Incidence of Clinical and Environmental *Vibrio parahaemolyticus* Isolates in Hat Yai City and Biosurfactants Produced by Soil Microorganisms against *Vibrio parahaemolyticus*

Junthip Thongjun

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

Prince of Songkla University

2013

Copyright of Prince of Songkla University

Thesis Title Incidence of Clinical and Environmental *Vibrio parahaemolyticus* Isolates in Hat Yai City and Biosurfactants Produced by Soil Microorganisms against *Vibrio parahaemolyticus*

Author Miss Junthip Thongjun

Major Program Microbiology

Major Advisor

……………………………….
(Prof.Dr.Varaporn Vuddhakul)

Co-advisor

…………………………………..
(Dr.Kanchana Srinitiwarawong)

Examining Committee :

…..…………………………...Chairperson
(Assoc.Prof.Dr.Pojana Sriburee)

…..………………………………
(Prof.Dr.Varaporn Vuddhakul)

…..………………………………
(Asst.Prof.Dr.Kanda Panthong)

…..………………………………
(Dr.Pimonsri Mittraparp-Arthorn)

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

……………………………………
(Assoc.Prof.Dr.Teerapol Srichana)

Dean of Graduate School

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

……………………..…………

(Prof.Dr.Varaporn Vuddhakul)

Major Advisor

…………………………………

(Miss Junthip Thongjun)

Candidate

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

……… ………………...

(Miss Junthip Thongjun)
Candidate

Thesis Title Incidence of Clinical and Environmental *Vibrio parahaemolyticus* Isolates in Hat Yai City and Biosurfactants Produced by Soil Microorganisms against *Vibrio parahaemolyticus*

Author Miss Junthip Thongjun

Major Program Microbiology

Academic Year 2012

Abstract

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium detected in marine environments worldwide. It is one of the leading causes of gastroenteritis after consumption of seafood. Transmission of this bacterium to human can be direct transmission via consumption of raw or undercooked seafood and indirect transmission via cross-contamination of *V. parahaemolyticus* to other foods during preparation. In this study, incidences of *V. parahaemolyticus* infection in Hat Yai Hospital and number of this bacterium in shellfish were investigated. In addition, biosurfactants derived from bacteria will be determined in order to decrease cross-contamination of this bacterium on surface area of cooking.

A total of 776 *V. parahaemolyticus* isolates obtained from patients in Hat Yai Hospital from 2006-2010 were determined. The highest number of this bacterium was detected in 2006 and the lowest number was in 2009. The bacterium was classified into four groups: tdh^+trh^-, tdh^+trh^+, tdh^-trh^- and tdh^-trh^+ based on presence or absence of the *tdh* or *trh* toxin genes. Most of the clinical *V. parahaemolyticus* isolates were in the tdh^+trh^-

group which was divided into pandemic (GS-PCR positive) and non-pandemic (GS-PCR negative) strains. The predominant isolates of *V. parahaemolyticus* in each year were pandemic strains of which highest number, 73.6%, and the lowest number, 51.3%, were from 2008 and 2010, respectively. The numbers of isolates in the other 3 groups, $tdh^{+}trh^{+}$, $tdh^{-}trh^{-}$ and $tdh^{-}trh^{+}$, were highest in 2007 (7.9%), 2010 (12.6%) and 2009 (2.7%), respectively. O3:K6 was the most predominant serotype of pandemic strains, however, the predominant serotypes of isolates from non-pandemic strains were O1:K56, O4:K8, O1:KUT and O4:K9. Comparison of the numbers of *V. parahaemolyticus* isolates in this 5 years study and those from the same hospital in 2000-2005 indicates that incidence of *V. parahaemolyticus* infection in this area does not decrease.

Shellfish especially mollusks seems to harbor high number of *V. parahaemolyticus* because they are filter feeders and they may be an important vehicle of seafood borne infection. In this study, using CHROMagar Vibrio, total number of vibrios and *V. parahaemolyticus* in each shellfish samples including shrimp, crab, mussel, squid, hard clam and bloody clam were investigated. The average total number of vibrios was highest in bloody clam at 7.7 x 10^{6} cfu/g. The average lowest number of vibrios was observed in squid at 1.6 x 10^{4} cfu/g. The highest number of *V. parahaemolyticus* was detected in mussel (4.7 x 10^{4} cfu/g) and the lowest number was detected in crab (1.0 x 10^{2} cfu/g). There was no correlation between the number of total vibrios and that of *V. parahaemolyticus***.** Virulence genes (*tdh* and *trh*) could be detected in none of these isolates. Diversities in the serotype and DNA fingerprints were confirmed among the isolated strains from a single shellfish sample. It is concluded that most of the single shellfish harbor heterogeneous population of *V. parahaemolyticus* and the concentrations of pathogenic strains are low.

In order to evaluate bacteria producing biosurfactants, 28 soil samples derived from mangrove and coastal areas were investigated. A total of 462 bacterial isolates were obtained. Using cross streak technique, 26

isolates inhibited growth of *V. parahaemolyticus*. One isolate designated as SM11 showed highest inhibitory activity against *V. parahaemolyticus* by agar well diffusion technique. Identification of 16S rDNA sequencing indicated that this strain is *Bacillus amyloliquefaciens*. Inhibition zone against *V. parahaemolyticus* of chloroform and methanol - crude extract derived from this bacterium at the concentration of 900, 1,800 and 2,600 μg/ml were 16.9±0.2, 18.4±0.5 and 25.0±0.1 mm, respectively. Confirmation of inhibitory activity of this substance by tube dilution method revealed the lowest MIC and MBC were 256 and 512 μg/ml, respectively. Crude extract of biosurfactants showed more than 60% of adhesion inhibition against *V. parahaemolyticus* at the concentration of 500 μg/ml. Fractions obtained from ion exchange chromatography and RP-HPLC were analyzed by ESI- Q-TOF MS spectrometry indicated that these biosurfactants were surfactins, iturin A and mycosubtilins.

ชื่อวิทยานิพนธ์ อุบัติการณ์ของเชื้อ *Vibrio parahaemolyticus* จากผู้ป่วยและสิ่งแวดล้อมในเมืองหาดใหญ่และการสร้างสารลดแรงตึงผิวจากจุลินทรีย์ดินต่อต้านเชื้อ *Vibrio parahaemolyticus*

ผู้เขียน นางสาวจันทร์ทิพย์ ทองจันทร์

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2555

บทคัดย่อ

Vibrio parahaemolyticus เป็นแบคทีเรียแกรมลบเจริญในสภาวะที่มีเกลือและพบได้ในสิ่งแวดล้อมบริเวณชายฝั่ง น้ำกร่อย น้ำทะเลแพร่กระจายอยู่ทั่วโลก เชื้อนี้เป็นสาเหตุของกระเพาะและลำไส้อักเสบหลังบริโภคอาหารทะเล การติดเชื้อของ *V. parahaemolyticus* เกิดโดยตรงเมื่อบริโภคอาหารทะเลดิบหรืออาหารทะเลที่ปรุงไม่สุกและการติดเชื้อทางอ้อมมีสาเหตุจากการปนเปื้อนเชื้อนี้ไปสู่อาหารอื่นระหว่างขั้นตอนการเตรียมอาหาร ในการทดลองนี้ได้ทำการศึกษาจำนวนและปัจจัยก่อโรคของเชื้อ *V. parahaemolyticus* ที่แยกจากผู้ป่วยของโรงพยาบาลหาดใหญ่และตรวจหาจำนวนของเชื้อนี้ในอาหารทะเลชนิดต่าง ๆ ที่อาจมีความเกี่ยวข้องกับการติดเชื้อในผู้ป่วย พร้อมทั้งแยกแบคทีเรียที่สร้างสารลดแรงตึงผิว เพื่อนำมาประยุกต์ใช้ลดการปนเปื้อนของเชื้อนี้บนพื้นผิวที่ประกอบอาหาร

จากการศึกษา *V. parahaemolyticus* จำนวน 776 ไอโซเลทที่แยกจากผู้ป่วยในโรงพยาบาลหาดใหญ่ในปี ค.ศ. 2006 ถึง 2010 พบจำนวนแบคทีเรียสูงสุดและต่ำสุดในปี ค.ศ. 2006 และ 2009 ตามลำดับ แบคทีเรียสามารถจัดเป็นกลุ่มได้ 4 กลุ่ม โดยอาศัยการตรวจพบยีน *tdh* และ *trh* คือ $tdh^{+}trh^{-}$, $tdh^{+}trh^{+}$, $tdh^{-}trh^{-}$ และ $tdh^{-}trh^{+}$ เชื้อที่ก่อโรคในผู้ป่วยส่วนใหญ่อยู่ในกลุ่ม $tdh^{+}trh^{-}$ ซึ่งแบคทีเรียกลุ่มนี้แบ่งเป็น pandemic strain เมื่อผลการทดสอบของ GS-PCR เป็นผลบวก และ non pandemic

strain เมื่อให้ผล GS-PCR เป็นลบ ในแต่ละปีจะตรวจพบ *V. parahaemolyticus* ในกลุ่ม pandemic strains เป็นจำนวนมากที่สุดโดยพบปริมาณสูงสุด (73.6%) และปริมาณต่ำสุด (51.3%) ในปี ค.ศ. 2008 และ 2010 ตามลำดับ จำนวนเชื้อที่พบในอีก 3 กลุ่ม คือ $tdh^{+}trh^{+}$, $tdh^{-}trh^{-}$ และ $tdh^{-}trh^{+}$ มีจำนวนสูงสุดในปี ค.ศ 2007 (7.9%), 2010 (12.6%) และ 2009 (2.7%) ตามลำดับ สายพันธุ์ pandemic *V. parahaemolyticus* ที่พบส่วนใหญ่เป็นซีโรไทป์ O3:K6 ส่วนสายพันธุ์ non pandemic ที่ตรวจพบส่วนใหญ่ได้แก่ ซีโรไทป์ O1:K56, O4:K8, O1:KUT และ O4:K9 โดยสรุปการติดเชื้อ *V. parahaemolyticus* ในผู้ป่วยโรงพยาบาลหาดใหญ่พบว่าจำนวนของเชื้อ *V. parahaemolyticus* ในช่วงเวลา 5 ปี (2006-2010) มีจำนวนที่ไม่แตกต่างจากจำนวนที่พบในช่วงปี ค.ศ 2000-2005 แสดงว่าการติดเชื้อ *V. parahaemolyticus* ในพื้นที่นี้ไม่ได้ลดลง

