The Use of Purple Nonsulfur Photosynthetic Bacteria to Maintain Water Quality, Sources of Single Cell Protein and Bioactive Compounds for Shrimp Cultivation

Supaporn Chumpol

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

Prince of Songkla University

2017

The Use of Purple Nonsulfur Photosynthetic Bacteria to Maintain Water Quality, Sources of Single Cell Protein and Bioactive Compounds for Shrimp Cultivation

Supaporn Chumpol

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

Prince of Songkla University

2017

Thesis Title	The Use of Purple Nonsulfur Photosynthetic Bacteria to Maintain Water Quality, Sources of Single Cell Protein and Bioactive Compounds for Shrimp Cultivation
Author	Miss Supaporn Chumpol
Major Program	Microbiology

Major Advisor

.....
(Prof. Dr. Duangporn Kantachote)

Co-advisor

.....
(Prof. Dr. Hiroshi Kanzaki)

.....
(Assoc. Prof. Dr. Teruhiko Nitoda)

.....
(Dr. Pattamarat Rattanachuay)

Examining Committee:

.....Chairperson
(Dr. Ampaitip Sukhoom)

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

.....Committee
(Prof. Dr. Hiroshi Kanzaki)

.....Committee
(Asst. Prof. Dr. Nugul Intrasungkha)

.....Committee
(Dr. Pattamarat Rattanachuay)

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

.....
(Assoc. Prof. Dr. Teerapol Srichana)
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
(Miss Supaporn Chumpol)
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

(Miss Supaporn Chumpol)

Candidate

ชื่อวิทยานิพนธ์ การใช้แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์เพื่อรักษาคุณภาพน้ำ เป็นแหล่งโปรตีนเซลล์เดียว และสารออกฤทธิ์ทางชีวภาพในการเลี้ยงกุ้ง

ผู้เขียน นางสาวสุภาภรณ์ ชุมพล

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2560

บทคัดย่อ

การศึกษานี้ได้แยกแบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ (Purple Nonsulfur Bacteria; PNSB) ซึ่งเป็นแบคทีเรียที่มีประโยชน์ จากบ่อเลี้ยงกุ้งในหลายพื้นที่ภาคใต้ของประเทศไทยเพื่อนำมาคัดเลือกหาสายพันธุ์ที่เด่นเพื่อนำไปใช้ในการเพาะเลี้ยงกุ้งจาก PNSB จำนวน 185 สายพันธุ์ที่แยกได้ เมื่อนำมาทดสอบคุณสมบัติความเป็นโปรไบโอติกส์ในหลอดทดลอง พบว่ามีเพียง 4 สายพันธุ์ ได้แก่ SS15/ S3W10/ TKW17 และ STW181 ที่มีความสามารถสูงในการผลิตเอนไซม์ย่อยอาหาร การผลิตวิตามินบี 12 และการสร้างสารยับยั้งเชื้อก่อโรคในกลุ่มวิบริโอ ผลการระบุสายพันธุ์ของเชื้อที่คัดเลือกได้พบว่าสายพันธุ์ SS15/ S3W10 และ TKW17 คือ *Rhodobacter sphaeroides* ส่วนสายพันธุ์ STW181 คือ *Afifella marina* ได้นำเชื้อดังกล่าวมาตรวจสอบความเป็นโปรไบโอติกส์ในกุ้งขาว (*Litopenaeus vannamei*) ระยะวัยอ่อน โดยใช้ในรูปเชื้อผสม (แต่ละสายพันธุ์ใช้ 1×10^8 เซลล์ต่อมิลลิลิตร) 3 ชุดการทดลองคือ T1 (S3W10+SS15) T2 (S3W10+TKW17) และ T3 (S3W10+STW181) โดยเติมลงไปในน้ำเลี้ยงกุ้งขาว ทุกสัปดาห์ (1-7) และเพาะเลี้ยงเป็นเวลา 60 วัน จากนั้นทดสอบความต้านทานของกุ้งต่อโรคตับวายเฉียบพลัน (AHPND) โดยการเติม *Vibrio parahaemolyticus* SR2 ที่ความเข้มข้น 4×10^4 เซลล์ต่อมิลลิลิตร และเลี้ยงต่อเป็นเวลา 10 วัน พบว่าทั้ง 3 ชุดการทดลองที่เติม PNSB กุ้งมีกิจกรรมเอนไซม์ย่อยอาหารสูงขึ้นและเจริญเติบโตเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ โดย PNSB สามารถเพิ่มจำนวนในทางเดินอาหารของกุ้งได้ แม้ว่าการอยู่รอดของกุ้งไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามการตายของกุ้งที่ได้รับสายพันธุ์รุนแรง AHPND (*V. parahaemolyticus* SR2) ลดลงมากในทุกชุดการทดลองที่เติม PNSB โดย

พบว่ากุ้งตาย 40% ในชุดควบคุม ขณะที่ในชุดการทดลอง T1 T3 และ T2 ตาย 21% 24% และ 32% ตามลำดับ นอกจากนี้ยังศึกษาความสามารถในการควบคุมคุณภาพน้ำโดยใช้โปรไบโอติกส์ PNSB ในรูปแบบผสมอัตราส่วน 1:1 เช่นเดียวกับการทดลองก่อนหน้านี้ พบว่าตลอด 8 สัปดาห์ของการเพาะเลี้ยงกุ้งขาว ชุดการทดลอง T1 และ T3 มีประสิทธิภาพในการลดปริมาณ NH_4^+ และส่งเสริมการเจริญเติบโตของกุ้งได้ดีที่สุด ดังนั้นจึงเลือกใช้แบคทีเรีย 3 สายพันธุ์ ได้แก่ SS15/ S3W10 และ STW181 (แต่ละสายพันธุ์ใช้ 1×10^8 เซลล์ต่อมิลลิลิตร) โดยเติมลงไปในน้ำเลี้ยงกุ้ง เพื่อทดสอบศักยภาพในการป้องกันการเกิดโรค AHPND ด้วยการเติม *V. parahaemolyticus* SR2 (1×10^5 เซลล์ต่อมิลลิลิตร) ในวันที่ 15 ของการเลี้ยง ซึ่งเลี้ยงกุ้งเป็นระยะเวลา 30 วัน พบว่าชุดที่เติม PNSB อย่างเดียว (ชุดควบคุมทางบวก) และชุดทดสอบที่เติมทั้ง PNSB และเชื้อก่อโรคสายพันธุ์ SR2 มีคุณภาพน้ำในการเพาะเลี้ยงกุ้งดีกว่าชุดควบคุมที่ไม่เติมเชื้อ (native control) และชุดที่เติมเฉพาะเชื้อก่อโรค SR2 (challenge test) ซึ่งการทดลองนี้ยืนยันความเป็นโปรไบโอติกส์ของ PNSB ที่สามารถครอบครองพื้นที่ภายในลำไส้ของกุ้ง และควบคู่กับการควบคุมคุณภาพน้ำส่งผลให้กุ้งมีการเจริญเติบโตดีขึ้น และการอยู่รอดของกุ้งเพิ่มขึ้น 11% ในชุดการทดลองที่ทำให้กุ้งรับเชื้อก่อโรค AHPND สายพันธุ์ SR2 สำหรับผลการศึกษาสภาวะที่เหมาะสมต่อการผลิตสารยับยั้งเชื้อวิบริโอของโปรไบโอติกส์ PNSB (SS15/ TKW17 และ STW181) พบว่าสภาวะที่เหมาะสมในการผลิตสารยับยั้งวิบริโอใกล้เคียงกับสภาวะที่ใช้ในการเลี้ยงกุ้ง สารยับยั้งวิบริโอที่ผลิตจาก PNSB มีส่วนประกอบหลักเป็นโปรตีน ไขมัน และคาร์โบไฮเดรตที่มีความเสถียรในช่วงค่าที่กว้างของ pH และอุณหภูมิซึ่งเหมาะกับการนำไปใช้ในการเพาะเลี้ยงกุ้ง สารยับยั้ง วิบริโอมีฤทธิ์แบบการฆ่าแบคทีเรียจากหลักฐานที่พบว่าเกิดรูจำนวนมากบนเซลล์ของเชื้อก่อโรค และยังพบว่ามีกิจกรรมที่ทำให้เซลล์ของเชื้อก่อโรคแตกสลาย การนำสารยับยั้งวิบริโอที่ผ่านการทำให้บริสุทธิ์ของสายพันธุ์ SS15 มาศึกษาลักษณะพบว่าเป็นสารที่มีขนาดโมเลกุลเล็กกว่า 3,000 ดัลตัน โดยมีประจุบวกชนิด weak cation ที่มีหมู่เอมีนเป็นองค์ประกอบ นอกจากการใช้ PNSB เป็นโปรไบโอติกส์ยังศึกษาศักยภาพของ PNSB เพื่อเป็นโปรตีนเซลล์เดียว (single cell protein; SCP) โดยสายพันธุ์ SS15 และ STW181 ถูกคัดเลือกมา ซึ่งพิจารณาจากปริมาณโปรตีน รงควัตถุที่ใช้ในการสังเคราะห์แสง และกรดอะมิโนที่จำเป็นต่อการเจริญเติบโตของกุ้ง นำ PNSB ทั้ง 2 สายพันธุ์ในรูปไลโอฟิไลซ์ผสมในอัตราส่วน 1: 1 และเติมลงในอาหารกุ้งทางการค้าคิดเป็น 1% 3% และ 5% ของน้ำหนักอาหารกุ้ง ได้

อาหารดัดแปลง 3 สูตร ได้แก่ อาหารสูตรที่ 1 สูตรที่ 2 และสูตรที่ 3 ตามลำดับ เพื่อใช้ในการเลี้ยงกุ้งตั้งแต่ระยะวัยอ่อนจนถึงกุ้งวัยรุ่นช่วงต้นซึ่งเพาะเลี้ยงเป็นเวลา 60 วัน พบว่าปริมาณของ NH_4^+ NO_2^- NO_3^- และ COD ในน้ำเลี้ยงกุ้งที่ให้อาหารสูตรที่ 2 และ 3 มีค่าสูงกว่าชุดควบคุมอย่างมีนัยสำคัญทางสถิติขณะที่น้ำเลี้ยงกุ้งจากบ่อที่ให้อาหารสูตรที่ 1 มีค่าพารามิเตอร์ดังกล่าวต่ำที่สุดเมื่อเทียบกับชุดที่ให้อาหารสูตรดัดแปลงด้วยกัน และมีค่าใกล้เคียงกับชุดควบคุม พบว่าการเจริญเติบโตของกุ้งที่ให้อาหารสูตรที่ 1 มีการเจริญเติบโตดีมากกว่ากุ้งที่ให้อาหารสูตรที่ 2 3 และกุ้งในชุดควบคุม การอยู่รอดของกุ้งสูงสุดพบในชุดการทดลองอาหารสูตรที่ 1 เท่ากับ 85% ซึ่งค่าที่ได้ไม่แตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับชุดการทดลองอื่น ซึ่งกุ้งในชุดควบคุมมีการอยู่รอด เท่ากับ 80% อีกทั้งยังพบว่ากุ้งในทุกชุดการทดลองที่ให้อาหารสูตรดัดแปลงมีการตอบสนองของระบบภูมิคุ้มกันสูงขึ้น (ปริมาณเม็ดเลือดรวม และกิจกรรมของเอนไซม์ฟีนอลออกซิเดส รวมทั้งเอนไซม์ซูเปอร์ออกไซด์ ดิสมิวเทส) รวมทั้งผลของเนื้อเยื่อตับและตับอ่อนของกุ้ง (hepatopancreas) ในทุกชุดที่ให้อาหารสูตรดัดแปลงมีลักษณะปกติ ซึ่งบ่งชี้ว่ากุ้งมีสุขภาพดี โดยสรุปผลงานวิจัยครั้งนี้แสดงให้เห็นว่า PNSB ทั้ง 4 สายพันธุ์ที่คัดเลือกได้จัดเป็นแบคทีเรียที่มีประโยชน์ที่มีศักยภาพสูงสำหรับใช้ในการเพาะเลี้ยงกุ้งขาว

คำสำคัญ: โปรไบโอติกส์ แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ การเพาะเลี้ยงกุ้ง โปรตีนเซลล์เดียว แบคทีเรียกลุ่มวิบริโอ คุณภาพน้ำ

Thesis Title The use of purple nonsulfur photosynthetic bacteria to maintain water quality, sources of single cell protein and bioactive compounds for shrimp cultivation.

Author Miss Supaporn Chumpol

Major Program Microbiology

Academic Year 2017

ABSTRACT

Purple nonsulfur bacteria (PNSB) as beneficial bacteria were isolated from various shrimp ponds in Southern Thailand for promising strains selection with the purpose to use them in shrimp cultivation. Among 185 isolated PNSB were screened based on probiotics properties *in vitro*; there were only 4 strains (SS15, S3W10, TKW17 and STW181) showed strong activities for producing digestive enzymes, vitamin B12 and antivibrio compounds. These selected PNSB were identified as *Rhodobacter sphaeroides* for strains SS15, S3W10 and TKW17 and *Afifella marina* for strain STW181. They were proved for their probiotic properties *in vivo* as a mixed culture (each at 1×10^8 cells mL^{-1}) for 3 sets (T1: S3W10+SS15, T2: S3W10+TKW17 and T3: S3W10+STW181), by inoculating into rearing water of postlarval *Litopenaeus vannamei* every week (1-7) for 60 days; and then challenge white shrimp with acute hepatopancreatic necrosis disease (AHPND) causing *V. parahaemolyticus* SR2 at 4×10^4 cells mL^{-1} by continuing cultivation for 10 days. All PNSB sets significantly enhanced the digestive enzyme activities and shrimp growth with their proliferation in shrimp gastrointestinal tract although the shrimp survival was not significantly different. However, the cumulative mortality of shrimp exposed to a virulent AHPND - *V. parahaemolyticus* SR2 was a remarkable decrease in all PNSB sets as 40% in control to 21, 24 and 32% in T1, T3 and T2 sets. The probiotic PNSB were also investigated for their ability to control water quality using a mixed culture at a ratio of 1: 1 with the same protocol as the previous

experiment. Throughout 8 weeks of white shrimp cultivation, the effective sets that reduced NH_4^+ and also promoted shrimp growth were T1 and T3. Hence, PNSB strains (SS15, S3W10 and STW181) were used as a mixed culture for each at 1×10^8 cells mL^{-1} by adding into rearing water for assessment their potential to prevent AHPND as all shrimp were challenged at day 15 with AHPND - *V. para-haemolyticus* SR2 (1×10^5 cells mL^{-1}) during shrimp cultivation for 30 days. Inoculated PNSB sets (positive control: only PNSB inoculation, and treatment: both PNSB and SR2 inoculations) produced a better water quality than that found in native control (no inoculation) and challenge test (only SR2 inoculation). This experiment confirmed their probiotic properties as they colonized in intestinal shrimp tract, and accompanying to control water quality for enhancement shrimp growth and increase 11% survival rate of challenged shrimp with AHPND strain SR2. Conditions for producing antivibrio compounds of probiotic PNSB (SS15, TKW17 and STW181) were investigated for obtaining optimal conditions and found that their optimum conditions were close to the conditions of shrimp cultivation. These antivibrio compounds mainly consisted of protein, lipid, and carbohydrate; and they were stable under wide variations of pH and temperature that was suitable for shrimp cultivation. The antivibrio compounds acted as bactericidal action as the evidence of damaged cells with many holes and also showed bacteriolytic activity. One of the purified antivibrio compounds from strain SS15 was characterized and found that it was a low molecular weight (< 3000 Da) with weak cationic compound containing - NH_2 group. In addition of using PNSB as probiotics they were also investigated for the potential to be used as single cell protein (SCP), and strains SS15 and STW181 were selected as SCP on the basis of protein, photopigments and essential amino acids contents. A mixture of both lyophilized cells (1:1) at 1, 3 and 5% (w/w) were mixed well with commercial shrimp feed to obtain modified shrimp feed recipes; Diet 1, Diet 2 and Diet 3, respectively for feeding white shrimp starting from postlarval until early juvenile for 60 days. Levels of NH_4^+, NO_2^-, NO_3^- and COD in rearing water from Diet 2 and 3 sets were significantly higher than control set; however, Diet 1 set showed the lowest levels of these water parameters among modified Diet sets and closed to a control set. Based on shrimp growth performance, Diet 1 set was better than other modified diet sets including control set. The

maximum shrimp survival as 85% was also observed in Diet 1 set although no significant difference with other sets as in the control set was 80%. Moreover, Shrimp in all modified shrimp diet sets showed increasing of immune responses (total hemocyte count, and activities of phenoloxidase and superoxide dismutase) and hepatopancreas histopathology of shrimp in a good condition as healthy shrimp. Overall this thesis proved that the four selected PNSB strains as beneficial microbes have the great potential to be used in white shrimp cultivation.

Keywords: probiotics, purple nonsulfur bacteria, shrimp cultivation, single cell protein, *Vibrio* spp., water quality

ACKNOWLEDGEMENTS

I would like to express my sincere grateful thanks and sincere appreciation to my advisor, Professor Dr. Duangporn Kantachote, for her helpful suggestion, valuable guidance and comments throughout my study. Sincere appreciation is expended to Professor Dr. Hiroshi Kanzaki, Associate Professor Dr. Teruhiko Nitoda and Dr. Pattamarat Rattanachuay, my co-advisors, for their valuable contributions and advice relating to this study.

I wish to express my gratitude to Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program; grant number PHD/0106/2554, which support this work and the Graduate School of Prince of Songkla University is also acknowledged for partly supported to conduct this work.

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

I would like to express my beloved friends at ST608 Microbiology Laboratory for their kindly and helpful advice during my entire study.

Finally, I wish to thank my beloved, parents, grandmother, brother and all friends for their helpful support and encouragement.

Supaporn Chumpol

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF TABLE

LIST OF TABLE (Continued)

LIST OF TABLE (Continued)

LIST OF FIGURE

LIST OF FIGURE (Continued)

LIST OF FIGURE (Continued)

LIST OF FIGURE (Continued)

LIST OF ABBREVIATIONS AND SYMBOLS

AHPND: Acute hepatopancreatic necrosis disease

ASW: Artificial sea water

BChl: Bacteriochlorophyll

BIM: Basic Isolation Medium

CFU: Colony forming units

CPD: Critical point dryer

DLE: Daily length gain

DO: Dissolved oxygen

DNS: 3,5-dinitrosalicylic acid

DWG: Daily weight gain

EMS: Early mortality syndrome

FCR: Feed conversion ratio

GA: Glutamate acetate

GIT: Gastrointestinal tract

H&E: Hematoxylin and eosin

LG: Length gain

MBC: The minimum bactericidal concentration

MIC: The minimum inhibitory concentration

NH_4^+: Ammonium

NO_2^-: Nitrite

NO_3^-: Nitrate

NSS: Normal saline solution

OD: Optical density

PL: Postlarval shrimp

PBS: Phosphate buffer solution

PO: Phenoloxidase

PNSB: Purple nonsulfur bacteria

RGR: Relative gain rate

R_f: Retention factor

SCP: Single cell protein

SEM: Scanning electron microscope

SGR: Specific growth rate

SOD: Superoxide dismutase

TCBS: Thiosulfate citrate bilesalt sucrose agar

THC: Total hemocyte count

TLC: Thin layer chromatography

TSB: Tryptic Soy Broth

WG: Weight gain

CHAPTER 1

INTRODUCTION

Rationale and background

Shrimp cultivation is one of the most important aquaculture businesses that are one of the international-economic trades in the world (FAO, 2014); and Thailand is a top three ranking for large shrimp producing that exported shrimp products around 400,000 tonnes in 2011 (Reed & Royales 2014). However, in Thailand the newly emerging disease named early mortality syndrome (EMS) reduced roughly 10% shrimp production in 2012 from the 2011. In 2013, EMS caused decrease the country's usual annual output to 300,000 tones and this shortage affected on almost largest companies in Thailand (Thai Frozen Foods Association, 2012; FAO, 2013). In 2014, the total production of shrimp was likely lower than 200,000 tonnes (FAO, 2014). Recently, total shrimp production in 2015 was likely increased 35,000 tonnes compared in 2014 production and it likely to be continued increase until 2016 (FAO, 2015). Shrimp products in Thailand are exported to many countries more than 90% of the products are in various forms such as chilled, frozen and processed shrimp. In 2015, the main markets for Thai shrimp exporting are US and Japanese as 63% and 66% that increased 3.4 %, dominated by value-added shrimp (FAO, 2016).

Among shrimp species, *Litopenaeus vannamei* also known as Pacific white shrimp, is commercially dominant species worldwide, including Thailand for avoiding white spot disease (WSD) outbreaks that cause a serious problem of *Penaeus monodon* farming (Thitamadee et al., 2016). *L. vannamei* is a

marine crustacean belonging to the order Decapoda and the family Penaeidae and always live in sea water (Treece, 2014). This species can grow well in a wide range of temperature (> 20 $^{\circ}$C) and resist to the wide range of salinity as 0.5 - 45 ppt (Briggs et al., 2004). Water quality for shrimp cultivation is very important for shrimp growth and health status as shrimp always variably follow the surround residence conditions (Xiong et al., 2016; Zhang et al., 2016). Poor water quality during cultivation causes directly adverse effects on shrimp such as stressful, weakness, low immunity and easily infected by pathogens that are the major factor contributing to outbreaks of shrimp diseases (Thakura and Lin, 2003). However, this problem is quite hard to control because most of commercially cultured using intensive shrimp farming system which culturing high shrimp stocking density roughly 500 postlarvae m^{-2} (Sandifer et al., 1987; Lightner et al., 2006).

Intensive shrimp farming has led shrimp exposure to stressful conditions, which increase opportunity for vibriosis infection such as *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. splendidus* and *V. anguillarum* (Dash et al., 2017). The serious pathogenic strains that have been reported to cause disease outbreaks in shrimp is *V. harveyi*, which is one of the most important luminous marine bacteria, and luminescence produced by this species is easily visible in the dark (Gunalan et al., 2014; Dash et al., 2017). In recent years, EMS technically term as acute hepatopancreatic necrosis disease (AHPND) has been the most serious disease outbreak; and it has caused huge global economic losses (Zorriehzahra and Banaederakhshan, 2015). This disease caused by *V. parahaemolyticus* which normally affects postlarval shrimp at days 20-35 in early cultivation with frequently causes up to 100% mortality (Tran et al., 2013; De Schryver et al., 2014; Joshi et al., 2014).

To solve serious problems cause by shrimp pathogenic vibrios, farmers normally use chemicals and antibiotics in shrimp cultivation for

adjustment water quality and help for controlling shrimp pathogens. However, both chemical agents and antibiotics used in shrimp production produced the risk associated with consumer health due to the high dose and also their residues. For instance, chloramphenicol has been banned for using in aquaculture because its residual remained in shrimp products and induce aplastic anemia in human (Serrano, 2005). In addition, residual of antibiotics remained in the environment and shrimp products, led to cause antibiotic resistant genes among opportunistic pathogens such as *Vibrio* spp. (Zokaeifar et al., 2012). Therefore, shrimp cultivation in terms of organic agriculture is very interesting because it emphasizes to use natural materials and avoid using synthetic materials for friendly environmental conditions. The use of microbes with probiotic properties has many benefits for both agriculturists and consumers because these probiotic microbes are able to adjust the equilibrium conditions and prevent shrimp diseases. Purple nonsulfur bacteria (PNSB) are one of probiotic bacteria to control water quality and stimulate growth of aquaculture animal has been reported (Shapawi et al., 2012). Up to date, there is a little research on antivibrio compounds produced by PNSB; thereby, in addition of searching probiotic PNSB, interesting of their antivibrio compounds should be not ignored.

PNSB are normally found in natural water with organic matter in the light zone. It has a wide range of growth conditions as they are able to grow under anaerobic-light conditions (phototrophic), some use of low sulfide levels and thiosulfate as an electron donor in photosynthesis or under aerobic-dark (chemotrophic) use organic matter, such as alcohol, fatty acids as a carbon source and/or electron donor. For example, some members of PNSB are *Rhodobacter*, *Rhodocyclus*, *Rhodomicrobium*, *Rhodopila*, *Rhodopseudomonas* and *Rhodospirillum* (Madigan and Jung, 2009). PNSB showed many benefits such as use in wastewaters treatment i.e. *Rps. palustris* reduced 65.3% ammonia in shrimp pond (Luo et al., 2012), use as a source of protein or single

cell protein (SCP) for animal feed (Kim and Lee, 2000; Azad et al., 2002) and so on. In addition, they can produce many important compounds such as glutathione, vitamin B12, ubiquinone (coenzyme Q10) and 5-aminolevulinic acid (ALA) (Sasaki et al., 2005; Noogkongbut et al., 2016) including carotenoids pigment with many types such as neurosporene, spirilloxanthin, chloroxanthin, spheroidene, spheroidenone, okenone and lycopene. These pigments help to stimulate the color of aquatic animals (Kiriratnikom, 2006).

According to above information, to solve the serious problem of shrimp diseases, especially AHPND by avoiding the use of chemicals and antibiotics, the use of probiotic bacteria, particularly probiotic PNSB would be approachable way to achieve for producing safe shrimp under environmentally friendly for sustainable shrimp cultivation to support shrimp farmers and Thai economic from shrimp exporting. The use of antivibrio compounds produced by probiotic PNSB is also an alternative way for controlling shrimp pathogens. In addition, PNSB could be considered as SCP replacement for some fish meal with more benefits to shrimp and also reducing cost of shrimp feed. Hence, to explore the benefits of PNSB strains as above described for successful applying in white shrimp (*L. vannamei*) cultivation and gaining new knowledge or better understanding for leading to organic shrimp cultivation; the objectives of this thesis are the following provided.

Objectives

1. To isolate and select PNSB for using as probiotics with ability to control shrimp pathogenic *Vibrio* spp., particularly AHPND-causing *V. parahaemolyticus*,

2. To investigate the roles of probiotic PNSB for controlling water quality and preventing AHPND for enhancing white shrimp growth with higher survival during cultivation,

3. To optimize culture conditions for production and characterization of antivibrio compounds produced by selected PNSB,

4. To investigate the possibility of selected PNSB to be used as SCP for supplementation in shrimp feed.

Scope of the study

Isolated PNSB strains from shrimp ponds were selected based on their ability to inhibit shrimp pathogenic *Vibrio* spp. to apply in white shrimp (*L. vannamei*) cultivation. The selected PNSB were investigated their probiotic properties for controlling AHPND-causing *V. parahaemolyticus*. They were further investigated their roles to control water quality and also prevent AHPND for promoting white shrimp growth with higher survival during cultivation. Optimal culture condition for producing antivibrio compounds by selected PNSB was investigated; and a purified antivibrio compound was characterized. The selected PNSB were also investigated for the potential to be used as SCP for producing modified shrimp feed.

Anticipated Outcomes

1. This study proved that selected PNSB strains are a good candidate as probiotics to facilitate shrimp growth and inhibit shrimp pathogenic vibrios for using in white shrimp cultivation.

2. This study explored that selected probiotic PNSB strains showed abilities to control water quality and also to prevent AHPND during white shrimp cultivation.

3. This study presented that selected PNSB strains under their optimal culture conditions are able to produce high antivibrio compounds against *Vibrio* spp. as causative agents of luminous vibriosis and AHPND.

4. This study suggested that biomass of selected PNSB has the great potential as SCP for supplementation in shrimp feed to promote shrimp growth and also increase survival rate with immunostimulants in white shrimp cultivation.

CHAPTER 2

LITERATURE REVIEW

Pacific white shrimp

Pacific white shrimp (*Litopenaeus vannamei*) also known as king prawn is a native in the eastern Pacific Ocean from the north of Mexico to the north of Peru (Ghaffari et al., 2014). Since the 1970, it has been cultured widely around the Latin America and especially since 2000, as it has become the principle shrimp species of cultivation in Asia (Ghaffari et al., 2014). This shrimp can greatly adapt itself in environmental conditions and resist to a wide range of salinity (0.5 - 45 ppt) and also temperatures that is higher than 20°C (Briggs et al., 2004). In addition, this shrimp is easy to culture and grow faster when compared with other shrimps and this causes more production for commercial in the world market.

Taxonomy of Pacific white shrimp (Roman, 2006)

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Dendrobranchiata
Family: Penaeidae
Genus: Litopenaeus
Species: *Litopenaeus vannamei*

Characteristic of *L. vannamei*

The external anatomy of *L. vannamei* is characterized by a cephalothorax with a characteristic hard rostrum (Figure 2.1). Pacific white shrimp has eight white segments body, rostrum likes triangle of the segment header and in the head region has antennules and antennae perform sensory functions. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen. Most internal organs like gills, heart, lymphoid organ, hepatopancreas (HP), and stomach are located in the cephalothorax, while the muscles concentrate in the abdomen. Male can reach a total length of 187 mm and 230 mm for female (Phuoc, 2008). In general, female grows faster and bigger than male shrimp (Holthuis, 1980).

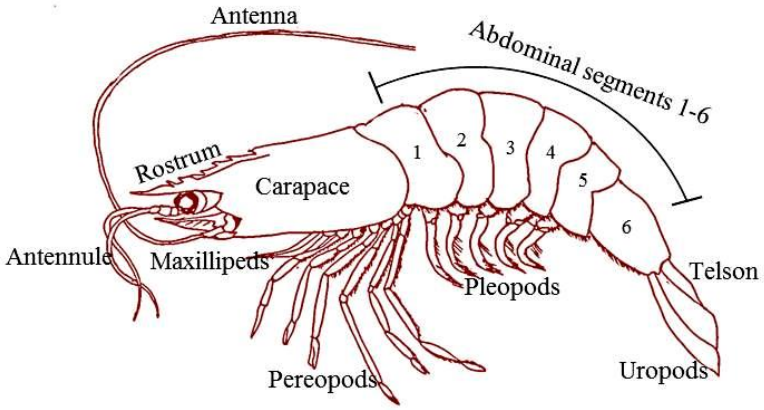


Figure 2-1. External anatomy of *Litopenaeus vannamei* (Modified from Phuoc, 2008)

Life cycle of *L. vannamei*

Shrimp has several stages for shrimp development as shown in Figure 2-2. The mature, mate, and spawn shrimp live in the offshore of the coastal areas at a temperature of 26-28°C and a salinity of about 35 ppt. The eggs hatch and the larvae develop as a part of the zooplankton. *L. vannamei* postlarvae moves inshore and settle to the bottom in shallow estuaries where the nutrition,

salinities and temperatures are suitable for growing. After several months in an estuary, juvenile shrimp return to the offshore marine environment, where sexual maturation, mating, and spawning occurrence (Phuoc, 2008).

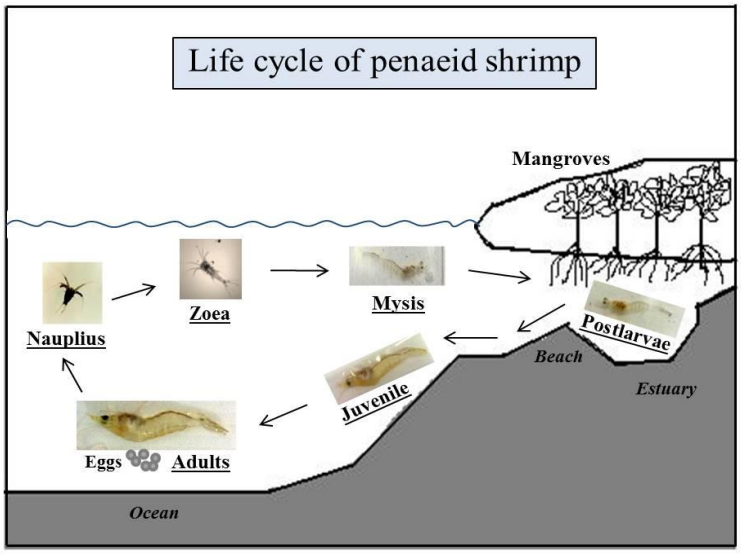


Figure 2-2. Life cycles of penaeid shrimp (Modified from Phuoc, 2008)

The processes of shrimp cultivation

The first process for shrimp cultivation is a selection of male and female mature shrimp about 6-7 months, weight not less than 40 grams, free-pathogen and resist to grow in environmental conditions. Broodstock are stocked in mature tanks at dark condition with filtered seawater. One eyestalk from each female is ablated, leading to repeated maturation and spawning. Female either spawns in communal or individual tanks (to avoid disease transmission). Shrimp is grown in a nursery pond about 1-5 weeks. After that they go to stages of zoea, postlarvae (PL), and juvenile, respectively. Shrimp can be harvested in 3-5 months as shown in Figure 2-3 (Lightner et al., 2006).

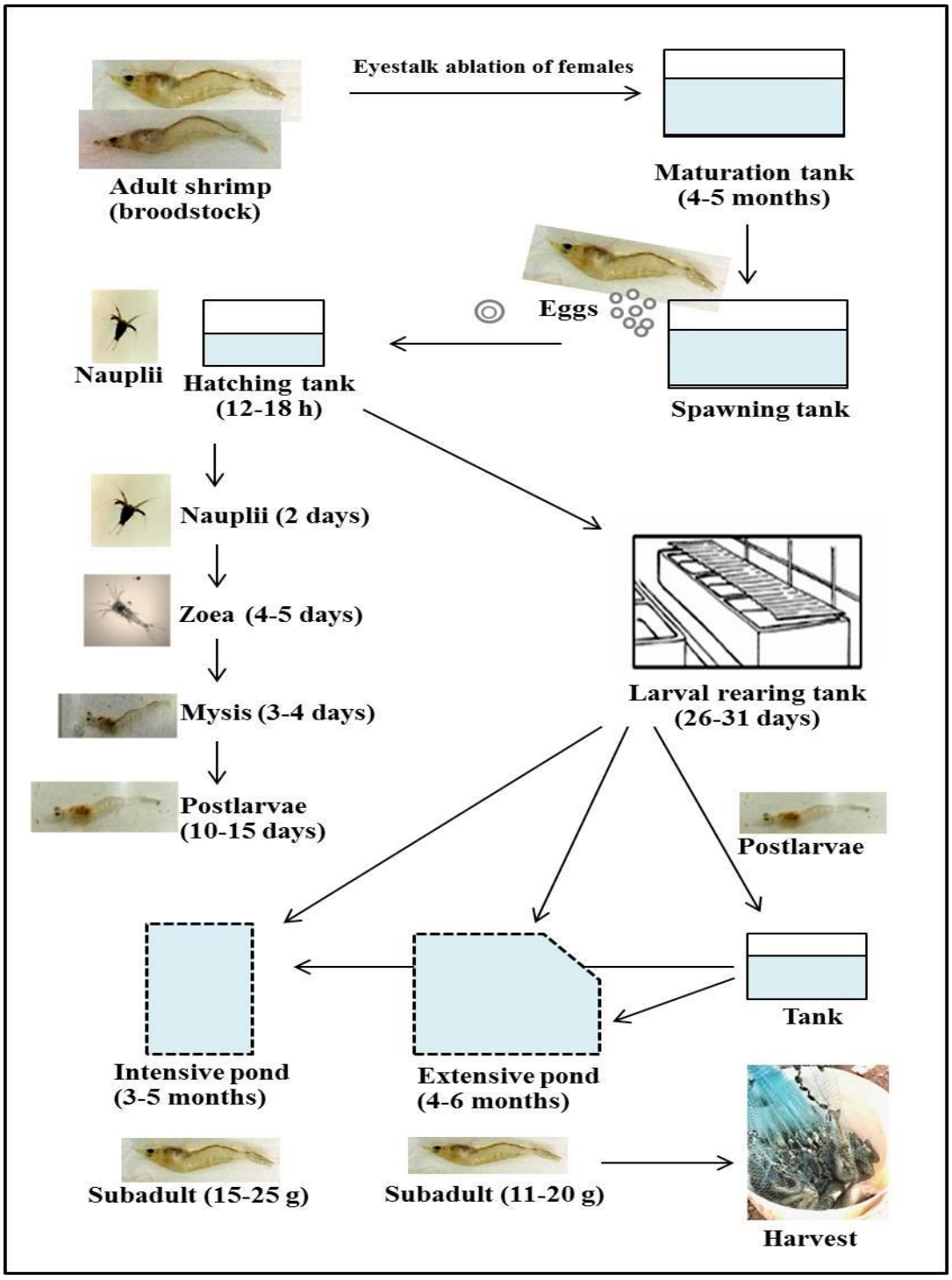


Figure 2-3. The processes of shrimp aquaculture (Modified from Lightner et al., 2006)

Water quality in *L. vannamei* cultivation

The appropriate water quality is very important in shrimp cultivation because the poor water quality directly affects on shrimp such as low growth rate, stress and weakness, finally cause easily infectious by pathogenic microorganisms (Lightner et al., 2006). Therefore, maintaining good water quality is always necessary and it is an important factor for shrimp cultivation. The intensive shrimp farming with high stocking densities in range from 500-1,000 PL m^{-2} have been used for shrimp cultivation (Sandifer et al., 1987; Lim, 1998; Lightner et al., 2006); this leads to give more feeding and supplying high aeration. However, shrimp can eat feed only 85% (Goddard, 1996) that causes residual feed in the pond with consequently making water quality unsuitable for shrimp growth (Rattanachuay et al., 2007).

The major nutrients found in shrimp feed are protein, lipid and carbohydrate with minor compositions of vitamin, mineral and water; and shrimp requires 35-55% protein from feed (Kanazawa, 1989; Goddard, 1996). Uneaten feed leads to residual protein in the water and also excretes feces in form of inorganic nitrogen (ammonia; NH_3). Hence, nitrogen cycle is an important process for shrimp ecological environment as organic degradation in shrimp cultivation mainly is protein. Proteolytic bacteria can digest protein to amino acids and release NH_3 by ammonifying bacteria that is called ammonification process. Ammonia is a toxic substance which affects on shrimp growth by increasing pH level in blood and shrimp cannot transport nutrients and oxygen for cell living (Goddard, 1996). However, toxicity of NH_3 depends on pH level as normally exists in a form of ammonium (NH_4^+) and changing to NH_3 at alkaline condition (Barajas et al., 2006). Additionally, a sufficient oxygen in the water column of shrimp pond allows NH_3/ NH_4^+ and nitrite (NO_2^-) are converted to nitrate (NO_3^-) by nitrifying bacteria and no toxicity on shrimp. On the other hand, if the water has high concentration of NO_3^-, it

causes shrimp under stress conditions so at the concentration of NO_3^- over than 50 mg L^{-1} the water must be discharged (Chuntapa et al., 2003).

Feeding and digestion in shrimp

Shrimp is typically carnivorous in feed habit. They feed primarily on small animals such as mollusks, crustaceans, and polychaetes which live on the surface layer of the pond bottom (Goddard, 1996). As commercial shrimp cultivation, shrimp feed has been developed to commercial feed for ready and appropriate use. The nutrients in shrimp feed, some are used to build and maintain tissues, while other supply energy. (Goddard, 1996). In case of the ingredients in feed containing low protein, shrimp always have a low growth rate and weight loss than normal shrimp because protein in muscle are used for replacing insufficient protein. In contrast, a high protein in shrimp feed leading to an excessive protein so it is used for supply energy as well, consequently more eliminating nitrogen in a form of NH_3 in the water.

Shrimp can consume both natural feed organism and formulated feed. Within the digestive gland the liquefied feed is treated with a full range of digestive enzymes: proteases, lipases, and amylases. The digestive process in shrimp is generally rapid as it has been reported to be completely within 4-6 h at 20°C. The volume of the digestive tract has been estimated as only 2-3% of the total body volume. This limits ability to store feed and couple to a rapid digestive process, means that actively growing shrimp must frequently feed. In case of shrimp consume on formulated feed; it may be largely lost due to shrimp cannot completely eat feed and remaining residual feed in the pond (Goddard, 1996).

Shrimp immunity

Shrimps are invertebrate animals; therefore they have lower immunity than vertebrate animals. Immunity in crustaceans, including shrimps depend on

innate immune responses to combat invading microbes; and lacking an adaptive immune system (lymphocytes and functional immunoglobulin) (Rowley and Powell, 2007). The innate immunity consists of the cellular innate immunity and humoral innate immunity (Rowley and Powell, 2007). The cellular immune mechanisms include phagocytosis, nodulation and encapsulation; whereas the humoral immune mechanisms involve the synthesis and release of several immune proteins such as antimicrobial peptides (AMPs), proteinase inhibitors, cytokine-like factor, etc. (Tassanakajon et al., 2012).

In terms of cellular mechanisms referred to hemocytes which play important roles in the immune mechanisms including phagocytosis, nodulation and encapsulation (Fagutao et al., 2011). Hemocytes are the initiation of wound repair/ blood coagulation to prevent pathogens penetrates into the main body cavity termed the hemocoel (Rowley and Powell, 2007). If this barrier is destroyed, the blood cells present in hemocoel can show phagocytosis and small invader digestion such as protozoans, bacteria, fungi, and viruses. Besides, covering multicellular parasites in a thick wall of hemocytes, a process termed encapsulation. In the final cellular defense mechanism, termed nodule formation, microorganisms are cleared from the hemocoel and become enmeshed in a central core of melanized hemocytes surrounding by a wall of flattened hemocytes, hence isolating such particles from the rest of the host (Rowley and Powell, 2007).

In terms of humoral mechanisms; there are a lot of immune molecules and the defense reactions such as AMPs, clotting, pattern recognition receptors or pattern recognition proteins, proteinase, melanization, apoptosis, antioxidant enzymes (Tassanakajon et al., 2012).

Antimicrobial peptides (AMPs) are key factors in the elimination and destruction of bacteria and fungi in invertebrates. AMPs are typically small in size, generally less than 150-200 amino acid residues. AMPs are naturally

derived or synthetic and are active against a wide range of microorganisms such as bacteria, viruses, yeasts, parasites and fungi; and they may also exhibit an anti-tumor activity. There are several families of shrimp AMPs such as penaeidins, lysozymes, crustins, antilipopolysacharide factor (ALFs) and stylicins. They are produced and stored in the hemocytes (Tassanakajon et al., 2012).

Clotting occurs to prevent the loss of hemolymph upon the injury and invasion of infected microorganisms. The rapid blood coagulation system presents at the site of injury that is a prominent immune mechanism (Sritunyalucksana and Soderhall, 2000; Tassanakajon et al., 2012).

The innate immune system recognizes invading microorganisms through a limited number of germline-encoded pattern recognition receptors (PRRs). PRRs bind pathogens which associated with molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PG) and beta-glucan (BGs) those are presented on the surface of microorganisms. Several penaeid shrimp PRRs such as LGBP, BG-binding protein (GBP) and lectinare have been reported (Tassanakajon et al., 2012).

Proteinases are part of several proteolytic cascades that are key components of the innate immune system and serve important roles in several related biochemical pathways such as apoptosis and melanization. The important members of a proteinase family is serine proteinase (SP) which is responsible for converting prophenoloxidase (proPO) to phenoloxidase (PO) (Tassanakajon et al., 2012; Zokaeifar et al., 2012).

Melanization is an important immune defense component of insects as well as in crustaceans. Melanin synthesis is achieved by the proPO system which is an enzymatic cascade involving several enzymes including the key enzyme PO. The proPO-activating system is initiated by the recognition and binding of the PRRs to microbial cell wall components. The complex triggers

the activation of the SP cascade that converts proPO to active PO. Active PO oxidizes phenols into quinine that can nonspecifically crosslink neighboring molecules become the melanin formation (Tassanakajon et al., 2012; Zokaeifar et al., 2012).

Apoptosis is a genetically regulating cell death program that eliminates leftover, damaged or harmful cells. Apoptosis plays a key role in the animal defense mechanism against viral infection (Tassanakajon et al., 2012).

Reactive oxygen species (ROS) are naturally produced in vertebrates and invertebrates as a defense mechanism against microbial infection during phagocytosis (Parrilla-Tayloret al., 2013). The members of ROS are superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). For example, in several stress conditions, such as environmental stresses (oxygen, pH and temperature), dietary toxicants and biological stresses (pathogen infection) lead to increase levels of ROS production. However, the increase of ROS levels can cause oxidative damage to important cellular macromolecules (lipids, proteins, carbohydrates and nucleotides) that are components of the membranes, cellular enzymes and DNA (Tassanakajon et al., 2012). Hence, most cells have protective mechanisms to balance ROS production and avoid oxidative stress, namely antioxidants. Antioxidant defenses include a variety of non-enzymatic molecules, such as glutathione and ascorbic acid, and also enzymatic system i.e. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione s-transferase (GST) (Liu et al., 2007). These processes play an important role in microbiocidal activity; O_2^- can be dismutated by SOD to H_2O_2, which can be further metabolized to H_2O and O_2 by CAT and GPx; thereby, deterring synthesis of hydroxyl radical and contributing to avoiding deleterious effects of ROS (Parrilla-Tayloret al., 2013).

Shrimp disease

Shrimp disease is one of the most serious problems in shrimp cultivation that causes shrimp productivity loss. There are many groups of infectiously pathogenic microorganisms such as bacteria, virus, fungi, protozoa and parasite; these pathogens increase the disease outbreaks that may be caused by either the introduction of new pathogens or changing the environment conditions (Sweet et al., 2015). In general, the intensive shrimp aquaculture where is easily stress shrimp as a high stock-density and occurring of inappropriate water quality, and quickly change ecological conditions. This causes shrimp weakness and finally become to disease. This study focused on bacterial pathogens, particularly *Vibrio* spp.

Shrimp pathogenic bacteria

Pathogenic *Vibrio* species have been affiliated as one of the major bacterial infections that cause shrimp diseases. Vibrios are Gram-negative halophilic bacteria, curved-rod shape (comma-shaped), ubiquitous in marine and estuarine ecosystems as well as aquaculture farms (Chatterjee and Haldar, 2012). They are chemoorganotroph and growing in facultative anaerobe. These bacteria damage shrimp in both of larval and adult stages by attachment with walking legs and maxillipeds that causes weak shrimp and abnormal movement (Munn, 2004). The penaeid shrimp are colonized by vibrios as the major normal microbiota roughly 38-81% of the bacterial biota (Kongnum and Hongpattarakere, 2012). However, *Vibrio* spp. are opportunistic pathogens; they become apparently lethal infection which response to extreme environmental stress, other infection diseases, nutritional disease and wounds (Kongnum and Hongpattarakere, 2012; Heenatigala and Fernando, 2016).

Shrimp diseases occur by bacterial pathogens

Vibriosis is a common disease in penaeid shrimp cultivation. It caused by *Vibrio* spp. such as *V. harveyi*, *V. parahemolyticus, V. vulnificus, V. alginilyticus, V. anguillarum, V. penaeicida*and, *V. splendidus.* (Rattanachuay et al. 2010; Chatterjee and Haldar, 2012). Shrimp is normally infected by these pathogens occurs via water during early stage prior to harvesting (Lightner et al., 2006). This is possible when the water quality and environmental conditions are not suitable in shrimp aquaculture that lead to stressful and become low shrimp immunity.

Luminescence disease is significantly damage disease in shrimp production; the dominant species causing luminous vibriosis is *V. harveyi*. This disease occurs during postlarval until adult stage of shrimp (Vandenberghe et al., 1999). The virulence of *V. harveyi* may up to 85% mortality in nauplii (Chatterjee and Haldar, 2012), but it depends on pathogenic strains, some strains can cause shrimp death up to 100% at 10^2 CFU mL^{-1}; whereas some strains at a concentration of 10^6 CFU mL^{-1} cannot cause shrimp disease (Munn, 2004). The symptoms of luminescence disease are less to eat or not eat, have tea brawn pigment in the gills, weakness swimming, opacity of abdominal muscle and shrimps have fluorescent phenomenon at night (Pasharawipas et al., 1998).

Early mortality syndrome (EMS) is a new emerging shrimp disease that caused huge losses productivity in Southeast Asia. Since 2012, there were widely spread to the Eastern part of Thailand and follow to other culture areas of Thailand (Zorriehzahra and Banaederakhshan, 2015). EMS also called acute hepatopancreas necrosis disease (AHPND) that causes by AHPND-strain *V. parahaemolyticus*; it contains plasmid that able to secrete toxin (Tran et al., 2013). The comparison of genome sequences among *V. parahaemolyticus* strains revealed that AHPND-causing *V. parahaemolyticus* has plasmid

containing two toxin genes similar with *Photorhabdus* insect related (Pir) toxin genes, the presence of this plasmid was found in all pathogenic strains but absence in non-pathogenic strains (Han et al., 2015). EMS/AHPND normally affects on postlarval stage of shrimp within the first 20-35 days in cultivation that leads to a remarkable mortality up to100% of shrimp populations (De Schryver et al., 2014; Han et al., 2015). As the lumen of each tubule in HP of shrimp surrounding by four types of epithelial cells are B-, R-, F-, and E-cells. B-cells are the largest cells which contain a large vacuole; locate mainly in distal part of tubules; while R-cells distribute throughout the tubule with the presence of numerous small lipid vacuoles. F-cells show a fibrillar appearance and usually have a central oval nucleus. E-cells are cuboidal and occur primarily in the distal part of hepatic tubules (Chupani et al., 2016). The symptoms of EMS/AHPND are hepatopancreas damage including the sloughing of HP cells, enlarged HP nuclei, lack of B, F, R cells and E cell mitosis (FAO, 2013).

Use of antibiotics in shrimp farm

Antibiotics have been widely used in animal aquaculture for treatment and control the diseases caused by bacteria. However, with the long term use leads to residual compounds in water and sediment; bacteria can develop more capable to resist antibiotics and become antibiotic resistance (Defoirdt et al., 2011). The causes of antibiotic resistance, antibiotics are normally less absorbed in the gut of the animals so the majority is excreted unchanged in faces and urine (Sarmah et al., 2006). Therefore, the antibiotics residues in the environment lead to increased bacterial antibiotic resistance genes (Zhang et al., 2011). Gram negative bacteria have more capable to resist antibiotics than Gram positive bacteria because the former group has a complex structure of cell wall and also outer membrane for drug barrier into cell (McEntire and Montville, 2007). The cell wall plays important roles in bacterial growth and

survival in environments to control cell shape, protect high internal osmotic pressure that against plasmolysis process (Lambert, 2002). Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antibiotics, while Gram-negative bacteria possess the outer membrane as significant barrier to the penetration of small hydrophilic molecules, their rate of penetration and excluding larger molecules (Lambert, 2002).

The survey of shrimp aquaculture in Thailand found that farmers used antibiotics 74% in the Andaman coast and 92% in the Gulf of Thailand. The group of commonly antibiotics used in shrimp aquaculture are divided into 4 groups; tetracyclines, quinolones, sulphonamide and other groups. Quinolones group was popular antibiotics, especially norfloxacin, subordinate was tetracyclines group (Holmstrom et al., 2003). However, some antibiotics such as oxytetracycline, it is hard to degrade therefore it accumulated in underwater that increased the antibiotic resistance genes and it may be accumulated in shrimp products (Vaseeharan et al., 2005). For example, *V. harveyi* was reported for its antibiotic resistance to ampicillin (10 µg), streptomycin (10 unit) and ciprofloxacin (5 µg) with 100%, 57% and 52% of the 21 isolates were tested, respectively (Vaseeharan et al., 2005). Hence, Thailand have been banned some of antibiotics such as chloramphenicol and nitrofuran group including furazolidone, nitrofurazone, nitrofurantion, nitroquine and nitrofuratel (Graslund et al., 2002) due to these drugs have affected to induce abnormal in the human body such as aplastic anemia and cancer (Serrano, 2005).

Organic marine shrimp farming

Definition

Organic marine shrimp farming means farm management practices for marine shrimp including cultivation, harvesting and transportation that introduces specific requirement to an organic standard. They are based on the holistic agricultural management, environmentally friendly and sustaining biodiversity. All input materials should be natural products, avoid using synthetic products and any genetically modified organisms or genetic engineering (Ministry of Agriculture and Cooperatives, 2007).

Requirements of organic shrimp farming (Ministry of Agriculture and Cooperatives, 2007)

- Production principle

Focusing on maintaining good water quality and soil, for maintaining the biodiversity of overall ecosystem and avoid all activities that may pollute environment.

