Characterization of Bioactive Compound Produced by *Pseudomonas* sp. W3 for Controlling Shrimp Pathogenic *Vibrio harveyi*

Pattamarat Rattanachuay

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Author	Miss Pattamarat Rattanachuay
Major Program	Microbiology

Major Advisor:

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

Co-advisor:

……
(Prof. Dr. Hiroshi Kanzaki)

……
(Assoc. Prof. Dr. Teruhiko Nitoda)

……
(Dr. Manee Tantirungkij)

Examining Committee:

……Chairperson
(Dr. Ampaithip Sukhoom)

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

……
(Dr. Manee Tantirungkij)

……
(Dr. Jumroensri Thawonsuwan)

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

……..……..……..
(Prof. Dr. Amornrat Phongdara)
Dean of Graduate School

ชื่อวิทยานิพนธ์	คุณสมบัติของสารออกฤทธิ์ทางชีวภาพผลิตโดย *Pseudomonas* sp. W3 สำหรับการควบคุมเชื้อก่อโรคกุ้ง *Vibrio harveyi*
ผู้เขียน	นางสาวปฐมารัตน์ รัตนช่วย
สาขาวิชา	จุลชีววิทยา
ปีการศึกษา	2553

บทคัดย่อ

การทดสอบฤทธิ์ของสารยับยั้งเชื้อกลุ่มวิบริโอที่ก่อโรคกับกุ้งซึ่งผลิตจากเชื้อ *Pseudomonas* sp. W3 โดยใช้ส่วนของน้ำเลี้ยงเชื้อสายพันธุ์ W3 หรือน้ำเลี้ยงเชื้อที่ผ่านการกรอง (0.45 ไมโครเมตร และ 0.22 ไมโครเมตร) โดยใช้วิธีการ agar well diffusion พบว่าส่วนของน้ำเลี้ยงเชื้อมีประสิทธิภาพในการยับยั้งการเจริญของเชื้อ *Vibrio. harveyi* PSU 2015 และ *V. cholerae* PSSCMI 0062 มากที่สุด รองลงมาคือน้ำเลี้ยงเชื้อที่ผ่านการกรองด้วยกระดาษกรองที่มีรูกรองขนาด 0.45 ไมโครเมตร และตามด้วยน้ำเลี้ยงเชื้อที่ผ่านการกรองด้วยขนาดรูกรอง 0.22 ไมโครเมตร นอกจากนี้สารยับยั้งเชื้อกลุ่มวิบริโอ สามารถทำให้เกิดการแตกของเซลล์ของเชื้อ *V. harveyi* PSU2015 ทั้งเซลล์เป็นและเซลล์ตาย โดยพบว่าสารออกฤทธิ์ส่วนใหญ่เป็นสารที่มีน้ำหนักโมเลกุลเล็กสามารถผ่านการกรองที่มีขนาดรูกรอง 0.22 ไมโครเมตรได้ และต้านทานต่อเอนไซม์ชนิดต่างๆ ที่ใช้ทดสอบ ทั้งยังทนค่า pH ในช่วง 4-8 และอุณหภูมิได้สูงถึง 121 องศาเซลเซียส เป็นเวลา 30 นาที และสำหรับค่า pH ที่เหมาะสมสำหรับสารต้านเชื้อกลุ่มวิบริโอของน้ำเลี้ยงเชื้อที่ผ่านการกรองด้วยขนาดรูกรอง 0.45 ไมโครเมตร อยู่ระหว่าง 6-7 เมื่อนำน้ำเลี้ยงเซลล์ที่ผ่านการกรองด้วยกระดาษกรองที่มีรูกรองขนาด 0.45 ไมโครเมตร ปรับ pH ให้มีค่าเท่ากับ 2 จากนั้นนำไปสกัดด้วย ethyl acetate (EtOAc) พบว่าสายพันธุ์ W3 ผลิตสารสีน้ำตาลออกเหลือง (EtOAc-W3) 82.15 มิลลิกรัม จากน้ำเลี้ยงเซลล์ 1 ลิตร โดยค่า MIC ของสารสกัดนี้กับเชื้อทดสอบ *V. harveyi* 18 สายพันธุ์มีค่าอยู่ระหว่าง 225-450 ไมโครกรัมต่อมิลลิลิตร โดยค่า MIC ของสารนี้ต่อสายพันธุ์ PSU 2015 ซึ่งเป็นสายพันธุ์ก่อโรคกุ้งซึ่งรุนแรงที่สุด เท่ากับ 450 ไมโครกรัมต่อมิลลิลิตร และเมื่อทดสอบฤทธิ์ในการยับยั้งสายพันธุ์ PSU2015 ของสารสกัด EtOAc-W3 ในน้ำทะเลเทียม (Artificial Sea Water; ASW) โดยใช้ความเข้มข้นที่ค่า MIC พบว่า สารสกัดมีฤทธิ์ในการฆ่าเชื้อ แต่อย่างไรก็ตามในน้ำทะเลธรรมชาติพบว่าสารสกัดมีฤทธิ์ในการยับยั้งการเจริญของเชื้อเท่านั้น การศึกษาความเป็นพิษของสารสกัด EtOAc-W3 ต่อตัวอ่อนของกุ้งขาววานาไมด์อายุ 21 วัน (*Litopenaeus vannamei*, PL 21) และฤทธิ์ต้านเชื้อวิบริโอสายพันธุ์ PSU 2015 ซึ่งการทดลองประกอบด้วยชุดควบคุม (น้ำทะเลเทียม) ชุด

ควบคุมสารสกัด (45 ไมโครกรัมต่อมิลลิลิตร) ชุดรับเชื้อโรคกุ้ง (6.0 x 10^6 cfu/ml) และ ชุดทดสอบ (เชื้อโรคกุ้ง 6.0 x 10^6 cfu/ml และสารสกัด 45 ไมโครกรัมต่อมิลลิลิตร) และได้ทำการทดลองซ้ำเพียงแต่เพิ่มความเข้มข้นของสารสกัดเป็น 90 ไมโครกรัมต่อมิลลิลิตร ผลการทดสอบทั้งสองความเข้มข้นของสารสกัด พบว่าสารสกัด EtOAc-W3 ไม่มีความเป็นพิษต่อกุ้งขาววานาไมด์ สำหรับอัตราการตายของกุ้งที่ติดเชื้อ *V. harveyi* PSU 2015 ในชุดรับเชื้อโรคของแต่ละการทดลองพบการตายของกุ้งประมาณ 80% โดยเริ่มปรากฏให้เห็นที่เวลาหลังจาก 72 ชั่วโมง แต่ในชุดที่มีสารสกัดที่ความเข้มข้น 45 และ 90 ไมโครกรัมต่อมิลลิลิตรพบการตายของกุ้งคิดเป็น 63% และ 23% ตามลำดับ และสำหรับการศึกษาส่วนประกอบของสารสกัด EtOAc-W3 พบว่าสารส่วนใหญ่ในสารสกัดคาดว่าเป็น 2-heptyl-4-quinolone (HHQ) จากนั้นได้ตรวจสอบฤทธิ์ของสารออกฤทธิ์ทางชีวภาพของสายพันธุ์ W3 ในการยับยั้งการเจริญของเชื้อก่อโรคในระบบทางเดินอาหาร พบว่าน้ำเลี้ยงเชื้อมีฤทธิ์ยับยั้งสูงสุดตามด้วยนำเลี้ยงเชื้อที่ผ่านการกรองด้วยขนาดรูกรอง 0.45 และ 0.22 ไมโครเมตรตามลำดับ สำหรับฤทธิ์ของนำเลี้ยงเชื้อสามารถยับยั้งการเจริญของเชื้อก่อโรคจากมากไปน้อยเป็นลำดับคือ *Clostridium perfringens* PSSCMI 0030 *Yersinia enterocolytica Bacillus cereus* ATCC 11778 *Vibrio cholera* PSSCMI 0062 *Listeria monocytogenes* 4553 *Bacillus cereus* 687 *Vibrio parahaemolyticus* AAHRC 1 *Vibrio parahaemolyticus* PSU 1681 สำหรับน้ำเลี้ยงเชื้อที่ผ่านการกรอง (0.45 และ 0.22 ไมโครเมตร) พบว่ามีสารออกฤทธิ์ทางชีวภาพที่สามารถยับยั้งการเจริญของเชื้อก่อโรคได้แต่ยับยั้งได้น้อยชนิดกว่าและมีฤทธิ์ยับยั้งน้อยกว่าด้วย ในทางตรงกันข้ามพบว่าส่วนของน้ำเลี้ยงเชื้อและน้ำเลี้ยงเชื้อที่ผ่านการกรองทั้ง 0.45 และ 0.22 ไมโครเมตร ไม่สามารถยับยั้งการเจริญของเชื้อ *Escherichia coli* ATCC 25922 *Staphylococcus aureus* PSSCMI 0006 *Salmonella typhimurium* PSSCMI 0034 และ *Vibrio vulnificus* PSU 3427 ท้ายสุดเมื่อนำเชื้อสายพันธุ์ W3 ไปเทียบเคียงโดยอาศัยการทดสอบทั้งแบบดั้งเดิมด้วยการใช้ชุดทดสอบ API 20NE และวิธีทางชีวโมเลกุลโดยใช้ยีนส์ 16S rRNA ที่มีขนาด 1,481 bp ผลการทดสอบทั้งหมดบ่งชี้ว่าไอโซเลท W3 เป็นสมาชิกของ *P. aeruginosa* และได้รับ accession number HQ 378506

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Author	Miss Pattamarat Rattanachuay
Major Program	Microbiology
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ABSTRACT

Antivibrio compounds in the culture supernatant or culture filtrates (0.45 µm and 0.22 µm) of *Pseudomonas* sp. W3 were tested by an agar well diffusion method on a number of shrimp pathogenic vibrios. The effectiveness of preparations from the isolate W3 against *Vibrio harveyi* PSU 2015, and *V. cholerae* PSSCMI 0062 was in the degree of culture supernatant > 0.45 µm culture filtrate > 0.22 µm culture filtrate. In addition, these extracellular antivibrio compounds also lysed both dead and living cells of the strain PSU 2015. It was found that most of the inhibitory compounds were of small molecular weight able to pass through a 0.22 µm filter and were resistant to treatment with various enzymes, pH values between 4-8 and temperatures up to 121ºC for 30 min. The optimum pH for the antivibrio activity in the 0.45 µm culture filtrate was between pH 6-7. A culture filtrate (0.45 µm) of the isolate W3 at a pH of 2 was extracted with ethyl acetate (EtOAc) to produce 82.15 mg/l of a yellow-brown extract (EtOAc-W3) that had MIC values of 225-450 µg/ml against the growth 18 shrimp pathogenic *V. harveyi* strains. The MIC of EtOAc-W3 against the most serious pathogenic strain PSU 2015 was 450 µg/ml and at this MIC value, EtOAc-W3 in artificial sea water (ASW) killed the strain PSU 2015. However in natural sea water, only a partial growth inhibition was observed. The toxicity to Pacific white shrimp (*Litopenaeus vannamei*, PL 21) and antivibrio activity of the

EtOAc-W3 were investigated by conducting an experiment with 4 sets; native control (commercial ASW), EtOAc-W3 control (45 µg/ml) , challenge (PSU 2015, 6.0 x 10^6 cfu/ml) and treatment (PSU 2015, 6.0 x 10^6 cfu/ml and EtOAc-W3, 45 µg/ml). The same experiment was repeated by increasing the dose of EtOAc-W3 to 90 µg/ml. Both dose tested of EtOAc-W3 had no toxicity to postlarval shrimp. A significant decrease in shrimp mortality was observed over a 72 h period as approximately 80% of the shrimp died in each challenge set, but only 63 and 23% died in the presence of 45 and 90 µg/ml EtOAc-W3, respectively. The major component of EtOAc-W3 was supposed to be 2-heptyl-4-quinolone (HHQ). Consequently, the broad spectrum of bioactive compounds from the strain W3 were examined with gastrointestinal tract pathogenic bacteria and the most effective inhibition was found in the culture supernatant followed by the culture filtrates of 0.45 and 0.22 µm, respectively. The culture supernatant of the strain W3 inhibited pathogenic bacteria in the order of *Clostridium perfringens* PSSCMI 0030 > *Yersinia enterocolytica* > *Bacillus cereus* ATCC 11778 > *Vibrio cholera* PSSCMI 0062 > *Listeria monocytogenes* 4553 > *Bacillus cereus* 687 > *Vibrio parahaemolyticus* AAHRC 1 > *Vibrio parahaemolyticus* PSU 1681. The results showed that both culture filtrates (0.45 and 0.22 µm) contained bioactive compounds that could inhibit some pathogenic bacteria tested but less than the culture supernatant both in variety of pathogens and antibacterial activity. In contrast, no growth inhibition of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* PSSCMI 0006, *Salmonella typhimurium* PSSCMI 0034 and *Vibrio vulnificus* PSU 3427 was observed by the culture supernatant and both culture filtrates. Finally, *Pseudomonas* sp.W3 was identified using the conventional methods with the API 20NE test kit and also the 16S rDNA sequence analysis with 1,481 bp. The overall results indicated that the strain W3 was a true member of the *Pseudomonas aeruginosa* with the accession number of HQ 378506.

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LIST OF ABBREVIATIONS AND SYMBOLS

ASW	=	Artificial Sea Water
cfu	=	Colony Forming Unit
°C	=	Degree Celsius
DMSO	=	Dimethyl sulfoxide
DO	=	Dissolve Oxygen
EC	=	Electrical Conductivity
EtOAc	=	Ethyl acetate
FAB-MS	=	Fast Atom Bombardment Mass Spectroscopy
g	=	Gram
MBC	=	Minimum Bactericidal Concentration
MeOH	=	Methanol
MIC	=	Minimum Inhibitory Concentration
mg	=	Milligram
ml	=	Milliliter
Na_2SO_4	=	Sodium sulfate
NMR	=	Nuclear Magnetic Resonance
OD	=	Optical Density
PMS	=	Phenazine methoxysulfate
TCBS	=	Thiosulfate Citrate Bile Salts Sucrose

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

TLC	=	Thin Layer Chromatography
TSA	=	Tryptic Soy Agar
TSB	=	Tryptic Soy Broth
µ	=	Micro
µm	=	Micrometer
µl	=	Microliter

Chapter I

Introduction

Shrimp cultivation is one of the most economically important aquacultural activities in Asia, South America and it exist worldwide (Nimrat et al., 2008). Thailand is one of the top countries of the shrimp farming as shrimp producing was 384,000 Metric tons in 2005 (Tanticharoen et al., 2008) and exporting 600,000 tons in 2007 (FAO, 2001; Holmström et al., 2003, Uddin, M. T., 2008). In the past decade, there were many farms, more than 20,000 which located on east coast and west coast in the south of Thailand; consequently farmers have faced with many serious problems and the main problem is shrimp's diseases (Geovanny D et al., 2007; Sharshar and Azab, 2008). Mainly coused by virus and bacteria. Disease due to bacterial infections, particularly luminous vibriosis is a major problem in shrimp cultivation in Asian countries (Walker and Subasinghe, 2000). The group of luminescent bacteria in *Vibrios* is the main cause of shrimp's death, especially *Vibrio harveyi*, which can infect larva in hatchery and shrimp in the soil pond (Vinod et al., 2005; Chari and dubey. 2006). The virulence of *V. harveyi* has caused mass mortalities of up to 100% in both hatcheries and cultivation ponds (Chythanya et al., 2002; Musa et al., 2008; Rahman et al. 2009). The effects of diseases lead of poverty to farmers and also destroy aquatic animals' cultures economic (Nash et al., 1992; Than et al., 2004; Austin and Zhang, 2006).

In shrimp, the symptoms of vibriosis are eye-lesions, gastro-enteritis, vasculitis, and luminous vibriosis (Teo et al., 2002). Normally, antibiotics are used to treat disease outbreaks or to prevent shrimp from infection by *V. harveyi* (Gräslund and Bengtsson 2001; Holmström et al. 2003). Antibiotics such as tetracycline, chloramphenical and nitrofurans (Holmström et al., 2003) are commonly used to control these shrimp diseases. In many cases, extensive use has led to a decrease in the therapeutic efficiency of the least expensive antibiotics, so many Asian aquaculture programs have gradually switched during the past decade to the use of

broad spectrum antibiotics such as chloramphenicol (Huys et al., 2007). Recently, in Thailand some antibiotics such as chloramphenicol and the group of nitrofurans (furazolidone, nitrofurazone and nitrofurantion) are not allowed to use in shrimp cultivation (Gräslund et al., 2002; Ponprateep et al., 2009). However, the farmers sometimes solve the problem of bacterial infection by using antibiotics like oxytetracycline, sulfadimethoxine and ormethoprim. The continued antibiotic use leads to selection of antibiotic-resistant microbes and eventually these might transfer to humans as pathogens (Holmström et al. 2003; Rahman et al. 2009). Based on a list of the antibiotics that can be used in aquaculture, only nalidixic acid and norfoxazine can effectively treat shrimp diseases (Musa et al. 2008; Rattanachuay et al. 2010; Teo et al. 2002). An alternative way for decreasing antibiotic problem is the use of microbial products such as enzymes, vitamins and bioactive compounds.

According to the above information, the use of compounds other than regular antibiotics is acceptable for shrimp cultivation especially if they are more cost effective and can maintain shrimp health. (Alavandi et al. 2004; Preetha et al. 2009). Therefore, exploring for new bioactive compounds against shrimp bacterial disease an agent like *V. harveyi* is an urgent priority. There has been much research to report that *Pseudomonas* spp. can produce a variety of bioactive compounds that can control shrimp pathogenic bacteria such as *Vibrio* spp. with no harm to the shrimp (Chythanya et al. 2002; Liu et al. 2000; Vijayan et al. 2006). It is well recognized that *Pseudomonas* spp. produce bioactive compounds such as phenazine compounds, quinolones, hydrogen cyanide, 2, 4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin (Ge et al. 2004; Mashburn-Warren et al. 2009).

Hence, *Pseudomonas* spp. are an attractive group of bacteria in a process of producing bioactive compounds in controlling *Vibrio* spp. and other pathogenic bacteria. This research was interested in investigating bioactive compounds produced by *Pseudomonas* sp. W3 with ability to control *V. harveyi* and foodborne bacteria. This bacterium was also identified by traditional method and 16S rRNA genes.

The objectives of this study

1. To investigate antibacterial activity of bioactive compounds produced by *Pseudomonas* sp. W3 against growth of *Vibrio harveyi* and pathogenic bacteria in seafood industry.
2. To extract, purify and identify a target bioactive compound from *Pseudomonas* sp. W3 that inhibits growth of *Vibrio harveyi*.
3. To study anti-vibrio activity of the bioactive compound and its toxicity to shrimp.
4. To identify *Pseudomonas* sp. W3.

The benefit of this thesis

The information of this thesis provided characteristics of the bioactive compound from *Pseudomonas* sp. W3 that controls growth of *V. harveyi* and the disease causes by *V. harveyi* in hatchery and shrimp cultivation. In addition, the bioactive compounds would be possible to use in a frozen seafood industry in order to control pathogenic bacteria and spoilage organisms.

The scope of this thesis

Bioactive compounds which produced by *Pseudomonas* sp. W3 were investigated to reveal their ability for controlling, *V. harveyi* and foodborne pathogenic bacteria including spoilage organism in a seafood industry. Extraction, purification and identification of bioactive compounds were also examined. Anti-vibrio activity of the bioactive compound produced by *Pseudomonas* sp. W3 and its toxicity to shrimp were carried out.

Chapter II

Literature review

1. Shrimps

Shrimps are an aquatic animal in the Kingdom of Animalia, Phylum Arthropoda and Class Crustacea. Shrimps use gills for breathe and have a long body with 10 legs on the chest. They have a small carapace that encloses and have a muscular abdomen for swimming (Waller et al., 1996). They can be found widely around the world in both freshwater and salt water. Most of them mature and breed in marine (i.e *Penaeus monodon*, *Penaeus setiferus*, *Litopenaeus vannamei*, *Penaeus aztecus* and *Penaeus duorarum*) (Gillett, 2008), although a small member of them mature and breed in freshwater such as *Macrobrachium rosenbergii* and *Macrobrachium americanum* (New, 2002). The features of shrimp and *Penaeid* shrimp life cycle are shown in Figure 2-1 and Figure 2-2, respectively. All shrimps are divided into two basic categories: marine; cold-water or northern and warm-water, tropical, or southern; and fresh-water (Whetstone et al., 2002).

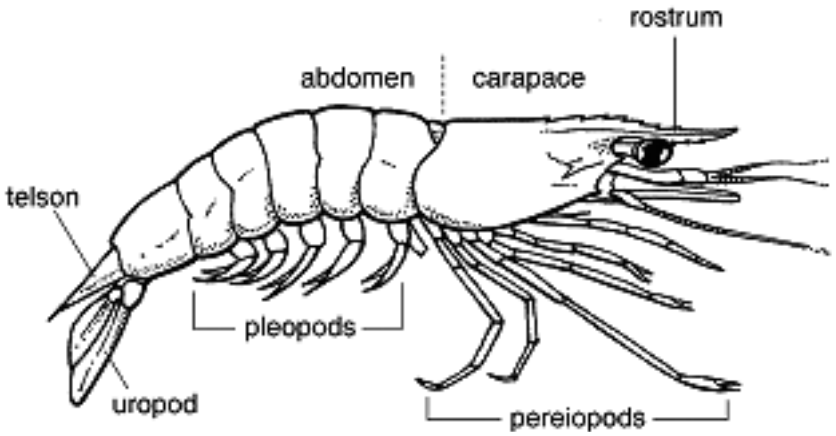


Figure 2-1 Feature of shrimp (King, 2007)

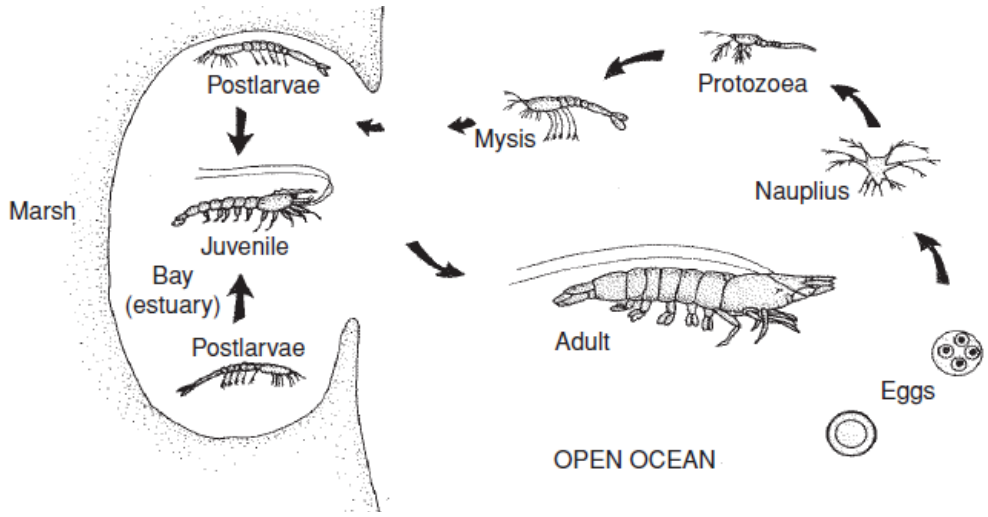


Figure 2-2 *Penaeid* shrimp life cycle (Whetstone et al., 2002)

1.1 Marine shrimp

1.1.1 Cold-water or northern

The cold-water shrimps inhabit in northern Atlantic and northern Pacific such as *Pandalus borealis* and *Pandalus jordani*, respectively. They are very small in size and can reach 36 mm carapace length. The common harvesting size is 24-30 mm carapace length (Lart and Green, 2009). The shrimps are live at 10 m to 500 m of deep sea and at 2°C to 6°C of sea water. The pandalid shrimp are commercial importance in the North Atlantic and the North Pacific Ocean (Bergström, 1992).

1.1.2 Warm-water, tropical or southern

The warm-water shrimps inhabit in many places like Gulf of Mexico, Florida, Alabama, Mississippi, Louisiana, Texas and also in Asia (Bailer, 1989). There are many kinds of shrimps in warm-water such as *Penaeus monodon*, *Penaeus*

merquiensis, Penaeus setiferus, and *Litopenaeus vannamei* (Farfante and Kensley, 1997; Holthuis, 1980). Warm-water shrimps are desirable in a world market and shrimps are cultured in tropical countries. The parameters for the tropical shrimp maturation are 27-36 ppt of salinity, 27-29°C of temperature, pH 7.8, and 5 ppm dissolved oxygen (DO) (Whestone et al., 2002).

1.2 Freshwater

The Fresh-water shrimps are found in lakes, rivers, swamps, irrigation ditches, canals, and ponds closed to the coast. Most species require brackish water in the initial stages of their life cycle although some complete their cycle in inland saline and freshwater lakes. Some species prefer rivers containing clear water, while others are found in extremely turbid conditions (New, 2002). The most popular species of freshwater shrimp is *Macrobrachium rosenbergii* or giant river prawn. This species is a tropical species native to Southeast Asia and introduce widely into other parts of the world where suitable temperatures naturally occur or environmental temperature can be controlled by the culturist (Stickney, 1994). This species is farmed in many countries. The major producers are Bangladesh, Brazil, China, Ecuador, India, Malaysia, Taiwan, Thailand, and Viet Nam (New, 2002).

Asia, Central America, and South America produce the shrimp and prawn about 80% of total world output since 1980 with remaining 20% of output representing cold-water shrimp and 60% representing warm-water shrimp (Poudel, 2008). In Asia, black tiger shrimp, *Penaeus monodon*, is the dominant species while the Pacific White shrimp, *Litopenaeus vannamei*, is dominant in the west. In 2007, *L. vannamei* production accounted for more than 75% of total world production and became the dominant species farmed in Thailand (Wyban, 2009).

1.3 *Penaeus monodon*

The common name of *Penaeus monodon* is black tiger shrimp, black tiger prawn or giant tiger prawn. This shrimp is in the Subclass Malacostraca, Order Decapoda, Suborder Dendrobranchiae, Superfamily Penaeoidea, Family Penaeidae, Genus *Penaeus*, and Species *monodon* (Farfante and Kensley, 1997; Holthuis, 1980). The black tiger shrimp has reddish brown to brown body, nine dark brown stripes on its back, reddish brown pereiopods (walking legs), bluish brown pleopods (swimming legs), seven to eight upper rostral spines, three lower rostral spines and inhabits on sandy mud, sand of coral and sand of shell. The average size of adult shrimp is maximum total length 336 mm and weight 60 g to 130 g (Holthuis, 1980; Kongkeo, 2010). This shrimp inhabits in Asia (i. e. India, Sri Lanka, Malaysia, Singapore, Indonesia, and Thailand), East coast of Africa (i. e. Red Sea, Madagascar, and Mauritius), and northern Australia (Farfante and Kensley, 1997; Holthuis, 1980).

1.4 *Litopenaeus vannamei*

Litopenaeus vannamei is a group of Pacific white shrimp found by Boone in 1931. This shrimp is in the Subclass Malacostraca, Order Decapoda, Suborder Natantia, Section Penacidea, Family Penaeidae, Genus *Penaeus litopenaeus* Subgenus *Penaeus litopenaeus*, and Species *vannamei* (Farfante and Kensley, 1997; Holthuis, 1980).

L. vannamei has rostrum armed with 7-10 dorsal and 2-4 ventral teeth, eight white joint stems, one head joint stem, big reddish brown rostral spine, eight upper rostral spines, two lower rostral spines, white or reddish pink carapace, white pereiopods (walking legs), five red pleopods (swimming legs), white or reddish pink shell, four dark red uropods, one telson, two red antenna flagella, and red eyes (Farfante and Kensley, 1997). Average size of adult shrimp is about 230 mm (from rotrum to telson) with body weigh about 120 g and 90 mm maximum carapace length (Figure 2-3). The female is faster growing and larger than male (Holthuis, 1980). *L.*

vannamei is native to Pacific coast of Mexico, Gulf of Panama, Central and South America as far south as Peru. It can grow in the area where water temperatures are over 20°C throughout year and tolerance a wide range of salinities as 0.5 – 45 ppt, but grows particularly well between 10 – 15 ppt (Briggs et al., 2004).

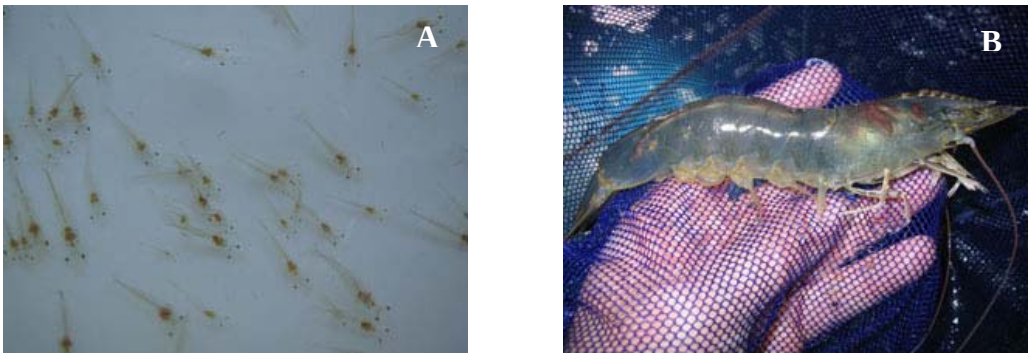


Figure 2-3 *Litopenaeus vannamei* or Pacific white shrimp

A: *Litopenaeus vannamei* postlarvae (Vannamei101)

B: Adult of *Litopenaeus vannamei* (Briggs, 2010)

2. Problems in marine shrimp cultivation

Modern shrimp farming was begun in the late 1960s and early 1970s when French researchers developed techniques for culture penaeid shrimp species (Gillett, 2008). In Thailand, three distinct types of shrimp farming were extensive farming, semi-intensive farming, and intensive farming. Semi-intensive shrimp farming was popular in 1972 and shifted to the Taiwanese-style intensive shrimp farming in 1986 (Pongsri and Sukumasavin, 2010). Marine shrimp has been classified as one main agricultural export products of Thailand (Poudel, 2008). Recently the shrimp export and shrimp cultivation has faced many problems. For instance low productivity of shrimp cultivation has been caused by diseases and poor sanitation of

shrimp ponds (Briggs et al., 2004). Moreover, un-proper management of shrimp cultivation has caused environmental damage and reflected to shrimp cultivation.

2.1 Pollution in shrimp pond

The management of parameters in shrimp ponds is very important to prevent shrimp diseases and poor sanitation of shrimp ponds. The intensive shrimp farming have been used to culture shrimp in Asian countries such as Thailand, Indonesia, Philippines, and Taiwan (Kongke, 1997). The intensive ponds are stocked at densities of shrimp about 50-100 larvae per m^2, fed with high quality artificial feed at 4-5 times a day and a little change of water (Pongsri and Sukumasavin, 2010), so water culture is one of the important processes during the shrimp culture period. The water quality parameters such as pH, salinity, dissolved oxygen, alkalinity, H_2S, and ammonia are measured (Brigg, 2010). A little change of water replacement and poor water quality management has caused unsuitable conditions for shrimp growth and might be the reasons for shrimp diseases (Soundarapandian et al., 2009). Outbreaks of the serious virus and bacteria occur after dramatic changes in water parameters such as temperature, DO, and the stress shrimp caused by deterioration in water quality and ponds bottom environment (Black, 2001; Kongkeo, 2010).

Consequently, farmers tried to solve a problem by antibiotics and chemicals to prevent infection of shrimp's pathogens and control water quality in shrimp ponds. Moreover, intensive shrimp cultivation and antibiotics residues have destroyed ecosystems around shrimp area and given adverse effects on shrimp cultivation (Chalo and Pornlert, 2004, in Thai).

2.2 Shrimp diseases outbreaks

Diseases are one of important factors to reduce the shrimp production. The disease problems in shrimp culture have escalated since the late 1970s. The

shrimp farming in China and Thailand has been affected by outbreaks of diseases. In 1996-1997, Thailand's total shrimp production was dropped and a similar boom-and–bust pattern occurred in Indonesia and Philippines (Primavera, 1997; Kautsky et al., 2000). The viral and bacterial diseases have caused devastating economic losses (Kautsky et al., 2000). The diseases of cultured shrimp syndromes with infectious (viral, fungal, and bacterial) and noninfectious diseases are also of importance for the food industry (Lightner and Rdman, 1998). The occurring of shrimp diseases in shrimp ponds have been caused by excessive stocking, poor water quality, and un-proper management farming areas. Viruses and bacteria are the main causes of shrimp diseases and shrimp mortality such as Hepatopancreatic Parvo Virus (HPV), Monodon Baculovirus (MBV), Lymphoid Organ Vacuolization Virus (LOVV) (Chalo and Pornlert, 2004 (In Thai)), and vibriosis (Chythanya et al., 2002). The luminous vibriosis '*Vibrio harveyi*' is a major problem in shrimp cultivation in Asian countries (Walker and Subasinghe, 2000) and they can completely destroy hatchery productivity (Nash et al., 1992; Vinod et al., 2005). The virulence of *V. harveyi* has caused 100% loss in shrimp production (Chythanya et al., 2002; Musa et al., 2008).