สัตว์ทะเลโดยเฉพาะหอย 2 ฝา (mollusk) เป็นสัตว์ที่พบจำนวนของ *V. parahaemolyticus* ในปริมาณสูง เพราะหอยเป็นสัตว์ที่กินอาหารโดยการกรองจึงมีโอกาสสะสมเชื้อ *V. parahaemolyticus* มากกว่าสัตว์อื่น จึงเป็นแหล่งเชื้อ *V. parahaemolyticus* ในการศึกษาจำนวนแบคทีเรียทั้งหมดและจำนวนเชื้อ *V. parahaemolyticus* ในตัวอย่างอาหารทะเลซึ่งประกอบด้วย กุ้ง ปู หอยแมลงภู่ ปลาหมึก หอยตลับ และ หอยแครง โดยใช้อาหาร CHROMagar Vibrio ในการเพาะเลี้ยง พบว่าหอยแครงและปลาหมึกเป็นสัตว์ที่พบจำนวนของเชื้อแบคทีเรียใน genus *Vibrio* โดยเฉลี่ยสูงที่สุด (7.7×10^{6} cfu/g) และต่ำที่สุด (1.6×10^{4} cfu/g) ตามลำดับ หอยแมลงภู่ และปูเป็นสัตว์ที่พบจำนวนของ *V. parahaemolyticus* สูงที่สุด (4.7×10^{4} cfu/g) และต่ำที่สุด (1.0×10^{2} cfu/g) ตามลำดับ อย่างไรก็ตามการพบจำนวนของเชื้อแบคทีเรียใน genus *Vibrio* ทั้งหมดไม่สัมพันธ์กับจำนวนของเชื้อ *V. parahaemolyticus* เชื้อ *V. parahaemolyticus* ที่แยกได้ทั้งหมดอยู่ในกลุ่ม tdh^{-} trh^{-} การศึกษาความหลากหลายของซีโรไทป์และลายพิมพ์ดีเอ็นเอของเชื้อ *V. parahaemolyticus* ที่แยกได้จากอาหารทะเลแต่ละตัว พบว่าส่วนใหญ่ประกอบด้วยสายพันธุ์ที่แตกต่างกัน (heterogeneous population)

เพื่อลดการปนเปื้อนของเชื้อ *V. parahaemolyticus* บนพื้นผิวปรุงอาหาร การศึกษานี้จึงแยกเชื้อแบคทีเรียที่สร้างสารลดแรงตึงผิวจากตัวอย่างดินในแหล่งต่างๆ รวม 28 ตัวอย่าง ได้แบคทีเรียจำนวน 462 ไอโซเลท และ 26 ไอโซเลท สามารถยับยั้งการเจริญของเชื้อ *V. parahaemolyticus* เมื่อทดสอบโดยเทคนิค cross streak จากนั้นจึงยืนยันผลการยับยั้งด้วยเทคนิค agar well diffusion กับเชื้อ 26 สายพันธุ์ พบว่าแบคทีเรีย SM11 มีกิจกรรมยับยั้งเชื้อ *V. parahaemolyticus* สูงสุด จากการศึกษาลำดับเบสของ SM11 ที่ตำแหน่ง 16S rDNA บ่งชี้ว่าแบคทีเรียนี้ คือ *Bacillus amyloliquefaciens* การศึกษาครั้งนี้พบว่าการใช้ chloroform และ methanol สกัดน้ำเลี้ยงเชื้อ *B. amyloliquefaciens* ที่ความเข้มข้นที่ 900, 1,800 และ 2,600 ไมโครกรัมต่อมิลลิลิตร ให้ผลยับยั้ง*เชื้อ V. parahaemolyticus* โดยมีเส้นผ่าศูนย์กลางของวงใส (inhibition zone) เท่ากับ 16.9±0.2, 18.4±0.5 และ 25.0±0.1 มิลลิเมตร ตามลำดับ เมื่อยืนยันผลการยับยั้งด้วยเทคนิค tube dilution ได้ค่า MIC และ MBC เท่ากับ 256 and 512 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ การทดสอบสารลดแรงตึงผิว crude extract ที่ความเข้มข้น 500 ไมโครกรัมต่อมิลลิลิตร พบว่าสามารถยับยั้งการเกาะติดของเชื้อ *V. parahaemolyticus* ได้ถึง 60% สารลดแรงตึงผิวถูกทำให้บริสุทธิ์ด้วยเทคนิค ion exchange chromatography และ RP-HPLC เมื่อนำไปวิเคราะห์หาน้ำหนักโมเลกุลด้วยเทคนิค ESI- Q-TOF MS spectrometry พบว่าสารลดแรงตึงผิวนี้ประกอบด้วย surfactins, iturin A และ mycosubtilins

CONTENTS

	Page
Abstract	v
Contents	xii
List of tables	xviii
List of figures	xx
List of abbreviations and symbols	xxiv
Chapter	
1. Introduction	
Background and rationale	1
Literature reviews	4
Vibrio parahaemolyticus characterization and distribution	4
Pathogenesis and virulent factors	5
Pandemic strain of *V. parahaemolyticus*	9
Infection by *V. parahaemolyticus*	9
Cross-contamination by *V. parahaemolyticus*	11
Cross-contamination control	12
Microbial bioactive compounds	13
Biosurfactants	13

CONTENTS (continued)

	Page
Biosurfactant lipopeptides synthesized by *Bacillus* spp.	16
Surfactin family	17
Surfactin	17
Iturin family	18
Iturin	19
Fengycin family	20
Environmental factors involved in biosurfactants production	21
Biosurfactants investigation techniques	22
Purification of biosurfactants	23
Antibacterial activity of biosurfactants	25
Pore forming	27
Anti-adhesion activity of biosurfactants	29
Objectives	31
2. RESEARCH METHODOLOGY	32
MATERIALS AND METHODS	32
Equipments	32
Media	33

CONTENTS (continued)

	Page
Enzymes, antibodies and other reagents	34
Primers	35
Other chemical substances	36
METHODS	37
Part I. Investigation and characterization of *V. parahaemolyticus* isolates from clinical and environmental samples	37
Part IA Investigation and characterization of *V. parahaemolyticus* isolates from patients in Hat Yai Hospital	38
Sample collection and bacteriology	38
Investigation of the *toxR* gene	38
Detection of *tdh* and *trh* genes	39
Serotyping	39
GS-PCR	40
Part IB Isolation and characterization of *V. parahaemolyticus* isolates from shellfish	40
Enumeration of *V. parahaemolyticus* in shellfish	40
Confirmation of *V. parahaemolyticus* and detection of virulence genes	41

CONTENTS (continued)

	Page
Serotyping	41
Arbitrarily primed polymerase chain reaction	41
Pulsed Field Gel Electrophoresis (PFGE)	42
Part II Isolation and characterization of biosurfactants that possess inhibitory activity against *V. parahaemolyticus*	43
Bacterial strains	44
Soil samples	44
Isolation of bacteria and screening for inhibitory activity to *V. parahaemolyticus*	45
Acid precipitation of biosurfactants and agar well diffusion technique	46
Identification of biosurfactants producing bacterium	46
Media and conditions of biosurfactants production	47
Kinetic production of biosurfactants	47
Surface tension measurement	47
Chloroform and methanol extraction, MIC and MBC determination	48

CONTENTS (continued)

	Page
Adhesion inhibition of crude biosurfactants against *V. parahaemolyticus*	48
Determination of ion of biosurfactants	49
Purification and identification of biosurfactants	49
DEAE-cellulose anion exchange chromatography	49
High-performance liquid chromatography (HPLC)	50
Mass spectrometry	50
Infrared spectroscopy analysis	51
3. RESULTS	52
Part I Investigation and characterization of *V. parahaemolyticus* isolates from clinical and environmental samples	52
Part IA Investigation and characterization of *V. parahaemolyticus* isolates from patients in Hat Yai Hospital	52
Part IB Investigation and characterization of *V. parahaemolyticus* isolates from shellfish	57
Part II. Isolation and characterization of biosurfactants that possess inhibitory activity against *V. parahaemolyticus*	63
4. DISCUSSIONS	79

CONTENTS (continued)

	Page
5. CONCLUSIONS	87
REFERENCES	89
APPENDIX	110
APPENDIX A	111
APPENDIX B	117
APPENDIX C	120
APPENDIX D	123
APPENDIX E	125
APPENDIX F	128
VITAE	129

LIST OF TABLES

Table **Page**

1 Biosurfactants obtained from various microorganisms. 15

2 Biosurfactants reported from *Bacillus* spp. 16

3 Amino acid composition of heptapeptides in surfactin family 17

4 Amino acid composition of heptapeptides in iturin family 19

5 Amino acid composition of decapeptides in fengycin family 20

6 Purification methods of biosurfactants 24

7 Bacterial strains used in this study 44

8 Soils samples collected from mangrove and coastal areas in southern Thailand 45

9 Characteristics of *V. parahaemolyticus* isolates from Hat Yai Hospital from 2006 to 2010 53

10 Serotypes of *V. parahaemolyticus* isolated between 2006 and 2010 55

11 Predominant serotypes of GS-PCR positive strains 57

12 Meat weight, total number of bacteria and *V. parahaemolyticus* detected on CHROMagar Vibrio from single shellfish samples 59

LIST OF TABLES (continued)

Table **Page**

13 Serotype of *V. parahaemolyticus* detected in single shellfish samples 60

14 Inhibitory activity against *V. parahaemolyticus* and surface tension of bacterial isolates from soil 64

15 Biochemical test of *Bacillus* sp.SM11 65

16 Comparison of 4 different media for biosurfactants producing by *B. amyloliquefaciens* 67

17 MIC and MBC of chloroform and methanol extract of *B. amyloliquefaciens* 69

18 Antimicrobial activity against *V. parahaemolyticus* of PSM1-5 fractions obtained from RP-HPLC 74

19 Mass spectrometric characteristic of PSM1-5 fractions after eluted from RP-HPLC 76

20 Tris-HCl buffer pH 8.0 (20 mM) supplemented with NaCl concentration 118

LIST OF FIGURES

Figure	Page
1 *V. parahaemolyticus* pathogenicity islands containing *tdh* and *trh* genes	5
2 T3SS is composed of a basal body, a needle structure and a needle tip	7
3 Comparison of pathogenicity island of *V. parahaemolyticus* between *V. parahaemolyticus* TH3996 *trh*-positive and *V. parahaemolyticus* RIMD2210633 *tdh*-positive	8
4 Structure of cyclic lipopeptide surfactin	18
5 Structure of cyclic lipopeptide iturin A	20
6 Structure of cyclic lipodecapeptides fengycin A	21
7 Drop collapse method	22
8 Antimicrobial activity of surfactin	26
9 Activity of surfactin to peptide molecule	26
10 Model of an iturin A connected to bilayer	28
11 Formation of ion channels of syringomycin (lipopeptide) in *Pseudomonas syringae* pv. *syringae*	29
12 Illustrate diagram of Part I investigation	37