- Farm location

Farm should not be located in prohibitive area for shrimp farming such as mangrove conservation areas. The area should be appropriate for organic shrimp farming with good sanitary in both water and soil such as the location of farm should not be located in contaminated areas and should have the appropriate of pH levels for preventing contamination.

- Farmer

Farmer should acquire knowledge or proper training in organic marine shrimp farming.

- Larvae selection

Farmer should use only healthy, disease resistance larvae and not use genetically modified shrimp.

- General farm management

Farm layout should be done according to farm management principle. Proper reservoir/treatment pond should be adequate for intensive shrimp farming system, use organic products or natural products. The following lists are prohibited substances in shrimp farm management; genetically modification microorganisms and also products derived from such microorganisms, synthetic substances for growth promotion, toxic substances derived from natural sources such as heavy metals which may impact to environment and human health and manure or composed manure derived from city waste. The important thing is water pump or aerator in ponds should be adequate for shrimp aquaculture.

- Feed management

Feed should be produced from natural raw or organic materials. The following lists are prohibited substances in shrimp farm management including, antibiotics, chemotherapeutics, pure amino acids and materials or products derived from genetically modification organisms.

- Shrimp health management

Farmer should regularly check shrimp health and water quality. The use of chemical substances, are permitted such as potassium permanganate, hydrogen peroxide, copper sulfate, chorine, rotenone, povidone-iodine and benzalkonium chloride.

- Effluent and sediment management

Farmer should not discharge effluent to natural fresh water sources and arable land. Then, the effluent discharge and sediment disposal should not affect natural water resources and surrounding environment.

- Harvesting, post harvest handlings and distribution

Before harvesting, random check chemical and antibiotic residues in shrimp should be done. The postharvest handlings and distribution should take place when shrimp are good health shrimp and be as quick as possible, in order

to maintain freshness of shrimp and organic status including use proper freezing in clean and cold water.

The use of probiotics in shrimp aquaculture

The use of antibiotics or chemotherapeutic agents in shrimp aquaculture leads to enhance bacterial drug resistance. Therefore, the use of environmentally friendly is very interesting and become increasingly important in shrimp aquaculture such as probiotics, prebiotics and antimicrobial peptides, (Hai and Fotedar, 2012).

Definition of probiotics

Probiotics are live microorganisms administrated in adequate amounts that confer beneficial health on host (Sanders, 2008).

Probiotic bacteria in shrimp cultivation

Bacteria have been proposed as the most commonly and successfully use as probiotics in aquaculture that belong to the genera of *Bacillus, Lactobacillus*, *Vibrio*, *Pseudomonas*, *Alteromonas.* In contrast, mainly strains of *Vibrio, Pseudomonas* and *Alteromonas* spp. are pathogenic bacterial strains that cause severe shrimp mortalities (Hai and Fotedar, 2012). Therefore, probiotic properties of microbes to be used for shrimp aquaculture must be investigated before using.

Bacillus sp. has been widely used as probiotics in shrimp aquaculture because this bacterium produces exoenzyme such as amylase, protease and lipase for feed digestion, nutrients absorption and control water quality in aquaculture (Liu et al., 2012). There are many research publications reported on the use of *Bacillus* sp. as probiotics as follows. The investigation of using *B. subtilis* E20 as probiotic by adding into rearing water at concentrations of 10^8

CFU mL^{-1}, the results found that this strain could stimulate fast development of shrimp and more resistant in inappropriate environmental conditions than the control group (Liu et al., 2010). Moreover, use of *B. subtilis* strains L10 and G1 for juvenile shrimp aquaculture by adding mixed strains into shrimp feed at concentrations of 10^8 CFU g^{-1} could increase growth rate and increase survival rate with low mortality when infected by *V. harveyi* (Zokaeifar et al., 2012). At a concentration of 10^8 CFU g^{-1} probiotics, the mortality rate of shrimp was only 20% while mortality rate in the control group was 63%, in addition, immune gene expression of shrimp including prophenoloxidase (proPO), lipopolysaccharides-β-1, 3-glucan-binding protein (LGBP) and serine protein (SP) in shrimp fed with mixed probiotics were increased indicating that probiotics *B. subtilis* could enhance immunity of shrimp (Zokaeifar et al., 2012).

Lactobacillus sp. has been widely used as probiotics in aquaculture, especially fish cultivation; however, it also applying in shrimp cultivation. The administration of *Lactobacilus plantarum* dried-cells in the diet at 10^{10} CFU kg $diet^{-1}$ induced immune modulation such as up-regulation of prophenoloxidase (proPO) and peroxinectin (PE) mRNA transcription levels, and enhancement of cellular and humoral immune responses; phenoloxidase (PO), superoxide dismutase (SOD) to enhance the immune ability of *L. vannamei* for resistance to *V. alginolyticus* infection (Chiu et al., 2007). In addition, the live cells of probiotic strain (*L. plantarum* MRO3.12) isolated from digestive tract of wild banana shrimp, was investigated its probiotic property by mixing into shrimp feed at 10^{10} CFU mL^{-1}, the results found that this bacterium well-adapted to establish in shrimp gut and significantly increased shrimp survival as shrimp more resistant to *V. harveyi* infection than the control (Kongnum and Hongpattarakere, 2012).

Some researchers studied on the possibility to use *Pseudomonas* sp. and *Vibrio* sp. as probiotics; however, a few strains of them that could be used as probiotics. *Pseudomonas* sp. PM 11 and *Vibrio fluvialis* PM 17 isolated from gut of sub-adult shrimp were selected as candidate probiotics, the results found that these strains presented desirable traits *in vitro*, such as abilities to produce extracellular enzymes and siderophores, but they did not enhance shrimp immune responses (Alavandi et al., 2004). The probiotic *Pseudomonas aeruginosa* PIC 4 was proved to be non-pathogenic to shrimp by pathogenicity test, the results showed that *Vibrio* counts in this probiotic fed shrimp and the rearing water were significantly lower compared to the control group and also increased shrimp survival (Jannakiram et al., 2014). Moreover, some studies investigated the effect of *Alteromonas* sp. on shrimp cultivation. For instance, *Alteromonas* sp. BY-9 was used as a probiotic in larval stage of *Penaeus monodon* and found that growth and survival rate of larvae fed with strain BY-9 were higher than that found in control set (Haryanti et al., 2001). *Alteromonas* sp. P7 isolated from hatchery larvae of *P. monodon*, this strain had ability to inhibit *V. harveyi* and *in vivo* test found that this bacterium could colonize in shrimp larvae and reduced shrimp mortality (Abraham, 2004).

Photosynthetic bacteria

Photosynthetic bacteria are divided into two groups as anoxygenic photosynthetic bacteria and oxygenic photosynthetic bacteria (Willey et al., 2008). Anoxygenic photosynthetic bacteria are divided into two groups as purple phototrophic bacteria and green phototrophic bacteria (Imhoff et al., 2005). These bacteria are Gram-negative, with various cell shapes such as coccus, rod, ovoid, and spiral. Colonies have different colors such as red-purple, orange-brown, brown or green. Most of these bacteria use binary fission for reproduction, but some genera use budding (Imhoff et al., 1984). Anoxygenic photosynthetic bacteria have a different photosynthesis from blue-

green algae (cyanobacteria) and plants as they use hydrogen sulfide (H_2S) and reduced sulfur compounds or organic compounds i.e. malate, acetate, pyruvate as electron donor; therefore no oxygen production in their photosynthesis (Thatoi et al., 2013). As mentioned above, anoxygenic photosynthetic bacteria are divided into two groups and each group is classified based on sulfur accumulation in their cells as shown in Table 2-1 (Willey et al., 2008); and in this study focuses on only purple nonsulfur phototrophic bacteria.

Table 2-1. The different characteristics of anoxygenic photosynthetic bacteria (Modified from Willey et al., 2008)

Anoxygenic Photosynthetic Bacteria				
Characteristic	**Green Sulfur**	**Green Nonsulfur**	**Purple Sulfur**	**Purple Nonsulfur**
Photosynthetic pigments	Bacteriochlorophylls *a* plus *c, d* or *e*	Bacteriochlorophylls *a* & *c*	Bacteriochlorophylls *a* & *b*	Bacteriochlorophylls *a* or *b*
Morphology of photosynthetic membranes	Photosynthetic system partly in chlorosomes that are independent of the plasma membrance	Chlorosomes present when grown anaerobically	Photosynthetic system contained in spherical or lamellar membrane complexes	Photosynthetic system contained in spherical or lamellar membrane complexes
Photosynthetic electron donors	H_2, H_2S, S	Usually organic molecules, H_2S, H_2	H_2, H_2S, S	Usually organic molecules; sometimes reduced sulfur compounds or H_2
Sulfur deposition	Outside of the cell	-	Inside the cell	Outside of the cell
General metabolic type	Obligately anaerobic photoautotrophs	Photoheterotrophs, Photoautotrophs, Chemoheterotrophs	Obligately anaerobic photoautotrophs	Photoheterotrophs, Photoautotrophs,Chemoheterotrophs
Motility	Non motile; some have gas vesicles	Gliding	Motile with polar flagella; some are peritrichously flagellated	Motile with polar flagella or nonmotile

Purple nonsulfur bacteria (PNSB)

Purple nonsulfur bacteria are divided into two groups as α-Proteobacteria and β-Proteobacteria (Imhoff et al., 2005). These bacteria grow under anaerobic-light conditions call phototrophic growth using organic matter as a carbon source and reducing molecules such as sulfide at low level, hydrogen, and organic matter as their electron donor. However, they also can grow under aerobic or microaerobic-dark conditions as chemotrophic growth using organic matter such as alcohol, fatty acids as electron donor and carbon source. Some members can use hydrogen sulfide or thiosulfate as an electron donor in photosynthesis but cannot use sulfur as an electron donor and cannot accumulate sulfur inside cells (Imhoff et al., 2005). PNSB present multiple cells shapes such as rod, coccus or spiral shape which different colors including orange-brown to red-purple and mostly contain bacteriochlorophyll *a* and carotenoids as photosynthetic pigments. (Kiriratnikom, 2006). Each bacteriochlorophyll can absorb light in a different wavelength as shown in Table 2-2 (Willey et al., 2008). The members of this group are *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodomicrobium*, *Rhodopila*, *Rhodocyclus*, and *Rubrivivax*.

Table 2-2. Light absorption wavelength of chlorophyll and bacteriochlorophyll by anoxygenic photosynthetic bacteria (Modified from Willey et al., 2008)

Pigments	**Long Wavelength (nm)**	
	In ether or acetone	**Range of values in cells**
Chlorophyll *a*	665	680-685
Bacteriochlorophyll *a*	775	850-910 (purple bacteria) 805-810 (green bacteria)
Bacteriochlorophyll *b*	790	1,020-1,035
Bacteriochlorophyll *c*	660	745-760
Bacteriochlorophyll *d*	650	725-745
Bacteriochlorophyll *e*	647	715-725

Beneficial applications of purple nonsulfur bacteria (PNSB)

Use of PNSB for controlling water quality and treating wastewater

Due to PNSB can grow under various conditions as previously described; thereby they are widely distributed in aquatic habitat and also wastewater plants. PNSB are attractive organism to be used for maintaining water quality in shrimp aquaculture as they normally found in shrimp ponds (Azad et al., 2002). High levels of NH_3, NO_2^- and NO_3^- are toxic to shrimp as previously described; therefore in rearing shrimp water they should be controlled under acceptable levels for shrimp growth.

Kim et al. (2004) studied on wastewater treatment in swine wastewater by *Rhodopseudomonas palustris*. The characteristics of swine wastewater containing 18,700 mg L^{-1}chemical oxygen demand (COD) and high nutrients of nitrogen and phosphorus became serious loads in water streams and major sources of eutrophication. Furthermore, volatile organic compounds (VOCs) are responsible for severe malodor generating during storage and treatment.

With the use of *Rps. palustris* under anaerobic-light conditions for 7 days reduced 50% of COD, 58% of phosphate and removed unpleasant odor.

Lu et al. (2011) used *Rhodobacter sphaeroides* for treating soybean wastewater under light-anaerobic conditions could reduce 40% total nitrogen (TN) and 95% COD; while Madukasi et al. (2011) used *R. sphaeroides* reduced 90% of COD in wastewater from soybean industry.

Luo et al. (2012) studied on the use of PNSB for treating wastewater from shrimp aquaculture. Inoculation of *Rps. palustris* at various concentrations; 5.0 $x10^6$, $1.0x10^6$, $2.0x10^6$, $4.0x10^6$ and $8.0x10^6$ CFU mL^{-1} into the wastewater for 4 days; it was found that at concentrations of $4.0x10^6$ and $8.0x10^6$ CFU mL^{-1} produced the most effective in reducing nutrients; total phosphate, TN and COD, especially at concentration of 4.0 x 10^6 CFU mL^{-1} reduced ammonia up to 65.3%.

Use of PNSB for sources of digestive enzymes

Oda et al. (2004) studied on *Rubrivivax gelatinosus* (KDDS1) for its proteinase enzyme by testing in Glutamate Malate medium (GM) containing 1% casein under microaerobic-light condition and found that this enzyme was serine-type proteinase with molecular weight of 32.5 kDa with the highest activity at pH 9.6, 45°C.

Munjam et al. (2005) studied on four PNSB (*Rps. palustris*, *R. sphaeroides*, *Rhodocyclus gelatinosus* and *Rhodocyclus tenuis*) for their lipase production and found that *Rc. gelatinosus* and *R. sphaeroides* were good lipase producers; but *Rps. palustris* and *Rc. tenuis* were poor in lipase secretion.

Rhodobacter capsulatus KU002 and *Rhodopseudomonas acidophila* KU001 were isolated from leather industry effluents (Merugu et al., 2010). Both PNSB produced the following enzymes; cellulase, hemicellulase, amylase, protease and lipase.

Use of PNSB as Single cell protein (SCP)

Getha et al. (1998) used *Rps. palustris* as shrimp feed. The chemical composition of cell showed high protein up to 53%; it was selected to test with shrimp larvae. The data showed shrimp larvae had survival rate in a range of 42-53% and stimulated growth up to 78-88%.

Kiriratnikom (2006) used PNSB as SCP for culturing Giant tiger prawn. The results showed that over 8 weeks shrimp fed with PNSB at a concentration of 5% PNSB did not enhance shrimp growth compared with control group. However, shrimp fed with 5% PNSB showed darker color than control group that presented good shrimp color.

Shapawi et al. (2012) applied *Rhodovulum* sp. as SCP in fish cultivation (*Lates calcarifer*) by mixing dry cell of bacterial biomass into commercial formulated seabass feed, the results demonstrated that fish fed with 0.3% *Rhodovulum* sp. could improve growth, survival rate and better feed conversion ratio (FCR) than the fish fed with control feed. At the end of experiment, weight gain and specific growth rate of fish in treatment groups were higher than control group. The most effective shrimp feed was found in fish fed 0.3% bacterial biomass showed FCR as 1.95 and 86.7% of survival rate.

Use of PNSB as a source of vitamin B12

Vitamin B12 or cobalamin is a complex structure and small molecule, found in prokaryote organism that can produce vitamin B12. Natural structure of vitamin B12 has adenosylcobalamin and methylcobalamin, both are important cofactor for methylmalonyl CoA mutase and methionine synthase, respectively. However, vitamin B12 used in commercial product as cyanocobalamin form as cyano group was used instead of adenosyl and methyl group because the process of extraction of vitamin B12 uses cyano group for

obtaining higher stable vitamin (Kang et al., 2012). Vitamin B12 is utilized in several ways including medicine, pharmacy and animal husbandry. Then, vitamin B12 is alsopopular mixing in animal feed in order to help accelerate the growth of baby animals.

Vitamin B12 is dark red crystals in a prism form and the molecular formula is $C_{63}H_{88}N_{14}O_{14}PCo$ or 5, 6-dimethyl benzimidazolylcobamide cyanide. Vitamin B12 has a complex structure, as the Co atom is equatorially coordinated by tetrapyrrol ring (porphyrin) with attractive interaction between N-atoms and Co-atom possessing seven amide side chains (Figure 2-4).

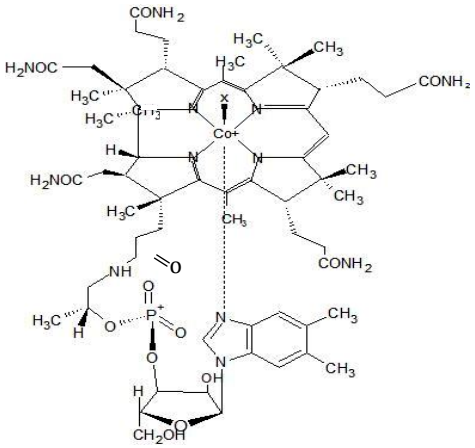


Figure 2-4. The structure of vitamin B12 (Modified from Randaccio et al., 2010)

Biosynthesis of vitamin B12 occurs in both aerobe and anaerobe conditions by the two main pathways together as shown in Figure 2-5. In oxygen-dependent pathway has precorrin 2 but in oxygen independent pathway, precorrin 2 was changed into sirohydrochlorin. Both pathways merge at Cob (II) yrinate a, c-diamind and continue to synthetic cobalamin (Kang et al., 2012).

There are many bacteria can produce vitamin B12 such as *Pseudomonas denitrificans, Propionibacterium freudenreichii, Bacillus*

megaterium, *Salmonella enteric* and *Rhodobacter sphaeroides* (Kang et al., 2012). Among PNSB, *R. sphaeroides* was classified as a good candidate to produce vitamin B12 as *R. sphaeroides* P47 produced 87 µg g^{-1} dry cells; while other such as *R. capsulatus* and *Rps. gelatinosa* produced 21-33 µg g^{-1} dry cells under anaerobic light conditions (Sasaki et al., 2005).

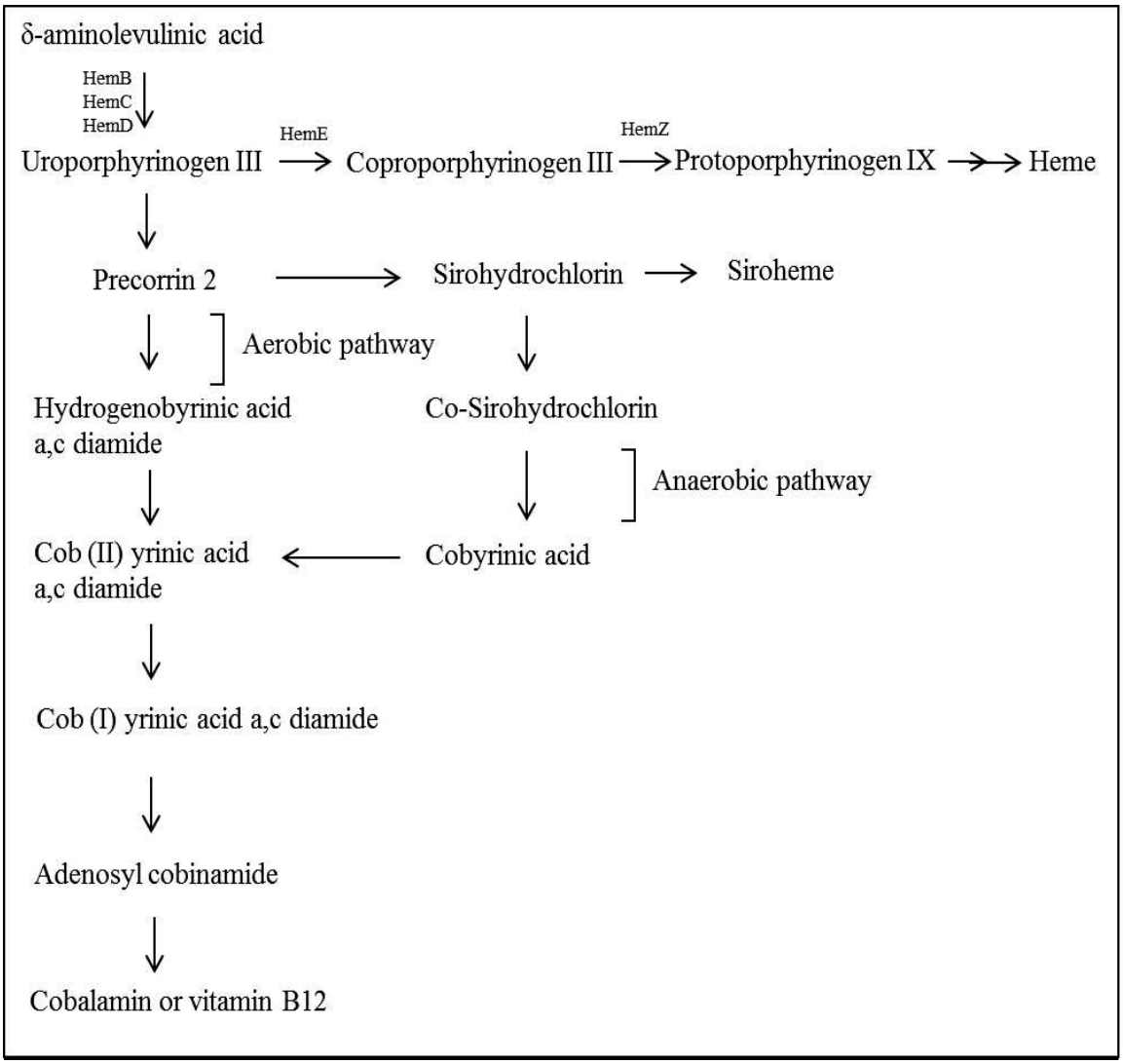


Figure 2-5. Pathways of vitamin B12 synthesis (Modified from Kang et al., 2012)

Use of PNSB as a source of antivibrio compounds

The outbreak diseases in shrimp cultivation are very serious problem for farmers. Farmers must follow a good practice for shrimp cultivation to protect shrimp against diseases. To avoid using antibiotics for controlling shrimp disease, bioactive compounds produced by microbes that are effective in inhibiting bacteria (*Vibrio* spp.) are very attractive to use instead of antibiotics.

Rattanachuay et al. (2007) studied on *Pseudomonas* sp. W3 that could produce protease enzyme and also control shrimp pathogenic bacterium *Vibrio harveyi* (Luminescent disease). By testing in agar well diffusion method, the results showed clear zone inhibition was 21.62 mm, and then testing in shrimp aquaria found that *V. harveyi* was decreased 1 Log cycle compared with the control group.

However, there still be a little information about antivibrio compounds produced by PNSB. Chandrasekaran and Ashok Kumar (2011) studied on the effect of bioactive compounds produced by *R. sphaeroides* BRP9 isolated from a shrimp pond. The result showed that bioactive compounds could inhibit *V. harveyi* and *V. fischerii* by agar well diffusion method with clear zones, 27 and 24 mm, respectively. In addition, the research of Juneius and Selvin (2012) isolated *R. sphaeroides* MSB 57 and *Rps. Palustris* MSB 55 from sponges and found that these bacteria showed ability to inhibit *V. parahaemolyticus* by cross-streak method.

Use of PNSB as a source of photopigments

PNSB are photosynthetic bacteria that contain photosynthetic pigments (bacteriolochlorophylls (Bchl) and carotenoids), this make PNSB are interesting bacteria as a source of pigments in animal feed (Kuo et al., 2012). Natural pigments are produced by many microorganisms and plants; among them carotenoids are an important in medicinal and biotechnological

application. Carotenoids have been used commercially as food colorants (red, orange) in animal feed supplements (Mukoyama et al., 2006). Carotenoids are major pigment in Crustaceans including shrimp (Latscha, 1989). As carotenoids are directly important roles for pigmentation that affect on the color of shrimp quality; therefore shrimp feeds require carotenoids as one of supplements that causes high cost of commercial feed (Wade et al., 2017). Moreover, carotenoids play an important role as antioxidants, source of pro-vitamin for enhancement growth and reproduction (Linan-Cabello et al., 2002).

R. sphaeroides is a potent organism to produce high yield of carotenoids. Biosynthesis of these pigments, first step is the condensation of two molecules of geranylgeranyl pyrophosphate to phytoene. Phytoene is colorless and incapable of photoprotection, and is followed by subsequent a series of de-saturations to convert into colored carotenoids by phytoene desaturase (CrtI) (Wu and Liu, 2011).

Liu et al. (2015) used *R. sphaeroides* strain ATCC17023 for studying carotenoid production and under its optimum condition this organism was able to produce high carotenoid yield (3.24 mg g biomass^{-1}); while carotenoid yield in control was only 1.90 mg g biomass^{-1}.

Chiu and Liu (2014) reported that *R. sphaeroides* WL-APD911 is a commercial strain to produce carotenoids as product name Lycogen™. The carotenoid composition of Lycogen™ includes neurosporene and β-carotene rather than lycopene, a traditional carotenoid found in plants and photobacteria. In addition, gamma-aminobutyric acid (GABA) and ubiquinone (Q10) were also found in Lycogen™. For applying, this product was mixed with commercial fish feed and found a better growth performance of seawater red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*) during cultivation for 7 weeks. The dietary supplementation with 1.0% Lycogen™ was the most effective level to enhance growth and innate humeral immunity of tilapia (Chiu and Liu, 2014).

CHAPTER 3

In vitro and *in vivo* selection of probiotic purple nonsulfur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease causing *Vibrio parahaemolyticus* and other vibrios

Abstract

Shrimp cultivation has been faced with huge losses in productivity caused by infectious shrimp pathogenic vibrios, especially *Vibrio parahaemolyticus* that causes acute hepatopancreatic necrosis disease (AHPND). Hence, purple nonsulfur bacteria (PNSB) were isolated from shrimp ponds for investigating their abilities to control shrimp pathogenic *Vibrio* spp. and their use as probiotics for sustainable shrimp cultivation. Based on their probiotic properties, strains S3W10 and SS15 were selected because of their strong abilities to produce amylase, gelatinase and vitamin B12. However, only three PNSB strains (SS15, TKW17 and STW181) strongly inhibited *V. harveyi* _KSAAHRC and *V. vulnificus*_KSAAHRC including *V. parahaemolyticus* AHPND strains by secreting antivibrio compounds. Four selected PNSB also grew in the presence of pancreatic enzymes, and they were identified as *Rhodobacter sphaeroides* for strains S3W10, SS15 and TKW17 and *Afifella marina* for strain STW181. The effects of a mixed culture were also investigated as follows: T1 (S3W10+SS15), T2 (S3W10+TKW17) and T3 (S3W10+STW181) on postlarval white shrimp (*Litopenaeus vannamei*) for 60 days by comparison with a control. All three probiotic PNSB sets significantly improved the digestive enzyme activities and shrimp growth with their proliferation in shrimp gastrointestinal tract although the shrimp survival was not significantly different. They also significantly reduced the cumulative

mortality of shrimp exposed to a virulent AHPND strain (*V. parahaemolyticus* SR2). This is the first to conclude that selected probiotic PNSB strains have great potential to be used for shrimp cultivation to control vibrios including AHPND strains.

Keywords: antivibrio compounds, early mortality syndrome, probiotics, purple nonsulfur bacteria, shrimp cultivation, *Vibrio* spp.

บทคัดย่อ

การเพาะเลี้ยงกุ้งต้องเผชิญกับปัญหาการสูญเสียผลผลิตสูงมาก เนื่องจากแบคทีเรียก่อโรคกุ้งกลุ่มวิบริโอ (*Vibrio* spp.) โดยเฉพาะอย่างยิ่ง *Vibrio parahaemolyticus* ซึ่งเป็นสาเหตุของโรคกุ้งตายด่วน หรือโรคตับตายเฉียบพลัน (Acute hepatopancreatic necrosis disease, AHPND) ดังนั้นจึงคัดแยกแบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ (Purple nonsulfur bacteria, PNSB) จากบ่อเลี้ยงกุ้ง เพื่อนำมาทดสอบความสามารถในการควบคุมเชื้อก่อโรค *Vibrio* spp. และความสามารถในการเป็นโปรไบโอติกส์เพื่อนำมาใช้ในการเพาะเลี้ยงกุ้งอย่างยั่งยืน เมื่อพิจารณาคุณสมบัติการเป็นโปรไบโอติกส์พบว่าไอโซเลท S3W10 และ SS15 มีความสามารถที่ดีในการผลิตเอนไซม์อะไมเลส เจลาติเนส และวิตามินบี 12 และมีจำนวน 3 ไอโซเลท ได้แก่ SS15 TKW17 และ STW181 ที่มีความสามารถสูงในการยับยั้งเชื้อก่อโรคกุ้ง *V. harveyi*_KSAAHRC *V. vulnificus*_KSAAHRC รวมทั้งเชื้อก่อโรคตับวายเฉียบพลัน (AHPND) *V. parahaemolyticus* โดยการหลั่งสารออกฤทธิ์ยับยั้งวิบริโอออกมานอกเซลล์ แบคทีเรีย PNSB ทั้ง 4 ไอโซเลท ที่คัดเลือกสามารถเจริญได้ในเอนไซม์แพนครีเอติน และเมื่อทำการเทียบเคียงสายพันธุ์พบว่า ไอโซเลท S3W10 SS15 และ TKW17 คือแบคทีเรีย *Rhodobacter sphaeroides* ส่วนไอโซเลท STW181 คือแบคทีเรีย *Afifella marina* และผลของการเติม PNSB แบบผสม 2 ไอโซเลทเข้าด้วยกันโดยมีชุดการทดลองดังนี้ T1 (S3W10 + SS15) T2 (S3W10 + TKW17) และ T3 (S3W10 + STW181) ซึ่งเปรียบเทียบกับชุดควบคุม ในการเพาะเลี้ยงกุ้งขาว (*Litopenaeus vannamei*) ระยะวัยอ่อน (postlarvae) เป็นเวลา 60 วัน พบว่า ทั้ง 3 ชุดการทดลองที่เติม PNSB มีผลทำให้กิจกรรมเอนไซม์ย่อยอาหารในตัวกุ้งเพิ่มมากขึ้นอย่างมีนัยสำคัญ และทำให้กุ้งมีการเติบโตเพิ่มขึ้น โดย PNSB สามารถเพิ่มจำนวนได้ในลำไส้กุ้ง แม้ว่าอัตราการอยู่รอดของกุ้งในแต่ละชุดการทดลองไม่แตกต่างกันอย่างมีนัยสำคัญ แต่ชุดทดลองที่มีการเติม PNSB สามารถลดอัตราการตายของกุ้งที่เกิดจากการทำให้ติดเชื้อก่อโรค AHPND (*V. parahaemolyticus* SR2) นี้เป็นการรายงานแรกสุดที่พบว่าแบคทีเรียโปรไบโอติกส์ PNSB ที่คัดเลือกได้มีศักยภาพในการประยุกต์ใช้ในการเพาะเลี้ยงกุ้งเพื่อควบคุมเชื้อก่อโรคในกลุ่มวิบริโอรวมถึงเชื้อก่อโรค AHPND ด้วย

คำสำคัญ : สารออกฤทธิ์ยับยั้งวิบริโอ โรคกุ้งตายด่วน โปรไบโอติกส์ แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ การเพาะเลี้ยงกุ้ง แบคทีเรียกลุ่มวิบริโอ

Introduction

Cultivation of shrimp is one of the most important economic activities in aquaculture and is one of the fastest growing animal food-producing sectors in the world (FAO, 2014). Thailand has become one of the world top countries for exporting shrimp: around 185,515 pounds to the United States in 2013 (Reed and Royales 2014). Intensive shrimp farming on an industrial scale has faced many problems, particularly the difficulty of growing shrimp in poor water quality as a result of high stock densities and unsuitable management practices that quickly leads to the development of shrimp diseases. Generally, the most serious diseases found in shrimp cultivation are caused by bacterial infections, in particular by *Vibrio* species such as *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Chatterjee and Haldar 2013). Since 2010, the early mortality syndrome (EMS) technically known as "acute hepatopancreatic necrosis disease" (AHPND), has been the most serious shrimp disease and it has caused huge economic losses, in the order of USD 1 billion in the Asian shrimp industries (De Schryver et al., 2014; Zorriehzahra and Banaederakhshan, 2015).

This disease has subsequently spread to the Western Hemisphere and it emerged in Mexico in early 2013 (Nunan et al., 2014). It frequently causes up to 100% mortality in early shrimp cultivation and research groups have established that the most virulent bacterial strains causing AHPND are invariably *V. parahaemolyticus* strains (Tran et al., 2013; De Schryver et al., 2014; Joshi et al., 2014). To help solve these serious problems, shrimp farmers must use chemicals and antibiotics to maintain shrimp water quality and eliminate shrimp pathogens. However, some of those compounds cause harmful effects to consumers health, in particular from the use of antibiotics as after long-term usage they accumulate in the shrimp pond sediments, and the bacteria can develop antibiotic resistance (Zhang et al., 2011). For example,

chloramphenicol has been banned for use in aquaculture because residual amounts remained in shrimp products, and it can then induce aplastic anaemia in humans (Serrano, 2005). Hence, for sustainable shrimp cultivation the use of antibiotics should be avoided. However, probiotics can act as biocontrol agents against pathogenic *Vibrio* species and they can be a more attractive way to achieve the target goal of using economical culture methods. In aquaculture, probiotics means the use of live microbes to help the host animal survive by providing it with beneficial effects for example by modifying the local microbial community, improving the animals ability to digest feed and/or enhance the ability of the host to resist disease (Verschuere et al., 2000; Zhou et al., 2007).

Among the probiotics used in shrimp aquaculture, purple nonsulpur bacteria (PNSB) are one of the groups with good potential because they can clean up water, reduce sulfide levels and act as probiotics to promote shrimp growth by secreting bioactive compounds that control shrimp pathogens (Qi et al., 2009; Luo et al., 2012). Purple nonsulfur bacteria are normally found in the light zone of natural water containing some organic matter. They have a wide range of growth conditions such as being photoautotrophic (electron donors, i.e. hydrogen or low level of sulfide) or photoorganotrophic under anaerobic-light conditions, and growing heterotrophically under aerobic-dark conditions using organic matter such as alcohols and volatile fatty acids (Lu et al., 2011). Some PNSB also produce various extracellular enzymes such as lipase, amylase, protease, cellulase and hemicellulase (Merugu et al., 2010). This is why PNSB have been applied to treat various wastewaters (Kantachote et al., 2005; Lu et al., 2011; Luo et al., 2012). Moreover, PNSB can secrete vitamin B12 and also produce antivibrio compounds that inhibit pathogenic *Vibrio* spp. (Chandrasekaran and Ashok Kumar, 2011).

However, there is still only limited information about the antivibrio activities of PNSB. It will therefore be of benefit for shrimp aquaculture to explore the possibility of using PNSB in shrimp cultivation as probiotics and for controlling shrimp pathogenic vibrios, in particular the *V. parahaemolyticus* causing AHPND. Hence, this study aimed to isolate and select PNSB from shrimp ponds to investigate their probiotic properties and their ability to control shrimp pathogenic *Vibrio* spp., especially *V. parahaemolyticus* that causes AHPND for the possibility to use them in shrimp cultivation. This could lead to an ability to cultivate shrimp using "organic farming" methods that will provide for a shrimp friendly environment and improves the safety for humans who consume them.

Materials and methods

Isolation of PNSB for investigating the probiotic properties *in vitro*

Samples of water and sediment were collected from 29 shrimp ponds in three provinces of Southern Thailand, including Trang, Songkhla and Surathani. They were used to isolate PNSB following the methods of Nunkaew et al. (2012); however, in this study BIM (basic isolation medium) (Brown, 2012) was used instead of glutamate acetate (GA) medium. All the PNSB used were isolated from various shrimp ponds on both the coastal shorelines in the south of Thailand with the exception of strain STW181 that had been isolated previously in our laboratory (Mukkata et al., 2015). The PNSB were grown in BIM except for strain STW181 that was grown in GA. The BIM consists of (in g L^{-1}) 1.0 $(NH_4)_2SO_4$, 0.5 K_2HPO_4, 0.2 $MgSO_4$, 5 $NaHCO_3$, 15 NaCl, 1.5 yeast extract, 1.5 mL glycerol and 0.03 L-cysteine, adjusted to pH 7. The GA medium consists of (g L^{-1}) 3.8 sodium-L-glutamate, 5.44 sodium acetate, 0.5 KH_2PO_4, 0.5 K_2HPO_4, 0.8 $(NH_4)_2HPO_4$, 0.2 $MgSO_4$, 2.0 yeast extract, 20 NaCl, and trace elements 0.053 $CaCl_2 \cdot 2H_2O$, 0.001 nicotinic acid, 0.001

thiamine hydrochloride, 0.01 biotin, 0.012 $MnSO_4 \cdot 5H_2O$, 0.025 ferric citrate and 0.95 $CoCl_2 \cdot 6H_2O$.

A point inoculation method on specific media, starch agar, Frazier gelatin and tributyrin agar, was used to investigate the production of the following digestive enzymes: amylase, gelatinase and lipase, as these enzymes would help to digest the feed and promote shrimp growth and also would help to clean up the water in the shrimp ponds. Isolated PNSB were grown in BIM broth plus 1.5% NaCl under microaerobic-light conditions for 48 h. The culture broth was adjusted to an optical density of 1.0 (OD_{660}), and a 10-µL aliquot was dropped onto the surface of each medium and incubated under aerobic-dark conditions at room temperature for 5 days. Clear zones around colonies on tributyrin agar indicated that PNSB digested lipid or tributyrin. For starch and gelatin digestions, these were tested by adding an iodine solution and Frazier's reagent (15%, w/v, $HgCl_2$ + 20%, v/v, HCl conc.) respectively onto the surface of the test plates to look for clear zones around the colonies.

The production of vitamin B12 by PNSB was tested by growing in GA broth containing 4 µM $CoCl_2$ (Cauthen et al., 1967) under microaerobic-light conditions at room temperature for 48 h. The culture broths were centrifuged at 10418 *g* for 15 min to obtain cell pellets, and vitamin B12 was extracted by cell disruption in a 0.1 N phosphate-buffered solution containing 0.01% KCN pH 5.5 at 121°C for 15 min. The suspensions were then centrifuged at 16278 g for 10 min to obtain the supernatants. The supernatants were tested for the dicyano form of B12 using a microplate reader (PowerWaveX; Biotek, Winooski, VT, USA) at a wavelength of 367 nm (Hugenschmidt et al., 2010). In addition, extracellular vitamin B12 was directly determined by checking their supernatants, and the same procedure was used as for the extracted intracellular vitamin B12. Both intracellular and extracellular vitamin B12 production were

determined to evaluate the possible use of PNSB for single cell protein and their use as probiotics.

Administering probiotics in adequate amounts confers a health benefit to the host; however, the probiotics must survive in the gastrointestinal tract (GIT) or digestive tract. Generally, the digestive process in shrimp is completed quickly within 4 h (Goddard, 1996) so a time of 240 min was suitable for testing the tolerance of the potential probiotics in this process. The ability of selected PNSB to survive the pancreatic enzymes was tested in vitro according to the method of Zhou et al. (2007) by growing PNSB in BIM broth under microaerobic-light conditions for 48 h. This experiment was carried out after secondary screening based on the antivibrio activity obtained from 1 mL of culture broth that had been centrifuged at 5860 g for 10 min and the cell pellet was washed three times with phosphate-buffered saline (PBS, pH 7.0), consisting of 0.02% KCl, 0.144% Na_2HPO_4, 0.8% NaCl and 0.024% KH_2PO_4. A cell count was also made on BIM agar using the spread plate method and incubated under aerobicdark conditions. The pancreatic enzyme preparations were made by mixing pancreatin (4x USP; Sigma, St. Louis, MO, USA) in sterile saline (0.5% NaCl, w/v) at a concentration of 1 g L^{-1} and at different pH values (7.2, 7.7, 8.3) to provide conditions similar to those present in the shrimp digestive tract. For testing, 0.2 mL of each cell suspension was added into a screw-cap Eppendorf tube containing 0.3 mL sterile saline and 1 mL of the pancreatic enzyme. This was then mixed well by vortex for 10 s and incubated at 30 °C for 0, 180 and 240 min. After that, survivor cells were counted using the same technique as above and compared with those found at zero time for each strain.

Isolation of shrimp pathogenic bacteria causing EMS/AHPND and vibrios used

V. parahaemolyticus strains that caused EMS/ AHPND were isolated by collecting shrimp (postlarval-15-stage) from an endemic area in the Ranot District of Songkhla Province (7°49053.9″N 100°21010.6″E). The shrimp sample was ground in TSB plus 1.5% NaCl and incubated at 35°C for 18 h to enrich the growth of *Vibrio* spp. The samples were then spread on TCBS agar with 1% NaCl and incubated at 35°C for 18 h. Green single colonies were picked and stabbed onto CHROMagarTM Vibrio. These plates were incubated at 35°C for 24 h to check for *V. parahaemolyticus* strains that produced a purple color on the medium (Nunan et al., 2014). As this study focused on *V. parahaemolyticus* that could cause EMS/AHPND, the PCR technique was used to confirm they were *V. parahaemolyticus* EMS strains using the AP2 specific primers, which included the forward and reverse primers AP2-F (5'-TCACCCGAATGCTCGCTTGTGG-3') and AP2-R (5'-CGTCGCTACTGTC TAGCTGAAG-3').

These primers had been released for the detection of bacterial isolates that cause AHPND (http://www.enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf). The PCR techniques were performed based on the optimum protocol, which included the following: pre-denaturation: 94°C for 5 min; denaturation: 94 °C for 30 s; annealing: 60 °C for 30 s; extension: 72°C for 60 s with 30 cycles; and a final extension: 72 °C for 10 min. The PCR products were checked by electrophoresis on 1% agarose gel and stained with ethidium bromide. When the EMS/ AHPND bands were detected at approximately 700 bp, this means that it was *V. parahaemolyticus* that had caused the EMS/ AHPND. In addition, these isolates were confirmed as *V. parahaemolyticus* strains using the PCR technique using the universal

primer (8F and 1492R); the PCR products were sequenced and submitted to the NCBI-BLAST (http://www.ncbi.nlm.nih.gov).

The isolated shrimp pathogenic bacteria (*Vibrio* spp.) included *V. vulnificus*_KSAAHRC, *V. vulnificus*_KSAAHRC2, *V. harveyi*_KSAAHRC, *V. harveyi*_KSAAHRC142 and *V. parahaemolyticus*_KSAAHRC46, all isolated from shrimp disease situations and that were provided by the Aquatic Animal Help Research Center (AAHRC), Prince of Songkla University (PSU). In addition, five strains of *V. parahaemolyticus* (PSU5427, PSU5428, PSU5429, PSU5433 and PSU5442) that caused EMS or AHPND in shrimp were provided by Prof. Dr. Varaporn Vuddhakul, from PSU. All pathogenic *Vibrio* spp. were cultured in tryptic soy broth (TSB) containing 1.5% NaCl unless otherwise stated or in thiosulfate-citrate-bile salts-sucrose (TCBS) agar containing 1% NaCl.

Selection of isolated PNSB able to inhibit shrimp pathogenic *Vibrio* spp. / causing AHPND

An overlay diffusion method was used for a preliminary screening of antivibrio activity of the PNSB isolates. The PNSB were cultured in BIM or GA broth and incubated under microaerobic-light conditions at room temperature for 48 h. Then, the OD_{660} was adjusted to 0.5 for obtaining roughly $1.2x10^8$ cell mL^{-1}. A 10-µL sample of each PNSB suspension was spotted onto TSA containing 1.5% NaCl and incubated under anaerobic-light conditions for 5 days. *V. harveyi*_KSAAHRC and *V. vulnificus*_KSAAHRC were used as the target shrimp pathogens in this experiment. Each shrimp pathogen was cultured in TSB containing 1.5% NaCl and shaken at 150 rpm min^{-1}, 35 $^{\circ}C$ for 18 h; then, the cell density was adjusted to $1.0x10^5$ CFU mL^{-1} (OD_{660} = 0.1). The pathogenic *Vibrio* sp. was mixed well with melted TSA containing 1.5% NaCl before pouring onto TSA plates previously spotted with PNSB. These plates

were incubated at 35 °C for 18 h, and clear zones around colonies were measured where no growth had occurred.

An agar well diffusion method was used for the secondary screening of the antivibrio activity of isolated PNSB by growing PNSB in BIM or GA medium under microaerobic-light conditions at room temperature for 7 days. Culture broths were centrifuged at 10418 *g*, for 15 min to obtain the culture supernatants and these were concentrated by lyophilization (Alpha 1-2 LD; Christ, Osterode am Harz, Germany). The lyophilized supernatants were each dissolved in 10% methanol to vary the concentrations to obtain a 5–15 times concentration than that of the initial material for testing their antivibrio activity using 10% methanol as a negative control. Either *V. harveyi*_KSAAHRC or *V. vulnificus*_KSAAHRC at a cell density of 1.0×10^5 CFU mL^{-1} was swabbed over the surface of a TSA plate containing 1.5% NaCl with 3 wells per plate (diameter, 8 mm per well). A 120-µL sample of each lyophilized supernatant was added into each well and incubated at 35 °C for 18 h prior to the measurement of the inhibition zones.

To test antivibrio activity of the selected PNSB against the growth of all shrimp pathogenic vibrios they were grown under microaerobic-light conditions with their optimal conditions for producing antivibrio compounds (Chumpol et al., our unpublished data) to obtain culture supernatants. The culture supernatants were concentrated to 15X and 20X their initial concentrations for testing their inhibitory effect on the vibrios including *V. parahaemolyticus* pathogen strains that caused EMS /AHPND using the agar well diffusion method as previously described.

Identification of selected probiotic PNSB using 16S rRNA gene sequencing

With regard to the results of the previous experiments, four PNSB strains were selected based on their antivibrio activities and their abilities to

produce digestive enzymes and vitamin B12 including their proliferation in the presence of pancreatic enzyme, so they were considered as probiotics with ability to inhibit vibrios. They were cultured in BIM or GA broth under microaerobic-light conditions for 48 h; then, their harvested cell pellets were prepared by centrifugation at 10418 *g* for 20 min. The cell pellet was used to extract DNA using the DNA isolation kit (PowerSoil; MO Bio, Carlsbad, CA, USA). The primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') were amplified for the 16 S rRNA genes using the PCR technique. There were three steps: the initial step, denaturation at 95 °C, 60 s; an annealing step at 55 °C, 30 s; and the final extension step at 72 °C, 120 s. The PCR products were purified using the Gel /PCR DNA fragment extraction kit (Geneaid, Qiagen, Taiwan) according to the manufacturer's instructions. Then, the purified PCR products were sequenced, and they were submitted to the NCBIBLAST for comparison with known sequences available in the GenBank database. The sequences analysis was performed using CHROMASPRO version 1.5 (Technelysium, South Brisbane, QLD, Australia), and the phylogenetic trees were constructed using MEGA 6.0 Free available software (Tamura et al., 2013).

Effects of probiotic PNSB on digestive enzymes, shrimp growth and their survivals

Postlarval (PL-19) white shrimp (*Litopenaeus vannamei*) were used to investigate the potential of selected probiotic PNSB for facilitating shrimp growth. Shrimp were acclimatized for 3 days in commercial artificial sea water (ASW), which adjusted to an initial pH of 7.8, an initial salinity of 15 g L^{-1} and a water temperature of between 25 and 27 °C. The shrimp were fed four times per day (7:00, 12:00, 16:00 and 20:00) with commercial shrimp feed roughly 2% of total body weight. Shrimp aquaria were made from acrylic plastic with a size of 12.5 cm x 12.5 cm x 25.5 cm, and 3.6 L capacity. Twenty shrimp were

randomly divided into each aquarium including control set and three treatment sets with three replications.

To establish the effects of four selected probiotic PNSB on digestive enzymes and PNSB survival in the shrimp GIT, shrimp growth including the survival rates of shrimp, the three treatment sets for each treatment had strain S3W10 mixed with an another strain: SS15 for T1, TKW17 for T2 and STW181 for T3. A mixed culture at a ratio of 1:1 of each treatment was inoculated into the shrimp culture water every week. Each PNSB strain was cultured under microaerobic-light condition for 48 h and centrifuged at 10418 *g* for 10 min to obtain cell pellets. The cell pellets were washed three times with 0.85% normal saline solution (NSS) and resuspended in NSS to adjust the cell density measured using a spectrophotometer at 660 nm. Each PNSB strain was inoculated into the culture water at a final concentration of 10^8 CFU mL^{-1} every week whereas a set without inoculation served as a control. Shrimp cultivation was carried out for 60 days and all parameter as previously mentioned were monitored at days 0, 30, 45 and 60. For each sampling time, two shrimp were randomly collected from each replicate of the control and treatment sets so a total of eight shrimp from each aquarium were sacrificed for monitoring the digestive enzymes and PNSB survival in the shrimp GIT after measuring shrimp growth (body weight and total length).

The whole GIT of the shrimps were separated out for weighing and homogenized with cold deionized water for measuring the activities of the protease, amylase and lipase enzymes. The GIT crude homogenate was centrifuged at 10418 *g* for 10 min to obtain the supernatant, and then, it was filtered through a 0.45-μm filter and kept in sterile Eppendorf tubes at 20 $^{\circ}$C for assay of the digestive enzymes (Zokaeifar et al., 2012). Bovine serum albumin was used as a standard for determination of the total protein. Total protease activity was determined using 0.65% casein as substrate in 50 mM

potassium phosphate buffer and reacting with Folin–Ciocalteu's reagent. The absorbance of reaction mixture was measured at 660 nm and compared with a tyrosine standard curve. One unit of protease activity was defined as the number of l M of tyrosine released per min at 37 °C. Total amylase activity was assayed using 1% soluble starch as substrate in 0.5 M sodium acetate and reacting with 3,5-dinitrosalicylic acid (DNS), and the absorbance was measured at 540 nm and compared to a maltose standard curve (Bernfeld, 1955). One unit of amylase activity was defined as the amount of l M maltose released per min at 37 °C. Total lipase activity was investigated using 20 mM *p*-nitrophenol palmitate as substrate. The absorbance of the reaction mixture was measured at 410 nm and compared with a *p*-nitrophenol standard curve (Pencreac'h and Baratti, 1996). One unit of lipase activity was defined as the presence of l M *p*-nitrophenol per min at 37 °C.

To determine PNSB survival, the whole of the GIT was ground in 0.85% NSS and diluted by tenfold serial dilutions. The pour plate technique was used to count PNSB using BIM and GA media specific to the PNSB used and incubated under aerobic-dark conditions for 3 days. Fortunately, on either aerobic-dark or microaerobic-light conditions, these probiotic PNSB having a color as red or pink depended on their species for indicating they were PNSB. The PNSB counts were recorded data as log CFU g^{-1} of the shrimp GIT.

Challenge test with *V. parahaemolyticus* AHPND strain

To follow real shrimp cultivation after 60 days of the previous experiment, the juvenile shrimp (average body weight, 0.555 ± 0.142 g) were continued without addition of probiotic PNSB in this step for a challenge with a virulent AHPND strain of *V. parahaemolyticus* SR2 that was isolated in this study. The juvenile shrimp with their body weight in the range of 0.5-5 g had been reported for their sensitivity to be infected by *V. parahaemolyticus* causing AHPND (Tran et al., 2013; Joshi et al., 2014; Lai et al., 2015). The

water in each aquarium was replaced by 75% ASW before starting the challenge test. This was because shrimp were dying during cultivation so at the beginning each aquarium had an average of eight shrimp in each aquarium, except T3 set which had only seven shrimp. As to the challenge the strain SR2 was added into each aquarium of the control and the treatment sets to obtain an initial cell density of approximately 4 x 10^4 CFU mL^{-1}. The cell density was designed by considering the infectious dosages of the *V. parahaemolyticus* caused AHPND during shrimp cultivation after shrimp were infected by AHPND strains in the range of 10^3-10^6 CFU mL^{-1} (Joshi et al., 2014). The percentage of the shrimp cumulative mortality was determined daily for up to 10 days (Zokaeifar et al., 2012).

Statistical analysis

All experiments in this study were conducted with three replicates, and all data were analysed using the one-way analysis of variance (ANOVA) with the exception that shrimp survival percentages were analysed using Kruskal-Wallis test because the data were not normally distributed. ANOVA was used to detect any significant differences between the means of more than two groups of data at a *P*-value < 0.05 and the mean comparisons were performed using the Duncan's multiple-range test. The data were analysed using the SPSS program analysis version 11.5 (Lead Technologies, Armonk, NY, USA).