Good management practices can often be used to keep diseases under control and reduce the loss of shrimp (Chalo and Pornlert, 2004, in Thai). A balanced normal bacteria composition may keep the pond healthy and reduce risks for rapidly spread of pathogenic bacteria. Sometime, the management of shrimp pond becomes more difficult and the farmer may sterilize the shrimp pond environment with some antibiotics, chlorine and formaldehyde (Primavera et al., 1993; Barg and Lavilla-Pitogo, 1996; Kautsky et al., 2000).

2.3 Residual antibiotics and chemicals in shrimp

The antibiotics and chemicals are used in marine aquaculture for prevention and treatment of diseases. Antibiotics such as tetracycline, chloramphenicol, and nitrofurans (Holmström et al., 2003) are used to control those shrimp diseases. In Philippine, oxytetracycline, oxolinic acid, chloramphenicol, and

furazolidone are incorporated in artificial feeds as treatment luminescent vibriosis (Tendencia and De la Peña, 2001). In the year 2002, chloramphenicol and a group of nitrofurans were found in shrimp exported from Thailand because most of farmers used those antibiotics to cure shrimp's diseases and to increase shrimp production. Shrimp exported products which had antibiotic residues were rejected and consequently caused many adverse effects to farmers and Thai's economy. Chloramphenical and a group of nitrofurans have been banned for cultivation of aquatic animals (Chalo and Pornlert, 2004, in Thai) because the toxic of antibiotics occurred to consumers. For example, the toxic effect of chloramphenicol on bone marrow. It can affect the production of blood corpuscles by reduction of blood cells pancytopenia caused by composition change in blood (Somjetlerdcharoen, 2002). In addition, widespread use of antibiotics (oxytetracycline, oxolinic acid, erythromycin, kanamycin, pencicillin G, and streptomycin) in marine shrimp cultivation has resulted in development of resistant strains of pathogenic bacteria i.e. *V. harveyi* and *V. splendides* (Tendencia and De la Peña, 2001; Tookwinas, 2003).

3. Shrimp diseases

3.1 Vibriosis

Vibriosis is a disease caused by *Vibrio* spp. including *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida*, and other *Vibrio* species (Ruangpan, 1998; Chythanya et al., 2002). These bacteria cause of marine shrimp mortality and marine crustacean culture. The infection of *Vibrio* sp. was occurred during the later stages of culture until the harvest due to poor water and pond bottom quality conditions (MPEDA/NACA, 2003). The symptoms of vibriosis were separated by *Vibrio* ssp. such as infection with *V. harveyi* or luminescence *Vibrio*, some farmers call it 'luminescence disease'.

3.2 Luminescence disease

The luminescence disease is very common in penaeid shrimp hatcheries and penaeid shrimp ponds. This disease is a serious disease that causes loss in the shrimp culture in South America and Asia (Defoirdt et al., 2007). Pollution in shrimp ponds causes of disease like *V. harveyi* which can infect shrimps. The fluctuations in environmental conditions have a significant effect on the virulence of *V. harveyi*. The low oxygen levels increase sensitivity being to vibriosis in penaeid shrimp (Kautsky et al., 2000). The symptoms of luminescence disease are black gills, weakness disoriented swimming, diffuse muscle opacity of the abdominal musculature, less to eat feed, more water around joint of head and body than normal shrimp, low blood cell, high pH in blood, and fluorescent phenomenon at night (Jareeporn et al., 2004). Shrimps that were infected by *V. harveyi* can easily be observed at night as the fluorescent shrimps like a firefly when farmers turn off the light. *V. harveyi* can be isolated from the fluorescent shrimp by using Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar as its colony on TCBS- green luminescence (Wanipa, 1996, in Thai).

3.3 Necrotising hepatopancreatitis

Necrotizing hepatopancreatitis (NHP), Texas necrotizing hepatopancreatitis (TNHP), Texas pond mortality syndrome (TPMS), and Peru necrotizing hepatopancreatitis (PNHP) are intracellular bacterial diseases of shrimp such as *Litopenaeus vannamei*, *Farantopenaeus aztecus*, *Litopenaeus setiferus*, *Litopenaeus stylirostris*, and *Farantopenaeus californiensis*. These diseases are caused by alpha proteobacteria, Gram-negative, pleomorphic, and rod-shaped of helical-shaped bacteria (Raidal et al., 2004). Shrimp pathogenic bacteria attacking the hepatopancrea cells, leads to varied symptoms including enlargement of hepatopancrease, pale-whitish or black hepatopancreas, weakness disoriented swimming, loss of appetite, abdominal muscle atrophy, soft exoskeleton, slow death, and generalized surface fouling (Raidal et al., 2004; Del Río-Rodríguez et al., 2006).

In addition, NHP does not seem to occur when salinity is below 10 ppt (McVey, 1993). The disease has caused mortality rate of shrimp between 20% and 80% in Mexico where the salinity are over 45 ppt (Del Río-Rodríguez et al., 2006).

3.4 Black gill disease

Black gill disease occurs in marine shrimp ponds. The disease occurs during the grow-out phase of shrimp culture (2-3 months). The gills are clogged with organic debris, overgrown with peritrichs, bacteria, protozoa, and filamentous algae settle on gill surfaces (Lester and Paynter, 1989). The black, green or brown pigment presented in the gill, appendage, and cuticle lesions is melanin, which is formed at the sites of hemocytic inflammation and tissue necrosis (Lightner et al., 2009).

4. *Vibrio* spp.

Vibrio is a Gram-negative, rod shape, non spore forming, motile by polar flagella, and produce luciferase that can show luminescence similarity with firefly. This genus belongs to the bacteria in a family of *Vibrionaceae* (Krieg and Holt, 1984). Vibrios can be found in brackish and sea water, often in association with invertebrates and fishes. Some species are pathogenic for fish, eels, and other aquatic animals' i. e. shrimps and molluscs (Ganesh et al., 2010). In aquatic animals, vibrios cause wound and generalized infections. Vibrios are chemoorganotrophic, facultatively anaerobic bacteria which produce different organic acids during fermentation such as formic, lactic, succinic and pyruvic acids, and produce iron chelating molecules' siderophores' for growth (Famer III et al., 2005). In 1999, Montero et al. reported that *V. harveyi* produced the extract that could break the red blood cell of sheep and horses. In addition, *V. harveyi* is a cause of aquatic animal disease such as fish, oyster, and shrimp (Defoird et al., 2006).

The luminescent bacterial diseases, especially in the shrimp farms of Asian countries, have caused the decrease in shrimp production and intensive rearing systems. Luminescent vibriosis is mainly caused by *V. harveyi* (Lavilla Pitogo et al., 1998; Soto-Rodriguez, et al., 2006). *V. harveyi* can infect some aquatic animals, larval shrimp in hatcheries and adult stages of shrimp (Gomez-Gil et al., 2000; Chiu et al., 2006; Vijayan et al., 2006). The symptom of infected shrimp normally shows fluorescence at night then shrimp's mortality (Jareeporn et al., 2004, in Thai). In the Philippines, virulent strain of *V. harveyi* has caused 100% losses in the larval production with bacterial cell densities as low as 10^2 cells/ml (Lavilla Pitogo et al., 1998). *V. harveyi* luminescent strain has been reported to cause major losses in the shrimp larviculture in South America and South East Asia (Robertson et al., 1998; Soto-Rodriguez, et al., 2006). It was reported that *V. harveyi* had caused 70–100% of shrimp larvae's death in southern parts of Thailand (Thaithongnum et al., 2006). Indeed, *V. harveyi* is the most important pathogen of black tiger shrimp in shrimp farms (Ruangsri et al., 2004; Thaithongnum et al., 2006).

5. Antibiotic and microbial products used in shrimp culture

In general, shrimps are cultivated in any intensive shrimp farms where there are many serious conditions such as high stocking density, poor water quality, and suddenly changes in environmental factors. These conditions have caused shrimp weakness and following by shrimp's diseases. Antibiotics have been used in aquaculture as additives feed to promote growth and cure shrimp diseases (Aarestrup et al., 2001). Besides, many products such as vitamins, antimicrobial agents, disinfectants, algaecide, and microbial products are added in shrimp cultivation to increase shrimp's health and to prevent disease occurrence (Boyd, 2002).

5.1 Antimicrobial agents

The antimicrobial agents are produced from any organisms, microorganisms, and chemical synthesis (Bentley, 2000). The bio-function of antimicrobial agents control microbes, particularly pathogens by inhibiting growth or death. Some antibiotics are produced by microbes such as oxytetracycline and chloramphenical, whereas some antibiotics are produced by chemical synthesis such as a group of sulfa and a group of quinolon. Moreover, some antibiotics are produced by combination of microbiological and chemical processes (semi-synthesis) such as ampicillin and amoxicillin. The antibiotics that have been accepted to use for aquatic animal cultures are the following (Gräslund et al., 2003);

- A group of tetracycline: oxytetracycline

- A group of sulfa: sulfamonomethoxcin, and sulfadimethoxcin

- A group of co-sulfa: trimethoprim-sulfamethoxazole

- A group of nitro: furanase, nifurpirinol, and furazolidone

- A group of quinolon: oxolinic acid, norfloxacin, sarafloxacin, siprofloxacin, and fefloxacin.

The antibiotics in shrimp farming have been spread into environments by discharge of wastewater and sediment. In general, antibiotics can treat shrimps which are infected by bacteria, but can not treat those infected with virus such as white spot and yellow head. *V. harveyi* that causes aquatic animal disease is sensitive to chloramphenical and novobiocin, but it is resistant to streptomycin (Sae-oui et al., 1987). In view of the massive use of antibiotics in aquaculture, many studies have mentioned resistant of luminescent vibriosis. In 1994, Karunasaka reported the mortality of shrimp larvae cased by *V. harve*yi with multiple resistances to cotrimoxazole, chloraphenicol, erythromycin, and streptomycin, so the global efforts are need to promote more judicious use of antibiotics in aquacultures and new strategies to control pathogenic bacteria are need to make the industries more

sustainable. The methods to protect marine aquacultures from luminescent vibriosis without using antibiotics are being developed and tested (Defoirdt et al., 2007).

In Thailand, some groups of antibiotic like chloramphenical and a group of nitrofurans have been banned for cultivation of aquatic animals. (i.e. furazolidone, nitrofurazone, nitrofurantion, nitroquine, and nitrofuratel) (Graslund et al., 2002). The poison of the drugs have been induced the symptom of aplastic anemia and induced cancer in human (Somjetlerdcharoen, 2002). However, in case of USA's import can accept the residue of oxytetracycline at concentrations ≤0.1 mg/l (Sermwatanakul, 1994).

5.2 Microbial products

There are many microbial products that containing microorganisms have been used by farmers. These have been used to treat water, sediment, and given directly to shrimps. Some of products used as feed additives also contained enzymes and other nutrients (Gräslund et al., 2003). *Bacillus* spp. and *Lactobacillus* spp. have been used as probiotics as well as used to control organic compounds in shrimp ponds. Gram-positive bacteria, *Bacillus* spp., produce a wide range of antagonistic compounds and exo-enzymes that are very efficient at breaking down large molecules. *Bacillus* spp. such as *B. subtilis* and *B. licheniformis* are suitable as commercial probiotics in aquaculture. *Bacillus* S11 used as a probiotic was mixed with shrimp feed. The mixed food can protect shrimp from infection of *V. harveyi* and the shrimps had a high survival rate as 74% (Rengpipat et al., 1998; Sunicha, 2002). In addition, microbial products used in shrimp cultivation have been reported, for instance, *Lactobacillus* spp. was mixed with shrimp food to stimulate immune system. Chythanya et al. (2002) found that *Pseudomonas* sp. I-2 could inhibit growth of *V. harveyi* and there was no sign of danger to shrimp larvae (PL-18). *Pseudomonas* sp. W3 used as inoculants has the potential for controlling *V. harveyi* during white shrimp (P-30) cultivation. Furthermore, many enzymes produced by bacteria have been used to control water quality in shrimp ponds. In addition, some products were extracted

from cell wall of *Saccharomyces* spp. (Leaño et al., 2005) such as polysaccharides and oligosaccharides to increase shrimp growth rate. Overall, microbial extracts and whole cell of microbes have been used in aquaculture as an alternative way to avoid use of antibiotics. (Defoirdt et al., 2007).

6. *Pseudomonas* spp.

Pseudomonas is a Gram-negative, aerobic rod belonging to a member of *Pseudomonadaceae* family, motile by one or several polar flagella; rarely non-motile. *Pseudomonas* spp. are respiratory and never fermentation (Garrity et al., 2005). However, some species are facultative anaerobes with nitrate as a final electron acceptor. *Pseudomonas* spp. dissimilate sugars through the Entner-Doudoroff pathway. Most species grow easily in chemically defined media without addition of growth factors, but some species (i.e. *P. iner*, *P. lanceolata* and *P. spinnosa*) require organic growth factors. (Krieg and Holt, 1984; Garrity et al., 2005). Pseudomonads are common inhabitants of the aquatic environments (fresh water, brackish water, and sea water) as well as shrimp culture ponds (Ott et al., 1999) and they are commonly associated with gills, skin and intestinal tract of live fish (Troller and Frazier, 1963; Daly et al., 1973). *Pseudomonas* spp. produce two types of soluble pigments, the fluorescent pigment pyoverdin, and the blue pigment pyocyanin (Krieg and Holt, 1984).

Some *Pseudomonas* species are or may be pathogens of humans, animals, and plants, while some species may be benefits to environments for applications. For example, denitrifying pseudomonad reduces nitrate to nitrous oxide (N_2O) and further reduce nitrous oxide (N_2O) to nitrogen gas (N_2) (Krieg and Holt, 1984). *P. denitrificans* has been used to produce vitamin B12 for commercial production (Crueger and Crueger, 1989). Besides, *Pseudomonas* spp. can degrade many xenobiotic compounds (Assinder et al., 1990), while some *Pseudomonas* spp. can produce bioactive compounds that can inhibit Gram-positive and Gram-negative bacteria (Nair and Simidu, 1987; Uzair et al., 2006). There are several bioactive

compounds such as phenazine from *P. aeruginosa* (Mavrodi et al., 2001; Parsons et al., 2004) and phenazine-1-carbox-amide from *P. aureofaciens* (Woeng et al., 1998; Siunova et al., 2002). In addition, *P. syringae* pv. *ciccaronei* NCPPB2355 produces bacteriocin that was sensitive to heat, protease, and non-polar organic solvent (Lavermicocca et al., 2000). *Pseudomas* spp. are a new source of antibiotics and bioactive compounds (Burkholder et al., 1966; Laue et al., 2000; Chythanya et al., 2002; Uzair et al., 2006).

7. Molecular biology of *Pseudomonas*

In 1990, Krawiec and Riley studied the component of *Pseudomonas* sp. genome and the effect of environments on *Pseudomonas* sp. genome. Bacterial chromosomes vary in size from 586 kb (*Mycoplasma genitalium*) to 9,454 kb (*Myxococcus xanthus*). Most of non-pathogenic bacteria so far measured have chromosome sizes in a range of 2,000 to 4,000 kb, but *Pseudomonas* species are stated at the upper end of that range (Holloway, 1992) such as *P. aeruginosa* has a chromosome size 5,862 kb (Ratnaingsih et al., 1990) while *P. putida* has a chromosome size 6,920 kb. *P. aeruginosa* PAO has two origins of chromosomal replication (*ori*C). Only one of these, *ori*C310, is a true origin while the other, *ori*C301, could be considered as a pseudo-origin. This origin (*ori*C) was shown by probing with appropriate cosmid clones obtain from a *P. aeruginosa* PAO library to be included in the 240-kb *Spe*I fragment 11. By use of the restriction enzymes *Ase*I, *Dra*I and *Xba*I, a more detailed map of *Spe*I fragment 11 had been prepared; by the use of DNA probes, it has been shown that the two *ori*C sequences are separated by about 10 kb (Holloway, 1992).

Some species of *Pseudomonas* produce bioactive compounds, thereby genes that produce bioactive compounds have been studied such as Pierson et al. (1994) found that *phz*R (phenazine regulator) gene controls the production of phenazine compound in *P. aurrofaciens* 30-84 and Brodhagen et al. (2005) concluded that the expression of the transport genes *pltI* and *pltJ* is, in turn, highly sensitive to

concentrations of extracellular pyoluteorin in *P. fluorescens* Pf-5. The pyoluteorin production was controlled by 9 genes as follows: *plt*F, *plt*I, *plt*J, *plt*K, *plt*N, *plt*O, *plt*P, *plt*R and *pltZ* (Nowak-Thompson et al., 1997; Nowak-Thompson et al., 1999).

Many studies have described the potential of *Pseudomonas* spp. to inhibit pathogens and degrade varieties of compounds (Spanggord et al., 1991; Widmer et al., 1998). A culturing-independent detection protocol for *Pseudomonas* spp. would represent a valuable tool in ecological and identify the genus. The molecular ecological techniques and taxonomy open ways to design highly specific PCR protocols, especially for detection of 16S (small-subunit) rRNA genes (Weidner et al., 1996). The rRNA genes are highly conserved and essential for the survival of living organisms (Hirano and Upper, 1983). Phylogenetic analysis based on the 16S rRNA genes became well established as a standard method for the identification of bacteria at the family, genera and species levels (Woese, 1987). The rRNA is amplified from DNA using PCR techniques with primers specific to conserved regions (Saunders and Saunders, 1993; Jeng et al., 2001). Tyler et al. (1995) used 16S-23S rRNA internal transcribed spacers (ITS) region to differentiate between eight species of pathogenic pseudomonads from human infection and twenty four *Pseudomonas* spp. from the American Type Culture Collection (ATCC).

8. Bioactive compounds and characteristic of bioactive compounds

Bioactive compounds can be produced by live cells such as algae, sponge, fungi, bacteria, plants, and so on. Bioactive compounds from nature are complex as they mixed with other compounds thereby identifying the compounds extraction, separation, and purification steps are required. Most of bioactive compounds are extracted by organic solvents such as hexane, chloroform, ethyl, acetate, whereas some bioactive compounds can be easily extracted by pure water, but this method can not make a pure residue like organic solvents. After the extraction step, it is normally followed by a separation step, which may have several steps to obtain a pure compound. Recently, HPLC is a general technique used to separate the

extracts depending on their retention times of each compounds (Bhakuni and Rawat, 2005).

Antibiotics, produced by microorganisms including bacteria, actinomycetes, and fungi, are secondary metabolites, which restrain the growth of many lives. Antibiotics can be divided by their chemical structures into 9 groups, divided by restraining any microbes into 2 groups, or divided by mode of actions into 5 groups, and a type of producing microbes (Garrod et al., 1981).

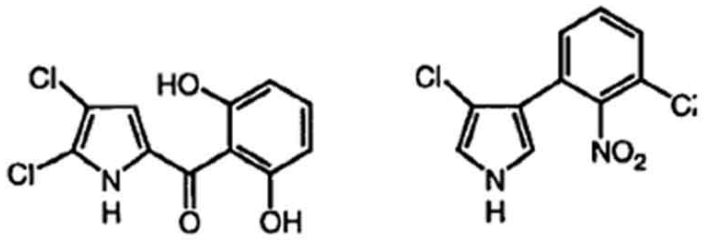
8.1 Bioactive compounds produced by *Pseudomonas* sp.

More than 3,800 biologically active microbial metabolites have been isolated from bacteria. In the group of bacteria, *Bacillus* and *Pseudomonas* species are the most frequent producer. *Pseudomonas* produces 795 bioactive substances, including 610 antibiotics and 185 substances with bioactive properties other than antibiotic activity (Bérdy, 2005; Isnansetyo and Kamei, 2009). There are numerous reasons of studies on bioactive production by pseudomonads as they are common inhabitants of the rhizosphere and phylloshere, easily isolated from natural environmental, utilize a wide range of substrates, easy to culture and manipulate genetically, making them more amenable to experimentation (Leisinger and Margraff, 1979; Whipps, 2001; De Souza, 2002). *Pseudomonas* biocontrol strains not only exhibit a wide range of diversity in the type but also in the number of antibiotics produced. Pseudomonas produced numerous bioactive compounds such as antimicrobial, antiviral, and cytotoxic agent (De Souza, 2002; Isnansetyo and Kamei, 2009).

8.1.1. Pyrroles

Pyrroles are heterocyclic aromatic organic compound and five membered rings with the formula C_4H_4NH. Substituted derivative are called pyrroles.

The pyrroles are antibiotic that produced by bacteria such as marine bacteria (*Pseudomonas bromoutilis* and *Actinomycete*). The pyrrole antibiotics which produced by *Pseudomonas bromoutilis* inhibit Gram-positive bacteria i.e. *Staphylococcus aureas*, *Diplococcus pneumonia*, and *Staphylococcus pyrogenes*, but pyrroles are not active against Gram-negative bacteria and fungus (Isnansetyo and Kamei, 2009). Brominated pyrroles are commonly separated compounds from bacteria in sea water and sponge. Derivative of brominated pyrroles and a group of bioactive compounds with low molecular weight are produced by bacteria (Azumi et al., 1990; Azumi et al., 1990). For example, using broth assay bromo-pyrrole which was produced by *Pseudomonas bromoutilis* at a concentration of 0.0063 µg/ml could inhibit Gram-positive bacteria and non-toxic in mice at 25 or 250 mg/kg (Burkholder et al., 1996; Bhakumi and Rawat, 2005). Burkholder et al. (1996) found that *P. bromoutilis* produced the pyrrole which has 5 bromines in 1 molecule of pyrrole. Whilst *P. pyrrocinice* produced pyrrolnitrin (chloropyrrole) with ability to control fungi in skin disease and plant diseases such as *Fusarium graminearum*, *F. culmorum*, and *Pyrenophora triticirepentis*. At the moment, the pyrrolnitrin (chloropyrrole) has been produced in commercial scale in Japan as trading in brand of PYRO-ACE to cure fungi's inflammation on skin (Bhakumi and Rawat, 2005). Figure 2-4 shows chemical structures of pyrroles that are produced by *Pseudomonas* spp.



Pyoluteorin **Pyrrolnitrin**

Figure 2-4 Chemical structures of pyrroles produced by *Pseudomonas* spp. (Gribbla, 1996).

8.1.2 Phenazines (PHZs)

PHZs have more than 6,000 types; fewer than 100 are of natural origin (Mavrodi et al., 2006). PHZs are redox and heterocyclic compounds which are substituted at different point around the ring. Small modifications of the PHZs structure give rise to full spectrum of color; lemon yellow of phenazine-1-carboxylic acid (PCA), green of phenazine carboxamide (PCN), bright blue of pyocyanin (PYO), and bright orange of 2-Hydroxyphenazine-1-carboxylic acid (2-OHPCA) (Price-Whelan et at., 2006). The broad-spectrum of PHZs are antibacterial, antifungal, antitumor, and parasitic activity (Saosoong et al., 2005; Mavrodi et al., 2006). The chemical structures of PHZs are shown in Figure 2-5.

PHZs are bioactive compounds produced by *Pseudomonas* and they have ability to control pathogenic bacteria and fungi (Mazzola et al., 1992; Pierson III et al., 1994). In 1988, Thomashaw and Weller found that *P. fluorescens* can also produce phenazine, which inhibited the growth of *Gaeumannomyces graminis var. tritici* that caused wheat root's disease. Phenazine-1-carboxamide (PCN) was produced by *P. aeruginosa* R and was able to control *E. coli* and *S. aeraus* (Hongthani, 2005), while *P. chlororaphis* PCL1391 produced phenazine-1-carboxamide (PCN) to inhibit the growth of *Fusarium oxysporum* in disease of tomato (Girard et al. 2006). Moreover, Chythanya et al. (2002) used culture broth of *Pseudomonas* sp. I-2 that was extracted by chloroform to inhibit *V. harveyi* and the extract was identified as pyocyanin, a group of phenazine. Normally *Pseudomonas* can create phenazine and has ability to control any diseases such as root rot from *Fusarium oxysporum* f. sp. *radicislycopersic*i (Chin-A-Woeng et al., 2001).

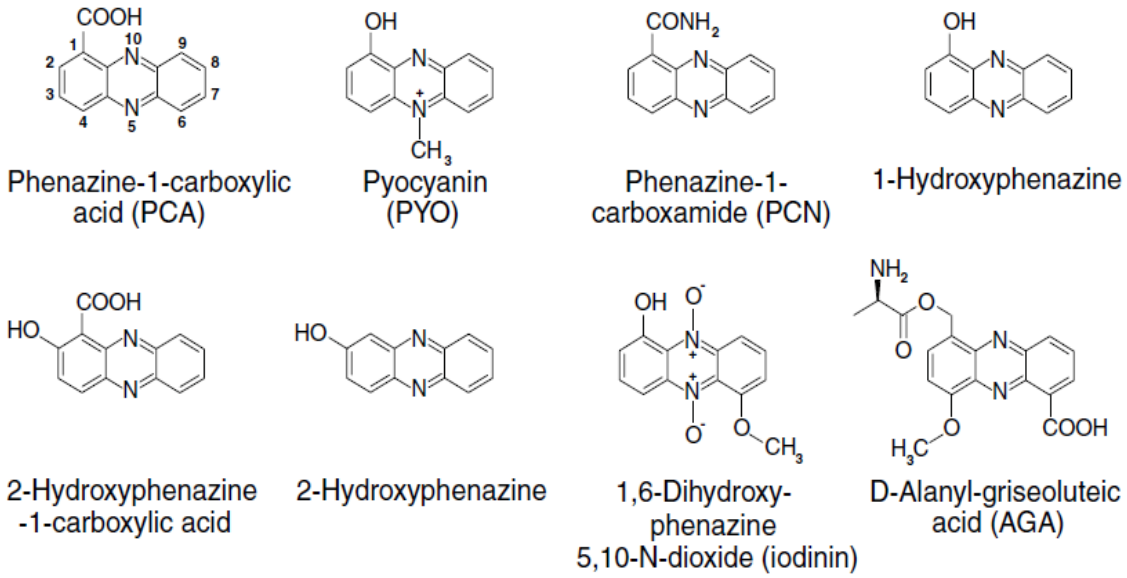


Figure 2-5 Chemical structure of PHZs produced by bacteria (Mavrodi et al., 2006).

8.1.3 Pyoluteorin (PLT)

PLT is a yellow aromatic polymatic polyketide antibiotic. PLT can soluble in chloroform, dichloromethane, water, and carbontetrachloride and can increase solubility when increasing temperature in four solvents (Wang et al., 2008). This compound was first isolated from *P. aeruginosa* and late from *P. aeruginosa* strain S10B2 and *P. fluorescens*. The broad-spectrum of PLT has bactericidal, herbicidal, fungicidal, and oomycidal activities (De Souza and Raaijmakers, 2003). Application of PLT was used for controlling plant pathogens such as the pure PLT was used to control *Pythium ultimum* in cotton seed. The structure of PLT is shown in Figure 2-6.

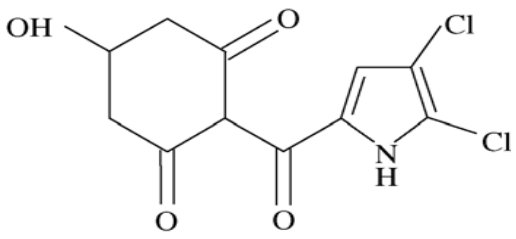


Figure 2-6 Chemical structure of PLT (Wang et al., 2005).

8.1.4 Biosurfactant antibiotics

Biosurfactants are the surface-active molecules that have amphiphatic properties. The structure composes of hydrophilic region and hydrophobic portion (Maneerat, 2005). Generally biosurfactants are classified into five major groups including glycolipids, phospholipids and fatty acid, lipopeptides (lipoprotein), polymeric biosurfactant, and particulate biosurfactant (Deka and Das, 2009). Many biosurfactants produced by microorganisms i. e. *Pseudomonaas* species. *Pseudomonas* sp. produces rhamnolipids, lipopolysaccharides, viscosin, particulate surfactant (PM), biosur PM, and a glycolipid type of biosurfactant (Rahman and Gakpe, 2008; Deka and Das, 2009). The structure of rhamnolipids are shown in Figure 2-7. Applications of biosurfactant were used in agricultural, food industry, and environment (Rahman and Gakpe, 2008). In term of antibiotic property, rhamnolipid was used to control pathogenic in plant such as in vitro assay; 2.50 mg/ml rhamnolipids reduced 62% of mycelia growth of *Pythium myriotylum* (Perneel et al., 2008). Rhamnolipild B that produced by *P. aeruginossa* B5 showed antifungal activity against hyphal growth of *Phytophthora capsici* and spore germination of *Colletotrichum orbicularie* at concentration of 50 µg/ml (Kim et al., 2000). In addition, Tensin (lepopeptide) produced by *P. fluorescens* strain 96.578 was reported to inhibit mycelia growth of *Rhizoctonia solani*, hyphal swelling and stimulating hyphal branching (Nielsen et al., 2000; De Souza, 2002).

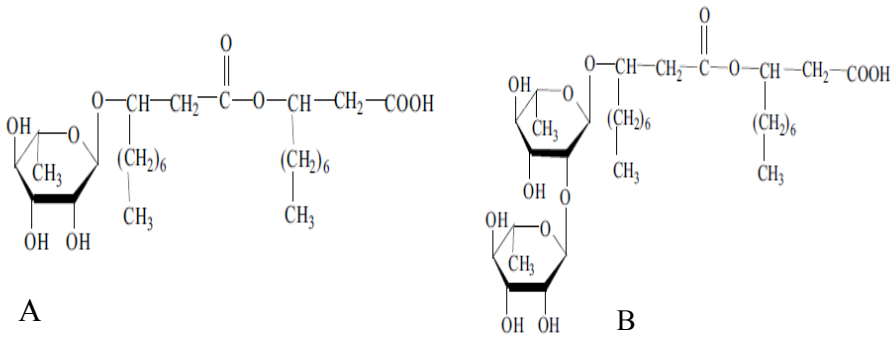


Figure 2-7 Chemical structure of rhamnolipids; A) mono rhamnolipid and B) di rhamnolipid (Pashynska, 2009).

8.1.5 Quinoline and Quinolone

Quinoline and quinolone are derivatives that are important organic compounds. These compounds occur widely in nature and possess interesting biological and pharmacological activities (Wang et al., 2001). *Pseudomonas* species such as marine *Pseudomonas* can produce quinoline and quinolone of which the properties are belonging to quorum sensing and antibiotic activity (Pesci et al., 1999; Isnansetyo and Kamei, 2009). The molecule for quorum sensing belongs to the 4-quinolone family and known as the antibiotic activity of many of its members such as 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-hydroxyquinoline-*N*-oxide produced by *P. aeruginosa* (Pesci et al., 1999). The 4-quinolones are potential therapeutic alternative infections caused by organisms, including *Neisseria gonorrhoeae*, *S. aureus*, common enteric pathogens and intracellular bacteria (Eggleston and Park, 1987). Wratten et al. (1977) reported that antibiotics which produced by *Pseudomonas* sp. isolated from sea water, named 4-hydroxybenzaldehyde, 2-n-heptyl-4-quinolinol, and 2-n-pentyl-4-quinolinol could inhibit *V. harveyi* at the concentration of 50 µg/disk. Bultel-Poncé et al. (1999) isolated *Pseudomonas* sp. 1531-E7 that produced 4 quinolones (2-undecyl-4-quinolone, 2-undecen-1'-yl-4-quinolone, 2-nonyl-4-quinolone, and 2-nonyl-4-hydroxyquinoline-*N*-oxide) from marine sponge and they had ability to control

Plasmodium falciparum. In addition, 2-undecyl-4-quinolone and 2-nonyl-4-hydroxyquinoline *N*-oxide were active against HIV-1 and *S. aureus*. The chemical structures of 4 quinolones produced by *Pseudomonas* sp. 1531-E7 are shown in Figure 2-8.

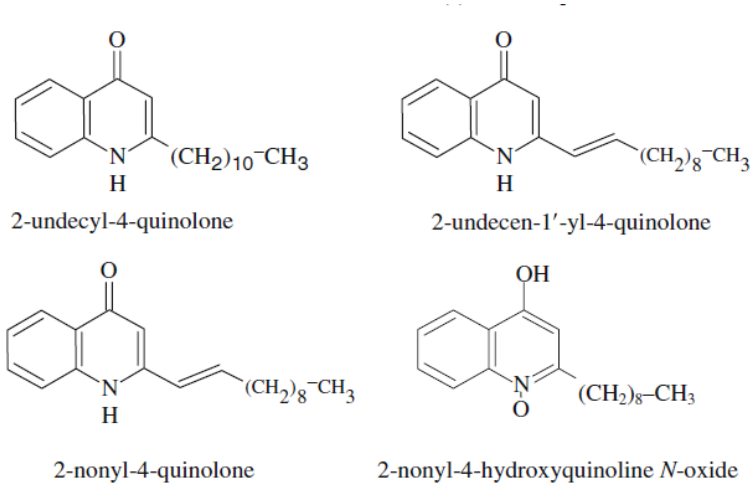


Figure 2-8 Chemical structures of quinoline and quinolone produced by *Pseudomonas* sp. (Bultel-Poncé et al., 1999; Isnansetyo and Kamei, 2009).

9. Chromatography technique

Chromatography technique is the collective term for the separation of mixtures. It involves passing a mixture dissolved in a mobile phase through a stationary phase, which separates the compound to be measured from other molecules in the mixture and allows it to be isolated (Rouessac and Rouessac, 2000).