LIST OF FIGURES (continued)

Figure **Page**

13 Illustrate diagram of Part II study 43

14 Numbers of total and pandemic strains of *V. parahaemolyitcus* isolated from patients in Hat Yai Hospital from 2000 to 2005 54

15 Comparison of number of *V. parahaemolyticus* isolates belong to 5 groups of toxin gene profile obtained from patients in Hat Yai Hospital between 2000 -2005 and 2006-2010 54

16 AP-PCR profile of *V. parahaemolyticus* isolates from shellfish DNA fingerprints of identical or nearly identical serotypes of *V . parahaemolyticus* isolates from shellfish were determined. The results were obtained with primer 2. 61

17 PFGE confirmation of *V. parahaemolyticus* isolates from mussel and hard clam. Both isolates of each serotype (O1:KUT and O8:KUT) of *V. parahaemolyticus* obtained from mussel and hard clam were investigated 62

18 Colony characteristic and Gram staining of *B. amyloliquefaciens* SM11 on TSA agar 65

LIST OF FIGURES (continued)

Figure **Page**

19 Neighbour-joining phylogenetic tree of *B. amyloliquefaciens* SM11 constructed by Mega 4.0.2 . 66

20 Conditions of biosurfactants production in Mckeen medium by *B. amyloliquefaciens*; A: inoculums size; B: temperature; C: pH and D: speed of shaker 67

21 Kinetic production of biosurfactants produced by *B. amyloliquefaciens* 68

22 Adhesion inhibition of biosurfactants derived from *B. amyloliquefaciens* to *V. parahaemolyticus* 70

23 Ionic charge of biosurfactants derived from *B. amyloliquefaciens*; CTAB: cetyltrimethylammonium (cationic surfactant); SDS: sodium dodecyl sulfate (anionic surfactant); DW: distilled water. 71

24 Biosurfactants purification by DEAE cellulose anion exchange chromatography 72

25 Active fractions (PSM1-PSM5) of biosurfactants detected by RP-HPLC 73

LIST OF FIGURES (continued)

Figure **Page**

26 ESI-Q-TOF MS spectrometric characteristic of PSM3 (A) and PSM4 (B) fractions. 75

27 ESI-Q-TOF MS spectrometric characteristic of PSM1 (A), PSM2 (B) and PSM5 (C) fractions. 75

28 Infrared spectrum analysis of RP-HPLC-purified bioactive PSM4 fraction to compare with those bonds in functional groups of surfactin (Sigma) (Das *et al.*, 2008) 77

29 Infrared spectrum analysis of RP-HPLC-purified bioactive PSM5 fraction to compare with those bonds in functional groups of surfactin (Sigma) (Das *et al.*, 2008) 78

LIST OF ABBREVIATIONS AND SYMBOLS

AP-PCR = Arbitrarily primed polymerase chain reaction

APW = alkaline peptone water

cfu = colony forming unit

CTAB = cetyltrimethylammoniumbromide

DEAE = diethylaminoethyl cellulose

dNTP = deoxynucleoside triphosphate

DW = distilled water

EDTA = ethylenediaminetetraacetic acid

EPS = extracellular polymeric substance

ESI- Q-TOF MS = Electrospray ionization quadrupole time-of-flight mass spectrometry

FTIR = Fourier transform infrared

g = gram

GS-PCR = Group specific polymerase chain reaction

HCl = hydrochloric acid

KP = Kanagawa phenomenon

IS = insertion sequence

LB = Luria-Bertani

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

MALDI-TOF MS = Matrix-assisted laser desorption ionization time of flight mass spectrometry

Mb = megabase

MBC = minimum bactericidal concentration

MHA = Mueller Hinton agar

MHB = Mueller Hinton broth

MIC = minimum inhibitory concentration

min = minute

mM = millimolar

MPN = Most probable number

MSSW = modified synthetic sea water medium

NaOH = sodium hydroxide

nm = nanometer

OD = Optical density

PAI = pathogenicity island

PBS = phosphate buffer saline

PCR = Polymerase chain reaction

PFGE = Pulsed field gel electrophoresis

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

RP-HPLC = Reverse phase high-performance liquid chromatography

SDS = sodium dodecyl sulfate

TBE = Tris borate EDTA

TCBS = Thiosulfate-citrate-bilesalts-sucrose

TDH = Thermostable direct hemolysin

tdh = thermostable direct hemolysin gene

TFA = trifluoroacetic acid

TRH = TDH-related hemolysin

trh = TDH-related hemolysin gene

TSA = trypticase soy agar

T3SS = Type III secretion system pathogenicity island

UT = untypeable

μg = microgram

μl = microliter

μM = micromolar

μm = micrometer

CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium detected worldwide. It is naturally found in marine environments including shellfish, mollusk, crustacean and seawater because it is a part of the normal microflora of coastal and estuarine waters (Yeung and Boor, 2004). Distribution and population dynamics of *V. parahaemolyticus* may be controlled by temperature and salinity. High density of total and pathogenic *V. parahaemolyticus* were observed with high water temperatures which could explain the seasonality of infections and being more abundant in warmer period of time (DePaola *et al.*, 2000; Geneste *et al.*, 2000 ; Kelly and Stroh, 1989). *V. parahaemolyticus* is a food-borne pathogen that causes mild to moderate gastrointestinal illness after consumption of raw or improper cooked seafood (Yeung and Boor, 2004). The infective dose of *V. parahaemolyticus* has been estimated to be 10^5 to 10^7 organisms contaminated in seafood (Sanyal and Sen, 1974). Clinical symptoms include abdominal pain, watery diarrhea, nausea, vomiting and is occasionally associated with headache, fever and chills (Yeung and Boor, 2004).

An important virulence factor of *V. parahaemolyticus* is thermostable direct hemolysin (TDH) that causes this bacterium to exhibit β-hemolysis on Wagatsuma agar (Nishibuchi and Kaper, 1995). It has been considered as a crucial marker to distinguish pathogenic from non pathogenic strains (Nishibuchi *et al.*, 1992). Another virulence factor is TDH-related hemolysin (TRH) (Honda *et al.*, 1988). TDH and TRH are encoded by *tdh* and *trh* genes, respectively. *toxRS* operon has been reported to involve in regulation of these virulence genes (Lin *et al.*, 1993; Reich and Schoolnik, 1994). Around 90% of clinical isolates of *V. parahaemolyticus* possess either *tdh*, *trh* or both genes. However, the pathogenic strains of *V. parahaemolyticus* (*tdh*$^+$ or *trh*$^+$) have been isolated less frequently from environments

with the number of 1 to 10% depending on geographic locations and detection techniques (Cook *et al.*, 2002; Johnson *et al.*, 2009; West *et al.*, 2013; Wootipoom *et al.*, 2007). Classification of *V. parahaemolyticus* by serotyping is based on difference in its lipopolysaccharide (O) and capsular (K) antigens (Joseph *et al.*, 1982). At present time only 13 O and 74 K serotypes have been established, while there are many environmental strains of *V. parahaemolyticus* that are untypeable (UT) because environmental strains have not been included in establishment of the O:K serotype (Okuda *et al.*, 1997).

In 1996, a new and unique clone of serotype O3:K6 *V. parahaemolyticus* (tdh^{+} trh^{-}) emerged in India (Matsumoto *et al.*, 2000; Okuda *et al.*, 1997). Group-specific PCR (GS-PCR) has been established to detect nucleotide variations within the 1,364 bp of *toxRS* region (Matsumoto *et al.*, 2000). This strain has spread globally to Europe, Africa, South America, the United States and especially in many Asian countries, such as India, Bangladesh, and Thailand (Matsumoto *et al.*, 2000; Okuda *et al.*, 1997). Thus, it has been assigned as a pandemic strain. This strain has increased globally in the last 10 years since 1996 (Hurley *et al.*, 2006). Later on distinct pandemic serotypes (O1:K25, O4:K68, O1:KUT etc.) have been identified. It has demonstrated that they have diverged from the O3:K6 original pandemic clone (Bhuiyan *et al.*, 2002; Matsumoto *et al.*, 2000).

In Thailand, O3:K6 *V. parahaemolyticus* pandemic strain was first isolated from patients in Hat Yai Hospital in 2000. In addition, one isolate of the pandemic strain was detected in shellfish (Vuddhakul *et al.*, 2000). Continue investigation of *V. parahaemolyticus* in this hospital for 6 years between 2000 and 2005 revealed that most of *V. parahaemolyticus* isolates obtained were pandemic strains. Consumption of shellfish especially cockle may be the major cause of infection (Vuddhakul *et al.*, 2006). Thus, in order to confirm that this area is endemic for *V. parahaemolyticus* and shellfish especially mollusk is the important vehicle for infection, continue evaluation infection due to *V. parahaemolyticus* in this hospital and enumeration of this bacterium in many kinds of shellfish should be performed.

Transmission of *V. parahaemolyticus* to human can be direct transmission via consumption of raw or undercooked seafood and indirect transmission via cross-contamination of *V. parahaemolyticus* to other foods by

surface contamination during preparation. Kim *et al*. (2012) demonstrated that fish fillet inoculated with of *V. parahaemolyticus* was able to transfer to cutting board and caused cross contamination of *V. parahaemolyticus* to cucumber. In China markets, crustaceans produced in freshwater ponds were cross-contaminated with *V. parahaemolyticus* (Yano *et al.*, 2006). Finally, in Thailand, acute gastroenteritis caused by *V. parahaemolyticus* O4:K55 in nursing students has been reported to be due to cross-contaminated with boiled eggs (Jatapai *et al.*, 2010).

Various techniques have been demonstrated to reduce *V. parahaemolyticus* contaminated in seafood, such as high pressure, thermal process and depuration (Ma and Su, 2011; Shen *et al.*, 2009; Su *et al.*, 2010). To eliminate *V. parahaemolyticus* on contact surfaces is also important to reduce cross contamination. Normally physical and chemical methods such as cleaning and disinfection procedures have been extensively used over the years. However, it has been reported that bacteria attached to surfaces were more resistant to sanitization than free-living cells (Hood and Zottola, 1995; Joseph *et al.*, 2001).

