 to 6 months to maintain effective use in real-world application (Figure 5-3D). This was because vacuum sealing removed all or almost the air from the packaging and therefore there was no or minimal oxygen or carbon dioxide available for bacterial metabolism (Fixen and Harwood, 2016). Therefore, vacuum sealing was a good practical procedure to protect biofertilizers including PNSB as it slowed the process of metabolism. It should be noted that this study investigated the effective dose of PNSB biofertilizer for application over storage time of 6 months and (Figure 5-3) therefore, longer storage times should be further investigated based on the effective dose to indicate the shelf life of PNSB biofertilizer.

Phytotoxicity of PNSB biofertilizers and enhancement of rice seedling growth

Comparison of the toxicity of solid and liquid forms of PNSB biofertilizer found that the solid PNSB biofertilizer was more toxic than the liquid PNSB biofertilizer (Figure 5-4). This is because the toxicity originated from the optimal mixed carrier (control) and this was reduced by dilution at 1: 1000 with little toxicity remaining. It should be noted that no phytotoxicity was found at dilutions of 1:100 and 1:1000 of PNSB biofertilizer from both solid and liquid formulations. This implies that, for reasons of economy and any adverse effect of the mixed carrier on organisms including the environment, the optimal dilution for application should be at 1:1000. In contrast, the most optimal dilution of biofertilizer in liquid form was 1:100. This dilution would reduce costs as no significant difference in a performance of PNSB biofertilizer existed between the dilutions at 1: 10 and 1: 100. The optimal condition of concentration of biofertilizer based on dilution was studied before use. This is also in accordance with the results of Kantha et al. (2015) for application of PNSB biofertilizer. In this study, all PNSB biofertilizers, either as individual or mixed cultures, clearly supported rice growth. However, strain TN110 was the most effective candidate as a plant growth promoter/biofertilizer (Figure 5-5). It should be noted that the different optimal dilutions should affect efficacy of PNSB concentration as the optimal dilution of strain TN110 at 1: 100 produced a PNSB population roughly 1 log cycle higher than strain TN114 including the mixed cultures (dilution 1: 1000). In addition, it could be possible that seed germination and rice growth improve at different cell densities. Therefore, before applying any PNSB biofertilizer on paddy fields, the suitability of equal cell density for both treatments should be investigated in paddy soil under greenhouse conditions to obtain the most effective PNSB biofertilizer.

Conclusions

As biofertilizer can be formulated either in liquid or solid form, this study demonstrated that the agricultural wastes RWA: DCC: RHA: SCG at the optimal ratio of 3:4:2:1 produced an optimal mixed carrier for maintaining PNSB survival (*R. palustris* TN110). The use of nylon-LLDPE packaging bags and vacuum sealing at 500 W prolonged shelf life of PNSB strain *R. palustris* TN110 up to tested 6 months. As no phytotoxicity was found, all tested PNSB biofertilizers (TN110, *R. gelatinosus* TN114 and a mix of both strains), whether single or mixed cultures from liquid and solid forms, effectively promoted rice growth in paddy soil. This suggests the prepared PNSB biofertilizers have potential for application in paddy fields.

CHAPTER 6

Abilities of Different Biofertilizer Formulations of Purple Non-Sulfur Bacteria on Growth Enhancement and Heavy Metal Accumulation Reduction in Rice (*Oryza sativa* L.) Grown in Cadmium-Contaminated Paddy Soil

Abstract

Purple non-sulfur bacteria are considered as biofertilizers and heavy metal bioremediation agents. This study aimed to investigate a performance of PNSB: *Rhodopseudomonas palustris* TN110, *Rubriviva xgelatinosus* TN414 and a mixed culture of both strains (equally combined) in both liquid and solid forms on rice (*Oryza sativa* L.) seedlings grown in cadmium (Cd)-contaminated paddy soil. Rice growth based on lengths of shoots and roots for treatment sets from both forms of PNSB biofertilizer(s) in either Cd-contaminated soil or control paddy soil were significantly higher than their controls (water and a mixed carrier without culture). The application of *R. palustris* TN110 in solid form to Cd-contaminated paddy soil significantly improved rice growth based on the maximum shoot dry weight and root length. All PNSB biofertilizer(s) in a solid form produced a remarkable less accumulation of both Cd and Zn in rice shoots and roots in Cd-contaminated soil compared with a liquid form. Strains TN110 and TN414 were great in Cd removal although the former strain was better in reducing Cd in root. However, strain TN414 was also powerful in Zn removal resulting in low accumulation of Cd and Zn in rice materials. The application of PNSB biofertilizer(s) has potential to promote rice growth in Cd-contaminated soil and to provide production of safe rice.

Keywords: Anoxygenic phototrophic bacteria, Biofertilizer, Cadmium, Contaminated soil, Paddy field, Zinc

บทคัดย่อ

แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์มีความน่าสนใจที่จะนำไปใช้เป็นทั้งปุ๋ยชีวภาพและตัวบำบัดโลหะหนักทางชีวภาพการศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบศักยภาพของแบคทีเรียกลุ่มนี้สายพันธุ์*Rhodopseudomonas palustris*TN110 และ*Rubrivivax gelatinosus*TN414 รวมถึงการผสมทั้ง 2 สายพันธุ์ในอัตราส่วนเท่ากัน เมื่อผลิตเป็นปุ๋ยชีวภาพทั้งที่อยู่ในรูปของเหลวและของแข็งโดยนำไปใช้ทดสอบในการปลูกข้าวในดินนาข้าวที่ปนเปื้อนแคดเมียม การเจริญของข้าวพิจารณาจากความยาวของลำต้นและรากพบว่าทุกชุดการทดลองที่มีการใช้ปุ๋ยชีวภาพทั้งสองรูปแบบในนาข้าวทั้งมีการปนเปื้อนแคดเมียมและไม่มีดีกว่าชุดควบคุม (น้ำก๊อก และวัสดุพยุง)อย่างมีนัยสำคัญ เมื่อใช้แบคทีเรียสายพันธุ์ TN110 ในรูปของแข็งในดินนาข้าวที่มีการปนเปื้อนของแคดเมียมให้ค่าน้ำหนักแห้งของลำต้นและรากข้าวสูงสุด ปุ๋ยชีวภาพทุกชุดในรูปของแข็งเมื่อใช้ในดินนาข้าวที่มีการปนเปื้อนแคดเมียมสามารถลดการสะสมของแคดเมียมและสังกะสีในลำต้นและรากข้าวได้ดีกว่าปุ๋ยชีวภาพในรูปของเหลว โดยสายพันธุ์ TN110 และสายพันธุ์ TN414 มีประสิทธิภาพในการลดการสะสมของแคดเมียมแม้ว่าสายพันธุ์แรกลดแคดเมียมในรากได้ดีกว่าอย่างไรก็ตามสายพันธุ์ TN414 มีประสิทธิภาพในการลดการสะสมของสังกะสีได้ดีมากทั้งในลำต้นและราก การประยุกต์ใช้ปุ๋ยชีวภาพดังกล่าวแสดงให้เห็นถึงความเป็นไปได้ของการส่งเสริมการเจริญของการปลูกข้าวในพื้นที่นาข้าวที่มีการปนเปื้อนแคดเมียมและนำไปสู่การได้ผลผลิตข้าวที่ปลอดภัย

คำสำคัญ : แคดเมียม ดินที่ปนเปื้อนแคดเมียม นาข้าว ปุ๋ยชีวภาพ แบคทีเรียสังเคราะห์แสงที่ไม่เกิดออกซิเจนจากการสังเคราะห์แสง สังกะสี

Introduction

Thailand is an agricultural country and rice is one of the main crops that produce income about ten billion baht (0.3 billion U.S. dollars) per year. The area for rice seedlings of Thailand is roughly 30 million acres and this consists of in-season rice field about 24 million acres, and off-season rice field about 6 million acres (Office of Agricultural Economics, 2017a). The major plant nutrients are nitrogen (N), phosphorus (P), and the potassium (K). Among them, nitrogen is the element that rice needs for the whole life of growing until harvesting; thereby it impacts the production and quality of rice grains (Dobermann and Fairhurst, 2000). In addition, phytohormones are one of plant stimulating factors; however, they are too expensive for rice cultivation. Plants can produce hormones such as auxins, cytokinins and gibberellins, but the amounts do not meet the demand for rice yield increase (Liu et al., 2011).

Chemical fertilizers or artificial fertilizers have been heavily used by rice farmers for many decades because they are simple to use and provide high productivity although they are expensive to increase rice cost production. These fertilizers have adverse effects on soil by decreasing soil quality such as acidity build up, high soluble toxic metals, and less water available in the soil (Wang and Yang, 2003). Moreover, chemical fertilizers like phosphate fertilizers can cause environmental impact as they are often contaminated with heavy metals, particularly cadmium (Cd) which originated from rock phosphate (Schipper et al., 2011).Asa limitation of land use for rice cultivation; therefore, some paddy fields have to be located near mines. This can lead to heavy metals contamination in the fields that caused by inadequately treated mining drainage discharge (Yang et al., 2006). Heavy metals slowly contaminated rice through the roots and then accumulated in the grain. However, most of the accumulation of heavy metals was observed in the roots more than the grains (Li et al., 2007). Römkens et al. (2009) reported that soils contaminated with Cd in a range of 0.1 to 30 mg Cd/kg of soil resulted in contamination of the grains at an average of 0.2 mg Cd/kg of grain.

The consumption of Cd-contaminated rice at 0.24 µg Cd/g of unpolished rice was likely to contribute a Cd dose of 2.2 µg/kg body weight/day for adult 60 kg weigh and a dose of 1.5 µg Cd/kg body weight/day for children 40 kg weigh(Yang et al., 2006). In Thailand, the contamination of Cd and zinc (Zn) in rice nearby a Zn mine was observed in Mae Sot District, Tak Province, and caused adverse effects on human health and environment. Jasmine rice was a major export product of the district; therefore, the government paid the farmers to refrain from planting rice in this area during 2005-2007 (Academic and Standard Groups, Office of Management and Environmental Remediation, Department of Primary Industries and Mines, 2004). To solve such serious problem, the chemical fertilizers should be reduced to apply by replacing with biofertilizers, and the Cd-contaminated areas should be bioremediated.