The separation of bioactive compounds that produced by *Pseudomonas* spp. is regularly extracted by organic solvents i.e. phenazine-1-carboxylic acid from *Pseudomonas* sp. was extracted with toluene (Chin-A-Woeng et al., 2001), whereas pyocyanin from *Pseudomonas* sp. I-2 was extracted with chloroform (Chythanya et al., 2002). Besides, 2-alkyl-4-quinolones (AHQs) from *P. aeruginosa* was extracted with ethyl acetate (Fletcher et al., 2007). The mixture of these compounds is resolved

by standard chromatographic techniques which are important techniques to purify bioactive compounds such as Thin layer chromatography (TLC) and High-performance liquid chromatography (HPLC) (Pingoud et al., 2002). Multiple chromatography is also used for active fraction before being concentrated to a state of purification (Bhakuni and Rawat, 2005). TLC is widely used to fractionate antibiotics recovered from natural materials. For example, the separation of phenazine-1-carboxylic acid from *Pseudomonas* sp. was extracted with organic solvent. The mixture was resolved by TLC Silica plate (butanol-acetone as a mobile phase) and then each fraction was resolved again by the HPLC technique (Hypersil octadecyl saline as column) (Chin-A-Waoeng et al., 2001).

9.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a widely-employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, modified silica gel, alumina, or cellulose on a flat, inert substrate. A rectangular plate made out of glass, plastic or aluminum. Compared to paper, it has the advantage of faster runs, better separations, and more choice of the adsorbents. Different compounds in the sample mixture travel different distances according to how strongly they interact with the adsorbent. This allows the calculation of an R_f value and can be compared to standard compounds to aid in the identification of an unknown substance (Rouessac and Rouessac, 2000). TLC can be a reasonable step in piloting an HPLC. This is becoming easier since almost all the stationary phase available for HPLC can be found spread on TLC plate as precoated plates (Grinberg, 1990).

9.2 Column chromatography

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample (Ettre, 1993).

9.3 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is also sometimes referred to as high-pressure liquid chromatography (Browning, 1996). HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed and the chromatography column. HPLC systems are composed of several components with defined functions that represent independent yet interrelated entities such as those one might find in a hi-fi audio system. The components can be, and often are, inserted in a common frame to form an integrated unit. The components within the HPLC system are interconnected by short segments of transfer tubing with a very small internal diameter (about 0.1 mm). Traditionally constructed from stainless steel, transfer tubes can also be made of polyethertherketone (PEEK®), a colored, flexible polymer that is less expensive to fabricate than stainless steel and is resistant to common solvents under elevated pressures (up to 350 bar) (Rouessac and Rouessac, 2000).

10. The structure elucidation of active compounds

The elucidation of the structure of bioactive compound with high biological activity is both stimulating and challenging. The first step in structure elucidation is to ascertain the skeleton and this can often to be narrow down by reference to phytochemical on related genera and species. Knowledge of biosynthesis of secondary metabolites is helpful in deducing the patterns once the basic structural is established. Nuclear Magnetic Resonance (NMR), Infrared (IR), Ultraviolet (UV) and mass spectrometry (MS) are determined and compared with those reported for compound which may be related on the basis of chemical and biosynthetic reasoning (Bhakuni and Rawat, 2005).

The bioactive compounds that produced by *Pseudomonas* sp. such as phenazine compounds are purified by chromatography techniques and studied structure. The structure of phenazine is obtained from the spectral analysis including UV-Visible (UV-Vis) spectroscopy, IR, NMR and MS (Kumar et al., 2005; Hongthani et al., 2007), accompanied with some physical characteristics of phenazines standard (Femllndez and Pizarro, 1997; Hongthani et al., 2007).

11. Pathogenic bacteria in food, water and frozen food

Foodborne diseases present a widespread and growing public health problem in developed and developing countries. A great of food poisoning and food-borne diseases can be attributed to contamination of food and drinking water with heavy metals, bacterial toxin, and pathogens (Sharif and Al-Malki, 2010). The main cause of these diseases comes from bacteria. The pathogenic bacteria i.e. *Clostridium botulinum*, *E. coli*, *Salmonella*, *Staphylococcus*, *Vibrio* spp., and *Bacillus cereus* are naturally present in the aquatic and the general environment. The major five microorganisms that cause food poisoning are *Salmonella*, *Campylobacter*, *Clostridium*, *Escherichia coli* O157 and *Listeria* (Postnote, 2003). The symptoms of any food-borne disease of gastrointestinal tract include stomach cramp or abdominal

pain, diarrhea, squeamish, exhaustion, and vomiting. However, the different food-borne diseases have many different symptoms, so there is no one syndrome that is food-borne illness (CDC, 2005). The virulent of the disease concern type and quantity of bacteria or toxin in foods.

Food safety issues associated with aquaculture products differ from region to region and from habitat and vary according to the method of production, management practices and environmental condition (Feldhusen, 2000). In the step of pre-harvest, the contamination with pathogens from the human or animal reservoir may pose a risk since in some cases a very low infective dose is required to cause illness.

11.1 Pathogenic bacteria in food and water

In general, gastrointestinal tract of human and warm blood animal have normal flora. One of bacterial groups found is enterobacteriaceae which are a Gram-negative rod and facultative anaerobe. Some members of this family are normally detected i. e. *Escherichia coli* and *Klebsiella* (WHO, 2001; Zhu et al., 2005). However, some of them are pathogenic bacteria such as *Salmonella* and *Shigella* (Montville, 2010). The enteropathogenic bacteria are contaminated in environmental areas and food chain by feces (CDC, 2008). Consequently, food and water are contaminated by pathogenic bacteria. Foods as a source of nutrients provide proliferation of microbes including pathogenic bacteria. Hence standards or quality criteria of foods and waters have been set for the safety of customers.

11.2 Pathogenic bacteria in frozen seafood

Seafood (i.e. fish, crab, shrimp, and squid) is high on the list of food transmitting disease. The more than 80% of seafood- born outbreaks are related to biotoxin, scombrotoxin or the consumption of raw molluscan shellfish (Huss et al.,

2000). Normally, the seafoods are easily infected by spoilage and pathogenic microbes, thereby food preservation methods must be ensured that they can control both organisms to provide food safety. The bacterial pathogens which associated in seafood-borne diseases can be categorized into three groups. Group I, there are bacteria which are normal components of the marine environment such as *Vibrio* spp., *Listeria monocytogenes*, *Clostridium botulinum*, and *Aeromonas hydrophila* (only virulent strain). Group II, there are enteric bacteria that are present due to faecal contamination such as *Salmonella* spp., *E. coli*, *Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica*. Group III, there are the bacteria contamination during processing such as *Bacillus cereus* (only toxigenic strains), *L. monocytogenes*, *Stapylococcus aureus*, and *Clostridium perfringens* (Feldhusen, 2000).

Nowadays, frozen foods are more popular in Thailand than it had been during the 1960s when the industry was established. There are many advantages of the frozen foods as they can easily be stored for long time (6-12 months), extend shelf life, limit microbial, and enzyme activity which cause deterioration and cause a little damage to nutrients and vitamins. Marine shrimps are the most important traded fishery products worldwide. Thailand is one of the largest shrimp exporting countries which 90% of shrimp total output and covering 18% of the world market of frozen shrimp (Oosterveer, 2006). Normally, the frozen seafood products are rejected when the products are contaminated with *V. cholera*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvalia*, *V. alginolyticus*, *Samonella* spp., *B. cereus*, *C. botulinum*, *S aureus*, *E. coli*, and *L. monocytogenes* (Feldhusen, 2000).

11.2.1 *Escherichia coli*

E. coli is a Gram-positive, rod shape, non-spore forming, and facultative bacterium that found in human and homeothermic animal gastrointestinal tract. Strains of *E. coli* were first accepted as a cause of gastroenteritis in early 1940s and the strain O157:H7 has become well established as food-borne pathogen (Jay, 1996: Bellara et al., 2000). The virulence of *E. coli* depends on its enterotoxin

production factors, which are the causes of diarrhea in human and animal, including Shiga toxins (Stx1 and Stx2), intimin (encoded by the *eae* gene), and the enterohaemolysin (Karch et al., 1998; García-Sánchez et al., 2007). The symptom of this disease that caused by *E. coli* are severe cramping (abdominal pain) and diarrhea without fever. 2% to 10% of Hemolytic uremic syndrome (HUS) was occurred in patients; usually a week after the diarrhea begins. HUS can affect all ages but is most common in children under 10 years old (CFSPH, 2004).

E. coli can contaminate on the surface of raw meat, fruits and vegetable, and also seafood (Feldhusen, 2000). This bacterium is also used as a common indicator of fecal contamination in water and food (APHA, 1998). For instance, acceptable level of *E. coli* in frozen shrimp is not more than 4 x 10^2 MPN/g and exceed 4 MPN/g not more than 3 food samples from 5 food samples (TISs 115-2529).

11.2.2 *Staphylococcus aureus*

S. aureus is a Gram-positive coccus, facultative anaerobic, and non-motile. Cells are spherical single or paired cocci, or form grape-like clusters. *S. aureus* can be found on skin, mucous membranes of human and homeothermic animal (Loir et al., 2003). It produces a wide variety of toxin including staphylococcal enterotoxin (SEs; SEA to SEE) with demonstrated emetic activity, and staphylococcal-like (SEI) proteins (Ángeles et al., 2010). The staphylococcal toxins are produced at high level while *S. aureus* in a late exponential or early stationary phase. A toxin level of less than 1 µg has been reported to cause staphylococcal intoxication, a concentration that is reached when *S. aureus* populations exceed 100,000 cells per gram (Bremer et al., 2004). The symptoms of staphylococcal food poisoning usually develop within 1-6 h after ingestion of contaminated food. The symptoms are nausea, vomiting, retching, abdominal cramping, and diarrhea. In severe cases, the symptoms are headache, muscle cramping, and transient changes in blood pressure and pulse rate (Bremer et al., 2004; CDC, 2006). *S. aureus* often

causes of pus, food poisoning and pus of operated wound. *S. aureus* can infect frozen seafood, thereby it must be checked in frozen sea food such as frozen shrimp. The maximum level is 5 x 10^3 colony per 1 gram of shrimp and not more than 3 sample in 5 sample could have 1 x 10^3 colony per 1 gram of shrimp (TISs 115-2529) .

11.2.3 *Salmonella*

Salmonella are a Gram-negative, rod shape, motile, and hydrogen sulfide production. They can be present in various environmental conditions outside living hosts and frequently found in the international tract of numerous animals, also fresh water culture catfish and brackish water pond (Feldhusen, 2000; Wan Norhana et al., 2010). More than 2,500 serovars are potential pathogens in animal and man (Wan Norhana et al., 2010). Salmonella are the most important causes of gastrointestinal diseases worldwide (Feldhusen, 2000). The disease that cause of *Salmonella* is called salmonellosis (CDC, 2005). The symptoms of this diseases are enteric fever such as typhoid fever (*S. typhi*) and paratyphoid fever (*S. paratyphi* A, B and C), food borne illness such as gastritis (*S. typhimurium*), gastroenteritis (*S. enteritidis*), and septicemia (*S. choleraesuis*) (FDA, 2001). In case of poor underlying health or weakened immune systems, Salmonella can invade the bloodstream and cause life-threatening infections (CDC, 2005).

The incident of *Salmonella* in seafood was reported in many countries such as Japan, Vietnam, India, Sri Lanka, Thailand, US, and UK (Feldhusen, 2000; Ponce et al., 2008). *Salmonella* enteric serovar Typhimurium was the most serovar causing human salmonellosis. However, *Salmonella weltevreden* has been reported as a frequent and increasing cause of human infection in Southeast Asia. This serotype was the most common serovar amongst 210 isolates from imported seafood from 20 countries during 2000 to 2005 (Ponce et al., 2008).

11.2.4 *Vibrio* spp.

Vibrio spp. is a Gram-negative, rod shape and non-spore forming. They are every common in estuarine and coastal environments. Several species of *Vibrio* are notable for clinically important human pathogenic abilities such as *V. cholerae* (cholera), *V. vulnificus* (food poisoning), and *V. parahaemolyticus* (gastroenteritis) (Davis et al., 1981; FDA, 2001). Vibrios are commonly associated with seafood and frozen seafood as natural contaminants. Vibrios are frequent isolate from seafood and 10-20% of seafood contaminated with *Vibrio* spp. (FDA/CFSAN, 2001). Most of foods borne infection are caused by *V. parahaemolyticus*, *V. cholera*, and *V. vulnificus* (FEHD, 2005; Cañigral et al., 2010). Check of these bacteria in frozen seafood as *Vibrio* spp. must not be found in 25 grams of frozen seafood (TISs 482-2525).

V. cholera is of most concern of its ability to cause cholera. *V. cholera* can be divided in to serogroups on the basis of the O antigen. O1 and O139 are associated with the epidemiological features and clinical syndrome of cholera and the other serogroups are associated infrequent case of foodborne outbreaks of gastroenteritis, but are not spread in epidemic form (FEHD, 2005). The virulence factor of *V. cholera* O1 and O139 is cholera toxin (Feldhusen, 2000). Cholera toxin remains a public health threat globally causing hundreds of thousand cases every year and this toxin is an acute intestinal infection (FEHD, 2005). Its incubation period ranges from a few hours to five days, usually two to three days. The symptom of disease are including sudden onset of profuse painless watery diarrhea that can quickly lead to rapid dehydration, acidosis, circulartory collapse, hypoglycaemia in children, renal failure, and death if treatment is not prompthy given. Nausea and vomiting also occurs early in the course of illness (ICMSF, 1996; FEHD, 2005).

V. parahaemolyticus causes gastrointertinal illness in humans. Clinical isolates are associated with Kanagawa phenomenon (KP). The reaction of Kanagawa is measured by ability of strains to produce β-haemolysis on special blood agar (Lake et al., 2003). The symptoms of this disease are watery diarrhea often with abdominal cramping,

nausea, vomiting, fever, and chill (Su and Liu, 2007). These symptoms are occurred within 24 h of ingestion and this disease usually resolves in 3 days (FEHD, 2005).

Vibrio vulnificus is an opportunistic pathogen which can cause wound infection and primary septicemia (FAO/WHO, 2005; FEHD, 2005). The virulence factors associated with *V. vulnificus* include a capsule, cytolysis, protease or elastase, and phospholipase (Strom and Paranjpye, 2000; FAO/WHO, 2005). The incubation period of the disease is 7 h to several days. The symptoms are fever, chills, nausea, and cardiovascular hypotension. Most patients do not significant diarrhea. For predisposed person, septicemia can presumably occur with doses of less than 100 total organisms (ICMSF, 1996; FEHD, 2005).

11.2.5 *Yersinia enterocolitica*

Yersinia spp. are a small rod shape or coccobacilli (0.8-3.0 μm), Gram-negative, oxidase-negative, catalase-positive, straight, facultative anaerobes, and non spore forming. They are motile at 22-30°C, but they are not motile at 37°C. *Yersinia* spp. include tree important species of human significance; *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (Percival et al., 2004). These bacteria inhabit in environments and foods (i.e. lake water, soil, raw meat, and raw milk). The disease which causes by *Y. enterocolitica* is called Yersiniosis (Pullela et al., 1997). The sero-groups which is the cause of Yersiniosis are O:3, O:5, O:8, O:9, and O:27 (Pullela et al., 1997; Percival et al., 2004). However, the infective dose is unknown. Illness onset is usually between 24 and 36 hours after ingestion and the duration of illness is usually 1-3 days (Pullela et al., 1997). The symptoms of this disease are a diarrhea, abdominal pain, fever, stomach cramp, squeamish, and blood in stools (Percival et al., 2004). The Foodborne Diseases Active Surveillance Network (FoodNet) reported that approximately one culture-confirmed *Y. enterocolitica* infection per 100,000 persons occurs each year. Children are infected more often than adults (CDC, 2005).

11.2.6 *Listeria monocytogenes*

The genus of Listeria includes six species as *L. innocua*, *L. ivanovii, L. seligeri*, *L. welshimeri*, *L. grayi*, and *L. monocytogenes* (Jemmi and Stephan, 2006). *L. monocytogenes* is a Gram-positive, non-spore forming and motile by flagella. This bacterium inhabits in environments (i.e. river and soil), waste, and contamination in seafood (Pearson and Marth, 1990). The disease causes by *L. monocytogenes* is called Listeriosis. The infective dose is unknown and estimates vary from 10^2 to 10^9 CFU, depending on the immunological status of host (Jemmi and Stephan, 2006). One-third of human *L. monocytogenes* infections are perinatal, involving pregnant women, and their unborn or newly born infants and the other two-thirds occur in non-pregnant people of all ages. However, Most *L. monocytogenes* infections occur in people whose immunity has been impaired by age, conditions such as cancer, organ transplantation, corticosteroid use or AIDS (ICMSF, 1996). The difference syndromes of these Listeriosis diseases are occurred in difference condition as pregnancy infection, granulomatosis infantiseptica, sepsis, meningoencephalitis, and focal infection.

- Pregnancy infection: Pregnancy infection is pregnant women who infected with *L. monocytogenes*. The symptoms of this disease are a severe illness, convulsions, muscle aches, headache and miscarriage. In perinatal period, the symptoms are generally only a mild fever in the mother with or without slight gastroenteritis or flu-type symptoms, but the consequences for the foetus or newborn are often major or fatal (ICMSF, 1996).

- Granulomatosis infantiseptica (infection of the newborn): Granulomatosis infantiseptica is babies who infected with *L. monocytogenes*. The patients have a blue skin, respiratory distress, heart failure, refusal to drink, vomiting, convulsions, soft whimpering, early discharge of meconium, and mucus stools (Pullela, 1997).

- Meningoencephalitis and septicemia: Meningoencephalitis is sepsis in newborn, adults and abortion in pregnant women. The symptoms of sepsis are a fever, meningitis, myelitis, convulsions, and pharyngitis (Pullela, 1997).

- Focal infection: The symptoms of sepsis are headache, squeamish, vomiting, and eye conjunctivitis (Pullela, 1997).

11.2.7 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, rod shaped, anaerobic (microaerophilic), spore-forming bacterium of the genus *Clostridium*, and non-motile. *C. perfringens* is grouped into five types; A, B, C, D, and E according to the exotoxins produced. The toxin types; A, B, C, and D are toxic to humans (CFSPH, 2004). The toxins of *C. perfringens* are shown in Table 2-1. The unique enterotoxin of *C. perfringens* types A and C are produced in significant amounts in the intestine only and is associated with spore formation. Small amounts may be produced in some food and may contribute to the early onset of symptoms in some cases of *C. perfringens* food poisoning (ICMSF, 1996). This bacterium is ubiquitous in nature and can be found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, and soil. Some strains of *C. perfringens* produce toxins which cause food poisoning (Sneath, 1986). The common form of perfringens poisoning is characterized by intense abdominal cramps, nausea and acute diarrhea which begin 8-24 hours after consumption of foods containing large numbers of those *C. perfringens* bacteria capable of producing the food poisoning toxin (ICMSF, 1996). The illness occurs when people swallow the bacteria or spores formed by them which then multiply and produce toxin in the intestine, or from eating the toxin already produced in food (Schneider et al., 2003). The illness is usually of short duration and full recovery within 24-48 h (ICMSF, 1996). *C. perfringens* must not be found in 0.1 grams of KAPI (Fermented shrimp paste) and dried fishery products such as dried seasoned squid or fish, salted shrimp and salted fish (TISs 1080-2535).

Table 2-1 Toxin produced by *C. perfringens* (CFSPH, 2004)

Strains of *C. perfringens*	Toxin
Type A	Alpha
Type B	Alpha, beta, epsilon
Type C	Alpha, beta
Type D	Alpha, epsilon
Type E	Alpha, iota

11.2.8 *Bacillus cereus*

Bacillus cereus is a Gram-positive, aerobic, spore forming, and can produce toxic which contaminate in food. This strain can grow at 30-40°C, but some strain can grow at 4-5°C (ICMSF, 1996). This bacterium is ubiquitous in nature and can be found in soil, dust, plant production such as rice, cereal, wheat flour, spices, meat production, raw food, and sauce (Rogers et al., 1978; Tay et al., 1982). The detection of *B. cereus* can be confirmed by the isolation of $\geq 10^5$ CFU/g from epidemiologically implicated food (CDC, 1994). The common from *B. cereus* poisoning is characterized by nausea, vomiting, abdominal cramp, diarrhea, headache, and fever (Tay et al., 1982; CDC, 1994). The bacillus poisoning occurs after the ingestion of foods which the organism has grown and form toxin. These are two types of intoxication. The first is diarrhoeagenic toxin. This toxin is characterized by diarrhea occurring 8-24 h after the ingestion of cells or toxin. The second is emetic toxin (cereulide). This toxin is characterized by emesis occurring within a short time (1-6 h). The amount of emetic toxin in food sample implicated in vomiting-type food poisoning cases ranged from 0.01-1.28 μg/g (Agata et al., 2002). The illness is relatively mild and recovery occurs within 12-24 h (ICMSF, 1996).

Chapter III

Inhibition of shrimp pathogenic vibrios by extracellular compounds from a proteolytic bacterium *Pseudomonas* sp. W3

Abstract

Pseudomonas sp. W3, a bacterium that produces an extracellular alkaline protease, secreted secondary metabolites that inhibited pathogenic bacteria responsible for shrimp luminous vibriosis disease. Antivibrio compounds in the culture supernatant or culture filtrates (0.45 µm and 0.22 µm) of the isolate W3 were tested using an agar well diffusion method on a number of pathogenic vibrios. *Vibrio harveyi* PSU 2015 was the most sensitive strain. The effectiveness of preparations from the isolate W3 against *V. harveyi* PSU 2015, and *V. cholerae* PSSCMI 0062 was in the order of culture supernatant > 0.45 µm culture filtrate > 0.22 µm culture filtrate. These extracellular antivibrio compounds also lysed both dead and living cells of *V. harveyi* PSU 2015. Results of partial characterization tests indicated that there was some particulate antivibrio compound that was destroyed by treatment with enzymes particularly α-chymotrypsin, autoclaving at 121^{o}C for 15 min and was mostly removed by filtration through a 0.22 µm filter. Most of the inhibitory compounds were of small molecular weight able to pass through a 0.22 µm filter and were resistant to treatment with various enzymes, pH values between 4-8 and temperatures up to 121ºC for 30 min. The optimum pH for the antivibrio activity in the 0.45 µm culture filtrate was between pH 6-7.

Keywords: *Pseudomonas* sp. W3, *Vibrio harveyi*, shrimp pathogenic bacteria, growth inhibitory compounds, bacteriolytic enzymes

Introduction

Shrimp cultivation is one of the most economically important aquacultural activities in Asia, and South America but is also practiced worldwide (Nimrat et al. 2008). However, shrimp cultivation has faced many serious problems such as shrimp diseases, unsatisfactory practices i.e. inadequate control of water quality, etc. Viruses and bacteria are the main causes of shrimp diseases. Disease due to bacterial infections, particularly luminous vibriosis, is a major problem for shrimp cultivation in Asian countries (Musa et al. 2008) and if they can completely destroy hatchery productivity for extended periods (Gomez et al. 2000; Vinod et al. 2005; Sharshar and Azab, 2008). The causative agents of vibriosis include *Vibrio harveyi*, *V. parahaemolyticus*, *V. cholerae* and others (Austin and Zhang 2006; Won and Park, 2008). Among vibrios, *V. harveyi* is the main cause of shrimp death, infecting larva in the hatchery as well as shrimp in the cultivation pond (Vinod et al. 2005; Chari and Dubey 2006). The virulence of *V. harveyi* has at times caused a 100% loss in shrimp production (Chythanya et al. 2002; Musa et al. 2008). *V. harveyi* is also a serious pathogen for a wide range of marine animals and in other aquaculture systems (Austin and Zhang 2006).

Antibiotics such as tetracycline, chloramphenical, oxolinic acid, and norfoxazine (Holmström et al. 2003) have been commonly used to control these shrimp diseases. In many cases, their extensive use has led to a decrease in the therapeutic efficiency of the least expensive antibiotics, so during the past decade many Asian aquaculture programs have gradually switched to the use of broad spectrum antibiotics such as chloramphenicol (Huys et al. 2007). However recently, in Thailand some antibiotics such as chloramphenical and the group of nitrofurans (furazolidone, nitrofurazone and nitrofurantion) have been banned for use in shrimp cultivation due to the retention of antibiotics residues in aquaculture products (Gräslund et al. 2002). There is also evidence of the development of bacterial resistance to a number of antibiotics i.e. ampicillin and sulphamethoxazole (Musa et al. 2008). Therefore, for the important shrimp pathogenic bacteria like *V. harveyi*, it is important to carry out laboratory testing for the sensitivity of an isolated know shrimp pathogen to different antibiotics and compounds, particularly those that could be used

in shrimp aquaculture. Based on the above information in order to limit the use of antibiotics, many workers have been exploring the use of new bioactive compounds for controlling bacterial diseases of shrimp, particularly that caused by *V. harveyi*.

Pseudomonads are attractive bacteria for testing as sources of new bioactive compounds including antibiotics, bacteriocins, biosurfactants and bacteriolytic enzymes (Vijayan et al. 2006; Kumar et al. 2008). They are widely distributed in soil and aquatic habitats including shrimp ponds (Sakami et al. 2008). There have been reports that *Pseudomonas* spp. produce bioactive compounds with ability to control vibrios such as *V. harveyi* and *V. parahaemolyticus*, and that have no effect on shrimp (Chythanya et al. 2002; Vijayan et al. 2006). One of our isolates, *Pseudomonas* sp. W3 is a proteolytic bacterium and is also able to control *V. harveyi* during white shrimp *(Litopenaeus vannamei)* cultivation with no harm to shrimp (Rattanachuay et al. 2007). The results prompted us to explore its ability to produce compounds that could control shrimp pathogens like vibrios. Hence, the aims of this study were to produce an antibiogram of pathogenic *V. harveyi* strains commonly found in Thailand, to investigate the spectrum of anti-vibrio compounds produced by *Pseudomonas* sp. W3 and to characterize their actions against *V. harveyi* including the possibility that they might be useful as alternative biocontrol agent in shrimp cultivation.

Materials and methods

3.1 Bacterial strains used

Pseudomonas sp. W3 was isolated on Frazier gelatin medium (FGM) (Frazier and Rupp, 1982; Furczak and Joniec, 2007) from a water sample collected from an intensive shrimp cultivation pond in Pattanee province, Thailand. The following shrimp pathogens; *V. harveyi* AAHRC 1, *V. harveyi* AAHRC 2, *V. parahaemolyticus* AAHRC 1, *V. harveyi* PSU 2015, *V. parahaemolyticus* PSU 1681 and *V. cholerae* PSSCMI 0062 were provided by the Aquatic Animal Health Research Center (AAHRC), Prince of Songkla University and Assoc. Prof. Dr. Varaporn

Vuddhakul, Department of Microbiology, Faculty of Science, Prince of Songkla University. All shrimp pathogens were isolated from shrimp samples infected by vibrios.

3.2 Preparation of culture samples for testing for antivibrio activity

The isolate W3 was grown in FGM plus 2% NaCl under optimal conditions (30°C, 150 rpm) for 18 h (Rattanachuay et al., 2007). The culture broth of *Pseudomonas* sp. W3 was centrifuged at 6000 rpm for 25 min (SANYO, Harrier 18/80 Refrigerator; U.K.) to remove bacterial cells and provide the culture supernatant. A portion of the culture supernatant was then filtered through either a 0.45 µm or 0.22 µm pore size cellulose acetate filter to provide the culture filtrates used for testing. It was hoped that the 0.22 µm filter would remove any particulate cell debris so that this culture filtrate would contain only truly soluble chemicals and colloidal organic matter. The inhibition of shrimp pathogenic bacteria, by these culture samples (culture supernatant and culture filtrates), were separately tested by the agar well diffusion method. The shrimp pathogenic bacteria were all cultivated in TSB (Tryptic soy broth; Difco, USA) plus 1.5% NaCl and shaken (Gallenkamp, orbital incubator) at 150 rpm, 30°C for 18 h to use for inoculation of the test plates.

3.3 Inhibition of shrimp pathogens by antibiotics and culture samples of *Pseudomonas* sp. W3

The antibiotics in Table 1, commonly used in aquaculture in Asian countries such as Malaysia and Thailand were chosen for investigating their effects on an isolated bacterial shrimp pathogen. Some of these antibiotics have recently been banned (furazolidone, chloraphenicol, oxolinic acid and tetracycline) (Gräslund et al. 2002; Holmström et al. 2003; Musa et al. 2008). Only three strain of *V. harveyi* were used to produce their antibiograms because they are the usual cause of vibriosis. They are virulent strains and frequently found in infected shrimp from hatcheries and cultivation ponds. Samples from *Pseudomonas* sp. W3 or antibiotics (Table 1) were

tested by the agar well diffusion method for inhibition of the vibrios as follows: shrimp pathogens in TSB plus 1.5% NaCl were individually adjusted to 0.5 McFarland standard (bio Merieux; 69280 Marcy l'Etoile, France) and then swabbed over the surface of TSA plus 1.5% NaCl plates. Antibacterial disks (Table 1) were placed evenly over the surface of the plate with forceps. Wells (diameter 7 mm) were punched in the TSA plate by a Pasteur pipette (3 wells/plate). 70 µl of the different culture samples from the isolate W3 was transferred into each well and the diffusion of culture sample into the agar could be visually observed. All plates were incubated at 30°C for 24 h and the diameter of the inhibition zones was measured using a Vernier caliper.

3.4 Effect of culture age on production of antivibrio compounds

Pseudomonas sp. W3 was cultured in FGM medium supplement with 2% NaCl under the optimal conditions as mentioned previously. The culture broth was sampled at 0, 4, 8, 12, 16, 18, 24 and 36 h to measure growth based on cell turbidity at 660 nm (OD 660) using a spectrophotometer and each sample was then filtered using a 0.45 µm cellulose acetate filter and tested by the agar well diffusion method for inhibitory activity against *V. harveyi* PSU 2015.

3.5 Assay of bacteriolytic activity

The 0.45 µm culture filtrate prepared from either an 18 h or 24 h culture of *Pseudomonas* sp. W3 was used to test its bacteriolytic activity using a method previously described by Than et al. (2004). A 48 h culture of *V. harveyi* PSU 2015 was centrifuged at 6000 rpm for 25 min to obtain the cell pellet. This was dispersed in 3 ml of sterile Artificial Sea Water (ASW) and incubated in a water bath at 50°C ± 0.5°C for 30 min to obtain heat-killed cells. A mixture consisting of 7 ml of the 0.45 µm culture filtrate from the isolate W3 and 3 ml of the heat-killed *V. harveyi* cell suspension in sterile ASW was adjusted to an initial absorbance of approximately 0.5 (OD 540 nm) using a spectrophotometer and then incubated at 30°C for

investigating cell lytic activity. The cell lytic activity was assayed by monitoring the absorbance at 540 nm at different times; 0, 2, 4, 6, 8, 10 and 22 h and the ASW was used as a blank. A 24 h culture filtrate W3 was also used to test its bacteriolytic activity against live cells of *V. harveyi* PSU 2015 by using the same method as for heat-killed cells, except that dead cells was replaced by live cells. Both live cells and dead cells of a target organism were used to investigate the lytic activity of the isolate W3 due to previous reports that different amounts of lytic activities were detected when comparing dead and live cells (Guilloux-Benatier et al. 2000; Ryazanova et al. 2005). In addition, another reason was that one of the aims of some study was to use lytic enzymes to break dead cells for use as a source of nutrients (Wahid et al. 2007) and in this study the main aim was to test for lytic activity in the hope that it might be used to control a shrimp pathogenic bacterium such as *V. harveyi*.

Lytic activity was ranked into four levels according to the percentage decrease in absorbance at 540 nm in comparison with the control: -, 0-24%; +, 25-49%; ++, 50-74% and +++, 75-100% (Niwa et al., 2005). In addition a scanning electron microscope (SEM) was used to observe the cells obtained from both live and dead cell experiments and a transmission electron microscope (TEM) for the live culture experiments using the manufacturers protocols.

3.6 Preliminary studies on the property of bioactive compounds from *Pseudomonas* sp. W3

The culture filtrates (0.45 and 0.22 µm) of *Pseudomonas* sp. W3 were separately tested for protein characteristics by treatment with proteinase K (1 mg/ml at 30°C) (Fluka), pronase (2 mg/ml at 30°C) (Fluka), α-chymotrypsin (5 mg/ml at 30°C) (Fluka), trypsin (50 mg/ml 30°C) (Fluka) and lysozyme (1 mg/ml at 25°C) (Fluka); for lipid characteristics using lipase (1 mg/ml at 37°C) (Fluka)) and for carbohydrate characteristics using α-amylase (1 mg/ml at 25°C) (Fluka) . Briefly for any enzymatic treatment, the amount of enzyme used as indication i.e. 1 mg of enzyme was added to 1 ml of culture filtrate, mixed well, and incubated at the appropriate temperature for 1 h. The remaining anti-*Vibrio harveyi* PSU 2015 activity

was then observed by the agar well diffusion method (Vijayan et al., 2006). Negative controls were also prepared using a similar procedure, except that the isolate W3 preparation was omitted (uninoculated medium). Positive controls were prepared without the addition of enzymes.

The heat stability of the anti-*Vibrio harveyi* compounds was separately tested by heating the culture filtrates (0.45 and 0.22 µm) of *Pseudomonas* sp. W3 in a water bath for 30 min at different temperatures; 50°C, 65°C, 75°C, 100°C; and was also autoclave at 121°C for 15 min and 30 min. For controls each culture filtrate was incubated at 30°C for 30 min. The anti-*Vibrio harveyi* PSU 2015 activity was tested by the agar well diffusion method (Vijayan et al., 2006).