Results

Selection of isolated PNSB with probiotic properties and their antivibrio activities

A total of 185 PNSB isolates were obtained from various shrimp ponds, 119 isolates from 56 water samples and 66 isolates from 23 sediment samples. When the isolated PNSB were tested for their abilities to produce

digestive enzymes, only six showed strong activity for producing digestive enzymes (Table 3-1). All these six PNSB strains digested gelatin (clear zones, 9.93 - 34.33 mm), and strain SS15 showed the highest gelatinase enzyme production. However, only strains S3W10 and S3W11 produced amylase with clear zones of 26.80 and 22.23 mm respectively. While strains SS15 and W164 produced clear zones of 6.83 and 11.94 mm on tributyrin agar so were lipase producers.

As a similar amount of vitamin B12 was produced intracellularly and extracellularly only the extracellular amount has been presented as the aim in this study was to use the isolates as probiotics that is secreting vitamin B12. Only 9.2% (17 isolates) of the isolated PNSB produced extracellular vitamin B12 over a wide range of from 43.81 to 136.50 μg mL^{-1} (Table 3-2). There were seven PNSB, including TKW31, S3W10, S11, S3W11, SS15, TKW1 and TPW71, that produced vitamin B12 in either good, very good or excellent amounts.

Table 3-1. Probiotic properties of purple nonsulfur bacteria (PNSB) isolated from various shrimp ponds in Southern Thailand for digestive enzymes production.

PNSB strain	Clear zones (mm)		
	Starch (starch agar)	**Gelatin (Frazier gelatin)**	**Lipid (tributyrin agar)**
S3W10	26.80 ± 0.44	27.40 ± 0.61	-
S3W11	22.23 ± 1.13	16.23 ± 0.42	-
SS15	-	34.33 ± 0.47	6.83 ± 0.78
STW181	-	28.85 ± 0.65	-
W164	-	13.53 ± 0.33	11.94 ± 0.20
TKW17	-	9.93 ± 0.32	-

Each value is mean of triplicate and its standard deviation (SD). "-" no digestion.

Table 3-2. Extracellular vitamin B12 production

Vitamin B12 (μg mL^{-1})					
Very low (43.81-51.17)	**Low (53.97-55.42)**	**Fair (58.06-66.01)**	**Good (71.85-74.47)**	**Very good (79.77-84.32)**	**Excellent (110.64-136.50)**
STW181, STS162, TPW21, SRW153	W164, TKW17, W161	S2W1, TPW55, TKW32	TKW31, S3W10	S3W11, S11	SS15, TKW1, TPW71

In parallel, 185 PNSB were firstly screened for their ability to inhibit the *Vibrio* species, *V. harveyi*_KSAAHRC and *V. vulnificus*_KSAAHRC. Only 10 isolates produced inhibition zones; however, their inhibition zones were not strong (< 15 mm) except for strain STW181 (27.20 ± 0.10 mm with *V. harveyi* _KSAAHRC, but only 13.20 ± 0.52 mm with *V. vulnificus*_KSAAHRC) (Figure 3-1A). Another strain W164 was the best to inhibit *V. vulnificus* _KSAAHRC (20 ± 0.13 mm); however, this strain did not inhibit *V. harveyi* _KSAAHRC. There were two strains (W164 and STW4) that did not inhibit *V. harveyi*_KSAAHRC. Hence, only eight PNSB were further studied for secondary screening.

To test for the secretion of antivibrio compounds by the eight selected PNSB, their culture supernatants were concentrated by lyophilization that varied from 5 to 15 times. Results showed that only the 15-fold-concentrated supernatant PNSB had a strong activity to inhibit both *Vibrio* spp. and the most effective strain was TKW17 (*V. harveyi*_KSAAHRC, 13.39 mm and *V. vulnificus*_KSAAHRC, 12.97 mm), followed by STW181 (Figure 3-1B). The antivibrio activity of strain SS15 was similar to the other strains but it also produced the best proteolytic activity and the most vitamin B12.

Regarding Figure 3-1B, only three PNSB (SS15, TKW17, STW181) had a strong ability to inhibit the shrimp pathogens and one strain S3W10 that produced high amounts of digestive enzymes and vitamin B12 (Tables 3-1 and 3-2) was selected to test its ability to tolerate pancreatin as it had the potential to act as a probiotics for shrimp cultivation. There was no significant difference for the initial cell densities of each selected PNSB at approximately 1.0 x 10^6 CFU mL^{-1}, and all selected PNSB not only survived but also grew in the simulated shrimp pancreatin at the various pH values (7.2, 7.7 and 8.3) as shown in Table 3-3. The proliferation of PNSB was in the order of the pH values 7.7 > 8.3 > 7.2, and their viable cells significantly increased during the incubation periods (0–240 min) at all the tested pH values. For instance, at pH 7.7 for all selected PNSB there were roughly 10^6 CFU mL^{-1} (t = 0), and this significantly increased to 1 x 10^7 CFU mL^{-1} after 240 min of incubation.

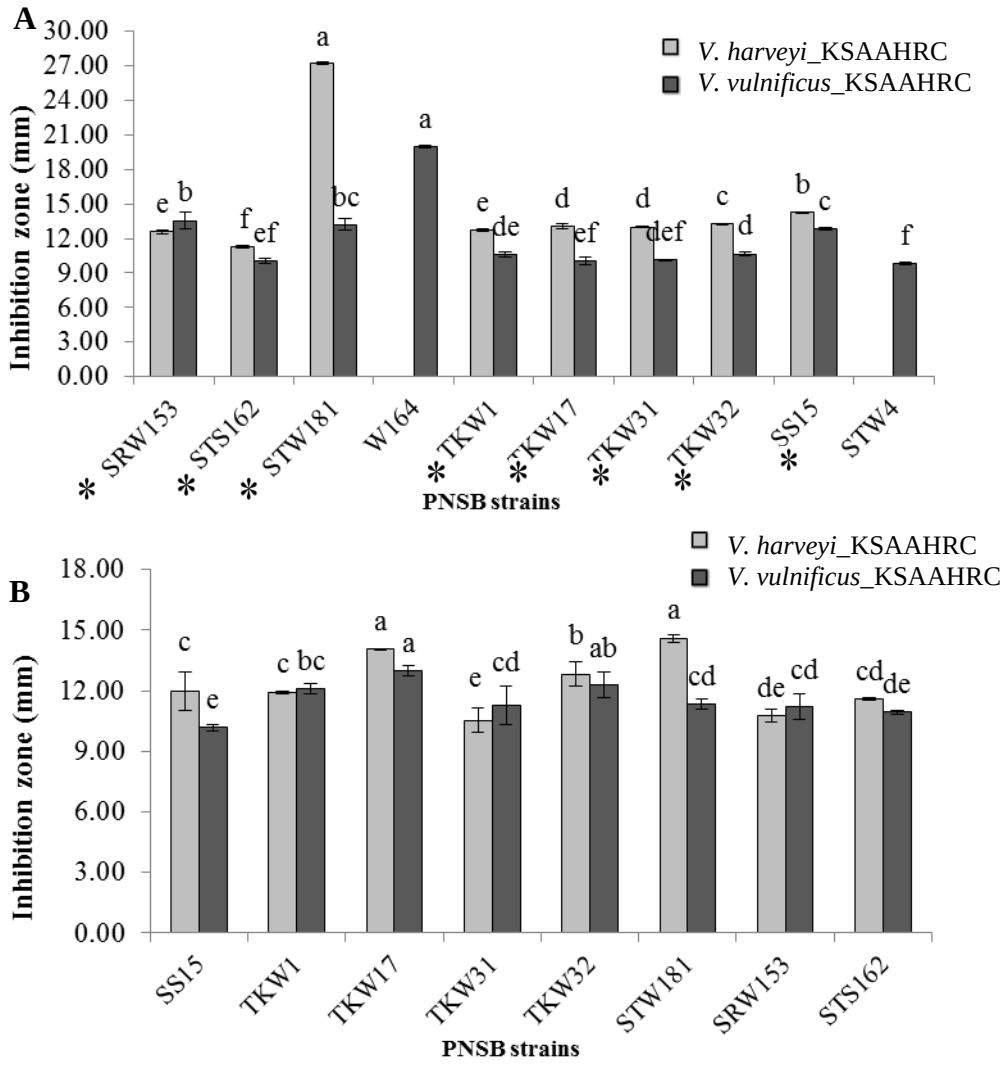


Figure 3-1. Antivibrio activity of PNSB against shrimp pathogenic *Vibrio* spp. (A) primary screening using an overlay diffusion method and (B) secondary screening using an agar well diffusion assay using 15X concentrated culture supernatants. Different lowercase letters above bars indicate significant differences ($P < 0.05$) and an asterisk in (*) indicates that the strain was selected for further studies.

Table 3-3. Effect of pancreatic enzymes under simulated shrimp gastro-intestinal tract (GIT) conditions on the viability of the selected PNSB strains

PNSB	pH of GIT	Viable count of PNSB (CFU mL^{-1})		
		0 min	180 min	240 min
SS15	7.2	$1.2 \times 10^{6\ Ac}$	$4.6 \times 10^{6\ Bb}$	$9.1 \times 10^{6\ ABa}$
	7.7	$1.1 \times 10^{6\ Ac}$	$7.5 \times 10^{6\ Ab}$	$1.3 \times 10^{7\ Aa}$
	8.3	$1.0 \times 10^{6\ Ac}$	$5.4 \times 10^{6\ ABb}$	$7.6 \times 10^{6\ Ba}$
S3W10	7.2	$1.3 \times 10^{6\ Ac}$	$7.0 \times 10^{6\ Cb}$	$1.2 \times 10^{7\ Ca}$
	7.7	$1.2 \times 10^{6\ Ac}$	$9.7 \times 10^{6\ Ab}$	$1.5 \times 10^{7\ Ba}$
	8.3	$1.0 \times 10^{6\ Ac}$	$8.4 \times 10^{6\ Bb}$	$1.9 \times 10^{7\ Aa}$
TKW17	7.2	$1.1 \times 10^{6\ Ab}$	$1.2 \times 10^{6\ Bb}$	$1.1 \times 10^{7\ Ca}$
	7.7	$9.8 \times 10^{5\ Ac}$	$2.1 \times 10^{6\ Ab}$	$1.7 \times 10^{7\ Aa}$
	8.3	$1.2 \times 10^{6\ Ac}$	$1.9 \times 10^{6\ ABb}$	$1.4 \times 10^{7\ Ba}$
STW181	7.2	$1.1 \times 10^{6\ Ac}$	$2.1 \times 10^{6\ Bb}$	$2.6 \times 10^{6\ Ca}$
	7.7	$1.0 \times 10^{6\ Ac}$	$6.0 \times 10^{6\ Ab}$	$1.4 \times 10^{7\ Aa}$
	8.3	$9.8 \times 10^{5\ Ac}$	$5.2 \times 10^{6\ ABb}$	$9.7 \times 10^{6\ Ba}$

Mean values of triplicate are shown, and significant differences are analysed at $P < 0.05$. Different uppercase and lowercase letters indicate significant differences for values in each column and each row respectively.

In vitro test for antivibrio activities by selected PNSB

A number of pathogenic *Vibrio* spp. was used for testing the activity of antivibrio compounds using a 15X concentrated lyophilized of selected PNSB. The most sensitive strain was *V. harveyi*_KSAAHRC as inhibition zones of 18.10 ± 0.75 mm, 17.13 ± 0.91 mm and 14.82 ± 0.41 mm were achieved by strains SS15, TKW17 and STW181 (Table 3-4A). Both *V. vulnificus* _KSAAHRC2 and *V. parahaemolyticus*_KSAAHRC46 were the most resistant

strains as all selected PNSB produced inhibition zones only in a range of 12.40 - 13.07 mm for the former and 12.55 - 12.73 mm for the latter target organism.

In this study, a total of 73 *Vibrio* strains were isolated and screened by PCR for causing EMS/ AHPND using the specific primer named AP2; however, only three isolates (SR1, SR2 and SR3) showed a DNA band at approximately 700 bp, the same as from a positive control and no band for the non-EMS/AHPND isolates when exposed to UV light (Figure 3-2). In addition, the presence of a 16S rRNA gene using the universal primer (8F and 1492R) was used to confirm bacterial identification. The three isolates (SR1, SR2 and SR3) that produced the 700-bp band were *V. parahaemolyticus* with a 99% similarity, and their accession numbers were KT006930, KT006931 and KT006932.

The ability of antivibrio compounds produced by a 20X concentration of lyophilized supernatant from selected PNSB against the growth of shrimp pathogenic *V. parahaemolyticus* that caused EMS/AHPND is shown in Table 3-4B. All the selected PNSB inhibited all the pathogens tested; however, the antivibrio activity was dependent on the target organisms and the selected PNSB strains. For example, antivibrio compounds produced by strains STW181 and TKW17 were the most effective to control *V. parahaemolyticus* PSU5427 and PSU5442. While, *V. parahaemolyticus* SR1, SR2 and SR3 were more resistant to antivibrio compounds produced by all selected PNSB than those found for all *V. parahaemolyticus* PSU strains. However, strains SS15 and TKW17 were the best to inhibit the more resistant strains, *V. parahaemolyticus* SR1, SR2 and SR3. Among resistant AHPND strains, strain SR2 was quite sensitive to antivibrio compounds produced by selected PNSB.

Table 3-4. Inhibitory effect of antivibrio compounds produced by selected PNSB under microaerobic-light conditions. (A) The effect of the 15X concentrated lyophilized supernatants against the growth of shrimp pathogenic *Vibrio* spp. (B) The effect of the 20X concentrated lyophilized supernatants against the growth of *V. parahaemolyticus* strains that can cause EMS/AHPND in shrimp.

Shrimp pathogenic bacteria	**Inhibition zone (mm) by PNSB**		
	SS15	**TKW17**	**STW181**
A			
*V. harveyi*_KSAAHRC	18.10 ± 0.75 ^{Aa}	17.13 ± 0.91 ^{Aa}	14.82 ± 0.41 ^{Ab}
*V. harveyi*_KSAAHRC142	13.15 ± 0.13 ^{Cb}	13.57 ± 0.65 ^{Bab}	14.42 ± 0.55 ^{Aa}
V. vulnificus KSAAHRC	14.80 ± 1.20 ^{Ba}	13.00 ± 0.20 ^{Bb}	12.78 ± 0.71 ^{Bb}
*V. vulnificus*_KSAAHRC2	12.40 ± 0.75 ^{Ca}	12.67 ± 0.36 ^{Ba}	13.07 ± 0.08 ^{Ba}
*V.parahaemolyticus*_KSAAHRC46	12.55 ± 0.18 ^{Ca}	12.73 ± 0.25 ^{Ba}	12.70 ± 0.09 ^{Ba}
B			
V. parahaemolyticus PSU5427	12.68 ± 0.32 ^{Ab}	13.21 ± 0.23 ^{Aa}	13.16 ± 0.29 ^{Aa}
V. parahaemolyticus PSU5428	12.43 ± 1.12 ^{Aa}	12.63 ± 0.58 ^{ABa}	12.43 ± 0.61 ^{Ba}
V. parahaemolyticus PSU5429	12.16 ± 0.70 ^{Aa}	12.78 ± 0.68 ^{ABa}	12.81 ± 0.32 ^{ABa}
V. parahaemolyticus PSU5433	12.43 ± 0.19 ^{Aa}	12.29 ± 0.41 ^{BCa}	12.63 ± 0.21 ^{Ba}
V. parahaemolyticus PSU5442	11.11 ± 0.86 ^{Bb}	12.66 ± 1.24 ^{ABa}	13.13 ± 0.29 ^{Aa}
V. parahaemolyticus SR1	11.34 ± 0.37 ^{Ba}	10.94 ± 0.32 ^{Dab}	10.64 ± 0.49 ^{Eb}
V. parahaemolyticus SR2	12.52 ± 0.50 ^{Aa}	12.65 ± 0.30 ^{ABa}	11.38 ± 0.49 ^{Db}
V. parahaemolyticus SR3	10.94 ± 0.37 ^{Bb}	11.59 ± 0.81 ^{CDab}	11.93 ± 0.37 ^{Ca}

Values are presented as a mean of three replicates ± SD, and significant differences are analysed at $P < 0.05$. Different uppercase and lowercase letters indicate significant differences for values in each column and each row respectively.

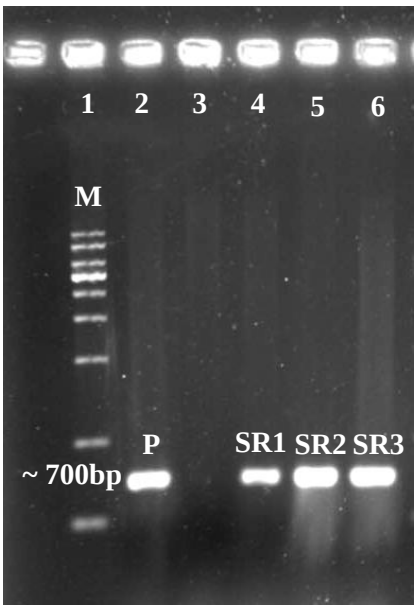


Figure 3-2. The PCR products of *V. parahaemolyticus* causing EMS /AHPND in shrimp as shown by gel electrophoresis. Lane 1 (M): 1-kb ladder DNA marker; lane 2 (P): positive control for EMS/ AHPND (*V. parahaemolyticus* PSU5427); lanes 3–6: isolated *V. parahaemolyticus* strains.

Identification of selected probiotic PNSB using 16S rRNA gene sequencing

The four selected probiotic PNSB strains (SS15, S3W10, TKW17 and STW181) were identified based on their 16S rRNA gene sequences by comparisons with the known sequences available in the GenBank database, and their accession numbers are KJ955372, KJ955374, KM387395 and KJ955373 respectively (Figure 3-3). Strains SS15, S3W10 and TKW17 had the closest relationship to *Rhodobacter sphaeroides* JA404 (FM177579), *R. sphaeroides* Dian 8-15 (GQ503895) and *R. sphaeroides* DBNRh12 (KJ776412) with the same 99% similarity respectively. While strain STW181 was most closely related to *Afifella marina* MS (KC205144) also with a 99% similarity.

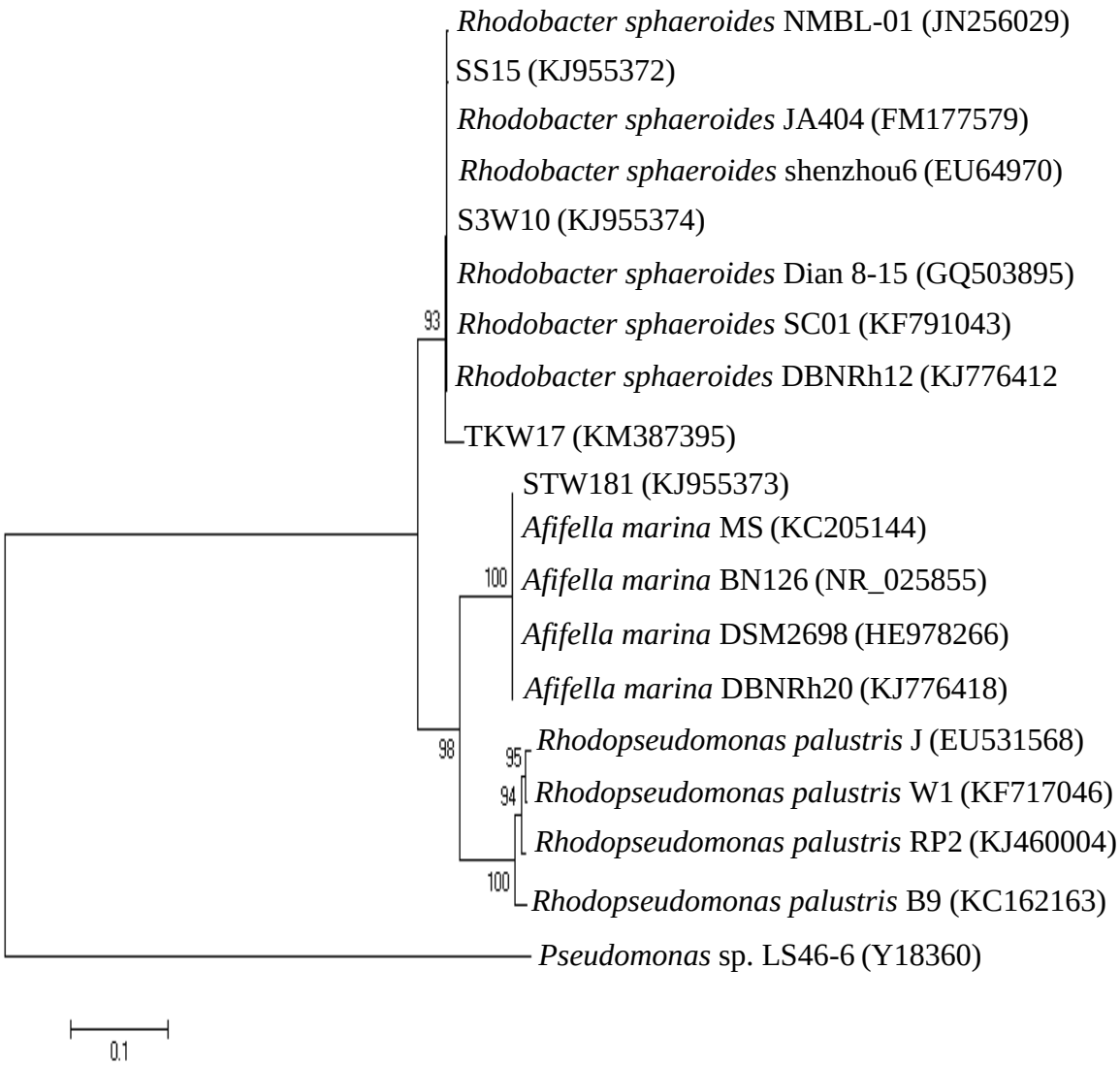


Figure 3-3. Phylogenetic tree of four selected probiotic PNSB strains, as inferred from neighbour-joining (NJ) trees based on Jukes-Cantor distances. The number on each branch indicates the bootstrap value using 1000 replications.

Effects of probiotic PNSB on digestive enzymes, shrimp growth and their survivals

Total protease, amylase and lipase activities were monitored during shrimp cultivation for 60 days to ensure the use of selected PNSB as probiotics (Table 3-5). The results demonstrate that all PNSB sets significantly improved ($P < 0.05$) compared with a control set, particularly for the protease activity. The highest protease activity was observed in the shrimp GIT of the T1 set (S3W10+SS15) from days 30 until 60, followed by T3 (S3W10+STW181) and T2 (S3W10+TKW17) sets. At day 60, the protease activity in T1, T2 and T3 sets (0.303 ± 0.027- 0.345 ± 0.014 U mg protein^{-1}) were significantly higher than the control set (0.191 ± 0.012 U mg protein^{-1}). For the amylase activity, the highest was presented in T3 set at days 45 and 60 (0.115 ± 0.009 0.249 ± 0.014 U mg protein^{-1}). However, the amylase activity of T1 and T2 sets also increased, but there were no significant differences, except at day 30 for the T1 set. The lipase activity in the shrimp GIT showed the same level of enzyme activity in all treatment sets and the control at days 0, 30 and 45. However, at 60 days of cultivation lipase activity in T2 and T1 sets (0.166 ± 0.005 and 0.162 ± 0.002 U mg protein^{-1}) significantly increased compared with the control set (0.146 ± 0.007 U mg protein^{-1}).

Viable cells of PNSB were counted throughout shrimp cultivation for 60 days to ensure that the probiotic PNSB were able to colonize in the GIT of shrimp. At day 0, PNSB could not be counted and was the same as a control set (Table 3-6). In contrast, a remarkable increase in PNSB population was found at day 30 and this regularly increased until the end of cultivation, and there was no detection of PNSB in the control set. Among the treatment sets, the highest PNSB population in the shrimp GIT was in the T3 set at days 30, 45 and 60 (3.89 ± 0.80, 4.98 ± 2.30 and 5.12 ± 1.24 Log CFU g^{-1}) although no significant difference was observed at day 45. The T1 set (5.03 ± 0.80 Log CFU g^{-1})

showed a significantly higher PNSB population than T2 set (4.50 ± 0.62 Log CFU g^{-1}) at only day 60.

The shrimp growth based on their average body weight and total length at day 60 in all PNSB sets was significantly higher than that in the control set (0.416 ± 0.034 g and 41.25 ± 1.57 mm), and the maximal was found in the T3 set (0.740 ± 0.012 g and 50.33 ± 1.54 mm) (Table 3-6). No shrimp died in all sets at day 30; however, during later cultivation, a few shrimp died in each set. Based on median values, the survival percentage at day 60 was in the degree of T1 = control (75) > T2 and T3 (58), but with no significant difference between the control and the PNSB sets throughout shrimp cultivation.

Table 3-5. Effects of probiotic PNSB strains on the digestive enzyme activities in the gastrointestinal tract of cultivated postlarval white shrimp (*Litopenaeus vannamei*).

	Control	T1	T2	T3
Protease activity (U mg protein^{-1})				
Day 0	0.164±0.009 ^{a}	0.170±0.017 ^{a}	0.168±0.030 ^{a}	0.163±0.012 ^{a}
Day 30	0.135±0.007 ^{b}	0.204±0.045 ^{a}	0.203±0.028 ^{a}	0.199±0.007 ^{a}
Day 45	0.167±0.009 ^{c}	0.286±0.003 ^{a}	0.256±0.013 ^{b}	0.267±0.011 ^{ab}
Day 60	0.191±0.012 ^{c}	0.345±0.014 ^{a}	0.303±0.027 ^{b}	0.343±0.005 ^{a}
Amylase activity (U mg protein^{-1})				
Day 0	0.030±0.004 ^{a}	0.031±0.005 ^{a}	0.032±0.003 ^{a}	0.030±0.004 ^{a}
Day 30	0.084±0.004 ^{b}	0.094±0.007 ^{a}	0.084±0.003 ^{b}	0.089±0.004 ^{ab}
Day 45	0.100±0.007 ^{b}	0.107±0.003 ^{ab}	0.106±0.001 ^{ab}	0.115±0.009 ^{a}
Day 60	0.157±0.021 ^{b}	0.181±0.024 ^{b}	0.219±0.057 ^{ab}	0.249±0.014 ^{a}
Lipase activity (U mg protein^{-1})				
Day 0	0.091±0.008 ^{a}	0.091±0.012 ^{a}	0.102±0.005 ^{a}	0.101±0.005 ^{a}
Day 30	0.089±0.003 ^{a}	0.102±0.019 ^{a}	0.095±0.003 ^{a}	0.100±0.008 ^{a}
Day 45	0.138±0.003 ^{ab}	0.147±0.004 ^{a}	0.146±0.008 ^{a}	0.136±0.004 ^{b}
Day 60	0.146±0.007 ^{b}	0.162±0.002 ^{a}	0.166±0.005 ^{a}	0.152±0.003 ^{b}

Values are presented as mean ± SD, and significant differences are analysed at $P < 0.05$. Different lowercase letters indicate significant differences for values in each row.

Table 3-6. Populations of PNSB detected in the shrimp gastrointestinal tract and the growth performance of shrimp under various treatments with probiotic PNSB.

	Control	T1	T2	T3
PNSB survival (Log CFU g^{-1}) *				
Day 0	nd	nd	nd	nd
Day 30	nd	3.58 ± 1.59^{Cb}	3.56 ± 0.71^{Cb}	3.89 ± 0.80^{Ba}
Day 45	nd	4.85 ± 0.44^{Ba}	4.79 ± 1.68^{Aa}	4.98 ± 2.30^{Aa}
Day 60	nd	5.03 ± 0.80^{Ab}	4.50 ± 0.62^{Bc}	5.12 ± 1.24^{Aa}
Weight (g) **				
Day 0	0.108 ± 0.031^{a}	0.111 ± 0.004^{a}	0.128 ± 0.001^{a}	0.136 ± 0.001^{a}
Day 30	0.186 ± 0.015^{ab}	0.177 ± 0.010^{b}	0.198 ± 0.016^{ab}	0.214 ± 0.020^{a}
Day 45	0.253 ± 0.070^{b}	0.360 ± 0.065^{a}	0.226 ± 0.025^{b}	0.241 ± 0.051^{b}
Day 60	0.416 ± 0.034^{d}	0.589 ± 0.008^{b}	0.477 ± 0.004^{c}	0.740 ± 0.012^{a}
Total length (mm) **				
Day 30	29.33 ± 1.51^{a}	29.75 ± 1.25^{a}	30.08 ± 2.82^{a}	31.25 ± 1.51^{a}
Day 45	30.08 ± 2.06^{b}	34.08 ± 1.83^{a}	30.25 ± 2.12^{b}	32.08 ± 1.53^{ab}
Day 60	41.25 ± 1.57^{d}	48.08 ± 1.28^{b}	44.25 ± 1.75^{c}	50.33 ± 1.54^{a}
Survival % **				
Day 30	100±0.0%	100±0.0%	100±0.0%	100±0.0%
Day 45	85.71±7.14%	89.29±4.12%	85.71±7.14%	78.57±4.12%
Day 60	75.00±9.62%	75.00±14.43%	58.33±19.25%	58.33±0.0%

*Values are presented as a mean ± SD, and significant differences are analysed at $P < 0.05$. Different uppercase and lowercase letters indicate significant differences for values in each column and each row respectively. nd, not detected.

†Values are presented a mean ± SD, and significant differences are analysed at $P < 0.05$. Different lowercase letters indicate significant differences for values in each row.

‡Survival percentages are presented as median ± SD because the data had a wide range of standard deviations, so they were analysed by Kruskal–Wallis test, with no significant difference at $P < 0.05$.

Challenge test with *V. parahaemolyticus* AHPND strain

After 60 days of cultivation, shrimps were challenged by *V. parahaemolyticus* strain SR2 causing AHPND with an exposure time of 10 days for monitoring the mean cumulative mortality. The mean cumulative mortality of shrimp after exposure with a virulent shrimp pathogenic strain significantly dropped ($P < 0.05$) in the PNSB sets compared with the control set (Figure 3-4). After 10 days of exposure, the mean cumulative mortality of shrimp was reduced in the order of T1 > T3 > T2 > Control. The mean cumulative mortality of shrimp in per cent was 20.83, 23.81, 32.00 and 40.00 for T1, T3, T2 and the control. In addition, the shrimp death was first found in the control set at day 3 of exposure, followed by sets of T1 and T2, and T3 at days 4 and 5 of exposure respectively.

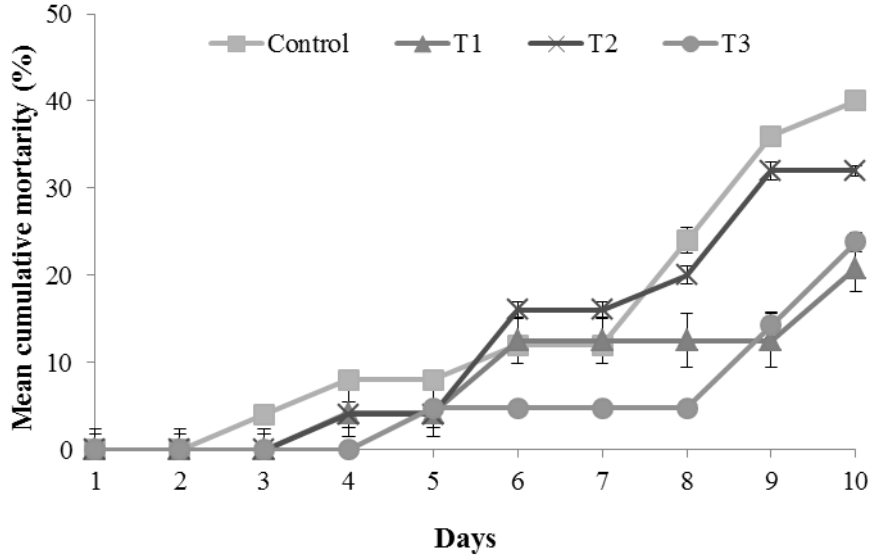


Figure 3-4. Cumulated mortality (%) in the challenge test on juvenile white shrimp (*Litopenaeus vannamei*) by *V. parahaemolyticus* SR2 (AHPND strain: accession number KT006931) for 10 days. Probiotic PNSB sets: T1 (S3W10+SS15), T2 (S3W10+TKW17), T3 (S3W10+STW181) and control (no added PNSB); values are presented as mean ± SD.

Discussion

In vitro test for probiotic properties of selected PNSB and their antivibrio activity

Purple nonsulfur bacteria are widely distributed in various aquatic habitats, such as fresh and marine water including sediments (Panwichian et al., 2010; Mukkata et al. 2015). A greater number of PNSB were isolated from sediment samples; this might be because generally shrimp ponds are shallow and allow for light to penetrate to the sediment. In addition, the sediment normally has little oxygen and this provides good growth conditions for PNSB as they grow best with anaerobic-light conditions (Nunkaew et al., 2012). One of the key properties of probiotic bacteria is their ability to produce digestive enzymes that can stimulate the digestive system of a host. There is normally a high content of protein in shrimp ponds because shrimp feed consists of 14% starch, 46% protein and 8% lipid components (Lin et al., 2004). So bacteria that produced proteases amylases and lipases would be expected to be present in their growth ponds. A comparison of the four selected PNSB strains (SS15, S3W10, TKW17 and STW181) found that strains S3W10 and SS15 were the best producers of amylase and gelatinase enzymes (Table 3-1) and strain SS15 also produced lipase. The isolates that produced only gelatinase were STW181 and TKW17 but at a much lower level than the previously mentioned isolates.

Vitamin B12 or cyanocobalamin is an important vitamin for maintaining good health of human and animal including shrimp; therefore, vitamin B12 is often added as a food supplement to stimulate the growth of various animals (Shiau and Lung, 1993). The addition of vitamin B12-producing PNSB to shrimp ponds together with an ability to produce digestive enzymes would provide benefits for shrimp growth; however, only 9% of the isolated PNSB in this study were able to produce vitamin B12. Among them strain SS15 was selected as the best vitamin B12 producer (136.50 μg mL^{-1})

(Table 3-2) and this isolate also showed high antivibrio activity (Figure 3-1A and B). Strain TKW17 and STW181 with high antivibrio activity were also selected although they produced much lower amounts of vitamin B12 at 55.37 and 43.81 μg mL^{-1}.

In this study, we tried to simulate nearly natural shrimp digestive tract (GIT) conditions by adjustment pH of pancreatic enzymes in a range of 7.2–8.3 (Omondi, 2005) for investigating survival of PNSB in the shrimp GIT. As proliferation of all selected PNSB under the simulated shrimp GIT (Table 3-3) suggests that they might have good potential as probiotics to colonize and survive in the GIT to perform positive effects on shrimp health such as providing digestive enzymes, vitamin B12 and antivibrio compounds. A similar result was reported by Zhou et al. (2007) in that *Rhodopseudomonas palustris* HZ0301 and *Rhodobacter sphaeroides* HZ0302 significantly increased the simulated small intestinal juices in the absence of bile salt and in 0.3% bile salt. Three of the four selected PNSB in this study are *R. sphaeroides* (SS15, S3W10 and TKW17). Up to date, our *Afifella marina* STW181 was the first of this genus to show for its probiotic properties for possible use in shrimp cultivation.

It is well recognized that most of the bacteria that cause pathogenic infections of shrimp are *Vibrio* spp. (Rattanachuay et al., 2010, 2011). The use of antibiotics for shrimp farming is not recommended for controlling disease (Serrano, 2005; Albuquerque et al., 2015) because of the potential to build up antibiotic resistant strains in the sediments (Defoirdt et al., 2011). Hence, this study has focused on the ability of extracellular antivibrio compounds especially from probiotic PNSB to inhibit shrimp pathogenic bacteria (*Vibrio* spp.). However, only 5.4% of the isolated PNSB showed antivibrio activity (Figure 3-1A), and this is one reason that probiotics should be used for shrimp cultivation. Strains SS15, TKW17 and STW181 were selected due to the ability

of their 15X concentrated culture supernatants to produce inhibition zones on both *V. harveyi*_KSAAHRC and *V. vulnificus*_KSAAHRC target organisms. Also there were no inhibition zone observed on both *Vibrio* spp. by a 15X concentrated uninoculated medium and a 10% methanol that was used for dissolving the concentrated culture supernatants (Figure 3-1B). These results indicate that the extracellular compounds produced by selected PNSB inhibited vibrios.

The aetiology of EMS/AHPND has been determined using Koch's postulates with four criteria and *V. parahaemolyticus* is the causative organism (Tran et al., 2013). Isolation of *V. parahaemolyticus* from diseased shrimp from local shrimp ponds, showed that only three strains or 4.1% (SR1, SR2 and SR3: KT006930, KT006931 and KT006932) were EMS/AHPND strains (Figure 3-2). It should be noted that there was a different metagenomic sequencing of the *V. parahaemolyticus* causing EMS/AHPND. This is the first report that antivibrio compounds produced by selected probiotic PNSB could inhibit eight strains of *V. parahaemolyticus* that caused EMS/AHPND with different degrees of inhibition (Table 3-4B). However, a higher dose was used to control *V. parahaemolyticus* (Table 3-4B) compared to the control of *V. harveyi* (Table 3-4A) using a 20X and 15X concentrated lyophilized supernatants respectively. This is in agreement with Rattanachuay et al. (2010) who reported that *V. parahaemolyticus* was more tolerant to antivibrio compounds than *V. harveyi*. This is perhaps one of the main reasons why shrimp farmers have been faced with the serious problem of EMS/AHPND. It is possible that our selected PNSB could be used to control this pathogen either in the form of probiotics or for the production of an extract containing antivibrio compounds from these PNSB. It is not surprising that our selected probiotic PNSB belonged to *Rhodobacter* and *Afifella* as both genera are marine PNSB and are frequently found in shrimp ponds (Mukkata et al., 2015).

Effects of probiotic PNSB on digestive enzymes, shrimp growth, survivals and challenge test with AHPND strain

As no significant differences were found at day 0, it means that during shrimp cultivation probiotic PNSB increased their digestive enzyme activities in the shrimp GIT by releasing extracellular enzymes, particularly protease because of the high protein content in shrimp feed (Table 3-5). Normally, shrimp require 30–57% protein in their feed so they need protease to break down protein into small molecules (Goddard, 1996). Among the three PNSB sets a mixed culture of strains SS15 and S3W10 (T1) was the most effective for proteolytic enzyme in the shrimp GIT. The result agreed with previous experiments that both strains had strong activity for protease activity (Table 3-1) so leading to an increased protease activity in shrimp. Activities of the amylase and lipase were not much higher in the PNSB sets this might be caused by the lower contents of starch and lipid in the shrimp feed. This study has clearly indicated that our probiotic PNSB were able to enhance digestive enzymes in the shrimp GIT.

Digestive processes and nutrient absorption including the protective functions of shrimp are related to the microbiota in the shrimp GIT (Tzuc et al., 2014). Among the microbiota in the shrimp GIT, PNSB as marine alpha proteobacteria have been detected (Johnson et al., 2008). However, in this study, no detection of PNSB in the control set was found throughout cultivation, but during shrimp cultivation, proliferation of PNSB was found in all the PNSB sets, particularly set T3 (Table 3-6). These results suggest that PNSB colonized the shrimp GIT and enhanced activities of digestive enzymes (Tables 3-5 and 6). This led to the greater shrimp growth in all PNSB sets with the biggest size found in the T3 set and the smallest size in the control (Table 3-6). However, this produced an adverse effect to cause some shrimp died in the T3 set as they were in a step of moulting and were attacked by other

shrimp. Hence, shrimp survival in this set was only 58% at day 60 compared to 79% at day 45. Fortunately, the survival of shrimp between control and all PNSB sets was not significantly different (Table 3-6); therefore, probiotic PNSB showed the possibility to be used in shrimp farming for enhancement shrimp growth.

EMS refers to an acute mortality in shrimp and the outbreaks have been extensively found in farmed pacific white shrimp (Joshi et al., 2014; Zorriehzahra and Banaederakhshan, 2015). *V. parahaemolyticus* is a causative agent of EMS /AHPND by colonizing the shrimp GIT to create a poison that causes tissue devastation and destruction of the digestive system known as the hepatopancreas (Zorriehzahra and Banaederakhshan, 2015). The protective defense against pathogens relies on the microbiota in the shrimp GIT as previously described. This could be used to explain the survival of shrimp after 10 days of exposure to a virulent *V. parahaemolyticus* SR2 causing AHPND that was in a range of 68–80% for the PNSB sets while 60% in the control (Figure 3-4). This proves that probiotic PNSB, particularly in the T1 and T3 sets did control the shrimp pathogen. Thus, it might be that both sets had a high amount of PNSB population and also maximal protease activity in the shrimp GIT (Tables 3-5 and 3-6), and strain SS15 strongly inhibited *V. parahaemolyticus* SR2 (Table 3-4B) while strain STW181 highly colonized in the shrimp GIT (Table 3-6).

Conclusions

Shrimp ponds are normal habitats for PNSB; however, only a few PNSB isolates can act as probiotics together with antivibrio activity for controlling shrimp pathogenic *Vibrio* spp. Therefore, the isolation and selection of PNSB that could be used for sustainable shrimp cultivation is important. Based by testing *in vitro* and *vivo*, it has been clearly demonstrated that *R.*

sphaeroides strains S3W10 and SS15 and *A. marina* strain STW181 are suitable isolates with great potential to facilitate shrimp growth and inhibit shrimp pathogenic vibrios including EMS/ AHPND-causing *V. parahaemolyticus*. They will now be tested in shrimp farming to help to facilitate shrimp growth.

CHAPTER 4

The roles of probiotic purple nonsulfur bacteria to control water quality and prevent acute hepatopancreatic necrosis disease (AHPND) for enhancement growth with higher survival in white shrimp (*Litopenaeus vannamei*) during cultivation

Abstract

This study aimed to investigate the potential of a mixed probiotic purple nonsulfur bacteria (PNSB) for controlling water quality and preventing acute hepatopancreatic necrosis disease (AHPND) to promote growth performance and increase survival of white shrimp (*Litopenaeus vannamei*) in postlarval stage during cultivation. Four probiotic PNSB (*Rhodobacter sphaeroides* strains SS15, S3W10, TKW17 and *Afifella marina* STW181) were investigated for controlling water quality using a mixed culture at a ratio of 1: 1 (roughly 1×10^8 cells mL^{-1} for each, every week 1-7) as follows; T1 (S3W10+SS15), T2 (S3W10+TKW17) and T3 (S3W10+STW181) and found that throughout 8 weeks shrimp cultivation the most effective sets to reduce NH_4^+ and promote shrimp growth (based on weight and total length) were T1 and T3, respectively. Hence, PNSB strains (SS15, S3W10 and STW181) were used as a mixed culture (1: 1: 1, roughly 1×10^8 cells mL^{-1} for each at weeks 1, 2 and 3) assessing their potential to prevent AHPND by challenge test on shrimp with a virulent strain AHPND causing *Vibrio parahaemolyticus* SR2 (roughly 1×10^5 cells mL^{-1} at day 15) during shrimp cultivation for 30 days. Inoculated PNSB sets (positive control: only PNSB inoculation, and treatment: both PNSB and SR2 inoculations) significantly decreased ($P < 0.05$) the levels of NH_4^+, NO_2^-, NO_3^- and chemical oxygen demand (COD) with significantly

higher ($P < 0.05$) of dissolved oxygen (DO) compared to native control (no inoculation) and challenge test (only SR2 inoculation). The maximal growth performance of shrimp was observed in the positive control; while no significant difference ($P > 0.05$) was observed for other sets. PNSB survived and colonized in intestinal shrimp tract to prevent AHPND by increasing 11% survival rate of infected shrimp by strain SR2. The correlation coefficient between PNSB population and shrimp survival showed a positive strong correlation, but a negative strong correlation between vibrios population and shrimp survival. Overall results proved that a mixed three probiotic PNSB are a good candidate for applying in white shrimp cultivation to maintain water quality and to protect shrimp diseases for promoting shrimp growth with higher survival rate.

Keywords: Acute hepatopancreatic necrosis disease, Probiotics, Purple nonsulfur bacteria, Shrimp cultivation, *Vibrio parahaemolyticus*, Water quality

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบศักยภาพของแบคทีเรียโปรไบโอติกส์แบบผสม โดยใช้แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ (Purple nonsulfur bacteria, PNSB) สำหรับควบคุมคุณภาพน้ำ และป้องกันโรคตับวายเฉียบพลันในกุ้ง (AHPND) เพื่อส่งเสริมการเจริญเติบโต และเพิ่มอัตราการอยู่รอดของกุ้งขาว (*Litopenaeus vannamei*) ในระยะวัยอ่อน (postlarvae) ของการเพาะเลี้ยง จึงนำแบคทีเรียโปรไบโอติกส์ PNSB จำนวน 4 ไอโซเลท ได้แก่ *Rhodobacter sphaeroides* SS15 S3W10 TKW17 และ *Afifella marina* STW181 มาทดสอบความสามารถในการควบคุมคุณภาพน้ำโดยใช้ PNSB 2 ไอโซเลทผสมกันในสัดส่วน 1: 1 (จำนวนอย่างละ 1×10^8 cells mL^{-1} โดยประมาณ) โดยเติม PNSB ลงในบ่อกุ้งทุกสัปดาห์ ตั้งแต่สัปดาห์ที่ 1-7 โดยมีชุดการทดลองดังนี้ T1 (S3W10+SS15), T2 (S3W10+TKW17) และ T3 (S3W10+STW181) ผลการทดลองพบว่าตลอดระยะเวลา 8 สัปดาห์ของการเลี้ยงกุ้ง ชุดการทดลองที่มีประสิทธิภาพสูงสุดในการลดปริมาณ NH_4^+ คือ ชุดการทดลอง T1 ส่วนชุดการทดลองที่สามารถส่งเสริมการเจริญเติบโตของกุ้งได้ดีที่สุด (พิจารณาจากค่าน้ำหนักตัว และความยาว) คือชุดการทดลอง T3 ด้วยเหตุนี้จึงเลือก PNSB จำนวน 3 สายพันธุ์ ได้แก่ SS15 S3W10 และ STW181 มาผสมกันในสัดส่วน 1: 1: 1 จำนวนอย่างละ 1×10^8 cells mL^{-1} โดยประมาณ โดยเติมโปรไบโอติกส์ PNSB ลงในบ่อกุ้งทุกสัปดาห์ ตั้งแต่สัปดาห์ที่ 1-3 เพื่อศึกษาศักยภาพในการป้องกันโรค AHPND ด้วยวิธีการเติมแบคทีเรียก่อโรค *Vibrio parahaemolyticus* SR2 จำนวน 1×10^5 cells mL^{-1} โดยประมาณในวันที่ 15 ของการทดลองและทำการเลี้ยงกุ้งเป็นเวลา 30 วัน โดยชุดทดลองที่เติม PNSB เพียงอย่างเดียว (positive control) และชุดที่เติม PNSB กับ SR2 (treatment set) สามารถลดปริมาณ NH_4^+, NO_2^-, NO_3^- และค่าปริมาณออกซิเจนที่ใช้ในการย่อยสลายสารอินทรีย์ในน้ำ (COD) อย่างมีนัยสำคัญ ($P < 0.05$) อีกทั้งปริมาณออกซิเจนที่ละลายในน้ำ (DO) สูงกว่าชุดควบคุมที่ไม่เติมแบคทีเรีย (native control) และชุดทดสอบที่เติมเฉพาะแบคทีเรียก่อโรค SR2 (challenge test) อย่างมีนัยสำคัญ ($P < 0.05$) นอกจากนี้ในชุดการทดลองที่มีการเติม PNSB เพียงอย่างเดียว ส่งเสริมให้กุ้งมีการเจริญเติบโตดีที่สุด แต่ไม่แตกต่างอย่างมีนัยสำคัญ ($P > 0.05$) เมื่อเทียบกับชุดอื่นๆ และยังพบว่าแบคทีเรีย PNSB สามารถอยู่รอดและตั้งถิ่นฐานภายในลำไส้กุ้งได้ส่งผลให้สามารถควบคุมแบคทีเรียก่อโรค AHPND ทำให้อัตราการอยู่รอดของกุ้ง

เพิ่มขึ้น 11% ในชุดการทดลองที่ทำให้ติดเชื้อก่อโรคด้วยสายพันธุ์ SR2 (treatment set) เมื่อวิเคราะห์ค่าสัมประสิทธิ์สหสัมพันธระหว่างจำนวน PNSB และการอยู่รอดของกุ้งพบความสัมพันธ์เชิงบวกสูง (positive strong correlation) แต่ค่าสัมประสิทธิ์สหสัมพันธ์ระหว่างจำนวน Vibrios และอัตราการรอดของกุ้งเป็นความสัมพันธ์เชิงลบสูง (negative strong correlation) ผลการศึกษาทั้งหมดแสดงว่าการผสมแบคทีเรียโปรไบโอติกส์ PNSB ทั้ง 3 ไอโซเลทมีศักยภาพที่ดีสำหรับการนำไปประยุกต์ในการเพาะเลี้ยงกุ้งขาว เพื่อรักษาคุณภาพน้ำและป้องกันการติดเชื้อจากแบคทีเรียก่อโรคกุ้ง ส่งเสริมการเจริญเติบโต และอัตราการรอดของกุ้งเพิ่มขึ้น

คำสำคัญ : โรคตับวายเฉียบพลันในกุ้ง โปรไบโอติกส์ แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ การเพาะเลี้ยงกุ้ง แบคทีเรีย *Vibrio parahaemolyticus* คุณภาพน้ำ

Introduction

Shrimp are commercially cultured using intensive shrimp farming system across the world by having a larger area in Eastern hemisphere and a smaller area in Western hemisphere (Lightner et al., 2006). The most serious economic losses in shrimp cultivation have been caused by shrimp diseases either virus or bacteria as the intensive shrimp farming always have many problems. Particularly the maintenance of water quality as resulted from high stocking densities which cause inappropriate conditions that quickly stimulate shrimp diseases (Boyd, 1990; Boyd and Fast, 1992; Biao et al., 2004). Among shrimp, *Litopenaeus vannamei* (Pacific white shrimp) is commercially the dominant species worldwide, including Thailand for avoiding white spot disease (WSD) outbreaks (Thitamadee et al., 2016). However, the most serious shrimp disease recently found in white shrimp is the early mortality syndrome (EMS), technically known as "acute hepatopancreatic necrosis disease" (AHPND) that causes by AHPND-causing *Vibrio parahaemolyticus* (Tran et al., 2013; Joshi et al., 2014). This disease normally affects postlarval shrimp within 20-35 days in early shrimp cultivation that leads to productivity losses and frequently causes up to 100% mortality (Tran et al., 2013; De Schryver et al., 2014). To solve this problem, the antibiotic agents have been applied to control pathogens in shrimp hatcheries (Zhang et al., 2011). However, this practice caused the loss of stable microbial balance, then providing empty surface for opportunistic pathogens such as *Vibrio* spp. (Xiong et al., 2016). Besides, long-term usages of antibiotics have resulted in development of the antibiotic resistance bacteria and consequently impacts on human health (Zhang et al., 2011; Singer and William-Nguyen, 2014). Therefore, the use of probiotics would be attractive in shrimp cultivation to avoid the treatment with antibiotics.

The major factor contributing to outbreaks of shrimp diseases is an inappropriate water quality during cultivation (Thakura and Lin, 2003) as the abundance of pathogenic bacteria in aquaculture are key factors for the present of shrimp diseases and are directly influenced to shrimp. As shrimp always live in rearing water the health status of shrimp depends on the environment (Xiong et al., 2016; Zhang et al., 2016). The increasing input of feed based upon the high shrimp densities in intensive farming system enhances the accumulation of residual food and fecal matter with devastating water quality to be unsuitable conditions for shrimp growth. The poor water quality directly influences on shrimp growth such as low growth rate, stress and weakness that easily stimulate pathogenic infection and consequently high mortality (Lightner et al., 2006). Better water quality management is recommended by increasing the decomposition of organic waste mainly protein that results in increasing concentrations of nitrogen and phosphorus in shrimp ponds (Boyd and Gross, 1998; Nimrat et al., 2012). Hence, a sufficient amount of oxygen in the water column of shrimp ponds is required for eliminating of toxic ions such as ammonium and nitrite by oxidation to nitrate using nitrifying bacteria. Nitrate is nontoxic form to shrimp; however, if the concentration of nitrate is higher than 50 mg L^{-1}, the water must be discharged (Chuntapa et al., 2003) as it causes algal bloom. In addition, rearing water after shrimp harvesting should be treated before discharge to protect the water quality and the environment in the surrounding area of shrimp farms (Thuyet et al., 2012).