Biofertilizers are an attractive to chemical fertilizers. Among biofertilizers that produce plant growth promoting substances and potentially alleviate heavy metals including Cd in contaminated areas are purple non-sulfur bacteria (PNSB)(Harada et al., 2005; Nookongbut et al. 2018;Sakpirom et al., 2017 and 2019). They are a group of bacteria that distribute in natural areas where sunlight can reach i.e. flooded paddy fields. They can produce certain phytohormones such as auxin and cytokinin including 5-aminolevulinic acid (ALA), a plant growth promoting substance (Mujahid et al., 2011; Nunkaew et al., 2015; Sakpirom et al., 2017; Nookongbut et al., 2018).Formulations of biofertilizers should be considered as they are important concern for providing survival cells during storage and ensuring their establishment in fields (Singh et al., 2011; Herrmann and Lesueur, 2013; Kantha et al., 2015). The biofertilizers in solid form are more convenient to handle and longer shelf life than liquid form (Herrmann and Lesueur, 2013). Nevertheless, the biofertilizers in solid form require a suitable carrier for formulation that might be toxic to plant and also biofertilizers (Herrmann and Lesueur, 2013).

Some members of PNSB can use hydrogen sulfide (H_2S) surrounding rice roots as an electron donor for photosynthesis; thereby, resulting in reduction of toxicity to rice, stronger roots, and consequently higher productivity (Lamer et al., 2013). Moreover, flooded paddy fields provide anoxic conditions which are suitable for production of H_2S by sulfate reducing bacteria (Lamers et al., 2013). When H_2S is transformed to sulfide (S^{2-}), it actively reacts with heavy metals ions contaminated in

paddy fields to produce a precipitated form of metal-sulfide nanoparticles (Castillo et al., 2012) that are also found in PNSB (Bai et al.,2009; Bai and Zhang, 2009; Sakpirom et al., 2019). The heavy metals as the precipitated form are less toxic than their soluble form (Reichman, 2002). It is well recognized that PNSB have been widely studied for removal of heavy metals ions, especially Cd bioaccumulation and biosorption (Smiejan et al., 2003; Watanabe et al., 2003; Panwichian et al., 2010b; Sakpirom et al., 2017) along with conversion of Cd to more stable forms like Cd sulfide (Fan et al., 2012; Sakpirom et al.,2019).

Therefore, this study aimed to investigate the performance of PNSB biofertilizers, *Rhodopseudomonas palustris* TN110 and *Rubrivivax gelatinosus* TN414, either as individual cultures or a mixed culture of both strains in both liquid and solid formulations on rice (*Oryza sativa* L.) growth in Cd-contaminated paddy soil. The ability of PNSB biofertilizer(s) to reduce Cd and Zn in rice materials was also evaluated.

Materials and methods

Preparation of PNSB inoculums

Rhodopseudomonas palustris TN110 and *Rubrivivax gelatinosus* TN414, isolated from saline paddy fields in Southern Thailand (Nunkaew et al., 2015), were used in this study based on their abilities to be plant growth promoters and biofertilizers, and to reduce both Cd and Zn (Sakpirom et al., 2017).To prepare PNSB inoculants, both PNSB strains were separately subcultured twice to obtain active inoculums, and 10% of each culture was transferred to GA broth in a screw cap test tube (Sakpirom et al., 2017). All culture tubes were incubated under their optimal microaerobic-light conditions for 48 h (Sakpirom et al., 2017). Afterwards, the culture broths were centrifuged at 6,000 rpm for 15 min to obtain cell pellets. Each biomass was adjusted to an optical density of 2.0 at a wavelength of 660 nm (OD_{660}) using 0.1% peptone water to provide a cell density of approximately 10^{10} CFU/mL

PNSB biofertilizer(s) preparation

Rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA) and spent coffee grounds (SCG) were used as a mixed carrier for supporting biofertilizer(s) in a solid form. These carriers and an optimal mixed carrier were characterized for their properties as described in Chapter 5. Each carrier was individually well ground and then filtrated through a sieve with 0.5 mm openning. The optimal proportion of RWA: DCC: RHA: SCG at 3: 4: 2: 1 (as reported in Chapter 5) was mixed and transferred (approximately 100 g) to sterile polypropylene (PP) bags (size 12 × 18 cm) for packaging. All bags were tightly tied using rubber bands prior to sterilize at 121 °C for 30 min, followed by drying in an oven at 60 °C until reaching a constant weight and then cooled down to room temperature (25±3 °C). The active inoculums (*R. palustris* TN110, *Ru. gelatinosus* TN414 and a mixed culture of both strains at 1: 1) were separately inoculated in the optimal mixed carrier with 40% moisture content using sterile mature coconut water for obtaining bacterial cell density approximately 10^{10} CFU/g. All PNSB biofertilizers and carrier control were mixed well by a Stomacher® blender and they were ready for use.

Influence of PNSB biofertilizer(s) on rice growth in Cd-contaminated paddy soil

Physico-chemical properties of paddy soils

Paddy soils in Mae Sot District, Tak province, Thailand were collected at 16°40'32.50"N, 98°35'44.52"E for Cd-contaminated paddy soil and for control paddy soil with an expectation to be uncontaminated soil at 16°41'25.43"N, 98°35'43.79"E. Both paddy soils were determined for their physico-chemical properties as follows. pH and electrical conductivity (EC) were measured by a pH and EC meter (SevenMulti, Mettler Toledo, Switzerland). The Walkley and Black method was used for characterization of organic matter and organic carbon. Total nitrogen (N) and total sulfur (S) were determined by a combustion and photometric method, respectively. The above standard methods used in this study are described in Sparks et al. (1996). Total phosphorus (P_2O_5), total potassium (K_2O), calcium (Ca), magnesium (Mg),

silicon (Si), sodium (Na), iron (Fe), manganese (Mn), copper (Cu), nickel (Ni), cobalt (Co), cadmium (Cd), zinc (Zn), molybdenum (Mo) and vanadium (V) were determined by an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Perkin Elmer, Germany).

Pot greenhouse experiment

The experimental design consisted of 6 treatments (3 PNSB; TN110, TN414 and a mixed culture × 2 forms of biofertilizer formulation (solid and liquid)) and two controls (tap water and carrier) as follows: Cd-contaminated paddy soils with inoculation of TN110, TN414 and a mixed culture in both forms, and their controls using sterile tap water and the optimal mixed carrier without PNSB. In addition, parallel experimental sets as above described were also conducted in un-contaminated paddy soil to serve as another control. The optimal dilution of each biofertilizer obtained from the previous study as described in Chapter 5 with both forms was designed for investigating their efficiency to enhance rice seedling growth in both paddy soils. Rice (*Oryza sativa* L.) seeds were separately submerged in 10 mL of each PNSB biofertilizer with its optimal dilution (Chapter 5) from both the liquid and solid formulations to provide10^8 cells/mL for strain TN110 and 10^7 cells/mL for TN414 and a mixed culture based on their optimal dilution at 1: 100 for TN110 and 1: 1,000 for TN414 and a mixed culture; whereas sterile tap water was used as a control. All submerged rice seeds were incubated overnight to ensure the attachments of bacterial cells on rice seed surface. A 500 g of paddy soil was added into each pot (size 12 × 12 × 25 cm). Tap water was added to flood fresh paddy soil at 1 cm from the top of the soil for 7 days before planting.

Nine germinated rice seeds were grown in each soil pot until day 7. Then, PNSB biofertilizer(s) in both forms were applied separately (30 mL for each form in each soil pot) to give concentrations of 10^8 cells/mL for strain TN110 and 10^7 cells/mL for TN414 and a mixed culture (Chapter 5). Sterile tap water and the optimal mixed carrier without PNSB served as the controls for liquid and solid forms of biofertilizer(s), respectively. The water level was maintained 1 cm above the surface

soil throughout the experiment for 28 days. The experiment was carried out in a greenhouse with three replicates (9 × 3 = 27 seedlings/set).

After 21 days of applying biofertilizer(s) either in liquid or solid form; seedling growth was measured based on lengths of shoots and roots and their dry weight. Rice root and shoot were dried in an oven at 60 °C overnight or until they reached a constant weight for measuring dry weight. Dried plant materials both shoot and root were also analyzed for Cd and Zn contents. After grinding, they were individually separated through a 2 mm sieve, then the portions smaller than 2 mm were extracted by acid digestion (HNO_3: HCl = 1: 3) and heated at 70 °C for 1 h by periodic shaking. Afterwards, each extract was diluted with 6 mL of deionized water and filtered through Whatman filter paper no.1 (Kim et al., 2002). The filtrate from shoot and root samples were analyzed for Cd and Zn using an inductively coupled plasma - optical emission spectrometer (ICP-OES) (PerkinElmer, Germany).

Data presentation and statistical analysis

All experiments were conducted in triplicate unless otherwise stated. Means and standard deviations are presented. Statistical differences among means were performed by the Duncan's multiple tests at $P \leq 0.05$.

Results

Influence of PNSB biofertilizer(s) on rice growth in Cd-contaminated paddy soil

Cd and Zn concentrations in Cd-contaminated paddy soil were 10.65 and 417.54 mg/kg; while control paddy soil contained Cd and Zn at 1.20 and 66.24 mg/kg (Table 6-1). The optimal dilution of each biofertilizer in both liquid and solid forms was investigated for their performance on growth parameters of rice seedlings grown in both paddy soils. Application of all PNSB -biofertilizer(s) in both forms significantly improved ($P < 0.05$) all plant growth parameters compared with their controls, with exception of shoot dry weight in sets of TN414 and mixed culture in liquid form (Figure 6-1A). The lowest plant growth performance was observed in

both controls (carrier and water controls), and no significant difference was found for both controls in both paddy soils (Figure 6-1A to D). It was found that almost all of the growth parameters on the basis of shoot and root dry weight, shoot and root length of rice seedlings from both paddy soils were significantly higher in set of *R. palustris* TN110 in solid form compared with other biofertilizers and itself in liquid form (Figure 6-1A to D). The shoot dry weight and root length with inoculation of *R. palustris* TN110 in solid form had significantly the highest values in Cd-contaminated paddy soil (Figure 6-1A and D). Moreover, all biofertilizers in the solid form significantly provided higher shoot dry weight than the liquid form and their controls in both paddy soils (Figure 6-1A). However, the solid and liquid forms of almost all the biofertilizes gave no significant difference in root dry weight and shoot length of rice seedlings for both paddy soils (Figure 6-1B and C).

Table 6-1. Physico-chemical properties of Cd-contaminated soil and control soil collected from paddy fields in Mae Sot District, Tak Province, Thailand.