The pH susceptibility of bioactive compounds from *Pseudomonas* sp. W3 against *V. harveyi* PSU 2015 was tested by adjusting the pH of each culture filtrate (0.45 and 0.22 µm) over the range of 1.0-10.0 with either 0.1 N HCl or 0.1 NaOH (Lee et al., 2004). Culture filtrates without adjustment of pH served as positive controls whereas negative controls were prepared using a similar procedure with treated set in uninoculated medium (FGM). The anti-*Vibrio harveyi* activity was tested by the agar well diffusion method.

3.7 Statistical analysis

Three replicates were used in each experiment, unless otherwise stated. All results were presented as means ± their standard deviations. A One-way ANOVA (SPSS for windows version 15) was used to analyze statistical differences at a *p*-value < 0.05 and mean comparisons were performed by the Duncan's multiple range test.

Results

3.1 Inhibitory effect of antibiotics and culture samples of *Pseudomonas* sp. W3 against shrimp pathogens

Antibiotic susceptibility testing of the shrimp pathogen, *V. harveyi* is shown in Table 3-1. All strains tested were susceptible to chloramphenicol, tetracycline, nalidixic acid, oxolinic acid and norfoxazine but resistant to kanamycin and ampicillin. Furazolidone and sulphamethoxazone gave intermediate zones of inhibition to strains AAHRC1 and AAHRC2. Table 3-2 shows that 70 µl of the culture supernatant from *Pseudomonas* sp. W3 inhibited the growth of the following shrimp pathogens; *V. harveyi* PSU 2015, *V. harveyi* AAHRC 1, *V. harveyi* AAHRC 2, *V. parahaemolyticus* AAHRC1, *V. cholerae* PSSCMI 0062 with clear zones in a size range of 13.7 ± 0.8 to 21.2 ± 0.4 mm. The most sensitive strain was *V. harveyi* PSU 2015 while *V. parahaemolyticus* PSU 1681 was the most tolerant strain with a zone size of only 6.5 ± 0.2 mm.

There were very little significant differences between the inhibitory characteristics of the 0.45 µm and the 0.22 µm culture filtrates but there was a tendency for the 0.22 µm filtrate to be less inhibitory than the 0.45 µm filtrate. Moreover, in most cases the 0.22 µm culture filtrate sample gave inhibitory zone sizes that were significantly less than those for the culture supernatant samples, especially the reductions of inhibition zone sizes with *V. parahaemolyticus* PSU1681, *V. cholerae* PSSCMI 0062 and *V. harveyi* PSU 2015 that were 100, 39 and 27%, respectively.

Table 3-1. The susceptibility of *Vibrio harveyi* strains to antibiotics (mm of inhibition zone)

Antimicrobial agent	Disk content (µg/disk)	*Vibrio harveyi*		
		PSU 2015	AAHRC1	AAHRC2
Ampicillin	10	R (0)	R (0)	R (0)
Kanamycin	30	R (13)	R (11)	R (12)
Furazolidone	15	S (18)	I (17)	I (17)
Sulphamethoxazone-trimethoprim	25	S (21)	I (13)	I (13)
Chloramphenicol	30	S (31)	S (25)	S (26)
Oxolinic acid	2	S (19)	S (18)	S (20)
Tetracycline	30	S (27)	S (23)	S (23)
Nalidixic acid	30	S (25)	S (23)	S (24)
Norfoxazine	10	S (21)	S (21)	S (23)

R (resistant); I (intermediate); S (susceptible)

Table 3-2 Inhibition of shrimp pathogens by samples from culture supernatant and culture filtrates of *Pseudomonas* sp. W3

Bacterial strain	Inhibition zone (mm)		
	supernatant	0.45 µm culture filtrate	0.22 µm culture filtrate
V. harveyi PSU 2015	21.16 ± 0.40 [a]	18.03 ± 0.42 [b]	15.39 ± 0.44 [c]
V. harveyi AAHRC 1	15.30 ± 0.44 [a]	15.31 ± 0.41 [a]	14.43 ± 0.65 [b]
V. harveyi AAHRC 2	14.51 ± 0.51 [ab]	14.73 ± 1.74 [a]	13.20 ± 0.58 [b]
V. parahaemolyticus AAHRC1	13.67 ± 0.78 [a]	12.83 ± 0.64 [b]	12.80 ± 0.39 [b]
V. parahaemolyticus PSU1681	6.51 ± 0.24 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
V. cholerae PSSCMI 0062	16.47 ± 0.61 [a]	10.92 ± 0.46 [b]	9.95 ± 0.59 [c]

Mean value ± standard deviation of nine observations. Different letters in the same row indicate significant differences ($p < 0.05$)

3.2 Effect of culture age on production of antivibrio compounds

The 0.45µm culture filtrates from *Pseudomonas* sp. W3 grown in FGM medium plus 2% NaCl under the optimal conditions showed that cells started to produce bioactive compounds that inhibited *V. harveyi* PSU 2015 when the cells were reaching the late log phase of growth at 12 h and the inhibition zone was 11.0 ± 0.4 mm) (Fig. 3-1). After that cells in the early stationary phase (18h) gave the highest activity (16.9 ± 0.8 mm) against the target organism and then significantly decreased ($p < 0.05$) to 15.8 ± 0.4 mm at the late stationary phase (24h).

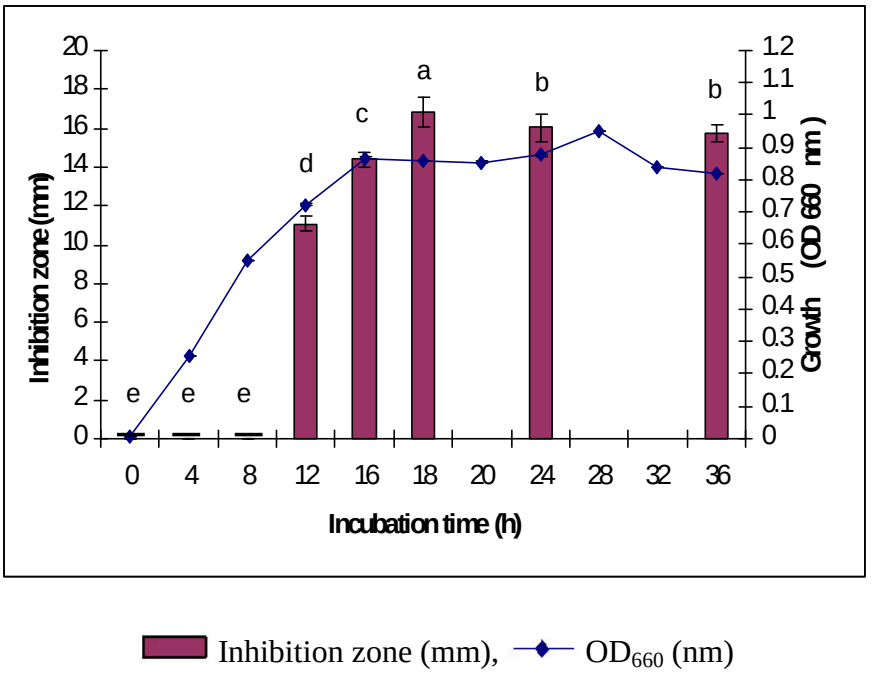


Figure 3-1 Growth of *Pseudomonas* sp. W 3 in FGM medium plus 2% NaCl and its inhibitory effect on the growth of *V. harveyi* PSU 2015.

3.3 Assay of bacteriolytic activity

The bacteriolytic activity of a 0.45µm culture filtrate from both 18 h and 24 h *Pseudomonas* sp. W3 culture against heat killed cells of *V. harveyi* PSU 2015 is shown in Table 3-3. The 18 h filtrate had a low lytic activity (+) that was observed at 6 h until the end of testing at 22 h. The 24 h culture filtrate W3 caused a low level of lysis after 4 h and this increased to a higher level (++) at 22 h. The bacterial cell-lytic activity of the culture filtrate W3 was confirmed by SEM photomicrography (Figure 3-2). There was no change of cell shape in the control set of heat killed cells throughout the 22 h testing period (Figure 3-2: A-B). Some modified dead cells of the treatment set after 22 h of incubation appeared as ovoid or spherical shapes and the arrow shows the presence of holes (Figure 3-2D) whereas a treatment set at 0 h showed most killed cells were rod shaped (Figure 3-2C).

The lytic activity of the 24 h culture filtrate W3 against live cells of *V. harveyi* PSU 2015 was evident after 4 h of incubation i. e. at the same time as with the

heat killed cells but after that a higher lytic activity was found (Table 3-3). SEM photomicrographies of live cells in a treatment set of 22 h incubation showed holes (Figure 3-2F) while normal live cell shapes were found in the treatment at 0 h (Figure 3-2E). TEM photomicrographs strongly supported the finding that culture filtrate W3 in the treatment set after 22 h incubation caused live cells of *V. harveyi* PSU 2015 to lyse (Figure 3-3D) whereas only normal cells were found in a control set at both 0 and 22 h of incubation and also in a treatment set taken at 0 h of incubation (Figur 3-3A-C).

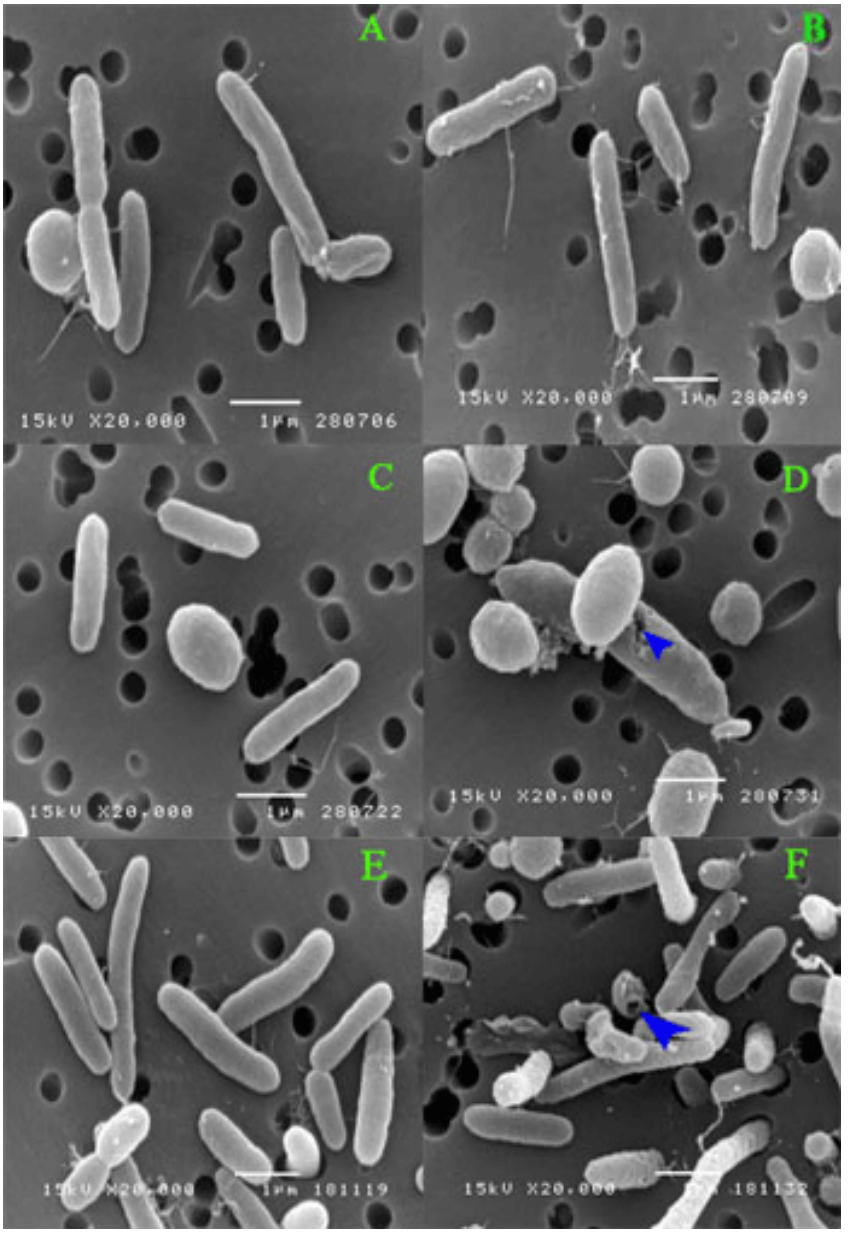


Figure 3-2 SEM photomicrographs showing lysis of heat killed cells (A, B, C and D) and live cells (E and F) of *V. harveyi* PSU 2015 by a 24 h culture filtrate (0.45µm) of *Pseudomonas* sp. W3; A, control (no addition) at 0 h; B, control at 22 h; C, treatment at 0 h; D, treatment after 22 h incubation; E, treatment at 0 h and F, treatment after 22 h incubation. The arrows show the presence of holes on cells.

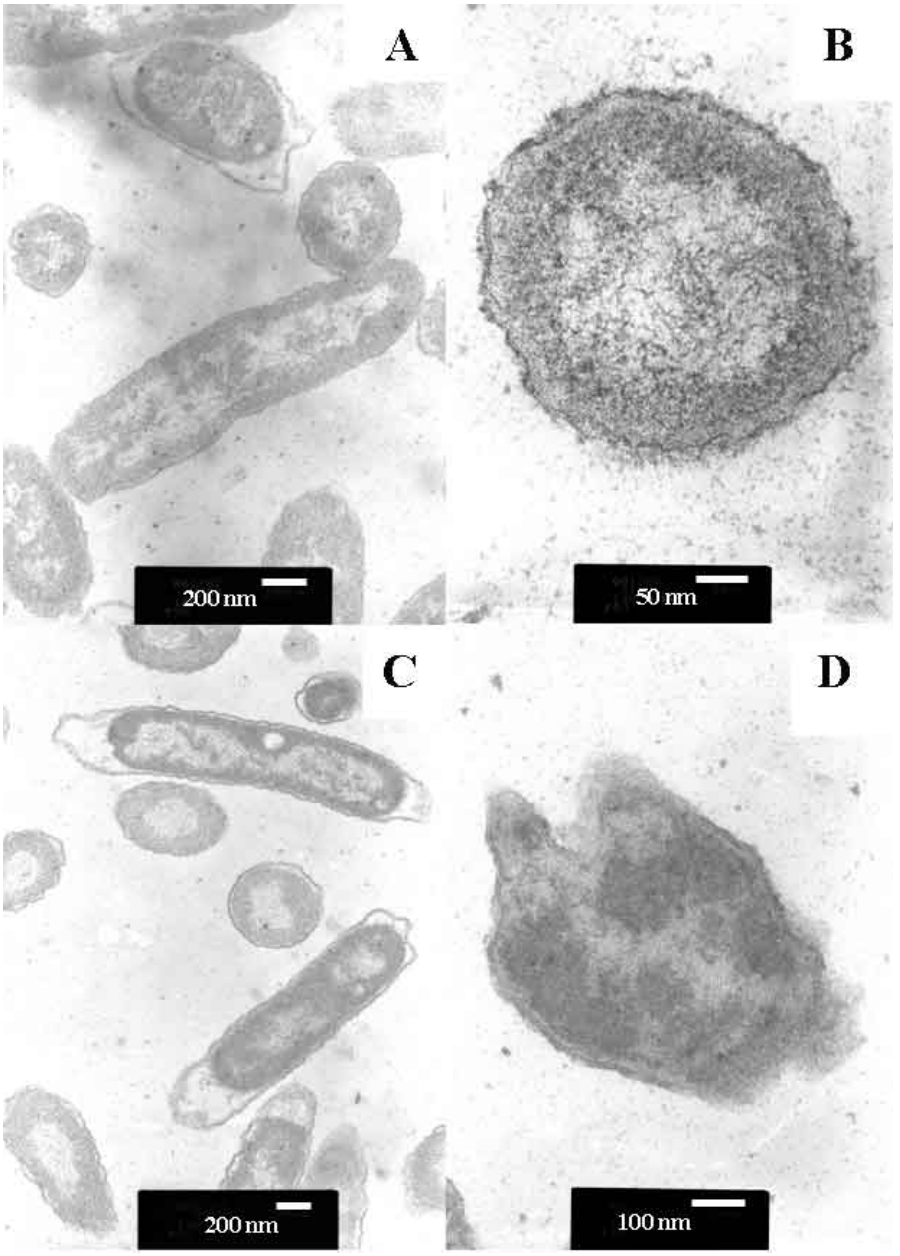


Figure 3-3. TEM photomicrographs showing altered structures of live cells (A, B, C and D) of *Vibrio harveyi* PSU 2015 by a 24 h culture filtrate (0.45 μm) of *Pseudomonas* sp. W3**;** (A) control (no addition) at 0 h; (B) control at 22 h; (C) treatment at 0 h; (D) treatment after 22 h incubation

Table 3-3. Comparison of the observation of lytic activity, against heat killed and live cells of *V. harveyi* PSU 2015 by a 0.45 µm culture filtrates obtained during growth of *Pseudomonas* sp. W3 culture

Incubation time (h)	Bacteriolytic activity				
	Dead cells of *V. harveyi* PSU2015			Live cells of *V. harveyi* PSU2015	
	Control	18 h culture filtrate	24 h culture filtrate	Control	24 h culture filtrate
0	-	-	-	-	-
2	-	-	-	-	-
4	-	-	+	-	+
6	-	+	+	-	++
8	-	+	+	-	++
10	-	+	+	-	++
22	-	+	++	-	++

Cell lytic activity when compared with a control set (no addition of 0.45 µm culture filtrate) was scored as follows: -, 0-24%; +, 25-49%; ++, 50-74 and +++, 75-100%.

3.4 Preliminary studies on the property of bioactive compounds from *Pseudomonas* sp. W3

Most of the enzyme treatments caused some loss of inhibitory activity against *V. harveyi* PSU 2015 and this loss was significantly less ($p < 0.05$) with the 0.22 µm filtrate than with the 0.45 µm filtrate (Table 3-4). We assume that some inhibitory compound, more sensitive to the enzyme treatments was removed by the smaller sized filter. This material was perhaps protein because treatment with any of the proteolytic enzymes caused a loss in inhibitory activity (19-27%) from the 0.45 µm filtrate; and the maximum loss of the 0.22 µm culture filtrate treated with α-chymotrypsin was only 8%. The biggest reduction (27% loss) occurred when the 0.45 µm filtrate was treated with α-chymotrypsin. However, in most cases for the 0.45 µm filtrate, more than 78% of the activity remained after enzyme treatment and for the

0.22 µm filtrate more than 90% of the activity remained after enzyme treatment. The other enzyme treatments lysozyme, lipase and α-amylase caused a 19, 17 and 16% loss of activity with the 0.45 µm filtrate and a 5, 5 and 8% loss with the 0.22 µm filtrate, respectively.

The inhibitory activity of either culture filtrate was not affected by heating up to 100^{o}C but heating in an autoclave at 121^{o}C for 30 min caused a 20% loss of activity in the 0.45 µm filtrate and a 9% loss in the 0.22 µm filtrate.

The optimum pH for the inhibitory activity of the 0.45 µm filtrate occurred at a pH between 6 and 7. At a pH of 4, 2 and 1 activity declined by 19%, 24% and 69% and at a pH of 8 and 10 activity declined 28% and 35%, respectively. The effect of the pH on the activity of the 0.22 µm filtrate was minimal, except for the pH 1 sample (40% loss of activity).

Table 3-4 Inhibition of *V. harveyi* PSU 2015 by 0.45 µm and 0.22 µm culture filtrates from *Pseudomonas* sp. W 3

Treatment	Inhibition zone (mm)	
	0.45 µm culture filtrate	0.22 µm culture filtrate
Enzyme		
Control	17.83 ± 0.27 [a]	15.19 ± 0.39 [a]
Proteinase K	13.78 ± 0.23 [bc]	14.73 ± 0.69 [abc]
Pronase	13.93 ± 0.43 [bc]	14.37 ± 0.46 [abc]
Pepsin	14.38 ± 0.15 [bc]	14.28 ± 0.21 [abc]
α-chymotrypsin	12.97 ± 0.23 [c]	13.96 ± 0.48 [c]
Trypsin	14.01 ± 0.55 [bc]	15.13 ± 0.73 [ab]
Lysozyme	14.48 ± 0.22 [bc]	14.84 ± 0.22 [abc]
Lipase	14.78 ± 0.42 [b]	14.43 ± 0.43 [abc]
α-amylase	15.09 ± 1.41 [b]	13.99 ± 0.43 [bc]
Temperature of incubation (°C)		
Control (30)	17.99 ± 0.45 [a]	15.39 ± 0.44 [a]
50	17.52 ± 0.48 [a]	15.33 ± 0.29 [a]
65	16.49 ± 0.36 [b]	14.68 ± 0.14 [ab]
75	15.88 ± 0.50 [b]	14.98 ± 0.38 [ab]
100	16.18 ± 0.30 [b]	14.68 ± 0.39 [ab]
Autoclave (121°C, 15 min)	15.14 ± 0.43 [c]	14.73 ± 0.21 [ab]
Autoclave (121°C, 30 min)	14.33 ± 0.22 [d]	14.00 ± 0.74 [b]
pH		
Control (pH 7.40)	17.14 ± 0.45 [a]	16.84 ± 0.66 [a]
1	5.31 ± 0.50 [f]	10.07 ± 0.68 [c]
2	13.08 ± 0.39 [cd]	15.48 ± 0.66 [b]
3	13.30 ± 0.50 [c]	15.39 ± 0.47 [b]
4	13.93 ± 0.46 [bc]	16.13 ± 0.31 [ab]
5	14.38 ± 0.45 [bc]	15.59 ± 0.98 [ab]
6	16.47 ± 0.32 [b]	15.98 ± 0.46 [ab]
7	16.34 ± 0.28 [b]	15.54 ± 0.81 [ab]
8	12.41 ± 0.40 [d]	15.54 ± 0.32 [ab]
9	12.23 ± 0.43 [d]	14.86 ± 0.60 [b]
10	11.08 ± 0.44 [e]	14.88 ± 0.75 [b]

Means value ± standard deviation of nine observations for enzyme treatments and pH activities; three observations for temperature. Different letters in the same column denote significant differences ($p < 0.05$)

Discussion

3.1 Inhibitory effect of commercial antibiotics and bioactive compounds produced by the isolate W3 against vibrios

Most of the antibiotics used could cause inhibition of all tested strains of *V. harveyi* (Table 3-1), hence antibiotics could be used to control *V. harveyi* infections that affect aquaculture in Thailand. However, some of them such as chloramphenicol, furazolidone, tetracycline and oxonilic acid are banned from use in aquaculture as previously mentioned. In general, β-lactam antibiotics such as ampicillin are preferred for use by shrimp farmers to treat luminous vibriosis disease since these groups of antibiotics do not cause significant side effects (Teo et al. 2002). Unfortunately, all tested strains in this study were resistant to ampicillin and this result is in agreement with Teo et al. (2002) who reported that many types of β-lactam antibiotics were no longer able to prevent vibriosis. In Thailand sulphamethoxazole is normally used in hatcheries against *Vibrio* sp.; however, in this study it showed only moderate ability to inhibit shrimp pathogens. Results in this study were similar to previous studies (Otta et al. 2001; Musa et al. 2008). Based on the above results, only a limited number of antibiotics, such as nalidixic acid and norfoxazine, could be used for treating shrimp diseases. However, resistance to quinolones in the form of nalidixic acid and norfoxazine has been observed in target pathogens due to the quinolones act against DNA-metabolism and repair attempts by the bacteria can lead to the formation of resistant mutant (WHO, 1998; Gräslund and Bengtsson, 2001). Therefore, the use of new inhibitory compounds needs to be investigated to combat luminous vibriosis.

Many studies have shown that compounds produced by bacteria could be used to inhibit bacterial pathogens in aquaculture systems (Alavandi et al. 2004; Vijayan et al. 2006). The use of such compounds to control shrimp pathogens by means other than by the use of antibiotics is now gaining acceptance in shrimp farming as it can be a better and more cost effective alternative than administering antibiotics to manage the health of shrimps (Gómez et al. 2007; Verschuere et al. 2000).

Late log phase cultures of *Pseudomonas* sp. W 3 inhibited a variety of shrimp pathogens tested *in vitro* and a maximum activity was present during their early stationary growth phase (Figure 3-1). This indicated that the antivibrio compounds could be “secondary metabolites”. The culture supernatant and culture filtrates (0.45 µm and 0.22 µm pore size filters) contained inhibitors of the growth of most shrimp pathogens tested with different degrees of inhibition although none of the culture filtrate inhibited the growth of one *V. parahaemolyticus* strain PSU1681 (Table 3-2). Production of extracellular antivibrio substances by another strain of pseudomonad, *Pseudomonas* I-2, was also observed (Chythanya et al. 2002). The use of the agar well diffusion technique indicated that some antivibrio compounds produced by the isolate W 3 were water soluble compounds as they readily diffused through the agar medium. Previous studies have reported that *Pseudomonas* spp. produce water soluble bioactive compounds that are secreted into the media such as a group of phenazines (Kerr 2000; Price-Whelan et al. 2006).

The unfiltered culture supernatant produced the best inhibition of *V. harveyi* PSU 2015 and *V. cholerae* PSSCMI 0062 followed by the 0.45 µm filtrate while the 0.22 µm culture filtrate was the least inhibitory. This indicated that some of the inhibitory activity was associated with particulate matter that was removed by the filtration process and only dissolved and soluble colloidal organic matter was present in the 0.22 filtrate. It has been recommended that a 0.22 µm membrane can be used to separate proteins of low molecular weight or peptides from larger molecules (Thermo Fisher Scientific Inc, 2008).

3.2 Preliminary studies on the property of bioactive compounds produced by the isolate W3

In this study, some of the growth inhibitory compounds may be bacteriolytic as rod shaped cells were changed to ovoid cells (Table 3-3, Figure 3-2 D and F). However, although the isolate W3 showed lysis against dead and live cells of *V. harveyi* PSU2015 neither were completely lysed after 22 h incubation. This might be due to an attack on the cell envelope structure of *V. harveyi* (Figure 3-2). In

contrast, a *Pseudomonas* sp. isolated from the surface seawater of Japan produced an enzyme that completely lysed the dead cells of *V. parahaemolyticus* VPHK-46 (Than et al., 2004). Our previous study indicated that *Pseudomonas* sp. W 3 produces an extracellular alkaline protease (Ratanachuay et al. 2007). There is evidence from this present study that at least some of the inhibitory activity may be associated with a proteolytic enzyme. This work was similar to that reported by Shastry and Prasad (2002) who showed that *Pseudomonas* sp. (CL 1457) produced an extracellular alkaline protease against heat killed cells of *Xanthomonas campestris*.

In general, bacteriolytic enzymes are classified into two major groups; peptidoglycan hydrolytic (i.e. Nacetylmuramidases, N-acetyl glucosamidases, etc.) and proteolytic enzymes (Than et al. 2004). Results in Table 3-4 indicate that some loss of anti-*Vibrio harveyi* activity of the isolate W3 was caused by proteolytic enzymes and lysozyme (N-acetylmuramidase). Hence, it may be possible the isolate W3 produced both types of lytic enzymes. The cell walls of Gram negative bacteria are surrounded by an outer membrane consisting of lipopolysaccharides, phospholipids and lipoproteins and thus they are less sensitive to bacteriolytic enzymes than Gram positive bacteria (Wang et al. 2008). Only a few lytic enzymes have been reported to lyse cells of Gram negative bacteria (Shastry and Prasad 2002); therefore, it is interesting that the isolate W3 produced lytic enzymes that acted to remove the outer membrane as some leaky cells can be seen by electron microscopy (arrows in Figure 2 D and F; Figure 3-3D). After removal of their outer membrane *Vibrio* cells become more easily damaged by other bioactive compounds produced by the isolate W3.

A higher lytic activity of the isolate W3 against *V. harveyi* PSU 2015 was found in a 24 h culture filtrate when compared to an 18 h culture filtrate. This may be caused by the lytic enzymes being produced during the early stationary phase of bacterial growth (Figure 3-1) in a similar way to the yeast lytic enzymes produced by *Oenococcus oeni* (Guilloux-Benatier et al. 2000). It was surprising to note that the isolate W3 showed a higher lytic activity on living cells compared to dead cells of *V. harveyi* PSU 2015 during the 6-10 h testing time (Table 3). This may imply that the lytic enzymes work better when cell walls are being produced actively. It has been

recognized that heating of live cells helps to disrupt the outer membrane of Gram negative bacteria, and this can allow hydrolytic enzymes a better access to the bacterial peptidoglycan (Ryazanova et al. 2005). However, the results in this study showed that no disruption of the outer membrane occurred in dead cells of the control at 0 and 22 h (Figure 2a and 2b). This might explain that live cells were killed by heating at only 50ºC for 30 min while Ryazanova et al. (2005) live cells were killed by autoclaving (121ºC). It will be necessary to carry out further studies to clearly identify the mode of action of these lytic enzymes.

The results from Table 3-4 indicate that some anti-*Vibrio harveyi* compounds present on particulate matter are destroyed by treating with proteolytic enzymes, lysozyme, lipase and amylase and are destroyed by autoclaving at 121^{o}C for 30 min. The inhibitory compounds left in the 0.22 µm filtrate were not protein, were possibly not related to carbohydrate or lipid moieties and not heat labile, had pH stability (pH 4-8) and are small molecular weight compounds (Tables 3-3 and 3-4). This was in agreement with Chythanya et al. (2002) who reported *Pseudomonas* I-2 produced an antibacterial compound responsible for the inhibition of vibrios *i.e. V. harveyi*, *V. parahaemolyticus*, etc. and that compound may be pyocyanine, which is a nonproteinacious, chloroform-soluble blue pigment, heat stable with low molecular weight. Vijayan et al. (2006) also found that *Pseudomonas* PS-102 inhibited the growth of *V. harveyi* in a similar manner to *Pseudomonas* I-2 by a cell free supernatant that contained a heat stable compound and it was resistant to proteolytic, lipolytic and amylolytic enzymes. The property of heat stability would be useful during the production of shrimp feed, as at certain stages of production it is exposed to higher temperatures. It is of interest that some different activities were found for the isolate W3 as this bacterium produced more than one bioactive compound that were active over a wide range of pH and also produced lytic enzymes (Table 3-2, Table 3-3, Table 3-4, Figure 3-2 and Figure 3-3). This means that the inhibitory compounds produced by the isolate W3 may be alternative compounds for use in shrimp cultivation.

Conclusions

The results from this study indicate that *Pseudomonas* sp. W3 produced more than one extracellular antibacterial substance responsible for inhibiting the growth of shrimp pathogenic bacteria and caused cell lysis of *V. harveyi*. It can be concluded that some of the compounds that inhibit the growth of *V. harveyi,* are proteinaceous while the most active are heat stable, pH tolerant and resistant to various enzymes (lysozyme, proteolytic, lipolytic and amylolytic enzymes) and have a small molecular weight. Our work is continuing to characterize the target of these inhibitory compounds and to further explore the potential of *Pseudomonas* sp. W3 for use in the shrimp aquaculture industry.

Chapter IV

Antivibrio compounds produced by *Pseudomonas* sp. W3: characterization and assessment of their safety to shrimps

Abstract

In order to explore compounds naturally inhibitory to shrimp pathogenic vibrios, a culture filtrate of *Pseudomonas* sp. W3 at a pH of 2 was extracted with ethyl acetate (EtOAc) to produce 82.15 mg/l of a yellow-brown extract (EtOAc-W3) that had MIC values of 225-450 μg/ml against the growth of 18 shrimp pathogenic *Vibrio harveyi* strains. The MIC of EtOAc-W3 against the most pathogenic strain PSU 2015 was 450 μg/ml and this strain had the lowest LD_{50} (50% lethal dose) to Pacific white shrimp (*Litopenaeus vannamei*, PL 21). At this MIC value, EtOAc-W3 in artificial sea water (ASW) killed strain PSU 2015; however in natural sea water, only a partial growth inhibition was observed. The toxicity to Pacific white shrimp and antivibrio activity of the EtOAc-W3 were investigated by conducting experiment with 4 sets; native control (commercial ASW), EtOAc-W3 control (MIC/10, 45 μg/ml), challenge (inoculation 6.0 x 10^6 cfu/ml PSU 2015) and treatment (6.0 x 10^6 cfu/ml PSU 2015 + 45 μg/ml EtOAc-W3). The same experiment was repeated by increasing the dose of EtOAc-W3 to 90 μg/ml (MIC/5). Both concentrations tested of EtOAc-W3 tested had no toxicity to postlarval shrimps. A significant decrease in shrimp mortality was observed over a 72 h period as approximately 80% of the shrimp died in each challenge set but only 63 and 23% died in the presence of 45 and 90 μg/ml EtOAc-W3. The major component of EtOAc-W3 was supposed to be 2-heptyl-4-quinolone (HHQ) by FAB-MS and ^{1}H-NMR analyses of the purified fraction.