Regarding to the information above the alternative way of using probiotics has been increased as the probiotics are live microorganisms so administration them in adequate amounts confer beneficial health on host (Sanders, 2008). They are effective microorganisms which provide many advantages to their host by the improvement of the digestive enzymatic activity, enhancement of immune response, and production of antimicrobial compounds against pathogenic bacteria (Verschuere et al., 2000; Balcazar et

al., 2006; Nimrat et al., 2012). In addition to produce safe shrimp, the use of probiotics also supports the ecological production as of no use of chemicals or antibiotics; this would achieve organic shrimp farming for sustainable shrimp cultivation. Among probiotics, purple nonsulfur bacteria (PNSB) are one of probiotic candidates that not only act as probiotics (Chumpol et al., 2017a) but also clean up water during shrimp cultivation (Qi et al., 2009; Luo et al., 2012). This leads to promote growth performance of shrimp and also shrimp survival.

PNSB are normally found in natural water including shrimp ponds as they have versatile growth modes such as photoautotroph or photoorganotroph under anaerobic-light or microaerobic-light conditions and heterotroph under aerobic-dark conditions (Panwichian et al., 2010; Mukkata et al., 2016). Intensive studies on the application of PNSB to treat various wastewaters have been reported (Luo et al., 2012; Kornochalert et al., 2014); however, there is still less application for use as probiotics to prevent shrimp diseases like AHPND and also cleaning water in shrimp cultivation. Our recent publication revealed that *Rhodobacter sphaeroides* strains SS15, S3W10, TKW17 and *Afifella marina* strain STW181 are outstanding strains promoting shrimp growth in early juvenile stage and also increasing shrimp survival after challenging with a virulent AHPND strain (Chumpol et al., 2017a). It would be worth to explore these PNSB strains for shrimp cultivation, particularly in postlarval stage which is the most sensitive to shrimp diseases like AHPND. Therefore, this study aimed to evaluate the efficiency of a mixed PNSB strains on the basis of controlling water quality and AHPND for promoting growth including the increasing survival of *L. vannamei* in postlarval stage during cultivation.

Materials and methods

Probiotics strains and cultivation

In this study probiotic PNSB, *R. sphaeroides* (SS15, S3W10, and TKW17) and *A. marina* STW181, isolated from shrimp ponds in the south of Thailand (Chumpol et al., 2017a) were used. Each PNSB strain was separately cultured in duran bottles containing 500 mL Glutamate-Acetate medium (GA) with supplementation of 2% NaCl and incubated under microaerobic-light conditions with tungsten light at 3500 lux for 48 h. Each culture broth was centrifuged at 10418 x *g* for 10 min (Sorval, RC 5C plus, USA) to obtain a cell pellet; and it was washed three times with 0.85% normal saline solution (NSS). Then, each pellet was re-suspended in NSS to adjust the cell density measured using a spectrophotometer at a wavelength of 660 nm for obtaining OD_{660} at 1.0 (roughly 10^{10} cells mL^{-1}) to use as a probiotic in white shrimp cultivation.

Shrimp cultivation and experimental designs

Experiment on water quality and shrimp growth

L. vannamei or white shrimp in the postlarval stage for 19 days (PL-19) was used to investigate the role of PNSB used on water quality in shrimp cultivation. According to the successful previous study which obtained 4 probiotic PNSB and used them as a two mixed culture (Chumpol et al., 2017a); however, we did not study their ability for controlling water quality that is one of its vital factors for shrimp growth including shrimp's diseases. Therefore, three treatment sets were designed by using a mixed culture (1:1) based on their abilities to inhibit vibrios, particularly AHPND strains and promote shrimp growth as follows; T1 (SS15 and S3W10), T2 (TKW17 and S3W10) and T3 (STW181 and S3W10) with a set without inoculation was served as a control. All shrimp aquaria were made from glass with a size of 3.6 L capacity (12.5 cm × 12.5 cm × 25.5 cm), and filled with 2.5 L commercial artificial sea water

(ASW) which adjusted to an initial pH of 7.8, an initial salinity of 15 ppt and awater temperature in a range of 25 and 27 °C depending on ambient temperature.

Shrimp were acclimated for 3 days with commercial ASW; and they were fed each time roughly 2% of total body weight with commercial shrimp feed for four times per day (7:00, 12:00, 16:00 and 20:00). To follow intensive cultivation (shrimp stocking 500-1000 postlarval m^{-2}) 20 shrimp were randomly divided into each aquarium (627 postlarval m^{-2}) for 1 week; and at week 1 each PNSB strain was inoculated into the rearing water at a final concentration of 10^8 cells mL^{-1} for 3 treatment sets as previously described. Each PNSB strain was inoculated as the same cell density into the rearing water every week until week 7. Aeration was provided throughout cultivation; and the rearing water was changed every week with 50% exchange. This experiment was carried out for 8 weeks with three replicates and parameters that reflect to water quality were monitored every week during shrimp cultivation. In addition, shrimp growth including shrimp survival was observed.

Experiment on water quality, shrimp growth and challenge test

Regarding the results of the first experiment a mixed three PNSB strains (SS15, S3W10 and STW181) was designed to know the roles of PNSB on water quality and anti-AHPND causing *V. parahaemolyticus* for stimulating shrimp growth in white shrimp postlarvae-15 stage (PL-15). Experimental design consisted of native control, challenge test (negative control), treatment and positive control. No addition of any probiotics or AHPND strains was a native control; while only AHPND-causing *V. parahaemolyticus* strain SR2 was inoculated at day 15 of shrimp cultivation was a challenge test. Only a mixed PNSB was inoculated for a positive control and both inoculations of strain SR2 and a mixed PNSB for a treatment set. This experiment was designed based on the infectious period of AHPND strains in the white shrimp

postlarval (30-45 stages) that were sensitive to AHPND strains during the first 15-35 days in grow-out ponds (FAO, 2013). The experiment was performed in a bigger size of glass aquarium (25 cm × 20 cm × 15 cm, and 7.5 L capacity) and each aquarium containing 4 L of ASW with an aerator as the same with the previous experiment. A forty shrimp (800 postlarvae m^{-2}) were randomly divided into each aquarium and shrimp was fed with the same protocol as previously mentioned and no changing of water throughout shrimp cultivation. This is because fluctuation of water quality from the previous experiment and also to investigate the efficiency of PNSB used.

Sets of positive and treatment were first inoculated at week 1 of a mixed three PNSB to obtain a final cell density of 10^8 cells mL^{-1} for each strain and further inoculations at weeks 2 and 3. Additionally, the treatment set was inoculated with strain SR2 only one time at cultivation day 15 (postlarval-30) for mimicking the infectious situation in the postlarval white shrimp. *V. parahaemolyticus* strain SR2 was grown in tryptic soy broth (TSB) supplemented with 1.5% NaCl) and incubated in a shaker at 150 rpm min^{-1}, 35 °C for 18 h, and centrifuged to obtain cell pellets. The cell density was adjusted to 1 × 10^8 cells mL^{-1} (OD_{660nm} = 0.4) and diluted with NSS for inoculation to obtain a final cell density of approximately 1 × 10^5 cells mL^{-1} in rearing water in sets of challenge and treatment as previously mentioned. This cell density was considered as an infectious dosage of AHPND-causing *V. parahaemolyticus* during shrimp cultivation that were infected in the range of 10^3-10^6 CFU mL^{-1} (Joshi et al., 2014). The percentage of shrimp cumulative mortality was determined daily from day 15 until the end of the experiment. This experiment was conducted for 30 days with three replicates; and water quality, bacterial count in water and shrimp including shrimp growth and their survival were monitored every week.

Water quality analysis in shrimp cultivation

Above both experiments, water samples were used to investigate the role of probiotic PNSB on water quality during white shrimp cultivation in both systems between 50% water exchange and without changing water. To investigate water quality, the following parameters including pH, salinity, temperature and dissolved oxygen (DO) were measured daily using pH meter (Sartorius AG, Germany), salinometer (Salinity Refractometer, Japan), thermometer and portable DO meter (SevenGo Pro, Mettler Toledo, Switzerland), respectively. In addition, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and chemical oxygen demand (COD) were determined using Spectroquant® photometric test kit (Merck, Germany) at the beginning of experiments and every week until the end of cultivation. These test kits are analogous with standard methods, including NH_4^+ (No. 1.14752.0001, EPA 350.1, APHA 4500-NH3 D), NO_2^- (No. 1.14776.0001, EPA 354.1, APHA 4500-NO_2^- B), NO_3^- (No. 1.14773.0001, APHA 4500 NO_3^- B) and COD (No.1.14540.001, EPA 4.10.4, APHA 5220 D). For COD measurement, NaCl in each water sample was eliminated by adding silver nitrate ($AgNO_3$) to form white precipitate (silver chloride; AgCl). Then, the clear solution was added into COD test kit to measure COD level.

Shrimp growth performance

Shrimp growth was observed for the first experiment; however, shrimp growth (body weight in g and total length in mm) was measured at only the end of experiment (shrimp in a stage of early juvenile) as the main aim to investigate the role of the PNSB used on the water quality. For the second experiment, shrimp growth was recorded every week from postlarval-15 until postlarval-45 stages by random sampling 6 shrimp (2 shrimp × 3 replicates /set /time). Body weight and total length were measured by a digital balance (Denver Instruments TB-214, New York) and vernier caliper, respectively.

Length gain, weight gain and specific growth rate (SGR) were calculated based on the following formulas; length gain (%) $=\left(\frac{Lt-Li}{Li}\right) x\ 100$, weight gain (%) = $\left(\frac{Wt-Wi}{Wi}\right) x\ 100$, SGR = $\left(\frac{In\ Wt-In\ Wi}{time}\right) x\ 100$, where Lt, Li represent the length at sampling and initial length, Wt, Wi represent the weight at sampling and initial weight, and time represent the cultivation day. These shrimp samples were also used to enumerate bacteria (*Vibrio* spp. and PNSB). In addition, the case of dying shrimp was recorded.

Enumeration of bacteria in rearing water and shrimp

In order to know the role of these bacteria on shrimp growth for the second experiment parameters of water quality and shrimp growth were recorded weekly. Samples of water and shrimp were used to count viable cells of *Vibrio* spp. and PNSB. The whole 6 shrimp for 1 set in each sampling time were grounded in 0.85% NSS and diluted by ten-fold serial dilutions; and the appropriate dilutions were used to count bacterial populations. Spread plate technique was used to count *Vibrio* spp. on thiosulfate citrate bile salt sucrose agar (TCBS) (Merck, Germany) and incubated at 35 °C for 24 h; while PNSB using pour plate technique on GA medium with addition of 2.0% NaCl and incubated under aerobic-dark conditions for 3 days as the PNSB used in this study showed red or pink color depending on their species.

Bacterial colonization in intestinal shrimp using scanning electron microscope (SEM)

For the second experiment the presence and colonization of bacteria in the intestinal tract of white postlarval-45 days from all experimental sets were investigated by scanning electron microscopy (SEM). The intestinal shrimp samples at day 30 of cultivation were separated by cross-section with sharp knives and immersed in 2.5% cold glutaraldehyde overnight. After that, these samples were removed from glutaraldehyde and washed three times with 0.1 M

phosphate buffer solution (PBS, pH 7.4) for 30 min in each step, then dehydrated samples two times with serial concentrations of ethanol (50%, 60%, 70%, 80%, 90% and 100% ethanol) at room temperature for 15 min in each step. Finally the samples were dried in a critical-point dryer (CPD) and demonstrated on specimen stub, coated with gold and observed under SEM (FEI Quanta 400, FEI company, USA) by following the instruction of manufacturing.

Statistical analysis

All data were conducted with three replicates unless otherwise stated and expressed as mean ± standard deviation (S.D.) or standard error of mean (S.E.). The one-way analysis of variance (ANOVA) was used to analyze the significant differences between the mean of data more than two sets at level of $P < 0.05$ and the pair-wise comparison were performed using the Duncan's multiple-range test (DMRT). However, the shrimp survival (%) was analyzed using Kruskal-Wallis test because the data was not in normal distribution. All statistics were analyzed using SPSS program version 11.5 (Lead Technologies, USA). Correlation and regression between the number of bacteria and shrimp survival (%) were analyzed by Pearson's product moment correlation coefficient using Microsoft Excel 2013; and the correlations were considered statistically significant at $P < 0.05$. Correlation coefficient (r) was used for indicating linear estimations of the strength and direction of the linear correlations between two parameters (shrimp survival and vibrios/ PNSB population) as “r” is always between −1 and +1 that means a perfect correlation, while 0 means the absence of a relationship (Kornboonraksa and Lee, 2009). Hence, r values < 0.4, 0.60–0.67 and 0.68–1.0 are taken to interpret weak, moderate and high correlations between the two parameters, respectively (Taylor, 1990).

Results

Water quality in shrimp cultivation

In experiment 1, over 8 weeks of shrimp cultivation with 50% water exchange there was no significant difference ($P > 0.05$) in temperature (25-26 °C) and salinity (15-17 ppt) in the rearing water of control and treatment sets (Table 4-1). However, values of DO (7.47 – 8.04 mg L^{-1}) and pH (7.53-8.14) significantly increased ($P < 0.05$) in treatment sets (Table 4-1). In contrast, the amount of NH_4^+ fluctuated throughout shrimp cultivation and at the end of the experiment the lowest concentration of NH_4^+ was found in T1 set (1.22 ± 0.02 mg L^{-1}) while the highest concentration was found in the control set (3.63 ± 0.02 mg L^{-1}) and lower concentrations in T2 and T3 sets (3.03 ± 0.02 to 3.13 ± 0.02 mg L^{-1}) (Figure 4-1A). The amount of NO_2^- increased along with cultivation times, in all sets, particularly the control set; and the highest NO_2^- levels were found at week 8 in the control (0.109 ± 0.003 mg L^{-1}) and in all treatment sets in a range of (0.087 ± 0.006 to 0.097 ± 0.005 mg L^{-1}) (Fig. 4-1B). A similar pattern for levels of NO_2^- and NO_3^- although NO_3^- levels dropped at week 3 in all sets; and the maximal NO_3^- levels were found at week 8 in the control set (4.23 ± 0.06 mg L^{-1}) followed by T1 set (4.13 ± 0.06 mg L^{-1}), T3 set (3.80 ± 0.10 mg L^{-1}) and T2 set (3.40 ± 0.10 mg L^{-1}) (Figure 4-1C). All sets produced a similar change of COD levels as not much change for 7 weeks cultivation and significantly increased ($P < 0.05$) at week 8 by the highest in the control set (57.60 ± 4.73 mg L^{-1}) followed by T1 set (42.27 ± 4.57 mg L^{-1}), T3 set (44.93 ± 5.83 mg L^{-1}) and the lowest in T2 set (38.53 ± 1.01 mg L^{-1}) (Figure 4-1D).

Table 4-1. Monitoring parameters of water quality during postlarval white shrimp (*L. vannamei*) cultivation for 8 weeks (1st experiment).

Parameter	Control	T1 (S3W10+SS15)	T2 (S3W10+TKW17)	T3 (S3W10+STW181)
DO (mg L^{-1})				
Week 0	7.73[a]	7.73[a]	7.76[a]	7.73[a]
Week 1	7.59[b]	7.71[a]	7.68[a]	7.69[a]
Week 2	7.60[b]	7.72[a]	7.75[a]	7.74[a]
Week 3	7.59[b]	8.04[a]	8.00[a]	8.01[a]
Week 4	7.47[c]	7.81[a]	7.71[ab]	7.66[b]
Week 5	7.56[b]	7.77[a]	7.87[a]	7.83[a]
Week 6	7.47[b]	7.68[a]	7.80[a]	7.67[a]
Week 7	7.63[b]	7.73[a]	7.72[a]	7.79[a]
Week 8	7.59[b]	7.69[a]	7.76[a]	7.74[a]
pH				
Week 0	7.53[a]	7.54[a]	7.54[a]	7.55[a]
Week 1	7.88[b]	8.05[a]	8.04[a]	8.00[a]
Week 2	7.84[b]	7.99[a]	7.98[a]	8.04[a]
Week 3	7.83[b]	8.02[a]	7.98[a]	7.98[a]
Week 4	7.65[b]	7.78[a]	7.87[a]	7.87[a]
Week 5	7.70[b]	8.11[a]	8.10[a]	8.14[a]
Week 6	7.58[b]	7.83[a]	7.91[a]	7.91[a]
Week 7	7.71[b]	7.95[a]	7.97[a]	7.90[a]
Week 8	7.67[b]	7.84[a]	7.89[a]	7.86[a]
Salinity (ppt)				
Week 0	15.0[a]	15.7[a]	15.3[a]	15.7[a]
Week 1	16.0[a]	16.3[a]	15.7[a]	16.3[a]
Week 2	17.3[a]	17.0[a]	16.3[a]	16.3[a]
Week 3	16.7[a]	16.7[a]	16.0[a]	16.3[a]
Week 4	16.0[a]	15.7[a]	16.3[a]	16.0[a]
Week 5	16.7[a]	17.0[a]	16.7[a]	17.3[a]
Week 6	16.3[a]	16.3[a]	16.0[a]	15.7[a]
Week 7	16.0[a]	15.7[a]	15.3[a]	16.0[a]
Week 8	16.7[a]	16.3[a]	16.3[a]	16.0[a]
Temperature (°C)				
Week 0	26.23[a]	26.17[a]	26.03[a]	25.93[a]
Week 1	25.93[a]	25.97[a]	25.87[a]	25.73[a]
Week 2	24.93[a]	24.80[a]	24.67[a]	24.67[a]
Week 3	25.47[a]	25.37[a]	25.37[a]	25.43[a]
Week 4	24.37[a]	24.23[a]	23.97[a]	24.03[a]
Week 5	25.07[a]	25.13[a]	25.09[a]	25.03[a]
Week 6	26.03[a]	26.01[a]	25.93[a]	25.90[a]
Week 7	25.83[a]	25.63[a]	25.70[a]	25.70[a]
Week 8	25.89[a]	25.93[a]	25.91[a]	25.85[a]

Values are presented as a mean ± S.D. (n = 3) and different lowercase letters indicate significant differences in each row at $P < 0.05$.

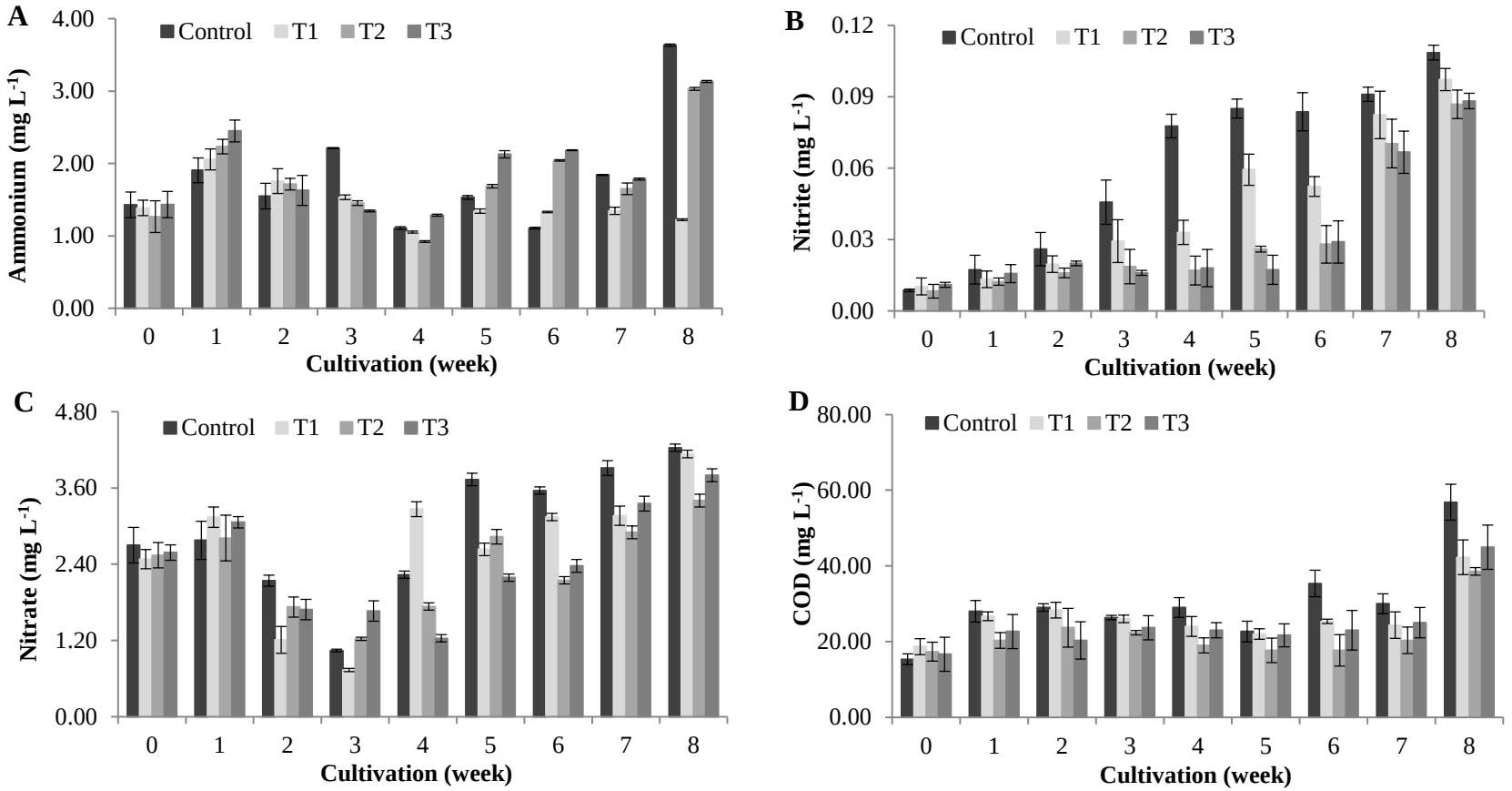


Figure 4-1. Effect of a two mixed probiotic PNSB (1: 1) on water quality; (A) NH_4^+, (B) NO_2^-, (C) NO_3^- and (D) COD in *L. vannamei* cultivation with 50% water exchange. A mixed probiotic PNSB; T1 (SS15+S3W10, T2 (TKW17+S3W10) and T3 (STW181+S3W10) and uninoculated set served as control. Error bars represent standard deviation of three determinations.

In experiment 2 without changing of water, no significant difference was found ($P > 0.05$) for salinity (15-17 ppt) and temperature (24-25 °C) in all experimental sets throughout shrimp cultivation (Table 4-2). However, in all experimental sets the DO value significantly decreased ($P < 0.05$) while pH, COD, NH_4^+, NO_2^- and NO_3^- significantly increased ($P < 0.05$) (Figure 4-2). For almost all parameters with exception of DO and pH, the values in sets of native control and challenge test were significantly higher than ($P < 0.05$) in inoculated PNSB sets (positive and treatment). During 4 weeks cultivation, the increasing of pH was observed with a higher pH found in both inoculated PNSB sets (7.50-8.25) than in sets of native control and challenge test (7.50 - 8.00); however, this was converse with NH_4^+ levels (Figure 4-2A). DO values (mg L^{-1}) were in order of the following sets; positive control (7.55 ± 0.02) > treatment (7.48 ± 0.04) > native control (7.31 ± 0.06) > challenge test (7.26 ± 0.05); and these were reverse to COD levels in their sets (Figure 4-2B). A sharp increase of COD, NH_4^+, NO_2^- and NO_3^- started at week 2 and continued until week 4; and the least of these values found in both inoculated PNSB sets at week 4 had COD (22.33 ± 3.06 mg L^{-1}) (Figure 4-2B), NH_4^+ (roughly 7.25-8.30 mg L^{-1}) (Figure 4-2A), NO_2^- (1.81 ± 0.09 mg L^{-1}) (Figure 4-2C) and NO_3^- (6.10 ± 0.01 mg L^{-1}) (Figure 4-2D).

Table 4-2. Changes of salinity and temperature of rearing water during postlarval white shrimp (*L. vannamei*) cultivation for 30 days (2nd experiment).

Time (week)	Salinity (ppt)				Temperature (°C)			
	Native control	Challenge test	Treatment	Positive control	Native control	Challenge test	Treatment	Positive control
0	15.33[a]	15.33[a]	15.00[a]	15.33[a]	25.03[a]	25.00[a]	24.97[a]	24.90[a]
1	16.00[a]	16.33[a]	15.00[a]	15.67[a]	25.07[a]	25.03[a]	25.07[a]	25.03[a]
2	16.33[a]	16.00[a]	16.00[a]	15.67[a]	24.10[a]	24.23[a]	24.20[a]	24.13[a]
3	17.00[a]	16.33[a]	16.67[a]	16.33[a]	24.23[a]	24.27[a]	24.30[a]	24.30[a]
4	17.33[a]	17.00[a]	16.67[a]	17.00[a]	24.07[a]	24.10[a]	24.10[a]	24.03[a]

Values are presented as a mean ± S.D. (n = 3) and all data are not significantly different in each row at $P > 0.05$. Native control (uninoculted set), challenge test (strain SR2 inoculation, roughly 1 x 10^5 cells mL^{-1} at day 15), treatment (inoculations of a mixed three culture PNSB and strain SR2) and positive control (PNSB inoculation, roughly 1 x 10^8 cells mL^{-1} for each at weeks 1, 2 and 3).

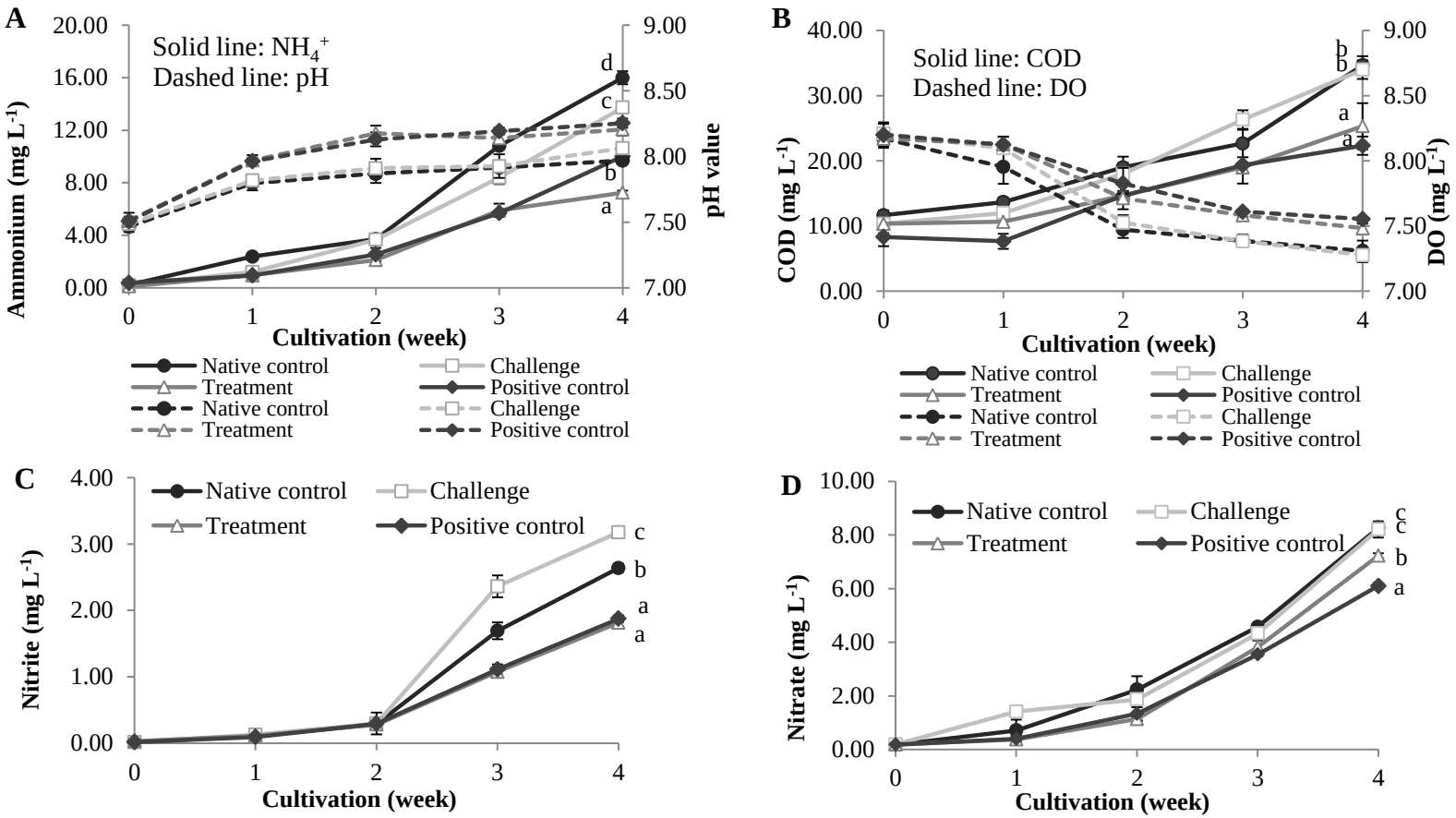


Figure 4-2. Effect of a three mixed probiotic PNSB (1: 1: 1) on water quality; (A) NH_4^+, pH; (B) COD, DO; (C) NO_2^-; and (D) NO_3^- in *L. vannamei* cultivation without water exchange. Native control (no inoculation), Challenge (inoculated strain SR2 at day 15), Positive control (PNSB inoculation at weeks 1, 2 and 3) Treatment (PNSB and SR2 inoculations). Error bars represent standard deviation of three determinations.

Shrimp growth

At the end of cultivation for the experiment 1, the shrimp growth based on their size and weight significantly increased ($P < 0.05$) in all treatment sets compared to a control as in the degree of T3 > T1 > T2 > control (see details in Table 4-3). However, no significant difference ($P > 0.05$) was found for shrimp survival among treatment and control sets (Table 4-3). For the experiment 2 as no significant differences ($P > 0.05$) in weight (g) and total length (mm) of shrimp at starting the experiment implied that the significant differences ($P < 0.05$) of the results should be governed by inoculating a mixed three probiotic PNSB (weeks 1, 2 and 3) and/or *V. parahaemolyticus* strain SR2 at day 15 of cultivation (Table 4-4). No significant difference ($P > 0.05$) for shrimp growth was observed in all sets for the first 2 weeks of cultivation. However, at week 3, shrimp growth in sets of positive (inoculated PNSB), treatment (inoculated PNSB and SR2) and native (no inoculation) were significantly higher ($P < 0.05$) than in a challenge test (inoculated SR2). At week 4, the shrimp in the positive control had the largest size (0.136 ± 0.027 g, 29.33 ± 2.30 mm), a medium size in treatment set (0.104 ± 0.005 g, 26.67 ± 1.88 mm) and native control (0.118 ± 0.027 g, 26.42 ± 3.37 mm), and the smallest size in challenge test (0.094 ± 0.006 g, 25.42 ± 2.60 mm). The results also showed significant increases ($P < 0.05$) in length gain, weight gain and SGR in the positive control compared to challenge and treatment sets. Growth performance in the treatment and native sets was higher than in the challenge test although no significant difference ($P > 0.05$).

Table 4-3. Effect of a mixed two probiotic PNSB (1: 1 at roughly 1 x 10^8 cells mL^{-1} for each for every weeks 1-7) on the growth of postlarval white shrimp (*L. vannamei*) and their survival at week 8 of cultivation.

Experimental set	Weight (g)	Total length (mm)	Shrimp survival (%)
Control	0.363 ± 0.028^{d}	38.27 ± 1.58^{c}	71.43 ± 4.12^{a}
T1 (S3W10 + SS15)	0.537 ± 0.012^{b}	44.35 ± 0.97^{a}	76.19 ± 4.76^{a}
T2 (S3W10 + TKW10)	0.412 ± 0.005^{c}	40.52 ± 1.05^{b}	73.81 ± 2.38^{a}
T3 (S3W10 + STW181)	0.601 ± 0.020^{a}	45.47 ± 1.14^{a}	69.05 ± 2.38^{a}

Each value is presented as a mean of 6 replicates (2 x 3) ± S.D. (standard deviation). Different lowercase letters indicate significant differences in each column at $P < 0.05$. Shrimp survival is presented as a mean of 6 replicates (2 x 3) ± S.E. (standard error of mean) and this data were analyzed by Kruskal-Wallis test, which no significant difference at $P > 0.05$.

Table 4-4. Influence of a mixed probiotic PNSB (SS15, S3W10 and STW181; 1: 1: 1 at roughly 1 x 10^8 cells mL^{-1} for each) inoculations at weeks 1, 2, and 3 on growth performance of *L. vannamei* in chalenge test with *V. parahaemolyticus* SR2 (1 X 10^5 cells mL^{-1} at day 15) during cultivation for 30 days.

Cultivation (week)	Native control (Uninoculated)	Challenge test (Inoculated SR2)	Treatment (Inoculated SR2+PNSB)	Positive control (Inoculated PNSB)
Weight (g)				
0	0.011 ± 0.001^{a}	0.011 ± 0.001^{a}	0.011 ± 0.001^{a}	0.011 ± 0.001^{a}
1	0.055 ± 0.005^{a}	0.053 ± 0.005^{a}	0.052 ± 0.007^{a}	0.056 ± 0.005^{a}
2	0.086 ± 0.005^{a}	0.090 ± 0.006^{a}	0.085 ± 0.009^{a}	0.090 ± 0.007^{a}
3	0.107 ± 0.009^{a}	0.096 ± 0.009^{b}	0.107 ± 0.005^{a}	0.108 ± 0.002^{a}
4	0.118 ± 0.027^{ab}	0.094 ± 0.006^{c}	0.104 ± 0.005^{bc}	0.136 ± 0.027^{a}
Total length (mm)				
0	9.08 ± 0.74^{a}	8.92 ± 0.58^{a}	8.67 ± 0.49^{a}	8.83 ± 0.82^{a}
1	17.17 ± 1.97^{a}	16.67 ± 2.23^{a}	16.67 ± 2.27^{a}	17.33 ± 1.99^{a}
2	21.25 ± 2.84^{a}	21.33 ± 2.04^{a}	21.25 ± 2.64^{a}	21.42 ± 4.02^{a}
3	26.25 ± 2.97^{a}	23.67 ± 2.42^{b}	26.58 ± 1.50^{a}	27.00 ± 2.20^{a}
4	26.42 ± 3.37^{ab}	25.42 ± 2.44^{b}	26.67 ± 1.88^{ab}	29.33 ± 2.30^{a}
Length gain (%)				
at week 4	190.38 ± 0.10^{b}	188.25 ± 0.25^{b}	199.53 ± 0.16^{b}	228.32 ± 0.29^{a}
Weight gain (%)				
at week 4	960.53 ± 1.56^{ab}	762.38 ± 0.66^{c}	849.89 ± 1.24^{bc}	1107.01 ± 1.48^{a}
Specific growth rate (SGR; %)				
at week 4	8.39 ± 0.01^{ab}	7.69 ± 0.00^{c}	8.13 ± 0.00^{bc}	8.87 ± 0.00^{a}

Values ± S.D. are presented as a mean of 6 replicates (2 x 3). Different lowercase letters indicate significant differences for values in each row at $P < 0.05$.

Bacterial populations in rearing water and shrimp

Bacterial populations (PNSB and *Vibrio* spp.) in samples of rearing water and shrimp for each experimental set are shown in Table 4-5. The PNSB population in samples of water and shrimp were found only in sets of treatment and positive control at week 1 until the end of experiment. PNSB population in water and shrimp samples of both sets significantly increased ($P < 0.05$) along with cultivation period with roughly 1 Log cycle (weeks 1- 4). In both sets the PNSB population in samples of water and shrimp was similar for each sampling time i.e. at weeks 1 and 4 were roughly 2.8 and 3.8 Log CFU mL^{-1} for water/ Log CFU g^{-1} for shrimp, respectively. The water samples of each experimental set showed significant differences ($P < 0.05$) for vibrios population. Particularly at week 4 their population (Log CFU mL^{-1}) was in the degree of positive set (2.86 ± 0.03) similar to the native set (3.07 ± 0.03), but lower than the treatment set (3.82 ± 0.05), and the challenge set (3.97 ± 0.04). In the case of shrimp samples, the *Vibrio* spp. in each experimental set was found at the starting of cultivation and significantly increased ($P < 0.05$) at week 1, then slightly increased until the end of cultivation. Vibrios population at week 3 in treatment and positive sets (5.02-5.04 Log CFU g^{-1}) was significantly lower ($P < 0.05$) than in challenge set (5.10 Log CFU g^{-1}). Later on, vibrios population in the positive control set (5.04 ± 0.07 Log CFU g^{-1}) was significantly lower ($P < 0.05$) than the native control set (5.12 ± 0.09 Log CFU g^{-1}).

The bacterial populations in the intestine of shrimp at day 30 of cultivation were observed by SEM. The results revealed that in shrimp of the native control set found various bacterial shapes such as spherical and rod (Figure 4-3A); while in the challenge set was found only rod-shaped like *V. parahaemolyticus* where SR2 was inoculated (Figure 4-3B). On the other hand, inoculations of both PNSB and SR2 in treatment set or only PNSB in

positive set mainly found ovoid shape; and also for some cells with ovoid shape showed binary fission on the inner surface of shrimp intestinal tract (Figure 4-3C and D). These results proved that probiotic PNSB strains used in this study were able to colonize and proliferate in the intestinal tract of shrimp.

Table 4-5. Influence of a mixed probiotic PNSB (SS15, S3W10 and STW181; 1: 1: 1 at 1 x 10^8 cells mL^{-1}) on bacterial populations in chalenge test with *V. parahaemolyticus* SR2, roughly 1 x 10^5 cells mL^{-1} at day 15, during shrimp cultivation for 30 days.

		Viable bacterial count (Log CFU mL^{-1}, Log CFU g^{-1})			
	Cultivation (week)	Native control (Uninoculated)	Challenge test (Inoculated SR2)	Treatment (Inoculated SR2+PNSB)	Positive control (Inoculated PNSB)
Vibrio spp.	0	nd	nd	nd	nd
in water	1	2.56 ± 0.07 ^{Ac}	2.60 ± 0.05 ^{Ac}	2.55 ± 0.09^{Ab}	2.62 ± 0.04 ^{Ab}
sample	2	2.89 ± 0.03 ^{Bb}	3.08 ± 0.07 ^{Ab}	2.72 ± 0.08 ^{BCb}	2.65 ± 0.19 ^{Cb}
	3	2.92 ±0.06 ^{Ab}	3.00 ± 0.04 ^{Ab}	2.93 ± 0.03 ^{Ab}	2.72 ±0.13 ^{Bab}
	4	3.07 ± 0.03^{Ca}	3.97 ± 0.04 ^{Aa}	3.82 ± 0.05 ^{Ba}	2.86 ± 0.03^{Ca}
PNSB in	0	nd	nd	nd	nd
water sample	1	nd	nd	2.89 ± 0.06 ^{Ad}	2.79 ± 0.05 ^{Ad}
	2	nd	nd	3.22 ± 0.01 ^{Ac}	3.26 ± 0.03 ^{Ac}
	3	nd	nd	3.54 ± 0.10^{Ab}	3.48 ± 0.05^{Ab}
	4	nd	nd	3.82 ± 0.07^{Aa}	3.78 ± 0.08^{Aa}
Vibrio spp.	0	4.18 ± 0.04 ^{Ac}	4.21 ± 0.03 ^{Ad}	4.17 ± 0.03 ^{Ad}	4.20 ± 0.02 ^{Ac}
in shrimp	1	4.97 ± 0.08 ^{ABb}	5.01 ± 0.06 ^{Ac}	4.87 ± 0.06^{Bc}	4.92 ± 0.06 ^{ABb}
sample	2	5.04 ± 0.07 ^{Aab}	5.03 ± 0.04 ^{Ac}	4.92 ± 0.03 ^{Ac}	4.98 ± 0.07^{Aab}
	3	5.06 ± 0.02 ^{Bab}	5.10 ± 0.05^{Ab}	5.04 ± 0.06 ^{Bb}	5.02 ± 0.03 ^{Ba}
	4	5.12 ± 0.09 ^{Ba}	5.22 ± 0.04^{Aa}	5.19 ± 0.05^{Aa}	5.04 ± 0.07^{Ca}
PNSB in	0	nd	nd	nd	nd
shrimp	1	nd	nd	2.87 ± 0.08 ^{Ad}	2.77 ± 0.09^{Ad}
sample	2	nd	nd	3.19 ± 0.03 ^{Ac}	3.16 ± 0.05^{Ac}
	3	nd	nd	3.63 ± 0.06^{Ab}	3.61 ± 0.05^{Ab}
	4	nd	nd	3.87 ± 0.03^{Aa}	3.80 ± 0.04^{Aa}

Values ± S.D. are presented as a mean of 6 replicates (2 x 3). Different uppercase and lowercase letters indicate significant differences for values in each row and each column, respectively at $P < 0.05$.

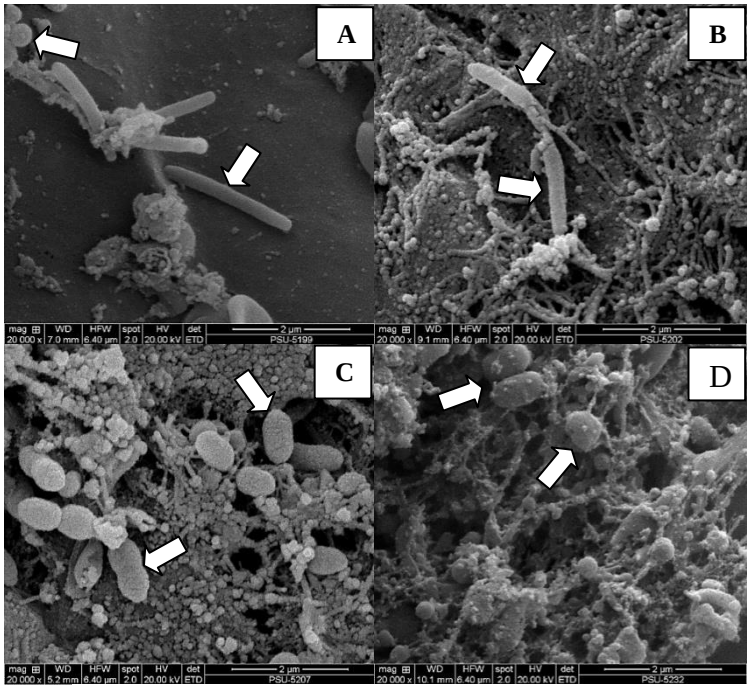


Figure 4-3. SEM photomicrographs showing bacteria colonized in intestinal tract of postlarval- 45 stage white shrimp at day 30 of cultivation and each picture was taken from random 6 shrimps in each set; (A) Native control, (B) Challenge test, (C) Treatment set, and (D) Positive control.

Shrimp survival with related to bacterial populations

At 15 days of cultivation, the shrimp were challenged by adding AHPND-causing *V. parahaemolyticus* SR2 (approximately 10^5 cells mL^{-1}) into the rearing water in challenge and treatment sets for monitoring shrimp survival after exposure time for 15 days (total 30 cultivation days). Numbers of shrimp survived in a step of inoculating SR2 (initial numbers of shrimp) in native, positive and challenge sets was roughly 35; whereas in treatment set was roughly 36. Five shrimp suddenly died at the day 1 of exposure in a challenge set; and only one shrimp died in a treatment set that corresponded to survival percentage at 90.74% and 97.22%, respectively (Figure 4-4). At the 15th exposure day, shrimp survival in the treatment set (73.12%) was significantly higher ($P < 0.05$) than in the challenge set (62.37%). A significant increase of shrimp survival in sets with no added pathogenic strain SR2 was observed as well as in the positive control set with added a mixed three probiotic PNSB (88.71%) and 79.57% for native control (Figure 4-4).

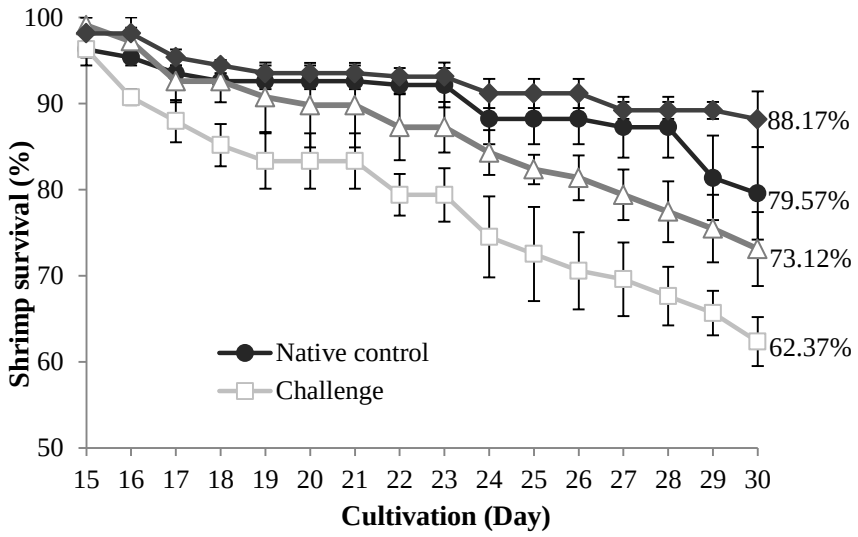


Figure 4-4. Effects of a three mixed probiotic PNSB (1: 1: 1) and AHPND-causing *V. parahaemolyticus* SR2 on white shrimp survival during 30 days of cultivation. Initial shrimp at day 15 for challenge test; native control (n = 34, 35, 35), positive control (n = 34, 36, 36), treatment (n = 35, 36, 36) and challenge (n = 34, 34, 36). Error bars represent standard error of mean.

The linear correlation between numbers of bacteria (Log CFU mL^{-1}/ Log CFU g^{-1}) in samples (water or shrimp), and percentage of shrimp survival during shrimp cultivation for 30 days is shown in Figure 4-5. The linear correlation coefficient (r) demonstrated a strong negative correlation between the numbers of *Vibrio* spp. and percent shrimp survival in water samples (r = -0.8680, $P < 0.05$) (Figure 4-5A) and in shrimp samples (r = -0.7188, $P < 0.05$) (Figure 4-5B). These results indicate that increasing numbers of *Vibrio* spp. resulted in decreasing percent shrimp survival. To know the effect of probiotic PNSB used on shrimp survival and also as no detection of PNSB in sets of native (no inoculation) and challenge (inoculated SR2) so a different shrimp survival between treatment (PNSB and SR2 inoculations) and challenge or between positive (inoculated PNSB) and native controls was used for investigating the correlation between the numbers of PNSB (samples of shrimp or water) and a difference percentage in shrimp survival of a pair test. Comparison between treatment and challenge sets found a strong positive correlation in both water (r = 0.9133) and shrimp (r = 0.9080) samples (Figure 4-5C, D and supplementary Table 4-6). A similar result was also observed in positive and native control sets for a strong positive correlation in water (r = 0.7235) and shrimp (r = 0.7891) samples (Figure 4-5E, F and Tables 4-6 to 4-8). These results suggest that increasing numbers of PNSB are followed by an increasing shrimp survival percentage.

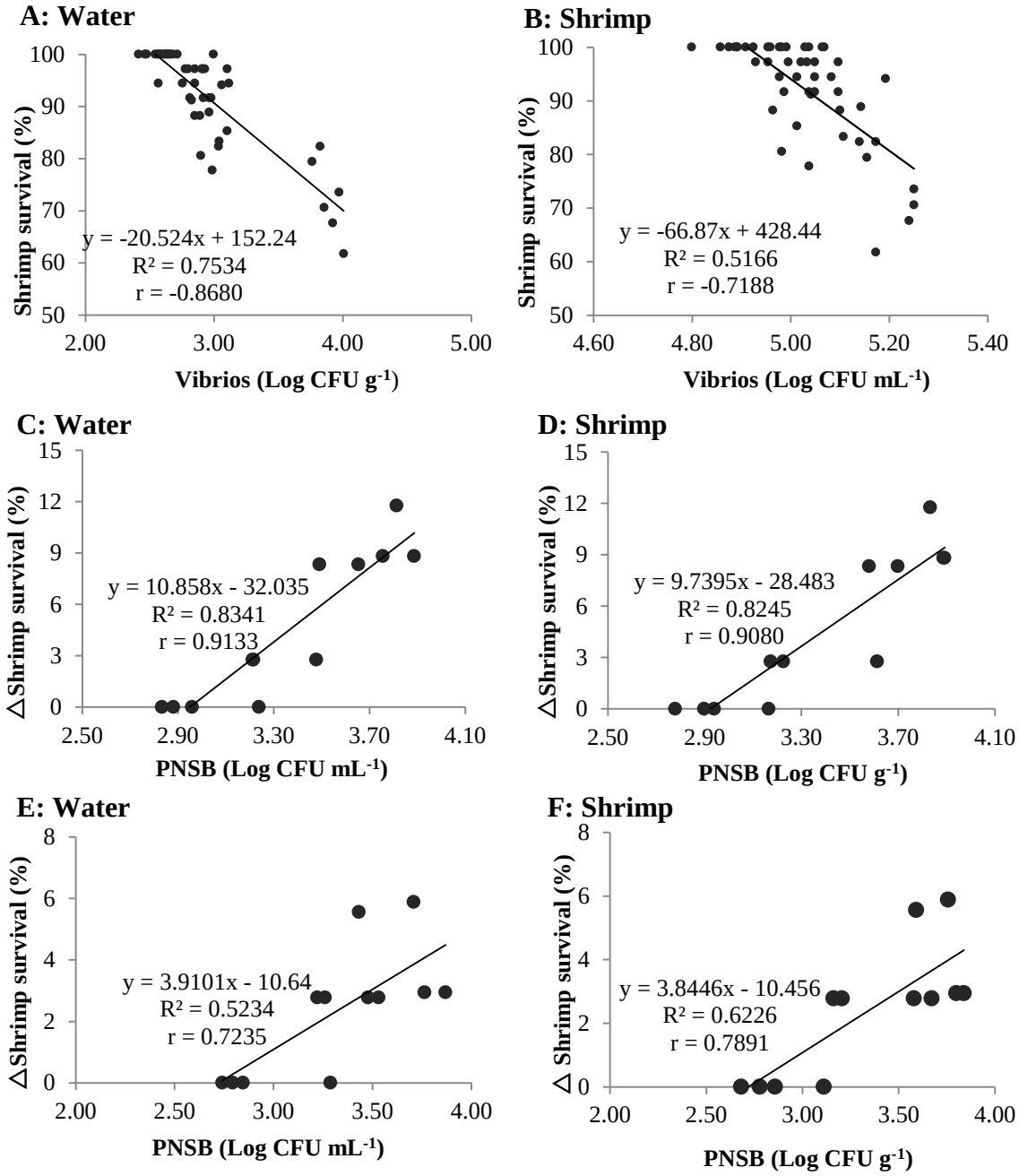


Figure 4-5. The linear correlation coefficient between numbers of bacteria and shrimp survival during white shrimp cultivation for 30 days; (A) Water samples (survival and vibrios count for all sets), (B) Shrimp samples (survival and vibrioscount in all sets), (C) Water samples (Δ survival rate; treatment - challenge, and PNSB count in treatment set), (D) Shrimp samples (Δ survival rate; treatment - challenge, and PNSB count in treatment set, (E): Water samples (Δ survival rate; positive - native, and PNSB count in positive control) and (F) Shrimp samples (Δ survival rate; positive - native, and PNSB count in positive control.

Table 4-6. The raw data associated with linear correlation that presented △ Shrimp survival (%) in sets of treatment and positive controls.