Biosurfactants are amphipathic molecules with hydrophilic and hydrophobic moieties and prefer to be between oil and water or water and air. They are widely used in many environmental applications (Desai and Banat, 1997). One of bioactive compounds classified as biosurfactant exhibits anti-adhesive activity (Janek *et al.*, 2012). The aims of this study are investgation of incidence of *V. parahaemolyticus* infection in Hat Yai Hospital, determination number *V. parahaemolyticus* presented in seafood especially shellfish and isolation of bacteria from different sources of soil to investigate their potential for effective producing biosurfactants to decrease *V. parahaemolyticus* adherence on surface.

LITERATURE REVIEWS

Vibrio parahaemolyticus characterization and distribution

Vibrio parahaemolyticus was first discovered in 1950 in Japan during a food-poisoning outbreak, it caused 279 illness and 20 deaths associated with consumption of sardines (Daniels *et al.*, 2000b). This organism is a Gram-negative, non spores forming, curved rod-shaped bacterium (0.5 - 0.8 µm in width and 1.4 - 2.4 µm in length). Its classified is in kingdom: Bacteria, phylum: Proteobacteria, class: Gamma-Proteobacteria, order: Vibrionales, family: Vibrionaceae, genus: *Vibrio* (Drake *et al.*, 2007). It is facultative anaerobe that can ferment glucose without gas production and positive for oxidase (Butt *et al.*, 2004). It has a polar flagellum and lateral flagella which enables it to move in liquid media and migrate across semi-solid surfaces (swarming), respectively (Stewart and McCarter, 2003). It is a moderate halophile and does not grow unless a salt concentration of at least 0.5% is present. In fact it can grow at salt levels as high as 8% (DePaola *et al.*, 2000).

V. parahaemolyticus can be detected at 1500 km offshore. Distribution and population dynamics of *V. parahaemolyticus* may be shaped by existence of an oceanic transport of this organism and zooplankton (Martinez *et al.*, 2012). *V. paraheamolyticus* is a part of normal microflora of coastal and estuarine water in tropical and almost all temperate regions. In temperate region, it may be present in high numbers when water temperature is high during summer (Martinez *et al.*, 2012). It is assumed that frequency of *V. parahaemolyticus* in environment is correlated to temperature and salinity. DePaola *et al*. (2000) observed that salinity affected *V. parahaemolyticus* level less than temperature. However, Rodriguez *et al.*, (2010) observed that salinity was the primary factor governing distribution of *V. parahaemolyticus* in mussel at Galicia in Spain.

Pathogenesis and virulent factors

Not all strains of *V. parahaemolyticus* are considered pathogenic, but almost all clinical *V. parahaemolyticus* isolates exhibit β-hemolysis on Wagatsuma agar which known as Kanagawa phenomenon (KP). The KP-positive strains are induced by a thermostable direct hemolysin (TDH) which produced by the organism and has been considered as a crucial marker to distinguish pathogenic from non pathogenic strains. TDH is encoded by *tdh* gene. Deletion of this gene resulted in loss of enterotoxic activity in laboratory investigation (Nishibuchi *et al.*, 1992). TDH is a protein toxin which composed of 165 amino acid residues, and it displays several biological activities, i.e., hemolytic activity, enterotoxicity, cytotoxicity, and cardiotoxicity (Takeda, 1982). The *tdh* gene is located on pathogenicity islands containing gene clusters including *toxR*, transposase gene, type III secretion system (T3SS)-related gene and other genes of pandemic *V. parahaemolyticus* on chromosome II as shown in Figure 1 (Chen *et al.*, 2011)**.**

Five *tdh* genes designated as *tdh*1 to *tdh*5 have been reported (Nishibuchi and Kaper, 1985). In addition, genes encoding *tdh* detected in 3 strains of *V. parahaemolyticus* and designated as *tdh*S, *tdh*A, *tdh*X and *tdh*/I were cloned (Iida and Yamamoto, 1990). It has been demonstrated that KP-positive *V. parahaemolyticus* usually contains two non-identical copies of the *tdh*1 and *tdh*2 but many KP-negative or weakly KP-positive *V. parahaemolyticus* containe only one *tdh* gene copy (Nishibuchi and Kaper, 1985).

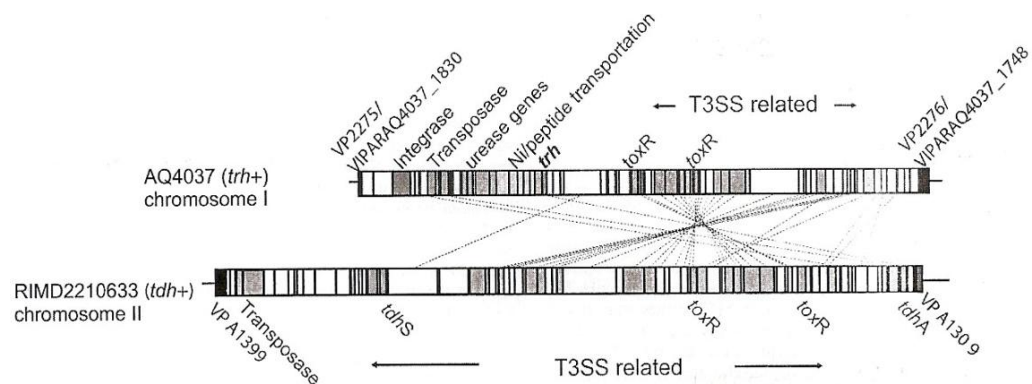

Figure 1 *V. parahaemolyticus* pathogenicity islands containing *tdh* and *trh* genes. [Source: From reference (Chen *et al.*, 2011)]

Investigation of an outbreak in Maldives in 1985 revealed that some clinical *V. parahaemolyticus* strains do not possess the *tdh* gene but carry the *tdh*-related hemolysin (*trh*) gene which responded in producing TDH-related hemolysin (TRH) (Nishibuchi *et al.*, 1989). TRH exhibits various biological activities similar to TDH, such as fluid accumulation in rabbit ileal loops, increase of rabbit skin vascular permeability, and cardiotoxicity on cultured myocardial cells. *trh* gene can be divided into two subgroups, *trh1* and *trh2* (Kishishita *et al.*, 1992). Molecular epidemiological studies have demonstrated that clinical isolates of *V. parahaemolyticus* possess *tdh*, *trh*, or both genes; but environmental isolates rarely carry these genes (Shirai *et al.*, 1990).

The *trh* gene is located on pathogenic island containing urease encoding gene (Figure 1) (Chen *et al.*, 2011). Thus, urease production is one factor that indicates the possession of *trh* gene. It has been reported that 59 out of 60 (98%) urease positive strains of *V. parahaemolyticus* isolated from patients on the West Coast of the United States, harbored the *trh* (either *trh*1 or *trh*2) gene and 54 strains (90%) carried the *tdh* gene (Okuda *et al.*, 1997). Thus, the presence of hemolysin genes is always considered as current markers of pathogenicity in *V. parahaemolyticus*. TRH induces Ca^{2+}activated Cl^{-} channnels with result in altered ion flux (Takahashi *et al.*, 2000a) and stimulates fluid secretion in the rabbit ileal loop test, which suggests a possible role of this toxin in inducing diarrhea and has an amino acid sequence that is approximately 67% homologous with *V. parahaemolyticus*-TDH (Honda *et al.*, 1988; Xu *et al.*, 1994). However, *V. parahaemolyticus* lacking both *tdh* and *trh* genes have been reported from some clinical specimens (Bhoopong *et al.*, 2007).

The *toxR* gene was first described as a regulatory gene of cholera toxin, however, it has been shown later to involve in regulation of many other genes in *Vibrio cholerae* (DiRita, 1992). The *toxR* is clustered with *toxS* in an operon. *toxRS* is encoded for transmembrane proteins that involved in regulation of virulence-associated genes and is well conserved in the genus *Vibrio* (Lin *et al.*, 1993; Reich and Schoolnik, 1994). In *V. parahaemolyticus*, the level of TDH production may be under the control of *toxRS* because *toxRS* promoted the expression of the *tdh*2 gene at the transcriptional level (Nishibuchi *et al.*, 1991). The *tdh*2 was enhanced up to five

fold by the activity of ToxR in a culture medium-dependent manner. In addition, a KP-positive strain caused less fluid accumulation in rabbit ileal loops when the *toxR* gene was inactivated. Thus, ToxR appears to play an important role in stimulating *tdh*, the gene encoding a major virulence factor of *V. parahaemolyticus*. The *toxR* is one of targeted PCR for identification of *V. parahaemolyticus* (Kim *et al.*, 1999).

Type III secretion system (T3SS) is a complex protein appendage similar to needle found in several Gram-negative pathogenic bacteria including *V. parahaemolyticus* (Figure 2). T3SS secretes and translocates bacterial virulence factor across membrane into cytoplasm of eukaryotic host (Hueck, 1998). The virulence factor proteins delivered by T3SS are capable of modulating and interfering with host cellular processes, which cause diseases in animals and plants (Park *et al.*, 2004b). Genes encoding T3SS are clustered, and usually located within the pathogenicity island (PAI) region on bacterial chromosome (Makino *et al.*, 2003).

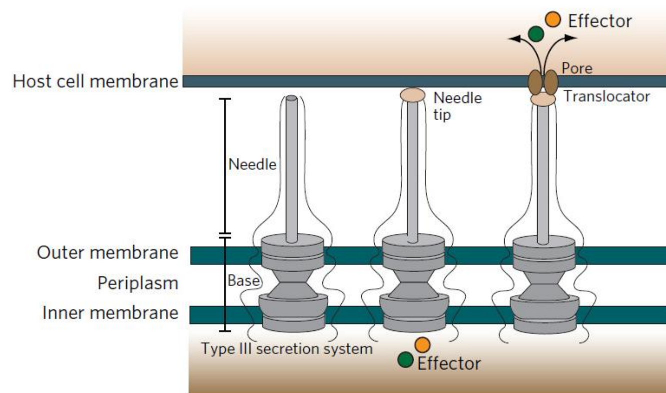

Figure 2 T3SS is composed of a basal body, a needle structure and a needle tip. [Source: From reference (Ashida *et al.*, 2011)]

Two sets of T3SS genes were identified in *V. parahaemolyticus* strain RIMD2210633. T3SS1 is located at positions 1.77 to 1.81 Mb from the replication origin on chromosome 1. Another set of genes for the T3SS were identified within the pathogenicity island (PAI) region at positions 1.38 to 1.47 Mb from the replication origin on chromosome 2 (Makino *et al.*, 2003). This region was obtained by horizontal gene transfer and was designated as T3SS2. Some KP positive *V.*

parahaemolyticus possesses both T3SS1 and T3SS2, while KP negative of *V. parahaemolyticus* possesses only T3SS1 (Park *et al.*, 2004b).