Keywords: Antivibrio compounds; *Pseudomonas* sp.; *Vibrio harveyi*; Shrimp; Toxicity

Introduction

Among shrimp diseases caused by bacteria, particularly those of luminous vibrios, *Vibrio harveyi* is the most serious causative agent as it can cause mass mortalities of up to 100% to larva in hatcheries and shrimp cultivation ponds (Chari and Dubey 2006; Chythanya et al. 2002; Vinod et al. 2006). Normally, antibiotics are used to treat disease outbreaks or to prevent shrimp infection by *V. harveyi* (Gräslund and Bengtsson 2001; Holmström et al. 2003); however, effective antibiotics such as chloramphenicol, furazolidone, oxonilic acid and tetracycline are not allowed for use in shrimp cultures in Thailand due to their persistence in aquaculture products (Gräslund et al. 2002). Continued antibiotic use also leads to selection of antibiotic-resistant microbes and eventually these might transfer to humans as pathogens (Holmström et al. 2003; Rahman et al. 2009). Based on a list of the antibiotics that can be used in aquaculture, only nalidixic acid and norfoxazine can be used treat shrimp diseases effectively (Musa et al. 2008; Rattanachuay et al. 2010; Teo et al. 2002). In addition, in Thailand sulphamethoxazole is extensively used in shrimp hatcheries, but this has only moderate ability to inhibit shrimp pathogens (Rattanachuay et al. 2010; previous chapter).

According to the above information, the use of compounds other than regular antibiotics could be acceptable for shrimp cultivation especially if they are more cost effective and can maintain shrimp health (Alavandi et al. 2004; Preetha et al. 2009). Therefore, exploring for new bioactive compounds against shrimp bacterial disease agents like *V. harveyi* is an urgent priority. There has been much research to report that *Pseudomonas* spp. can produce a variety of bioactive compounds that can control shrimp pathogenic bacteria such as *Vibrio* spp. with no harm to the shrimp (Chythanya et al. 2002; Liu et al. 2000; Vijayan et al. 2006). It is well recognized that *Pseudomonas* spp. produce bioactive compounds such as phenazine compounds, quinolones, hydrogen cyanide, 2, 4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin (Ge et al. 2004; Mashburn-Warren et al. 2009).

However, there have been only a few reports on work that has attempted to identify antivibrio compounds produced by *Pseudomonas* spp. and most

of them have reported that a siderophore is the antivibrio compound (Vijayan et al. 2006). Recently, a bioactive compound, produced by *Pseudomonas* MCCB 102 and 103, that inhibited *V. harveyi* was identified as N-methyl-1-hydroxyphenazine, a phenazine antibiotic (Preetha et al. 2009). Our previous work also showed that one of most active antivibrio compounds of one of our isolates, *Pseudomonas* sp. W3, has a small molecular weight, is heat stable, pH resistant and mostly tolerant to a variety of enzymes such as lysozyme, proteolytic, lipolytic and amylolytic enzymes (Rattanachuay et al. 2010). Hence, in the present study, the crude extracted compounds produced by strain W3 were investigated for their ability to control *V. harveyi* and also their toxicity to shrimps for possible use in shrimp farming. Characterization of the antivibrio compound was also investigated.

Materials and methods

4.1 Bacteria used and culture conditions

Pseudomonas sp. W3 has been proven to secrete secondary metabolites, particularly those of a small molecular weight that inhibit vibrios i.e. *V. harveyi* (Rattanachuay et al. 2010; previous chapter). The W3 isolate was grown in Frazier gelatin medium (Frazier and Rupp, 1982) containing 2% NaCl under optimum growth conditions: 30°C on a shaker (Gallenkamp, orbital incubator), shaking speed 150 rpm, time 18 h and the culture broth was used to extract antivibrio compounds.

The following shrimp pathogens; *V. harveyi* AAHRC 1 and *V. harveyi* AAHRC 2, were provided by the Aquatic Animal Health Research Center (AAHRC), Prince of Songkla University while *V. harveyi* PSU 2015 and other strains as shown in Table 4-1 were provided by Assoc. Prof. Dr. Varaporn Vuddhakul, Department of Microbiology, Faculty of Science, Prince of Songkla University. All the shrimp pathogenic bacteria were cultivated in TSB (Tryptic soy broth) containing 1.5% NaCl and shaken (Gallenkamp, orbital incubator) at 150 rpm, 30°C for 18 h and were tested for their sensitivity to bioactive compounds produced by *Pseudomonas* sp. W3.

4.2 Shrimp used

Post larvae (PL-19) Pacific white shrimp (*Litopenaeus vannamei*) and commercial shrimp food were kindly provided by Charoen Pokphand Group (CP), Tha Bon, Ranot district, Songkhla province. Before starting the experiment for investigating the toxicity of the crude extracted antivibrio compounds to shrimp, the white shrimp collected from the farm were maintained to acclimatize in a storage tank for 2 days. Commercial artificial sea water (ASW) was adjusted to an initial pH of 7.8, an initial salinity of 15 ppt and a water temperature of between 25 and 27°C depending on the ambient temperature. The commercial ASW (Deep Blue Sea, Imipape sea salts, Medifish Product Bangkok, Thailand) was used because its composition is similar to the natural sea water used for shrimp cultivation. For the shrimp experiment, all white shrimp were incubated in commercial ASW using the same conditions used in the acclimatizing medium in the storage tank. They (20 shrimps per aquarium) were fed with 0.01 g food per aquarium 4 times per day. All the aquaria used for testing shrimp toxicity were made from acelic plastic with a size of (12.5 cm x 12.5 cm x 25.5 cm, and 3.6 liter capacity).

4.3 Solvent extraction of the culture filtrate of *Pseudomonas* sp. W3

An 18 h culture of *Pseudomonas* sp. W3 was centrifuged at 6000 rpm (SANYO, Harrier 18/80 Refrigerator; U.K.) for 25 min. The culture supernatant was filtered through a 0.45 μm pore size cellulose acetate filter to remove cells and then extracted with chloroform. A 300 ml portion of the cell-free culture filtrate (culture filtrate) without adjustment of pH (7.8) was extracted with 90 ml chloroform. The chloroform layer (blue color) was evaporated with a flash evaporator at 45°C. A similar protocol was used for the ethyl acetate (EtOAc) extraction. The amount of each dried extract was weighed and the antivibrio activity of each extract was tested with the shrimp pathogens.

These chloroform/EtOAC extracts did not show significant antivibrio activity, even though the blue color of the chloroform extract was associated with a

phenazine derivative. However as Preetha et al. (2009) had previously identified *N*-methyl-1-hydroxyphenazine as an antivibrio compound from the blue-colored chloroform extract of a *Pseudomonas* culture filtrate we further investigated the possibility that the antivibrio compounds produced by *Pseudomonas* sp. W3 were phenazine derivatives. Since extraction with ethyl acetate at an acidic pH is usually employed for isolation of phenazines, the following extraction procedure was used. A freeze dried sample (5 g) from 162.50 ml of a culture filtrate of *Pseudomonas* sp. W3 was added to 200 ml of distilled water and the pH adjusted to 2 with 1N HCl. The sample was extracted with 100 ml of EtOAc, 3 times (100 ml x 3). The EtOAc layer was combined and dried over anhydrous sodium sulfate (Na_2SO_4). The EtOAc extract was named EtOAc-W3 and weighed to calculate the amount of the extract per volume of culture broth and further tested for antivibrio activity.

4.4 Antivibrio activity of extracts of the culture filtrate of *Pseudomonas* sp. W3

The minimal inhibitory concentration (MIC) of both initial dried extracts (chloroform and EtOAc) against virulent strains of shrimp pathogens; *Vibrio harveyi* AAHRC 1, AAHRC 2 and PSU 2015 was determined using a broth dilution method in test tubes (each test tube contained 0.5 ml test solution and 0.5 ml culture suspension). The extracts were separately dissolved in 10% DMSO (Dimethyl sulphoxide) to provide final concentrations in the range of 10-20 mg/ml. Freeze dried samples of an untreated culture filtrate of *Pseudomonas* sp. W3 was also tested for testing antivibrio activity. However, the final concentrations of the freeze dried sample were in the range of 12.5-100 mg/ml. A negative control using 10% DMSO was also included in the test. Three virulent strains of *V. harveyi* were grown in TSB as previously described, their cell density was then adjusted to 0.4 at $OD_{660\ nm}$ (1.2 x 10^8cfu/ml) and the cell suspension was diluted 10 times in TSB containing 1.5% NaCl (1.2 x 10^7cfu/ml) before adding to each test tube to obtain a final cell density of 6.0 x 10^6 cfu/ml. The cell density (6.0 x 10^6 cfu/ml) used in this experiment was designed based on the infectious dose of *V. harveyi* to post larvae shrimps at 10^6 – 10^8 cfu/ml (Ruangpan 1998).

The antivibrio activity of the yellow-brown EtOAc extract (EtOAc-W3) was assessed for its MIC and MBC (minimum bactericidal concentration) using a microtiter plate (96 wells) according to the standard technique (NCCLS, 2003) and in this experiment a total of 18 strains *V. harveyi* were used as target organisms (Table 4-1). A 5.40 mg quantity of EtOAc-W3 was dissolved in 600 µl methanol to obtain the stock solution at a concentration of 9000 µg/ml. Then 20 µl of the stock solution was serially diluted by twofold dilutions to obtain final concentrations in the range of 1.76 µg/ml-900 µg/ml. The methanol was evaporated and 20 µl of 10% DMSO was added to each well to dissolve the residue. Then, 80 µl of TSB containing 1.5% NaCl was added into each well followed by 100 µl (1.2 x 10^7cfu/ml) of bacterial cells to achieve a final cell density of 6.0 x 10^6 cfu/ml. Each concentration was conducted in three replicates. Bacterial cells in 10% DMSO without addition of the EtOAc-W3 served as a negative control set, while bacterial cells in concentrations of nalidixic acid of 0.391-800 µg/ml served as a positive control set. This antibiotic was used as the positive control due to the report by Rattanachuay et al. (2010; previous chapter) that shrimp pathogenic strains of *V. harveyi* were sensitive to nalidixic acid and in Thailand, this antibiotic is allowed for use in shrimp farming and hatcheries. The 96-well plate was incubated at 30°C for 24 h and the bacterial growth was measured as turbidity by a microplate reader at a wavelength of 660 nm. The lowest concentration of the EtOAc-W3 to prevent bacterial growth was reported as its MIC. Afterwards, all wells with no visible growth were streaked on TSA containing 1.5% NaCl plates to check for survivors. The minimum concentration of the EtOAc-W3 that allowed no growth was interpreted as the MBC.

4.5 Pathogenicity of strains of *Vibrio harveyi* to post larvae shrimps

Since the pathogenesis of the strains of *V. harveyi* used in this study has not been previously reported, the LD_{50} (lethal dose at 50%) of the following strains; PSU 2015, AAHRC 1 and AAHRC 2 were determined prior to the start of the next experiment. These three strains were chosen for this study because they produced a higher mortality to shrimp than other strains isolated from the farm and hatcheries

including their having a higher tolerance to the antivibrio compounds (EtOAc-W3) produced by the isolate W3 when compared with other pathogenic strains of *V. harveyi* (Table 4-1). Briefly, pathogenic strains were prepared to obtain cell pellets as previously described and then each strain was adjusted to 0.4 at a wavelength of 660 nm (OD_{660nm}) ($1.2x10^8$ cfu/ml) in commercial ASW (15 ppt). For the treatment sets; 1, 10 and 100 ml cell suspension were inoculated into 999, 990 and 900 ml commercial ASW to achieve final cell densities at 1×10^5, 1×10^6 and 1×10^7 in each aquarium. Then, 20 healthy post larval shrimps (PL21) were transferred into each aquarium including one set that had no inoculation of pathogen to serve as a control set. The number of 20 post larvae shrimp was used to interpret the results of the toxicity to small animals (Peterson and Talcott, 2006). Survival of post larval shrimp were monitored at 0, 24, 48, 72 and 96 h for investigating the LD_{50} by Probit-analysis (Finney and MLS methods; see appendix B) and this experiment was conducted in triplicate.

4.6 Antivibrio activity of EtOAc-W3 in artificial sea water and natural sea water

The inhibitory effect of EtOAc-W3 against the growth of *V. harveyi* PSU 2015 was investigated in ASW (Than et al. 2004) and also in natural sea water (in order to evaluate the possible use of the extract in the field). Only the strain PSU 2015 was chosen in this experiment due to it causing a higher mortality to post larvae shrimp in a hatchery when compared with other strains and it also had the lowest LD_{50} values at various exposure times (Table 4-2). The EtOAc-W3 was prepared in sterile ASW at a concentration of 4,500 μg/ml and 20 μl of the test solution was transferred into each well of a 96 well plate and followed by 80 μl of ASW. Then a 100 μl cell suspension was transferred into each well to achieve 450 μg/ml (MIC, Table 4-1) of the test compound and 6.0×10^6 cfu/ml of the pathogen. This cell density was designed after evaluating the results in Table 4-2. The cell suspension was prepared as previously described; however, in this work the cell pellet was washed twice with 0.85% NaCl. No plasmoptysis of vibrio cells was observed as viable cells show no significant differences ($p > 0.05$) after being washed twice with

0.85% NaCl when compared with no washing and being washing with ASW and natural sea water (our preliminary work).

The well plate was incubated at 30°C and bacterial growth in each well was measured at OD_{660nm} by a microplate reader. Any wells that showed no visible growth were used to check for survivor cells by streaking onto plates of TSA containing 1.5% NaCl. This experiment also examined for activity at various pH values and at 0, 12, 24, 36, 48, 60 and 72 h. The biotic control contained 100 µl of *V. harveyi* PSU 2015 and 100 µl of ASW to assess if the culture could survive in ASW while the abiotic control consisted of 20 µl of test solution (test compound, 450 µg/ml) and 180 µl of ASW to help to detect any antivibrio activity of the EtOAc-W3. The inhibitory effect of EtOAc-W3 against the growth of *V. harveyi* PSU2015 in natural sea water was also conducted using the same protocol but with sterile natural sea water instead of sterile ASW.

4.7 Assessment of antivibrio activity and shrimp toxicity of EtOAc-W3

EtOAc-W3 prepared in sterile commercial ASW at a concentration of 450 µg/ml was used as a stock solution. Preliminary results had shown that EtOAc - W3 at 450 µg/ml (MIC) was toxic to post larval shrimp so that the concentration of the test extract was diluted to obtain a MIC/10 of 45 µg/ml. There were 4 sets, designed to assess the antivibrio activity and shrimp toxicity of EtOAc-W3 as follows: challenge set or inoculation set (200 ml pathogen suspension + 200 ml commercial ASW, treatment set (40 ml EtOAc-W3, 45 µg/ml + 160 ml commercial ASW + 200 ml pathogen suspension), native control (only 400 ml commercial ASW) and EtOAc-W3 control (40 ml EtOAc-W3 + 360 ml commercial ASW). A cell suspension of *V. harveyi* PSU 2015 was prepared in the commercial ASW that is normally used for shrimp farming. A 200 ml cell suspension containing 1.2×10^7 cfu/ml was transferred to each aquarium of challenge and treatment sets to make a final cell concentration of 6.0×10^6 cfu/ml. Non sterile commercial ASW was used in this experiment, except for the stock solution of EtOAc-W3 as previously mentioned.

20 healthy Pacific white shrimp (PL21) were added to each aquarium of each set. The experiment was conducted in triplicate and the following parameters; water temperature, dissolved oxygen (DO), electrical conductivity (EC), pH, salinity, a numbers of living shrimp were monitored every 12 h for 72 h. All water samples were also collected to count viable bacteria using the spread plate method with TCBS agar. The TCBS plates were incubated at 30°C for 24 h and only glowing green-colored colonies were counted as *V. harveyi*. In order to investigate if reduced mortality was concentration dependent, the experiment was repeated with a higher dose set (MIC/5, 90 μg/ml). Results are presented as a mean ± standard deviations from three determinations unless otherwise stated. Statistical analysis using one way ANOVA to analyse statistical differences at a $P < 0.05$ and mean comparisons were performed by the Duncan' s multiple range test.

4.8 Characterization of the antrivibrio compound

The yellow-brown EtOAc extract or EtOAc-W3 was subjected to thin layer chromatography (TLC) on silica gel 60F 254 aluminum sheets (Merck, Germany). The EtOAc-W3 was dissolved in 80% methanol and 1 μl of the sample (4.45 mg/ml) was spotted onto the TLC plate by a capillary tube. Two developing solvents systems were used: system A, methanol/chloroform (2: 8 v/v), system B, methanol/ethyl acetate (1: 9 v/v). After separation the TLC sheets were dried and exposed to UV radiation at 254 nm to detect any spots and their Rf values were calculated. Phenazine methoxysulfate (PMS) was used as an authentic standard. Any visible spots of the sample obtained from the TLC plates were scraped off and dissolved in 10% DMSO to test for antivibrio activity.

Based on the TLC result the yellow-brown extract or EtOAc-W3 was further analyzed as follows. A 20 μl sample of the EtOAc-W3 (0.2 mg/ml) in 80% methanol (MeOH) was analyzed using an analytical HPLC system (LaChrom Elite, Hitachi, Japan) with an Inertsil ODS-3 column (4.6 x 250 mm, GL Sciences, Japan). MeOH:water (70: 30, v/v) was used as an eluent with a flow rate of 0.53 ml/min. Detection was performed at 234 nm using an L-7455 diode array detector (Hitachi,

Japan). Later 1.5 ml of the EtOAc-W3 (6.89 mg/ml) in 80% MeOH was purified by preparative HPLC on an Inertsil ODS-3 column (20 x 250 mm, GL Sciences, Japan) using a Waters 600 controller (Waters, USA) equipped with Waters 616 pump (Waters, USA) and Waters 486 Tunable Absorbance Detector (Waters, USA). MeOH: water (70: 30) was used as an eluent with a flow rate of 10 ml/min. The purified fraction was subjected to FAB-MS (fast atom bombardment mass spectrometry) and ^{1}H-NMR (nuclear magnetic resonance spectroscopy) analyses. FAB-MS analysis was performed on a JMS-SX102A mass spectrometer (JEOL, Japan) using 4-nitrobenzyl alcohol as a matrix. ^{1}H-NMR analysis was performed on a Varian Unity INOVA AS600 (600 MHz, Varian, USA) in CD_3OD at 30°C.

Results

4.1 Solvent extraction of the culture filtrate of *Pseudomonas* sp. W3

Antivibrio compounds obtained from the different organic solvent extractions are presented in Table 4-1. The color of the chloroform extract was green, whereas that of the EtOAc extract was brown. In contrast, the freeze dried sample was white. However, a crude yellow-brown extract was obtained by extracting a culture filtrate with EtOAc after adjusting the pH to 2, this was named EtOAc-W3. Based on the initial dry weight of 5 g of a freeze dried sample (162.5 ml culture filtrate) this produced 13.35 mg of EtOAc-W3 (see appendix B). Thus the yield of the yellow-brown EtOAc extract was 2.67 mg/g and equivalent to 82.15 mg/l of culture filtrate. Purification of the crude EtOAc extract (13.35 mg) by preparative HPLC yielded 0.45 mg of a yellow powder (2.77 mg/l of culture filtrate). The purified fraction was subjected to FAB-MS and ^{1}H-NMR analyses.

Table4-1 The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of antivibrio compounds produced by *Pseudomonas* sp. W3

V. harveyi strain	Antivibrio compound (Color)				
	MIC (mg/ml)				MBC (mg/ml)
	Freeze dried sample (white)	Chloroform extract (green)	EtOAc extract (brown)	EtOAc-W3* (yellow-brown)	EtOAc-W3* (yellow-brown)
AAHRC 1	100	15	20	0.450	0.900
AAHRC 2	na	20	na	0.450	0.900
PSU 2015	100	10	20	0.450	0.900
PSU 3280	nd	nd	nd	0.450	0.450
PSU 3284	nd	nd	nd	0.450	0.900
PSU 3289	nd	nd	nd	0.450	0.900
PSU 3292	nd	nd	nd	0.450	0.900
PSU 3293	nd	nd	nd	0.450	0.900
PSU 3295	nd	nd	nd	0.450	0.450
PSU 3297	nd	nd	nd	0.450	0.450
PSU 3299	nd	nd	nd	0.450	0.450
PSU 3300	nd	nd	nd	0.450	0.900
PSU 3302	nd	nd	nd	0.450	0.450
PSU 3304	nd	nd	nd	0.450	0.450
PSU 3306	nd	nd	nd	0.450	0.450
PSU 3311	nd	nd	nd	0.225	0.450
PSU 3314	nd	nd	nd	0.450	0.450
PSU 3316	nd	nd	nd	0.450	0.450

Maximum concentrations used were 20 and 100 mg/l for the chloroform/EtOAc extracts and freeze dried sample, respectively. *Culture filtrate was adjusted to pH 2 before extraction with EtOAc. 10% DMSO without EtOAc-W3 served as a negative control. Nalidixic acid was used as a positive control and its MIC and MBC values (μg/ml) against the target organisms were in a range of 0.780-3.125 (2.343±1.354) and 0.780-6.500 (4.593±3.302). na = not applicable, nd = not determined

4.2 Antivibrio activity of extracts of the culture filtrate of *Pseudomonas* sp. W3

The antivibrio activities of the crude extracts and freeze dried sample are shown in Table 4-1. The chloroform extract had a higher inhibitory effect against the growth of strains of *V. harveyi* tested (AAHRC 1, AAHRC 2 and PSU 2015) (MIC values, 10-20 mg/ml) than that found for the brown EtOAc extract (MIC values; 20mg/ml to not applicable). The antivibrio activities of all extracted compounds were about 5-10 times higher than for the freeze dried sample.

The antivibrio activity of the yellow-brown EtOAc extract (EtOAc-W3) against the growth of all strains tested (18 strains) was determined and gave MIC and MBC values of 225-450 μg/ml and 450-900 μg/ml, respectively (Table 4-1). As the ratio of MBC to MIC of the EtOAc-W3 was only 2, it shows that its action at the high concentrations of 450 or 900 μg/ml was bactericidal because the MBC/MIC was <4 (NCLLS 2003). The control antibiotic used in this study (nalidixic acid) showed a better ability to control all strains of shrimp pathogens than that found for the EtOAc-W3 (Table 4-1). MIC values of nalidixic acid against all the tested strains of *V. harveyi* were in a range of 0.780-3.125 μg/ml while its MBC values were between 0.780-6.500 μg/ml (Table 4-1).

4.3 Pathogenicity of strains *Vibrio harveyi* to post larval shrimp

None of post larval shrimps died in a control set during the 96 h of testing time. In contrast post larval shrimps started to die at 48 h and onwards in all treatment sets that were challenged with shrimp pathogenic strains at varying cell densities, although no shrimp died at 24 h. However, other gross features of the diseased shrimp; weakness, swimming, disorders and loss of appetite, eventually leading to death was observed in roughly 50% of shrimp in treatment with infectious doses at 10^6 and 10^7 cfu/ml with all strains tested. Among the 3 strains of shrimp pathogen *V. harveyi* tested, it was found that strain PSU 2015 was the most virulent strain due to its lowest LD_{50} at 48, 72 and 96 h were $1.1x10^7$, $5.5x10^6$ and $5.1x10^6$ (Table 4-2). In contrast LD_{50} of strains AAHRC1 and AAHRC2 at 72 h were $2.4x10^7$

and $1.2x10^7$, respectively (see details of LD_{50} in Table 4-2). *V. harveyi* PSU 2015 was chosen for further studies due to this strain causing a higher mortality to postlarval shrimp in the hatchery when compared with other strains and also from the results obtained in this study as previously described.

Table 4-2 Pathogenicity of virulent strains *Vibrio harveyi* to post larvae Pacific white shrimp (PL21) infected by different cell densities.

Time (h)*	Lethal Dose 50% (LD_{50}; cfu/ml)		
	PSU 2015	AAHRC 1	AAHRC 2
48	1.1×10^7	2.8×10^7	3.4×10^7
72	5.5×10^6	2.4×10^7	1.7×10^7
96	5.1×10^6	2.4×10^7	1.8×10^7

* No post larvae shrimp died at a 24 h of exposure time

4.4 Antivibrio activity of EtOAc-W3 in artificial sea water and natural sea water

The pH values in a biotic control (without addition of the test extract), abiotic control (without addition of the pathogen) and a test set (EtOAc-W3, MIC value, 450 μg/ml) were almost identical over the testing times from 0 to 72 h (Table 4-3). All pH values in the sets of ASW and natural sea water were alkaline, except in the abiotic control over a 36 h period (Table 4-3). After 72 h the pH values of the ASW sets had fallen from 8.0 to 7.2, with an exception of a pH of 7.0 in the abiotic control, whereas that in the natural sea water had increased from 7.8 to 8.3 in both controls and the treatment. However, all wells that contained EtOAc-W3 at a concentration of 450 μg/ml had clear solutions and thus the solution in each well was streaked onto plates of TSA containing 1.5% NaCl. The EtOAc-W3 at the MIC value in ASW completely inhibited *V. harveyi* PSU 2015 as no growth was observed (Table 4-3). In contrast, the same concentration of the EtOAc-W3 in natural sea water gave only a partial inhibition of *V. harveyi* PSU 2015 due to bacterial growth occurring 48 h after being streaked onto agar plates whereas the positive control grew after 24 h.

Table 4-3 Inhibitory effect of the yellow-brown EtOAc extract (EtOAc-W3) (MIC, 450 μg/ml) against growth of *V. harveyi* PSU 2015 in Artificial Sea Water (ASW) and natural sea water

Time (h)	Artificial Sea Water			Natural Sea Water		
	Biotic control	Abiotic control	EtOAc-W3	Biotic control	Abiotic control	EtOAc-W3
0	++ (7.80)	– (7.70)	– (8.00)	++ (7.90)	– (7.80)	+ (7.80)
12	++ (7.70)	– (7.50)	– (7.80)	++ (8.00)	– (7.90)	+ (7.90)
24	++ (7.30)	– (7.20)	– (7.70)	++ (8.30)	– (8.20)	+ (8.30)
36	++ (7.30)	– (6.90)	– (7.60)	++ (8.20)	– (8.20)	+ (8.20)
48	++ (7.40)	– (7.00)	– (7.60)	++ (8.30)	– (8.20)	+ (8.20)
60	++ (7.40)	– (7.10)	– (7.40)	++ (8.20)	– (8.30)	+ (8.30)
72	++ (7.30)	– (7.00)	– (7.20)	++ (8.30)	– (8.30)	+ (8.30)

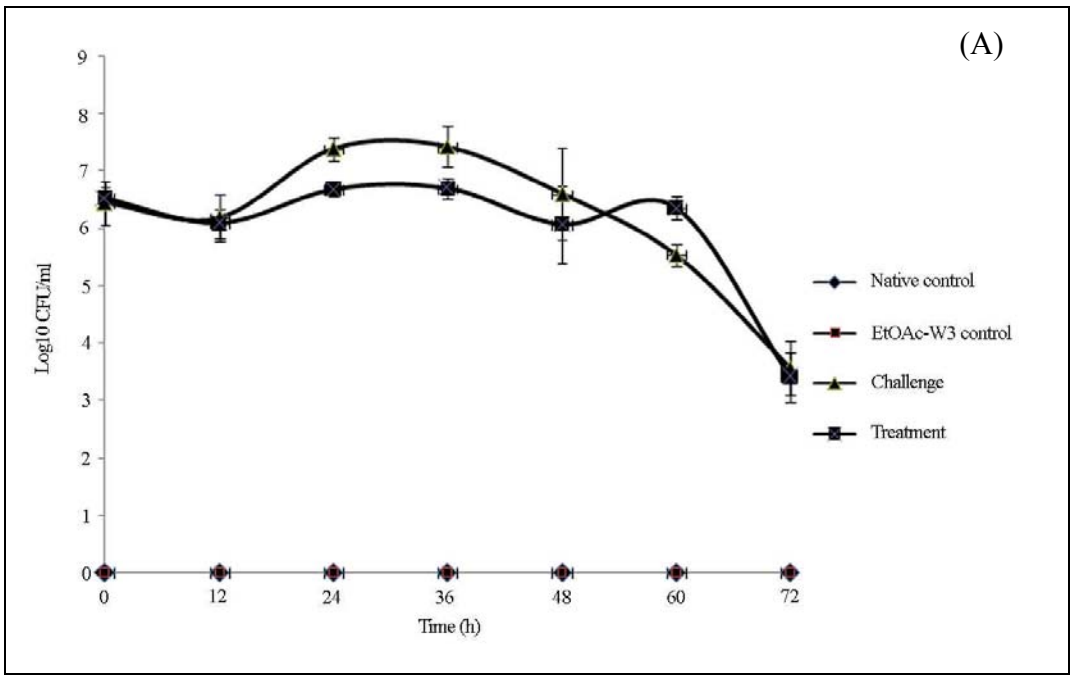
+ = Growth on TSA containing 1.5% NaCl after 48 h incubation, ++ = Growth on TSA containing 1.5% NaCl after 24 h incubation, - = No growth, Number in parenthesis is the pH value. The biotic control consisted of *V. harveyi* PSU2015 (6.0 x 10^6 cfu/ml) and ASW/natural sea water. The abiotic control consisted of crude extract at 450 μg/ml and ASW/natural sea water.

4.5 Assessment of antivibrio activity and shrimp toxicity of EtOAc-W3

Two experiments were conducted at different times to assess the antivibrio activity and shrimp toxicity of the EtOAc-W3, a low dose of 45 μg/ml (MIC/10,) and a high dose of 90 μg/ml (MIC/5). The physicochemical properties of the water column in aquaria that were used to test the safety of EtOAc-W3 at both concentrations on the post larvae of shrimp and also antivibrio activity are shown in Table 4-4. There was no significant difference found for any of the monitored parameters in each treatment and both control sets (temperature, DO, pH, EC and salinity).

The inhibitory effect of EtOAc-W3 in a low dose set against growth of *V. harveyi* PSU 2015 is presented in Figure 4-1A. As expected, *V. harveyi* was not detected in any water samples collected from either the EtOAc-W3 control and native control sets over testing time of 72 h. On the other hand, *V. harveyi* PSU 2015 was present in all samples of the challenge and the treatment sets up to 72 h with some differences (0.7-0.8 log unit) between them. Up to 48 h the counts in the challenge set counts were moderately higher but then decreased at a faster rate than in the treatment set. There was a little evidence for an antibacterial action of EtOAc-W3 under these conditions or in the high dose set. In the case of the high dose set a similar result was found, except that the cell numbers in the treatment set remained high after 72 h (Figure 4-2A).

Effects of EtOAc-W3 in the low dose set and the high dose set on post larval shrimps and *V. harveyi* PSU 2015 are shown in Figure 4-1B and Figure 4-2B. Both experiments showed that only a few postlarval shrimp died (maximum <5% at 72 h) in the control sets (native and EtAOc-W3) with no significant difference between them. The highest postlarval shrimps mortality was found in the challenge sets and the treatment sets. In the low dose set more than 50% and 80% of the postlarval shrimps died in the challenge set after 48 h and 72 h, respectively. In the treatment set, 63% of the shrimps were dead at 72 h. When the high dose set was tested, about 80% of the shrimps were dead at 72 h in the challenge set but only 23% died in the treatment set (Figure 4-2B). The protection was therefore concentration dependent.