Week	Shrimp survival (%)				△Shrimp survival (%)	
	Native control	Challenge	Treatment	Positive control	Treatment (Treatment-Challenge)	Positive control (Positive-Native controls)
1	100.000	100.000	100.000	100.000	0	0
	100.000	100.000	100.000	100.000	0	0
	100.000	100.000	100.000	100.000	0	0
2	97.222	94.444	97.222	100.000	2.78	2.78
	97.222	97.222	100.000	97.222	2.78	0
	97.222	100.000	100.000	100.000	0	2.78
3	88.889	83.333	91.667	94.444	8.33	5.56
	91.667	88.889	97.222	94.444	8.33	2.78
	91.667	77.778	80.556	94.444	2.78	2.78
4	82.352	67.647	79.411	88.235	11.76	5.88
	85.294	73.529	82.352	88.235	8.82	2.94
	91.176	61.764	70.588	94.117	8.82	2.94

The value of each plot was calculated from shrimp survival (%) by presenting △ Shrimp survival (%) in treatment (comparison between treatment and challenge sets) and positive control (comparison between positive and native controls) that related with Fig. 4-5C, D, E and F.

Table 4-7. Explanation how to build the linear correlation in case of treatment (comparison between treatment and challenge sets) from data between the numbers of PNSB (in both of water and shrimp samples) and percentage of △ Shrimp survival.

Week	Water samples		Shrimp samples		Explain how to obtain the results of linear correlation (r values)
	Number of PNSB (Log CFU mL^{-1})	△Shrimp survival (%)	Number of PNSB (Log CFU g^{-1})	△Shrimp survival (%)	
1	2.83	0	2.90	0	The correlation values were calculated using Microsoft Excel 2013 by following these steps; Open Microsoft Excel 2013 > Press Data > Press Data Analysis > Press Correlation > Select raw data > put in "Input Range" > put one blank cell in "Output Range" for the result > Press OK Fig. 5C, r = 0.9133 Fig. 5D, r = 0.9080
	2.96	0	2.94	0	
	2.88	0	2.78	0	
2	3.21	2.78	3.23	2.78	
	3.21	2.78	3.17	2.78	
	3.24	0	3.16	0	
3	3.49	8.33	3.58	8.33	
	3.65	8.33	3.70	8.33	
	3.48	2.78	3.61	2.78	
4	3.81	11.76	3.83	11.76	
	3.89	8.82	3.89	8.82	
	3.76	8.82	3.89	8.82	

These data were put in Microsoft Excel 2013 and created XY (scatter) chart; X axis presents the number of PNSB and Y axis presents △ shrimp survival (%). Then, these data were used to calculate r value by Pearson's product moment correlation using Microsoft Excel 2013.

Table 4-8. Explanation how to build the linear correlation in case of positive (comparison between positive and native controls) from data between the numbers of PNSB (in both of water and shrimp samples) and percentage of △ Shrimp survival.

Week	Water samples		Shrimp sample		Explain how to obtain the results of linear correlation (r values)
	Number of PNSB (Log CFU mL^{-1})	△Shrimp survival (%)	Number of PNSB (Log CFU g^{-1})	△Shrimp survival (%)	
1	2.74	0	2.78	0	The correlation values were calculated using Microsoft Excel 2013 by following these steps; Open Microsoft Excel 2013 > Press Data > Press Data Analysis > Press Correlation > Select raw data > put in "Input Range" > put one blank cell in "Output Range" for the result > Press OK Fig. 5E; r = 0.7235 Fig. 5F; r = 0.7891
	2.79	0	2.86	0	
	2.85	0	2.68	0	
2	3.22	2.78	3.16	2.78	
	3.29	0	3.11	0	
	3.26	2.78	3.20	2.78	
3	3.43	5.56	3.59	5.56	
	3.48	2.78	3.67	2.78	
	3.53	2.78	3.58	2.78	
4	3.71	5.88	3.76	5.88	
	3.87	2.94	3.84	2.94	
	3.76	2.94	3.80	2.94	

These data were put in Microsoft Excel 2013 and created XY (scatter) chart; X axis presents the number of PNSB and Y axis presents △ shrimp survival (%). Then, these data were used to calculate r value by Pearson's product moment correlation using Microsoft Excel 2013.

Discussion

Water quality in shrimp cultivation

The use of probiotic PNSB in this study suggests that inoculated PNSB population suspended in rearing water during shrimp cultivation significantly improved ($P < 0.05$) the water quality by reducing levels of NH_4^+, NO_2^-, NO_3^- and COD compared to a control set without PNSB inoculation (Figures 4-1 and 4-2). However, in the first experiment with 50% water exchange in every week, the changes of all monitored parameters in each experimental set were in acceptable ranges for shrimp cultivation (Boyd and Fast, 1992; Cohen et al., 2005; Mishra et al., 2008). It is well recognized that NH_4^+ and NO_2^- are toxic to shrimp; and their maximum levels were found at week 8 in all sets with exception of T1 set in case of NH_4^+ (Figure 4-1A). In all treatment sets at the end of shrimp cultivation found the lowest levels of NH_4^+ in T1 set, NO_2^- in T2, T3 sets, NO_3^- in T2 set and COD in T1, T2 sets (Figure 4-1). Among 3 treatment sets, T1 set (S3W10, SS15) produced a sharp decrease of NH_4^+ (1.22 ± 0.02 mg L^{-1}) compared to T2 (S3W10, TKW17) and T3 (S3W10, STW181) sets (3.13 ± 0.02 mg L^{-1}) (Figure 4-1A) and also reduced COD in the same level with T2 (Figure 4-1D). It seems to be that NH_4^+ levels in all experimental sets were quite high; however, they were in acceptable ranges for rearing water of white shrimp as the safe levels of NH_4^+ should be 6.52 mg L^{-1} for juvenile stage (Frías-Espericueta et al., 1999; Audelo Naranjo et al., 2012). As ability to reduce NO_2^- in T2 and T3 sets did not differ (Figure 4-1B); therefore, strains in sets of T1 and T3 (SS15, S3W10 and STW181) were selected for being used as a mixed culture in further studies. In addition, the strain STW181 was selected instead of the strain TKW17 because shrimp growth in set T3 was better than set T2 (Table 4-3).

In the second experiment with no exchange of water within 4 weeks of white shrimp cultivation, the results demonstrated that all monitored parameters in each aquarium were higher than that found in the first experiment (Figures 4-1 and 4-2). However, this experiment was conducted by following the stage of the production cycles as no water exchange in the first month of cultivation (Biao et al., 2004). During weeks 2 until 4, inoculated PNSB in treatment and positive control sets significantly reduced ($P < 0.05$) levels of NH_4^+, NO_2^-, NO_3^- and COD with significantly higher ($P < 0.05$) of DO compared with native and challenge sets as shown in Figure 4-2. The results suggest that PNSB in treatment set might compete with *V. parahaemolyticus* SR2 to control water quality as no significant difference was found between sets of treatment and positive control for NO_2^- and COD. In addition, the treatment set produced a higher efficiency to reduce NH_4^+ at week 4 (7.25 ± 0.02 mg L^{-1}, pH 8.21) than in the positive control set (10.01 ± 0.01 mg L^{-1}, pH 8.25) (Figure 4-2A), but showed less efficiency in case of NO_3^- (Figure 4-2D). At week 4 in all experimental sets had high levels of NH_4^+; however, the toxicity depends on various factors including species tolerance, water parameters (e.g. pH, DO, salinity, temperature) and cultivation period (Mishra et al., 2008). Toxicity of NH_4^+ increase significantly ($P < 0.05$) under alkaline conditions (pH > 9) as the mortality of postlarval *L. vannamei* was lower than 1.0% under NH_4^+ concentrations in a range of 0-18 mg L^{-1} at pH 8, 26 °C and salinity 38 ppt; but the same concentrations at pH 9, 30 °C caused high shrimp mortality (Barajas et al., 2006). It should be noted that NH_4^+ levels in both experiments of this study were high. This is due to the fact that ASW was used for shrimp cultivation in aquaria. Hence, the population of nitrifiers might be low and this caused high NH_4^+ levels. Based on the results in the experiment 1, this supports that a mixed probiotic PNSB, particularly strain SS15 could rapidly utilize NH_4^+ for its growth (Figure 4-1A and Table 4-3).

The good water quality is a very important factor for the shrimp growth as previously described; thereby the properties of good inoculants should enhance the natural processes such as the degradation of organic matter, ammonification, nitrification and sulfide oxidation in shrimp cultivation (Boyd and Gross, 1998). Normally shrimp requires high protein for their growth; however, shrimp can eat feed only 85% and uneaten feed leads to residual protein in the rearing water and also excrete feces in form of inorganic nitrogen (Goddard, 1996). A sufficient amount of DO by aeration in shrimp cultivation allows microbes to degrade protein, a main organic matter in COD, and release NH_4^+; and this ion is oxidized to NO_2^- and NO_3^- by nitrifying bacteria. Regarding to above results demonstrating that the mixed probiotic PNSB used in this study could have synergistic acting with normal flora to maintain water quality for shrimp growth during cultivation. In addition of controlling water quality for shrimp growth, the use of a mixed PNSB culture in this study might allow shrimp effluent for meeting the guidelines of aquaculture effluent or easier for treatment.

Shrimp growth and survival with related to bacterial populations

According to the results in the experiment 1 showed each experimental set had water quality in the acceptable levels for shrimp growth although levels of NH_4^+ were quite high (Figure 4-1). It is interesting that shrimp growth in all a mix of two probiotic PNSB sets was better than in the control (Table 4-3). This suggests that a mix of two probiotic PNSB could promote the growth of shrimp and it would be better to use a mix of three probiotic PNSB as previously discussed. This might be that the growth performances of shrimp are associated not only with environmental conditions but also health status that relies on the roles of intestinal microbiota such as promoting host nutrient (Xiong et al., 2016). Our results are in accordance with several researchers as probiotics were able to improve the growth performance in zoea, mysis,

postlarval and juvenile stages of shrimp (Kongnum and Hongpattarakere, 2012; Nimrat et al., 2012; Silva et al., 2013).

The greatest performance of shrimp growth (weight, length and SGR) was found in a positive control that only inoculated with mixed three probiotics PNSB (Table 4-2). Shrimp in treatment set (mixed three probiotic PNSB and *V. parahaemolyticus* SR2) had higher growth performance than that found in the challenge set although no significant difference ($P > 0.05$) (Table 4-4). These results indicate that the use of probiotic PNSB in white shrimp cultivation significantly improved shrimp growth ($P < 0.05$); however, their efficiency was reduced by shrimp pathogenic strain SR2. This is supported by our previous study that PNSB are good candidates to be used as probiotics by promoting the activities of host-digestive system with their digestive enzymes such as protease, amylase and lipase to stimulate shrimp growth (Chumpol et al., 2017a). It should be noted that inoculation either PNSB or PNSB and strain SR2 together could be colonized in the intestinal tract of shrimp (Figure 4-3B, C and D). Therefore, a mixed three probiotic PNSB should increase survival of infected shrimp by strain SR2, which will be discussed later.

No detection of *Vibrio* spp. in water samples of all experimental sets at the beginning of cultivation; however, after that this bacterium was found in all sets both in water and shrimp samples until the end of cultivation (Table 4-5). It should be noted that native and positive sets were uninoculated of strain SR2 but found *Vibrio* spp. in shrimp body throughout cultivation. This indicates that *Vibrio* spp. are normal flora in shrimp cultivation as this organism was detected approximately 4.0 Log CFU g^{-1} in shrimp body in each experimental set at starting the experiment (Table 4-5). This suggests that pathogenic strain SR2 could retard shrimp growth in challenge set compared with positive control (Table 4-4). One of the important criteria for probiotics should have the ability to adhere and colonize on epithelial surface of intestinal tract so that it would

be possible for producing greater benefits to host with various mechanisms for competitive exclusion against pathogenic strains at attachment sites, nutrient and production of antibacterial substances (Guo et al., 2009; Merrifield et al., 2010). Regarding the PNSB population found in both rearing water and shrimp samples only in sets of a mixed three probiotic PNSB inoculation (treatment and positive) with significant increases after inoculating at weeks 1, 2 and 3 (Table 4-5). It means that PNSB could survive in rearing water and some infected into shrimp body for colonization and proliferation in shrimp digestive tract as an evidence of photomicrograph from SEM (Figure 4-3C and D). Hence, the probiotic PNSB used in our study produced a remarkable increase of shrimp growth in the positive control was due to PNSB acting to control water quality and on the basis of their probiotics promoting shrimp growth during cultivation as previously described (Figures 4-2, 4-3 C-D and Table 4-4). This is in accordance with the overview by Xiong et al. (2016) that the successful application of probiotic strains for shrimp cultivation the probiotics should be established in both of the rearing water and in intestinal shrimp.

EMS/AHPND is a new emerging shrimp disease that still one of the most serious shrimp diseases in farmed shrimp in several countries such as in China, Vietnam, Thailand and Mexico (Zorriehzahra and Banaederakhshan, 2015). Normally after 20-35 days of white shrimp postlarval stocking into grow out ponds are sensitive to be infected by AHPND-causing *V. parahaemolyticus* that leads to mass mortality. Therefore, this study investigated the effect of probiotic PNSB on shrimp survival after exposure with *V. parahaemolyticus* SR2 during shrimp cultivation (postlarval 30-45 stages). The results proved that a mixed three probiotic PNSB was able to protect shrimp either healthy or infected shrimp with strain SR2 by increasing shrimp survival roughly 9% and 11%, respectively (Figure 4-4). This is supported by the strong positive correlation coefficients between PNSB population (shrimp and water samples) and the difference of shrimp survival in a pair test (treatment and challenge set,

r = 0.9133 and 0.9080 for water and shrimp) or (positive and native set, r = 0.7235 and 0.7891 for water and shrimp) (Figure 4-5C–F). These data proved that a mixed three probiotic PNSB were very effective to control pathogenic *V. parahaemolyticus* SR2 that caused AHPND as they are more productive with infected shrimp by SR2 than that found in shrimp with no infection of strain SR2 (Figures 4-4 and 4-5).

Conclusions

Overall results stated that a mixed probiotic PNSB (*R. sphaeroides* SS15, S3W10 and *A. marina* STW181) had a great potential for applying in white shrimp (*L. vannamei*) cultivation for preventing shrimp diseases, particularly AHPND and maintaining water quality to increase shrimp survival and stimulate shrimp growth. This would lead to organic shrimp farming for producing safe shrimp and environmental friendly for sustainable shrimp cultivation.

CHAPTER 5

Optimization of culture conditions for production and characterization of antivibrio compounds from probiotic purple nonsulfur bacteria against serious shrimp pathogenic *Vibrio* spp.

Abstract

Shrimp farmers have been faced with pathogenic bacterial infection, especially *Vibrio harveyi* and *Vibrio parahaemolyticus* as causative agents of luminous vibriosis and acute hepatopancreatic necrosis disease (AHPND), respectively. Hence, exploring of antivibrio compounds that are biodegradable and environmental friendly to prevent both serious shrimp diseases has been investigated. Purple nonsulfur bacteria (PNSB); particularly for probiotic strains are attractive to be used for producing antivibrio compounds against shrimp pathogenic vibrios. Three probiotic PNSB; *Rhodobacter sphaeroides* (SS15, TKW17) and *Afifella marina* STW181 released antivibrio compounds to perform the maximal antivibrio activity under their optimum conditions that were closed to the conditions of shrimp cultivation. The antivibrio compounds from these PNSB mainly consisted of protein, lipid and carbohydrate with stability under wide ranges of pH between 3 to 10 and high temperature up to 121 ºC for 20 min, except for strain STW181. All antivibrio compounds acted as bactericidal action as the evidence of damaged cells with many holes and also showed bacteriolytic activity as the highest found in strain STW181. One of the purified antivibrio compounds from strain SS15 was a low molecular weight (< 3000 Da) weak cationic compound with containing-NH_2 group. It can be concluded that the antivibrio compounds from all probiotic PNSB tested

have the great potential to be used for controlling both serious shrimp pathogenic vibrios.

Keywords: Bacteriolytic activity, Bioactive compounds, Early mortality syndrome (EMS), Phototrophic bacteria, *V. harveyi*, *V. parahaemolyticus*

บทคัดย่อ

เกษตรกรผู้เลี้ยงกุ้งมักเผชิญกับปัญหากุ้งติดเชื้อแบคทีเรียก่อโรค โดยเฉพาะอย่างยิ่ง *Vibrio harveyi* และ *Vibrio parahaemolyticus* ซึ่งเป็นสาเหตุของโรคเรืองแสง และโรคตับวายเฉียบพลันดังนั้นจึงมีการศึกษาค้นหาสารยับยั้งเชื้อวิบริโอที่ย่อยสลายได้ และเป็นมิตรต่อสิ่งแวดล้อมเพื่อป้องกันโรคกุ้งที่ร้ายแรงแบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ (Purple nonsulfur bacteria, PNSB) ที่มีคุณสมบัติเป็นโปรไบโอติกส์มีความน่าสนใจในการนำมาใช้สำหรับผลิตสารยับยั้งเชื้อก่อโรคในกลุ่ม *Vibrio* spp. แบคทีเรียโปรไบโอติกส์ PNSB 3 สายพันธุ์ ได้แก่ *Rhodobacter sphaeroides* (SS15/ TKW17) และ *Afifella marina* STW181 ต่างก็มีความสามารถในการสร้างสารยับยั้งเชื้อวิบริโอที่ปล่อยออกมานอกเซลล์ได้สูงสุด ภายใต้สภาวะที่เหมาะสมต่อการผลิตซึ่งมีความใกล้เคียงกับสภาวะในการเลี้ยงกุ้ง สารยับยั้งวิบริโอผลิตจากแบคทีเรีย PNSB มีส่วนประกอบหลักเป็นโปรตีน ไขมัน และคาร์โบไฮเดรตที่มีความเสถียรในช่วงกว้างที่ค่า pH ระหว่าง 3-10 และทนต่ออุณหภูมิสูงถึง 121 ºC เป็นเวลา 20 นาที ยกเว้นสารที่ผลิตจากสายพันธุ์ STW181 สารยับยั้งวิบริโอที่สร้างจากแบคทีเรีย PNSB ทั้ง 3 สายพันธุ์มีฤทธิ์แบบการฆ่าแบคทีเรียซึ่งมีหลักฐานที่พบว่าทำให้เกิดรูจำนวนมากบนเซลล์แบคทีเรียก่อโรค และยังพบว่ามีกิจกรรมที่ทำให้เซลล์แบคทีเรียก่อโรคแตกโดยสายพันธุ์ STW181 มีกิจกรรมสูงสุด เมื่อนำสารยับยั้งวิบริโอที่สร้างจากสายพันธุ์ SS15 มาทำให้บริสุทธิ์พบว่าเป็นสารที่มีขนาดโมเลกุลเล็กกว่า 3,000 ดัลตันซึ่งมีประจุบวกชนิด weak cation ที่มีหมู่เอมีนเป็นองค์ประกอบ จากการศึกษานี้สามารถสรุปได้ว่าสารยับยั้งเชื้อวิบริโอที่สร้างจากแบคทีเรียโปรไบโอติกส์ PNSB ทั้ง 3 สายพันธุ์ มีศักยภาพสูงสำหรับการควบคุมเชื้อก่อโรคกุ้งทั้งสองที่รุนแรง

คำสำคัญ: กิจกรรมที่ทำให้เซลล์แบคทีเรียแตก, สารออกฤทธิ์ทางชีวภาพ, โรคกุ้งตายด่วน, แบคทีเรียสังเคราะห์แสง, แบคทีเรีย *V. harveyi*, แบคทีเรีย *V. parahaemolyticus*

Introduction

Recently, *Litopenaeus vannamei* is one of the most economically dominant species among cultured shrimp cultivation. Shrimp production in Southeast Asia steadily averaged 6.0% annual growth from 2008 to 2011; however, the production declined from 3.45 million metric tons (MMT) to 3.25 MMT in 2012 (down 5.8%) and 3.21 MMT in 2013 (down 1.1%) due to the impact of early mortality syndrome (EMS) in China, Thailand, Vietnam and Malaysia (Andeson, 2016). EMS is also called acute hepatopancreatic necrosis disease (AHPND) which demonstrated an infectious symptom, including the sloughing of hepatopancreas (HP) cells, enlarged HP nuclei, lack of B, F, R cells and E cell mitosis (FAO, 2013). The outbreak of AHPND/ EMS in Thailand was first reported in late 2012 in pacific white shrimp (*L. vannamei*) farming on the eastern coast and then spreading to the western coast of the Gulf of Thailand (Flegel, 2012; Joshi et al., 2014). It caused 100% mortality in early shrimp growing around 20-30 days of cultivation; and *V. parahaemolyticus* is recognized as the bacterial strain caused AHPND/EMS (Tran et al., 2013).

There are five growth stages in the development of penaeid shrimp; including egg, larvae (nauplius, zoea and mysis), postlarvae, juvenile and adult. In larval stages, especially from the zoea to postlarvae periods which have low immunity and are susceptible to be infected by many pathogens resulted in mass mortalities in the shrimp hatchery (Vandenberghe et al., 1999; Zheng et al., 2016). Most diseases occur in shrimp cultivation that caused by bacterial infection, particularly *Vibrio* species such as *V. harveyi*, *V. paraheamolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. anguillarum*, and *V. splendidus* (Chatterjee and Haldar, 2012). *V. harveyi* is dominant species causing luminous vibriosis which is the most important *Vibrio* infections during postlarval stage until adult stage of shrimp (Prayitno and Latchford 1995; Vandenberghe et al., 1999). The virulence of *V. harveyi* caused 100% shrimp death in larvae of *Penaeus*

monodon cultivation (Musa et al., 2008; Rattanachuay et al., 2010). To solve these problems, shrimp farmers normally use antibiotics to eliminate shrimp pathogenic bacteria; however, antibiotics cause harmful effects to consumer health. In addition, long term usages of antibiotics lead to residual compounds in sediment and water; consequently bacteria can adapt themselves to resist antibiotics by developing antibiotic resistant genes (Zhang et al., 2010).

To sustain shrimp cultivation, organic farming is an attractive way to produce safe shrimp and also environmental friendly; thereby, probiotics as biocontrol agents against shrimp pathogenic *Vibrio* species and/or their antivibrio compounds have been explored to avoid the use of antibiotics. It is well recognized that purple nonsulfur phototrophic bacteria (PNSB) are generally distributed in aquatic system including shrimp farm; and some of them have been proved as probiotic bacteria to stimulate growth of aquatic animals and also to control water quality (Shapawi et al., 2012; Chumpol et al., 2017a, b). PNSB perform a wide range of growth modes depending on environmental conditions such as phototrophic growth under anaerobic/ microaerobic-light conditions and chemotrophic growth under aerobic-dark conditions (Panwichian et al., 2010). Up to date there is still little information about antivibrio compounds produced by these bacteria. For instance, *Rhodobacter sphaeroides* was able to produce bioactive compounds against *V. harveyi* and *V. fischerii* (Chandrasekaran and Kumar, 2011). Interestingly, PNSB isolated from shrimp ponds in our previous studies had been proved to act as probiotics for controlling shrimp pathogenic vibrios such as luminous vibriosis and AHPND strains, and also stimulating shrimp growth with higher survival (Chumpol et al., 2017a, b). Hence, it would be worth to explore the potential antivibrio compounds produced by our probiotic PNSB as they might be used for controlling shrimp pathogenic vibrios, including AHPND strains instead of their cells. According to above information, the aims of this study were to investigate optimal conditions for producing antivibrio compounds by

the probiotic PNSB and to characterize the antivibrio compounds in order to gain information to support the use for organic farming.

Materials and methods

Bacteria used and growth conditions

The PNSB strains *Rhodobacter sphaeroides* (SS15, TKW17) and *Afifella marina* STW181 isolated from shrimp ponds were used in this study due to their properties to act as probiotics and showing antagonistic activity against shrimp pathogenic *Vibrio* spp. (Chumpol et al., 2017a, b). Strains SS15, TKW17 were grown in basic isolation medium (BIM + 1.5% NaCl); while strain STW181 was grown in glutamate acetate medium (GA + 2% NaCl). These bacteria were cultured under microaerobic-light conditions with 3,500 lux of tungsten lamps for 48 h to use for inoculum preparation. For the shrimp pathogen, *Vibrio harveyi*_KSAAHRC was kindly provided by the Aquatic Animal Help Research Center (AAHRC), Prince of Songkla University. AHPND-causing *Vibrio parahaemolyticus* strains; SR1, SR2 and SR3 were isolated from infected shrimp in Songkhla province, Thailand by our previous study (Chumpol et al., 2017a). All shrimp pathogenic *Vibrio* spp. were grown in tryptic soy broth (TSB + 1.5% NaCl) and shaken at 150 rpm min^{-1}, 35 $^{\circ}$C for 18 h (Chau et al., 2011) prior to use for preparing inoculums.

Inhibition of shrimp pathogenic vibrios by extracellular compounds from probiotic PNSB

Agar well diffusion method was used to test antivibrio activity of samples i.e. lyophilized culture supernatants at 15 times concentration (15X) from three probiotic PNSB. Each probiotic PNSB was grown under microaerobic-light conditions as previously described and centrifuged at 10,418 *g* for 15 min to obtain culture supernatant. Each culture supernatant was

concentrated by lyophilizer (Alpha 1-2 LD, Christ, Osterode am Harz, Germany). The lyophilized culture supernatant was dissolved in 10% methanol to adjust for a design dose at 15X of its initial concentration; and 10% methanol was served as a negative control. The target organism or shrimp pathogen was adjusted to a final cell density at $1.0x10^5$ CFU mL^{-1} (OD_{660} = 0.1) and swabbed over the surface of TSA containing 1.5% NaCl with 3 wells (diameter 8 mm per well) in triangle for each plate. A 120 µL sample of each lyophilized culture supernatant at 15X was added into each well and incubated at 35 °C for 24 h and then the inhibition zones were measured using a vernier caliper.

Investigating optimal conditions of antivibrio compounds produced by probiotic PNSB

Due to the ability of PNSB to grow with various growth modes such as either phototrophic or heterotrophic, the probiotic PNSB strains were investigated for their optimum conditions to produce antivibrio compounds by varying the culture age, initial pH, light intensity and aeration (only in aerobic dark conditions). The probiotic PNSB were grown in BIM/ GA under microaerobic-light conditions (dark for aerobic conditions); and their 15X concentrated lyophilized supernatants were used to test for antivibrio activity. Antivibrio compounds produced by probiotic PNSB were considered on the basis of antivibrio activity against shrimp pathogenic vibrios.

Samples of the culture broths from the probiotic PNSB were taken every 10 h. The initial pH values of the medium used were adjusted over the range of 6.5, 7.0, 7.5, 8.0 and 8.5 by growing the probiotic PNSB until it reached its optimal culture age for producing antivibrio compounds. Light intensity was varied by varying the settings to provide 3,000, 3,500, 4,000 and 4,500 lux for incubating the probiotic PNSB in the medium used with their optimal initial pH values. Aeration was set by varying the shaking speeds at 50,

100, 150 and 200 rpm to grow the probiotic PNSB in the medium used for their optimal initial pH values under aerobic-dark conditions and incubating at 30 $^{\circ}$C to reach their optimal culture ages. The target pathogen used in this experiment was *V. harveyi*_KSAAHRC to investigate optimal conditions for antivibrio compounds production from three probiotic PNSB using agar well diffusion method as previously described.

Preliminary studies on the property of antivibrio compounds from probiotic PNSB

Lyophilized culture supernatants (15X) containing antivibrio compounds produced by probiotic PNSB were tested for protein property by treatment with the following enzymes; proteinase K (1 mg mL^{-1} at 30°C), pronase (2 mg mL^{-1} at 30°C), α-chymotrypsin (5 mg mL^{-1} at 30°C) and trypsin (50 mg mL^{-1} at 30°C), and lysozyme (1 mg mL^{-1} at 25°C). Lipid and carbohydrate properties were tested using lipase (1 mg mL^{-1} at 37°C) and α-amylase (1 mg mL^{-1} at 25°C). In enzymatic treatment test, the amount of enzyme used as equivalent level such as 1 mg of enzyme added to 1 mL of lyophilized supernatant, mixed well and incubated at the appropriate temperature of the enzyme for 1 h. The remaining antivibrio activity was observed by the agar well diffusion method. Uninoculated medium at 15X concentrated by lyophilization served as negative control; while positive controls were prepared as the same as treatment sets with exception as no addition of any enzymes (Rattanachuay et al., 2010; Ahmad Rather et al., 2013).

The pH susceptibility of a 15X lyophilized culture supernatant was investigated by adjusting the pH values in a range of 1.0-10.0 with 0.1-0.5 N HCl for acidity or 0.1-0.5 N NaOH for alkalinity. The samples without adjustment of pH value were served as positive controls; whereas negative

controls were prepared using uninoculated medium lyophilized supernatant. The antivibrio activity was determined by the agar well diffusion.

The heat stability of a 15X lyophilized culture supernatant was investigated its antivibrio activity. All samples were incubated in a water bath for 30 min at different temperatures; 50°C, 65°C, 75°C and 100°C, including autoclaved at 121°C for 15 and 20 min. For control set, the sample was incubated at 30°C for 30 min; and the remaining antivibrio activity in treatment and control sets were tested by the agar well diffusion method.

Inhibitory effect of lyophilized PNSB supernatant against AHPND-causing *V. parahaemolyticus* strains

Due to the success of probiotic PNSB to inhibit shrimp pathogenic *V. harveyi*_KSAAHRC that causing luminous vibriosis including the properties of their antivibrio compounds; thereby, they were further studied for their ability to inhibit AHPND-causing *V. parahaemolyticus*. A 400 mg of each lyophilized culture supernatant from three probiotic PNSB was separately dissolved in 1.5 mL distilled water and filtered through a 0.45 µm pore size cellulose acetate filter to obtain stock solution at concentration of 266.67 mg mL^{-1}. Chloramphenicol was used as a positive control by dissolving 6.40 mg chloramphenicol in 10 mL distilled water to obtain stock solution at 640 µg mL^{-1}. The shrimp pathogens; AHPND strains SR1, SR2 and SR3 were used as target organisms; and they were grown in TSB containing 1.5% NaCl under incubating at 28°C for 18 h. Each culture broth was adjusted for obtaining cell density of 1.2 x 10^8 CFU mL^{-1} ($OD_{660\ nm}$ = 0.4) by a spectrophotometer. A cell density was diluted 10-fold serial dilutions twice with normal saline solution (NSS; 0.85% NaCl) to obtain the cell density roughly 1.2 x 10^6 CFU mL^{-1}. For testing, 2-fold serial dilutions of lyophilized culture supernatant was prepared using TSB plus 1.5% NaCl; 80 µL of the lyophilized culture supernatant was added into the first well that contained 80 µL of TSB medium, mixed well and

continued 2-fold serial dilutions. Then, in each well of testing remained 80 µL of lyophilized culture supernatant in each diluted concentration, then adding 20 µL of each strain of *V. parahaemolyticus* into each well (final cell density as 2.4 x 10^5 CFU mL^{-1}); and this made total volume of testing per well was 100 µL.

The positive control was investigated as 10 µL (640 µg mL^{-1}) of chloramphenicol was added into the first well containing 150 µL of TSB medium and then diluted using 2-fold serial dilutions. After that, 20 µL of *V. parahaemolyticus* was added into each well so a total volume per well was 100 µL. All microtiter plates were incubated at 28°C for 24 h and observed ability of lyophilized culture supernatant to inhibit shrimp pathogens. Minimum inhibitory concentration (MIC) is the lowest concentration of the antivibrio compounds to prevent the visible growth of *V. parahaemolyticus*; and any wells with no visible growth were streaked on thiosulfate citrate bile salt sucrose agar. The lowest concentration that showed no growth was interpreted as the minimum bactericidal concentration (MBC). Both MIC and MBC were reported for presenting antivibrio activity of bioactive compounds from probiotic PNSB.

Assay of bacteriolytic activity of antivibrio compounds from probiotic PNSB

To know the mechanisms of antivibrio compounds produced by probiotic PNSB, their bacteriolytic activity was also investigated as each probiotic PNSB was separately cultured in BIM/ GA medium under microaerobic light conditions for 48 h and centrifuged to obtain culture supernatant. To obtain cell free supernatant, the culture supernatant was filtered through a 0.45 µm pore size cellulose acetate filter; and it was used for testing bacteriolytic activity on shrimp pathogenic, *V. parahaemolyticus* SR2. The strain SR2 was selected to use in this experiment based on the results of

previous experiment as no different for MIC/MBC values among PNSB tested; and this strain caused more serious on shrimp disease from our observation in shrimp ponds.

The pathogen was grown in TSB with 1.5% NaCl for 48 h and centrifuged at 10,418 *g* for 20 min to obtain cell pellet. The cell pellet was added into 3 mL of sterile artificial sea water (ASW) and incubated in water bath at 50 °C ± 0.5 °C for 30 min to obtain heat-killed cells (Rattanachuay et al., 2010). A mixture consisted of 7 mL of lyophilized cell free supernatant containing antivibrio compounds (106 mg mL^{-1}) and 3 mL of the heat-killed *V. parahaemolyticus* SR2 cell suspension was adjusted to an initial absorbance of 0.4 ($OD_{660\ nm}$) using a spectrophotometer and incubated at 30 °C for 0, 2, 4, 6, 8, 10 and 22 h to investigate cell lytic activity. ASW was used as a blank. In addition, live cells of *V. parahaemolyticus* SR2 was also parallel used for testing cell lytic activity using the same method as heat killed cells was replaced by live cells (Rattanachuay et al., 2010). Cell lytic activity is divided into four levels according to the percentage decrease in absorbance at 660 nm in comparison with the control: 0-24% (-), 25-49% (+), 50-74% (++) and 75-100% (+++) (Niwa et al., 2005).

Altered cells of *V. parahaemolyticus* SR2 were checked after exposure with cell free lyophilized supernatant containing antivibrio compounds. SR2 cells were immersed in 2.5% cold glutaraldehyde for 1 h and then removed glutaraldehyde and washed three times with 0.1 M phosphate buffer solution, pH 7.4 for 30 min in each step. The samples were dehydrated two times with serial concentrations of ethanol (50%, 60%, 70%, 80%, 90% and 100% ethanol) at room temperature for 15 min in each step. Finally the samples were dried in a critical-point dryer (CPD) and demonstrated on specimen stub, coated with gold and observed under scanning electron microscope (SEM) (FEI Quanta 400, FEI company, USA) by following the instruction of manufacturing.

Some characterization of antivibrio compounds from probiotic PNSB

Among three probiotic PNSB, antivibrio compounds produced by strain SS15 was chosen for characterization as the most effective to resist on various enzymes tested. Culture supernatant of probiotic SS15 was firstly extracted with ethyl acetate (EtOAc) to obtain the antivibrio compounds as 1 mL of lyophilized supernatant (266.66 mg mL^{-1}) was added into a centrifuge tube and followed by 1 mL of EtOAc. The compound was mixed using a vortex (20 times) and centrifuged at 3,000 *g* for 5 min and then it was separated into three fractions; EtOAc layer, emulsion layer and water layer. The EtOAc layer (upper layer) was removed into a new test tube; while 1 mL of EtOAc was added into the emulsion and water layers and mixed well using vortex. The solution was centrifuged again and clear EtOAc layer was removed and then combined into the clear EtOAc tube. EtOAc was evaporated at 30 ºC for 30 min, the remaining compounds were dissolved in 70% MeOH and tested for antivibrio activity. In addition, the water layer was also checked for antivibrio activity by determination of MIC values as previous description.

In a second step, the molecular weight estimation of antivibrio compounds was studied using ultra-centrifugal filter (3 kDa) (Amicon® Merck millipore). Sample was added into the Amicon filter device and place capped filter device into centrifuge rotor, spin the device at 3,000 *g* for 10-20 min. To recover the compounds that passed through the filter, insert a pipette into the bottom of the filter device and withdraw the sample. Re-centrifuge again until the retentate fraction remain only 150 µL and adjusted the volume of this fraction into 300 µL with distilled water (Concentrated 16.67X). Retentate and permeate fractions were tested for the antivibrio activity. As a third step, the crude antivibrio compound was purified using Oasis WCX (Weak Cation eXhance and reversed-phase sorbent) cartridge that suitable for high polar

compound. At the beginning, the column was cleaned using difference percent concentrations of MeOH (100, 80, 60, 40 and 20) and finally with water.

The sample was load into the cartridge and washed with 10 mL of 5% NH_4OH, followed by 10 mL of 100% MeOH**.** The sample was eluted with 10 mL of 2% HCOOH in 25% MeOH and followed by 10 mL of 2% HCOOH in 75% MeOH. The sample and 5% NH_4OH fractions were lyophilized to eliminate water. The washing and eluting fractions were evaporated to eliminate MeOH and then lyophilized to eliminate 2% HCOOH in water. All fractions were tested for the antivibrio activity to check which the fraction is able to inhibit *V. parahaemolyticus* SR2. Furthermore, thin layer chromatography (TLC) was used to determine the preliminary functional groups of the active fraction. An aliquot of the active fraction was spotted onto the TLC sheet (TLC silica gel 60 F_{254}, Merck) and allowed to dry for a few min. Afterwards, the plate was developed with butanol: acetic acid: water (4: 1: 1, v/v/v) as mobile phase in a saturated glass chamber. The developed plate was dried and sprayed with ninhydrin solution and heat at 110 $^{\circ}$C until found maximal visualization of the spots. The R_f (retention factor) values of the active fraction and standard were calculated and compared for interpreting the results.

Statistical analysis

Data were conducted with three replicates and expressed as mean ± standard deviation (S.D). The one-way analysis of variance (ANOVA) was used to analyze the significant differences between data at level of $P < 0.05$ and the pairwise comparison were performed using the Duncan's multiple-range test (DMRT) by using SPSS program analysis version 11.5 (Lead Technologies, USA).

Results

Optimal conditions of antivibrio compound production by probiotic PNSB

All three culture supernatants in the form of 15X lyophilize produced antivibrio activity; the maximal activity was observed at different culture ages (Figure 5-1A). *R. sphaeroides* strains SS15 and TKW17 produced the highest inhibition zones against *V. harveyi*_KSAAHRC (13.84 ± 0.99 and 14.33 ± 0.94 mm) with the same culture age at 20 h as their middle log phase (data not shown). The strongest inhibition by *A. marina* STW181 (13.65 ± 0.53 mm) was observed at 40 h of incubation (late log phase).

The optimum initial pH value for producing the antivibrio compounds by probiotic PNSB to inhibit the target organism was between 6.5-8.0 for *R. sphaeroides* SS15, 6.5-8.5 for *R. sphaeroides* TKW17 and 7.0-8.5 for *A. marina* STW181 (Figure 5-1B). For instance, inhibition zones at pH 7.5 against *V. harveyi*_KSAAHRC were 15.03 ± 0.99, 12.85 ± 0.86 and 14.91 ± 0.99 mm by strains; SS15, TKW17 and *A. marina* STW181, respectively.

The different probiotic PNSB strains required different optimum light intensities for producing antivibrio compounds. The highest inhibition zones against the target organism were 4,500, 3,000-3,500 and 3,000-4500 lux for *R. sphaeroides* SS15, TKW17 and *A. marina* STW181, respectively (Figure 5-1C).

There was no effect of agitation speed from 100 to 200 rpm for producing the antivibrio compounds against the target organism by all probiotic PNSB under aerobic-dark conditions (12.85 ± 0.24 -14.93 ± 0.94 mm) (Figure 5-1D). In addition, the effect of the antivibrio compounds produced by all probiotic PNSB under conditions of microaerobic-light and aerobic-dark were similar (Figure 5-1).

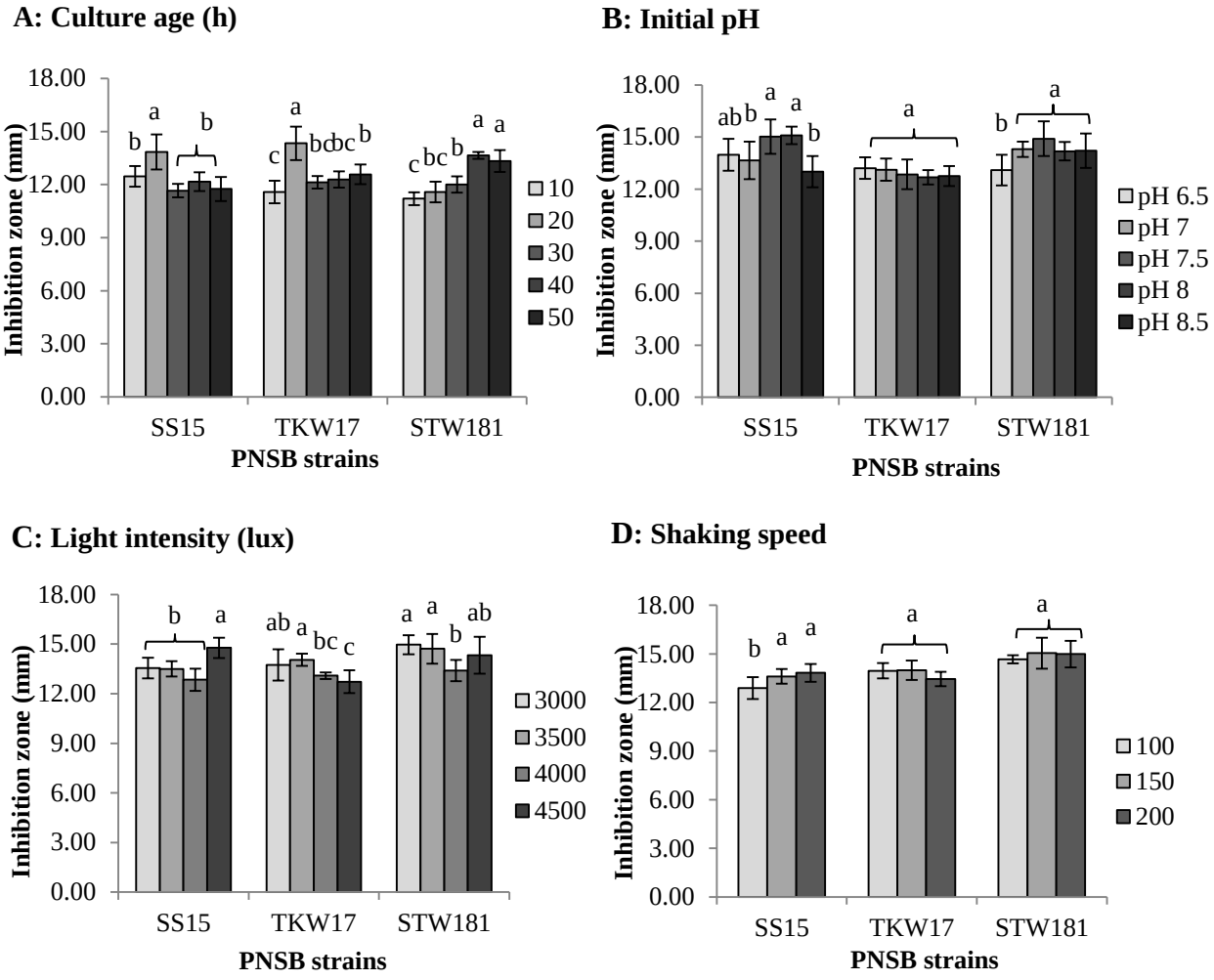


Figure 5-1. Antivibrio activity by 15X lyophilized culture supernatant of three probiotic PNSB against *V. harveyi*_KSAAHRC under various culture conditions; (A) Culture age, (B) Initial pH, (C) Light intensity and (D) Shaking speed. Different lowercase letters above bars indicate significant differences ($P < 0.05$).

Preliminary studies on the property of antivibrio compounds from probiotic PNSB

The property of antivibrio compounds was investigated by treating with several enzymes and the results showed that most of the enzyme treatments caused some loss of inhibitory activity against *V. harveyi* _KSAAHRC (Table 5-1). The antivibrio compounds from *R. sphaeroides* SS15 was the most sensitive to lipase (10.92%), pronase (10.33%) and α-amylase (9.94%) as shown in Table 5-1A and 5-1B. On the other hand, the antivibrio compounds from *R. sphaeroides* TKW17 was significantly reduced in the order of pronase > lipase, α-chymotrypsin, and amylase as 17.49%, 14.86%, 14.23%, and 12.75%, respectively (Table 5-1A and 5-1B). The antivibrio compounds from *A. marina* STW181 was the least inhibitory effect by α-chymotrypsin as 15.99% reduction, followed by proteinase K, lipase and trypsin (13.48%, 13.38% and13.17%), respectively (Table 1 5-A and 5-1B).

The effects of pH values on the inhibitory activity against *V. harveyi* _KSAAHRC were investigated. Table 5-2 shows that the optimal pH of the antivibrio compounds from all three probiotic PNSB was between 3 and 4 as a remarkable increase the inhibition zones compared with positive controls (the origin pH 7.5-7.8). The inhibition zones at pH3 were 15.54 ± 0.85, 17.64 ± 0.31 and 21.68 ± 1.34 mm for strains SS15, TKW17 and STW181, respectively. In contrast, negative control (15X lyophilized uninoculated medium) was also adjusted to pH 3 did not show inhibition zone around the wells. At pH 1 and 2 inhibitory activity of antivibrio compounds from strains SS15, TKW17 and STW181 dramatically reduced (40-68%). However, at pH 5 up to 10 the inhibitory activity in all three antivibrio compounds was not much different and closely to their positive controls (unadjusted pH). The original pH values of antivibrio compounds were in a range of 7.5-7.8; it was surprisingly, the antivibrio compounds from all probiotic PNSB presented maximal

inhibitory activity at a low pH of 3 and 4. However, at pH 1 and 2 the antivibrio compounds were mostly destroyed as presented low inhibitory activity. It should be noted that, higher pH values from 5 to 10 produced the inhibitory activity similar with the controls although a little decrease was found.

The effects of high temperature on antivibrio activity against *V. harveyi*_KSAAHRC of bioactive compounds from probiotic PNSB are shown in Table 5-3. The antivibrio compounds from strain SS15 significantly decreased at 65°C that showed significant reduction ($P < 0.05$) of inhibition zone (11.67 ± 0.48 mm) compared with positive control at 30°C (13.08 ± 0.12 mm). Whilst, the antivibrio compounds from strain TKW17 significantly reduced ($P < 0.05$) inhibition zones by heating up to 75°C (12.82 ± 0.51 mm) compared to 30°C, 50°C and 65°C (14.08 ± 0.94, 13.68 ± 0.26 and 13.53 ± 0.41 mm). The antivibrio activity of antivibrio compounds from strain STW181 significantly decreased ($P < 0.05$) at 50°C as less inhibition zone (14.30 ± 0.19 mm) compared with the control (16.11 ± 0.32 mm). It was surprising that at 121°C for 15 and 20 min, the antivibrio compound from *R. sphaeroides* SS15 and TKW17 could still inhibit *V. harveyi*_KSAAHRC. In contrast, the antivibrio compounds from *A. marina* STW181 could inhibit shrimp pathogen only at 121°C for 15 min but could not inhibit the shrimp pathogen at 121°C for 20 min.

Table 5-1. Effects of enzymes on inhibitory activity against *V. harveyi*_ KSAAHRC of antivibrio compounds from probiotic PNSB.

A: Antivibrio activity

Treatment	Inhibition zone (mm)		
	R. sphaeroides SS15	*R. sphaeroides* TKW17	*A. marina* STW181
α-amylase	12.23 ± 0.35 ^{c}	13.80 ± 0.58^{bc}	14.02 ± 0.45 ^{bc}
proteinase K	12.62 ± 0.34^{bc}	13.98 ± 0.50 ^{b}	13.80 ± 0.15 ^{cd}
pronase	12.18 ± 0.68 ^{c}	13.05 ± 0.59 ^{c}	13.90 ± 0.10 ^{cd}
α-chymotrypsin	13.52 ± 0.20 ^{a}	13.57 ± 0.32 ^{bc}	13.40 ± 0.28 ^{d}
trypsin	13.23 ± 0.65^{ab}	15.15 ± 0.44 ^{a}	13.85 ± 0.13 ^{cd}
lysozyme	13.08 ± 0.16 ^{ab}	14.03 ± 0.60 ^{b}	14.48 ± 0.12 ^{b}
lipase	12.10 ± 0.31 ^{c}	13.47 ± 0.44 ^{bc}	13.82 ± 0.16 ^{cd}
Positive control	13.58 ± 0.23 ^{a}	15.82 ± 0.35 ^{a}	15.95 ± 0.64 ^{a}

Values are means ± S.D. of six determinations and values followed by different letters in each column are significantly different ($P < 0.05$).

B: Reduction percentage of antivibrio activity

PNSB	Reduction of inhibition zone (%)						
	α-amylase	proteinase K	pronase	α-chymotrypsin	trypsin	lysozyme	lipase
R. sphaeroides SS15	9.94	6.11	10.33	0.47	2.58	3.68	10.92
R. sphaeroides TKW17	12.75	11.59	17.49	14.23	4.21	11.28	14.86
A. marina STW181	12.12	13.48	12.85	15.99	13.17	9.20	13.38

Table 5-2. Effects of various pH values on inhibitory activity against *V. harveyi*_ KSAAHRC of antivibrio compounds from probiotic PNSB.

Treatment	Inhibition zone (mm)		
	R. sphaeroides SS15	*R. sphaeroides* TKW17	*A. marina* STW181
pH 1	4.86 ± 0.56 ^{g}	5.21 ± 0.24 ^{g}	7.25 ± 1.01 ^{g}
pH 2	4.81 ± 0.50 ^{g}	7.13 ± 0.57 ^{f}	9.84 ± 0.57 ^{f}
pH 3	15.54 ± 0.85 ^{a}	17.64 ± 0.31 ^{a}	21.68 ± 1.34^{a}
pH 4	14.40 ± 0.62 ^{b}	15.96 ± 0.59 ^{bc}	18.23 ± 0.59 ^{b}
pH 5	12.06 ± 0.57 ^{f}	14.17 ± 0.46 ^{e}	15.48 ± 1.37 ^{d}
pH 6	12.97 ± 0.57 ^{de}	13.83 ± 0.68 ^{e}	13.67 ± 0.57 ^{e}
pH 7	13.43 ± 1.05 ^{cd}	16.13 ± 0.38 ^{b}	12.75 ± 0.33 ^{e}
pH 8	12.63 ± 0.79 ^{ef}	15.27 ± 0.70 ^{cd}	14.69 ± 0.30 ^{d}
pH 9	14.03 ± 0.76 ^{bc}	15.01 ± 0.91 ^{d}	13.18 ± 0.19 ^{e}
pH 10	12.94 ± 0.35 ^{de}	13.71 ± 0.53 ^{e}	13.40 ± 0.25 ^{e}
Positive control (7.5-7.8)	13.23 ± 0.22 ^{de}	16.08 ± 0.09 ^{bc}	16.43 ± 0.61 ^{c}

Values are means ± S.D. of six observations and values followed by different letters in each column are significantly different ($P < 0.05$).

Table 5-3. Effect of heat on inhibitory activity against *V. harveyi*_KSAAHRC of antivibrio compounds from probiotic PNSB.

Treatment	Inhibition zone (mm)		
	R. sphaeroides SS15	*R. sphaeroides* TKW17	*A.marina* STW181
50 °C	13.08 ± 0.56 [a]	13.68 ± 0.26 [ab]	14.30 ± 0.19 [b]
65 °C	11.67 ± 0.48 [bc]	13.53 ± 0.41 [ab]	13.52 ± 0.76 [c]
75 °C	11.95 ± 0.71 [bc]	12.82 ± 0.51 [cd]	13.20 ± 0.12[c]
100 °C	11.78 ± 0.22 [bc]	12.47 ± 0.31 [d]	12.67 ± 0.18 [d]
121 °C15 min	12.23 ± 0.55 [b]	11.87 ± 0.58 [bc]	12.56 ± 0.31 [d]
121 °C 20 min	11.38 ± 0.38 [c]	11.65 ± 0.36 [e]	0 ± 0[e]
Positive control (30°C)	13.08 ± 0.12 [a]	14.08 ± 0.94 [a]	16.11 ± 0.32 [a]

Values are means ± S.D. of six observations and values followed by different letters in each column are significantly different ($P < 0.05$).