Parameter	Cd-contaminated paddy soil (16°40'32.50"N, 98°35'44.52"E)	Control paddy soil (16°41'25.43"N, 98°35'43.79"E)
pH	7.61	7.37
EC (µS/cm)	79.80	117.70
Organic matter (%)	3.52	6.01
Organic carbon (%)	2.05	3.48
Total nitrogen (%)	0.15	0.32
Total phosphorus (%)	0.03	0.04
Total potassium (%)	0.10	0.10
Total sulfur (%)	0.01	0.02
Extractable elements (mg/kg)		
Ca	2300.00	3700.00
Mg	900.00	1900.00
Si	38.83	37.12
Na	74.16	87.59
Fe	7700.00	8900.00
Mn	192.17	300.46
Cu	12.61	14.81
Ni	8.46	7.16
Co	4.86	6.13
Cd	10.65	1.20
Zn	417.54	66.24
Mo	Not detected	Not detected
V	16.138	25.974

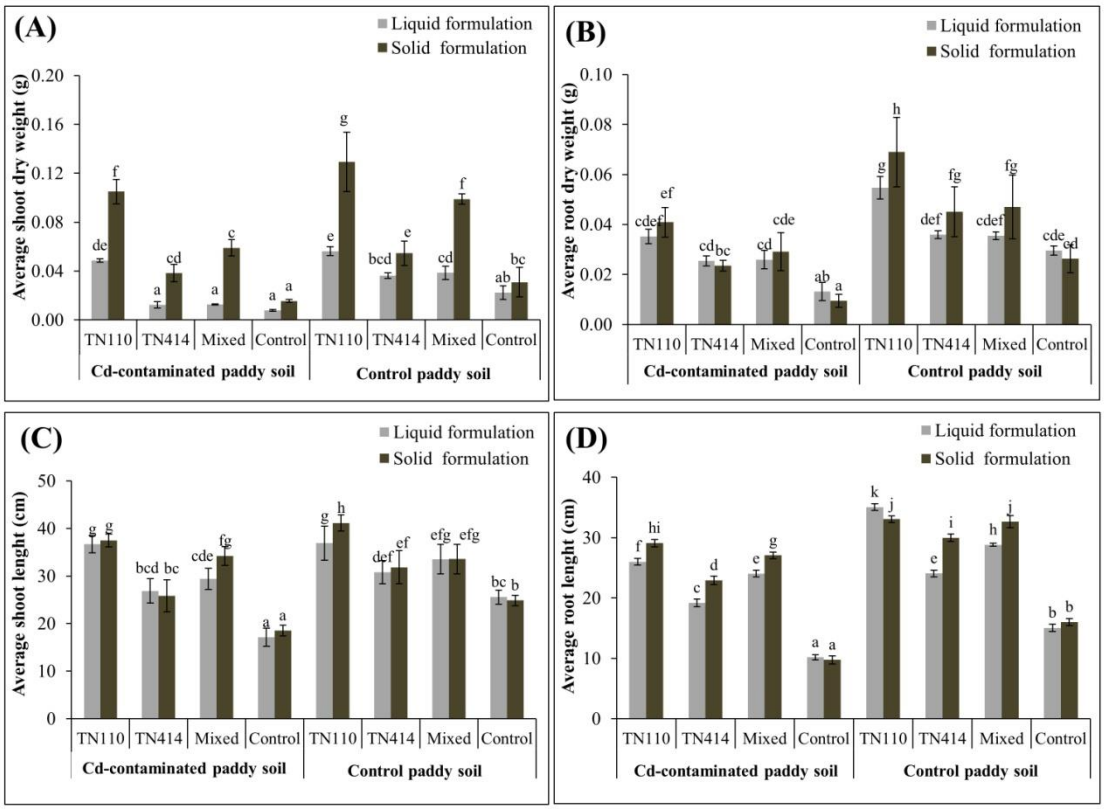

Figure 6-1. Influence of various PNSB biofertilizers in liquid and solid forms on rice seedling grown in pots with Cd-contaminated and control paddy soils under greenhouse condition for 28 days. Rice growth parameters are (A) shoot dry weight, (B) root dry weight, (C) shoot length and (D) root length. Mean values (n = 27) with the different lowercase letters are significantly different at $P \leq 0.05$.

Influence of PNSB biofertilizer(s) on heavy metal accumulation in rice seedlings

All PNSB biofertilizer(s) either in liquid or solid form significantly reduced Cd in root and shoot from Cd-contaminated paddy soil to lower than the control sets in a range of 3.07 -16.27 times (Figure 6-2A). On the other hand, Zn accumulation in both rice shoot and root from contaminated soil of all inoculated sets was lower than the control sets in a range of 1.62 - 10.37 times (Figure 6-2B). A similar trend was also found for both Cd and Zn in plant materials (shoot and root) from control paddy soil (Figure 6-2A to B) that contained both heavy metals (Table 6-1), although they are significantly different for only some sets. No significant difference was observed for Cd removal efficiency in shoot from Cd-contaminated paddy soil for all PNSB biofertilizers in a solid form (Figure 6-2A). On the other hand, all PNSB biofertilizers in solid form showed higher efficiency than liquid form in reducing Cd in rice materials from Cd-contaminated soil.

In contrast, strain TN414 in both forms was the most effective in reducing Zn content in both root and shoot of rice seedlings from both paddy soils, followed by a mixed culture (Figure 6-2B).The results of control set without inoculation showed that the Cd-contaminated paddy soil led to accumulation of Cd and Zn in rice seedling higher than the control paddy soil up to 88% and 72% for Cd in shoot and root, and 30% and 62% for Zn in shoot and root. Nevertheless, all sets inoculated with PNSB biofertilizer(s) had lower accumulations of both heavy metals in Cd-contaminated than their controls without PNSB inoculation. Similar to Cd removal, all PNSB biofertilizers in solid form in Cd contaminated soil were more effective in reducing Zn in both rice shoot and root than in liquid form.

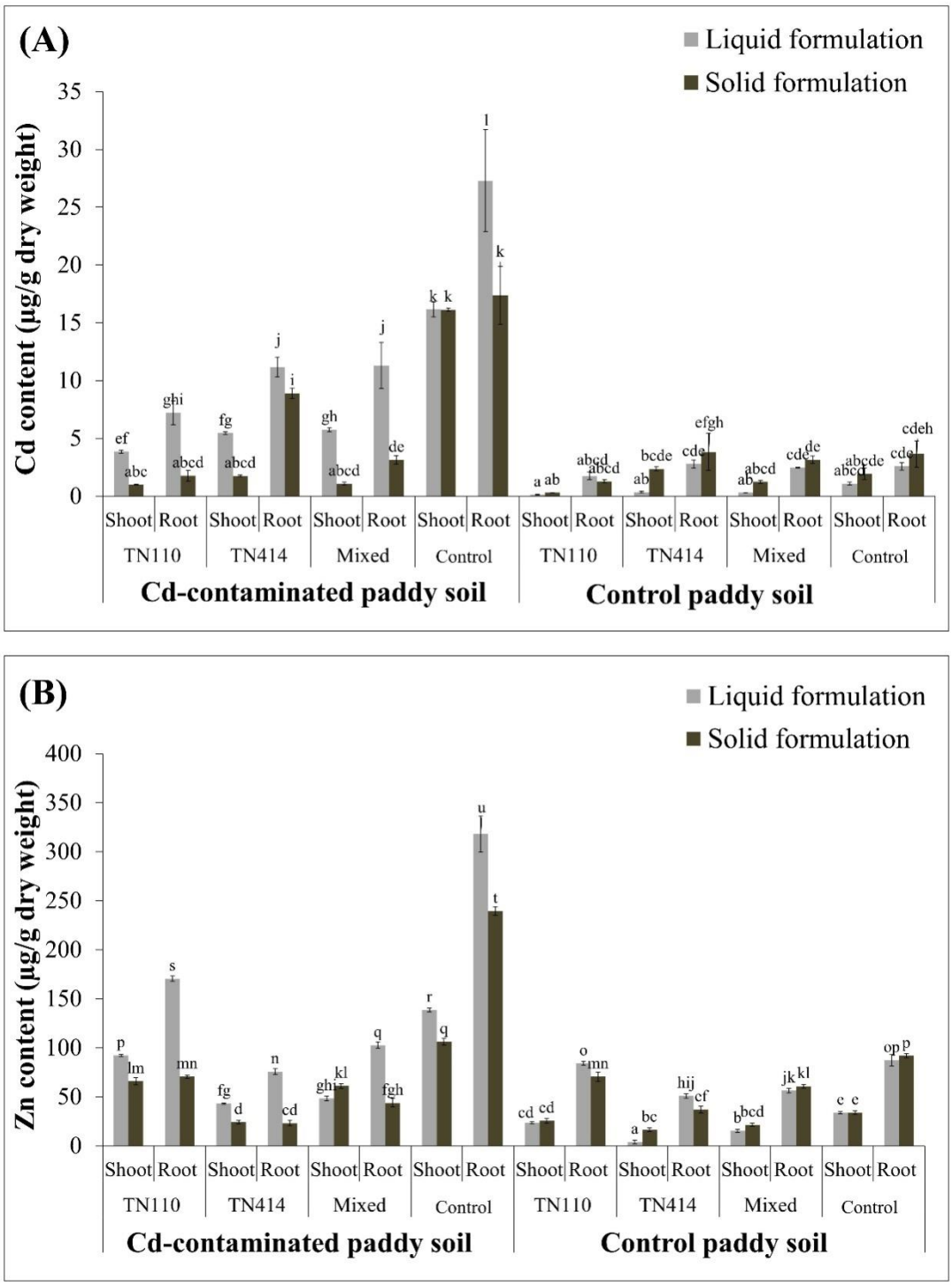

Figure 6-2. Influence of various PNSB biofertilizer(s) in liquid and solid forms on (A) Cd and (B) Zn accumulation in rice seedlings grown in pots containing Cd-contaminated and control paddy soils under greenhouse condition for 28 days. Mean values (n = 27) with different lowercase letters are significantly different at $P \leq 0.05$.