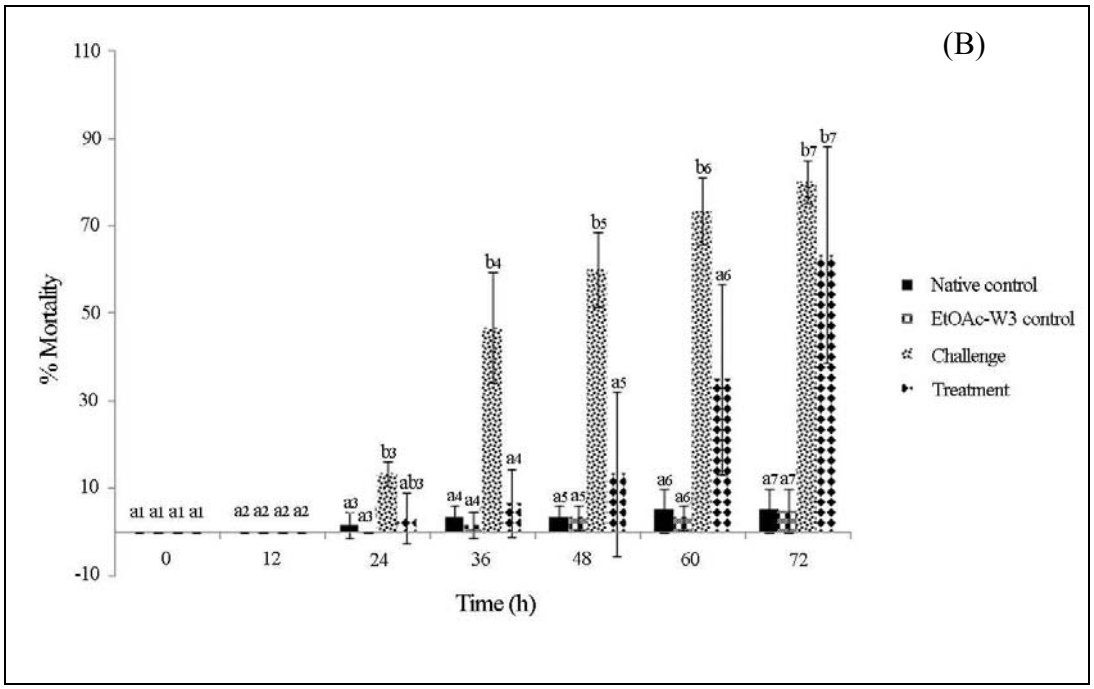
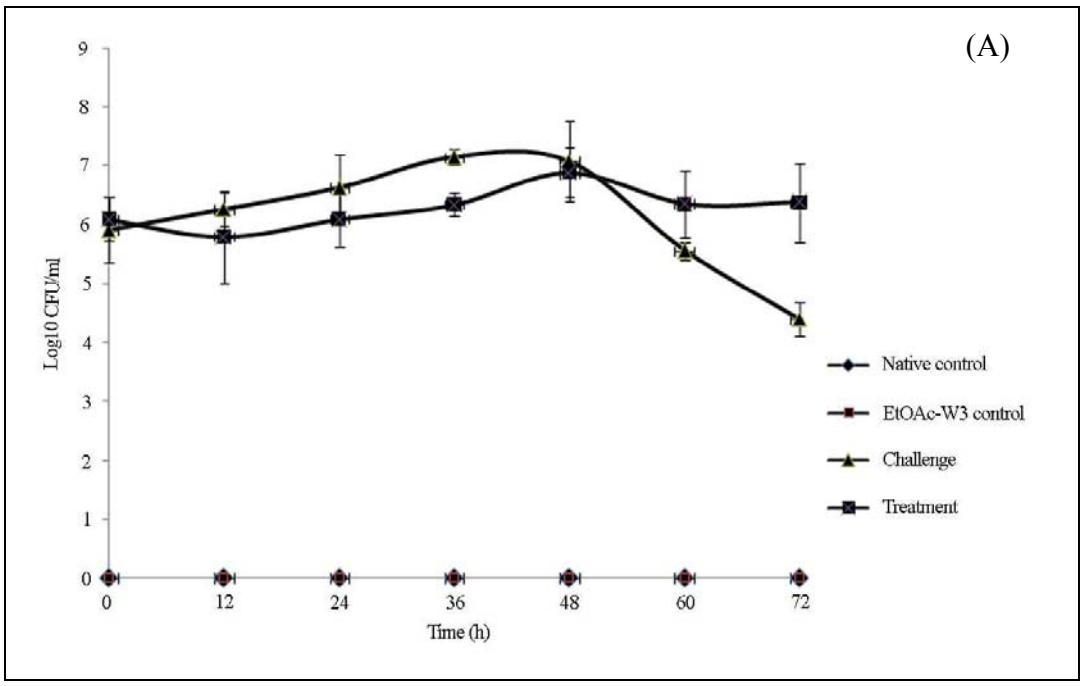


Figure 4-1 Effects of the EtOAc-W3 from *Pseudomonas* sp. W3 in a low dose set (MIC/10, 45 µg/ml) on (A) population change of *V. harveyi* PSU 2015 and (B) mortality of Pacific white shrimp (PL21). Each value is a mean of triplicate measurements ± standard deviation.



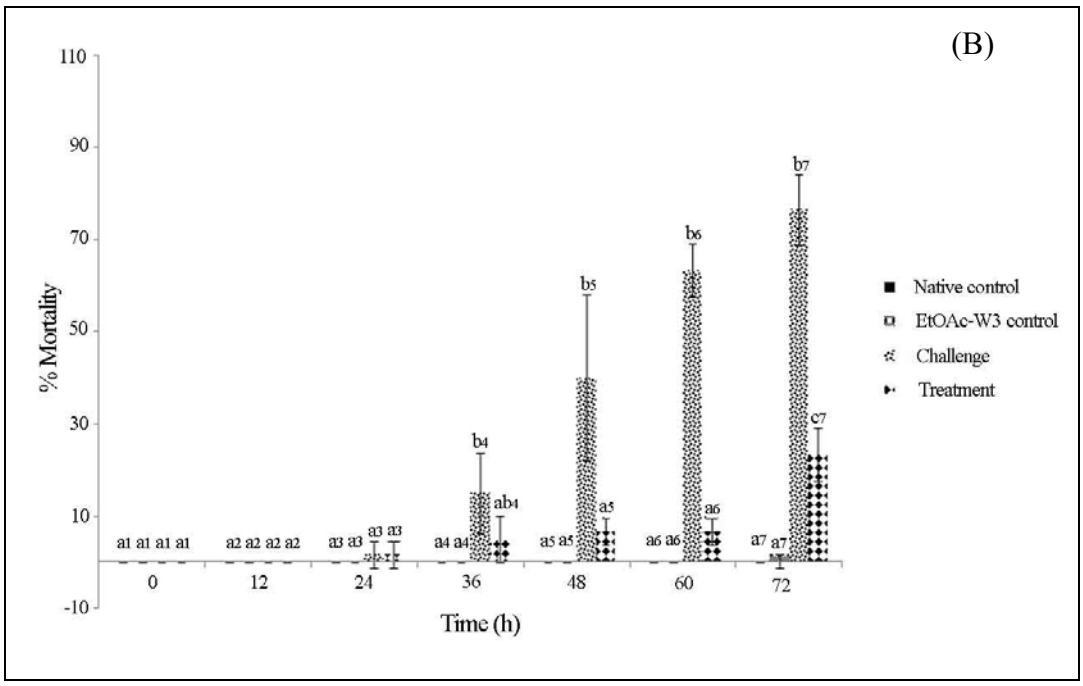


Fig. 4-2 Effects of the EtOAc-W3 from *Pseudomonas* sp. W3 in a high dose set (MIC/5, 90 μg/ml) on (A) population change of *V. harveyi* PSU 2015 and (B) mortality of Pacific white shrimp (PL21). Each value is a mean of triplicate measurements ± standard deviation.

Table 4-4 Physicochemical properties of the water column in aquaria of each set for testing shrimp toxicity and antivibrio activity of EtOAc-W3 produced by *Pseudomonas* sp.W3 at a low dose (MIC/10, 45 μg/ml) and a high dose (MIC/5, 90 μg/ml)

Parameter	**Native control set**	**EtOAc-W3 control set**	**Challenge set**	**Treatment set**
Low dose				
Temperature (°C)	25.77 ± 1.09^{a1}	25.40 ± 1.13^{a1}	25.57 ± 1.25^{a1}	25.58 ± 1.25^{a1}
DO (mg/l)	6.56 ± 0.39^{a2}	6.52 ± 0.26^{a2}	6.33 ± 0.21^{a2}	6.30 ± 0.26^{a2}
pH	7.76 ± 0.07^{a3}	7.73 ± 0.08^{a3}	7.75 ± 0.08^{a3}	7.71 ± 0.06^{a3}
EC (mS/cm)	19.46 ± 5.69^{a4}	18.03 ± 5.30^{a4}	22.80 ± 4.11^{a4}	20.33 ± 5.76^{a4}
Salinity (ppt)	16.95 ± 1.56^{a5}	16.86 ± 1.46^{a5}	16.86 ± 1.46^{a5}	16.86 ± 1.46^{a5}
High dose				
Temperature (°C)	25.66 ± 1.37^{a6}	25.31 ± 1.33^{a6}	25.34 ± 1.30^{a6}	25.44 ± 1.19^{a6}
DO (mg/l)	5.87 ± 0.48^{a7}	5.93 ± 0.60^{a7}	5.91 ± 0.52^{a7}	5.99 ± 0.46^{a7}
pH	7.72 ± 0.07^{a8}	7.72 ± 0.09^{a8}	7.70 ± 0.09^{a8}	7.67 ± 0.10^{a8}
EC (mS/cm)	23.62 ± 5.50^{a9}	24.84 ± 4.00^{a9}	20.59 ± 8.55^{a9}	23.86 ± 4.54^{a9}
Salinity (ppt)	19.71 ± 1.80^{a10}	19.71 ± 1.80^{a10}	19.62 ± 1.67^{a10}	19.67 ± 1.73^{a10}

Each value is a mean value ± standard deviation from 21 observations (3 replicates x 7 sampling times). Lowercase letters with number i.e. ^{a1} in each row indicates no significant difference when the same symbol was used ($p > 0.05$).

4.6 Characterization of the antivibrio compound

Two solvent systems used for analyzing EtOAc-W3 by TLC. In system A, there was one spot for PMS (phenazine methoxysulfate) with an Rf value of 0.90, whereas the EtOAc-W3 had an Rf value of 0.86 (Figure 4-3). In system B, the Rf values were 0.81 and 0.89 for PSM while the Rf value for the EtOAc-W3 was 0.64 (Figure 4-3). Spots for the EtOAc-W3 after being separated by either systems A or B showed antivibrio activity to *V. harveyi* PSU 2015 (data not shown). Based on results, system B seemed to be the best choice for a solvent system that would separate and help to purify EtOAc-W3 by TLC.

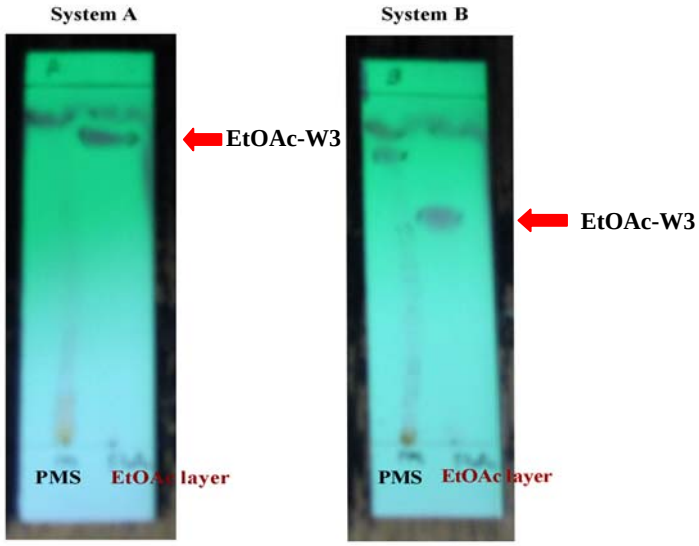


Figure 4-3 Thin layer chromatography of PMS and EtOAc-W3 in system A and system B. Standard, PMS = Phenazine methoxysulfate

The yellow-brown extract (EtAOc-W3) was analyzed by HPLC analysis. The major peak with a retention time of 30.0 min (Figure 4-4A) showed an intensive absorption maximum at 234 nm (Figure 4-4B). EtAOc-W3 was purified by

preparative HPLC to obtain the compound corresponding to the major peak of EtOAc and used for structural analysis.

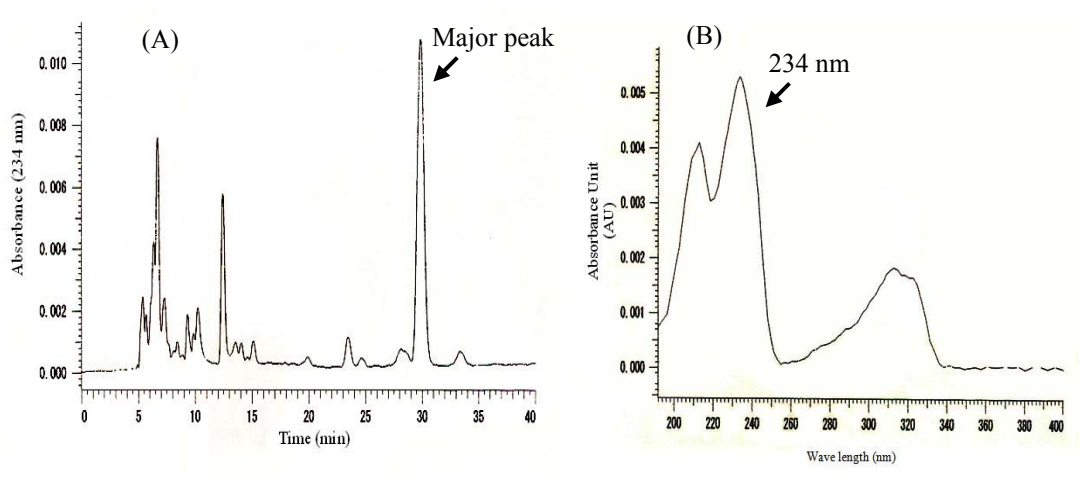


Figure 4-4 HPLC analysis of (A) EtOAc-W3 (detection at 234 nm) and (B) the UV spectrum of the major peak on the HPLC diode array detector.

FAB-MS of the major compound of EtOAc-W3 showed an $[M + H]^+$ peak at *m/z* 244 and $[M - H]^-$ peak at *m/z* 242 in positive and negative ion mode, respectively, to indicate that the molecular weight of the compound was 243. The odd number molecular weight indicates the compound had an odd number of nitrogen atoms. ^{1}H-NMR of the active compound showed the characteristic signals at δ8.35 (1H, dd, *J*=8.1, 1.4 Hz), δ8.19 (1H, br. s),δ7.57 (1H, ddd, *J*=8.4, 7.0, 1.4 Hz),δ7.32 (1H, dd, *J*=8.1, 7.0 Hz),δ7.29 (1H, d, *J*=8.4 Hz), δ6.18 (1H, s), δ2.62 (2H, t, *J*=7.8 Hz), and δ1.73 (2H, quint, *J*=7.6 Hz). In addition, methylene and methyl protons were detected at δ1.6-1.2; 2: 1) and δ0.88 (t, *J*=7.0 Hz), although the number of protons belonging to these signals could not be determined due to overlapping signals from other minor metabolites. From these results, the active compound was supposed to be 2-heptyl-4-quinolone (HHQ) (Figure 4-5) which is a member of the 2-alkyl-4-quinolons reported to be antibacterial metabolites of *Pseudomonas aeruginosa* and acts as a quorum-sensing signaling molecule (Diggle et al. 2007).

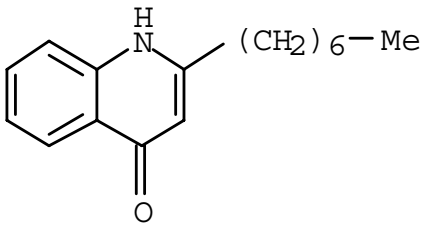


Figure 4-5 The structure of 2-heptyl-4-quinolone (HHQ).

Discussion

4.1 Antivibrio compounds produced by *Pseudomonas* sp. W3

It is well recognized that diverse bioactive compounds are produced by *Pseudomonas* spp. such as pyroles, pseudopeptides, phenazine, quinoline, quinolone, and so on (Isnansetyo and Kamei 2009; Preetha et al. 2009). However, there has been a little work to investigate the ability of these bioactive compounds to inhibit the growth of shrimp pathogenic vibrios even though the use of antibiotics to control disease caused by e.g. *V. harveyi* has been limited by legislation. In addition, shrimp pathogens readily become resistant to conventional antibiotics in aquaculture. Hence, novel bioactive compounds that are environmentally friendly and effective might help to solve the problem and most of the work has been focused on *Pseudomonas* spp. because they are coinhabitants of the water habitat of shrimp (Sakami et al. 2008). Some of the bioactive compounds produced by *Pseudomonas* have small molecular weights and thus can be easily degraded by microbes and therefore create no environmental problems.

In this study, antivibrio compounds produced by *Pseudomonas* sp. W3 were extracted using chloroform and EtOAc. The chloroform extract had a higher antivibrio activity than the brown EtOAc extract carried out at a pH of 7.8 (Table 4-1). This finding in the case of the chloroform extract is consistent with the work of

Preetha et al. (2009) who reported that an antivibrio compound produced by *Pseudomonas* MCCB 102 and MCCB 103 was detected only in the chloroform extract from a neutral pH while no antivibrio activity was found in an EtOAc extract. According to Preetha et al. (2009) the blue colored fraction obtained from a chloroform extract of a culture filtrate of *Pseudomonas* spp. at a neutral pH was N-methyl-1-hydroxyphenazine, a phenazine antibiotic and we suspected that we had a similar compound in our chloroform extract and also in the ethyl acetate extract after adjustment of the pH to 2. As the pH of the culture filtrate was adjusted to 2 and extracted with EtOAc, an antivibrio activity of a yellow-brown extract was obtained that had a 44 fold increase based on MIC (Table 4-1). However, TLC results showed that EtOAc-W3 was not a phenazine derivative (Figure 4-3). In addition, an EtOAc extract produced by *Pseudomonas aeruginosa* that strongly inhibited the growth of *Microsystis aeruginosa* was reported by Ren et al. (2010).

It is not surprising that the inhibitory effect of the antibiotic used as a control (nalidixic acid) against the shrimp pathogens tested was higher than that the EtOAc-W3 (Table 4-1) and our strains tested were more sensitive to this antibiotic (MIC, 0.780-3.125 μg/ml) when compared with *V. harveyi* M3 (MIC, 6.25 μg/ml) (Abraham 2004). However, the use of antibiotics is not environmentally friendly as previously mentioned, and also shrimp pathogenic strains that are resistant to nalidixic acid have been reported. Resistance to quinolones in the form of nalidixic acid has been observed in target pathogens because the quinolones act against DNA-metabolism and then bacteria try to repair and that can lead to the formation of resistant mutants (Gräslund and Bengtsson, 2001). Hence, the use of new antivibrio compounds needs to be explored to combat luminous vibriosis.

The main compound in EtOAc-W3 (Fig. 4-4A) was supposed to be 2-heptyl-4-quinolone (HHQ) (Figure 4-5). It has long been known that HHQ is a small molecule produced by *Pseudomonas aeruginosa* that is released into the environment and then assimilated by neighboring cells and converting to PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone (Diggle et al, 2006; Diggle et al. 2007). PQS acts as a quorum sensing signal that regulates numerous virulence genes including those involved in the production of rhamnolipids, pyocyanin (a blue-green phenazine

pigment) and iron-scavenging compounds (Diggle et al. 2007). PQS but not HHQ is able to disrupt the chrome azurol S-iron complex by chelating iron (III) (Diggle et al. 2007). However, *Pseudomonas* sp. W3 may have lost its ability to convert HHQ to PQS as it did not produce siderophores for iron chelation (data not shown) and thus could explain why a large quantity of HHQ is released into the culture medium (82.15 mg/l).

4.2 Assessment antivibrio activity and shrimp toxicity of EtOAc-W3

Results in Tables 4-1 and 4-3 demonstrate the anti *V. harveyi* PSU 2015 activities of the yellow- brown EtOAc extract (EtOAc-W3) in TSB, ASW and natural sea water. It may not be surprising that the MIC value of EtOAc-W3 against vibrio in ASW was higher than that found in natural sea water (Table 4-3). Sea water has much inorganic matter, particularly the ions of salts such as Cl^-, SO_4^{2-}, Na^+, Mg^{2+}, Ca^{2+} and so on (Cotruvo, 2005) and some dissolved organic compounds (DOC) that are very important for biological life processes (Ogawa and Tanoue 2003). In addition, the composition of natural sea water may provide an alkaline system for supporting growth of *V. harveyi* (Table 4-3) as this pathogen is a marine bacterium that prefers to live in a slightly alkaline to alkaline conditions. In contrast, sterile ASW (Than et al. 2004) has no DOC. Although the MIC values for EtOAc-W3 in TSB, ASW and natural sea water, were each 450 μg/ml (MIC) only cells from natural sea water and TSB survived on TSA plates. We suggest that this might be due to the presence of organic compounds that protected the cells from lysis by the test extract. However, the pH might have had an effect because after 72 h the pH values of the ASW in the sets of treatment, biotic and abiotic were 7.2, 7.3 and 7.0 while that of all sets of the natural sea water was 8.3. The results indicate that the ASW lacks a system to maintain alkaline conditions for supporting growth of the pathogen.

The toxicity of EtOAc-W3 to postlarval shrimps was investigated at concentrations of 45 μg/ml (MIC/10) for a low dose set and 90 μg/ml (MIC/5) for a high dose set. The following physicochemical parameters; temperature, pH, DO, EC and salinity were measured for all treatments and control conditions throughout the

experiment (Table 4-4). Results showed the conditions remained almost identical and were always in a suitable range for shrimp growth and survival (Kannapiran et al. 2009; Liu et al. 2000; McGraw et al. 2001). However, in this study the salinity was set a little lower (15 ppt) due to the CP Group growing shrimp larvae following a real situation in the shrimp pond during a rainy season. In general, postlarval shrimps can tolerate a wide range of salinity so no effect was expected on their survival or growth. Therefore, it can be claimed that any observed differences were due to the presence of EtOAc-W3.

Throughout the testing time of the two sets of experiments the numbers of postlarval shrimp that died in both control sets was very small (<5%) (Figure 4-1B and Figure 4-2B). The difference in counts of *V. harveyi* PSU 2015 between the test and challenge was no more than about 1 log cfu/ml, during 24-48 h for the low dose set (Figure 4-1A) and 12-36 h for the high dose set (Figure 4-2A). Thus at these low concentrations of EtOAc-W3 vibrio cells were not completely killed. Results in this study, show that LD_{50} values of the strain PSU 2015 at 48-96 h were between 10^6-10^7 cfu/ml (Table 4-2) and this was similar to the results of Robertson et al. (1998) and Aguirre-Guzman et al. (2001) who reported that infection of shrimp larvae with *V. harveyi* at 10^5-10^7 cfu/ml caused larval disease and shrimp death and thus postlarval shrimps died in the challenge and treatment sets caused by the shrimp pathogen, *V. harveyi* PSU 2015 (6.0×10^6 cfu/ml). However, there was a significant reduce shrimp mortality in the presence of EtOAc-W3, and the reduction was concentration dependent, as only 23% died in the high dose set compared to 63% in the low dose set. Abraham (2006) reported that Indian white shrimp larvae infected by *V. harveyi* at 10^6 cfu/ml had a cumulative mortality rate of 100% after 72 h. *V. harveyi* is a serious pathogen for a wide range of marine animals, particularly shrimp as this bacterium attaches to host cell, and damages the hepatopancreas and the digestive system of the shrimp (Austin and Zhang 2006). Based on above results the antivibrio compounds produced by *Pseudomonas* sp. W3 that includes 2-heptyl-4-quinolone (HHQ) may be alternative compounds for use to control vibrio infections in shrimp hatcheries.

Conclusions

This study demonstrated that *Pseudomonas* sp.W3 produced antivibrio compounds and one of the compounds is supposed to be HHQ. Furthermore, the crude EtOAc extract was proven to have a potential for use in shrimp hatcheries for controlling *Vibrio harveyi* with no harm to the postlarval shrimps.

Chapter V

The Inhibition of pathogenic bacteria in sea food by *Pseudomonas* sp.W3

Abstract

Pseudomonas sp. W3 produces bioactive compounds that inhibited the growth of gastrointestinal tract pathogenic bacteria. The broad spectrum of bioactive compounds of the strain W3 for those pathogenic bacteria in culture supernatant and culture filtrates (0.45 µm and 0.22 µm pore size cellulose acetate filter) was observed. The culture supernatant of this strain (70 µl) inhibited pathogenic bacteria in the order of *Clostridium perfringens* PSSCM I0030 > *Yersinia Enterocolytica* > *Bacillus cereus* ATCC 11778 > *Vibrio cholera* PSSCMI 0062 > *Listeria monocytogenes* 4553 > *Bacillus cereus* 687 > *Vibrio parahaemolyticus* AAHRC 1 > *Vibrio parahaemolyticus* PSU 1681. A similar result was observed in the 0.45µm culture filtrate that inhibited the growth of *Clostridium perfringens* PSSCMI 0030 > *Bacillus cereus* ATCC 11778 > *Bacillus cereus* 687 > *Vibrio parahaemolyticus* AAHRC 1 > *Vibrio cholera* PSSCMI 0062. In contrast, only *Clostridium perfringens* PSSCMI 0030, *Vibrio parahaemolyticus* AAHRC1 and *Vibrio cholera* PSSCMI 0062 were inhibited by the 0.22 µm culture filtrate. No growth inhibition of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* PSSCMI 0006, *Salmonella typhimurium* PSSCMI 0034 and *Vibrio vulnificus* PSU 3427 was found by the culture supernatant and both culture filtrates.

Keywords: Gram positive bacteria, Gram negative bacteria, Pathogenic bacteria, *Pseudomonas* sp. W3, Culture filtrates, Culture supernatant

Introduction

Foodborne illnesses are a problem of growing in developed and developing countries. The Centers for Disease Control and Preventtion (CDC) estimates that 76 million people a year suffer from food-borne illnesses (CDC, 2005). The great of food poisoning and food-borne diseases can be attributed to contamination of food and drinking water with bacterial toxin and pathogens (Sharif and Al-Malki, 2010). Most common bacterial foodborne pathogens are *Clostridium perfringens*, *Salmonella*, *Escherichia coli* and the other bacteria are *Bacillus cereus*, *Listeria monolytogenes*, *Yersinia enterocolytica* and *Vibrio* spp. The bacteria that produce exotoxins are *Clostridium botulinum*, *Clostridium perfrigens*, *Staphylococcus aureus* and *Bacillus cereus* (CDC, 2005; USDA, 2010). The main cause of food-borne illnesses is bacteria such as *Clostridium botulinum*, *Escherichia coli*, *Salmonella*, *Staphylococcus*, *Vibrio* spp., *Yersinia* spp. and *Bacillus cereus*. These bacteria are normally found in the aquatic ecosystem and the general environment (ICMSF, 1996). The major five microorganisms that cause food poisoning are *Salmonella*, *Campylobacter*, *Clostridium*, *Escherichia coli* O157 and *Listeria*. The most common symptoms are diarrhea, squeamish, exhaustion, vomiting and abdominal pain (Postnote, 2003). The different food-borne diseases have many different symptoms, so there is no one syndrome that is food-borne illness (CDC, 2005).

Seafoods are high on the list of food transmitting disease and are easily to spoilage. Sea foods spoilage is caused by microorganisms, enzymes and chemical action, with bacteria being the major cause of spoilage of most aquatic food production (Böhme et al., 2010). In the spoilage bacteria, there are species that are quite important in sea food spoilage bacteria such as *V. parahaemolyticus* that has been reported responsible for one-fourth of all gastrointertinal pathologies caused by seafood (Flick, 2007), thereby food preservation methods must be ensured that they can control spoilage organisms and pathogens to provide food safety.

Food safety issues associated with aquaculture products differ from region to region and from habitat and vary according to the methods of production, management practices and environmental conditions (Feldhusen, 2000). The pre-harvest step, the

contamination with pathogens from the human or animal may pose a risk since in some cases a very low infective dose is required to cause illness. The high quality foods are desired for consumers. The desires, along with tighter legislation regarding current preservatives, have challenged the food industry and have led to increased research into and use of bioactive compounds and antimicrobial agents to prevent the growth of microorganisms that may cause human disease or food spoilage (McLay et al. 2002; Fitzgerald et al. 2003; Miranda et al., 2010). This study was conducted in order to investigate the broad spectrum of bioactive compounds produced by *Pseudomonas* sp. W3 to inhibit food borne pathogen in sea food industry.

Materials and methods

1. Bacterial strains and cultivation

Pseudomonas sp. W3 has been proven to secrete secondary metabolites, particularly those of a small molecular weight that inhibit vibrios i.e. *V. harveyi* (Rattanachuay et al. 2010). It was grown in Frazier gelatin medium (Frazier and Rupp, 1982) plus 2% NaCl under optimum growth conditions: 30°C on a shaker (Gallenkamp, orbital incubator), shaking speed at 150 rpm, 18 h and the culture broth was used to test the inhibition of gastrointestinal tract pathogenic bacteria.

Food borne pathogens; *V. vunificus* PSU 3427, *V. parahaemolyticus* AAHRC 1 and *V. parahaemolyticus* PSU 1681, *V. cholerae* ATCC 14104, *C. perfringens* ATCC 27057, *S. typhimurium* PSSCMI 0034, *Y. enterocolytica*, *E. coli* ATCC 25922, *L. monocytogenes* No.6, *S. aureus* PSSCMI 0004 and *B. cereus* ATCC 11778 were provided by the Aquatic Animal Help Research Center (AAHRC) and Department of Microbiology, Faculty of Science, Prince of Songkla University. All pathogens were grown in Tryptic Soy Broth (TSB) and shaken at 150 rpm 30°C for 18 h, except *Vibrio* sp. and *Yersinia* sp. were grown in TSB with 1.5% NaCl and shaken at 150 rpm at 30°C for 18 h. The food borne pathogen cultures were tested for their sensitivity to bioactive compounds produced by *Pseudomonas* sp. W3.

2. Preparation of culture supernatant and culture filtrates for testing antibacterial activity.

The culture broth of *Pseudomonas* sp. W3 was centrifuged at 6,000 rpm, for 25 min (SANYO, Harrier 18/80 Refrigerator; U.K.) to remove cells and provide the culture supernatant. The culture supernatant was then filtered through either a 0.45 µm or 0.22 µm pore size cellulose acetate filter to provide the cell-free supernatant (culture filtrate). The inhibition of gastrointestinal tract pathogenic bacteria, bioactive compounds of the strain W3, was separately tested by the agar well diffusion method according to the method of Rattanachuay et al. (2010); previous chapter.

3. The inhibition of gastrointestinal tract pathogenic bacteria by culture supernatant and culture filtrates of *Pseudomonas* sp.W3

The culture supernatant collected from the strain W3 was tested for its inhibition to the gastrointestinal tract pathogenic bacteria by the agar well diffusion method as follows: the target organisms, gastrointestinal tract pathogenic bacteria**,** in TSB and TSB with 1.5% NaCl were individually adjusted to 0.5 McFarland standard (bio Merieux; 69280 Marcy l'Etoile, France; cell densities: 1.5 x 10^8 cell/ml) and then swabbed over the surface of TSA and TSA with 1.5% NaCl plates. Wells (diameter 7 mm) were punched in the TSA plate by a pasture pipette (3 wells/plate). 70 µl of the culture supernatant from *Pseudomonas* sp. W3 was transferred into each well in each plate and the plates were incubated at 30°C for 24 h. The diameter of the inhibition zones was measured using a Vernier caliper.

The cell-free supernatants that filtered through either a 0.45 µm or 0.22 µm pore size cellulose acetate filter were tested for their inhibition to the gastrointestinal tract pathogenic bacteria as the same method of culture supernatant of *Pseudomonas* sp. W3.

Results

1. The inhibition of gastrointestinal tract pathogenic bacteria by culture supernatant of *Pseudomonas* sp.W3

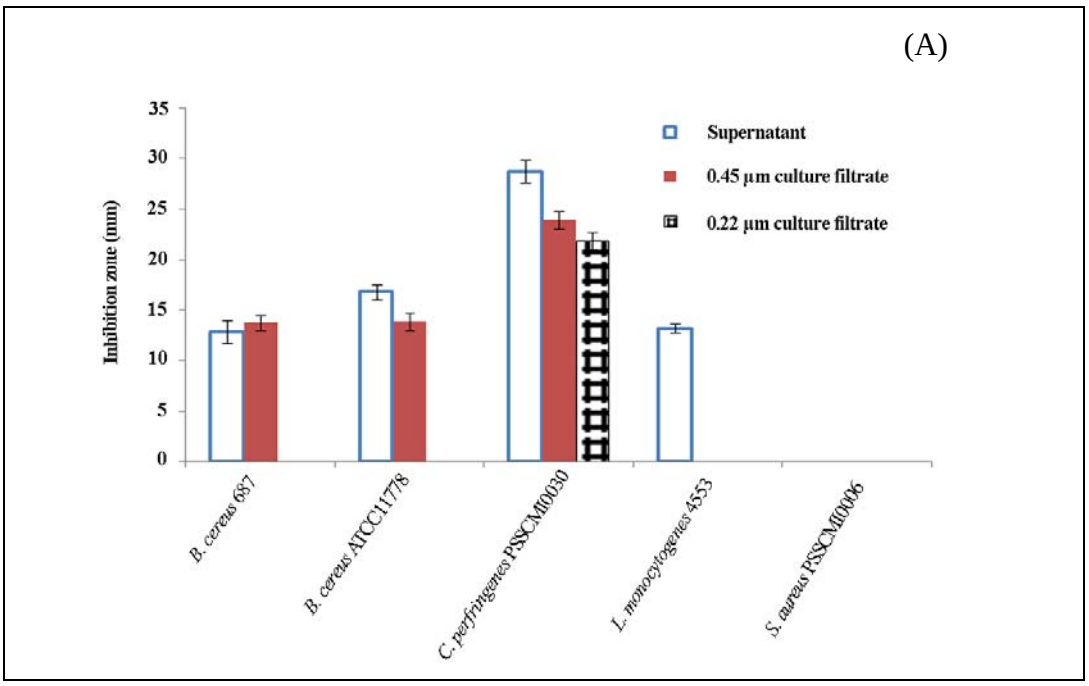
The culture supernatant from *Pseudomonas* sp. W3 (70 µl) inhibited the growth of pathogenic bacteria; *B. cereus* 687, *B. cereus* ATCC11778, *C. perfringens* PSSCMI0030, *L. monocytogenes* 4553, *V. cholera* PSSCMI0062, *V. parahaemolyticus* AAHRC1, *V. parahaemolyticus* PSU1681 and *Y. enterocolytica* with clear zone in a size range of 6.51 ± 0.24 to 28.77 ± 1.15 mm (Figure 5-1). The most sensitive strain was *C. perfringens* PSSCMI0030 with clear zone 28.77 ± 1.15 mm, while *V. parahaemolyticus* PSU1681 was the most tolerant strain with zone size 6.51 ± 0.24 mm. The culture supernatant from *Pseudomonas* sp. W3 could inhibit most pathogenic bacteria tested, although *E. coli* ATCC25922, *S. aureus* PSSCMI0006, *S. typhimurium* PSSCMI0034 and *V. vulnificus* PSU3427 were not inhibited by culture supernatant from *Pseudomonas* sp. W3 (Figure 5-1).