Inhibitory effect of PNSB supernatant against AHPND-causing *V. parahaemolyticus*

R. sphaeroides (SS15, TKW17) and *A. marina* STW181 produced the same MIC and MBC values against all strains tested of AHPND-causing *V. parahaemolyticus* (SR1, SR2 and SR3) at 106.67 mg mL^{-1} (Table 5-4). This means that antivibrio compounds from three probiotic PNSB acted as bactericidal action to control AHPND-causing *V. parahaemolyticus* tested. *V. parahaemolyticus* strain SR2 as a virulent strain to shrimp so it was selected for further studies as previously stated. Positive control, chloramphenicol showed MIC value as 1.0 µg mL^{-1} in three strains of *V. parahaemolyticus* SR1, SR2 and SR3, whereas MBC values of these strains as 2.0, 1.0 and 1.0 µg mL^{-1} , respectively (Table 5-4).

Bacteriolytic activity of antivibrio compounds from three probiotic PNSB was tested in both of live and heat killed cells of target organism (*V. parahaemolyticus* SR2). It was found that the antivibrio compounds from three PNSB strains showed low cell lytic activity that was observed from 2 h until 22 h at the end of treatment (Table 5-5). At 22 h of incubation, the highest cell lytic activity was found in heat killed cells (41.43%, +) and live cells (18.67%, -) which treated with antivibrio compounds from strain STW181.

The heat killed cells that treated with antivibrio compounds from *A. marina* STW181 were continually observed at the surface of *V. parahaemolyticus* SR2 using SEM. Figure 5-2 shows SEM profiles that *V. parahaemolyticus* SR2 had many holes or pits, particularly for heat killed cells (Figure 5-2D); this stated that one of the actions by antivibrio compounds was breaking cells.

Table 5-4. MIC and MBC values of antivibrio compounds from probiotic PNSB against AHPND-causing *V. parahaemolyticus*.

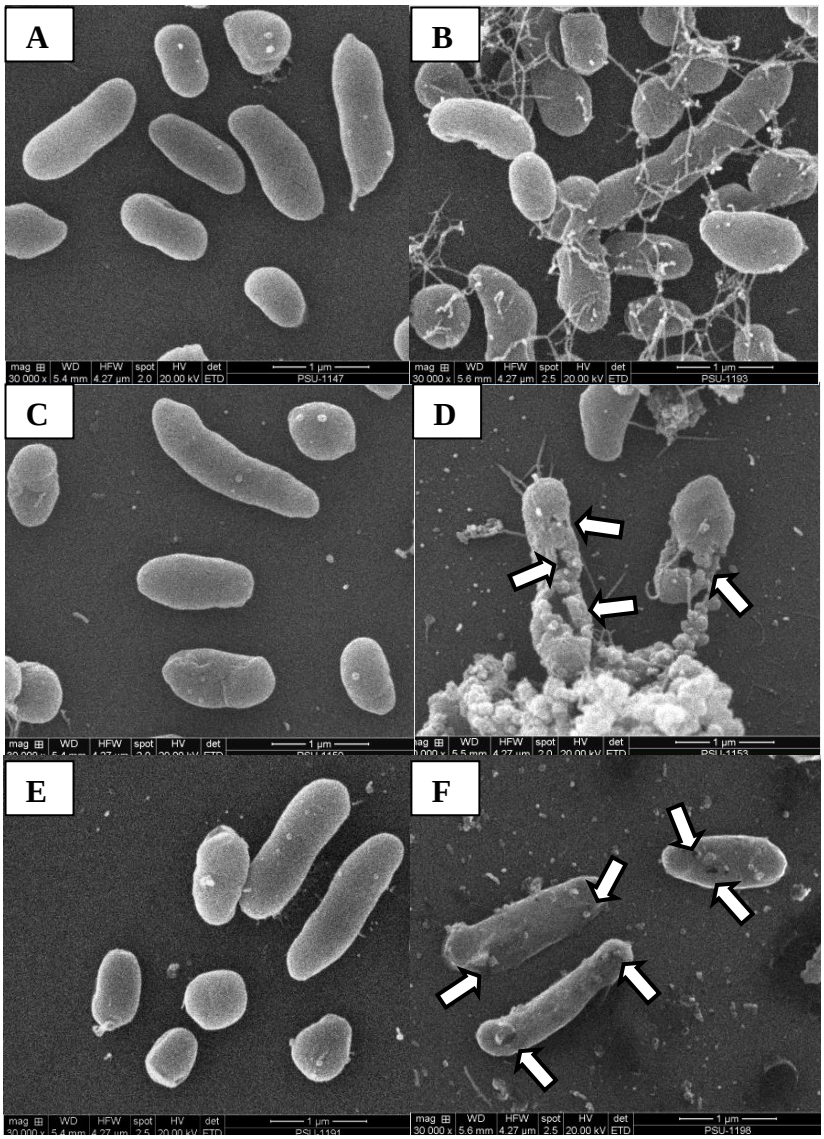
AHPND strains	MIC and MBC values (mg mL^{-1})					
	R. sphaeroides SS15		*R. sphaeroides* TKW17		*A. marina* STW181	
	MIC	MBC	MIC	MBC	MIC	MBC
V. parahaemolyticus SR1	106.67	106.67	106.67	106.67	106.67	106.67
V. parahaemolyticus SR2	106.67	106.67	106.67	106.67	106.67	106.67
V. parahaemolyticus SR3	106.67	106.67	106.67	106.67	106.67	106.67

*Chloramphenicol showed MIC value as 1.0 µg mL^{-1} in three strains of *V. parahaemolyticus* SR1, SR2 and SR3, whereas MBC values of these strains as 2.0, 1.0 and 1.0 µg mL^{-1}, respectively.

Table 5-5. The bacteriolytic activity of antivibrio compounds from probiotic PNSB against heat killed cells and live cells of *V. parahaemolyticus* SR2.

Incubation time (h)	Bacteriolytic activity					
	Heat killed cells			Live cells		
	R. sphaeroides SS15	*R. sphaeroides* TKW17	*A. marina* STW181	*R. sphaeroides* SS15	*R. sphaeroides* TKW17	*A. marina* STW181
0	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	- (2.93%)	- (2.38%)	nd
4	nd	nd	- (4.34%)	- (4.03%)	nd	- (1.41%)
6	nd	nd	- (8.24%)	- (5.49%)	- (4.57%)	- (2.61%)
8	nd	nd	- (10.41%)	- (2.01%)	- (0.75%)	- (11.24%)
10	- (1.30%)	nd	- (16.05%)	- (1.83%)	- (0.91%)	- (17.27%)
22	- (13.66%)	- (12.58%)	+ (41.43%)	- (4.21%)	- (4.02%)	- (18.67%)

Cell lytic activity was scored into four levels as follows; 0-24% (-), 25-49% (+), 50-74% (++) and 75-100% (+++).



⟼, at 30,000 X of magnifications, arrows indicate holes on bacterial cells. 1 µm

Figure 5-2. Scanning electron microscope photographs showing bacteriolytic activity of lyophilized cell free supernatant from *A. marina* STW181 against AHPND *V. parahaemolyticus* SR2; A-D: heat-killed cells and E-F: live cells; A and B for control at 0 and 22 h; C and E for treatments at 0 h; D and F for treatments at 22 h.

Some characterization of antivibrio compounds from probiotic PNSB

Culture supernatant of each probiotic PNSB strain was extracted with EtOAc and the results demonstrate that the antivibrio activity was found only in water layer of extraction. In the present study, the antivibrio compounds produced from the strain SS15 was chosen to study the characterization of antivibrio compounds because the water layer from this strain presented a better inhibitory activity than other strains at the concentration of 53.33 mg L^{-1}. The use of ultra-centrifugal filter to cut off the MW (3,000 Da) found that the permeate fraction had the MW less than 3,000 Da with ability to inhibit *V. parahaemolyticus* SR2. Oasis WCX (Weak Cation eXhance and reversed-phase sorbent) cartridge that suitable for high polar compound was used to purify the antivibrio compounds. The inhibitory activity of purified antivibrio compounds was observed in 2% HCOOH in 25% MeOH fraction including the sample fraction (unpurified compounds).

The results showed that the purified antivibrio compounds had more than one compound that showed the antivibrio activity. As the compounds were eluted in 2% HCOOH in 25% MeOH fraction, it means the compounds are weak cationic compounds; and MIC of the purified antivibrio compounds was 10.08 mg mL^{-1}. The specific activity of this fraction is higher 10X compared to the antivibrio compounds before purification. In addition, the sample fraction (MIC 106.67 mg mL^{-1}) also found the antivibrio activity; this suggests that some antivibrio compounds were able to pass through Oasis® WCX cartridge. It states the compounds from this fraction were not weak cation compound that may be neutral compound. The results of developed TLC plates found that the purified weak cation compound was an organic compound with $-NH_2$ group because the spot presented when sprayed with ninhydrin reagent (Figure 5-3). The R_f value of the antivibrio compound was 0.35, which showed different R_f values from reference standards (L-proline, R_f = 0.23 and Guanidine-HCL, R_f = 0.50)

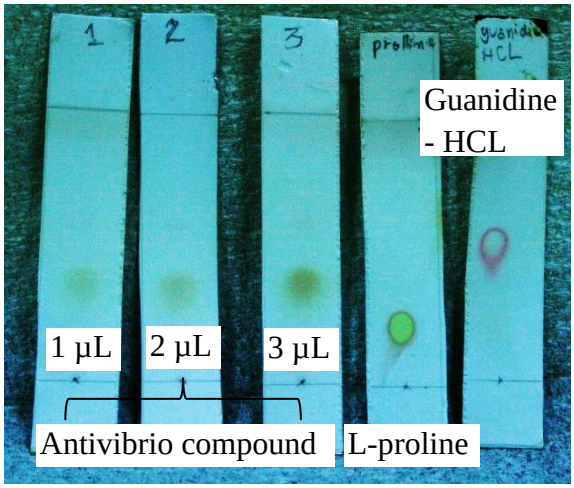


Figure 5-3. Thin layer chromatography of purified antivibrio compounds from *R. sphaeroides* SS15 and standards (L- proline and Guanidine-HCL) presented under developing solvents with butanol: acetic acid: water (4: 1: 1, v/v/v) as mobile phase and sprayed with ninhydrin solution.

Discussion

Optimal conditions of antivibrio compound production by probiotic PNSB

It is well recognized that natural products are any substances produced by living organisms such as animals, plants or microorganisms (Mann et al., 1994). They consist of primary and secondary metabolites; primary metabolite products are the fundamental units of all living organisms such as carbohydrates, proteins, fats, and nucleic acids (Dewick et al., 2009). On the other hand, secondary metabolites commonly are not essential for growth and reproduction, but they rather influence long-term survival by affecting the organism's interactions with its surrounding environment (Williams et al., 1989). In general, the production of bioactive compounds is not detected during log phase because microorganisms grow fast when nutrient are abundant and bioactive compound production takes place only after rapid growth has

deceased (Carvalho et al., 2010). However, some research reported that bioactive compound production could start in the mid log, late log phase and continued in the stationary phase (Bundale et al., 2015). In this study, all three probiotic PNSB produced antivibrio compounds in the log phase (middle and late) as previously described. This is a good sign for applying these probiotics in shrimp cultivation to protect shrimp during cultivation from vibrios as the evidences in our previous studies (Chumpol et al., 2017a, b).

As PNSB can grow as both phototrophs (autophototrop /photoheterotroph) and heterotrophs depending on the environmental conditions (Uffen and Wolfe, 1970; Lu et al., 2011); thereby, the optimum conditions for producing antivibrio compounds by probiotic PNSB were determined under both microaerobic-light and aerobic-dark conditions. It should be noted that no significant differences ($P > 0.05$) for antivibrio activity against the growth of *V. harveyi*_KSAAHRC was observed for both growth conditions by all probiotic PNSB tested (Figure 5-1). Indeed, initial pH was not critical point for probiotic PNSB tested to produce antivibrio compounds because a range of initial pH test allowed the growth of these PNSB; and they produced the antivibrio compounds in their log phase (Figure 5-1 A-B). Due to these pH values are those normally occur in shrimp cultivation (Anh et al., 2010). However, different probiotic PNSB preferred different light intensities; However strain STW181 producing the highest inhibition against both target organisms over a wide range of light intensity from 3,000-4,500 lux. All probiotic PNSB either single or as a mixed culture should be good candidates for controlling shrimp pathogenic *Vibrio* spp. as their antivibrio activity might be increased under optimized conditions which were with the same as those required for shrimp cultivation based on our previous study (Chumpol et al., 2017a, b).

Preliminary studies on the property of antivibrio compounds from probiotic PNSB

Up to date there is no report about the principle properties of antivibrio compounds from PNSB. According to the proteolytic, lipase and α-amylase enzymes caused a remarkable antivibrio activity compared with other enzymes, the present study revealed that most components of antivibrio compounds were proposed as protein, lipid and carbohydrate for strain SS15 while protein and lipid for strain TKW17 and STW181 (Table 5-1A and 5-1B). The antivibrio compounds from strain SS15 was probably glycolipoprotein; while strains TKW17 and STW181 were perhaps lipoprotein or glycolipoprotein components due to the compounds treated with the pronase and α-chymotrypsin caused loss of the inhibitory activity roughly 17 and 16%, respectively. These compounds were also presented as lipid component because the compounds treated with lipase lost the inhibitory activity approximately 11%, 15% and 13% for strains SS15, TKW17 and STW181, respectively (Table 5-1B).

This study indicates the antivibrio compounds from all three probiotic PNSB were stable at low pH as 3 (Table 5-2) and heat (Table 5-3) that was similar with weissellin, a bacteriocin produced by *Weissella confuse* (Goh and Philip, 2015). PNSB such as *R. capsulatus*, *R. sphaeroides* and *Rhodopseudomonas palustris* were reported to have ability for producing bacteriocins (Lee et al., 2009). In general, property of bacteriocins from any bacteria including PNSB is active against only strains of the same or related species (Kaspari and Klemme, 1977); so it should have more information to support by characterization of antivibrio compounds from our probiotic PNSB.

Inhibitory effect of PNSB supernatant against AHPND-causing *V. parahaemolyticus*

Strains SS15 and TKW17 showed the same level for bacteriolytic activity against *V. parahaemolyticus* that was much lower than strain STW181. This might be that both strains (SS15 and TKW17) were the same genus and species as *R. sphaeroides*. It suggests that *A. marina* STW181 was more effective for bacteriolytic activity than that found in *R. sphaeroides.*

The antivibrio compounds from strain STW181 damaged on the heat-killed cells rather than the live cells (Table 5-5, Figure 5-2D and 5-2F). It might be that the lytic enzyme activity normally produced stronger damage on heat killed cells than live cells (Zhang et al., 2014) and this study proved that heating at $\geq 50\ ^{\circ}C$ for 30 min was sufficient to kill *V. parahaemolyticus* SR2.

All probiotic PNSB produced low lytic enzymes against *V. parahaemolyticus* SR2 (Table 5-5). This might be that cell wall of Gram negative bacteria consist of single layer of peptidoglycan and surround by outer membrane to act as a protective mechanism against bioactive compounds or drugs into the cells; so this make them more resistant to bacteriolytic enzymes than Gram positive bacteria (Rattanachuay et al., 2010; Wang et al., 2008). At 22 h of incubation, the highest cell lytic activity was found in treated cells (both dead and live cells) with lyophilized cell free supernatant from *A. marina* STW181 although with low efficiency. This suggests that in antivibrio compounds from probiotic PNSB had bacteriolytic activity to inhibit shrimp pathogenic vibrios.

Some characterization of antivibrio compounds from probiotic PNSB

This is the first report for the characterization of antivibrio compounds derived from PNSB that the active fractions presented in the water layer after extraction. This means that the antivibrio compounds are high polar

compounds. The antivibrio compounds from *R. sphaeroides* SS15 containing more than one compound to inhibit shrimp pathogenic strain. One active fraction from Oasis®WCX cartridge was a weak cation compound and this fraction also presented amino groups (-NH_2). In this present study, incomplete characterization of the antivibrio compound from strain SS15 that will be further studied. It should be noted that, antibacterial compound produced by *Rhodobacteraceae bacterium* isolated from sponge had the chemical structure as a new amino compound namely N-benzyl-2-methoxy-N-(2-(4-nonylphenoxy) ethanamine which showed moderate activity against pathogenic *Vibrio eltor*, *Bacillus subtilis* and *Staphylococcus aureus* (Murniasih et al., 2014).

Conclusions

Three probiotic PNSB (*R. sphaeroides* SS15, TKW17 and *A. marina* STW181) are able to produce antivibrio compounds against *Vibrio* spp. as causative agents of luminous vibriosis and AHPND; and their optimal conditions for producing the antivibrio compounds like shrimp growth conditions. Most components of antivibrio compounds from all PNSB tested were protein, lipid and carbohydrate; and their antivibrio activity was stable under wide variation of pH from 3 up to 10, and high temperature up to 121 ºC for 20 min with exception of strain STW181. The antivibrio compounds acted as bactericidal action and also showed bacteriolytic activity. One of antivibrio compounds from strain SS15 was proven as a weak cationic compound with presenting-NH_2 group. These antivibrio compounds have the possibility to be used for controlling serious pathogenic vibrios in shrimp cultivation.

CHAPTER 6

Administration of purple nonsulfur bacteria as single cell protein by mixing with shrimp feed to enhance growth, immune response and survival in white shrimp (*Litopenaeus vannamei*) cultivation

Abstract

Single cell protein (SCP) is an alternative way to increase nutrients for animal consumption; and purple nonsulfur bacteria (PNSB) should be considered as SCP due to their rich sources of protein, vitamins and photopigments. Hence, the aim of this study was to investigate the potential of promising PNSB to be used as SCP by mixing with commercial shrimp feed for white shrimp cultivation starting from postlarval until early juvenile stages for 60 days. PNSB strains, *Rhodobacter sphaeroides* SS15 and *Afifella marina* STW181, were selected to use as SCP at a ratio of 1:1; and their lyophilized cells at 1, 3 and 5% (w/w) were mixed well with commercial shrimp feed to obtain modified shrimp feed recipes; Diet 1, Diet 2 and Diet 3, respectively. Levels of NH_4^+, NO_2^-, NO_3^- and COD in rearing water from Diet 2 and 3 sets were significantly higher than control set. However, Diet 1 set showed the lowest levels of these water parameters among modified diet sets as no significant difference levels of NO_3^- and COD between Diet 1 and control sets. Shrimp growth performance on the basis of relative gain rate and other growth parameters found that Diet 1 set was much better than sets of Diet 2, Diet 3 and control with the lowest found in the control set. In addition, the maximum shrimp survival was observed in Diet 1 set (85%) although no significant difference among them as in control set (80%). Shrimp in all modified shrimp diet sets showed increases all of immune responses (total haemocyte count, and

activities of phenoloxidase, superoxide dismutase); the results of hepatopancreas (HP) histopathology analysis also showed a good condition of HP as healthy shrimp. PNSB biomass as SCP has the potential not only to enhance shrimp growth but also to increase immunostimulants for increasing shrimp survival.

Keywords: Growth performance, Immune response, Purple nonsulfur bacteria, Shrimp, Single cell protein, Water quality.

บทคัดย่อ

โปรตีนเซลล์เดียว (Single cell protein; SCP) เป็นทางเลือกหนึ่งที่น่าสนใจในการเพิ่มคุณค่าสารอาหารให้แก่สัตว์ และแบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ (Purple nonsulfur bacteria; PNSB) เป็นกลุ่มแบคทีเรียที่ควรพิจารณานำมาใช้เป็น SCP เนื่องจากเซลล์มีปริมาณโปรตีนสูง และยังมีรงควัตถุสังเคราะห์แสงด้วย ดังนั้นวัตถุประสงค์ในการศึกษานี้เพื่อตรวจสอบศักยภาพของ PNSB เพื่อใช้เป็น SCP โดยผสมกับอาหารกุ้งขาว และนำมาใช้เพาะเลี้ยงกุ้งขาวตั้งแต่ระยะวัยอ่อน (Postlarvae) จนถึงระยะวัยรุ่น (Early juvenile) เป็นเวลา 60 วัน โดยคัดเลือกได้ PNSB 2 สายพันธุ์ ได้แก่ *Rhodobacter sphaeroides* SS15 และ *Afifella marina* STW181 มาใช้เป็น SCP ผสมกันในอัตราส่วน 1:1 โดยใช้ในรูปไลโอฟิไลซ์เซลล์ผสมกับอาหารกุ้งทางการค้าที่ 1% 3% และ 5% ของน้ำหนักอาหารกุ้ง ได้อาหารดัดแปลง 3 สูตร ได้แก่ อาหารสูตรที่ 1 สูตรที่ 2 และสูตรที่ 3 ตามลำดับ ผลการทดลองพบว่าปริมาณ NH_4^+ NO_2^- NO_3^-และ COD ของน้ำเลี้ยงกุ้งจากบ่อที่ให้อาหารสูตรที่ 2 และ 3 มีค่าสูงกว่าชุดควบคุมอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามน้ำเลี้ยงกุ้งจากบ่อที่ให้อาหารสูตรที่ 1 มีค่าพารามิเตอร์ดังกล่าวต่ำที่สุดเมื่อเทียบกับชุดที่ใช้อาหารสูตรดัดแปลงด้วยกัน และค่า NO_3^{-1}และ COD ของน้ำเลี้ยงกุ้งจากบ่อที่ให้อาหารสูตรที่ 1 และชุดควบคุมมีค่าไม่แตกต่างอย่างมีนัยสำคัญทางสถิติ การเจริญเติบโตของกุ้งพิจารณาจากอัตราการเพิ่มสัมพัทธ์ (relative gain rate) และพารามิเตอร์การเจริญเติบโตอื่นๆ พบว่ากุ้งที่ให้อาหารสูตรที่ 1 มีการเจริญเติบโตดีมากกว่ากุ้งที่ให้อาหารสูตรที่ 2 3 และกุ้งในชุดควบคุม โดยพบว่ากุ้งในชุดควบคุมมีการเจริญเติบโตต่ำสุด นอกจากนี้เปอร์เซ็นต์การอยู่รอดของกุ้งสูงสุดพบในชุดอาหารที่ 1 เท่ากับ 85% แต่ค่าที่ได้ไม่แตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มอื่นของชุดการทดลอง ซึ่งกุ้งในชุดควบคุมมีเปอร์เซ็นต์การอยู่รอด เท่ากับ 80% โดยกุ้งในทุกชุดการทดลองที่ให้อาหารสูตรดัดแปลงพบว่ามีการตอบสนองของระบบภูมิคุ้มกันสูงขึ้น (ปริมาณเม็ดเลือดรวมและกิจกรรมของเอนไซม์ฟีนอลออกซิเดส รวมทั้งเอนไซม์ซูเปอร์ออกไซด์ ดิสมิวเทส) นอกจากนี้ผลของเนื้อเยื่อตับและตับอ่อนของกุ้ง (Hepatopancreas) ในทุกชุดที่ให้อาหารสูตรดัดแปลงมีลักษณะปกติ ซึ่งบ่งชี้ได้ว่ากุ้งมีสุขภาพดี ดังนั้นชีวมวล PNSB ที่ใช้เป็น SCP ไม่เพียงแต่มีศักยภาพในการส่งเสริมการเจริญเติบโตของกุ้ง แต่ยังส่งผลกระตุ้นระบบภูมิคุ้มกันให้สูงขึ้นทำให้เพิ่มการอยู่รอดของกุ้ง

คำสำคัญ: การเจริญเติบโต ระบบภูมิคุ้มกันกุ้ง แบคทีเรียสังเคราะห์แสงสีม่วงที่ไม่สะสมซัลเฟอร์ กุ้ง โปรตีนเซลล์เดียว คุณภาพน้ำเลี้ยงกุ้ง

Introduction

Aquaculture is one of the important sources of food, nutrition, and livelihoods for hundreds of million people around the world. Amongst them, shrimp production is second ranked of the most-traded production in these decades; whereas fishery production ranks first. Asia's shrimp farms show fast growing for supporting people demand, although they face with a decline in output because of shrimp diseases (FAO, 2016). Recently, productions of shrimp broodstock and shrimp larvae are characterized by the universal use of a combination of live and/or processed natural feed items, including marine polychaetes, hermit crabs, Artemia biomass, oysters, and formulated dry (Tacon, 2017). Despite the widespread use of live and fresh unprocessed shrimp feeds, but there are many diseases that can transmit to shrimp through the use of raw materials such as hermit crabs present a risk factor for white spot syndrome virus (WSSV) through vertical virus transmission to shrimp cultivation because hermit crab is a common natural host of WSSV (Chang et al., 2012). For the risk of shrimp diseases from the current use of live feeds, it is clear that the commercial shrimp feed manufacturing industry must step up to produce nutritionally complete formulated feeds for the entire shrimp production cycle, from first feeding larvae to maturing broodstock as replacement for the use of live feeds (Tacon, 2017).

Shrimp feed consists of protein, lipid and carbohydrate as a major composition with a minor composition of vitamin, mineral and water; and shrimp requires 35 – 55% protein in their feed (Kanazawa, 1989; Goddard, 1996). Shrimp growth in each stage requires high protein diet although at different concentrations such as 48% for a juvenile stage and only 32% for a sub-adult stage of *Litopenaeus vannamei* (Kureshy and Davis, 2002). The low protein content in shrimp feed causes low growth rate and weight loss because protein in muscle is used as no sufficient of protein in feed; while high protein

content, shrimp has an excessive protein that is used for supplying energy and eliminating nitrogen in a form of ammonia/ammonium ion in rearing water (Goddard, 1996). High amount of biologically complete protein is required in shrimp feed which leads to high cost as its source normally originate from fish meal protein rather than plant protein; thereby, exploring sources of protein for producing inexpensive shrimp feed with high efficiency to enhance shrimp growth has been extensively studied.

According to above information, microbes should be considered to use as a protein source in order to replace the sources of common protein in shrimp feed as their high protein that consist of essential amino acids including vitamins. Single cell protein (SCP) is the protein production from biomass, originating from many species of microorganisms, including algae, fungi and bacteria (Nasseri et al., 2011). Among microbes, bacteria are the most attractive for use as a source of SCP because they rapidly grow in various substrates such as by-products from agro-industry and wastewaters (Ravinda, 2000). Purple nonsulfur bacteria (PNSB) are one of candidates that can be utilized as SCP because they are nutritious and non-toxic to host (Azad et al., 2002). Their cells are not only high in biologically complete protein but also in photopigments and vitamins (Kim and Lee, 2000; Kantachote et al., 2005; Kornochalert et al., 2014). As versatile organisms, PNSB are able to grow in a variety of growth modes such as photoautotroph/ photoheterotroph under anaerobic/ microaerobic-light conditions and heterotroph under aerobic-dark conditions (Panwichian et al., 2010; Kornochalert et al., 2014); so it would be possible to produce mass biomass from PNSB for using as SCP with low cost.

Water quality during shrimp cultivation is a very important factor to concern for a design diet formulation which approaches to balance feed utilization and feed wastage for healthy shrimp and also an environmentally friendly maintenance (Cho et al., 1994). It is well recognized that health status

of shrimp depends on rearing water (Xiong et al., 2016; Zhang et al., 2016; Chumpol et al., 2017a, b) as inappropriate water quality directly causes low growth rate, stressful and weak shrimp that stimulate pathogenic infection and high mortality (Lightner et al., 2006). Shrimps are invertebrate animals that depend on innate immune responses to combat invading microbes and lack of an adaptive immune system of no lymphocytes and functional immunoglobulin (Rowley and Powell, 2007). Major immune reactions of shrimp take place in hemolymph as several immune molecules are produced and stored in the granules of hemocyte before releasing into the hemolymph such as phenoloxidase (PO), antimicrobial peptides (AMPs) and superoxide dismutase (SOD) (Tassanakajon et al., 2013). It would be great to induce strong innate defense mechanisms for resistance to pathogens on the basis of immunostimulants by bacterial preparation in dietary feed (Barman et al., 2013; Wang et al., 2017). Therefore, this study aimed to investigate the possibility of promising PNSB to be used as SCP by mixing with commercial shrimp feed at different levels and their effects on water quality, growth performance and immunity of white shrimp (*Litopenaeus vannamei*) cultivation.

Materials and methods

Bacterial strains used and culture preparation

To investigate PNSB for considering as a potential SCP, PNSB used in this study included *Rhodobacter sphaeroides* strains; SS15, S3W10, TKW17 and *Afifella marina* STW181. Regarding our previous studies, they were isolated from shrimp ponds in the south of Thailand and were proved for their probiotic PNSB (Chumpol et al., 2017a, b). In this study, these PNSB were tested as a source of protein for expecting a novel shrimp feed to provide another choice for using promising PNSB for shrimp cultivation. Each PNSB strain was separately cultured in duran bottles containing 500 mL of basic

isolation medium (BIM + 1.5% NaCl) for *R. sphaeroides* and glutamate acetate medium (GA + 2% NaCl) for *A. marina* (Chumpol et al., 2017a) and incubated under microaerobic-light conditions with tungsten light at 3,500 lux for 48 h. Each culture broth was centrifuged at 10418 x *g* for 10 min (Sorval, RC 5C plus, USA) to obtain a cell pellet and then washed three times with 0.85% normal saline solution (NSS), and finally lyophilized to obtain dried cells (lyophilized cells) for the use as SCP in this study.

Proximate analysis of PNSB and shrimp feeds

Pure culture of each PNSB strain was determined its proximate analysis including crude protein, crude lipid, crude fiber, moisture and ash using standard methods (AOAC, 2010). Crude protein was analyzed using Kjeldahl Method; while crude fat was analyzed by soxhlet extraction method. Fiber analyzer (Anikom^{200}, USA) was used to analyze crude fiber. Moisture content was analyzed by drying at 135 ± 2 ºC; and ash was analyzed by burning at 550-600 ºC. Carbohydrate content was calculated by deduction from a total 100% of nutrient contents (protein + fat + moisture + ash). The different shrimp feeds, commercial shrimp feed (control) and a mixture of control with different amounts of PNSB addition, were investigated for their proximate analysis according to the methods as previously described. Among 3 strains of *R. sphaeroides* only one strain that showed maximal protein content was selected for the analysis of its amino acid profile. However, *A. marina* STW181 was also selected to analyze the amino acid composition as it might have a different amino acid profile from *R. sphaeroides*.

Amino acid profiles of selected PNSB

Each PNSB was grown as previously mentioned in section 2.1. Amino acid analysis was carried out by using 0.2 g of lyophilized cells to digest by adding 5 ml 6 N HCl and incubated at 110 °C for 22 - 24 h under vacuum. The hydrolyzed sample was adjusted to 10 mL by 0.1 N HCL and filtered through

Whatman No. 40, and finally diluted with sodium citrate buffer. A 20 µL of sample solution was injected to HPLC-LC-10ADvp (model LC-20A Series, Shimadzu, Japan, Shim-pack ISC-07/S 1504 Na) by following the conditions according to Kantachote et al. (2015).

Pigment analysis of PNSB

PNSB were cultured under microaerobic-light conditions for 48 h to obtain cell pellets as previously described in section 2.1. Each cell pellet collected from 10 mL of culture broth was washed twice with distilled water to remove residual medium and it was used for bacteriochlorophyll *a* (Bchl *a*) extraction using 1 mL acetone/methanol solvent (7:2, v/v). A mixture was left for 1 h at room temperature in dark; and Bchl *a* was measured by UV-visible spectrophotometer (Genesys 10S, Thermo scientific) at a wavelength of 771 nm. The amount of Bchl *a* was calculated according to the following formula; Bchl *a* content (mg L^{-1}) = ($ADV_1 /76V_2$) where A: the absorbance of Bchl *a* in diluted extract solution at 771 nm, D: the dilution ratio, V_1: the volume of extracted solvent added, V_2: the volume of PNSB culture broth (Zhou et al., 2014). The determination of extracted carotenoids was also investigated by acetone solvent as acetone was added in a tube and ultrasonic power was set at 390 W for 6 min to break cells, and the tube was kept in water bath, 20 °C for 10 min. The mixture was centrifuged at 10418 *g* for 10 min; and supernatant was used to determine content of total carotenoids using UV-visible spectrophotometer at 480 nm with the formula as follows. Carotenoids yield (mg L^{-1}) = $ADV_1 /0.16V_2$, where A: the absorbance of diluted extract solution at 480 nm, D: the dilution ratio, V_1: the volume of acetone added, 0.16 is extinction coefficient of carotenoids, and V_2: the volume of PNSB culture broth (Zhang and Hu, 2015).

Modified shrimp feed with SCP from PNSB for white shrimp cultivation

PNSB preparation for use as SCP in shrimp feed

R. sphaeroides SS15 and *A. marina* STW181 were selected to use as SCP because the former showed the highest amount of protein in cell composition; but the latter showed higher levels of six essential amino acids. Hence, a mixture of both strains at a ratio 1: 1 was designed with levels of 1, 3 and 5% for mixing with commercial shrimp feed; and lyophilized cells were used as SCP. To prepare modified shrimp feed, lyophilized cells of each selected PNSB was mixed with a commercial shrimp feed at the designed concentration and conjugated with 1% soy bean oil for Diet 1: 1% SCP (commercial shrimp feed mixed with both selected strains at each 0.5%). Diet 2 and Diet 3 were prepared as the same as Diet 1 by mixing 3 and 5% SCP with commercial shrimp feed. These diets are called modified shrimp feed with PNSB.

Experimental design

A postlarvae-15 stage of white shrimp (*Litopenaeus vannamei*) was used in this study. All shrimp aquaria made from glass with a size of 25 cm x 20 cm x 15 cm, and 7.5 L capacity, thirty shrimp were added randomly (600 shrimp m^{-2}; intensive cultivation) in each aquarium. There were four sets, including one control set (commercial shrimp feed) and three treatment sets of modified shrimp feed recipes (Diet 1, Diet 2 and Diet 3 represent for 1, 3 and 5% SCP). Modified shrimp feed recipes and the commercial shrimp feed were used to investigate the effects on growth performance immunity of shrimp and water quality. Shrimp were acclimated for 3 days in commercial artificial sea water (ASW) which adjusted to an initial pH of 7.8, an initial salinity of 15 ppt and a water temperature of between 23 and 25 $^{\circ}$C. They were fed four times per day (7.00 am, 12.00 am, 16.00 pm and 20.00 pm) at roughly 2% of total body weight with the commercial shrimp feed and modified shrimp feed recipes as

previously stated. This experiment was carried out for 60 days of shrimp cultivation under three replicates

Water quality during shrimp cultivation

Prior to exchange water, water samples were collected every week including at the starting for investigating the effect of modified shrimp feed with PNSB on water quality during shrimp cultivation with a 50% water exchange in each week. The following parameters; pH, salinity, temperature and dissolved oxygen (DO) were measured daily using pH meter (Sartorius AG, Germany), salinometer (Salinity Refractometer, Japan), thermometer and DO meter (SevenGo Pro, Mettler Toledo, Switzerland). Moreover, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and chemical oxygen demand (COD) were determined using Spectroquant® photometric test kit (Merck, Germany) at the beginning of experiment and every week until the end of cultivation. These test kits are analogous with standard methods, including NH_4^+ (No. 1.14752.0001, EPA 350.1, APHA 4500-NH_3 D), NO_2^- (No. 1.14776.0001, EPA 354.1, APHA 4500-NO_2^- B), NO_3^- (No. 1.14773.0001, APHA 4500-NO_3^- B) and COD (No. 1.14540.001, EPA 4.10.4, APHA 5220 D). For COD measurement, NaCl in water sample was eliminated as previously described (Chapter 4).

Growth performance of white shrimp and survival rate

Shrimp growth performance was evaluated by measurements on the parameters as follows: weight (g), total length (mm), shrimp survival for every 15 days and also calculations; weight gain (WG; g), diary weight gain (DWG; g day^{-1}), length gain (LG; mm), daily length gain (DLG; mm day^{-1}), relative gain rate (RGR; %), feed conversion ratio (FCR) and specific growth rate (SGR; % day^{-1}) at the end of experiment by following these formulas (Wang, 2011; Zokaeifar et al, 2012);

WG (g) = Wt - Wi

DWG (g day^{-1}) = $\left(\frac{Wt - Wi}{Wi}\right)/t$

LG (mm) = $\left(\frac{Lt - Li}{Li}\right) x\ 100$

DLG (mm day^{-1}) = $\left(\frac{Lt - Li}{Li}\right)/t$

RGR (%) = $\left(\frac{Wt - Wi}{Wi}\right) x\ 100$

FCR = Total Feed Given (g)/ Weight Gain (g)

SGR (%) = $\left(\frac{In\ Wt - In\ Wi}{time}\right) x\ 100$

Survival rate (%) = (Final numbers/Initial numbers) x 100

where Lt, Li represent the length at sampling and initial length, Wt, Wi represent the weight at sampling and initial weight, and time represent the cultivation day (t = 60).

Shrimp immunity

All shrimp in each set at day 60 was used to study immune responses, including total hemocyte count, phenoloxidase and superoxide dismutase as follows.

Total hemocyte count (THC)

Hemolymph was sampled individually withdrawn from the ventral sinus of each shrimp into a 1 mL sterile syringe needle (27 G x $\frac{1}{2}$) containing anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55). The anticoagulant-hemolymph was well mixed with 0.15% trypan blue and placed on a hemacytometer to measure THC (Liu and Chen, 2004).

Phenoloxidase activity (PO)

PO activity was measured using spectrophotometric method by recording the formation of dopachrome produced from L-

dihydroxyphenylalanine (L-DOPA; 4 mg mL^{-1}). Hemolymph was centrifuged at 1000 *g* at 4 °C for 20 min to obtain pellet; and it was re-suspended gently in cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and centrifuged again. The pellet was then re-suspended with 200 µL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 0.01 M $CaCl_2$, 0.26 M $MgCl_2$, pH 7.0). Then a 100 µL suspension was mixed with 50 µL trypsin (1 mg mL^{-1}), which served as an activator, at 25 °C for 10 min; then 50 µL of L - DOPA was added, followed by 800 µL of cacodylate buffer for 5 min. PO measurement was measured the optical density at 490 nm using a spectrophotometer (Genesys 10S, Thermo scientific). The control solution consisted of 100 µL of suspension, 50 µL of cacodylate buffer (to replace the trypsin) and 50 µL of L-DOPA, were used to measure the background phenoloxidase activity in all test solutions. The optical density of the shrimp's PO activity was expressed as dopachrome formation in 50 µL of hemolymph (Lin and Chen, 2004).

Superoxide dismutase (SOD)

Hemolymph was prepared as the same as for determination PO activity to obtain the pellet that was re-suspended with 0.2 mL cacodylate buffer and then adding the compounds as following provided to assay superoxide dismutase (SOD). The reaction system contained 1.2 mL 50 mM potassium phosphate buffer (pH 7.4), 0.1 mL 33 mM phenazinemethosulfate (PMS), 0.3 mL 50 µM nitrobluetetrazolium (NBT), 0.2 mL hemolymph sample, and water in a total volume of 2.8 mL, and finally added 0.2 mL of NADH. These tubes were incubated at 30°C for 90 sec and stopped by the adding 1.0 mL of glacial acetic acid. Then, adding 4 mL of butanol into the reaction mixture and vortex vigorously and kept for 10 min. The SOD activity was measured the optical density at 560 nm by a spectrophotometer (Genesys 10S, Thermo scientific). Blank was also conducted with the use of 0.2 mL of 50 mM potassium phosphate buffer instead of the sample. One unit of SOD defined as

requirement of SOD concentration to inhibit the optical density of 50% reduction of chromogen (Kakkar et al., 1984).

Histological analysis of hepatopancreas from white shrimp

A total of 24 shrimp bodies from all experimental sets (2 shrimp x 3 replication x 4 sets), after hemolymph collection, were used for preparing hepatopancreas as each shrimp was separated out and fixed in Davidson's AFA (330 mL 95% ethanol, 220 mL 100% formalin, 115 mL acid acetic, 335 mL distilled water and pH 4) for overnight (Andrade et al., 2008). After that, these tissues were transferred to 70% ethanol and passed through a dehydrating series of ethanol. Tissues were embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E) (Catap et al., 2003). All samples were histologically investigated for the HP by a light microscope (Olympus BX51, model BX51TRF, Japan).

Statistical analysis

All data were obtained from three replicates and are expressed as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used to analyze the significant differences among data at the level of $P < 0.05$; and the pairwise comparison was performed using the Duncan's multiple-range test (DMRT). However, the shrimp survival (%) was analyzed using Kruskal-Wallis test because the data was not in normal distribution. All statistics were analyzed using SPSS program version 11.5 (Lead Technologies, USA).

Results

Proximate and photopigment analyses of PNSB used

Table 6-1A shows proximate analysis (% dry basis, 100 g^{-1}) of four PNSB and significant differences ($P < 0.05$) as the maximal protein content in strain SS15 (53.98 ± 0.08%), followed by strain TKW17 (49.06 ± 0.10%), strain S3W10 (47.86 ± 0.08%) and strain STW181 (46.38 ± 0.06%). Ash content in each PNSB was in the same pattern with protein content. In contrast, lipid content showed the opposite results with protein and ash as the highest was observed in the order of strains S3W10 > STW181 > TKW17 > SS15. Fiber content in strain TKW17 was significantly higher than that found in other strains (see detail in Table 6-1A).

Bacteriochlorophyll *a* and carotenoid contents in four PNSB strains are presented in Table 6-1B; and significant differences were found among them. The results showed that strain SS15 produced the highest amount of bacteriochlorophyll *a* and also carotenoid (9.26 ± 0.72 and 6.87 ± 0.04 mg L^{-1}), followed by strain TKW17 (4.68 ± 0.38 and 4.60 ± 0.11 mg L^{-1}), strain S3W10 (3.43 ± 0.04 and 3.87 ± 0.12 mg L^{-1}) and strain STW181 (3.48 ± 0.07 and 2.27 ± 0.10 mg L^{-1}).

Table 6-1. Proximate analysis of cell composition and pigment analysis in PNSB strains.

	PNSB strain			
	***R. sphaeroides* SS15**	***R. sphaeroides* S3W10**	***R. sphaeroides* TKW17**	***A. marina* STW181**
A: Proximate analysis (% Dry basis; g 100 g^{-1})				
Protein	53.98 ± 0.08^{a}	47.86 ± 0.08^{c}	49.06 ± 0.10^{b}	46.38 ± 0.06^{d}
Lipid	0.34 ± 0.01^{d}	1.94 ± 0.08^{a}	0.57 ± 0.15^{c}	1.51 ± 0.16^{b}
Moisture	7.22 ± 0.16^{c}	14.18 ± 0.01^{b}	2.87 ± 0.11^{d}	16.31 ± 0.03^{a}
Fiber	0.12 ± 0.02^{b}	0.20 ± 0.09^{b}	0.86 ± 0.07^{a}	0.12 ± 0.02^{b}
Ash	20.73 ± 0.04^{a}	18.34 ± 0.03^{c}	20.26 ± 0.04^{b}	15.41 ± 0.01^{d}
B: Pigment analysis (mg L^{-1})				
Bacteriochlorophyll *a*	9.26 ± 0.72^{a}	3.43 ± 0.04^{c}	4.68 ± 0.38^{b}	3.48 ± 0.07^{c}
Carotenoids	6.87 ± 0.04^{a}	3.87 ± 0.12^{c}	4.60 ± 0.11^{b}	2.27 ± 0.10^{d}

Values are presented as a mean of three replicates ± S.D., and significant differences are analyzed at $P < 0.05$. Different letters indicate significant differences for values in each row.

Amino acid profiles of selected PNSB

As previously described in section 2.2, there were selected only strains SS15 and STW181 for investigating their amino acid profiles. This is because strain SS15 had the highest amount of protein. However, strain STW181 had the lowest protein content and it belongs to *A. marina* that it might have different amino acid profiles from *R. sphaeroides* strains; SS15, S3W10 and TKW17. The pattern of ten essential amino acids (EAA) requirement for shrimp of 2 selected PNSB (Table 6-2), presented that strain SS15 showed four EAA in g 100 g^{-1} dry weight with higher levels than strain STW181, including valine (2.54), threonine (2.45), isoleucine (1.54) and tryptophan (0.53). On the other hand, the amino acid profiles of strain STW181 that had six EAA levels higher than strain SS15 included 3.83 leucine, 2.91 arginine, 2.91

phenylalanine, 2.14 lysine, 1.07 methionine and 0.92 histidine. According to its EAA profile, strain STW181 was consider to use as SCP by a combination with strain SS15 at a ratio of 1: 1.

Proximate analysis of modified shrimp feed recipes

The proximate analysis of modified shrimp feed recipes and control (commercial shrimp feed) showed no significant difference ($P > 0.05$) for protein and ash contents among 4 diets (Table 6-3). The major nutrient content is protein in a range of 42.18-46.60% as the lowest was observed in the control. However, for lipid contents of modified shrimp diets, Diet 2 and Diet 3, (7.63-7.83%) were significantly lower than the control diet (8.49%) and Diet 1 (8.23). Fiber content of all modified shrimp diets (4.52-5.29%) were significantly higher ($P < 0.05$) than the control diet (3.89%). In contrast, moisture content of all modified shrimp diets (7.21-7.37) was significantly lower than control diet (7.51%).

Table 6-2. Essential amino acids (EAA) in bacterial cell component of *R. sphaeroides* SS15 and *A. marina* STW181.

Amino acid (g 100 g^{-1} dry cell weight)	**PNSB strain**		**Standard Guideline**
	***R. sphaeroides* SS15**	***A. marina* STW181**	**EAA requirements for shrimp (Ahamad-Al, 2006)**
Leucine *	3.65	3.83	2.16
Methionine *	0.19	1.07	0.96
Isoleucine *	1.54	1.53	1.40
Phenylalanine *	1.92	2.91	1.60
Histidine *	0.86	0.92	0.84
Lysine *	1.97	2.14	2.12
Arginine *	2.26	2.91	2.32
Tryptophan *	0.53	0.46	0.32
Valine *	2.54	2.04	1.60
Threonine *	2.45	2.19	1.44
Aspartic acid	3.89	4.49	ND
Serine	1.58	1.99	ND
Glutamic acid	5.18	5.41	ND
Proline	1.78	1.79	ND
Glycine	2.69	2.35	ND
Alanine	4.94	3.42	ND
Cystine	0.29	0.20	ND
Tyrosine	2.93	3.42	ND

* EAA requirements for aquatic animals. ND = no data for non-essential amino acids.

Table 6-3. Proximate analysis of different diet recipes of shrimp feed among commercial feed as control and supplementation of lyophilized PNSB (SS15 and STW181, 1: 1) in commercial shrimp feed.

Proximate analysis (% Dry basis; g 100 g^{-1})	**Shrimp feed recipe**			
	Control (Commercial feed)	**Diet 1 (1% PNSB)**	**Diet 2 (3% PNSB)**	**Diet 3 (5% PNSB)**
Protein	42.18 ± 0.12 [a]	42.29 ± 0.19 [a]	42.56 ± 0.21 [a]	42.60 ± 0.25 [a]
Lipid	8.49 ± 0.23 [a]	8.23 ± 0.09 [a]	7.83 ± 0.06 [b]	7.63 ± 0.30 [b]
Moisture	7.51 ± 0.02 [a]	7.37 ± 0.09 [b]	7.21 ± 0.08 [c]	7.26 ± 0.12 [c]
Fiber	3.89 ± 0.10 [c]	5.16 ± 0.03 [a]	5.29 ± 0.01 [a]	4.52 ± 0.33 [b]
Ash	12.51 ± 0.01 [a]	12.51 ± 0.03 [a]	12.55 ± 0.09 [a]	12.59 ± 0.01 [a]

Values are presented as a mean of three replicates ± S.D., and significant differences are analyzed at $P < 0.05$. Different letters indicate significant differences for values in each row.

Water quality during white shrimp cultivation

With 50% water exchange every week, no significant difference was observed for temperature (23-25 °C) and salinity (15-18 ppt) in rearing water of all sets throughout shrimp cultivation (Table 6-4). There were significant differences were found for DO and pH values as starting from week 6 until week 8 as a little drop of DO and changes of pH (see detail in Table 6-4). DO values of all experimental sets at week 8 were in a range of 7.44 to 7.68 mg L^{-1} compared to the starting point when the range was between 7.79-8.01 mg L^{-1}. In all sets at weeks 6-8, pH ranged from 7.82 to 8.22; and the average pH value throughout shrimp cultivation was approximately 8.0.

Amount of NH_4^+ in all sets significantly increased as longer cultivation and NH_4^+ levels among shrimp modified feeds were not much different (Figure 6-1A). However, at week 8, the lowest level of NH_4^+ (mg L^{-1}) was found in the control set (4.76 ± 0.14 mg L^{-1}) followed by modified shrimp feed Diet 1 (5.78 ± 0.05), Diet 2 (6.25 ± 0.13) and Diet 3 (6.89 ± 0.03) sets. The amount of NO_2^- slightly increased along cultivation times in all sets; however, a remarkable increase was found from week 7 to week 8 (Figure 6-1B). At week 8, the lowest NO_2^- level was found in the control set (0.23 ± 0.03 mg L^{-1}). Diet 1 and Diet 2 sets showed no significant differences of NO_2^- level; but significant difference was found in set of Diet 3 (0.37 ± 0.03 mg L^{-1}). NO_3^- levels started to increase from week 3 until the end of cultivation (Figure 6-1C). NO_3^- levels from week 6 until week 8, almost modified shrimp diets were higher than control set; however, no significant difference was found in control and Diet 1 sets with 6.27 ± 0.10 mg L^{-1} as the lowest NO_3^- level. All sets in this experiment produced a similar pattern of COD levels that slightly increased with longer cultivation period; and a little higher COD levels in sets of Diets compared to control set (Figure 6-1D). At week 8, COD level in the control set (49.33 ± 3.51 mg L^{-1}) was significantly lower than sets of Diet 2 (59.00 ± 4.58 mg L^{-1}) and Diet 3 (62.33 ± 2.08 mg L^{-1}), with exception for Diet 1 (56.00 ± 2.65 mg L^{-1}).

Table 6-4. Monitoring parameters of water quality during shrimp cultivation for 60 days under different shrimp feed recipes.

Parameter	Control	Diet 1	Diet 2	Diet 3
DO (mg L^{-1})				
Week 0	7.79 [a]	7.81 [a]	7.81 [a]	7.81 [a]
Week 1	7.74 [a]	7.75 [a]	7.72 [a]	7.74 [a]
Week 2	7.72 [a]	7.67 [a]	7.70 [a]	7.65 [a]
Week 3	7.73 [a]	7.70 [a]	7.73 [a]	7.70 [a]
Week 4	7.69 [a]	7.65 [a]	7.66 [a]	7.63 [a]
Week 5	7.58 [a]	7.56 [a]	7.55 [a]	7.50 [a]
Week 6	7.68 [a]	7.65[ab]	7.58[b]	7.57[b]
Week 7	7.54 [a]	7.48[ab]	7.46[b]	7.44[b]
Week 8	7.68[a]	7.67[a]	7.63[ab]	7.56[b]
pH				
Week 0	8.15 [a]	8.15 [a]	8.13 [a]	8.10 [a]
Week 1	8.23 [a]	8.17 [a]	8.16 [a]	8.22 [a]
Week 2	8.23 [a]	8.22 [a]	8.23 [a]	8.27 [a]
Week 3	7.88 [a]	7.87 [a]	7.92 [a]	7.94 [a]
Week 4	7.93 [a]	7.94 [a]	7.95 [a]	7.97 [a]
Week 5	8.14 [a]	8.15 [a]	8.16 [a]	8.18 [a]
Week 6	7.98[b]	8.09 [a]	8.08 [a]	8.12 [a]
Week 7	7.82[b]	7.87[b]	7.89[b]	8.03 [a]
Week 8	8.12[b]	8.15[ab]	8.21 [a]	8.22 [a]
Salinity (ppt)				
Week 0	15.0 [a]	15.0 [a]	15.0 [a]	15.0 [a]
Week 1	17.0[a]	16.7 [a]	17.0 [a]	17.0 [a]
Week 2	16.3 [a]	16.3 [a]	16.3 [a]	16.7 [a]
Week 3	15.3 [a]	16.0 [a]	16.3 [a]	15.7 [a]
Week 4	16.3 [a]	15.7 [a]	17.0 [a]	16.7 [a]
Week 5	16.7 [a]	16.7 [a]	17.3 [a]	17.0 [a]
Week 6	16.0 [a]	16.3 [a]	16.7 [a]	16.0 [a]
Week 7	16.7 [a]	16.7 [a]	16.7 [a]	17.0 [a]
Week 8	17.3 [a]	17.7 [a]	17.7 [a]	17.7 [a]
Temperature (°C)				
Week 0	24.9 [a]	24.9 [a]	24.6 [a]	24.5 [a]
Week 1	24.7 [a]	24.5 [a]	24.5 [a]	24.2 [a]
Week 2	25.1 [a]	24.8 [a]	24.7 [a]	24.6 [a]
Week 3	24.7 [a]	24.7 [a]	24.6 [a]	24.5 [a]
Week 4	24.5 [a]	24.4 [a]	24.5 [a]	24.3 [a]
Week 5	24.9 [a]	24.9 [a]	24.6 [a]	24.6 [a]
Week 6	24.3 [a]	24.1 [a]	23.9 [a]	23.7 [a]
Week 7	23.9 [a]	23.9 [a]	23.8 [a]	23.6 [a]
Week 8	25.2 [a]	24.9 [a]	24.8 [a]	24.7 [a]

Values are presented as a mean ± S.D. (n = 3) and different letters indicate significant differences in each row at $P < 0.05$.