Discussion

Influence of PNSB biofertilizer(s) on the growth of rice seedlings

For rice seedling growth in Cd-contaminated soil pots for 28 days, the solid form of PNSB biofertilizers was obviously higher than the liquid form for shoot dry weight and root length (Figure 6-1A and D). This might be because of no phytotoxicity found in biofertilizers in solid form compared to liquid form as no significant difference was observed for both controls (water and carrier) on rice growth in both soils used (Figure 6-1). In addition, the solid form containing a mixed carrier which had plant nutrients (Chapter 5) and therefore should stimulate rice growth (Figure 6-1A and D). Another reason is the solid PNSB biofertilizer(s) was able to reduce accumulation of Cd and Zn in root and shoot of rice (Figure 6-2A and B); this led to increase rice growth (Figure 6-1). Interestingly, PNSB biofertilizers in both forms significantly enhanced rice growth compared with the controls. This is due to the fact that all PNSB biofertilizer(s) in both forms were able to reduce the toxicity of both metal ions (Cd and Zn) by decreasing accumulated metals in rice shoot and root (Figure 6-2A and B), and this consequently promoted rice growth (Figure 6-1A to D). Moreover, it might be possible that PNSB released $NH_4^+$and/or plant growth promoting substances such as ALA and IAA (Sakpirom et al., 2017) as well as exopolymeric substances for preventing metals into rice through entrapment (Nguyen et al. 2018; Nookongbut et al. 2018). The PNSB biofertilizer(s) were applied on rice seeds at day 0 and also later at day 7 of cultivation at cell densities of roughly 10^7-10^8 cells/mL which were sufficient for plant growths (Lupwayi et al., 2000; Kantha et al., 2015; Nunkeaw et al., 2015; Noogkongbut et al. 2018). Both controls performed the least effective to promote rice growth as the less plant nutrients in the carrier control or no nutrient for the water control. Overall results proved that PNSB biofertilizer(s) have the potential to enhance rice growth in paddy soils contaminated with Cd at high or low concentration by preventing heavy metals from getting into rice and also stimulating rice growth.

Influence of PNSB biofertilizer(s) on heavy metal accumulation in rice seedlings

It should be noted that almost all of the treatments with the solid forms of PNSB biofertilizer(s) showed dramatic decreases in Cd and Zn accumulations in rice shoots and roots than the liquid forms for rice seedlings grown in Cd-contaminated soil (Figure 6-2A and B). The efficiency to reduce accumulations of Cd and Zn was governed by PNSB and the mixed carrier as previously discussed. This is in accordance with previous studies that the carriers used for biofertilizer(s) could mitigate heavy metal accumulation into plants by adsorption on their carrier surfaces (Hasan et al., 2000; Masae et al., 2013). The results in this study (Figure 6-2A and B) confirmed that both PNSB, *R. palustris* TN110 and *Ru. gelatinosus* TN414, are candidates for Cd and Zn removal particularly strain TN414 (Sakpirom et al., 2017). Overall results indicate that PNSB used in this present study not only acted as biofertilizers but also bioremediation agents for removal of heavy metals from Cd-contaminated paddy soil. This should be further investigated by looking at applications PNSB biofertilizer(s) in paddy fields for producing safe rice and decreasing use of chemical fertilizers.

Conclusions

R. palustris TN110 and *Ru. gelatinosus* TN414 are biofertilizers and as either a single or a mixed culture in both liquid and solid forms were able to enhance rice growth and to reduce accumulated heavy metals in rice shoot and root. However, the solid form is more beneficial than that the liquid form to rice in Cd-contaminated paddy soil for promoting rice growth and preventing both heavy metals from getting into rice. This should be a combination effect of PNSB and a mixed carrier used for supporting PNSB played the role to prevent both metals into rice.

CHAPTER 7

Effects of Light/Dark Cycles and Formulation Types of Purple Non-Sulfur Bacteria Biofertilizers on Mitigation of Greenhouse Gas Emission from Rice Straw Biodegradation in Paddy Soil Slurry

Abstract

Purple non-sulfur bacteria (PNSB) are attractive biofertilizers for rice cultivation and for reduction of methane emission in paddy fields by competition with methanogens. The light/dark cycle was investigated for the abilities of PNSB and methanogens under different cycles; 0/24, 8/16, 12/12, 16/8 and 24/0 h to release CH_4 and CO_2 from rice straw biodegradation in soil slurry. Under all light/dark cycles, inoculation of *Rhodopseudomonas palustris* TN110 from liquid form was the most effective in reducing CH_4 and CO_2 emissions compared with other sets (*Rubrivivax gelatinosus* strain TN414 and a mixed culture of both strains at 1: 1, carrier control, rubber wood ash: RWA, decanter cake: DCC, rice husk ash: RHA, spent coffee grounds: SCG and native control).All PNSB biofertilizer(s) in liquid form under all light/dark cycles throughout 10 day-incubation showed higher efficiencies in reducing total daily gas volume than a solid form. Strain TN110 in liquid form under a natural light/dark cycle at 12/12 h at day 10 reduced 72.27% CH_4 and 34.38% CO_2 emissions. PNSB biofertilizers, particularly strain TN110 can potentially be used in paddy fields for reducing greenhouse emissions.

Keywords: Dark, Light, Methane, Methanogens, Paddy field, Phototrophic bacteria

บทคัดย่อ

แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มที่ไม่สะสมซัลเฟอร์ (Purple non-sulfur bacteria; PNSB) หนึ่งในกลุ่มของแบคทีเรียสังเคราะห์แสงที่ไม่เกิดออกซิเจนจากการสังเคราะห์แสง เป็นกลุ่มของแบคทีเรียที่น่าสนใจในการนำไปใช้เป็นปุ๋ยชีวภาพในการปลูกข้าวและยังสามารถลดการปล่อยก๊าซมีเทนในนาข้าวโดยการแข่งขันกับจุลินทรีย์กลุ่มที่สร้างก๊าซมีเทน (Methanogens) ดังนั้นจึงตรวจสอบผลการให้แสง/ไม่ให้ต่อวันแตกต่างกันที่ 0/24 8/16 12/12 16/8 และ 24/0ชั่วโมง ต่อการแข่งขันการเจริญระหว่าง PNSB และ Methanogensซึ่งส่งผลต่อการปลดปล่อยก๊าซเรือนกระจก (ก๊าซมีเทนและคาร์บอนไดออกไซด์) ในการย่อยฟางข้าวที่ผสมกับดินนาข้าว ภายใต้สภาวะที่ให้แสงต่อวันแตกต่างกันตามที่กล่าวมา ผลการทดลองพบว่า สายพันธุ์ *Rhodopseudomonas palustris*TN110 ในรูปแบบของเหลวมีประสิทธิภาพดีที่สุดในการลดการปลดปล่อยก๊าซมีเทนและคาร์บอนไดออกไซด์เมื่อเปรียบเทียบกับชุดการทดลองอื่นๆ (*Rubrivivax gelatinousus* TN414 ชุดเชื้อผสมที่อัตราส่วน 1: 1) ชุดควบคุมที่เป็นวัสดุพยุง ขี้เถ้าเหลือใช้จากกระบวนการผลิตยางพาราแผ่น กากปาล์มน้ำมัน ขี้เถ้าจากแกลบข้าว และกากกาแฟ รวมถึงชุดควบคุมที่ไม่มีการเติม ตลอดการทดสอบ 10 วัน ปุ๋ยชีวภาพทุกชุดการทดลองในรูปของเหลวมีการปลดปล่อยก๊าซสะสมแต่ละวันต่ำกว่าปุ๋ยชีวภาพในรูปของแข็ง โดย ปุ๋ยชีวภาพในรูปของเหลวสายพันธุ์ TN110 ในวันที่ 10 ภายใต้สภาวะการให้แสงที่12/12 ชั่วโมง เปรียบเสมือนระบบแสงตามธรรมชาติสามารถลดการปลดปล่อยก๊าซมีเทนและคาร์บอนไดออกไซด์ได้ร้อยละ 72.27 และ 34.38 ตามลำดับ ปุ๋ยชีวภาพ PNSB โดยเฉพาะอย่างยิ่งสายพันธุ์ TN110 มีความเป็นไปได้สูงในการลดการปลดปล่อยก๊าซเรือนกระจกเพื่อป้องกันภาวะโลกร้อน

คำสำคัญ : นาข้าว แบคทีเรียสังเคราะห์แสง มีเทน มีแสง เมทาโนเจน ไม่มีแสง

Introduction

Rice cultivation in flooded paddy fields in developing countries has been charged as a source of greenhouse gases, particularly methane (CH_4) (Zhang et al., 2012; Begumet al., 2019; Chen et al., 2019). CH_4 is more potent than carbon dioxide (CO_2) approximately 34 times as a cause of global warming (IPCC, 2013). Ahmad et al. (2009) found that rice cultivation without the use of chemical fertilizers released CH_4 at 34.24 g/m^2; whereas that with the use of chemical fertilizers significantly emitted more CH_4 at 65.96 g/m^2. CH_4 is produced under anaerobic conditions in paddy fields which is a suitable condition for the growth of methanogens (De Vrieze et al., 2012). The continuously flooded rice fields emitted 0.19 g CH_4/m^2/day into the atmosphere (Wang et al., 2018). CH_4 is the end-product of anaerobic degradation of organic matter in the flooded soil. Several studies have shown that the produced CH_4 is derived from the degradation of rice straw, root exudates and soil organic matter (Whitman et al., 2006; Begum et al., 2019; Jakrawatana et al., 2019). Incorporation of rice straw into soil as a fertilizer which is a source of acetate is a common practice in rice cultivation. Therefore, this agricultural waste is one of the main carbon sources in flooded paddy soil for CH_4 production and emission (Smakgahn et al., 2018; Begum et al., 2019; Jakrawatana et al., 2019; Maneepitak et al., 2019). It is well recognized that light is one of the most important factors influencing the source strength for anoxygenic phototrophic bacteria to compete with methanogens in paddy fields that could be reduced atmospheric CH_4 emission (Tada et al., 2005).

Methanogens or methanogenic bacteria are a large group of archaea that are responsible to CH_4 emission and very diverse in their classification. They are classified into the domain archaea which are closely related to the eubacteria, especially the photosynthetic eubacteria (Costaand Leigh, 2014). There are 3 groups of methanogens based on their substrates (H_2, acetate and both substrates) used for producing CH_4 by methanogenesis pathways (Whitman et al., 2006). Methanogens are very sensitive to oxygen, so they can be found anywhere in the environment without oxygen. Interestingly, they are different from other kinds of archaea because they are normally found in the normal conditions such as mesophile, neutral pH and low salinity (Whitman et al., 2006, Chen et al., 2019). Generally, they can be detected

in anaerobic digestive system, gastrointestinal tract, anaerobic sediment and flooded soil (Paul et al., 2012; Wen et al., 2017; Islam et al., 2018; Suzuki et al., 2018). Under anaerobic conditions in the presence of terminal electron acceptors i.e. NO_3^-, Fe^{3+} and SO_4^{2-} would inhibit methanogens by competing in the use of electron donors i.e. H_2 and acetate by other microorganisms such as sulfate-reducing bacteria as well as anoxygenic phototrophic bacteria (Whitman et al., 2006).