2. The inhibition of gastrointestinal tract pathogenic bacteria by culture filtrates of *Pseudomonas* sp.W3

The culture supernatant from *Pseudomonas* sp. W3 was filtrated through either a 0.45 µm or 0.22 µm pore size cellulose acetate filter to provide the cell-free supernatant, results of broad spectrum for pathogenic bacteria are shown in Figure 5-1. The culture filtrate (0.45 µm pore size) inhibited the growth of *B. cereus* 687, *B. cereus* ATCC11778, *C. perfringens* PSSCMI0030, *V. cholera* PSSCMI0062 and *V. parahaemolyticus* AAHRC1 with a clear zone ranged from 10.92 ± 0.46 to 23.99 ± 0.89 mm. *C. perfringens* PSSCMI0030 was the most sensitive strain (23.99 ± 0.89 mm), while the least sensitive strain was *V. cholera* PSSCMI0062 (Figure 5-1). However, the following pathogenic bacteria; *L. monocytogenes* 4553, *V. parahaemolyticus* PSU1681 and *Y. enterocolytica* were not inhibited by the culture filtrate (0.45 µm pore size).

The culture filtrate (0.22 µm pore size) inhibited the growth of 3 pathogenic bacteria strains; *C. perfringens* PSSCMI0030, *V. cholera* PSSCMI0062 and *V.*

parahaemolyticus AAHRC1. The clear zone sizes are shown in Figure 5-1. The most sensitive strain was *C. perfringens* PSSCMI0030 (21.90 ± 0.88 mm) and the least sensitive was *V. cholera* PSSCMI0062 (9.95 ± 0.59 mm).



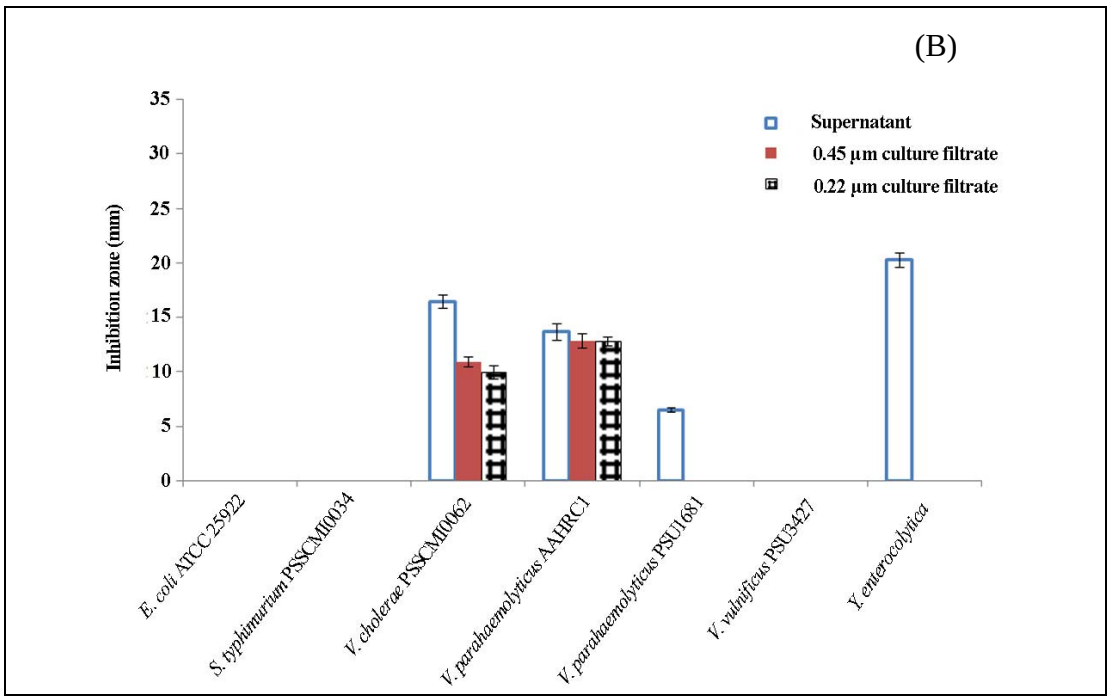


Figure 5-1 Inhibition of pathogenic bacteria by culture supernatant and culture filtrates of *Pseudomonas* sp. W3. A) Gram-positive pathogenic bacteria, B) Gram-negative pathogenic bacteria, each bar represents mean ± standard deviation of nine observations.

Discussion

Pseudomonas spp. can produce bioactive compounds that could be used to inhibit pathogenic bacteria (Gram, 1993; Ha, 2007). For example, phenazine, pyrrolnitrin and pyoluteorin are secondary metabolites that have been investigated for inhibiting a wide range of pathogenic bacteria (Vijayan et al., 2006; Uzair et al., 2006). One of our previous studies proved that *Pseudomonas* sp.W3 produced bioactive compounds with ability to control shrimp pathogens vibrios, particularly *Vibrio harveyi* (Rattanachuay et al., 2010; previous chapter). In this study, bioactive compounds in the culture supernatant and both culture filtrates (0.45 µm and 0.22 µm pore size filters) from *Pseudomoas* sp. W3 contained antibacterial compounds against the growth of the gastrointestinal tract pathogenic bacteria. Bioactive compounds in the culture supernatant from the strain W3 had a board spectrum to control both Gram-positive and Gram-negative pathogenic bacteria (Figure 5-1). However, bioactive compounds produced by this bacterium could not inhibit *S. aureus* PSSCMI0006 and this was differ from previous work by Isnansetyo and Kamei (2009) who reported that the bioactive compound of *P. bromoutilis* (pyrrole) inhibited *S. aureas*, *Diplococcus pneumoniae* and *Streptococcus pyrogenes*. Normally, lysozyme can destroy cell wall of Gram-positive bacteria (Salton, 1958) but some Gram-positive bacteria such as *S. aureus*, the cell wall can resist the action of lysozyme due to O-acetyl groups on carbon-6 of some muramic acid (MA) residues (Bera et al., 2007).

The production of extracellular bioactive compounds from *Pseudomonas* sp. that inhibited Gram-negative bacteria such as *Vibrio* spp. was also reported by Vijayan et al. (2006). They reported that the compounds that produced by *Pseudomonas* PS-120 was able to control varieties of vibrios such as *V. parahaemolyticus* and *V. vulnificus*. However, bioactive compounds produced by the strain W3 could not inhibit *V. vulnificus*, Suggesting that the main bioactive compound of both organisms should be different. One of our previous studies proved that the main compound from the strain W3 obtained by extraction with EtOAc under acidic condition should be HHQ (Rattanachuay et al., 2010). The HHQ is a small molecule and can inhibit vibrios especially *V. harveyi*. In addition, Gram-negative bacteria contain outer membrane that consists of phospholipids and lipopolysaccharides. The lipopolysaccharides in the outer membrane of Gram-negative bacteria can do adsorption and uptake bioactive compounds into the

cell and thus could be responsible for any differences of sensitively that occur between different bacteria (Russell, 2003).

The results in Figure 5-1 show that the culture filtrates contained bioactive compounds that could inhibit some pathogenic bacteria but less than culture supernatant both in variety of pathogens and antibacterial activity. In the present study, the culture supernatant of *Pseudomonas* sp. W3 was filtered with a 0.45 µm or a 0.22 µm pore size filters with the aim to remove cells and any particulate cell debris of bacterium; thereby the culture filtrates, particularly 0.22 µm culture filtrate contained only soluble chemicals and colloidal organic matter. This indicates that some compounds in a form of particulate matter were removed and it made the culture filtrates had less antibacterial activity.

Conclusions

The broad spectrum of bioactive compounds from *Pseudomonas* sp.W3 could inhibit most of Gram-positive and Gram-nagative pathogenic bacteria in seafood. Therefore, these compounds are interested for using against pathogenic bacteria in seafood and prevention seafood spoilage.

Chapter VI

Identification of *Pseudomonas* sp.W3

Abstract

Pseudomonas sp.W3 isolated from a shrimp pond has a great potential to inhibit shrimp pathogens such as *Vibrio harveyi* and the strain identification according to the biochemical and molecular properties was conducted in this study. The biochemical properties of strain W3 was determined by the API 20NE test kit (bioMerieux, France), and the results analyzed via the bioMeriex database indicated that the strain W3 was similar to *P. aeruginosa*. In addition, the analysis of 16S rDNA sequence and phylogenetic relationships were used for identification. The similarity levels of the sequences between *Pseudomonas* sp. W3, *P. aeruginosa* FJ864676, *P. aeruginosa* EU344794 and *P. aeruginosa* Z76651 were between 98.9%-99%. Moreover, Phylogenetic trees, constructed using Neighbor Joining (NJ) and Maximum Parsimony (MP), showed the closed relatedness of *Pseudomonas* sp. W3 to the tested taxa of *P. aeruginosa*. Hence, *Pseudomonas* sp. W3 is identified to be *P. aeruginosa* W3.

Keywords: *Pseudomona*s sp. W3, *Pseudomonas aeruginosa*, 16S rDNA, phylogenetic tree, Neighbor Joining, Maximum Parsimony

Introduction

Pseudomonas is a Gram-negative, non-spore forming and aerobic rod belonging to a member of Pseudomonadaceae family. *Pseudomonas* spp. can motile by one or several polar flagella and most species do not require organic growth factors. However, some species are facultative anaerobes with nitrate as a final electron acceptor. *Pseudomonas* spp. dissimilate sugars through the Entner-Doudoroff pathway. *Pseudomonas* spp. are common inhabitants of the aquatic environments (fresh water, brackish water, and sea water) as well as shrimp culture

ponds (Otta et al., 1999) and they are commonly associated with gills, skin and intestinal tract of live fish (Daly et al., 1973; Yagoub, 2009). *Pseudomonas* spp. produce two types of soluble pigments, the fluorescent pigment (pyoverdin) and the blue pigment (pyocyanin) (Krieg and Holt, 1984). Some *Pseudomonas* spp. are pathogens of humans, animals and plants, while some species are benefits to environments for applications. For example, denitrifying pseudomonad reduces nitrate to nitrous oxide (N_2O) and further reduce nitrous oxide (N_2O) to nitrogen gas (N_2) (Garrity et al., 2005). *P. denitrificans* has been used to produce vitamin B12 for commercial production (Crueger and Crueger, 1989). Besides, *Pseudomonas* spp. can degrade many xenobiotic compounds (Assinder et al., 1990), while some *Pseudomonas* spp. can produce bioactive compounds that can inhibit Gram-positive and Gram-negative bacteria (Nair and Simidu, 1987; Uzair et al., 2006). Therefore, *Pseudomomas* spp. become a new source of antibiotics and bioactive compounds (Burkholder et al., 1966; Laue et al., 2000; Chythanya et al., 2002; Uzair et al., 2006) such as phenazine from *P. aeruginosa* (Mavrodi et al., 2001; Parsons et al., 2004), phenazine-1-carbox-amide from *P. aureofaciens* (Woeng et al., 1998; Siunova et al., 2002). In addition, *Pseudomonas* sp. W3 was isolated from shrimp pond and it has potential as a biocontrol agent against shrimp pathogens, vibrios, particularly *Vibrio hareyi* (Rattanachuay et al., 2010; previous chapter).

Conventional method based on biochemical tests and physiological properties has been used to study the characterization and identification of bacteria. Recently, the molecular technique using 16S rRNA genes has been used for classification and identification of the bacteria and it is able to show definitive name of Archea, Bacteria and Eukarya (Woese et al 1990; Attitalla et al 2006). The molecular techniques and taxonomy open ways to design highly specific PCR protocols, especially for detection of 16S (small-subunit) rRNA genes (Weidner et al., 1996). rRNA is highly conserved and essential for the survival of living organisms (Hirano and Upper, 1983). Phylogenetic analysis based on the 16S rRNA gene became well established as a standard method for the identification of bacteria at the family, genera and species levels (Woese, 1987). In our previous study, the bacterial strain W3 was identified using biochemical tests and partial 16S rDNA sequence. The

results showed that the bacterial strain W3 was a *Pseudomonas* sp. (Rattanachuay et al., 2007). In order to know a species name for the strain W3 in this study, the conventional API 20NE test kit (BioMerieux, France) and full length of 16S rDNA analysis were used for identification and clarifying the classification of the *Pseudomonas* sp.W3. The evolution relationship between the strain W3 and the other *Pseudomonas* sp. was studied by phylogenetic tree analysis.

Materials and methods

6.1 Bacterial strains and growth media

Pseudomonas sp. W3 was isolated using Frazier gelatin medium (FGM) (Frazier and Rupp, 1982) from a water sample collected from an intensive shrimp cultivation pond in Pattanee province, Thailand. The pure culture of *Pseudomonas* sp. W3 was grown on FGM agar while *Pseudomonas aeruginosa* PSSCMI 0048 and *Pseudomonas aeruginosa* PSSCMI 0049 were grown on Nutrient agar (NA) for identification by a commercial test kit (API 20NE). Both strains were provided by Department of Microbiology, Faculty of Science, Prince of Songkla University. For 16S rDNA sequence method, the pure culture of *Pseudomonas* sp.W3 was cultured in Luria-Bertani broth (LB broth).

6.2 Identification of *Pseudomonas* sp.W3 by using conventional API 20NE test kit (BioMerieux, France)

Pseudomonas sp. W3, *P. aeruginosa* PSSCMI 0048 and *P. aeruginosa* PSSCMI 0049 were grown in media as previously described and incubated at 30°C for 24 h. Each inoculum was adjusted to 0.5 McFarland standard (1.5×10^8 cells/ml) in API AUX Medium and then 120 µl of cell suspension was transferred into cupules of API 20NE test. The cupules were incubated at 30°C for 24 h, and then the results were read and recorded in a record sheet. The results were analyzed with a software

for conventional API 20NE to compare each bacterium with the standard bacteria in API database (bioMerieux database).

6.3 Molecular identification of *Pseudomonas* sp.W3 by 16S rDNA sequence analysis

6.3.1 Genomic DNA extraction

DNA extraction was carried out following the Cetytrimethyl Ammonium Bromide (CTAB) method (Sambrook et al., 1989). *Pseudomonas* sp.W3 was cultured in 5 ml of Luria-Bertani broth (LB broth) and then shaken at 150 rpm at 30°C for 18 h. Culture broth (1.5 ml) was transferred to a microcentrifuge tube and centrifuged for 2 min at 12,000 rpm. The bacterial cells were collected and resuspended in 567 μl of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) with lysozyme (1 mg/ml), mixed well by vortex and incubated at 30°C for 1 h. Then 30 μl of 10% SDS and 3 μl of proteinase K (20 mg/ml) were added and incubated at 30°C for 1 h. After that, 100 μl of 5 NaCl and 80 μl of CTAB/NaCl were added in a microcentrifuge tube and incubated at 30 °C for 1 h. The chloroform:isoamyl alcohol (24:1) was added to a microcentrifuge tube as 0.7 – 0.8 fold of total volume of the mixture and mixed gently. After mixing, the solution was centrifuged for 10 min at 12,000 rpm and the aqueous phase (DNA solution) was transferred to a new microcentrifuge tube and extracted again using phenol:chloroform: isoamyl alcohol (25:24:1) and then centrifuged for 10 min at 12,000 rpm. The aqueous phase was transferred to a new microcentrifuge tube and DNA was precipitated by adding 0.6 volume of isopopanol**,** then washing with 70% ethanol, and dissolving in 200 μl TE buffer (10 mM, pH8, 1 mM EDTA). Then, RNase was added into the dissolved DNA to a final concentration at 20 μg/ml. The mixture was incubated at 30°C for 30 min and then extracted again using phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), respectively. The DNA was again precipitated by adding 1/10 volume of 3 M sodium acetate and 2-2.5 volume of absolute ethanol.

DNA was dissolved in 100 μl TE buffer (10 mM, pH8, 1 mM EDTA) and stored at 4°C.

6.3.2 The quantity and quality analysis methods

The quantity of DNA was carried out following the UV spectrophotometric method. The DNA solution was diluted with TE buffer (10 mM, pH8, 1 mM EDTA) and then measured the absorbance at 260 and 280 nm. The quantity of DNA was calculated as followed:

OD_{260} = 1.0 = 50 μg of DNA double helix/ml

OD_{280} = 1.0 = 40 μg of DNA double helix/ml

OD_{260} = 1.0 = ~ 20 μg of oligonucleotide/mm

The ratio of OD_{260}/OD_{280} was determined to assess the purity of the DNA. The ratio of OD260/OD280 should be ~1.8 – 2.0; lower ratios indicate protein contamination, whereas higher ratios indicate chloroform or phenol contamination. Agarose gel electrophoresis (0.8% agarose in 1X TAE buffer) was performed for DNA quantification and quality analysis. The DNA was mixed with 5X loading dye buffer and subjected to electrophoresis at 100 volts for 35 min. Lambda DNA digested with *Hin*dIII was used as a DNA marker. The gel was stained in ethidium bromide solution (0.5 μg/ml) for 15 min and then washed with distilled water for 10 min. The band of DNA was visualized and photographed using GEL LOGIC 100 IMAGING SYSTEM (Kodak, Japan).

6.3.3 Polymerase Chain Reaction (PCR) amplification of 16S rDNA

The chromosomal DNA of *Pseudomonas* sp. W3 was used as a DNA template for 16S rDNA amplification. Oligonucleotide primers were EUB27 (5'-ATTGGATCCGTTTGAGC(A/C)TGGCTGAG-3') as a forward primer and 1392RH (5'-CGGAAGCTTACGGGCGGTGTGT(A/G)C-3') as a reversed primer. Amplification reaction was performed in 25 μl of total volume containing 50 ng of

DNA template, 2.5 µl of 10 X PCR buffer, 2 µl of $MgCl_2$ 2.0 mM, 0.2 µl of each 25 mM dNTP, 2 µl of 10 pmol forward primer, 2 µl of 10 pmol reverse primer, 0.5 µl of *Taq* DNA polymerase 2.5 U (Invitrogen, UK) and 11.8 µl of sterilized distilled water. The amplification was performed in a Thermal Cycler. A total of 35 cycles of amplification was performed with the template DNA initial denaturation at 95°C for 120 sec, the template DNA denaturation at 94°C for 20 sec, the primer annealing at 55°C for 30 sec, the primer extension at 72°C for 120 sec and final extention at 72°C for 600 sec. The PCR product (16S rDNA sequence) was separated with electrophoresis method (1% agarose in 1X TAE buffer) and visualized using GEL LOGIC 100 IMAGING SYSTEM (Kodak, Japan). The DNA band with the expected size of 1,500 bp was cut, transferred to a microcentrifuge tube and purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). Five hundred microliter of DF buffer was transferred to the sample, mixed by vortex and incubated at 60°C for 10-15 min until the gel slice had been completely dissolved. The sample was cool down at room temperature and then transferred to DF column that placed in a 2 ml collection tube. The DF column was centrifuged at 13,000 rpm for 30 sec. The supernatant in the collection tube was discarded and then the DF column was placed back in the collection tube. After that, 600 µl of washing buffer was added in the column and centrifuged at 13,000 rpm for 30 sec. The supernatant in the collection tube was discarded and centrifuged at 13,000 rpm for 3 min to dry the column matrix. The DF column was transferred to a microccentrifuge and added 15-50 µl of elution buffer in the center of the column. The DF column was centrifuged at 13,000 rpm for 2 min. The supernatant containing 16S rDNA was collected and stored at 4°C.

6.3.4 Cloning 16S rRNA gene

The obtained 16S rDNA was ligated with pGEM®T-Easy vector (TA vector; Promega, USA). Ligation reaction for 16S rDNA of *Pseudomonas* sp.W3 was performed in 10 µl of total volume that containing 5 µl of 2X Rapid Ligation Buffer, 0.5 µl of 50 ng pGEM®T-Easy vector, 2 µl of 50 ng PCR product, 1 µl of T4 DNA ligase (3 weiss units/µl) and 1.5 µl of sterilized distilled water**.** The mixture was

incubated overnight at 4 °C and used for the transformation into *E. coli* JM 109 by heat shock method (Sambrook et al., 1989). The recombinant *E. coli* JM 109 was spread on LB agar with 50 μg/ml of ampicilin, 100 mM of isoprophy thio-β- D galactoside (IPTG) and 50 mg/ml of x-gal and incubated at 37°C for 24 h. The white colonies which is the recombinant *containing* pGEM®T-Easy vector harboring the inserted DNA were selected for plasmid extraction and confirmation of the ligation of 16S rDNA with pGEM®T-Easy vector. Plasmid DNA was extracted by alkaline lysis method. The selected clones were cultured in 5 ml of LB broth supplemented with 50 μg/ml of ampicilin and then shaken at 150 rpm at 30°C for overnight. The culture broth was transferred to a microcentrifuge tube and centrifuged for 30 sec at 12,000 rpm. The bacterial cell pellet was resuspended in 100 μl of cold solution I (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA pH 8.0; see the Appendix B), mixed well by a vortex, then added 200 μl of solution II (0.2 N NaOH, 1% SDS; see the Appendix B) and mixed gently by inverting. After that, 150 μl of cold solution III was added and shaken gently for 10 sec. The tube was soaked in the ice box for 3 min and centrifuged at 12,000 rpm for 5 min. The supernatant was collected and transferred to a new microcentrifuge. The mixture was added with the equal volume of phenol:chloroform: isoamyl alcohol (25:24:1), mixing well by a vortex and centrifuged at 12,000 rpm for 2 min. Again the supernatant was collected and transferred to a new microcentrifuge. The plasmid DNA was precipitated with 2 volumes of absolute ethanol and incubated at room temperature for 2 min and then centrifuged for 5 min at 12,000 rpm. The plasmid DNA was washed with 1ml of 70% ethanol and centrifuged for 5 min at 12,000 rpm. The plasmid DNA was dried at room temperature and dissolved in 50 μl TE buffer (10 mM, pH8, 1 mM EDTA), mixing well by a vortex and stored at -20 °C. The extracted plasmid DNA was digested with a restriction enzyme, *EcoR*I. The DNA digestion was performed in 10 μl of total volume containing 1 μl of plasmid DNA that contained 1 μg DNA, 1 μl of 10X *EcoR*I buffer, 0.5 μl of *EcoR*I, 0.1 μl of 10 μg/ml RNase and 7.4 μl of sterilized distilled water. The mixture was incubated at 37°C for 2 h and then was subjected to agarose gel electrophoresis (0.8% agarose in 1X TAE buffer) to confirm the presence of the 1.5 kb inserted DNA.

6.3.5 Nucleotide sequence analysis

The 1.5 kb inserted DNA was sequenced by the service of Genome Institute (GI), BIOTECH. Oligonucleotide primers, T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') were used for the analysis by BigDye Terminator Cycle Sequencing and ABI PRISM 3100 Genetic Analyzer. Length of the read is 750 bases at the 98.5% base calling accuracy with less than 2% ambiguity. The obtained sequences were compared with those in BLAST database of National Center for Biotechnology Information (NCBI); Genbank (http://www.ncbi.nih. nlm.gov) by using Blastn 2.2.18 (Altschul *et al*., 1997). The conserved regions were analyzed by using BioEdit version 7.0.0.

6.3.6 Determination of phylogenetic relationships

The phylogenetic relationships were analyzed by the Phylogenetic Analysis program, using Parsimony (PAUP*) version 4.0 beta 10 in Macintosh and Window versions (Swofford, 2002). Phylogenetic trees were constructed using Neighbor Joining (NJ) and Maximum Parsimony (MP). The robustness of the internal braches of the tree was estimated by bootstrap analyses using 1,000 replications. Bootstrap majority-rule (> 50%) consensus trees were obtained. Descriptive tree statistics (Tree Length, CI, RI) were calculated for trees generated under different optimality. Kishino-Hasegawa (KH) test was performed in order to determine whether tree were significantly different (Kishino and Hasegawa, 1989). The sequence alignments have been deposited in GenBank database.

Results

6.1 Identification of *Pseudomonas* sp.W3 using a conventional API 20NE test kit (BioMerieux, France)

Identification of *Pseudomonas* sp.W3 using a conventional API 20NE test kit was performed together with *P. aeruginosa* PSSCMI 0048 and *P. aeruginosa*

PSSCMI 0049 and the results were presented in Table 6-1. All strains could reduce nitrate to nitrogen and use L-arginine, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, urea, gelatin (bovine origin) and D-glucose (assimilation). For N-acelyl-glucosamine which could be utilized by *P. aeruginosa* PSSCMI 0048 and *P. aeruginosa* PSSCMI 0049, *Pseudomonas* sp. W3 could not use this substrate. The results of *Pseudomonas* sp. W3 was compared to those of *P. aeruginosa* PSSCMI 0048, *P. aeruginosa* PSSCMI 0049 and the standard bacteria '*P. aeruginosa'* in API database (bioMerieux database) using the analysis software for the conventional API 20NE and 99.9% identity of three strains were observed. The results showed the three strains of *Pseudomonas* were similar to the standard *P. aeruginosa* in bioMerieux database. Therefore, *Pseudomonas* sp. W3 was further confirmed with the molecular characteristics based on 16S rDNA sequences.

6.2 Molecular identification of *Pseudomonas* sp.W3 by 16S rDNA sequence analysis

The total genomic DNA of *Pseudomonas* sp. W3 was successfully extracted using CTAB method and the approximate size of genomic DNA was 23 kb (Figure 6-1 A). The quantity of genomic DNA was 168 µg/ml. The ratio of OD_{260}:OD_{280} was 1.98, showing the purity of the prepared genomic DNA, which was suitable to be used as a DNA. PCR amplification of 16S rDNA was performed as mentioned in Materials and methods and the size of PCR product was 1.5 kb as expected (Figure 6-1 B). After subcloning of the PCR product into pGEM-T easy vector, the inserted fragment was sequenced using T7 and SP6 primers. The sequence comprised of 1,481 bp was submitted to Genbank with the accession number of HQ378506. The obtained sequence was subjected to BLAST search of National Center for Biotechnology Information (NCBI); Genbank (http://www.ncbi.nlm.nih.gov) and compared with other sequences in library database of Genbank. The BLAST results showed that the obtained sequence was corresponded to the sequences of *Pseudomonas aeruginosa*. Strains of *P. aeruginosa* from BLAST results used for comparison were shown in Table 6-2.

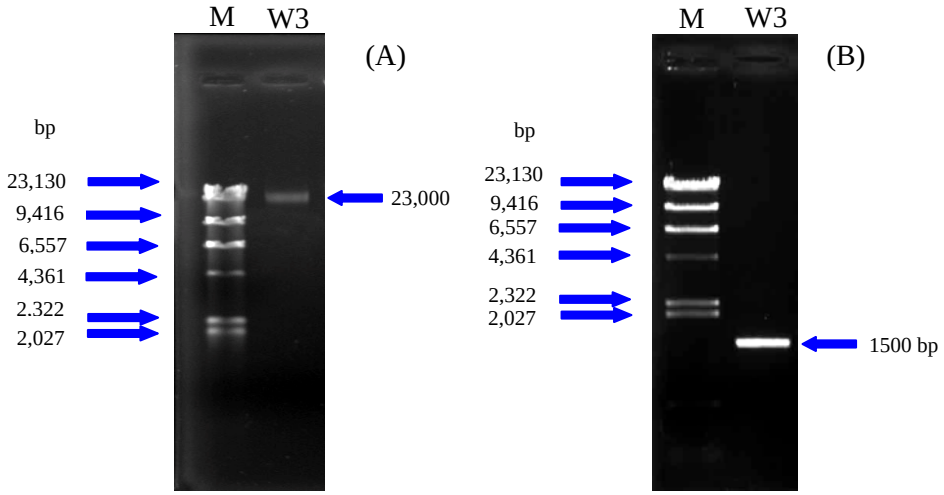


Figure 6-1 Size of genomic DNA (A) and PCR amplified product of 16S rDNA (B) of *Pseudomonas* sp.W3. Lambda DNA/*Hin*dIII (23 kb) was used as DNA marker (lane M).

The 16S rDNA alignment consisted of 8 taxa in *Pseudomonadaceae*; *P. aeruginosa* Z76651, *P. aeruginosa* FJ864676, *P. aeruginosa* EU344794, *P. fluorescens* GU726880, *P. fluorescens* GU726863, *P. fluorescens* GU048851, *P. putida* GU726875 and *P. putida* GQ280048, 3 taxa in *Acetobacteraceae*; *A. cerevisiae* NR025512, *A. indonesiensis* AB052715 and *A. ghanensis* AB470920 and *Azotobacter chroococcum*; EF 634040, EF634037 and EF634038 as an outgroup. Maximum parsimony (MP) analysis yields 1 MPT. Tree as estimated by K-H test is shown in Figure 6-2 with tree length, Consistency (CI) and Retention index (RI) of 526 steps, 0.8878 and 0.9174, respectively. The Neighbor-joining (NJ) tree had an almost identical to MP tree (Fig. 6-3). *Pseudomonas* sp. W3 was closely related to *P. aeruginosa* with short branch length and 50% bootstrap values from MP and NJ analyses. The similarity of the sequences among the strains of *Pseudomonas* sp. W3, *P. aeruginosa* FJ864676, *P. aeruginosa* EU344794 and *P. aeruginosa* Z76651 was between 99.3%-99.4%. Hence, *Pseudomonas* sp. W3 was identified to be *P. aeruginosa* W3.

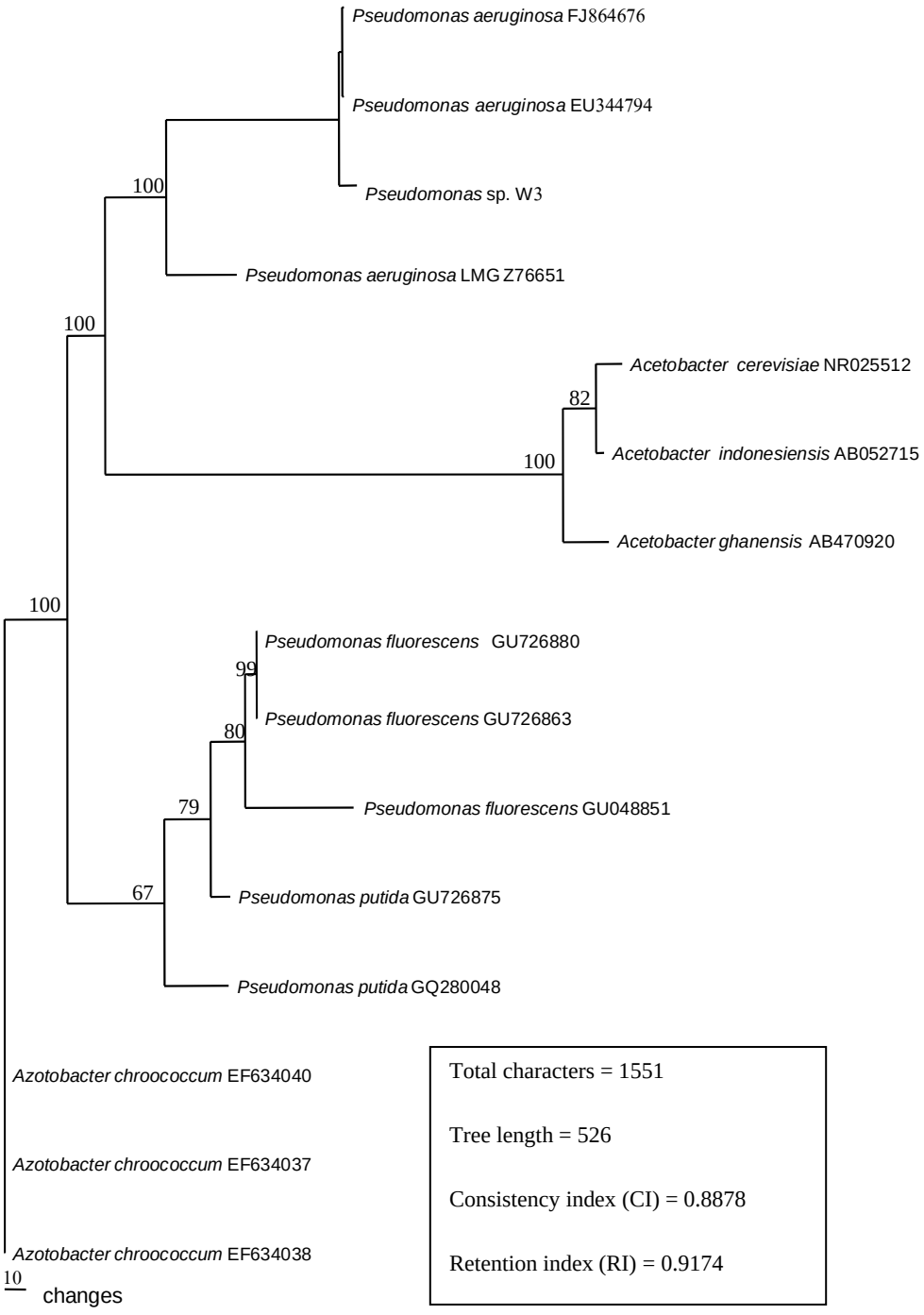


Figure 6-2 Maximum parsimony (MP) phylogenetic tree of *Pseudomonas* sp. W3. Bootstrap values calculated from 1,000 resamplings using MP is shown at the respective nodes when the calculated values were 50% or greater.