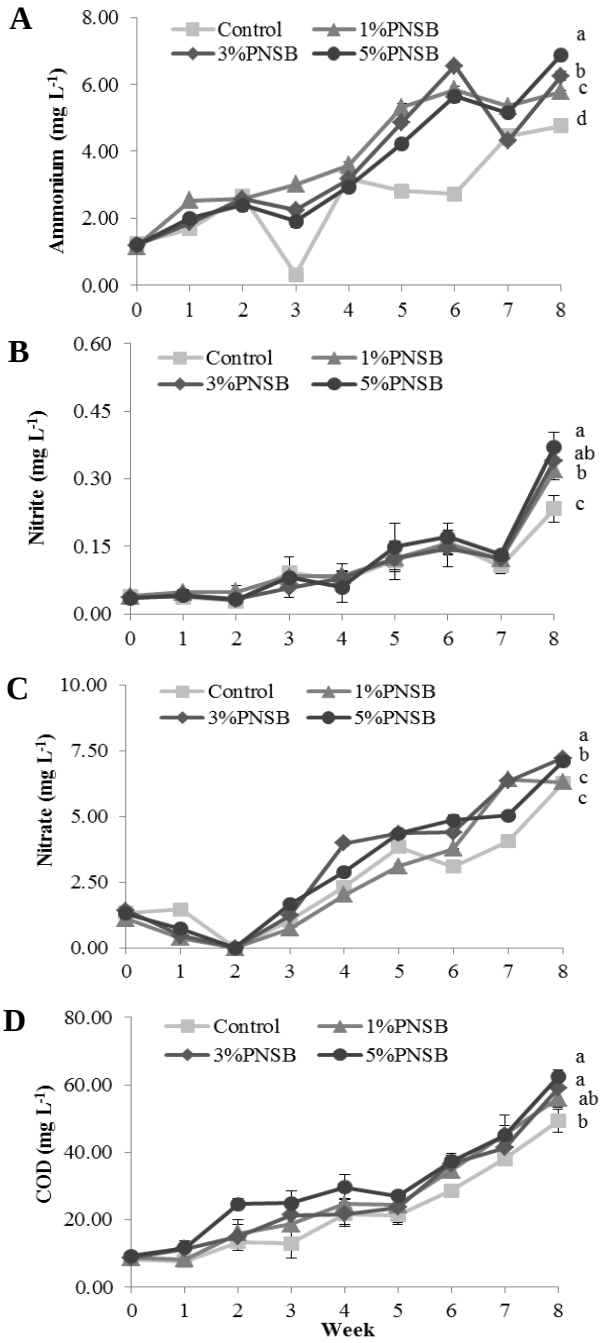


Figure 6-1. Effects of modified shrimp feed supplemented with various doses of PNSB-SCP (SS15 and STW181, 1: 1) at 1, 3 and 5% for Diet 1, Diet 2 and Diet 3, and commercial shrimp feed as control on water quality during 60 days of white shrimp cultivation; A: ammonium (NH_4^+), B: nitrite (NO_2^-), C: nitrate (NO_3^-) and D: chemical oxygen demand (COD).

Growth performance and survival of white shrimp

At the beginning, there were no significant differences in the initial weight and initial length between control and modified shrimp diet sets; however, significant differences were observed at day 60 for shrimp weight and at day 30 for shrimp length (Table 6-5). At the final shrimp weight (g) at day 60, all modified shrimp diet sets showed higher values than control set. However, only shrimp weight (0.671 ± 0.117 g) in Diet 1 set (1% SCP as PNSB) was significantly higher ($P < 0.05$) than control set (0.420 ± 0.090 g). For the final shrimp length (mm), a better result was also observed in modified shrimp diet sets; but it was significantly higher ($P < 0.05$) in sets of Diet 1 and Diet 2 (50.25 ± 3.55 and 46.25 ± 3.00 mm) as of no significant difference in sets of Diet 3 and control (45.92 ± 3.56 and 42.17 ± 3.76 mm). Growth performance based on weight gain, DWG, length gain, DLG and RGR showed that Diet 1 set was significantly greater than other sets including control and these parameters corresponded to FCR values (see details in Table 6-5). The control set showed the lowest growth performance for all parameters with exception of SGR values; however, there was no significant difference among control, Diet 2 and Diet 3 was found.

Although no significant difference was found for shrimp survival throughout cultivation; all modified shrimp feed recipes performed higher survival than control set, except in Diet 3 set at day 60 day (Figure 6-2). At the end of cultivation, shrimp survival was in the degree of sets Diet 1 (84.85 ± 5.25%) > Diet 2 (81.82 ± 4.55%) > Control (80.30 ± 6.94%) > Diet 3 (78.79 ± 2.62%).

Table 6-5. Influences of shrimp feed containing 1, 3 and 5% lyophilized PNSB (SS15: STW181, 1: 1) and no added PNSB on growth performance of white shrimp during 60-day cultivation.

Growth performance	**Control**	**Diet 1 (1% PNSB)**	**Diet 2 (3% PNSB)**	**Diet 3 (5% PNSB)**
Initial weight (g)	0.055 ± 0.003^{a}	0.058 ± 0.008^{a}	0.056 ± 0.005^{a}	0.060 ± 0.017^{a}
Day 15	0.086 ± 0.025^{a}	0.088 ± 0.011^{a}	0.090 ± 0.032^{a}	0.092 ± 0.012^{a}
Day 30	0.192 ± 0.045^{a}	0.247 ± 0.069^{a}	0.216 ± 0.041^{a}	0.230 ± 0.043^{a}
Day 45	0.304 ± 0.075^{a}	0.325 ± 0.074^{a}	0.358 ± 0.091^{a}	0.298 ± 0.054^{a}
Day60	0.420 ± 0.090^{b}	0.671 ± 0.117^{a}	0.526 ± 0.061^{b}	0.520 ± 0.129^{b}
Initial length (mm)	13.83 ± 0.52^{a}	13.58 ± 1.86^{a}	13.83 ± 1.03^{a}	13.92 ± 0.80^{a}
Day 15	17.83 ± 1.63^{a}	19.25 ± 1.25^{a}	19.42 ± 2.33^{a}	19.42 ± 1.16^{a}
Day30	30.75 ± 2.19^{b}	33.33 ± 2.23^{a}	32.83 ± 1.13^{a}	33.08 ± 1.63^{a}
Day 45	34.67 ± 4.12^{b}	38.08 ± 2.29^{a}	39.25 ± 2.89^{a}	36.50 ± 1.70^{ab}
Day60	42.17 ± 3.76^{c}	50.25 ± 3.55^{a}	46.25 ± 3.00^{b}	45.92 ± 3.56^{bc}
Weight gain (g)	0.365 ± 0.095^{b}	0.613 ± 0.113^{a}	0.470 ± 0.029^{ab}	0.460 ± 0.095^{ab}
DWG (g day^{-1})	0.006 ± 0.002^{b}	0.010 ± 0.002^{a}	0.008 ± 0.000^{ab}	0.008 ± 0.001^{ab}
Length gain (mm)	28.33 ± 3.75^{b}	36.67 ± 1.51^{a}	32.42 ± 0.76^{ab}	32.00 ± 2.39^{ab}
DLG (mm day^{-1})	0.47 ± 0.06^{b}	0.61 ± 0.02^{a}	0.54 ± 0.01^{ab}	0.53 ± 0.04^{ab}
RGR (%)	670.12 ± 166.78^{b}	1053.15 ± 213.25^{a}	845.23 ± 93.96^{ab}	784.01 ± 117.98^{ab}
FCR	4.17 ± 12.76^{a}	2.41 ± 4.64^{b}	3.07 ± 1.83^{ab}	3.23 ± 7.53^{ab}
SGR (% day^{-1})	3.35 ± 0.44^{a}	4.05 ± 0.33^{a}	3.74 ± 0.16^{a}	3.62 ± 0.66^{a}

Values are presented as a mean ± S.D. (n = 6) and different letters indicate significant differences in each row at $P < 0.05$. DWG, DLG, RGR, FCR and SGR represent for daily weight gain, daily length gain, relative gain rate, feed conversion ratio and specific growth rate, respectively.

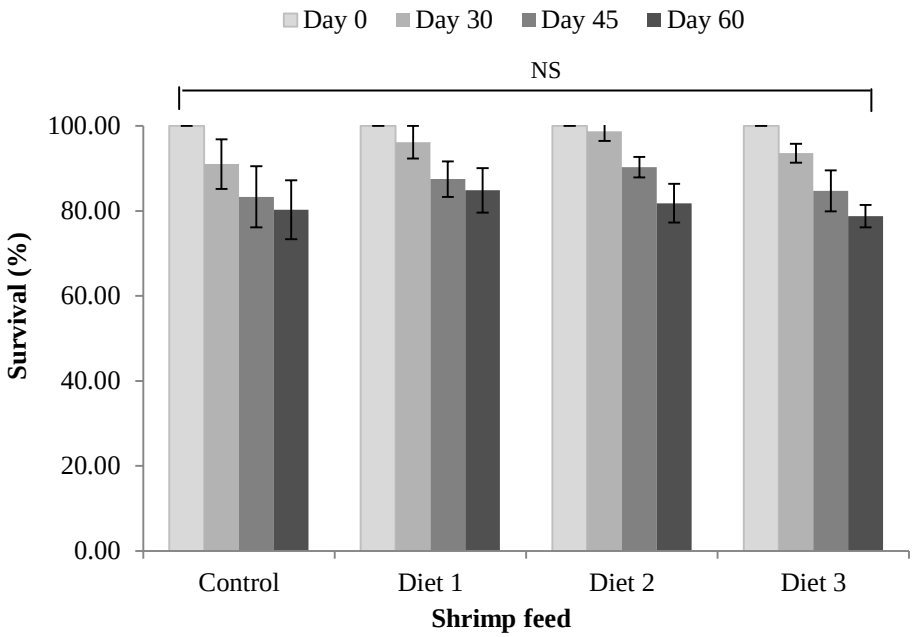


Figure 6-2. Influences of shrimp feed supplemented with various doses of PNSB-SCP (1, 3 and 5% for Diet 1, Diet 2 and Diet 3, respectively; commercial shrimp feed as control) on white shrimp survival during 60 days of cultivation. Shrimp survivals are presented as mean ± S.D. There were no significant differences ($P > 0.05$) among them in each sampling time that analyzed by Kruskal-Wallis test.

Shrimp immunity

At end of cultivation, all early juvenile of white shrimp were measured their immunological parameters and found that THC in shrimp from all sets of modified shrimp diets were higher than shrimp in control set although there were no significant differences among them (Figure 6-3A). The highest value of THC was found in Diet 2 set (5.40 x 10^5 cell mL^{-1}) and the lowest in the control (3.83 x 10^5 cell mL^{-1}). The PO activity (Unit mL^{-1}) in sets of Diet 3 (0.066 ± 0.006) and Diet 2 (0.061 ± 0.010) was significantly higher than Diet 1 (0.051 ± 0.004) and control sets (0.042 ± 0.007) (Figure 6-3B). Activity of SOD (Unit mL^{-1}) in Diet 1 set (0.029 ± 0.010) was significantly higher than Diet 2 and control sets as the lowest was found in control set (0.021 ± 0.006).

No significant differences were observed in sets of Diet 1 and Diet 3 although the highest level was found in Diet 1 set (Figure 6-3C).

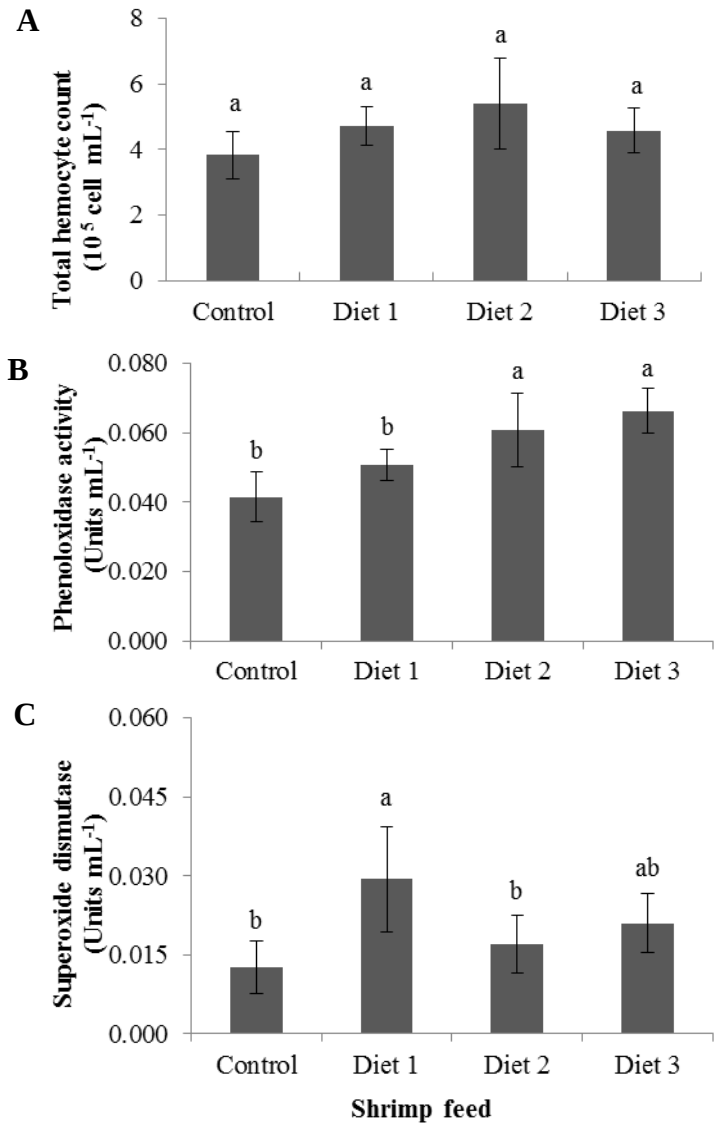


Figure 6-3. Effects of PNSB-SCP at 1, 3 and 5% mixed with commercial shrimp feed (control) for Diet 1, Diet 2 and Diet 3 on representative immune responses of shrimp at day 60. A: Total haemocyte count (10^5 cell mL^{-1}), B: Phenoloxidase activity (PO; Units mL^{-1}) and C: Superoxide dismutase (SOD; Units mL^{-1}).

Histological analysis of hepatopancreas (HP) from white shrimp

Based on the histological results, it was found that the HP of shrimp in all diets at the end of cultivation showed normal structures of tubule epithelial cells including secretory (B-cells), absorptive (R-cells), fibrillar (F-cells) and embryonic (E-cells) cells (Figure 6-4 A-D). Additionally, no pathogenic infection nor any damage by PNSB-SCP were observed in the HP histology of shrimp in three modified shrimp diet sets (Diets 1, 2 and 3) when compared with the HP of shrimp in control set (Figure 6-4 A-D). A number of B-cells in HP of shrimp in all modified shrimp diet sets were higher than that found in the control set (Figure 6-4 A-D).

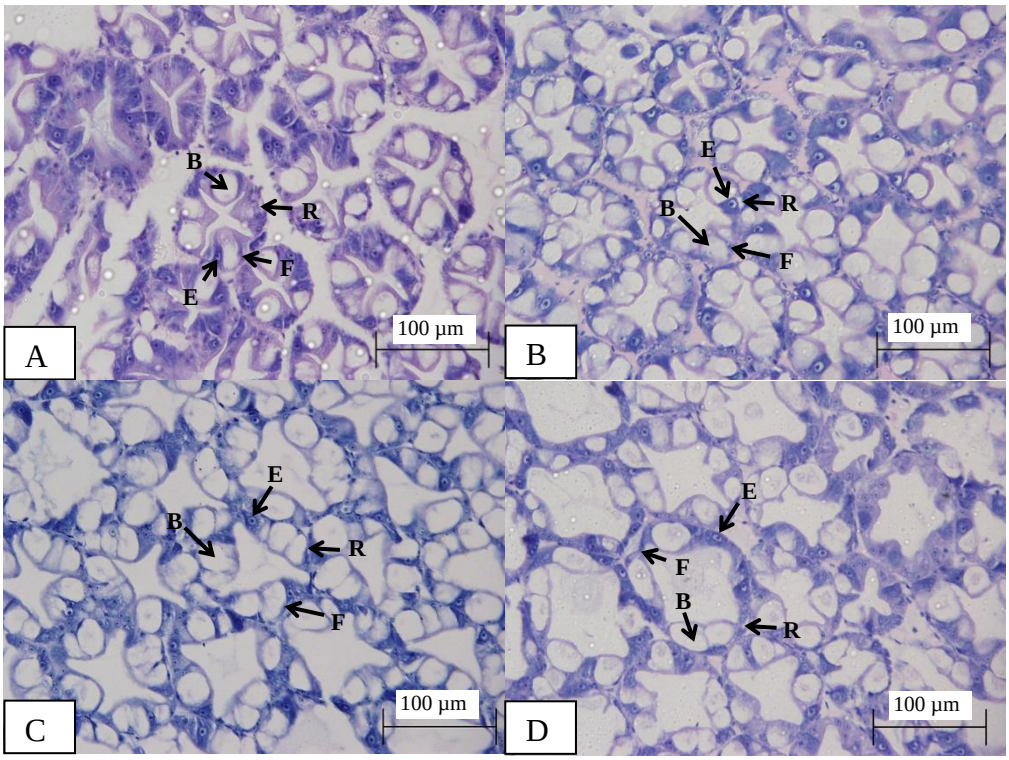


Figure 6-4. Effects of modified shrimp diets with PNSB-SCP on histological analysis of hepatopancreas of white shrimp at day 60; A: Control; B: Diet 1; C: Diet 2 and D: Diet 3. The magnification views of shrimp were observed by 200X. Arrows indicate the tubule epithelial cells in shrimp hepatopancreas including secretory (B-cells), absorptive (R-cells), fibrillar (F-cells) and embryonic (E- cells) cells.

Discussion

Considerations on the use of biomass from PNSB as SCP in shrimp feed

It is well recognized that PNSB could be a great potential source as feed supplement for aquatic animals because their biomass enriched with protein and nutrients (Kim and Lee, 2000; Azad, et al., 2002; Shapawi et al., 2012). This study found the maximum protein content (53.98%) in *R. sphaeroides* SS15 (Table 6-1A). This indicates that our 4 PNSB strains had a moderate-protein level compared to some previous studies which reported higher protein contents in a range of 60 - 75 % (Kim and Lee, 2000; Azad et al., 2002; Kantachote et al., 2005). However, our strains had been proved for their safety to use in shrimp cultivation as the probiotics PNSB to enhance shrimp growth and inhibit shrimp pathogenic vibrios including EMS /AHPND-causing *V. parahaemolyticus* (Chumpol et al., 2017a, b). It should be noted that strain SS15 and *A. marina* STW181 were selected to consider as SCP based on their protein and 10 EAA contents as previously explained (Table 6-2). Both PNSB strains, particularly strain STW181 provided all EAA in higher amounts than the recommended levels for each EAA; so they could be classified as biologically complete protein for shrimp feed (Table 6-2, Ahamad-Ali, 2006). Hence, the results guarantee to use them as SCP for shrimp cultivation.

The photopigment analysis showed that strain SS15 was the greatest producer of bacteriochlorophyll *a* and carotenoids (9.26 ± 0.72 and 6.87 ± 0.04 mg L^{-1}, respectively) (Table 6-1B). Carotenoids are major pigment found in Crustaceans including shrimp (Latscha, 1989). The carotenoids play an important role in shrimp pigmentation that directly influence on shrimp product quality, and the cost component of shrimp feeds (Wade et al., 2017). In addition of pigmentation, carotenoids act as antioxidants, source of pro-vitamin A to protect cells from photodynamic damage for enhancement growth and

reproduction (Linan-Cabello et al., 2002). As for more advantages of PNSB from their photopigments in addition of protein; this leads to strongly support that biomass of PNSB is the suitable source of SCP for shrimp cultivation. This study suggests that biomass of strains SS15 and STW181 enriched in protein with high EAA and photopigments should be a good source of SCP for supplementation in shrimp feed.

Our formulated 3 modified shrimp feed recipes with SCP from PNSB (strain SS15 and STW181, 1: 1) found no significant differences for protein and ash contents compared to control (Table 6-3). However, primary concern is the proper formulation and supplementation of feeds with adequate lipids, phosphorus and amino acids sources, to overcome the nutritional imbalances that arise when aquatic animal meals are removed from the formulation (Amaya et al., 2007). Among three modified shrimp feed recipes, Diet 1 showed the most closest for its proximate analysis to commercial shrimp feed (Control) followed by Diet 2 and Diet 3 (Table 6-3). Regarding the results all modified shrimp feed recipes should provide the optimum dietary nutrients that are necessary for white shrimp cultivation as the optimum dietary nutrients for *L. vannamei* were 25-45% of crude protein and 3-7% of lipid content (Cuzon et al., 2004).

Shrimp growth performance and their survival related to diets and water quality

The present study found that levels of NH_4^+, NO_2^-, NO_3^- and COD in all modified shrimp feed sets (Diets 1, 2 and 3) were higher than the control set (Figure 6-1 A-D). However, all monitored parameters in all sets throughout cultivation were in acceptable ranges for shrimp growth (Cohen et al., 2005; Mishra et al., 2008). It should be noted that water quality in Diet 1 set (1 % PNSB, 0.5 % strain SS15 + 0.5% strain STW181) was closest to the control set (Figure 6-1 A-D). This might be one of the reasons that shrimp in Diet 1 set

showed both the maximal shrimp growth performance and also survival at 85% compared to control at 80% (Table 6-5 and Figure 6-2).

The NH_4^+ levels in all experimental sets were quite high; however, they were in acceptable ranges, except Diet 3 (6.89 mg L^{-1}) for rearing water of white shrimp as the safety levels of NH_4^+ should be less than 6.52 mg L^{-1} for juvenile stage (Audelo-Naranjo et al., 2012). However, toxicity of NH_4^+ significantly increases under alkaline conditions (pH > 9), the concentrations at 0-18 mg L^{-1} of NH_4^+ at pH 8 caused postlarval *L. vannamei* mortality lower than 1.0%, while the same concentrations at pH 9 caused high shrimp mortality (Barajas et al., 2006; Chumpol et al., 2017b). Hence, the toxicity of NH_4^+ on shrimp in Diet 3 set might be reduced as the pH of this set was roughly 8.0; and this supports by the result of shrimp survival in this set was 79% (Table 6-4 and Figure 6-2).

The greatest shrimp growth performance was found in Diet 1 set as the lowest of FCR to indicate the most effective for conversion feed to shrimp body (Table 6-5). This suggests that modified shrimp feed with 1% PNSB was the most effective diet formulation for providing maximum growth and also better water quality (Table 6-5; Figure 6-1). However, the increasing of PNSB levels caused lower growth performance for white shrimp including water quality as previously mentioned. The results indicate that Diet 2 and Diet 3 with 3 and 5% PNSB were unsuitable diet formulations as lower significance of lipid content compared to control and Diet 1 (Table 6-3). It might be possible that shrimp utilized protein as a source of energy so reduced the amount of available protein for growth and tissue deposition (Kureshy and Davis, 2004). Moreover, the environmental stressors such as the levels of NH_4^+, NO_2^-, NO_3^- and COD were quite higher than control and Diet 1 sets (Figure 6-1) although in acceptable level for shrimp growth. This might have adverse effects on shrimp i.e. stress, weakness and low growth rate (Lightner et

al., 2006) as the evidence of shrimp growth performance (Table 6-5). Overall result proved that shrimp fed with Diet 1 at 1% PNSB not only produced better growth performance but also provided higher survival rate than a commercial shrimp feed or control (Table 6-5; Figure 6-2).

Shrimp immunity

Invertebrate animals including shrimp use innate immune responses to protect them from harmful microorganisms; and the innate immunity consists of the cellular innate immunity and humoral innate immunity (Chang et al., 2003b; Tassanakajon et al., 2013). The blood cells of shrimp are the primary effectors for host defenses and their involvement in numerous immune processes (Campa-Cordova et al., 2002). The cellular immune mechanism includes phagocytosis, nodulation and encapsulation, whereas the humoral immune mechanism involves biosynthesis and release of several immune proteins such as AMPs and melanization through the activation of the prophenol oxidase (pro PO) cascade (Fagutaoet al., 2011; Tassanakajon et al., 2012), and the active form as PO is responsible for initiating melanin biosynthesis (Hernandez-Lopez et al., 1996). THC is a useful indicator of shrimp health to defend itself against invading pathogens (Wilson et al., 2003). A lower of THC in crustaceans correlate with a reduced resistance to pathogens such as THC in shrimp significantly decreased when it was infected by WSSV (Chang et al., 2003). This might be one of the reasons to explain higher survival percentages in sets of Diet 1 and Diet 2 compared to control and Diet 3 sets (Figures 6-2 and 6-3A).

In addition, activities of PO and SOD in shrimp fed with modified shrimp feed recipes; Diet 1, 2 and 3 were higher than that found in shrimp from control set (Figure 6-3 B-C). PO activity in shrimp from sets of Diet 2 and Diet 3 did a remarkable increase that might be due to higher added PNSB at 3 and 5% (Figure 6-3B). Normally, stress conditions can create an

immunosuppressive state by reducing PO activity as found in *Litopanaeus schmitti* under low salinity (Lamela et al., 2005). Hence, the increase of PO activity in sets of modified shrimp feed diets suggests that PNSB biomass could help shrimp to resist stress conditions such as water quality as in this study no challenge shrimp with any shrimp pathogens, and water quality in the control set seemed to be better than other sets (Figures 6-1 and 6-3B).

Reactive oxygen species (ROS) i.e. superoxide anion radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) normally found in organisms inhabiting in aerobic environment as occurring of ROS by oxygen. Formation of ROS causes oxidative stress, and SOD is an oxidoreductase that converts O_2^- to H_2O_2 and O_2 to avoid the direct toxic effects from ROS (Chang et al., 2003b). The increased levels of SOD may enable shrimp to maintain good health by scavenging the radicals of ROS production (Li et al., 2008; Parrilla-Taylor and Zenteno-Savín, 2011) as the evidence of maximal shrimp survival found in Diet 1 set with the highest of SOD activity (Figures 6-2 and 6-3C). The results suggests that all modified shrimp diets containing SCP from PNSB were able to enhance THC, PO and SOD values that were related to healthy shrimp as bigger size and more survival compared to control set. It would be worth to further investigate why PNSB biomass could increase immune responses in addition of photopigments like carotenoids that act as antioxidant. Hence, adding PNSB biomass in shrimp diet might be not only acting as SCP but also as the effectiveness of potential immunostimulants for shrimp cultivation.

Histological analysis of HP

The HP is an important organ in shrimp that serves as the main energy reserve for growth, moulting and determination of health status (Stumpf et al., 2014). Occurring of lesions in HP can be considered as a damage response by some compounds or microorganism infection as related to impaired health

status of shrimp (Chupani et al., 2016). The lumen of each tubule in HP of shrimp is surrounded by four types of epithelial cells; B-, R-, F-, and E-cells. B-cells are the largest cells which contain a large vacuole; located mainly in distal part of tubules. R-cells distribute throughout the tubule with the presence of numerous small lipid vacuoles. F-cells show a fibrillar appearance and usually a central oval nucleus. E-cells are cuboidal and occur primarily in the distal part of hepatic tubules (Chupani et al., 2016). This present study found that numbers of B-cells in shrimp HP fed with modified shrimp feeds (Diet 1, Diet 2 and Diet 3) were higher than those fed with commercial feed. Therefore, higher shrimp growth rates were observed in all modified shrimp diet sets (Table 6-5) as more absorption and digestion of nutrients due to higher number of B-cells (Wang et al., 2016).

Conclusions

This study explored that PNSB, *R. sphaeroides* SS15 and *A. marina* STW181, are suitable biomasses to be used as SCP based on their protein and EAA contents including photopigments. Mixing them at 1% (w/w) with commercial shrimp feed provided the most effective modified shrimp feed (Diet 1) to enhance shrimp growth with higher survival as more immunostimulants and no adverse effects on HP shrimp and water quality.

CHAPTER 7

CONCLUSIONS

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

Among 185 PNSB isolated from various shrimp ponds in Southern Thailand, results *in vitro* test found that there were only 4 strains (SS15, S3W10, TKW17 and STW181) showed probiotic properties by secreting amyase, gelatinase, vitamin B12 including antivibrio compounds for promoting shrimp growth. This suggests that PNSB probiotics should be used for shrimp cultivation to obtain healthy shrimp. Moreover, only 5.4% of isolated PNSB (strains SS15, TKW17 and STW181) showed antivibrio activity against the growth of *Vibrio* spp.; and higher concentrated supernatants should be used to control *V. parahaemolyticus* causing EMS/AHPND. This points out that this shrimp pathogen is more resistant to antivibrio compounds than other vibrios such as *V. harveyi* and *V. vulnificus* that why it causes huge loses of shrimp production by EMS/AHPND. Four selected PNSB strains were identified as *R. sphaeroides* for strains SS15, S3W10 and TKW17 and *A. marina* for strain STW181.

Four selected PNSB strains were proved for their potential probiotics for white shrimp (*Litopenaeus vannamei*) cultivation by inoculating a mixed of two cultures at 1: 1 ratio (each at $1x10^{8}$ CFU mL^{-1}) into rearing water every week for 7 weeks. Among the three PNSB sets a mixed culture of strains SS15

and S3W10 (T1 set) was the most effective for secreting proteolytic enzyme in the shrimp GIT that related to their strong protease activity. Proliferation of PNSB found in all sets, particularly for T3 set (S3W10 + STW181) during shrimp cultivation suggests that inoculated PNSB could colonize in the shrimp GIT for increasing activities of digestive enzymes. This led to the greater shrimp growth in all PNSB sets with the biggest size found in the T3 set. In addition, all of the mixed PNSB sets, especially T1 set showed a remarkable decrease the cumulative mortality of shrimp exposed to a virulent AHPND strain (*V. parahaemolyticus* SR2). Hence, the results demonstrated that *R. sphaeroides* SS15 and S3W10 and *A. marina* STW181 are probiotic PNSB to enhance shrimp growth and inhibit *Vibrio* spp. including EMS /AHPND strains. As beneficial bacteria, another property of selected PNSB in addition of probiotic properties was investigated for supporting shrimp cultivation.

Four probiotic PNSB were used to investigate their ability for controlling water quality with the same protocol as a previous study and in this experiment 50% of rearing water was exchanged every week. The results found that throughout 8 weeks shrimp cultivation the most effective set to reduce level of NH_4^+ was T1 set; while T1 and T3 sets provided a better shrimp growth. As the same trend for their probiotic and controlling water quality; therefore, strains in sets of T1 and T3 (SS15, S3W10 and STW181) were selected for being used as a mixed culture (each at $1x10^8$ cells mL^{-1}) by inoculating every week in a challenge test without water exchange. *V. parahaemolyticus* SR2 at $1x10^5$ cells mL^{-1} was inoculated at day 15 of cultivation. During weeks 2 until 4, inoculated PNSB in treatment (PNSB and AHPND-*V. parahaemolyticus* SR2) and positive control (only PNSB) sets showed a better water quality for shrimp growth than that found in native (no inoculation) and challenge (AHPND-*V. parahaemolyticus* SR2) sets. The results suggest that probiotic PNSB in the treatment set might compete with *V. parahaemolyticus* SR2 to control water quality as no significant difference was

found between sets of treatment and positive control. As a positive strong correlation between PNSB population and shrimp survival supports that colonization of probiotic PNSB in intestinal shrimp tract could help to prevent AHPND by increasing 11% survival rate of infected shrimp by strain SR2.

Regarding above information points out that in addition of releasing antivibrio compounds into surrounding environment, probiotic PNSB might release antivibrio compounds during their colonization in shrimp GIT. Hence, it would be great to apply PNSB not only as probiotics for protecting shrimp from pathogens but also for treating infected shrimp by vibrios for avoiding the use of antibiotics. This led to optimize conditions for producing antivibrio compounds of probiotic PNSB (SS15, TKW17 and STW181) and their optimum conditions were closely to the conditions of shrimp cultivation. Characterization of antivibrio compounds found that main components were protein, lipid and carbohydrate; and their antivibrio activity was stable under wide variations of pH and temperature that could be used for shrimp cultivation. Antivibrio compounds produced by probiotic PNSB acted as bactericidal action as the evidence of damaged cells with many holes and also showed bacteriolytic activity. The results provide the answer why probiotic PNSB could promote shrimp growth and increase survival of infected shrimp by *V. parahaemolyticus* SR2. Hence, one of the purified antivibrio compounds from strain SS15 was characterized and found that it was a low molecular weight (< 3000 Da) weak cationic compound with containing -NH_2 group. This means that it would be easily to degrade so no remaining of its residual after using.

To reduce cost of shrimp feed, probiotic PNSB strains were also investigated their potential to be used as SCP by mixing with commercial shrimp feed. According to levels of protein and photopigments including EAA, strains SS15 and STW181 were suitable to be used as SCP at a ratio of 1: 1 by

mixing with shrimp feed at 1, 3 and 5% for Diet 1, Diet 2 and Diet 3, respectively for feeding white shrimp for 60 days. Water quality in Diet 1 set was very closed to control set; while Diet 2 and 3 sets were worse than the control although it still be acceptable for shrimp growth. This led to the maximum shrimp survival and also maximum shrimp growth performance were observed in Diet 1 set followed by other modified shrimp feeds as the lowest was found in the control set. This is because shrimp in all modified shrimp diet sets showed increases all of immune responses (total haemocyte count, and activities of PO and SOD); and these results related to the results of HP histopathology analysis as in a good condition to indicate healthy shrimp. The results state that PNSB biomass as SCP has the potential not only enhance shrimp growth but also increase immunostimulants for increasing shrimp survival.

Overall of this thesis provides attractive tools to sustain shrimp cultivation with the use of beneficial PNSB (strains SS15, S3W10, STW181) as probiotics in white shrimp cultivation to maintain water quality and prevent infection of shrimp pathogenic *Vibrio* spp., including enhance shrimp growth performance. It should be noted that the biomass of strains SS15 and STW181 are also the great potential of SCP for replacement some common protein source in shrimp feed with effective for promoting shrimp growth with higher survival as to increase immunity with no adverse effects on HP of shrimp. Hence, probiotic PNSB obtained in this study could be possible to be used for supporting organic shrimp farming to produce safe shrimp with environmentally friendly.

Future research needs

Although this thesis gave better understanding on the use of promising PNSB for shrimp cultivation; there are some issues need to be explored as follows.

1. In chapter 3 to support shrimp farmers for access inexpensive probiotic PNSB, a lower cost of PNSB inoculants should be studied for instance using cheaper raw materials or agricultural wastes as sources of carbon and nitrogen for preparing culture medium.

2. In chapter 4 for better understanding on the roles of probiotic PNSB and normal flora in white shrimp intestine, bacterial community should be investigated by molecular approaches such as using denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH). Besides, the effect of probiotic PNSB on immune responses in shrimp should be studied for explanation why they promote shrimp growth and show healthy.

3. In chapter 5, it would be worth to continue characterization of a purified antivibrio compound from probiotic PNSB to know its structure for the possibility to synthesize for commercial and use as biocontrol agents against shrimp pathogenic *Vibrio* spp.

4. In chapter 6, it should be investigated to assess the advantages of using a combination between probiotics PNSB and modified shrimp feed with PNSB on white shrimp cultivation.

5. With the use of probiotic PNSB and/or modified shrimp feed with probiotic PNSB, shrimp should be cultivated until harvesting for assessment achievement testing and doing cost analysis prior to apply in shrimp farms.

BIBLIOGRAPHY

Abraham, T. I. 2004. Antibacterial marine bacterium deterluminous vibriosis in shrimp larvae. NAGA, World Fish Center Quarterly 27(3): 28-31.

Ahamad-Ali, S. 2006. Protein, lipid and energy requirements of shrimp and fish. In Training manual on shrimp and fish nutrition and feed management. CIBA special publication No. 29: 10-20.

Ahmad, R. I., Seo, B. J., Kumar, V. J., Choi, U. H. and Choi, K.H. 2013. Isolation and characterization of a proteinaceous antifungal compound from *Lactobacillus plantarum* YML007 and its application as a food preservative. Letters in Applied Microbiology 57: 69-76.

Alavandi, S. V., Vijayan, K. K., Santiago, T. C., Poornima, M., Jithendran, K. P., Ali, S.A. and Rajan, J. J. S. 2004. Evaluation of *Pseudomonas* sp. PM 11 and *Vibrio fluvialis* PM 17 on immune indices of tiger shrimp, *Penaeus monodon*. Fish & Shellfish Immunology 17: 115-120.

Albuquerque- Costa, R. A., Araujo, R.L., Souza, O.V. and Vieira, R.H.S.D.F. 2015. Antibiotic-resistant vibrios in farmed shrimp. BioMed Research International, 1-4 Doi: 10.1155/2015/505914.

Amaya, E., Davis, D. A and Rouse, D. B. 2007. Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 262: 419-425.

Anderson, J. M. 2016. Global shrimp survey: Goal 2016. Global Aquacultue Advocate.http://advocate.gaalliance.org/global-shrimp-survey-goal-2016/#sthash.viqjDMFO. dpuf (Accessed 7 March 2017).

Andrade, T. P. D., Redman, R. M. and Lightner, D. V. 2008. Evaluation of the preservation of shrimp samples with Davidson's AFA fixative for infectious myonecrosis virus (IMNV) in situ hybridization. Aquaculture 278: 179-183.

Anh, P.T., Kroeze, C., Bush, S. R. and Mol, A.P.J. 2010. Water pollution by intensive brackish shrimp farming in south-east Vietnam: cause and options for control. Agricultural Water Management 97: 872-882.

AOAC. 2010. Official Methods of Analysis. 18th Edition, Association of Official Analytical Chemists, Washington DC.

Audelo Naranjo, J. M., Martínez-Córdova, L. R., Gómez Jiménez, S., Voltolina, D. 2012. Intensive culture of *Litopenaeus vannamei* without water exchange and with an artificial substrate. Hidrobiológica 22(1): 1-7.

Azad, S.A. 2002. Phototrophic bacteria as feed supplement for rearing *Penaeus monodon* larvae. Journal of the world aquaculture society 33(2): 158-168.

Balcazar, J. L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D.,Muzquiz, J. L.2006. The role of probiotics in aquaculture. Veterinary Microbiology 114: 173-186.

Barajas, F. M., Villegas, R.S., Clark, G. P., Mosqueda, J. G. and Moreno, B. L. 2006. Daily variation in short-term static toxicity of unionized ammonia in *Litopenaeus vannamei* (Boone) postlarvae. Aquaculture Research 37: 1406-1412.

Barman, D., Nen, P., Mandal, S. C. and Kumar, V. 2013. Immunostimulants for aquaculture health management. Journal of Marine Science: Research and Development 3:134. Doi: 10.4172/2155-9910.1000134.

Bernfeld, P. 1955. Amylase, alpha and beta. Methods in Enzymology 1: 149-158.

Biao, X., Zhuhong, D. and Xiaorong, W. 2004. Impact of the intensive shrimp farming on the water quality of the adjacent coastal creeks from Eastern China. Marine Pollution Bulletin 48: 543-553.

Boyd, C. E. 1990. Water quality in ponds for aquaculture. Research and Development Series No. 23, Birmingham publishing company, Birmingham, Alabama, pp. 274-282.

Boyd, C. and Fast, A.W. 1992. Pond monitoring and management. In: Fast A.W. and Lester L.J. (Eds). Marine shrimp culture: principles and practices. Developments in aquaculture and fisheries science, volume 23. Elsevier Science Publisher B.V., The Netherlands.

Boyd, C. E. and Gross, A. 1998. Use of probiotics for improving soil and water quality in aquaculture ponds. In Flegel TW (Ed) Advances in shrimp biotechnology. National center for genetic engineering and biotechnology, Bangkok, 101-105.

Briggs, M., Funge-Smith, S., Subasinghe, R. and Phillips, M. 2004. Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the

Pacific. Food and Agriculture Organization of the United Nations, Regional Office for Asia and the Pacific. RAP Publication. pp. 1–92.

Brown, J.W. 2012. Enrichment and isolation of purple nonsulfur bacteria, Retrieved from http://www.mbio.ncsu.edu/MB452/purple_nonsulfurs/purples.html. (Accessed 15 November 2013).

Bundale, S., Begde, D., Nashikkar, N., Kadam, T., Upadhyay, A. 2015. Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil. Advances in Microbiology 5: 441-451.

Campa-Cordova, A. I., Hernandez-Saavedra, N. Y., De-Philippis, R. and Ascencio, F. 2002.Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to *β*-glucan and sulphated polysaccharide. Fish & Shellfish Immunology 12: 353-366.

Carvalho, A. L. U., Oliveira, F. H. P. C., Mariano, R. L. R., Gouveia, E. R., Souto-Maior, A. M. 2010. Growth, sporulation and production of bioactive compounds by *Bacillus subtilis* R14. Brazilian Archives of Biology and Technology 53: 643-652.

Catap, E. S., Lavilla-Pitogo, R., Maeno, Y. and Travina, R. D. 2003. Occurrence, histopathology and experimental transmission of hepatopancreatic parvovirus infection in *Penaeus monodon* postlarvae. Diseases of Aquatic Organisms 57: 11-17.

Cauthen, S. E., Pattison, J. R. and Lascelles, J. 1967. Vitamin B12 in Photosynthetic bacteria and methionine synthesis by *Rhodopseudomonas spheroides*. Biochemical Journal: 774-778.

Chandrasekaran, R. and Ashok Kumar, G.V. 2011. Antagonistic activity of purple non-sulfur bacterial extracts against antibiotic resistant *Vibrio* sp. Malaysian Journal of Microbiology 7(1): 54-56.

Chang, C. F., Su, M. S., Chen, H. Y. and Liao, I. C. 2003. Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish & Shellfish Immunology 15: 297-310.

Chang, Y. S., Liu, W. J., Chen, T. C., Chan, T. Y., Liu, K. F., Chuang, J. C., Kou, G. H., Lo, C. F. and Wang, H. C. 2012.Feeding hermit crabs to shrimp

broodstock increases their risk of WSSV infection. Diseases of Aquatic Organisms98: 193-199.

Chau, N. T. T., Quang, P.H., Lan, P. T. N., Matsumoto, M. and Miyajima, I. 2011. Identification and characterization of *Pseudomonas* sp. P9 antagonistic to pathogenic *Vibrio* spp. isolated from shrimp culture pond in ThuaThien Hue–Viet Nam. Journal of the Faculty of Agriculture, Kyushu University 56 (1): 23-31.

Chatterjee, S and Haldar, S. 2013. *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. Journal of Marine Science: Research & Development 3: 1–7.

Chiu, C. H., Guu, Y, K., Liu, D, H., Pan, T, M. and Cheng, W. 2007. Immune responses and gene expression in white shrimp *Litopenaeus vannamei* induced by *Lactobacillus plantarum*. Fish & Shellfish Immunology 23: 364–377.

Chiu, K. H. and Liu, W. S. 2014. Dietary administration of the extract of *Rhodobacter sphaeroides* WL-APD911 enhances the growth performance and innate immune responses of seawater red tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus*). Aquaculture 418-419: 32-38.

Cho, C. Y., Hynes, J. D., Wood, K.R. and Yoshida, H. K. 1994. Development of high-nutrient-dense, low-pollution diets and prediction of aquaculture wastes using biological approaches. Aquaculture 124: 293-305.

Cohen, J. M., Samocha, T. M., Fox, J. M., Gandy, R. L., Lawrence, A. L., 2005. Characterization of water quality factors during intensive raceway production of Juvenile *Litopenaeus vannamei* using limited discharge and biosecure management tools. Aquacultural Engineering 32: 425-442.

Chupani, L., Zuskova, E., Stara, A, Velisek, J. and Kouba, A. 2016. Histological changes and antioxidant enzyme activity in signal crayfish (*Pacifastacus leniusculus*) associated with sub-acute peracetic acid exposure. Fish & Shellfish Immunology 48: 190-195.

Chuntapa, B., Powtongsook, S. and Menasveta, P. 2003. Water quality control using *Spirulina platensis* in shrimp culture tanks. Aquaculture 220: 355-366.

Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017a. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquaculture Research 48: 3182–3197.

Chumpol, S., Kantachote, K., Nitoda, T. and Kanzaki, H. 2017b. The roles of probiotic purple nonsulfur bacteria to control water quality and prevent acute hepatopancreatic necrosis disease (AHPND) for enhancement growth with higher survival in white shrimp (*Litopenaeus vannamei*) during cultivation. Aquaculture 473: 327–336.

Cohen, J. M., Samocha, T. M., Fox, J. M., Gandy, R. L. and Lawrence, A. L. 2005. Characterization of water quality factors during intensive raceway production of Juvenile *Litopenaeus vannamei* using limited discharge and biosecure management tools. Aquacultural Engineering 32: 425-442.

Cuzon, G., Lawrence, A., Gaxiola, G., Rosas, C. and Guillaume, J. 2004. Nitrition of *Litopenaeus vannamei* reared in tanks or in ponds. Aquculture 235: 513- 551.

Dash, P., Avunje, S., Tandel, R. S., Sandeep, K. P. and Panigrahi, A. 2017. Biocontrol of Luminous Vibriosis in Shrimp Aquaculture: A Review of Current Approaches and Future Perspectives, Reviews in Fisheries Science & Aquaculture. DOI:10.1080/23308249.2016.1277973.

Defoirdt, T., Sorgeloos, P. and Bossier, P. 2011. Alternatives to antibiotics for the control of bacterail disease in aquaculture. Current opinion in microbiology 14: 251-258.

De Schryver, P., Defoirdt, T., Sorgeloos, P., 2014. Early mortality syndrome outbreaks: a microbial management issue in shrimp farming. PLoS Pathog. 10(4), e1003919. http://dx.doi.org/10.1371/journal.ppat.1003919.

Dewick, P. M. 2009. Secondary metabolism: The building blocks and construction mechanisms. Medicinal natural products: John Wiley & Sons, Ltd.

Fagutao, F. F., Kondo, H., Aoki, T. and Hirono, I. .2011. Prophenoloxidase has a role in innate immunity in penaeid shrimp. Diseases in Asian aquaculture. VII. Fish health section. Asian Fisheries Society, Selangor, Malaysia, 171-176.

FAO. 2013. Report of the FAO/MARD Technical Workshop on Early Mortality

Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (Under TCP/VIE/3304). Hanoi, Viet Nam, on 25–27 June 2013. No. 1053, Rome. 54 pp.

FAO. 2014. International shrimp trade grows, supported largely by increased imports by the US and East Asian markets. http://www.fao.org/inaction/globefish/market-reports/resource-detail/en/c/338031/. (Accessed 9 May 2016).

FAO. 2015. Weak market prices persist in 2015 but import demand remains disappointing. http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/357932/. (Accessed 21 March 2016).

FAO. 2016. World production of farmed shrimp could be 10% lower in 2015 than in 2014 due to falling prices. http://www.fao.org/in-action/globefish/marketreports/ resource-detail/en/c/383163/. (Accessed 25 January 2017).

FAO. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

Flegel, T. W. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. Journal of Invertebrate Pathology 110 (2):166-173.

Frías–Espericueta, M. G., Harfush–Melendez, M., Osuna–López, J. I., F. and Páez–Osuna F. 1999. Acute toxicity of ammonia to juvenile shrimp *Penaeus vannamei* Boone, Bulletin of Environmental Contamination and Toxicology 62: 646–652.

Ghaffari, N., Sanchez-Flores, A., Doan, R., Garcia-Orozco, K. D., Chen, P. L., Ochoa-Leyva, A., Lopez-Zavala, A. A., Carrasco, J. S., Hong, C., Brieba, L. G., Rudino-Pinera, E., Blood, P. D., Sawyer, J. E., Johnson, C. D., Dindol, S. V., Sotelo-Mundo, R. R. and Criscitiello, M. F. 2014. Novel transcriptome assembly and improved annotation of the white leg shrimp (*Litopenaeus vannamei*), a dominant crustacean in global seafood mariculture. Scientific Reports. 4: 7081, 1-10. DOI: 10.1038/srep07081.

Goddard, S. 1996. Feed management in intensive aquaculture. The United States of America, 194 pp.

Goh, H. F. and Philip, K. 2015. Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. PLOS ONE 10(10): e0140434. doi:10.1371/journal.pone.0140434: 1-17.

Getha, K., Chong, V.C. and Vikineswary, S. 1998. Potential use of the phototrophic bacterium, *Rhodopseudomonas palustris*, as an aquaculture feed. Asian Fisheries Science 10: 223-232.

Graslund, S., Karin, K., Wongtavatchai, J. 2002. Responsible use of antibiotic in shrimp farming. Aquaculture Asia. 7(3): 17.

Guo, J. J., Liu, K. F., Cheng, S.H., Chang, C.I., Lay, J. J., Hsu, Y. O., Yang, J. Y. and Chen, T. I. 2009. Selection of probiotic bacteria for use in shrimp larviculture. Aquaculture Research 40: 609-618.

Gunalan, B., Soundarapandian, P., Anand, T., Kotiya, A. S. and Tabitha, S. 2014. Disease occurrence in *Litopenaeus vannamei* shrimp culture system in different geographical regions of India. International Journal of Aquaculture 4(4): 24-28.

Kang, Z., Zhang, J., Zhou, J., Qi, Q., Du, G. and Chen, J. 2012. Recent advances in microbial production of 8-aminolevulinic acid and vitamin B12. Biotechnology Advances 30(6): 1533-1542.

Hai, N.V and Fotedar, R. 2010. A review of probiotics in shrimp aquaculture. Journal of applied aquaculture 22(3): 251-266.

Han, J. E., Tang, K. F. J., Tran, L. H. and Lightner, D. V. 2015. *Photorhabdus* insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Diseases of Aquatic Organisms 113: 33-40.

Haryanti, K., Sugama, S., Tsumura, S. and Nishijima, T. 2001. Enhance production of Black Tiger shrimp *Penaeus monodon* postlarvae by probiotic bacterium *Alteromonas* sp. IFR Journal 7(1): 1-6.

Heenatigala, P. P. M. and Fernando, M. U. L. 2016. Occurrence of bacteria species responsible for vibriosis in shrimp pond culture systems in Sri Lanka and assessment of the suitable control measures. Sri Lanka Journal of Aquatic Sciences 21(1): 1-17.

Hernandez-Lopez, J., Gollas-Galvan, T., Vargas-Albores, F., 1996. Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensis* Holmes. Comparative Biochemistry and Physiology 113C: 61–66.

Holmstrom, K., Garaslund, S., Wahlstrom, A., Poungshompoo, S., Bengtsson, B.E. and Kautsky, N. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. International Journal of Food Science and Technology 38: 255-266.

Holthuis, L. B., 1980. FAO species catalogue. Vol. 1. Shrimps and prawns of the world. An annotated catalogue of species of interest to fisheries. FAO Fisheries Synopsis 1: 1–261.