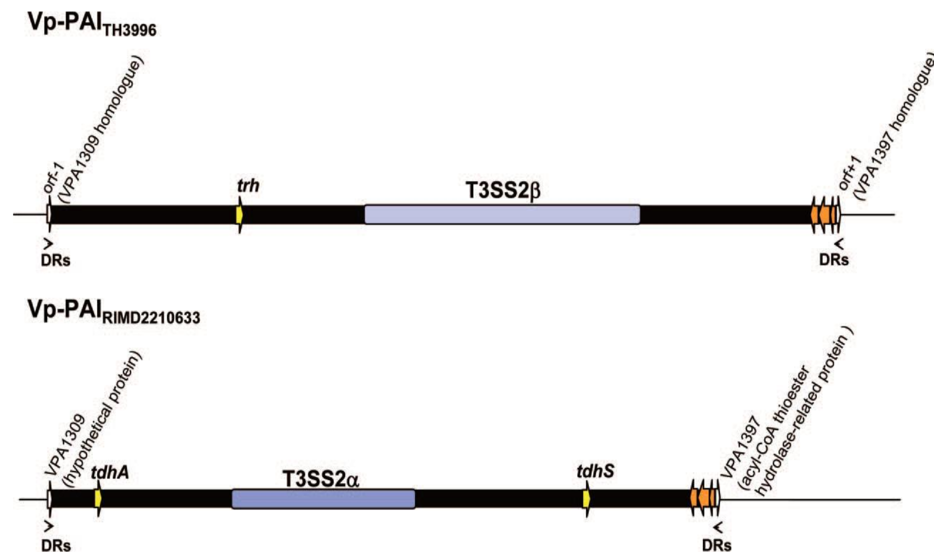

Figure 3 Comparison of pathogenicity island of *V. parahaemolyticus* between *V. parahaemolyticus* TH3996 *trh*-positive and *V. parahaemolyticus* RIMD2210633 *tdh*-positive. [Source: From reference (Okada *et al.*, 2009)]

Investigation of *V. parahaemolyticus* TH3996 (*tdh*$^-$*trh*$^+$) and *V. parahaemolyticus* RIMD2210633 (*tdh*$^+$*trh*$^-$) revealed T3SS2β and T3SS2α, respectively on chromosome 2 (Okada *et al.*, 2009) (Figure 3). Determination of T3SS2α and T3SS2β in 130 and 12 environmental and clinical *V. parahaemolyticus* isolates, respectively using *vopB2* probe revealed all *tdh*$^+$*trh*$^-$ *V. parahaemolyticus* isolates were positive for T3SS2α whereas, T3SS2β was detected in 109 isolates of *trh*$^+$ *V. parahaemolyticus* (Noriea Iii *et al.*, 2010).

Pandemic strain of *V. parahaemolyticus*

Since 1996, *V. parahaemolyticus* O3:K6 serotype carrying *tdh* gene has been reported from patients in many Asian countries including Europe, the United States and Africa (Matsumoto *et al.*, 2000; Okuda *et al.*, 1997). Comparison of *V. parahaemolyticus* isolates obtained before and after 1996 revealed differences in *toxRS* nucleotide sequence which used for PCR detection of pandemic strain designated as group specific PCR (GS-PCR) (Matsumoto *et al.*, 2000). Later on other serotypes (O1:K25, O4:K68, O1:KUT etc.) of pandemic strains have been reported and they are considered as clone of O3:K6 pandemic clone (Okura *et al.*, 2004; Wong *et al.*, 2005).

Infection by *V. parahaemolyticus*

V. parahaemolyticus causes mild to moderate gastrointestinal illness (Levine and Griffin, 1993). Primary mode of transmission is through ingestion of raw, undercooked, or contaminated shellfish (McLaughlin *et al.*, 2005). Most sporadic infections and outbreaks were linked to consumption of contaminated, raw molluscan shellfish (Daniels *et al.*, 2000a). The regulation of food hygiene in Japan indicates number of *V. parahaemolyticus* is less than 10^2 MPN/g in raw consumption of seafood (Yano *et al.*, 2006). However, number of *V. parahaemolyticus* indicated by FDA in molluscan shellfish is 10^4per gram (Cook *et al.*, 2002).

The Centers for Disease Control and Prevention of the United State (CDC) indicated that between 1973 and 1998, 40 outbreaks of *V. parahaemolyticus* infections caused more than 1000 illnesses. Most of these outbreaks caused by seafood, particularly shellfish and occurred during warm months. Between 1988 and 1997, 345 sporadic *V. parahaemolyticus* infections were reported, variety of infections included gastroenteritis (59%), wound infections (34%), septicemia (5%), and other exposures (2%) (Daniels *et al.*, 2000a). Forty-five percent of patients suffering from these conditions were hospitalized, and 88% of patients with acute gastroenteritis consumed raw oysters before their illnesses occurred.

Skin infection due to *V. parahaemolyticus* is less common than seafood-borne disease but it may occur when open wounds are exposed to warm seawater (Butt *et al.*, 2004). Swelling, redness, and pain may develop at the site of infection. This is generally more severe than gastrointestinal illness and requires hospitalization. Additionally, swimming or working in *V. parahaemolyticus* contaminated areas can lead to infections of eyes, ears or open cut areas (Penland *et al.*, 2000).

On rare occasions, infection can spread to bloodstream and causes septicemia with low blood pressure and shock (Butt *et al.*, 2004). There was 3 cases of septicemia with 2 deaths in Louisiana and Mississippi after Hurricane Katrina in 2005 (Su and Liu, 2007). The organism has not been shown to spread directly from one person to another. Contact with an infected person is not a risk of infection. Mechanism of *V. parahaemolyticus* infections has not been fully elucidated (Baffone *et al.*, 2005). However, incubation period ranges from 2 to 48 h. The common symptoms include diarrhea, abdominal pain, nausea, vomiting, fever and bloody stools. Duration of illness usually lasts for about 2 to 5 days (Daniels *et al.*, 2000a). Infection is usually self-limiting. In a volunteer feeding study, the infective dose of *V. parahaemolyticus* was determined to be between 10^5 and 10^7 organisms. Participants were healthy young adults who were given antacid tablets before being fed with *V. parahaemolyticus* (Sanyal and Sen, 1974).

Detection of *V. parahaemolyticus* in foods is commonly performed by most probable number (MPN) technique and culture on Thiosulfate-Citrate-Bilesalts-Sucrose (TCBS) agar, however, this method cannot differentiate between *V. parahaemolyticus,* and other *Vibrio* spp. such as *V. vulnificus, V. mimicus* because all of them are non-sucrose fermenter. Polymerase chain reaction (PCR) targeted to *toxR* or *tlh* genes is normally used to confirmed *V. parahaemolyticus*. DNA hybridization with specific probes, and chromogenic medium can also distinguish *V. parahaemolyticus* from other *Vibrio* spp.. Using chromogenic medium, CHROMagar Vibrio, colony of *V. parahaemolyticus* exhibits purple color (Hara-Kudo *et al.*, 2001). For epidemiological investigation, serotyping and DNA fingerprinting are required for confirmation the source of outbreaks (Okuda *et al.*, 1997; Vuddhakul *et al.*, 2000).

Treatment of *V. parahaemolyticus* can be performed by oral rehydration which is usually sufficient because the illness is usually mild and self-limited. Antimicrobial therapy may be helpful for patients with severe or prolonged diarrhea and can be life-saving for those with wound infections or septicemia (Yeung and Boor, 2004).

V. parahaemolyticus has been shown to susceptible to many antibiotics such as doxycycline, ciprofloxacin, tetracycline, ceftriaxone, chloramphenicol, imipenem, ofloxacin, nitrofurantoin, meropenem, oxytetracycline, fluoroquinolones, third generation cephalosporins, and aminoglycosides (Baker-Austin *et al.*, 2008; Butt *et al.*, 2004). Erythromycin may be used for pregnant women and children. Some antibiotics resistance has been reported for penicillin, ampicillin, apramycin, cephalothin, gentamycin, trimethoprim, and streptomycin (Baker-Austin *et al.*, 2008).

Cross-contamination by *V. parahaemolyticus*

Outbreaks of *V. parahaemolyticus* occured along coastal regions during summer and early fall when high water temperatures increased high level of bacteria in seafood (DePaola *et al.*, 2000). Transmission of *V. parahaemolyticus* to human has been demonstrated in 2 ways, direct transmission is through consumption of raw or undercooked seafood and indirect transmission causes by cross-contamination between seafood and other foods (Aarestrup and Wegener, 1999).

V. parahaemolyticus can be transferred from seafood to other foods, either by direct contact or indirect contact through food handlers, contact surfaces, or utensils. Cross-contamination has been reported as a main factor in some outbreaks. It has been demonstrated that steamed crabs and crab salad prepared from canned crabmeat were the vehicle of *V. parahaemolyticus* infection in Maryland outbreak. The steamed crabs was cross-contaminated after cooking with live crabs, surfaces contaminated with *V. parahaemolyticus* during preparation was suspected (Dadisman *et al.*, 1972). Outbreak of foodborne disease associated with passenger in ship has been demonstrated (Rooney *et al.*, 2004). Illness in one-third of passengers were caused by seafood whereas the rest was implicated with salads, eggs, poultry, and red

meat. Another outbreak, on a cruise ship was associated with shrimp contaminated with other foods during preparation (Rooney *et al.*, 2004). Two outbreaks of food poisoning caused by *V. parahaemolyticus,* were associated with the use of seawater from the ship's fire system (Lawrence *et al.*, 1979).