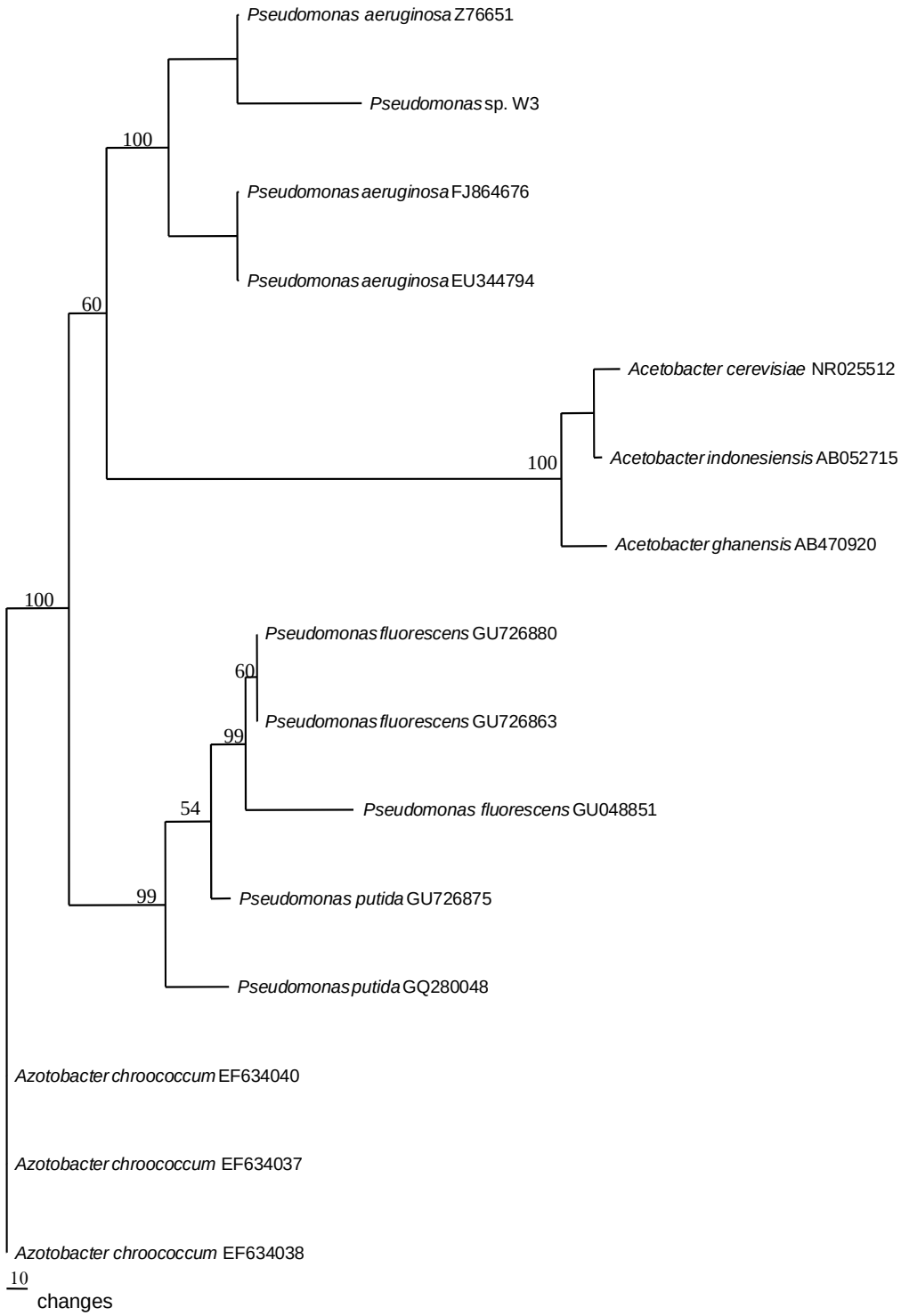


Figure 6-3 Neighbor-joining (NJ) phylogenetic tree of *Pseudomonas* sp. W3. Bootstrap values calculated from 1,000 resamplings using NJ is shown at the respective nodes when the calculated values were 50% or greater.

Table 6-1 Characteristic of *Pseudomonas* sp. W3 and references strains using a conventional API 20NE test kit (bioMerieux)

Active ingredients	Bacterial strains		
	Pseudomonas sp. W3	*Pseudomonas aeruginosa* PSSCMI0048	*Pseudomonas aeruginosa* PSSCMI0049
Potassium nitrate	+	+	+
L-tryptophane	-	-	-
D-glucose (fermentation)	-	-	-
L-arginine	+	+	+
Urea	+	+	+
Esculin ferric citrate	-	-	-
Gelatin (bovine origin)	+	+	+
4-nitrophenyl-βD-galactopyranoside	-	-	-
D-glucose (assimilation)	+	+	+
L-arabinose	-	-	-
D-mannose	-	-	-
D-mannitol	+	+	+
N-acetyl-glucosamine	**-**	**+**	**+**
D-maltose	-	-	-
Potassium gluconate	+	+	+
Capric acid	+	+	+
Adipic acid	+	+	+
Malic acid	+	+	+
Trisodium citrate	+	+	+
Phenylacetic acid	-	-	-
% Identity	99.9	99.9	99.9

+ = positive, **-** = negative

Table 6-2 The molecular identification results of *Pseudomonas* sp. W3 using 16S rDNA sequence analyses

Phylum	Class	Order	Expected species	Accession number	Similarity level with strain W3 (%)
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonas aeruginosa*	FJ864676	99.3
			Pseudomonas aeruginosa	EU344794	99.4
			Pseudomonas aeruginosa	Z76651	99.3
			Pseudomonas fluorescens	GU048851	88.6
			Pseudomonas fluorescens	GU726880	94.0
			Pseudomonas fluorescens	GU726863	94.0
			Pseudomonas putida	GU726875	95.3
			Pseudomonas putida	GQ280048	95.5
			Azotobacter chroococcum	EF634040	94.8
			Azotobacter chroococcum	EF634038	94.9
			Azotobacter chroococcum	EF634037	94.9
	Alphaproteobacteria	Rhodospirillales	*Acetobacter cerevisiae*	NR025512	78.8
			Acetobacter indonesiensis	AB052715	78.6
			Acetobacter ghanensis	AB470920	78.0

Discussion

The results on biochemical properties of the strain W3 using a API 20NE test kit (bioMerieux, France) show that the strain W3 was *P. aeruginosa* with 99% similarity. Hence, a full length of 16S rDNA sequence of *Pseudomonas* sp.W3 was analyzed in order to confirm the results of biochemical tests.

Sequence analysis of the small ribosomal RNA subunit gene (16S rDNA) is generally considered as a valuable tool for assigning bacterial strains to genus or species levels (Radice et al., 2006). The basic methods for phylogenetic analysis consist of distance method such as Neigbour-Joining (NJ) and discrete method such as Maximum Parsimony (MP) including Maximum Likelihood (Buatong, 2010). In this study, NJ and MP analyses were used for phylogenetic characterization. The full length of 16S rDNA and phylogenetic relationships were used to specify the species name of *Pseudomonas* sp.W3. Results in Figures 6-2 and 6-3 demonstrated that *Pseudomonas* sp.W3 was closely related to *P. aeruginosa* at 99.3%-99.9% similarity scores of *Pseudomonas* sp. W3, *P. aeruginosa* FJ864676, *P. aeruginosa* EU344794 and *P. aeruginosa* Z76651. However, the mismatch of some base pairs among the tested strains may occur during their adaptation to be survived in the environment. Moreover, Consistency index (CI) and Retention index (RI) of phylogenetic tree (Figure 6-2) showed high values, referring that the dataset had a little homoplasy and more synapomorphy (Rungjindamai, 2005). Therefore, the molecular results indicate that *Pseudomonas* sp. W3 is *P. aeruginosa*.

Normally, *Pseudomonas* spp. have been found in natural habitats such as aquatic as well as marine ecosystems (Krieg and Holt, 1984; Vijayan et al., 2006) and *P. aeruginosa* have been reported to be a normal flora in shrimp culture ponds (Ott et al., 1999; Chythanya et al., 2002; Bhakuni and Rawat, 2005). According to the results of API 20NE test kit and the full length of 16S rDNA analysis and the habitat of strain W3, there is not surprising that *Pseudomonas* sp. W3 is identified as *P. aeruginosa* W3.

Chapter VII

Conclusions

7.1 Conclusions

Pseudomonas spp. produce many kinds of bioactive compound such as siderophores, phenazines, pyoluteorin, quinolone, pyrroles and also biosurfactant. However, the information during the past on the applications of bioactive compounds from *Pseudomonas* spp. has been mainly focused on the potential biocontrol agents of plant diseases caused by various fungi. This study consequently aimed to examine the properties of bioactive compounds produced by *Pseudomonas* sp. W3 and its potential application as the biocontrol agents of aquatic animal diseases. Therefore, the antibacterial activity of bioactive compounds produced by *Pseudomonas* sp. W3 against vibrios and pathogenic bacteria in seafood, the characterization of the antivibrio compound, toxicity of antivibrio compounds to shrimp and finally the identification of the strain were investigated. In this final chapter, attempts to integrate the conclusions drawn from each experiment and also identify the future research needs were mentioned.

In chapter 3, the inhibition of shrimp pathogenic vibrios by extracellular bioactive compounds from *Pseudomonas* sp. W3 was investigated and concluded that *Pseudomonas* sp.W3 produced bioactive compounds that could inhibit shrimp pathogenic bacteria. Results show that *V. harveyi* PSU 2015 was the most sensitive strain whereas *V. parahaemolyticus* PSU 2015 was the most resistant strain. Inhibition of *V. harveyi* PSU 2015 and *V. cholerae* PSSCMI 0062 was greatest by the culture supernatant followed by the culture filtrate from 0.45 µm then the 0.22 µm culture filtrate. This bacterium caused lysis to *V. harveyi* cells and produced more than one extracellular antibacterial substance. Most of the active substances are heat stable (autoclaving at 121^{o}C for 30 min), pH tolerance and mostly resistance to various enzymes (lysozyme, proteolytic, lipolytic and amylolytic enzymes) and have a

small molecular weight but some are proteinaceous. It is interestingly to explore antivibrio compounds for protecting post larval shrimp in the hatcheries.

Chapter 4 focused on characterization of antivibrio compounds produced by *Pseudomonas* sp. W3 and assessment of their safety to shrimp. *Pseudomonas* sp. W3 produced 82.15 mg/l of a yellow-brown extract (EtOAc-W3) and its MIC against shrimp pathogenic *V. harveyi* (18 strains) was between 225-450 □g/ml. The toxicity to pacific white shrimp (*Litopenaeus vannamei*, PL 21) and antivibrio activity of the EtOAc-W3 against the most virulent strain, *V. harveyi* PSU2015, were investigated with 45 µg/ml (MIC/10) and 90 µg/ml (MIC/5). Approximately 80% shrimp death was observed in each challenge set but only 63% and 23% of shrimp died in sets of 45 □g/ml and 90 □g/ml, respectively. Therefore, EtOAc-W3 has a potential for controlling *V. harveyi* in shrimp hatcheries with no harm to postlarval shrimp. Identification result of EtOAc-W3 shows that the molecular weight of the compound was 243 and the compound was supposed to be HHQ (2-heptyl-4-quinolone) and thus should be confirmed to know exactly. Normally, HHQ is released into the environment and then assimilated by neighboring cells and converting to PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone) by *Pseudomonas aeruginosa*. Hence, why the strain W3 released large amount of HHQ should be further study

Chapter 5, we focused on the broad spectrum of bioactive compounds from *Pseudomonas* sp. W3 against pathogenic bacteria in seafood. As the results in chapter 3, the bioactive compounds from the strain W3 were interested for investigating possibility to control pathogenic bacteria in seafood because vibrios are the major pathogens in seafood. Inhibition of bioactive compounds against pathogenic bacteria was in the order of culture supernatant > culture filtrate from 0.45 µm > culture filtrate from 0.22 µm. The bioactive compounds had broad spectrum by inhibiting both Gram-negative (*V. cholera*, *V. parahaemolyticus*, and *Y. enterocolytica*) and Gram- positive *(B. cereus*, *C. perfringens*, and *L. monocytogenes*) *Y. enterocolytica* and *C. perfringens* were the most sensitive strains for Gram-negative and Gram-positive bacteria, respectively. Results indicate that bioactive compounds produced by the strain W3 have the potential for controlling foodborne

pathogen commonly detected in seafood and it is worth for further investigation to use in seafood industry.

In chapter 6, the species level of *Pseudomoas* sp.W3 was identified by using both biochemical properties (API 20NE test kit BioMerieux) and 16S rDNA sequence analysis as *Pseudomonas aeruginosa* W3. Phylogenetic trees were constructed using Neighbor Joining (NJ) and Maximum Parsimony (MP). The Phylogenetic tree estimated by K-H test as shown with tree length, Consistency (CI) and Retention index (RI) of 526 steps, 0.8878 and 0.9174, respectively. The sequence of *Pseudomonas* sp. W3 shows 99.3%-99.4% similarity sequence of *P. aeruginosa* FJ864676, *P. aeruginosa* EU344794 and *P. aeruginosa* Z76651. Due to some strain of this organism may be opportunistic pathogen; however, the strain W3 was isolated from the shrimp pond and it is one of normal flora in shrimp culture ponds have been reported. Therefore, this organism is safe to use in shrimp farming although its pathogenicity should be further investigated.

In the light of the results presented in this thesis provided the answers for the questions posed in the introduction part. Bioactive compounds produced by *Pseudomonas aeruginosa* W3 have antibacterial activity to control both vibrios and foodborne in seafood. The bioactive compounds consist of more than one compounds and the main compound supposed to be HHQ. The main compound was proved for its activity to control the most virulent strain, *V. harveyi* PSU2015 and it has the potential to use in hatcheries and shrimp cultivations as the biocontrol agent with no harm to postlarval shrimp. In addition, the bioactive compounds are possible to use in a frozen seafood industry in order to reduce and/or cease the contamination of pathogenic bacteria and spoilage organisms in seafood.

7.2 **Suggestions for future work**

Based on above conclusions it is clear that many questions remain unanswered. Hence, some specific suggestions for further research needs arising out of the current studied are the following provided.

1. Optimization conditions for producing bioactive compounds of the strain W3 should be further studied.

2. Inhibition of shrimp pathogenic vibrios by the main compound produced by the strain W3 should be investigated for its ability to control shrimp pathogens in the field.

3. Confirmation that the main compound from the strain W3 is HHQ or not.

4. If the main compound is HHQ; it needs to find out why the strain W3 released large amount of HHQ into the medium.

5. Inhibitory effect of bioactive compounds from the strain W3 against foodborne pathogens of seafood industry should be studied *in vivo*.

6. Pathogenicity of the *P. aeruginosa* W3 should be investigated for more application of this organism as it is a very good proteolytic bacterium that could be used for cleanup water in aquaculture as well.

7. Genes which control producing bioactive compounds by the strain W3 should be studied for the aim to transfer the genes into a probiotic bacterium.

Appendix A

Biochemical and other tests for identification of bacterium

1 Media

1.1 Nutrient agar (NA) medium (Difco, USA)

Composition per liter:

Beef extract	3.0 g
Peptone	5.0 g
Agar	15 g

1.2 Frazier gelatin medium (FGM) with 2% NaCl (Frazier and Rupp, 1982)

Composition per liter:

NaCl	20.0 g
K_2HPO_4	1.5 g
KH_2PO_4	0.5 g
Gelatin	4.0 g
Dextrose	0.05 g
Peptone	0.1 g
Beef extract	5.0 g

1.3 Luria-Bertani (LB) broth

Composition per liter:

Peptone	10 g
Yeast extract	5 g
NaCl	5 g

All media are sterilized by the autoclave at 121°C, 15 min and 15 pounds per square inch.

2. Chemicals

2.1 1 M Tris EDTA (TE) buffer

Tris base	60.5 g
Distilled water	500.0 ml

pH of this solution was adjusted to 8.0 with conc. HCl and then sterilized by the autoclave at 121°C, 15 min and 15 pounds per square inch.

2.2 5 M Ethylenediaminetetraacetic acid (EDTA)

$Na_2EDTA.2H_2O$	1.861 g
Distilled water	10.0 ml

pH of this solution was adjusted to 8.0 with 10 M NaOH and then sterilized by the autoclave at 121°C, 15 min and 15 pounds per square inch.

2.3 10% Sodium Dodecyl Sulfate (SDS)

SDS	1.0 g

Distilled water	10.0 ml

2.4 10% Cetyl trimethyl Ammonium Bromide (CTAB)

CTAB	1.0 g
Distilled water	10.0 ml

2.5 0.7 M NaCl

NaCl	0.4095 g
Distilled water	10.0 ml

This solution was sterilized by the autoclave at 121°C, 15 min and 15 pounds per square inch.

2.6 Solution I (100 ml)

1 M glucose	5.0 ml
1 M Tris-HCl	2.5 ml
0.5 M EDTA	2.0 ml
Distilled water	90.5 ml

The final concentration; 50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA pH 8.0

2.6 Solution II (100 ml)

10 N NaOH	2.0 ml
10% SDS	10.0 ml

Distilled water	88.0 ml

The final concentration; 0.2 N NaOH, 1% SDS

2.7 Solution III (100 ml)

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

Full length of 16S rDNA sequence analysis of *Pseudomonas* sp. W3

```
LOCUS       Pseudomonas sp. W3          1481 bp    DNA     linear
DEFINITION  No definition line found.
ACCESSION   HQ 378506
VERSION     HQ 378506
KEYWORDS    .
SOURCE      Unknown.
  ORGANISM  Unknown.
            Unclassified.
FEATURES             Location/Qualifiers
     source          1..1481
                     /mol_type="unassigned DNA"
BASE COUNT      372 a    342 c    463 g    303 t      1 others
ORIGIN
        1 attagatccg tttgagcatg gctcagattg aacgctggcg gcaggcctaa cacatgcaag
       61 tcgagcggat gaagggagct tgctcctgga ttcagcggcg gacgggtgag taatgcctag
      121 gaatctgcct ggtagtgggg gataacgtcc ggaaacgggc gctaataccg catacgtcct
      181 gagggagaaa gtgggggatc ttcggacctc acgctatcag atgagcctag gtcggattag
      241 ctagttggtg gggtaaaggc ctaccaaggc gacgatccgt aactggtctg agaggatgat
      301 cagtcacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
      361 tggacaatgg gcgaaagcct gatccagcca tgccgcgtgt gtgaagaagg tcttcggatt
      421 gtaaagcact ttaagttggg aggaagggca gtaagttaat accttgctgt tttgacgtta
      481 ccaacagaat aagcaccggc taacttcgtg ccagcagccg cggtaatacg aagggtgcaa
      541 gcgttaatcg gaattactgg gcgtaaagcg cgcgtaggtg gttcagcaag ttggatgtga
      601 aatccccggg ctcacctggg aactgcatcc aaaactactg agctagagta cggtagaggg
      661 tggtggaatt tcctgtgtag cggtgaaatg cgtagatata ggaaggaaca ccagtggcga
      721 aggcgaccac ctggactgat actgacactg aggtgcgaaa gcgtggggag caacaggatt
      781 agataccctg gtagtcccgc cgtaaacgat gtcgactagc cgtnggggatc cttgagatct
      841 tagtggcgca gctaacgcga taagtcgacc cgcctgggga gtacggccgc aaggttaaaa
      901 ctcaaatgaa ttgacggggg cccgcacaag cggtggagca tgtggtttaa ttcgaagcaa
      961 cgcgaagaac cttacctggc cttgacatgc tgagaacttt ccagagatgg attggtgcct
     1021 tcgggaactc agacacaggt gctgcatggc tgtcgtcagc tcgtgtcgtg agatgttggg
     1081 ttaagtcccg taacgagcgc aacccttgtc cttagttacc agcacctcgg gtgggcactc
     1141 taaggagact gccggtgaca aaccggagga aggtggggat gacgtcaagt catcatggcc
     1201 cttacggcca gggctacaca cgtgctacaa tggtcggtac aaagggttgc caagccgcga
     1261 ggtggagcta atcccataaa accgatcgta gtccggatcg cagtctgcaa ctcgactgcg
     1321 tgaagtcgga atcgctagta atcgtgaatc agaatgtcac ggtgaatacg ttcccgggcc
     1381 ttgcacacac cgcccgtaag cttccgaatc actagtgaat tcgcggccgc ctgcaggtcg
     1441 accatatggg agagctccca acgcgttgga tgcatagctg a
```

APPENDIX B

Determination of LD_{50} by Probit analysis

Probit Analysis

Dose (Stimulus)	Actual Percent (%)	Probit (Y)	Weight (Z)	X*Z	X*X*Z	Y*Z	X*Y*Z
100,000	0.1167	3.8081	2.9242	292,420.2502	29,242,025,019.1114	11.1356	1,113,556.0513
1,000,000	0.1833	4.0974	3.6948	3,694,755.934	3,694,755,934,018.5986	15.1388	15,138,811.5575
10,000,000	0.8667	6.1108	3.1675	31,675,157.1424	316,751,571,424,164.75	19.3561	193,561,440.1874
		Sum	**9.7865**	**35,662,333.3266**	**320,475,569,383,202.438**	**45.6305**	**209,813,807.7962**

LD50	**5,120,573.517**	*LD16*	744,237.0298	*Beta*	0
Standard Error LD50	461,306.3702	*LD84*	9,496,910.0042	*Alfa*	3.8299
LD50 LCL	4,210,275.15	*LD100*	11,685,078.2478	*Standard Error Beta*	0
LD50 UCL	6,030,871.884	*Significance Level*	0.05		
LD10	-488,702.8067				

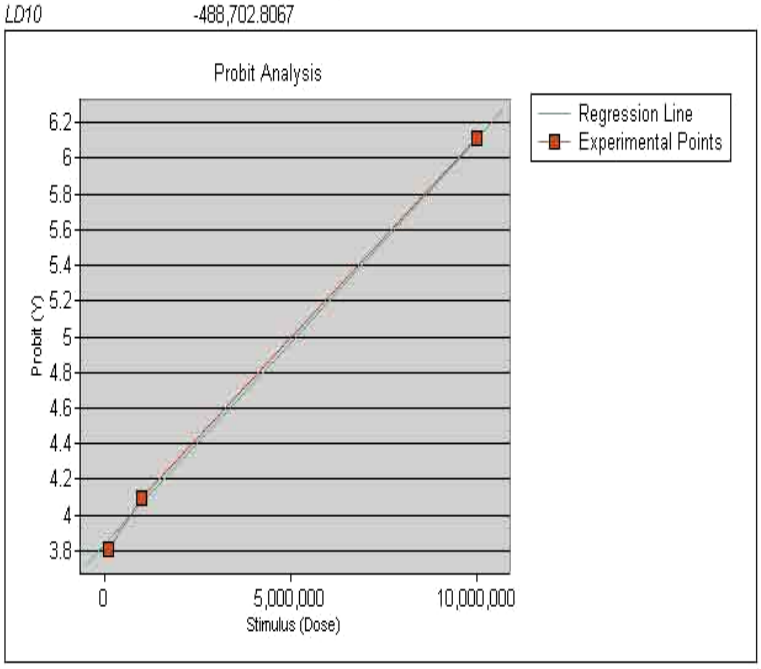


Figure B-1 Lethal Dose 50% (LD50) of *V. harveyi* PSU2015 in Pacific white shrimp (PL21) at 96 h

Probit Analysis							
Dose (Stimulus)	Actual Percent (%)	Probit (Y)	Weight (Z)	X'Z	X'X'Z	Y'Z	X'Y'Z
100,000	0.2	4.1585	3.8171	381,708.6565	38,170,865,652.9043	15.8735	1,587,351.9695
1,000,000	0.2333	4.2723	4.0447	4,044,691.1112	4,044,691,111,209.4756	17.2803	17,280,318.0928
10,000,000	0.3167	4.5234	4.5234	45,233,799.4354	452,337,994,353,614.312	20.461	204,609,661.1359
		Sum	**12.3852**	**49,660,199.2031**	**456,420,856,330,476.688**	**53.6148**	**223,477,331.1982**
LD50	**24,321,383.3383**	*LD16*	-5,947,470.6231	*Beta*	0		
Standard Error LD50	3,190,617.356	*LD84*	54,590,237.2996	*Alfa*	4.1965		
LD50 LCL	18,025,320.7723	*LD100*	69,724,664.2803	*Standard Error Beta*	0		
LD50 UCL	30,617,445.9042	*Significance Level*	0.05				
LD10	-14,475,077.2104						

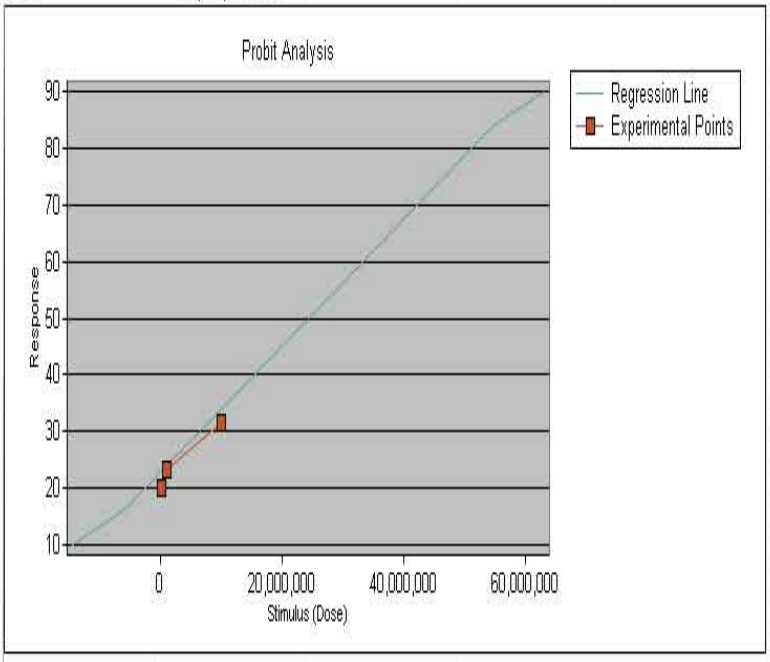


Figure B-2 Lethal Dose 50% (LD50) of *V. harveyi* AAHRC1 in Pacific white shrimp (PL21) at 96 h

Probit Analysis

Dose (Stimulus)	Actual Percent (%)	Probit (Y)	Weight (Z)	X'Z	X'X'Z	Y'Z	X'Y'Z
100,000	0.1333	3.8892	3.1675	316,751.5714	31,675,157,142.4165	12.319	1,231,901.3124
1,000,000	0.2167	4.2167	3.9334	3,933,425.933	3,933,425,933,019.4834	16.5861	16,586,128.1346
10,000,000	0.3333	4.5697	4.5697	45,697,082.4109	456,970,824,109,427.875	20.8822	208,822,334.0872
		Sum	**11.6706**	**49,947,259.9154**	**460,935,925,199,589.75**	**49.7874**	**226,640,363.5342**

LD50	**17,655,001.6853**	*LD16*	-568,249.02	*Beta*	0
Standard Error LD50	1,920,899.2867	*LD84*	35,878,252.3906	*Alfa*	4.0312
LD50 LCL	13,864,480.685	*LD100*	44,989,877.7432	*Standard Error Beta*	0
LD50 UCL	21,445,522.6856	*Significance Level*	0.05		
LD10	-5,702,262.7807				

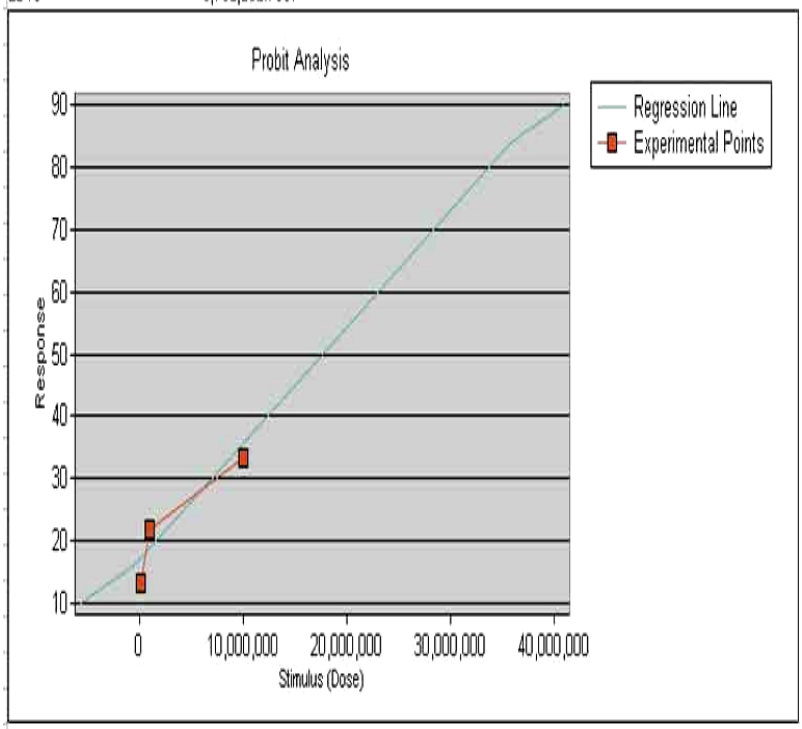


Figure B-3 Lethal Dose 50% (LD50) of *V. harveyi* AAHRC2 in Pacific white shrimp (PL21) at 96 h

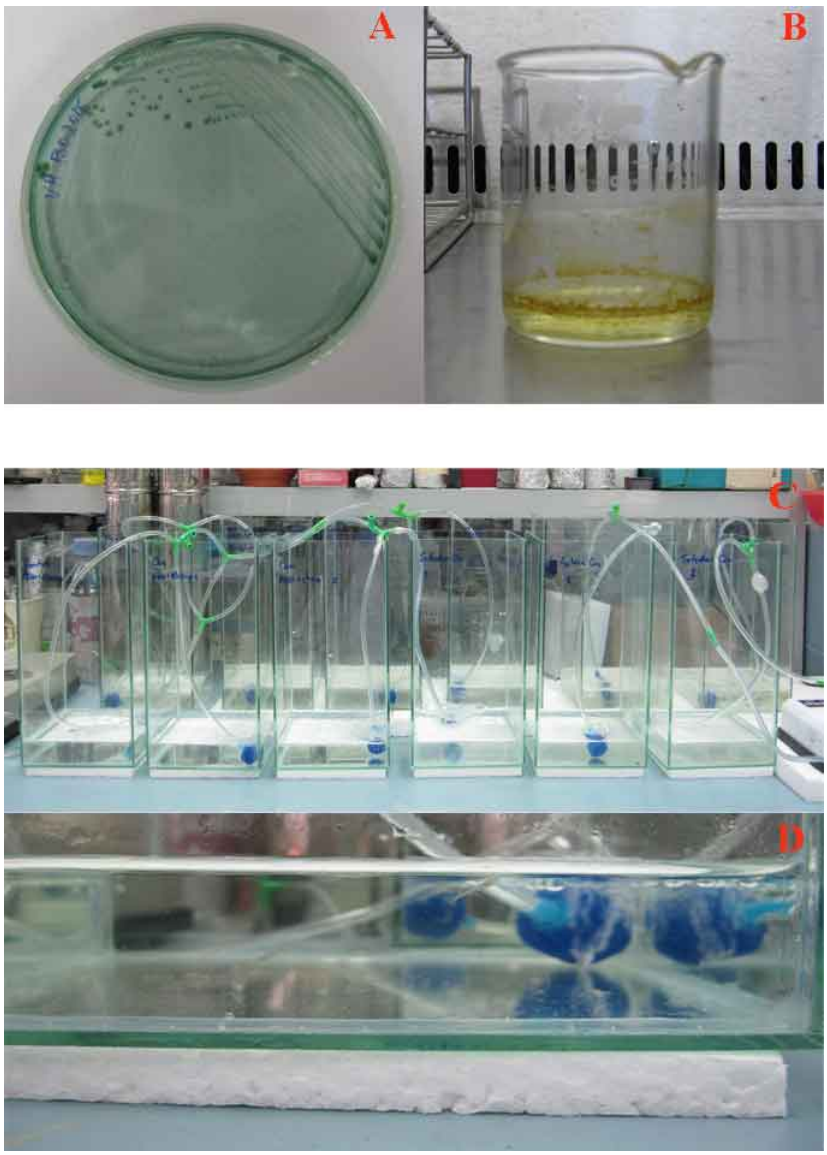


Figure B-4 Growth of *Vibrio harveyi* PSU2015 on TCBS agar (A), EtOAC-W3 (B) and Shrimp cultivation (C and D) in the investigating of shrimp mortality and antivibrio compounds by EtOAc-W3.

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VITAE

Name Miss Pattamarat Rattanachuay

Student ID 4910230022

Educational Attainment

Degree	Name of Institution	Year of Graduation
B. Sc.	Biotechnology	2004
M. Sc.	Microbiology	2006

Scholarship Awards during Enrolment

Graduate School Songklanakarin Scholarship 2007-2009

Teacher assistant (TA) fund From Faculty of Science

JSPS (Japan Society for the Promotion of Sciences) Exchange Program for East Asian Young Researchers, 'Collaborative research on development of useful microbial resources in tropical environments with South-East Asian young researchers'

List of Publication and Proceeding

1. Rattanachuay, P., Tantirungkij, M., Kuntachote, D. 2008. Partial Characterization of Bioactive compounds Produced by *Pseudomonas* sp. W3 with Ability to Inhibit *Vibrio harveyi*. Proceeding of The 10th National Graduate Research Conference. (Poster presentation).

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