Purple non-sulfur bacteria (PNSB) are one group of anoxygenic phototrophic bacteria with potential capabilities to reduce CH_4 emission by competing with methanogens for carbon sources, to act as biofertilizers to fix N_2, and to release plant growth promoting substances (Harada et al., 2001 and 2005; Kantachote et al. 2016; Sakpirom et al., 2017). Biofertilizers can be produced in either liquid or solid form with some advantages and disadvantages. It has long been known that PNSB growth under light relies on photosynthesis, but under dark they grow without photosynthesis. Therefore, light/dark cycles should be investigated for proliferation between PNSB and methanogens populations to elucidate how the conditions affect CH_4 emission in flooded paddy soil. Kantachote et al. (2016) reported that biofertilizer carriers are a source of CH_4 emission as well; thereby, any carriers that were used should be evaluated for their CH_4 emission.

The objectives of this study were to investigate effect of light/dark cycles, biofertilizer types (liquid and solid) and carriers on greenhouse gases, CH_4 and CO_2, emissions from rice straw biodegradation in paddy soil to obtain a better understanding on utilization of PNSB as biofertilizers as well as agents to reduce greenhouse gas emissions.

Materials and methods

Preparation of PNSB inoculums

Rhodopseudomonas palustris TN110 and *Rubrivivax gelatinosus* TN414, members of PNSB, isolated from saline paddy fields in Southern Thailand (Nunkaew et al., 2015) were used in this study based on their ability to be plant growth promoters and biofertilizers (Sakpirom et al., 2017). To prepare PNSB inoculums, both PNSB strains were separately subcultured twice, and 10% of each culture was transferred to GA broth in a screw cap test tube (Sakpirom et al., 2017). All culture tubes were incubated under their optimal microaerobic-light conditions for 48 h (Sakpirom et al., 2017). Afterwards, the culture broths were centrifuged at 6,000 rpm for 15 min to obtain cell pellets. Each biomass was adjusted to an optical density of 2.0 at a wavelength of 660 nm (OD_{660}) using 0.1% peptone water to obtain a cell density of approximately 10^{10} CFU/mL.

PNSB biofertilizer(s) preparation

Rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA) and spent coffee grounds (SCG) were used as a mixed carrier for supporting biofertilizer(s) in a solid form. These carriers and an optimal mixed carrier were characterized for their properties as described in Chapter 5. Each carrier was individually well ground and then filtrated through a sieve with 0.5 mm openning. The optimal proportion of RWA: DCC: RHA: SCG at 3: 4: 2: 1 (as reported in Chapter 5) was mixed and transferred (approximately 100 g) to sterile polypropylene (PP) bags (size 12 × 18 cm) for packaging. All bags were tightly tied using rubber bands prior to sterilize at 121 °C for 30 min, followed by drying in an oven at 60 °C until reaching a constant weight and then cooled down to room temperature (25±3 °C). The active inoculums (*R. palustris* TN110, *Ru. gelatinosus* TN414 and a mixed culture of both strains at 1: 1) were separately inoculated in the optimal mixed carrier with 40% moisture content using sterile mature coconut water for obtaining bacterial

cell density approximately 10^{10} CFU/g. All PNSB biofertilizers and carrier control were mixed well by a Stomacher® blender and they were ready for use.

Paddy soil characterization and rice straw preparation

Fresh paddy soil collected from Khuan Khanun District, Phatthalung Province, Thailand (7°45'11.0"N, 100°06'57.0"E) was used in this experiment and its characteristics are shown in Table 7-1. To determine pH, electrical conductivity (EC), organic matter, organic carbon, total N and total S, standard methods as described by Sparks et al. (1996) waere used. The fresh paddy soil was stored in the dark for prevention of photosynthesis at 4 ℃ until used. The rice straw was ground and then screened by a sieve with 0.5 mm opening and stored under the same condition as the paddy soil until used.

Paddy soil slurry for rice straw biodegradation model

Paddy soil slurry was prepared in a 120 mL transparent serum bottle by adding 10 g of fresh paddy soil into each serum bottle, 0.3 g of rice straw and finally 90 mL of distilled water. Ten mL inoculum of each PNSB serving as a liquid formulation (*R. palustris* TN110, *Ru. gelatinosus* TN414 and a mixed culture) was added into the soil slurry and the bottle was sealed with a rubber stopper and superimposed aluminum cap. On the other hand, 10 g of each solid formulation (*R. palustris* TN110, *Ru. gelatinosus* TN414, a mixed culture, and a carrier control) were diluted with sterile distilled water at a ratio of 1:100 (%w/v), and then separately added with 10 mL into soil slurry. A final concentration of PNSB population from both liquid and solid formulations was roughly 10^8 CFU/mL. Moreover, 10 g of each individual carrier (RWA, DCC, RHA and SCG) and an optimal mixed carrier was diluted with sterile distilled water at ratio of 1:100 (%w/v) and then 10 mL each was separately added into soil slurry. The paddy soil slurry was a closed microaerobic system at the beginning and later to become anaerobic conditions (based on preliminary work). The system was connected with a syringe to collect gas emission from rice straw biodegradation.

All serum bottles were incubated at room temperature (25±3 °C) with 3,000 lux light intensity for 10 days. The incubation was carried out under different light/dark cycles for periods of 0/24, 8/16, 12/12, 16/8 and 24/0 h. Simultaneously the incubation of the paddy soil slurry without inoculation was set under the same condition to serve as a control (native control). Ten-mL gas collection syringe was inserted through the septum by connecting with a hollow needle to maintain the inside at atmospheric pressure during the experiment and to observe a total gas volume every day for 10 days. A volume of total gas released was indicated by a space volume of the gas collection syringe. At day 10, the collected gases were analyzed for CH_4 and CO_2 this was due to the fact that total gas volume slightly increased from days 8 to 10 for all experimental sets.

The gas samples collected from the gas collection syringe were analyzed for CH_4 and CO_2 by a gas chromatograph (GC) as described in Sakpirom et al. (2017). In brief, a GC (7890A GC systems, Agilent Technologies, USA) equipped with a thermal conductivity detector (TCD) and HP-PLOT/Q column (length 30 m, diameter 0.53 mm, film thickness 40 µm) (Agilent Technologies, USA) was used to measure CH_4. The inlet and detector temperature, and the oven temperature were set at 250 °C and 60 °C (hold 3 min, 9 psi). The pressure of the carrier gas (high purity He), H_2, and air was approximately 75 psi. A biogas standard consisting of 60% (v/v) CH_4, 35% CO_2 and 5% N_2 was used for GC calibration. The CH_4 and CO_2 percentages were compared with the native control and percent reduction was then calculated.

Data presentation and statistical analysis

All experiments were conducted in triplicate. Means and standard deviations are presented. Statistical differences among means were determined by the Duncan's multiple tests at $P \leq 0.05$.

Results

Effect of light/dark cycles and formulation types of biofertilizer(s) on greenhouse emissions from rice straw biodegradation model

The results of soil used in this study showed that pH of the paddy soil was slightly acidic with pH of 5.29 and contained 9.09% of organic matter including macro- and micro-nutrient such as nitrogen (N), potassium (P), phosphorus (K), sulfur (S), calcium (Ca), magnesium (Mg), etc. (Table 7-1).

Table 7-1. Characteristics of paddy soils collected from Khuan Khanun District, Phatthalung Province in Southern Thailand.

Parameter	Method	Soil characteristic
pH	pH meter	5.29
EC (µS/cm)	EC meter	72.90
Organic matter (%)	Walkley and Black	9.09
Organic carbon (%)	Walkley and Black	5.27
Total N (%)	Combustion method	0.46
Total P_2O_5 (%)	ICP-OES	0.10
Total K_2O (%)	ICP-OES	0.04
Total S (%)	Photometric method	0.01
Extractable elements (mg/kg)		
Ca	ICP-OES	1000.00
Mg	ICP-OES	700.00
Mn	ICP-OES	50.53
Cu	ICP-OES	12.17
Fe	ICP-OES	3300.00
Zn	ICP-OES	20.86
Mo	ICP-OES	0.00
V	ICP-OES	19.89
Ni	ICP-OES	6.43
Co	ICP-OES	2.48
Si	ICP-OES	44.87
Na	ICP-OES	181.15
Cd	ICP-OES	0.10

The paddy soil was prepared as paddy soil slurry for studying CH_4 emission from rice straw biodegradation model in serum bottles under anaerobic-light/dark cycles for 10 days. The total gas volume collected by a collecting gas syringe under different supplements was observed daily for 10 days. PNSB biofertilizers both solid and liquid forms (*R. palustris* TN110, *Ru. gelatinosus* TN414 and a mixed culture of both strains, carrier control (optimal mixed carrier)) including each carrier (RWA, DCC, RHA and SCG) were supplemented to paddy soil slurry. All experimental sets under a light/dark cycle at 0/24 h released the highest total daily gas volume (Figure 7-1A to E). In contrast, under a cycle of 24/0 h of light/dark, the lowest total daily gas volume was observed for the first 5 days of the test (Figure 7-1E). The total daily gas volume released under 0/24 and 8/16 h of the light/dark cycles significantly increased with time throughout incubation (Figure 7-1A and B). In case of 12/12 h for light/dark cycle which is a natural light cycle for tropical zone, the total daily gas volume was constantly released after 5 days (Figure 7-1C). However, no significant difference was found for the total daily gas volume released under 12/12, 18/6 and 24/0 h of the light/dark cycles especially after 5 days (Figure 7-1C to E).

A maximum total of daily gas volume was obtained in the paddy soil slurry supplemented with SCG in all light/dark cycles investigated; whereas a minimum total daily gas volume was found in the paddy soil slurry supplemented with either RWA or RHA or liquid formulation of either strain TN110 or a mixed culture (Figure 7-1A to E). Moreover, the total daily gas volume of all light/dark cycles was significantly lower in liquid form than solid form of all PNSB biofertilizers (Figure 7-1A to E) with outstanding in light/dark cycle of 0/24 h (Figure 7-1A). Figure 7-1 also shows that a mixed carrier without PNSB inoculation (carrier control) for solid formulation emitted the total daily gas volume significantly higher than both formulations containing PNSB.