Yano *et al*, (2006) investigated cross-contamination of *V. parahaemolyticus* in six species of crustaceans collected from freshwater ponds in China market. The number of *V. parahaemolyticus* contaminated in those crustaceans were between 1.1 -5.1 log MPN/ml. In Thailand, acute gastroenteritis caused by *V. parahaemolyticus* O4:K55 in nursing students has been reported due to cross-contaminated in boiled eggs (Jatapai *et al.*, 2010). Cross-contamination due to cutting board has been documented (Kim *et al.*, 2012). Fish fillets inoculated with initial populations of *V. parahaemolyticus* without and with 3% (w/v) salt were 6.10 and 6.57 log cfu/g, respectively. After 1h, levels of *V. parahaemolyticus* transferred to cutting board surfaces without and with 3% salt were 2.8–4.1 and 1.6–3.6 log cfu/g, respectively. In addition, transfer levels of *V. parahaemolyticus* from the cutting boards to cucumber were 3.36 and 3.27 log cfu/unit area on the wood cutting boards at 25°C indicating high levels of this bacterium could cross-contaminate to cucumber.

Thus, it is important to train food handlers in good hygiene and promotes their awareness in cross-contamination between seafood and other foods to reduce risk of *V. parahaemolyticus* infection.

Cross-contamination control

Cross-contamination of food can occur in the final stages of food preparation, packaging, storage, transport to market and restaurant or cook processing at home.

Therefore, raw seafood should be separated, either physically or by time, from ready-to-eat foods, with effective intermediate cleaning, where appropriate. Surfaces, utensils, equipment, fixtures, and fittings should be thoroughly cleaned after raw seafood has been handled (Rooney *et al.*, 2004).

Proper cleaning and sanitizing to eliminate or reduce levels of microbiological contamination are required (Commission and Programme, 2003).

Kim *et al*. (2012) demonstrated that a combined cleaning method of wipping cutting board with an alcohol paper towel followed by running water was the most effective to eliminate *V. parahaemolyticus*. However, alcohol may be hazardous and activate enzyme reaction in seafood. Therefore, anti-adhesive property of biosurfactants may be an alternative way to decrease *V. parahaemolyticus* on surface to reduce cross-contamination (Falagas and Makris, 2009; Janek *et al.*, 2012; Meylheuc *et al.*, 2001; Rivardo *et al.*, 2009).

Microbial bioactive compounds

Since 1920s when the first antibiotic penicillin was discovered from soil, it was believed that soil microorganisms are the largest source of novel bioactive compounds. Around 18% of microorganisms including bacteria, fungi and algae obtained from marine and soil environments are sources of biomedical compounds (Blunt *et al.*, 2006). Most of them are aerobic spore-forming bacteria in the genera *Bacillus* and *Streptomyces* and fungi in the genera *Penicillium* and *Cephalosporium* (Okulate, 2009). However, some strains of marine bacteria showed antimicrobial activity and were in genera *Alteromonas*, *Pseudomonas* and *Flavobacterium* (Zheng *et al.*, 2005).

Bioactive compounds produced by microorganisms are depended on certain physico-chemical factors, oxygen concentration, temperature and pH. It was found that these parameters enhanced their metabolite production (Olano *et al.*, 2008). Some bacteria produced certain compounds that helped in cleaning and protecting their environment. They were classified as biosurfactants producers (Das *et al.*, 2009).

Biosurfactants

Biosurfactants are surface-active compounds that usually extracellular released from bacteria, yeast or fungi. They are capable of reducing surface and interfacial tension and have a wide range of industrial and environmental applications (Mukherjee and Das, 2010). Biosurfactant molecules have both hydrophilic and hydrophobic moieties that partite preferentially at interface between fluid phases with

different degrees of polarity and hydrogen bond. The polar moiety can be a carbohydrate, an amino acid, a phosphate group, or some other compounds. The non-polar moiety is mostly a long-carbon-chain fatty acid (Banat, 1995). Those characteristics allow it to be excellent detergent, emulsifying and foaming. Their low toxicity and environmental friendly nature make them to be useful in industrial applications, in bioremediation, health care and food processing (Cameotra and Makkar, 2004). Several patents have been issued on biosurfactants produced by *Acinetobacter* spp., *Bacillus* spp. and *Pseudomonas* spp. (Shete *et al.*, 2006).

Biosurfactants can be separated into high and low molecular weight molecules such as lipopeptides, glycolipids, phospholipids, emulsan and polysaccharides (Table 1) (Rosenberg and Ron, 1999). Low molecular weight molecules efficiently reduce surface and interfacial tension whereas high molecular weight molecules or polymers stabilize emulsions but do not decrease much surface tension (Neu, 1996; Rosenberg and Ron, 1999).

Table 1 Biosurfactants obtained from various microorganisms.

Biosurfactants	Producing microorganisms	References
Low molecular weight		
Lipopeptide	*Nocardiopsis* sp.B4 (sponge in marine actinomycetes)	(Khopade *et al.*, 2012)
Lipopeptide	*Lactobacillus paracasei* ssp. *paracasei* A20	(Gudina *et al.*, 2010)
Lipopeptide	*Brevibacterium aureum* MSA13 (marine actinobacterium)	(Seghal Kiran *et al.*, 2010)
Trehaloselipids	*Rhodococcus erythropolis* 51T7	(Marques *et al.*, 2009)
Glycolipid	*Ustilago maydis*	(Hewald *et al.,* 2005)
Serrawettin W1	*Serratia marcescens*	(Li *et al.*, 2005)
Rhamnolipids	*Pseudomonas* spp.	(Zhang and Miller, 1992)
Surfactin (lipopeptides)	*Bacillus subtilis*	(Neu and Poralla, 1990)
Sophorolipids	*Candida borgoriensis*	(Cutler and Light, 1979)
High molecular weight		
Biosurfactant	*Aspergillus ustus* MSF3 (marine endosymbiotic fungi)	(Kiran *et al.*, 2009)
Extracellular polymeric substance (EPS)	*Planococcus maitriensis* Anita I	(Kumar *et al.*, 2007)
Exopolysaccharide	*Halomonas maura*	(Bouchotroch *et al.*, 2001)
Food emulsifier	*Candida utilis*	(Shepherd *et al.*, 1995)
Thermophilic emulsifier	*Bacillus stearothermophilus*	(Gurjar *et al.*, 1995)
Alasan	*Acinetobacter radioresistens* KA53	(Navon-Venezia *et al.*, 1995)
Biodispersan	*Acinetobacter calcoaceticus* A2	(Rosenberg, 1993)

Biosurfactants have been reported in some *Bacillus* spp. (Table 2). Members of this genus produce both types of antimicrobial peptides, ribosomally synthesized bacteriocins such as subtilin and non-ribosomally synthesized lipopeptides such as surfactin (Banerjee and Hansen, 1988; Moyne *et al.*, 2001).

Table 2 Biosurfactants reported from *Bacillus* spp.

Bacillus spp.	Family of biosurfactants	References
B. amyloliquefaciens CCMI 1051	surfactin	(Caldeira *et al.*, 2011)
B. subtilis LSFM-05	fengycin	(de Faria *et al.*, 2011)
B. amyloliquefaciens GA1	surfactin, iturin A, fengycin	(Arguelles *et al.*, 2009)
B. subtilis natto TK-1	surfactin	(Cao *et al.*, 2009)
B. polyfermenticus KJS-2	surfactin	(Kim *et al.*, 2009)
B. circulans	surfactin	(Das *et al.*, 2008)
B. subtilis C-1.	surfactin, iturin A, fengycin	(Vater *et al.*, 2002)
B. amyloliquefaciens RC-2	iturin	(Yoshida *et al.*, 2001)
B. licheniformis BAS50	lichenysin (surfactin)	(Yakimov *et al.*, 1995)
B. subtilis	iturin	(Maget-Dana and Peypoux, 1994)

Biosurfactant lipopeptides synthesized by *Bacillus* spp.

Members of the *Bacillus* genus are often considered microbial factories for the production of lipopeptide biosurfactants including 3 major families; surfactin, iturin and fengycin. These biosurfactants have been used in biotechnology and biopharmaceutical applications based on their surfactant properties (Ongena and

Jacques, 2008). A recently work demonstrated a *Bacillus* sp. also produced a mixture of closely related cyclic lipopeptide isoforms of biosurfactants (Snook *et al.*, 2009)**.**

Surfactin family

Surfactin and closely related other variants based on type and amino acid position such as esperin, lichenysin and pumilacidin have been isolated from *B. amyloliquefaciens* CCMI 1051, *B. mesentericus*, *B. licheniformis* BAS50 and *B. pumilus*, respectively (Ito and Ogawa, 1959; Naruse *et al.*, 1990; Yakimov *et al.*, 1995), (Table 3). They are heptapeptides molecules and belong to surfactin family (Cooper and Zajic, 1980).

Table 3 Amino acid composition of heptapeptides in surfactin family

Surfactin family	Amino acid composition
Surfactin	**L-Glu**$_1$**-L-XS**$_2$-D-Leu$_3$-**L-XS**$_4$-L-Asp$_5$-D-Leu$_6$-**L-XS**$_7$
Esperin	**L-Glu**$_1$**-L-Leu**$_2$-D-Leu$_3$-**L-Val**$_4$**-**L-Asp$_5$-D-Leu$_6$-L-**Leu**$_7$
Pumilacidin	**L-Glu**$_1$**-L-Leu**$_2$-D-Leu$_3$-**L-Leu**$_4$-L-Asp$_5$-D-Leu$_6$-**L-XP**$_7$
Lichenysin	**L-XL**$_1$**-L-XL**$_2$-D-Leu$_3$-**L-XL**$_4$-L-Asp$_5$-D-Leu$_6$-**L-XL**$_7$

XS_2 = Val, Leu or Ile; XS_4 = Ala, Val, Leu or Ile and XS_7 = Val, Leu or Ile; XP_7 = Val or Ile; XL_1 = Gln or Glu; XL_2 =Leu or Ile and XL_7 = Val or Ile
[Source: From reference (Ongena and Jacques, 2008)]

Surfactin

Surfactin is the most powerful biosurfactants. Surfactin is heptapeptides chain linked to a β-amino fatty acid chain with a variable length of 12 to 15 carbon atoms (Figure 4) (Arima *et al.*, 1968). Surfactin is amphoteric, possessing both a positive and negative charge of amino acid in heptapeptide chain and hydrophobic charge of fatty acid chain. It exhibited the lowers surface tension of water from 72 to 27 mN/m (Cooper and Zajic, 1980). Antibiotic activity of surfactin

is based on its ability to alter cell membrane in a dose-dependent manner (Bernheimer and Avigad, 1970). This causes surfactin displayed haemolytic activity, antiviral (Kracht *et al.*, 1999), anti-mycoplasma (Vollenbroich *et al.*, 1997b) and antibacterial activities (Yakimov *et al.*, 1995).