Hugenschmidt, S., Schwenninge, S.M., Gnehm, N. and Lacroix, C. 2010. Screening of a natural biodiversity of lactic and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate. International Dairy Journal 20: 852-857.

Imhoff, J. F., Truper, H. G. and Pfennig, N. 1984. Rearrangment of the species and genera of phototrophic purple nonsulfur bacteria. International Journal of Systematic Bacteriology 34(3): 340-343.

Imhoff, J.F., Hiralshi, A. and Suling, J. 2005. Anoxygenic phototrophic purple bacteria. In Bergey's manual of systematic bacteriology. (2nd ed). Brenner, D.J., Krieg, N.R. and Staley, J.T. editors. Springer, USA, pp. 119-132.

Jannakiram, P., Veerendra-kumar, M., Jayasree, I. and Sivaprasad, B. 2014. Probiotic activity of *Pseudomonas aeroginosa* (PIC-4) isolated from Visakhapatnam coast, Bay of Bengal, India, against *Vibrio harveyi* in *Penaeus monodon*. International Journal of Fisheries and Aquatic Studies 1(6): 199-207.

Johnson, C.N., Ogle, S.B.J., and Grimes, D.J. 2008. Microbial community analysis of water, foregut, and hindgut during growth of Pacific white shrimp, *Litopenaeus vannamei*, in closed-system. Journal of the World Aquaculture Society 39: 251-258.

Joshi, J., Srisala, J., Truong, V.H., Chen, I.T., Nuangsaeng, B., Suthienkul, O., Lo, C.F., Flegel, T.W., Sritunyalucksana, K., Thitamadee, S. 2014. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture 428–429: 297–302.

Juneius, C.E.R. and Selvin, J. 2012. Identification, phylogenetic characterization and

preliminary screening of primary and secondary metabolites producing bacteria associated with marine sponge *Axinella donani*. Indian Journal of Drugs and Diseases 1(1): 1-9.

Kakkar, P., Das, B. and Viswanathan, P. N. 1984. A modified spectrophotometric assay of superoxide dismutase. Indian Journal of Biochemistry & Biophysics 21: 130-132.

Kanazawa, A. 1989. Protein requirements of penaeid shrimp. In: Advances in Tropical Aquaculture, Workshop at Tahiti, French Polynesia, 20 Feb-4 Mar 1989: 261-270.

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

Kantachote, D., Nunkaew, T., Ratanaburee, A. and Klongdee, N. 2016. Production of a meat seasoning powder enriched with γ-aminobutyric acid (GABA) from mature coconut water using *Pediococcus pentosaceus* HN8. Journal of Food Processing and Preservation 40: 733-742.

Kaspari, H. and Klemme, J. H. 1977. Characterization of antivibrio activities produced by *Rhodopseudomonas sphaeroides*. FEM Letters: 59-62.

Kim, J. K. and Lee, B. K. 2000. Mass production of *Rhodopseudomonas palustris* as diet for aquaculture. Aquacultural Engineering 23: 281-293.

Kim, M.K., Choi, K.M., Yin, C.R., Lee, K.Y., Im, W.T., Lim, J.H. and Lee, S.T. 2004. Odorous swine wastewater treatment by purple non-sulfur bacteria, *Rhodopseudomonas palustris*, isolated from eutrophicated ponds. Biotechnology Letters 26: 819-822.

Kiriratnikom, S. 2006. Evaluation of possible application of photosynthetic bacteria in black tiger shrimp (*Penaeus monodon*). Ph. D. Prince of Songkla University.

Kongnum, K. and Hongpattarakere, T. 2012. Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*. Fish & Shellfish Immunology 32: 170-177.

Kornboonraksa, T. and Lee, S. H. 2009. Factors affecting the performance of membrane bioreactor for piggery wastewater treatment. Bioresource Technology 100: 2926-2932.

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

Kuo, F. S., Chien, Y. H. and Chen, C. J. 2012. Effects of light sources on growth and carotenoid content of photosynthetic bacteria *Rhodopseudomonas palustris*. Bioresource Technology 113: 315-318.

Kureshy, N. and Davis, D.A. 2002. Protein requirement for maintenance and maximum weight gain for the Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 204: 125-143.

Lambert, P. A. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Journal of Applied Microbiology Symposium Supplement 92: 46-54.

Lai, H.C., Ng, T.H., Ando, M., Lee, C.T., Chen, I.T., Chuang, T.C., Mavichak, R., Chang, S.H., Yeh, M.D., Chiang, Y.A., Takeyama, H., Hamaguchi, H.O., Lo, C.F., Aoki, T. and Wang, H.C. 2015. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish & Shellfish Immunology 47: 1006-1014.

Latscha, T. 1989. The role of astaxanthin in shrimp pigmentation. In: Advances in Tropical Aquaculture, Workshop at Tahiti, French Polynesia, 20 Feb-4 Mar 1989. 319-325 pp.

Lee, S. S., Oh, T. J., Kim, J., Kim, J. B. and Lee, H. S. 2009. Bacteriocin from purple nonsulfur phototrophic bacteria, *Rhodobacter capsulatus*. Journal of Bacteriology and Virology 39: 269–276.

Li, E., Chen, L., Zeng, C., Yu, N., Xiong, Z., Chen, X. and Qin, J. G. 2008. Comparison of digestive and antioxidant enzymes activities, haemolymph oxyhemocyanin contents and hepatopancreas histology of white shrimp, *Litopenaeus vannamei*, at various salinities. Aquaculture 274: 80-86.

Lightner, D., Chen, S.N., Flegel, T. and Walker, P. 2006. Cultured aquatic species information Programme (*Penaeus vannamei*). http://www.fao.org/fishery/ culturedspecies/ *Litopenaeus_vannamei*/en (Access 19 July 2012).

Lim, C. E. 1998. Feeding Penaeid shrimps, In Nutrition and feeding of fish, second edition, Ed. Tom Lovell, Springer Science + Business Media, New York, 227- 248 pp.

Linan-Cabello, M. A., Paniagua-Michel, J. and Hopkins, P. M. 2002. Bioactive roles of carotenoids and retinoids in crustaceans. Aquaculture Nutrition 8: 299-309.

Lin, H.Z., Guo, Z., Yang, Y., Zheng, W. and Li, Z. 2004. Effect of dietary probiotics on apparent digestibility coeffecients of nutrients of white shrimp *Litopenaeus vannamei* Boone. Aquaculture Research 35: 1441-1447.

Liu, C. H and Chen, J. C. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. Fish & Shellfish Immunology 16: 321-334.

Liu, C. H., Tseng, M. C. and Cheng, W. 2007. Identification and cloning of the antioxidant enzyme, glutathione peroxidase, of white shrimp, *Litopenaeus vannamei*, and its expression following *Vibrio alginolyticus* infection. Fish & Shellfish Immunology 23: 34-45.

Liu, K.F., Chiu, C.H., Shiu, Y.L., Cheng, W. and Lui, C.H. 2010. Effects of the probiotic, *Bacillus sultilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish immunology 28: 837-844.

Liu, C. H., Chiu, C. H., Wang, S. W. and Cheng, W. 2012. Dietary administration of probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. Fish & Shellfish Immunology 33: 669-706.

Liu, S., Li, S., Zhang, G. and Zhang, J. 2015. Optimization of influencing factors on biomass accumulation and 5-aminolevulinic aacid (ALA) yield in *Rhodobacter sphaeroides* wastewater treatment. Journal of Microbiology and Biotechnology 25(11): 1920-1927.

Lu, H., Zhang, G., Wan, T. and Lu, Y. 2011. Influences of light and oxygen conditions on photosynthetic bacteria macromolecule degradation: Different metabolic pathways. Bioresource Technology 102: 9503-9508.

Luo, W., Deng, X., Zeng, W. and Zheng, D. 2012. Treatment of wastewater from shrimp farms using a combination of fish, photosynthetic bacteria, and vegetation. Desalination and Water Treatment 47: 221-227.

Madigan, M. T. and Jung, D. O. 2009. An overview of purple bacteria: systematics, physiology, and habitats. In the purple phototrophic bacteria. Springer Netherlands. pp. 1-15.

Madukasi, E.I., Chunhua, H. and Zhang, G. 2011. Isolation and application of a wild strain photosynthetic bacterium to environment waste management. International Journal of Environmental Science and Technology 8(3): 513-522.

Mann, J., Davidson, R. S., Hobbs, J. B., Banthorpe, D. V. and Harborne, J. B. 1994. Natural products - their chemistry and biological significance, Longman Scientific & Technical, London.

McEntire, J.C. and Montville, T.J. 2007. Antimicrobial resistance. Food Microbiology: Fundamentals and Frontiers, (3rd Ed): 23-27.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, T.M., Bogwald, J., Castex, M and Ringo, E. 2010. The current status and future focus of probioitic and prebiotic application for salmonids. Aquaculture 302(1-2): 1-18.

Ministry of Agriculture and Cooperatives. B.E. 2550 (2007). Thai Agricultural Standard; Organic marine shrimp farming (TAS 7413-2007). National Bureau of Agricultural Commodity and Food Standards.

Mishra, J. K., Samocha, T. M., Patnaik, S., Speed, M., Gandy, R. L. and Ali, A. M. 2008. Performance of an intensive nursery system for the Pacific white shrimp, *Litopenaeus vannamei*, under limited discharge condition. Aquacultural Engineering 38: 2-15.

Merugu, R.C., Girisham, S. and Reddy, S.M. 2010. Extracellular enzyme of two Anoxygenic phototrophic bacteria isolated from leather industry effluents.

The BioChemistry : An Indian Journal 4(2): 86-88.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S., Mallavarapu, M. and Naidu, R. 2015. Distribution of mercury in shrimp ponds and volatilization of Hg by isolated resistant purple nonsulfur bacteria. Water, Air, & Soil Pollution 226:148. Doi: 10.1007/s11270-015-2418-2.

Mukkata, K., Kantachote, D., Wittayaweerasak, B., Techkarnjanaruk, S. and Boonapatchareon, N. 2016. Diversity of purple nonsulfur bacteria in shrimp ponds with varying mercury levels. Saudi Journal of Biological Sciences 23: 478-487.

Mukoyama, D., Takeyama, H., Kondo, Y. and Matsunaga, T. 2006. Astaxanthin formation in the marine photosynthetic bacterium *Rhodovulum sulfidophilum* expressing crtI, crtY, crtW and crtZ. FEMS Microbiology Letters 265(1): 69-75.

Munjam, S., Girisham, S. and Reddy, S. M. 2005. Production of lipases by four anoxygenic purple non-sulfur phototrophic bacteria. Hindustan antibiotics bulletin 47-48: 32-35.

Munn, C.B. 2004. Marine microbiology: ecology and applications. Taylor and Francis Group, New York, 282 pp.

Murniasih, T., Kosela, S., Kardono, L. B. S, Hanafi, M. and Priyono, W. 2014. An antibacterial compound isolated from sponge-associated bacteria *Rhodobacteracea bacterium*. J. Med. Sci. 14(2): 75-80. DOI: 10.3923/jms.2014.75.80.

Musa, N., WEI, L. S. and WEE, W. 2008. Phenotypic and genotypic characteristics of *Vibrio harveyi* isolated from black tiger shrimp (*Penaeus monodon*). World Applied Sciences Journal 3 (6): 885-902.

Nasseri, A.T, Rasoul-Amini, S, Morowvat, M. H. and Ghasemi, Y. 2011. Single cell protein: production and process. American Journal of Food Technology 6(2): 103-116.

Nimrat, S., Suksawat, S., Boonthai, T. and Vuthiphandchai, V. 2012. Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology 159: 443-450.

Niwa, T., Kawamura, Y., Katagiri, Y. and Ezaki, T. 2005. Lytic enzyme, labiase for a broad range of Gram-positive bacteria and its application to analyze functional DNA/RNA. Journal of Microbiological Methods 61 (2): 251-260.

Noogkongbut, P., Kantachote, D. and Megharaj, M. 2016. Arsenic contamination in areas surrounding mines and selection of potential As-resistant purple nonsulfur bacteria for use in bioremediation based on their detoxification mechanism. Annals of Microbiology 66: 1419-1429.

Nunan, L., Lightner, D., Pantoja, C. and Gomez-Jimenez, S. 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Diseases of Aquatic Organisms 111: 81-86.

Nunkaew, T., Kantachote, D., Nitoda, T. and Kanzaki, H. 2012. The use of rice straw broth as an appropriate medium to isolate purple nonsulfur bacteria from paddy fields. Electronic Journal of Biotechnology 15, http://dx.doi.org/ 10.2225/vol15- issue6-fulltext- 8, p. 12.

Oda, K., Tanskul, S., Oyama, H. and Noparatnaraporn, N. 2004. Purification and Characterization of alkaline serine proteinase from photosynthetic bacterium, *Rubrivivax gelatinosus* KDDS1. Bioscience, Biotechnology, and Biochemistry 63(3): 650-655.

Omondi, J.G. 2005. Digestive endo-proteases from the midgut glands of the Indian white shrimp, *Penaeus Indicus* (Decapoda: *Penaeidae*) from Kenya. Western Indian Ocean. Journal of Marine Science 4: 109-121.

Panwichian, S., Kantachote, D., Wittayaweerasak, B. and Mallavarapu, M. 2010. Isolation of purple nonsulfur bacteria for the removal of heavy metals and sodium from contaminated shrimp pond. Electronic Journal of Biotechnology 13(4): 1-12 DOI: 10.2225/vol13-issue4-fulltext-8.

Panwichian, S., Kantachote, D., Wittayaweerasak, B. and Mallavarapu, M. 2010. Factors affecting immobilization of heavy metals by purple nonsulfur bacteria isolated from contaminated shrimp ponds. World Journal of Microbiology and Biotechnology 26 (12): 2199-2210.

Pasharawipas, T., Sriurairatana, S., Direkbusarakom, S., Donayadol, Y., Thaikua, S.,

Ruangpan, L. and Flegel, T.W. 1998. Luminous *Vibrio harveyi* associated with tea brown gill syndrome in black tiger shrimp. Advances in shrimp biotechnology. In National Center for Genetic Engineering and Biotechnology, Bangkok. Flegel TW (ed): 213-216 pp.

Parrilla-Taylor, D. and Zenteno-Savín, T. 2011. Antioxidant enzyme activities in Pacific white shrimp (*Litopenaeus vannamei*) in response to environmental hypoxia and reoygenation. Aquaculture 318: 379-383.

Parrilla-Taylor, D. P., Zenteno-Savin, T. and Magallon-Barajas. 2013. Antioxidant enzyme activity in pacific white leg shrimp (*Litopenaeus vannamei*) in response to infection with white spot syndrome virus. Aquaculture 380-383: 41-46.

Pencreac'h, G. and Baratti, J.C. 1996. Hydrolysis of *p*-nitrophenyl palmitate in n-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. Enzyme and Microbial Technology 18: 417-422.

Phuoc, L.H. 2008. Single and dual experimental infection of specific pathogen-free *Litopenaeus vannamei* shrimp with White Spot Syndrome Virus and *Vibrio* species. Ph.D, Ghent University, Belgium.

Prayitno, S. B. and Latchford J. W. 1995. Experimental infections of crustaceans with luminous bacteria related to Photobacterium and *Vibrio*. Effect of salinity and pH on infectiosity. Aquaculture 132(1-2): 105-112.

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

Randaccio, L., Silvano, G., Demitri, N. and Wuerges. J. 2010. Vitamin B12: unique metaloganic compounds and the most compounds and the most complex vitamins. Molecules15: 3228-3259.

Rattanachuay, P., Kantachote, D. and Suntinanalert, P. 2007. Selection of proteolytic bacteria with ability to inhibit *Vibrio harveyi* during white shrimp (*Litopenaeus vannamei*) cultivation. Songklanakarin Journal of Science and Technology 29(2): 235-243.

Rattanachuay, P. 2010. Characterization of bioactive compound produced by

Pseudomonas sp. W3 for controlling shrimp pathogenic *Vibrio harveyi*. Ph.D. Prince of Songkla University.

Rattanachuay, P., Kantachote, D., Tantirungkij, M., Nitoda, T. and Kanzaki, H. 2010. Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3. Electron Journal of Biotechnology 13: 1-11.

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

Ravinda, A.P. 2000. Value – added food: Single cell protein. Biotechnology Advances 18: 459-479.

Reed, K. and Royales, S. 2014. Shrimp disease in Asia resulting in high U.S. import prices. Beyond the Numbers Washington, DC: Bureau of Labor Statistics 3: 1-8.

Roman, J. M. T. 2006. Assessment of genetic variability in two lots of white shrimp, *Litopenaeus vannamei* (Boone, 193) introduced to Cuba. Master thesis. International Fisheries Management, Department of Aquatic Biosciences Norway, University of Chicago.

Rowley, A.F. and Powell, A. 2007. Invertebrate immune system-specific, quasi-specific or nonspecific. Journal of immunology 179: 7209-7214.

Sarmah, A.K., Meyer, M.T. and Boxall, A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 56: 725-759.

Sanders, M. E. 2008. Probiotics: definition, sources, selection, and uses. Clinical Infectious Diseases 46: 58-61.

Sandifer, P., Hopkins, S. and Stokes, A. D. 1987. Intensive cuture potential of *Penaeus vannamei.* Jounal of the world aquaculture society 18 (2): 94-100.

Sasaki, K., Watanabe, M., Suda, Y., Ishizuka, A. and Noparatnaraporn, N. 2005. Applications of Photosynthetic bacteria for medical fields. Journal of Bioscience and Bioengineering 100(5): 481-488.

Serrano, P.H. 2005. Responsible use of antibiotics in aquaculture. Management Service Information Division FAO, Itary, 97 pp.

Shapawi, R., Ting, T.E. and Al-azad, S. 2012. Inclusion of purple non-sulfur bacteria biomass in formulated feed to promote growth, feed conversion ratio and survival of asian seabass *Lates calcarifer* juveniles. Journal of Fisheries and Aquatic Science: 1-6.

Shiau, S. Y. and Lung, C. Q. 1993. Estimation of the vitamin B12 requirement of the grass shrimp, *Penaeus monodon*. Aquaculture 117: 157-163.

Silva, E. F., Soares, M. A., Calazans, N. F., Vogeley, J. L., do Valle, B. C., Soares, R. and Peixoto, S. 2013. Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquaculture Research 44: 13–21.

Singer, R. S. and William-Nguyen, J. 2014. Human health impacts of antibiotic use in agriculture: A push for improved causal inference. Current Opinion in Microbiology 19: 1-8.

Sritunyalucksana, K. and Soderhall, K. 2000. The proPO and clotting system in crustaceans. Aquaculture 191: 53-69.

Stumpt, L., Diaz, F. C., Viau, V. E., Valenti, W. C. and Greco, L. S. L. 2014. Effect of food shortage on growth, energetic reserves mobilization, and water quality in juveniles of the redclaw crayfish, *Cherax quadricarinatus*, reared in groups. Journal of Crustacean Biology 34 (5): 639-646.

Sweet, M. J. and Bateman, K. S. 2015. Diseases in marine invertebrates associated with mariculture and coomercial fisheries. Journal of sea research 104: 16-32.

Tacon, A. G. J. 2017. Biosecure shrimp feeds and feeding practices: guidelines for future development. Journal of the World Aquaculture Society 1-12. Doi:10.1111/jwas.12406.

Taylor, R. 1990. Interpretation of the correlation coefficient: A basic review. Journal of Diagnostic Medical Sonography 6(1): 35–39.

Tassankajon, A., Somboonwiwat, K., Supungul, P. and Tang, S. 2013. Discovery of immune molecules and their crucial function in shrimp immunity. Fish &

Shellfish Immunology 34: 954-967.

Thai Frozen Foods Association. 2012. Thailand seafood industry overview. The situation of Thai Shrimps in 2012 and its trend in 2013. http://www.thai-frozen.or.th/thailand_ seafood_02_en.php. (Accessed 15 May 2016).

Thakura, D. P. and Lin, C. K. 2003. Water quality and nutrient budget in close shrimp (*Penaeus monodon*) culture systems. Aquaculture Research 27(3): 159-176.

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

Thitamadee, S., Prachumwat, A., Srisala, J., Jaroenlak, P., Salachan, P. V., Sritunyalucksana, K., Flegel, T. W. and Itsathiphaisarn, O. 2016. Review of current disease threats for cultivated penaeid shrimp in Asia. Aquaculture 454: 69-87.

Thuyet, D. B., Luong-Van, J. and Austin, C. M. 2012. Impact of shrimp farm effluent on water quality in coastal areas of the world heritage-listed Ha Long Bay. American Journal of Environmental Sciences 8(2): 104-116.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K. and Lightner, D.V. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 105: 45-55.

Treece, G. 2014. White leg shrimp (*Litopenaeus vannamei*). The Monterey Bay Aquarium Seafood Watch® https://www.seafoodwatch.org/-/m/sfw/pdf/reports/s /mba _seafoodwatch_ usfarmedshrimpreport.pdf. (Accessed 11 May 2017).

Tzuc, J.T., Escalante, D.R., Herrera, R.R., Cortés, G.G. and Ortiz, M.L.A. 2014. Microbiota from *Litopenaeus vannamei*: digestive tract microbial community of Pacific white shrimp (*Litopenaeus vannamei*). SpringerPlus 3: 1-10. Doi: 10.1186/2193-1801-3-280.

Uffen, R. and Wolfe, R. S. 1970. Anaerobic growth of purple non sulfur bacteria under dark conditions. Journal of Bacteriology 104: 462-472.

Vandenberghe, J., Verdonck, L., Arozarena, R. R., Rivera, G. and Bolland, A. 1999. Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstockand hatchery probionts. Applied and Environmental Microbiology 65: 2592–2597.

Vaseeharan, B., Ramasamy, P., Murugan, T. and Chen, J. C. 2005. In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. Internation Journal of Antimicrobial Agents 26: 285-291.

Verschuere, L., Rombaul, G., Sorgeloos, P. and Verstrate, W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64: 655-671.

Wade, N. M., Cheers, S., Bourne, N., Irvin, S., Blyth, D. and Glencross, B. D. 2017. Dietary astaxanthin levels affect colour, growth, carotenoid digestibility and the accumulation of specific carotenoid esters in the Giant Tiger Shrimp, *Penaeus monodon*. Aquaculture Research 48: 395-406.

Wang, S., Kong, J. and Zhang, X. 2008. Identification and characterization of the two-component cell lysis cassette encoded by temperate bacteriophage ϕPYB5 of *Lactobacillus fermentum*. Journal of Applied Microbiology 105 (6): 1939-1944.

Wang, Y. 2011. Use of probiotics *Bacillus coagulants*, *Rhodopseudomonas palustris* and *Lactobacillus acidophilus* as growth promoters in grass carp (*Ctenopharyngodon idella*) fingerlings. Aquaculture Nutrition 17: 372-378.

Wang, X., Li, E., Xu, C., Qin, J. G., Wang, S., Chen, X., Cai, Y., Chen, K., Gan, L., Yu, N., Du, Z. Y. and Chen, L. 2016. Growth, body composition, ammonia tolerance and hepancreas histology of white shrimp *Litopenaeus vannamei* fed diets containing different carbohydrate sources at low salinity. Aquaculture Research 47: 1932-1943.

Wang, W., Sun, J., Liu, C. and Xue, Z. 2017. Application of immunostimulants in aquaculture: current knowledge and future perspectives. Aquaculture Research 48: 1-23.

Willey, J.M. Sherwood, L.M. and Woolverton, C.J. 2008. Bacteria: the deinococci and nonproteobacteria gram negative. Prescott, Harley, and Klein's Microbiology. (7th ed): 465-486.

Williams, D. H., Stone, M. J., Hauck, P. R. and Rahman, S. K. 1989. Why are secondary metabolites (natural products) biosynthesized?. Journal of Natural Products 52(6): 1189-1208.

Wilson, K., Knell, R., Boots, M. and Koch-Osborne, J. 2003. Group living and investment in immunedefence: an interspecific analysis. Journal of Animal Ecology 72: 133-143.

Wu, W. T. and Liu, W. S. 2011. Anti-inflammatory property of biomaterial carotenoids production by *Rhodobacter sphaeroides* WL-APD911. Advanced Materials Research 287-290: 2028-2031.

Xiong, J., Dai, W. and Li, L. 2016. Advances, challenges, and directions in shrimp disease control: the guidelines from an ecological perspective. Applied Microbiology and Biotechnology 100: 6947-6954.

Zhang, Y.B., Li, Y. and Sun, X.L. 2011. Antibiotic resistance of bacteria isolated from shrimp hatcheries and cultural ponds on Donghai Island, China. Marine Pollution Bulletin 62(11): 2299-2307.

Zhang, H., Kong, C., Wang, Y. and Shen, X. 2014. Effects of temperature on growth and survival of Pathogenic *Vibrio parahaemolyticus*. International Journal of Food Science & Technology 2(4): 106-109.

Zhang, H. and Hu, Q. P. 2015. Isolation, identification and physiological characteritics of high carotenoids yield *Rhodopseudomonas faecalis* PSB-B. International Journal of Recent Scientific Research 6(5): 3893-3899.

Zhang, H., Sun, Z., Liu, B., Xuan, Y., Jiang, M., Pan, Y., Zhang, Y., Gong, Y., Lu, D., Kumar, D., Hu, X., Cao, G., Xue, R. and Gong, C. 2016. Dynamic changes of microbial communities in *Litopanaeus vannamei* cultures and the effects of environmental factors. Aquaculture 455: 97-108.

Zheng, Y., Yua, M., Liua, Y., Sua,Y., Xu, T., Yu, M. and Zhang, X. H. 2016.

Comparison of cultivable bacterial communities associated with Pacific white shrimp (*Litopenaeus vannamei*) larvae at different health statuses and growth stages. Aquaculture 451: 163-169.

Zhou, X.X., Pan, Y.J., Wang, Y.B. and Li, w.f. 2007. In vitro assessment of gastrointestinal viability of two photosynthetic bacteria, *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides*. Journal of Zhejiang university science b 8(9): 686-692.

Zhou, Q., Zhang, P. and Zhang, G. 2014. Biomass and carotenoid production in photosynthetic bacteria wastewater treatment: effects of light intensity. Bioresource Technology 171: 330-335.

Zokaeifar, H., Balcázar, J. B., Saad, C. R., Kamarudin, M. S., Sijam, K., Arshad, A. and Nejat, N. 2012. Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish & Shellfish Immunology 33: 683-689.

Zorriehzahra, M. J. and Banaederakhshan, R. 2015. Early mortality syndrome (EMS) as new emerging threat in shrimp industry. Advances in Animal and Veterinary Sciences 3: 64–72.

APPENDIX

A. Media

Basic Isolation medium

$(NH_4)_2SO_4$	1.0	g
K_2HPO_4	0.5	g
$MgSO_4$	0.2	g
$NaHCO_3$	5.0	g
NaCl	15.0	g
Yeast extract	1.5	g
Glycerol	1.5	mL
L-cysteine	0.03	g
Distilled water	1,000	mL
pH	7	

Glutamate-Acetate medium

Sodium L- glutamic acid	3.8	g
Sodium acetate	5.4	g
Yeast extract	2.0	g
KH_2PO_4	0.5	g
K_2HPO_4	0.5	g
$(NH_4)_2HPO_4$	0.8	g
$MgSO_4$	0.2	g
NaCl	20.0	g
$CaCl_2 \cdot 2H_2O$	0.053	g
Nicotinic acid	0.001	g
Thiamine hydrochloride	0.001	g
Biotin	0.01	g
$MnSO_4 \cdot 5H_2O$	0.012	g
Ferric citrate	0.025	g
$CoCl_2 \cdot 6H_2O$	0.95	g
Distilled water	1,000	mL
pH	6.8	

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

LOCUS	KJ955372 1201 bp DNA linear BCT 30-JAN-2015
DEFINITION	*Rhodobacter sphaeroides* strain SS15 16S ribosomal RNA gene, partial sequence.
ACCESSION	KJ955372
VERSION	KJ955372.1
KEYWORDS	.
SOURCE	*Rhodobacter sphaeroides*
ORGANISM	*Rhodobacter sphaeroides* Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Rhodobacter*.
REFERENCE	1 (bases 1 to 1201)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios.
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1201)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JUN-2014) Microbiology, Prince of Songkla University, Kanjanavanit, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1201 /organism="*Rhodobacter sphaeroides*"

/mol_type="genomic DNA"
/strain="SS15"
/isolation_source="shrimp pond sediment"
/db_xref="taxon:1063"
rRNA <1..>1201
/product="16S ribosomal RNA"
ORIGIN

1 ggacttagcg gcggacgggt gagtaacgcg tgggaacgtg ccctttgctt cggaatagcc
61 ccgggaaact gggagtaata ccgaatgtgc cctatggggg aaagatttat cggcaaagga
121 tcggcccgcg ttggattagg tagttggtgg ggtaatggcc taccaagccg acgatccata
181 gctggtttga gaggatgatc agccacactg ggactgagac acggcccaga ctcctacggg
241 aggcagcagt ggggaatctt agacaatggg cgcaagcctg atctagccat gccgcgtgat
301 cgatgaaggc cttagggttg taaagatctt tcaggtggga agataatgac ggtaccacca
361 gaagaagccc cggctaactc cgtgccagca gccgcggtaa tacggagggg gctagcgtta
421 ttcggaatta ctgggcgtaa agcgcacgta cgcggatcgg aaagtcagag gtgaaatccc
481 agggctcaac cctggaactg cctttgaaac tcccgatctt gaggtcgaga gaggtgagtg
541 gaattccgag tgtagaggtg aaattcgtag atattcggag gaacaccagt ggcgaacgcg
601 gctcactggc tcgatactga cgctgaggtg cgaaagcgtg gggagcaaac aggattagat
661 accctggtag tccacgccgt aaacgatgaa tgccagtcgt cgggcagcat gctgttcggt
721 gaccacctaa cggattagca ttcgcctggg gagtacggcc gcaaggttaa aactcaaagg
781 aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgaagc aacgcgcaga
841 accttaccaa cccttgacat ggcgatcgcg gttccagaga tggttccttc agttcggctg
901 gatcgcacac aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgt tcggttaagt
961 ccggcaacga gcgcaaccca cgtccttagt tgccagcatt cagttgggca ctctagggaa
1021 actgccggtg ataagccgga ggaaggtgtg gatgacgtca agtcctcatg gcccttacgg
1081 gttgggctac acacgtgcta caatggcagt gacaatgggt taatcccaaa aagctgtctc
1141 agttcggatt ggggtctgca actcgacccc atgaagtcgg aatcgctagt aatcgcgtaa
1201 c

LOCUS	KJ955374 1319 bp DNA linear BCT 30-JAN-2015
DEFINITION	*Rhodobacter sphaeroides* strain S3W10 16S ribosomal RNA gene, partial sequence.
ACCESSION	KJ955374
VERSION	KJ955374.1
KEYWORDS	.
SOURCE	*Rhodobacter sphaeroides*
ORGANISM	*Rhodobacter sphaeroides* Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Rhodobacter*.
REFERENCE	1 (bases 1 to 1319)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios.
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1319)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (09-JUN-2014) Microbiology, Prince of Songkla University, Kanjanavanit, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1319 /organism="*Rhodobacter sphaeroides*" /mol_type="genomic DNA" /strain="S3W10"

/isolation_source="shrimp pond water"
/db_xref="taxon:1063"
rRNA <1..>1319
/product="16S ribosomal RNA"
ORIGIN

1 cagtcgagcg agtcttcgga cttagcggcg gacgggtgag taacgcgtgg gaacgtgccc
61 tttgcttcgg aatagccccg ggaaactggg agtaataccg aatgtgccct atgggggaaa
121 gatttatcgg caaaggatcg gcccgcgttg gattaggtag ttggtggggt aatggcctac
181 caagccgacg atccatagct ggtttgagag gatgatcagc cacactggga ctgagacacg
241 gcccagactc ctacgggagg cagcagtggg gaatcttaga caatgggcgc aagcctgatc
301 tagccatgcc gcgtgatcga tgaaggcctt agggttgtaa agatctttca ggtgggaaga
361 taatgacggt accaccagaa gaagccccgg ctaactccgt gccagcagcc gcggtaatac
421 ggagggggct agcgttattc ggaattactg ggcgtaaagc gcacgtaggc ggatcggaaa
481 gtcagaggtg aaatcccagg gctcaaccct ggaactgcct ttgaaactcc cgatcttgag
541 gtcgagagag gtgagtggaa ttccgagtgt agaggtgaaa ttcgtagata ttcggaggaa
601 caccagtggc gaaggcggct cactggctcg atactgacgc tgaggtgcga aagcgtgggg
661 agcaaacagg attagatacc ctggtagtcc acgccgtaaa cgatgaatgc cagtcgtcgg
721 gcagcatgct gttcggtgac acacctaacg gattaagcat tccgcctggg gagtacggcc
781 gcaaggttaa aactcaaagg aattgacggg ggcccgcaca agcggtggag catgtggttt
841 aattcgaagc aacgcgcaga accttaccaa cccttgacat ggcgatcgcg gttccagaga
901 tggttccttc agttcggctg gatcgcacac aggtgctgca tggctgtcgt cagctcgtgt
961 cgtgagatgt tcggttaagt ccggcaacga gcgcaaccca cgtccttagt tgccagcatt
1021 cagttgggca ctctagggaa actgccggtg ataagccgga ggaaggtgtg gatgacgtca
1081 agtcctcatg gcccttacgg gttgggctac acacgtgcta caatggcagt gacaatgggt
1141 taatcccaaa aagctgtctc agttcggatt ggggtctgca actcgacccc atgaagtcgg
1201 aatcgctagt aatcgcgtaa cagcatgacg cggtgaatac gttcccgggc cttgtacaca
1261 ccgcccgtca caccatggga attggttcta cccgaaggcg gtgcgccaac ctcgcaaga

LOCUS	KM387395 1280 bp DNA linear BCT 31-MAR-2015
DEFINITION	*Rhodobacter sphaeroides* strain TKW17 16S ribosomal RNA gene, partial sequence.
ACCESSION	KM387395
VERSION	KM387395.1
KEYWORDS	.
SOURCE	*Rhodobacter sphaeroides*
ORGANISM	*Rhodobacter sphaeroides* Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Rhodobacter*.
REFERENCE	1 (bases 1 to 1280)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio*
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1280)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (25-AUG-2014) Microbiology, Prince of Soongkla University, Kanjanawanit, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1280 /organism="*Rhodobacter sphaeroides*" /mol_type="genomic DNA" /strain="TKW17" /isolation_source="water of shrimp pond"

/db_xref="taxon:1063"

rRNA <1..>1280

/product="16S ribosomal RNA"

ORIGIN

1 acgcgtggga acgtgccctt tgcttcggaa tagccccggg aaactgggag taataccgaa
61 tgtgccctat gggggaaaga tttatcggca aaggatcggc ccgcgttgga ttaggtagtt
121 ggtggggtaa tggcctaccc agccgacgat ccatagctgg tttgagagga tgatcaccca
181 cactgcgact gagacacgac ccaaactcct actcgaggcc gctgtgggga atcttagaca
241 atgagtccta gcctgatcta gccatgccgc gcgatcgatg agggccttag ggttgcaaag
301 atctttcagg tgggaagata atgacggcac caccagaaga agccccggct aactccgtgc
361 cagcagccgc ggtaatacgg agggggctag cgaccttcgg aattactggg cgtaaagcgc
421 acgtacgcgg atcggatagt cagaggtgaa atcgcaggac tcaaccctgg aactgccttt
481 gaaactcccg atcttgaggt cgagagaggt gagtggaatt ccgagtgtag aggtgaaatt
541 cgtagatatt cggaggaaca ccagtggcga aggcggctca ctggctcgat actgacgctg
601 aggtgcgaaa gcgtggggag caaacaggat tagataccct ggtagtccac gccgtaaacg
661 atgaatgcca gtcgtcgggc agcatgctgt tcggtgacac acctaacgga ttaagcattc
721 cgcctgggga gtacggccgc aaggttaaaa ctcaaaggaa ttgacggggg cccgcacaag
781 cggtggagca tgtggtttaa ttcgaagcaa cgcgcagaac cttaccaacc cttgacatgg
841 cgatcgcggt tccagagatg gttccttcag ttcggctgga tcgcacacag gtgctgcatg
901 gctgtcgtca gctcgtgtcg tgagatgttc ggttaagtcc ggcaacgagc gcaacccacg
961 tccttagttg ccagcattca gttgggcact ctagggaaac tgccggtgat aagccggagg
1021 aaggtgtgga tgacgtcaag tcctcatggc ccttacgggt tgggctacac acgtgctaca
1081 atggcagtga caatgggtta atcccaaaaa gctgtctcag ttcggattgg ggtctgcaac
1141 tcgaccccat gaagtcggaa tcgctagtaa tcgcgtaaca gcatgacgcg gtgaatacgt
1201 tcccgggcct tgtacacacc gcccgtcaca ccatgggaat tggttctacc cgaaggcggt
1261 gcgccaacct cgcaagagga

LOCUS	KJ955373 1277 bp DNA linear BCT 30-JAN-2015
DEFINITION	*Afifella marina* strain STW18-1 16S ribosomal RNA gene, partial sequence.
ACCESSION	KJ955373
VERSION	KJ955373.1
KEYWORDS	.
SOURCE	*Afifella marina*
ORGANISM	*Afifella marina* Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhodobiaceae; *Afifella*.
REFERENCE	1 (bases 1 to 1277)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio*
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1277)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JUN-2014) Microbiology, Prince of Songkla University, Kanjanavanit, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1277 /organism="*Afifella marina*" /mol_type="genomic DNA" /strain="STW18-1" /isolation_source="shrimp pond water"

/db_xref="taxon:1080"

rRNA <1..>1277

/product="16S ribosomal RNA"

ORIGIN

```
   1 catgcagtcg acgcactctt cggagtgagt ggcagacggg tgagtaacgc gtgggaatct
  61 acccagtggt acgggataac ccgaggaaac tcgagctaat accgtatacg cccttcgggg
 121 gaaagattta ttgccattgg atgagcccgc gtcggattag cttgttggtg gggtaacggc
 181 ctaccaaggc aacgatccgt agctggtctg agaggatgat cagccacact gggactgaga
 241 cacggcccag actcctacgg gaggcagcag tggggaatct tggacaatgg gggaaaccct
 301 gatccagcca tgccgcgtga gtgaagaagg ccctagggtt gtaaagctct ttcagcgggg
 361 aagataatga cggtacccgc agaagaagcc ccggctaact tcgtgccagc agccgcggta
 421 atacgaaggg ggctagcgtt gttcggaatt actgggcgta aagcgcgcgt aggcggattg
 481 ttaagtcagg ggtgaaatcc cagagctcaa ctctggaact gcctctgata ctggcaatct
 541 cgagtccgga agaggttggt ggaattccga gtgtagaggt gaaattcgta gatattcgga
 601 ggaacaccag aggcgaaggc ggccaactgg tccgagactg acgctgaggc gcgaaagcgt
 661 ggggagcaaa caggattaga taccctggta gtccacgccg taaacgatgg atgctagccg
 721 ttggtgggta tactcatcag tggcgcagct aacgcattaa gcatcccgcc tggggagtac
 781 ggtcgcaaga ttaaaactca aaggaattga cgggggcccg cacaagcggt ggagcatgtg
 841 gtttaattcg aagcaacgcg cagaacctta ccagctcttg acatcccgat cgcggttacc
 901 ggagacggtt tccttcagct aggctggatc ggtgacaggt gctgcatggc tgtcgtcagc
 961 tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aaccctcgcc cttagttgcc
1021 agcattcagt tgggcactct aaggggactg cgcggtgata agccgagagg aaggtgggga
1081 tgacgtcaag tcctcatggc ccttacgggc tgggctacac acgtgctaca atggcggtga
1141 cagtgggaaa atccccaaaa accgtctcag ttcggattgt cctctgcaac tcgggggcat
1201 gaaggtggaa tcgctagtaa tcgtggatca gcatgccacg gtgaatacgt tcccgggcct
1261 tgtacacacc gcccgtc
```

LOCUS	KT006930 1333 bp DNA linear BCT 11-NOV-2015
DEFINITION	*Vibrio parahaemolyticus* strain SR1 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT006930
VERSION	KT006930.1
KEYWORDS	.
SOURCE	*Vibrio parahaemolyticus*
ORGANISM	*Vibrio parahaemolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*.
REFERENCE	1 (bases 1 to 1333)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio*
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1333)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JUN-2015) Microbiology, Prince of Songkla University, Kanjanavanit Road, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1333 /organism="*Vibrio parahaemolyticus*" /mol_type="genomic DNA" /strain="SR1"

/isolation_source="shrimp disease"
/db_xref="taxon:670"
rRNA <1..>1333
/product="16S ribosomal RNA"

ORIGIN

1 gagttatctg aaccttcggg gaacgataac ggcgtcgagc ggcggacggg tgagtaatgc
61 ctaggaaatt gccctgatgt gggggataac cattggaaac gatggctaat accgcatgat
121 gcctacgggc caaagagggg gaccttcggg cctctcgcgt caggatatgc ctaggtggga
181 ttagctagtt ggtgaggtaa gggctcacca aggcgacgat ccctagctgg tctgagagga
241 tgatcagcca cactggaact gagacacggt ccagactcct acgggaggca gcagtgggga
301 atattgcaca atgggcgcaa gcctgatgca gccatgccgc gtgtgtgaag aaggccttcg
361 ggttgtaaag cactttcagt cgtgaggaag gtagtgtagt taatagctgc attatttgac
421 gttagcgaca gaagaagcac cggctaactc cgtgccagca gccgcggtaa tacggagggt
481 gcgagcgtta atcggaatta ctgggcgtaa agcgcatgca ggtggtttgt taagtcagat
541 gtgaaagccc ggggctcaac ctcggaattg catttgaaac tggcagacta gagtactgta
601 gaggggggta gaatttcagg tgtagcggtg aaatgcgtag agatctgaag gaataccggt
661 ggcgaaggcg gccccctgga cagatactga cactcagatg cgaaagcgtg gggagcaaac
721 aggattagat accctggtag tccacgccgt aaacgatgtc tacttggagg ttgtggcctt
781 gagccgtggc tttcggagct aacgcgttaa gtagaccgcc tggggagtac ggtcgcaaga
841 ttaaaactca aatgaattga cgggggcccg cacaagcggg ggagcatgtg gtttaattcg
901 atgcaacgcg aagaacctta cctactcttg acatccagag aactttccag agatggattg
961 gtgccttcgg gaactctgag acaggtgctg catggctgtc gtcagctcgt gttgtgaaat
1021 gttgggttaa gtcccgcaac gagcgcaacc cttatccttg tttgccagcg agtaatgtcg
1081 ggaactccag ggagactgcc ggtgataaac cggaggaagg tggggacgac gtcaagtcat
1141 catggccctt acgagtaggg ctacacacgt gctacaatgg cgcatacaga gggcggccaa
1201 cttgcgaaag tgagcgaatc ccaaaaagtg cgtcgtagtc cggattggag tctgcaactc
1261 gactccatga agtcggaatc gctagtaatc gtggatcaga atgccacggt gaatacgttc
1321 ccgggccttg tac

LOCUS	KT006931 1395 bp DNA linear BCT 11-NOV-2015
DEFINITION	*Vibrio parahaemolyticus* strain SR2 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT006931
VERSION	KT006931.1
KEYWORDS	.
SOURCE	*Vibrio parahaemolyticus*
ORGANISM	*Vibrio parahaemolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*.
REFERENCE	1 (bases 1 to 1395)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio*
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1395)
AUTHORS	Chumpol, S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JUN-2015) Microbiology, Prince of Songkla University, Kanjanavanit Road, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1395 /organism="*Vibrio parahaemolyticus*" /mol_type="genomic DNA" /strain="SR2"

/isolation_source="shrimp disease"
/db_xref="taxon:670"
rRNA <1..>1395
/product="16S ribosomal RNA"
ORIGIN

1 caagtcgagc ggaacgagtt atctgaacct tcggggaacg ataacggcgt cgagcggcgg
61 acgggtgagt aatgcctagg aaattgccct gatgtggggg ataaccattg gaaacgatgg
121 ctaataccgc atgatgccta cgggccaaag agggggacct tcgggcctct cgcgtcagga
181 tatgcctagg tgggattagc tagttggtga ggtaagggct caccaaggcg acgatcccta
241 gctggtctga gaggatgatc agccacactg gaactgagac acggtccaga ctcctacggg
301 aggcagcagt ggggaatatt gcacaatggg cgcaagcctg atgcagccat gccgcgtgtg
361 tgaagaaggc cttcgggttg taaagcactt tcagtcgtga ggaaggtagt gtagttaata
421 gctgcatcat ttgacgttag cgacagaaga agcaccggct aactccgtgc cagcagccgc
481 ggtaatacgg agggtgcgag cgttaatcgg aattactggg cgtaaagcgc atgcaggtgg
541 tttgttaagt cagatgtgaa agcccggggc tcaacctcgg aattgcattt gaaactggca
601 gactagagta ctgtagaggg gggtagaatt tcaggtgtag cggtgaaatg cgtagagatc
661 tgaaggaata ccggtggcga aggcggcccc ctggacagat actgacactc agatgcgaaa
721 gcgtggggag caaacaggat tagataccct ggtagtccac gccgtaaacg atgtctactt
781 ggaggttgtg gccttgagcc gtggctttcg gagctaacgc gttaagtaga ccgcctgggg
841 agtacggtcg caagattaaa actcaaatga attgacgggg gcccgcacaa gcggtggagc
901 atgtggttta attcgatgca acgcgaagaa ccttacctac tcttgacatc cagagaactt
961 tccagagatg gattggtgcc ttcgggaact ctgagacagg tgctgcatgg ctgtcgtcag
1021 ctcgtgttgt gaaatgttgg gttaagtccc gcaacgagcg caacccttat ccttgtttgc
1081 cagcgagtaa tgtcgggaac tccagggaga ctgccggtga taaaccggag gaaggtgggg
1141 acgacgtcaa gtcatcatgg cccttacgag tagggctaca cacgtgctac aatggcgcat
1201 acagagggca gccaacttgc gaaagtgagc gaatcccaaa aagtgcgtcg tagtccggat
1261 tggagtctgc aactcgactc catgaagtcg gaatcgctag taatcgtgga tcagaatgcc
1321 acggtgaata cgttcccggg ccttgtacac accgcccgtc acaccatggg agtgggctgc
1381 aaaagaagta ggtag

LOCUS	KT006932 1356 bp DNA linear BCT 11-NOV-2015
DEFINITION	*Vibrio parahaemolyticus* strain SR3 16S ribosomal RNA gene, partial sequence.
ACCESSION	KT006932
VERSION	KT006932.1
KEYWORDS	.
SOURCE	*Vibrio parahaemolyticus*
ORGANISM	*Vibrio parahaemolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*.
REFERENCE	1 (bases 1 to 1356)
AUTHORS	Chumpol, S.
TITLE	*In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio*
JOURNAL	Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. *In vitro* and *in vivo* selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquac. Res. 48: 3182–3197.
REFERENCE	2 (bases 1 to 1356)
AUTHORS	Chumpol,S.
TITLE	Direct Submission
JOURNAL	Submitted (04-JUN-2015) Microbiology, Prince of Songkla University, Kanjanavanit Road, Hatyai, Songkhla 90110, Thailand
FEATURES	Location/Qualifiers
source	1..1356 /organism="*Vibrio parahaemolyticus*" /mol_type="genomic DNA" /strain="SR3"

/isolation_source="shrimp disease"
/db_xref="taxon:670"
rRNA <1..>1356
/product="16S ribosomal RNA"

ORIGIN

1 gataacggcg tcgagcggcg gacgggtgag taatgcctag gaaattgccc tgatgtgggg
61 gataaccatt ggaaacgatg gctaataccg catgatgcct acgggccaaa gaggggacc
121 ttcgggcctc tcgcgtcagg atatgcctag gtgggattag ctagttggtg aggtaagggc
181 tcaccaaggc gacgatccct agctggtctg agaggatgat cagccacact ggaactgaga
241 cacggtccag actcctacgg gaggcagcag tggggaatat tgcacaatgg gcgcaagcct
301 gatgcagcca tgccgcgtgt gtgaagaagg ccttcgggtt gtaaagcact ttcagtcgtg
361 aggaaggtag tgtagttaat agctgcatca tttgacgtta gcgacagaag aagcaccggc
421 taactccgtg ccagcagccg cggtaatacg gagggtgcga gcgttaatcg gaattactgg
481 gcgtaaagcg catgcaggtg gtttgttaag tcagatgtga aagcccgggg ctcaacctcg
541 gaattgcatt tgaaactggc agactagagt actgtagagg ggggtagaat ttcaggtgta
601 gcggtgaaat gcgtagagat ctgaaggaat accggtggcg aaggcggccc cctggacaga
661 tactgacact cagatgcgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca
721 cgccgtaaac gatgtctact tggaggttgt ggccttgagc cgtggctttc ggagctaacg
781 cgttaagtag accgcctggg gagtacggtc gcaagattaa aactcaaatg aattgacggg
841 ggcccgcaca agcggtggag catgtggttt aattcgatgc aacgcgaaga accttaccta
901 ctcttgacat ccagagaact ttccagagat ggattggtgc cttcgggaac tctgagacag
961 gtgctgcatg gctgtcgtca gctcgtgttg tgaaatgttg ggttaagtcc cgcaacgagc
1021 gcaaccctta tccttgtttg ccagcgagta atgtcgggaa ctccagggag actgccggtg
1081 ataaaccgga ggaaggtggg gacgacgtca agtcatcatg gcccttacga gtagggctac
1141 acacgtgcta caatggcgca tacagagggc agccaacttg cgaaagtgag cgaatcccaa
1201 aaagtgcgtc gtagtccgga ttggagtctg caactcgact ccatgaagtc ggaatcgcta
1261 gtaatcgtgg atcagaatgc cacggtgaat acgttcccgg gccttgtaca caccgcccgt
1321 cacaccatgg gagtgggctg caaaagaagt aggtag

VITAE

Name Miss Supaporn Chumpol

Student ID 5510230037

Educational Attainment

Degree	Name of Institution	Year of Graduation
B. Sc. Microbiology (1^{st} Class Honors)	Prince of Songkla University	2012

Scholarship Awards during Enrolment

2013-2017 Royal Golden Jubilee (RGJ) Ph.D. Program Scholarship, The Thailand Research Fund, Thailand

List of Publication and Proceeding

Chumpol, S., Kantachote, D., Rattanachuay, P., Vuddhakul, V., Nitoda, T. and Kanzaki, H. 2017. In vitro and in vivo selection of probiotic purple nonsulphur bacteria with an ability to inhibit shrimp pathogens: acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* and other vibrios. Aquaculture Research 48: 3182–3197.

Chumpol, S., Kantachote, K., Nitoda, T. and Kanzaki, H. 2017. The roles of probiotic purple nonsulfur bacteria to control water quality and prevent acute hepatopancreatic necrosis disease (AHPND) for enhancement growth with higher survival in white shrimp (*Litopenaeus vannamei*) during cultivation. Aquaculture 473: 327–336.

Chumpol S, Kantachote D, Rattanachuay P, Nitoda T. and Kanzaki, H. 2016. Selection of purple nonsulfur bacteria isolated from shrimp ponds with an ability to inhibit shrimp pathogenic *Vibrio* spp. RGJ-Ph.D. Congress XVII. June 8-11. Jomtien Plam Beach Hotel & Resort, Pattaya, Thailand. (Poster Presentation with outstanding poster presentation awards).

Chumpol, S., Kantachote, D., Rattanachuay, P., Nitoda, T. and Kanzaki, H. Optimization of culture conditions for production and characterization of antivibrio compounds from probiotic purple nonsulfur bacteria against serious shrimp pathogenic *Vibrio* spp., *Manuscript*, will be submitted.

Chumpol, S., Kantachote, D., Nitoda, T. and Kanzaki, H. Administration of purple nonsulfur bacteria as single cell protein by mixing with shrimp feed to enhance growth, immune response and survival in white shrimp (*Litopenaeus vannamei*) cultivation, *Manuscript,* will be submitted.