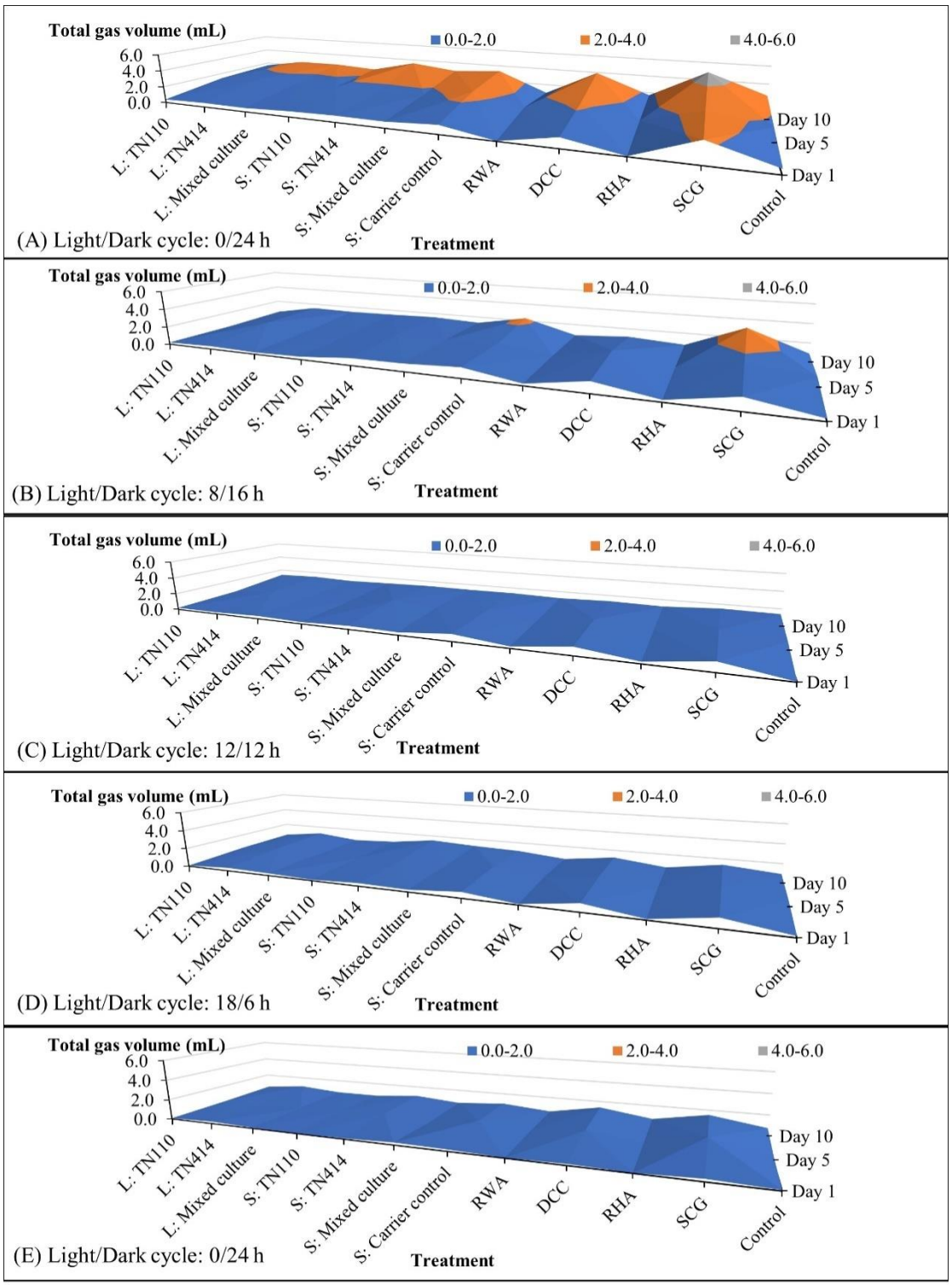

Figure 7-1. Total daily gas volume under different supplements in paddy soil slurry. A capital letter "L" in the figure description refers to a liquid formulation, whereas "S" represents a solid formulation.

The results of total daily gas volume were used to calculate a total gas volume for 10 days as presented in Table 7-2. All experimental sets under a light/dark cycle under 0/24 h for 10 days gave the maximum accumulated total gas volume (8.48 - 32.87 mL), while under a 24/0 h of light/dark cycle obviously showed the minimum accumulated daily gas volume (3.10 – 11.88 mL). The accumulated total gas volume ranging from maximum to minimum was in order of light/dark cycle: 0/24, 8/16, 12/12, 16/8 and 24/0 h (Table 7-2). The paddy soil slurry supplemented with strain TN110 in liquid form released significantly the lowest accumulated gas volume (3.10 - 6.89 mL) under light/dark cycles of 12/12, 16/8 and 24/0 h. The paddy soil slurry supplemented with RWA significantly released the lowest accumulated gas volume compared with other carriers (3.30 and 7.14 mL, respectively) (Table 7-2). On the other hand, the paddy soil slurry supplemented with SCG obviously released the highest accumulated gas volume (11.88 - 32.87 mL) in all light/dark cycles compared with other experimental sets (Table 7-2).

The total gas volume at day 10 from all experimental sets was determined for CH_4 and CO_2 emissions and calculated for reduction percentages by comparing with native control (no supplement) as shown in Figure 7-2. Among them under different light/dark cycles, 0/24 h, removal of CH_4 and CO_2 emissions were much lower than the others ranging from 0.16 to 31.58 % for CH_4 and 1.70 to 16.90% for CO_2 (Figure 7-2A and B). Nevertheless, the paddy soil slurry supplemented with either RWA or RHA showed no significant difference in CH_4 and CO_2 removal under all light/dark cycles and had the lowest the gas removal at only 1 - 5 % (Figure 7-2A and B). The paddy soil slurry supplemented with strain TN110 in liquid form reduced CH_4 emission up to 84.94% under light/dark cycle at 24/0 (Figure 7-2A). Interestingly, under a natural light/dark cycle of 12/12 h, this set could reduce 72.27% CH_4 and 34.38% CO_2 emissions. However, the PNSB biofertilizer(s) from both liquid and solid forms including addition of either DCC or SCG in the paddy soil slurry were not evidently different for CO_2 removal in all light/dark cycles tested, except 0/24 cycle (Figure 7-2B).

Table 7-2. Accumulated total gas volume (mL) for 10 days under different supplements in paddy soil slurry for rice straw biodegradation model.

Paddy soil supplementation	Light/Dark (h)				
	0/24	8/16	12/12	16/8	24/0
L: TN110	13.10^{cE}	5.60^{bcC}	6.89^{aD}	4.27^{aB}	3.10^{aA}
L: TN414	17.42^{gE}	11.33^{hD}	8.96^{hC}	8.02^{hB}	4.90^{gA}
L: Mixed culture	15.53^{dE}	8.37^{dD}	7.57^{dC}	4.73^{dB}	3.75^{eA}
S: TN110	15.89^{eE}	8.58^{deD}	7.68^{deC}	5.51^{eB}	3.73^{eA}
S: TN414	20.06^{iE}	12.02^{iD}	10.97^{iC}	8.91^{iB}	5.59^{hA}
S: Mixed culture	17.78^{hE}	11.28^{gD}	8.94^{gC}	7.30^{gB}	3.79^{fA}
S: Carrier control	24.12^{jE}	14.51^{jD}	11.63^{jC}	9.56^{jB}	7.56^{iA}
RWA	8.48^{aE}	5.25^{aC}	7.14^{bD}	4.29^{bB}	3.30^{bA}
DCC	25.30^{kE}	16.19^{kD}	11.80^{kC}	10.81^{kB}	9.56^{jA}
RHA	9.94^{bE}	5.56^{bC}	7.33^{cD}	4.65^{cB}	3.51^{cA}
SCG	32.87^{lE}	20.65^{lD}	13.27^{lC}	12.22^{lB}	11.88^{kA}
Control	17.00^{fE}	8.93^{fCD}	8.56^{fC}	7.17^{fB}	3.55^{dA}

A capital letter "L" refers to a liquid formulation, whereas "S" repersents a solid formulation. RWA, DCC, RHA and SCG stand for rubber wood ash, decanter cake, rice husk ash and spent coffee grounds, respectively. Values with the different lowercase letters in the same column are significantly different at $P \leq 0.05$ among treatments; whereas the different uppercase letters in the same row are significantly different at $P \leq 0.05$ at the different light/dark cycles. Control means no supplement, while carrier control means an optimal mixed carrier with no inoculation.

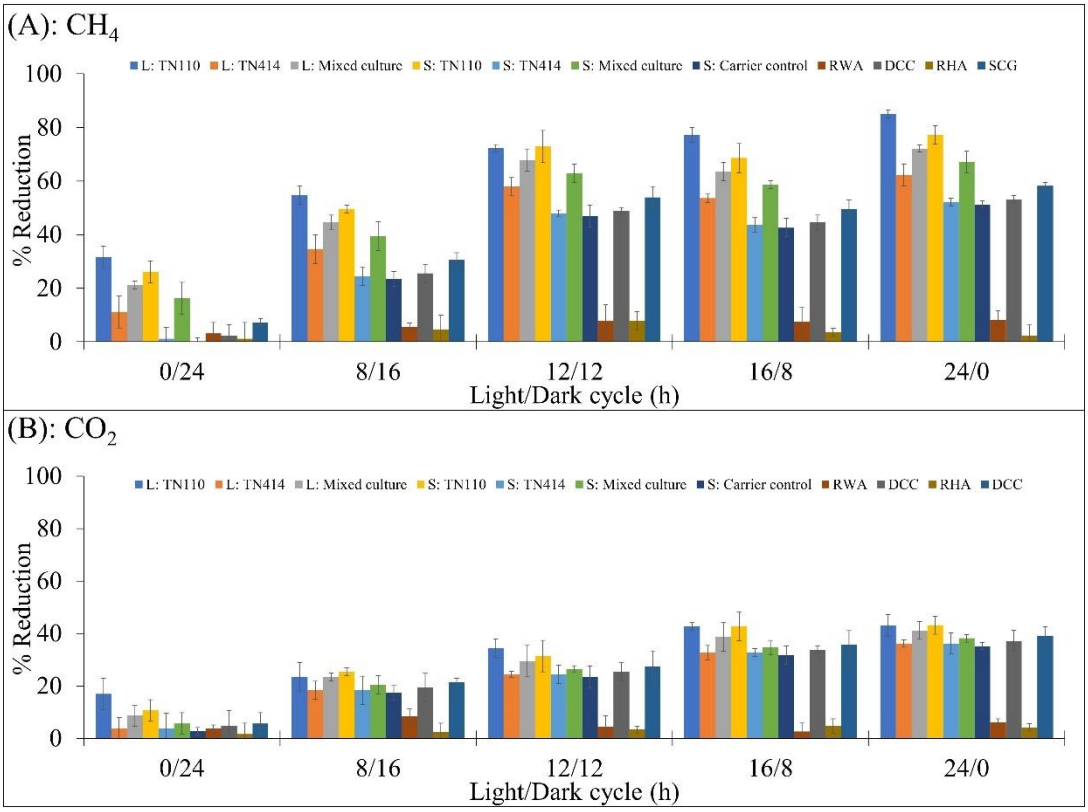

Figure 7-2. Effect of PNSB biofertilizer(s) from both solid and liquid forms on reduction of CH_4 and CO_2 emissions at day10. Capital letter "L" refers to a liquid formulation, whereas "S" represents as a solid formulation.