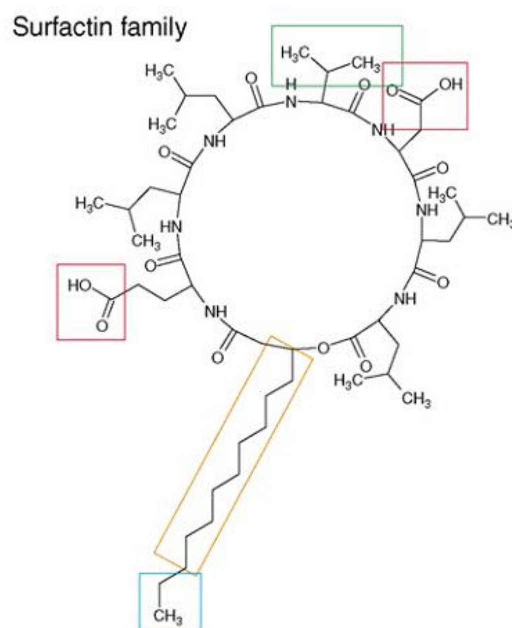

Figure 4 Structure of cyclic lipopeptide surfactin
[Source: From reference (Ongena and Jacques, 2008)]

Iturin family

Iturin A and C, bacillomycin D, F, L and LC (bacillomycin) and mycosubtilin possess different type and position of amino acid (Table 4), but they belong to iturin family.

Table 4 Amino acid composition of heptapeptides in iturin family

Iturin family	Amino acid composition
Iturin A	**L-Asn$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Gln$_4$-L-Pro$_5$-D-Asn$_6$-L-Ser$_7$**
Iturin C	**L-Asp$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Gln$_4$-L-Pro$_5$-D-Asn$_6$-L-Ser$_7$**
Bacillomycin D	**L-Asn$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Pro$_4$-L-Glu$_5$-D-Ser$_6$-L-Thr$_7$**
Bacillomycin F	**L-Asn$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Gln$_4$-L-Pro$_5$-D-Asn$_6$-L-Thr$_7$**
Bacillomycin L	**L-Asp$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Ser$_4$-L-Gln$_5$-D-Ser$_6$-L-Thr$_7$**
Bacillomycin LC	**L-Asn$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Ser$_4$-L-Glu$_5$-D-Ser$_6$-L-Thr$_7$**
Mycosubtilin	**L-Asn$_1$**-D-Tyr$_2$-D-Asn$_3$-**L-Gln$_4$-L-Pro$_5$-D-Ser$_6$-L-Asn$_7$**

[Source: From reference (Ongena and Jacques, 2008)]

Iturin

Iturin is heptapeptides chain linked to a β-amino fatty acid chain with a length of 14 to 17 carbon atoms (Ongena and Jacques, 2008) (Figure 5). It strongly display haemolytic and antifungal activities, but its antibacterial activity is restricted to some bacteria such as *Micrococcus luteus* (Aranda *et al.*, 2005; Maget-Dana and Peypoux, 1994). However, antiviral activity was not observed (Moyne *et al.*, 2001; Zeriouh *et al.*, 2011). Mechanisms of iturin are based on formation of ion-conducting pores and solubilization (Aranda *et al.*, 2005).

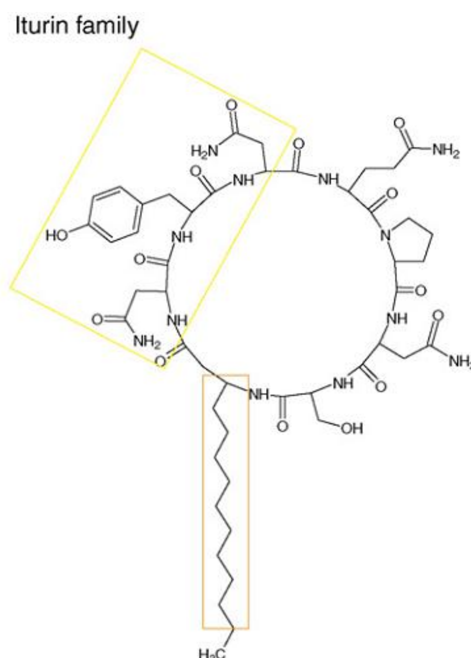

Figure 5 Structure of cyclic lipopeptide iturin A
[Source: From reference (Ongena and Jacques, 2008)]

Fengycin family

Fengycin A and B belong to fengycin family. They possess similarity of amino acid pattern, but type of amino acid at sixth position are different (Table 5) (Ongena and Jacques, 2008).

Table 5 Amino acid composition of decapeptides in fengycin family

Fengycin family	Amino acid composition
Fengycin A	L-Glu$_1$-D-Orn$_2$-D-Tyr$_3$-D-aThr$_4$-L-Glu$_5$-**D-Ala**$_6$-L-Pro$_7$-L-Gln$_8$-L-Tyr$_9$-L-Ile$_{10}$
Fengycin C	L-Glu$_1$-D-Orn$_2$-D-Tyr$_3$-D-aThr$_4$-L-Glu$_5$-**D-Val**$_6$-L-Pro$_7$-L-Gln$_8$-L-Tyr$_9$-L-Ile$_{10}$

[Source: From reference (Ongena and Jacques, 2008)]

These molecules are decapeptides chain linked to a β-amino fatty acid chain with a length of 14 to 18 carbons (Ongena and Jacques, 2008) (Figure 6). Fengycins exhibit less haemolytic activity than iturin and surfactin but retain a strong fungi toxic activity, specifically against filamentous fungi (Hofemeister *et al.*, 2004; Vanittanakom *et al.*, 1986).

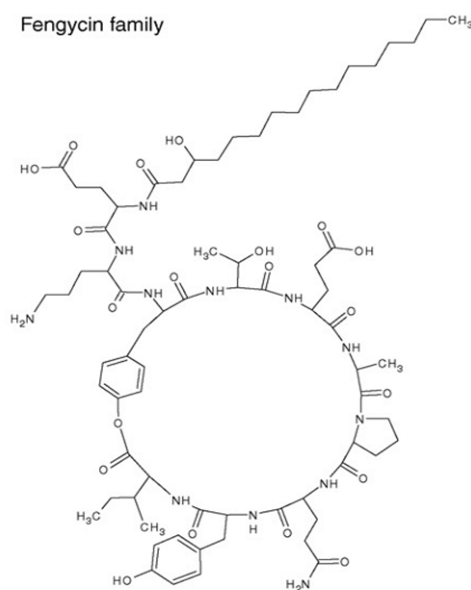

Figure 6 Structure of cyclic lipodecapeptides fengycin A
[Source: From reference (Ongena and Jacques, 2008)]

Environmental factors involved in biosurfactant production

Banat *et al*. (1995) demonstrated that salt concentration affected biosurfactant production. *Bacillus licheniformis* BAS50 was grown and produced a lipopeptide when cultured in variety of substrates and salinities. Biosurfactant production was optimal at 5% NaCl (Yakimov *et al.*, 1995). *Bacillus* spp. No.4 showed high salt tolerance and produced biosurfactant in a vast range of pH and temperature. However, some biosurfactants and their surface activity were not affected by environmental conditions. Surface tension reduction by biosurfactant derived from *Bacillus subtillis* C9 was stable at pH 5.0-9.5 (Tabatabaee *et al.*, 2005). McInerney *et al*. (1990) reported that lichenysin from *B. licheniformis* JF-2 was not affected by temperature (up to 50°C), pH (4.5-9.0), NaCl and Ca^{2+} (concentrations up

to 50 and 25g/l), respectively. A lipopeptide from *B. subtilis* LB5 was stable after autoclave (121°C/5 min) and after being kept for 6 months at 18°C (Ruangwong *et al.*, 2012).

Biosurfactants investigation techniques

Detection of surface tension of substances is the best way to indicate whether substances are biosurfactants. Determination of surface tension can be performed by drop collapse, oil spreading and Du Nouy ring tensiometer techniques (Morikawa *et al.*, 2000). However, oil spreading and Du Nouy ring tensiometer techniques are time-consuming techniques and are not suitable for screening a large number of biosurfactants. Drop collapse is the most reliable for screening biosurfactants (Bodour *et al.*, 2003). For this technique, test sample will be dropped to surface of oil and collapse of sample will be determined by visualization of sample spreading over the surface (Figure 7) (Tugrul and Cansunar, 2005). However, positive sample by this technique needed to be confirmed by Du Nouy ring tensiometer technique.

A B

Figure 7 Drop collapse method: A = not collapsed, B = collapsed
[Source: From reference (Tugrul and Cansunar, 2005)]

Purification of biosurfactants

Several methods for purification of biosurfactants, such as acid precipitation, organic solvent extraction, ammonium sulfate precipitation and centrifugation, have been reported (Table 6) (Desai and Banat, 1997). Acid precipitation, organic solvent extraction and centrifugation are popular techniques for crude extraction of biosurfactants.

However, for further purification, ion-exchange chromatography is normally performed. For final biosurfactant purification and identification, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is needed after biosurfactants have been seperated by reverse phase high-performance liquid chromatography (RP-HPLC) technique. MALDI-TOF MS is the technique that determine molecular mass of biosurfactants. Thus, molecule structures of biosurfactants will be illustrated.

Table 6 Purification methods of biosurfactants

Methods	Biosurfactants property responsible for separation	Advantages	References
Acid precipitation	Biosurfactants become insoluble at low pH values	Low cost, efficient in crude biosurfactant recovery	(Sen and Swaminathan, 2004)
Organic solvent extraction	Biosurfactants are soluble in organic solvent due to the presence of hydrophobic end	Efficient in crude biosurfactant recovery and partial purification	(Dubey *et al.*, 2005; Reiling *et al.*, 1986)
Centrifugation	Insoluble biosurfactants get precipitated because of centrifuge force	Reusable, effective in crude biosurfactant recovery	(Nitschke and Pastore, 2006)
Ion-exchange chromatography	Charge biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Highly pure, reusability, fast, recovery	(Reiling *et al.*, 1986)
Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry	A novel method for identification and structural characterization of microbial substance	High cost, efficient in crude or pure biosurfactant (whole cells are used as targets)	(Vater *et al.*, 2002)

Antibacterial activity of biosurfactants

Several lipopeptide surfactants are potent antibiotics (Yakimov *et al.*, 1995). Surfactin from *B. licheniformis* BAS50 exhibited antibacterial activity against *S. aureus* (Yakimov *et al.*, 1995). Crude biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20 showed activity against pathogenic microorganisms including *Candida albicans*, *Escherichia coli*, *S. aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae* at the concentration of MIC and MBC between 25 and 50 mg/ml (Gudina *et al.*, 2010). Conidial germination of fungus was completely inhibited by culture filtrate of *B. amyloliquefaciens* strain RC-2 (Yoshida *et al.*, 2001).