Discussion

The experiment of rice straw biodegradation in paddy soil slurry under different light/dark cycles for 10 days demonstrated that methanogens from fresh paddy soil were active in releasing CH_4. Not only rice straw but also DCC and SCG can be used as the substrates for CH_4 production due to their high organic matter contents (Chavalparit et al., 2006; Cruz et al., 2012). This is also supported by the results of this study as supplementation of DCC or SCG showed the highest total gas emission (Table 7-2) and the lowest removal of CH_4 and CO_2 (Figure 7-2). This suggests that they should be avoided as carriers for producing solid form of biofertilizers or used minimally as necessary.

Light is known as one of the important factors for the reduction of methanogenesis process (Tada et al., 2005). The methanogenesis is an anaerobic

process by methanogens that work together with other microorganisms including hydrolytic bacteria, fermentative acidogenic bacteria and syntrophic bacteria to generate CH_4 (Conrad, 2002). The CH_4 production of methanogens uses a competitive substrate such as acetate followed by H_2 in paddy fields (Conrad, 2002). On the other hand, PNSB can use both acetate and H_2 as electron donors for their photosynthesis (Nunkeaw et al., 2015; Sakpirom et al., 2017). The results suggest that under light condition a remarkable decrease of both CH_4 and CO_2 emissions were observed compared with no light application (Figures 7-1 and 7-2). Kantha et al. (2015) reported that PNSB biofertilizer, *R. palustris* PP803, under microaerobic light conditions was set at the starting with salt stress for 10 days delivered 100% and 46.88% for CH_4 and CO_2 removal, respectively. In this study, PNSB biofertilizer, *R. palustris* TN110, in liquid form at 24/0 h for light/dark cycle presented the maximum CH_4 and CO_2 removal at 84.94% and 43.03%, respectively (Figure 7-2). This is because different PNSB strains have different efficiencies in reducing greenhouse gases (Sakpirom et al., 2017).

Overall, the results demonstrated that all PNSB biofertilizers in both forms (liquid and solid), especially *R. palustris* TN110 greatly competed with the indigenous methanogens in paddy soil slurry, particularly under light condition. This is evident based on red color bloom in sets with PNSB biofertilizers indicating their higher proliferation than methanogens in rice straw biodegradation. It should be noted that the maximum removal percentages of total greenhouse gas (CH_4 and CO_2) emissions were observed under natural light cycle (12/12), and they were similar to reduction percentages of the total greenhouse gas emissions of light/dark cycles at 16/8 and 24/0 (Figure 7-2). This means that the use of PNSB biofertilizer(s) has great potential for the reduction of greenhouse gas emissions to prevent global warming. It should be noted that *R. palustris* TN110 from both formulations (liquid and solid) was able to reduce roughly 72% CH_4 and 34% CO_2 emissions under natural light cycle although the liquid form gave a higher efficiency (Figure 7-2A to B).

Conclusions

Rice straw biodegradation by soil microbes in paddy soil slurry under different light/dark cycles indicated that light directly affected the competition between PNSB and methanogens as suppression of the latter by the former was observed under light condition. Under natural light cycle (12/12) *R. palustris* TN110 from both biofertilizer formulations (liquid and solid) was remarkable in reducing both CH_4 and CO_2 emissions. In addition, to minimize greenhouse gas emissions using carrier with the minimum nutrients for solid formulation should be considered. Both formulations of PNSB biofertilizer(s) could be used in paddy fields to reduce greenhouse gas emissions, particularly CH_4.

CHAPTER 8

Conclusions

The discoveries of this thesis are summarized in this chapter. Moreover, this chapter also provides the recommendations for future work.

8.1 Conclusions

Purple non-sulfur bacteria (PNSB) are N_2 fixers and versatile organisms that can grow as phototrophs or chemotrophs. So, they have high potential to be applied as biofertilizers for supporting rice (*Oryza sativa* L.) growth. Therefore, this thesis aimed firstly to explore PNSB isolated from various paddy fields including Cd and Zn contaminated paddy fields based on their biofertilizer properties. Secondly, their potential as bioremediation agents for heavy metals (Cd and Zn) in Cd-contaminated paddy soil was investigated. Because the heavy metals in the studied paddy fields around the mine areas are major concern as they can cause unsafe rice products. Furthermore, the PNSB were also studied their potential to alleviate greenhouse gas emissions in the paddy soil through competing proliferation with methanogens responsible for greenhouse gas production.

Among 235 isolated PNSBs, strain TN110 was the most effective for plant growth promoting substances (PGPS) production including the releases of NH_4^+, 5-aminolevulinic acid (ALA) and indole-3-acetic acid (IAA), and reducing CH_4 emission up to 80%. This strain had *nifH*, *vnfG* and *anfG*, which are the Mo, V and Fe nitrogenase genes encoded for the key nitrogenases for N_2 fixation under different conditions. This strain also was able to reduce 84% Cd and 55% Zn in Cd-contaminated soil. Another potent isolate, TN414, not only produced PGPS but also was effective in removing both Cd and Zn up to 70%. Based on 16S rDNA sequencing, strain TN110 was identified as *Rhodopseudomonas palustris*; while strain TN414 was similar to*Rubrivivax gelatinosus*. This study obtained promising PNSB with various abilities as biofertilizers, greenhouse gas mitigators and heavy metal

bioremediation agents. Hence, these two strains were further investigated for their potential application in paddy fields, particularly Cd-contaminated paddy fields for producing safe rice and greenhouse gas mitigation.

For heavy metal bioremediation, only *R. palustris* TN110 was able to bioremediate Cd through the biosynthesis of CdS nanoparticles and to simultaneously fix N_2. It was found that strain TN110 synthesized CdS nanoparticles to reduce Cd toxicity under microaerobic-light conditions by converting 25.61% of 0.2 mM $CdCl_2$ to CdS nanoparticles under its optimal condition (pH 7.5, 30 °C and 3,000 lux). Cysteine desulfhydrase responsible for nanoparticle formation had similar activity levels under different metal cofactors for N_2 fixation. Two genes associated with N_2 fixation: Mo-Fe nitrogenase gene (*nifH*) and V-Fe nitrogenase gene (*vnfG*) were up-regulated 2.83- and 2.27-time changes, respectively by applying the produced CdS nanoparticles at IC_{50} (1.76 mM). The amounts of ammonia released by the strain corresponded with the gene expression results. Simultaneously, the CdS nanoparticles serve as a photosensitizer to increase N_2 fixation. Strain TN110 exhibited great potential to serve simultaneously as a cadmium bioremediation agent and a N_2 fixer. Hence, it was selected to evaluate its possibility to be used as biofertilizer in both liquid and solid forms. In addition, Strain TN414 was also considered because of its capabilities as previously described.

To produce PNSB biofertilizer(s) in solid form, carrier formulation and packaging are important factors during storage to maintain efficiency until application. The optimal mixed carrier of rubber wood ash (RWA), decanter cake (DCC), rice husk ash (RHA), and spent coffee grounds (SCG) at the ratios of 3: 4: 2: 1 was identified. With the mixed carrier containing roughly 10 log CFU/g *R. palustris* TN110 packed in nylon-LLDPE bags by vacuum sealed at 500 W, bacterial population decreased approximately 3 log cycles after 6 month-storage at room temperature (25±3 °C). All PNSB biofertilizers either a single culture (strain TN110 or strain TN414) or a mixed culture of both strains at 1: 1 in both solid and liquid forms at their optimal dilutions showed no phytotoxicity to rice. However, the optimal mixed carrier as carrier control was slightly toxic. At their optimal dilutions, all PNSB biofertilizers in either liquid or solid form significantly increased rice growth compared with their controls (tap water and carrier control). Soil pot house

experiments for investigating PNSB biofertilizer(s) on rice (*Oryza sativa* L.) seedling growth was then conducted.

All PNSB biofertilizers were tested in liquid and solid forms by applying at their optimal dilutions on rice seedlings grown in pot experiment with Cd-contaminated paddy soil and control paddy soil. The results demonstrated that all plant growth parameters (dry weight and length of shoots and roots) in inoculated sets from both forms of PNSB biofertilizer(s) in both Cd-contaminated and control paddy soilswere much higher than their controls. The application of *R. palustris* TN110 in solid form to Cd-contaminated paddy soil significantly improved rice growth with the maximum shoot dry weight and root length. The solid form of all biofertilizers gave remarkable Cd and Zn accumulation decreases in rice shoot and root compared with the liquid form. *R. palustris* TN110 was great in Cd removal; whereas *Ru. gelatinosus* TN414 also provided high Zn removal resulting in low accumulation of Cd and Zn contents in rice shoot and root. The results present that the application of PNSB biofertilizer(s) to support rice growth is possible. However, for Cd-contaminated soil the solid form of PNSB biofertilizer(s) was more suitable to enhance rice growth and reduce heavy metals for producing safe rice. This is because the mixed carrier used for supporting PNSB also acted as a source of plant nutrients and adsorbents to reduce metal toxicity. Finally, the role in reducing greenhouse gas (CH_4 and CO_2) emissions in paddy soil was next explored.

CH_4 is an important greenhouse gas which is contributed by methanogens in flooded paddy fields. As light/dark cycle influences photosynthesis in paddy soil slurry supplemented with rice straw and in turn PNSB and methanogens populations, different light/dark cycles; 0/24, 8/16, 12/12, 16/8 and 24/0 h were investigated for greenhouse gas emissions. The results indicated that at 24/0 h was the best condition for CH_4 and CO_2 mitigation up to 84.94% and 43.03%, respectively. Interestingly, similar results were observed with a natural light cycle (12/12 h). The paddy soil slurry inoculated with *R. palustris* TN110 in liquid form was clearly effective in reducing CH_4 and CO_2 emissions than other experimental sets under all light/dark cycles compared with the native control without inoculation/supplementation. The light is an important factor for methanogenesis reduction. Organic matters such as rice straw, DCC and SCG increased CH_4 production. These results prove that for rice

straw biodegradation by soil microbes in paddy soil slurry under different light/dark cycles, the light period directly affected competition between PNSB and methanogens, and natural light cycle could support PNSB proliferation to reduce greenhouse gas emissions. PNSB biofertilizer(s) in liquid form should be considered over solid form as the carriers contributed to greenhouse gas emissions.

This thesis demonstrated that PNSB: *R. palustris* TN110 and *Ru. gelatinosus* TN414 are usable as biofertilizers and bioremediation agents for enhancement of rice (*Oryza sativa* L.) cultivation in Cd-contaminated paddy field to produce safe rice and reduce global warming. Strain TN110 showed the great potential to alleviate Cd toxicity and also greenhouse gas emissions. On the other hand, strain TN414 was better than strain TN110 for Zn removal. Hence, use of both strains as a single culture or a mixed culture would be able to achieve the targets of producing safe rice and lowering greenhouse gas emissions. In addition, for uncontaminated paddy fields, the liquid form of PNSB biofertilizers is suggested for obtaining higher efficiency in reducing greenhouse gas emissions. In contrast, the solid PNSB biofertilizers form is recommended for Cd-contaminated paddy fields for achieving higher efficacy in reducing accumulated heavy metals in rice. The application of PNSB biofertilizers is a greener alternative to chemical fertilizers, and the agents for heavy metal bioremediation and greenhouse gas mitigation in paddy fields, especially for rice cultivation in heavy metal contaminated areas including Cd and Zn. The applications would have direct implications in the fields of environmental and agricultural sciences leading to production of safe rice along with soil quality improvement and global warming mitigation.

8.2 Future research recommendations

This thesis provided a better understanding on the use of promising PNSB as biofertilizers and bioremediation agents by reduction of heavy metals in rice plant and greenhouse gas emissions for rice cultivation. However, several aspects/issues related to this thesis research have been identified for future work as follows.

1. Screening part to select PNSB should cover more capacities for plant growth promotion such as phosphate solubilization and siderophore production. Moreover, production of exopolymeric substance (EPS) by the PNSB should be investigated because EPS is beneficial for heavy metal remediation. These aspects will elucidate more on the performances of the PNSB on plant growth promotion.

2. Effect of CdS nanoparticles on the production of plant growth promoting substances such as phytohormones and ALA should be investigated because mechanisms for productions also needs energy that the CdS nanoparticles might be supported. This future work will give more information on the effect of CdS nanoparticles on the productions of other plant growth promoting substances.

3. Not only concentration of CdS nanoparticles at IC_{50} to PNSB: *R. palustis* TN110, but also lower or higher concentrations than its IC_{50} should be investigated for performances of strain TN110 on N_2 fixation at various concentrations that could not be predicted when the strain is applied in the Cd-contaminated paddy fields. In addition, effect of carriers used for PNSB biofertilizer formulation should be also observed on CdS nanoparticle biosynthesis by strain TN110. This research should provide potential use of CdS nanoparticles biosynthesis by strain TN110 as PNSB biofertilizer in solid form and its effect on N_2 fixation under different states.

4. An individual and an optimal mixed carrier (RWA, DCC, RHA and SCG) for formulation of PNSB biofertilizer(s) in solid form should be investigated for their properties on degradation rates. Additionally, their ability for heavy metal removal should be examined.

5. Storage-time longer than 6 months for PNSB biofertilizer in solid form should be investigated. The shelf life information will be useful for competition with other commercial biofertilizer(s).

6. Field experiments for application of PNSB biofertilizer(s) in either solid and/or liquid form on rice cultivation should be performed in Cd-contaminated and un-contaminated paddy fields. The field experiments would confirm the laboratory scale results to ensure that the PNSB biofertilizer(s) can be used as biofertilizers and bioremediation agents to enhance rice growth by removal of Cd and to reduce greenhouse gas emissions.

7. Diversity of microorganisms under different light/dark cycles should be analyzed by a metagenomic technique for comparing PNSB and methanogen populations. It will unveil the role of microbial diversity on greenhouse gas emissions under different light period conditions.

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APPENDIX

A. Culture Media

Glutamate-Acetate (GA) Medium

Sodium glutamate	3.8 g
Sodium acetate monohydrate	5.44 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
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	53 mg
$CoCl_2 \cdot 6H_2O$	1.2 mg
$MnSO_4 \cdot 5H_2O$	1.2 mg
Biotin	0.01 mg
Ferric citrate	2.5 mg
Nicotinic acid	1 mg
Distilled water	1,000 mL
pH	7.0

Nitrogen-Free Medium

Sodium acetate	1.0 g
K_2HPO_4	0.9 g
KH_2PO_4	0.6 g
$MgSO_4$	0.2 g
$FeSO_4$	0.012 g
EDTA	0.018 g
$CaCl_2$	0.075 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
$MnSO_4 \cdot 4H_2O$	2.8 mg
$ZnSO_4$	0.75 mg
$CuSO_4$	0.24 mg
H_3BO_3	0.016 mg
Biotin	0.001 mg
Distilled water	1,000 mL
pH	7.0

B. Report of Bacterial Identification by 16S rDNA sequence analysis

LOCUS KU935455 1158 bp DNA linear BCT 31-MAR-2017

DEFINITION *Rhodopseudomonas palustris* strain TN110 16S ribosomal RNA gene, partial sequence.

ACCESSION KU935455

VERSION KU935455.1

KEYWORDS

SOURCE *Rhodopseudomonas palustris*

ORGANISM *Rhodopseudomonas palustris*

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; *Rhodopseudomonas*.

REFERENCE 1 (bases 1 to 1158)

AUTHORS Sakpirom, J.

TITLE The use of purple nonsulfur bacteria as biofertilizers for reducing heavy metals in rice plants and global warming

JOURNAL Sakpirom, J., Kantachote, D., Nunkaew, T. and Khan, E. 2017. Characterizations of purple non-sulfur bacteria isolated from paddy fields, and identification of strains with potential for plant growth-promotion, greenhouse gas mitigation and heavy metal bioremediation. Res. Microbiol. 168(3): 266-275.

REFERENCE 2 (bases 1 to 1158)

AUTHORS Sakpirom, J.

TITLE Direct Submission

JOURNAL Submitted (16-MAR-2016) Microbiology, Prince of Songkla University, 15 Karnjanavanich Rd., Hat Yai, Songkhla 90110, Thailand

FEATURES Location/Qualifiers

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DEFINITION *Rubrivivax gelatinosus* strain TN414 16S ribosomal RNA gene, partial sequence.
ACCESSION KU935456
VERSION KU935456.1
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SOURCE *Rubrivivax gelatinosus*
ORGANISM *Rubrivivax gelatinosus*
Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; *Rubrivivax*.
REFERENCE 1 (bases 1 to 1374)
AUTHORS Sakpirom, J.
TITLE The use of purple nonsulfur bacteria as biofertilizers for reducing heavy metals in rice plants and global warming
JOURNAL Sakpirom, J., Kantachote, D., Nunkaew, T. and Khan, E. 2017. Characterizations of purple non-sulfur bacteria isolated from paddy fields, and identification of strains with potential for plant growth-promotion, greenhouse gas mitigation and heavy metal bioremediation. Res. Microbiol. 168(3): 266-275.
REFERENCE 2 (bases 1 to 1374)
AUTHORS Sakpirom, J.
TITLE Direct Submission
JOURNAL Submitted (16-MAR-2016) Microbiology, Prince of Songkla University, 15 Karnjanavanich Rd., Hat Yai, Songkhla 90110, Thailand
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VITAE

Name Mr. Jakkapan Sakpirom

Student ID 5510230043

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science in Microbiology (First Class Honors)	Prince of Songkla University	2012

Scholarship Awards during Enrolment

2013-2018 Royal Golden Jubilee (RGJ) Ph.D. Scholarship, The Thailand Research Fund, Thailand

List of Publications and Proceedings

Sakpirom, J., Kantachote, K. and Khan, E. 2015. Screening of purple nonsulfur bacteria for their plant growth promoting activities. RGJ-Ph.D. Congress XVI "ASEAN: Emerging Research Opportunities", June 11-13, Jomtien Palm Beach Hotel & Resort, Pattaya, Thailand. Poster presentation.

Sakpirom, J., Kantachote, K., Nunkaew, T. and Khan, E. 2017. Characterizations of purple non-sulfur bacteria isolated from paddy fields, and identification of strains with potential for plant growth-promotion, greenhouse gas mitigation and heavy metal bioremediation. Research in Microbiology 168: 266-275.

Sakpirom, J., Kantachote, D., Siripattanakul-Ratpukdi, S., McEvoy, J. and Khan, E. 2019. Simultaneous bioprecipitation of cadmium to cadmium sulfide nanoparticles and nitrogen fixation by *Rhodopseudomonas palustris* TN110. Chemosphere 223:455-464.

Sakpirom, J., Kantachote, D., McEvoy, J. and Khan, E. 2019. Bioprecipitation of Zinc to Zinc Sulfide Nanoparticles by Phototrophic Bacterium: *Rubrivivax gelatinosus* TN414. 8th Annual Summit on Microbiology "The Latest Advances in Microbiology", May 17-18, Hilton Philadelphia Airport Hotel, Philadelphia, Pennsylvania, USA. Oral presentation.

Sakpirom, J. and Kantachote, K. Preservation method for photosynthetic bacteria in a solid form. Petty Patent submitted.

Sakpirom, J., Kantachote, K., Nunkaew, T. and Khan, E. Investigating optimal proportion of a combined agro-industrial wastes and packaging for purple non-sulfur bacteria biofertilizer(s) and their abilities to enhance rice (*Oryza sativa* L.) seedling grown in paddy soil. Manuscript will be submitted.

Sakpirom, J., Kantachote, K. and Khan, E. Influences of different biofertilizer formulations of purple non-sulfur bacteria to enhance growth and reduce accumulated heavy metals in rice (*Oryza sativa* L.) grown in cadmium-contaminated paddy soil. Manuscript will be submitted.

Sakpirom, J., Kantachote, K. and Khan, E. Effects of light/dark cycles and formulation types of purple non-sulfur bacteria biofertilizer(s) to mitigate greenhouse gas emission from rice straw biodegradation in paddy soil slurry. Manuscript will be submitted.