Bacillus sp. strain IBA 33 inhibited growth of *Penicillium digitatum*, *Geotrichum candidum*, *Aspergillus clavatus*, *Aspergillus niger* and *Fusarium moniliforme* (Gordillo *et al*., 2009). Bioactive fractions of biosurfactant obtained from marine *Bacillus circulans* possessed antimicrobial activity against various Gram-positive and Gram-negative bacteria such as *Micrococcus flavus, Bacillus pumilis, Mycobacterium smegmatis, E. coli, Serratia marcescens, Proteus vulgaris, Citrobacter freundii, Proteus mirabilis*, *Alcaligenes faecalis, Acetobacter calcoaceticus, Bordetella bronchiseptica, Klebsiella aerogenes* and *Enterobacter cloacae* (Das *et al.*, 2008). Antimicrobial activities of biosurfactants are cell membrane alteration, pore forming and anti-adhesion. Surfactin is an example of biosurfactant that can alter bacterial cell membrane. Interaction between surfactin divalent cations and bacterial membrane negative charges will facilitate it to penetrate into bacterial membrane. At low concentration of surfactin, phospholipids of bacterial membrane will be penetrated (A), at intermediate concentration, surfactin forms small micelles within phospholipid bilayer (B), whereas at high concentration it behaves like a detergent leading to bacterial cell disruption (Figure 8) (Bernheimer and Avigad, 1970).

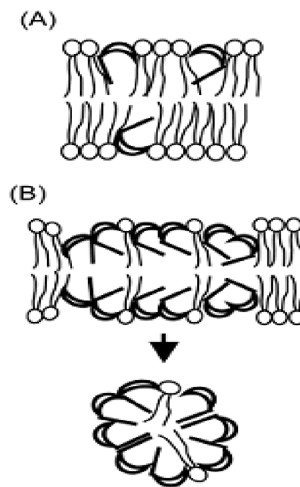

Figure 8 Antimicrobial activity of surfactin
[Source: From reference (Bernheimer and Avigad, 1970)]

For peptide molecule, surfactin can form a sheet-structure of synthetic peptide molecule and this depends on concentration of surfactin. The mechanism can be divided into three regions (Han *et al.*, 2008) (Figure 9).

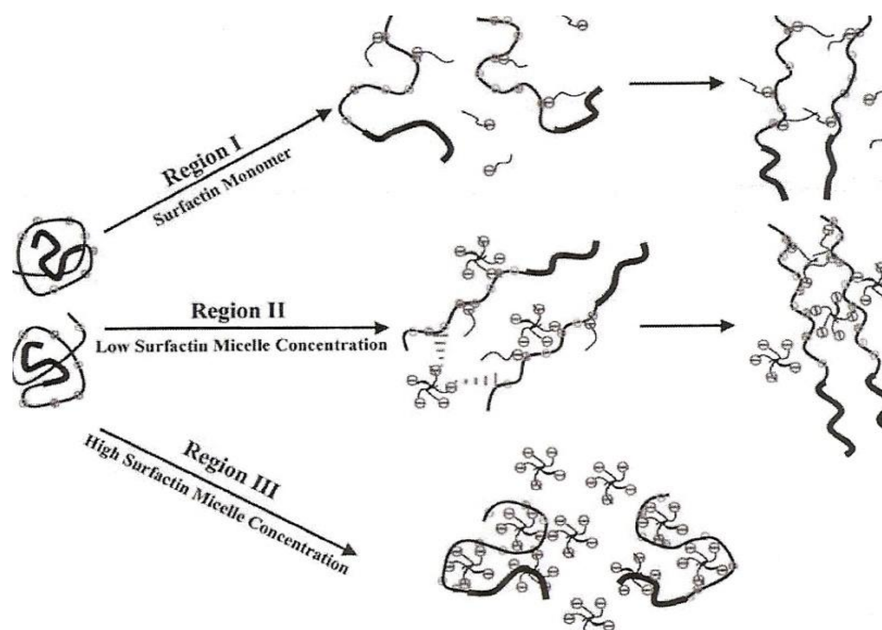

Figure 9 Activity of surfactin to peptide molecule
[Source: From reference (Han *et al.*, 2008)]

Region I. Low concentration of surfactin (Figure 9)

Binding between negative charge of surfactin monomer and positively charge molecule of peptide chain causes charges to be balanced and increase electrostatic repulsion among negative charges within peptide molecule. Then the peptide molecule will be extended and changed the conformation. Finally, hydrophobic interaction between hydrophobic chains (tail) of surfactin and peptide makes conformation of peptide to be sheet structure.

Region II. Medium concentration of surfactin

Surfactin starts to form micelles and binds to peptide chains. Increasing of negative charges on peptide chains causes peptides to unfold. On the other hand, surfactin can form hydrogen bonds with peptide chains and causes different peptide chains to attach to each other. These results in transformation of peptides to be sheet structure. Additionally, hydrophobic interaction among surfactin monomers that bound to peptides may increase the formation of the sheet structure.

Region III. High concentration of surfactin

At high concentration of surfactin, excessive surfactin micelles are formed and cannot bind to peptides. The separated peptide chains cannot interact to other peptide chains. However, unfold peptide chains can be formed by binding to the surfactin micelles and causes peptide aggregation instead of forming sheet (Han *et al.*, 2008).

Pore forming

Some biosurfactants can cause pore forming to target cells for example iturin. Iurin A increased ion permeability of lipid membranes by forming ion conducting pores (Maget-Dana and Ptak, 1990). Over the critical micellar concentration, iturin molecules form a fully connected to bilayer (Figure 10), where

each hydrocarbon tail span to the entire hydrocarbon width of the bilayer. This result in membrane destabilizing and causes leakage of protein and nucleic acids of cell (Fickers *et al.*, 2009; Grau *et al.*, 2001).

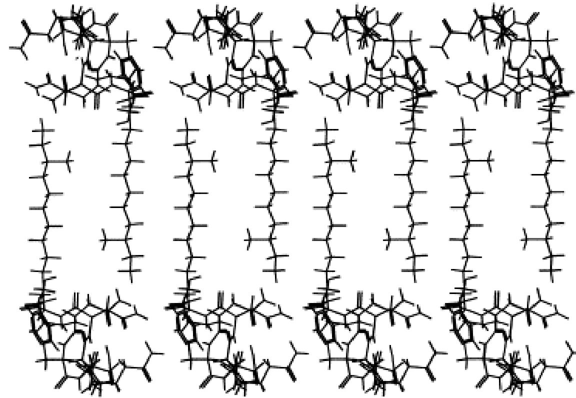

Figure 10 Model of an iturin A connected to bilayer
[Source: From reference (Grau *et al.*, 2001)]

Characteristics of these pores depend on lipid composition of the membrane, especially cholesterol (Nasir *et al.*, 2010). Pores of membrane are anion whereas polar groups of iturin are cation. Their binding can lead to the leakage of K^+ ion (Aranda *et al.*, 2005). Another example is formation of ion channels of biosurfactant syringomycin derived from *Pseudomonas syringae* pv. *syringae*. It generated a influx of Ca^{2+} and H^+ and efflux of K^+ in plasma membrane of plant. (Hutchison *et al.*, 1995) (Figure 11).

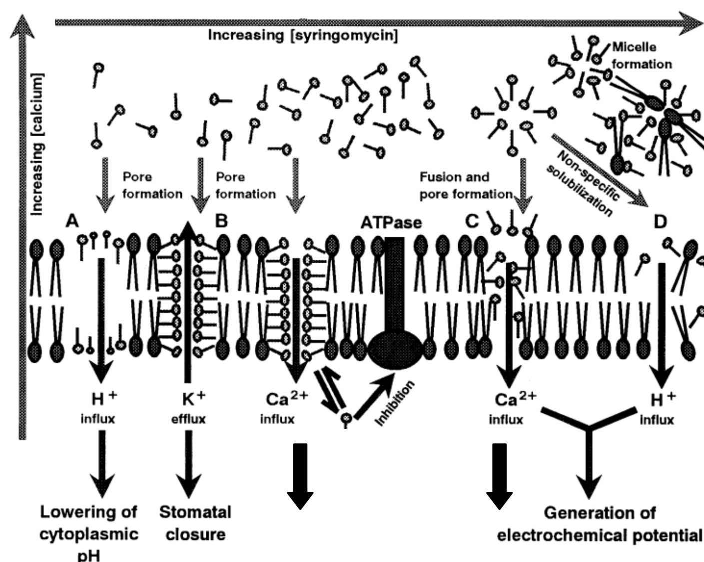

Figure11 Formation of ion channels of syringomycin (lipopeptide) in *Pseudomonas syringae* pv. *syringae* [Source: From reference (Hutchison *et al.*, 1995)]

Anti-adhesion activity of biosurfactants

Biosurfactant can increase surface area of substrates and regulate attachment of microorganisms to surface area such as release them from biofilm formation (Rosenberg and Ron, 1999). It was found that culture supernatants of *Bacillus pumilus* and *Bacillus indicus* inhibited initial attachment and biofilm formation of *Vibrio* spp. (Nithya *et al.*, 2010). In addition, the bacterial supernatants also reduced surface hydrophobicity of *Vibrio* spp. which is required for biofilm development. Recently, effect of temperature on anti-adhesive activity of surfactin has been demonstrated (Zeraik and Nitschke, 2010). Using polystyrene surface, attachment of *S. aureus*, *L. monocytogenes*, and *M. luteus* was inhibited by surfactin and its activity increased with the decrease in temperature.

Different concentrations of surfactin derived from *B. subtilis* and rhamnolipids from *Pseudomonas aeruginosa* reduced adhesion of *L. monocytogenes* and *Salmonella enterica* serovar Enteritidis to polystyrene surface (De Araujo *et al.*,

2011; Nitschke *et al.*, 2009). Stainless steel surfaces were conditioned by immersion in surfactin solution (0.25%) for 24 h, followed by rinsing for 15 min with demineralized water reduced by 90.0% the adhesion of *L. monocytogenes* (Meylheuc *et al.*, 2001). Biosurfactants obtained from Gram-negative bacterium (*Pseudomonas fluorescens*) and Gram-positive bacterium (*Lactobacillus helveticus*) inhibited adhesion of four listerial strains by reduction both total adhering flora and viable and cultivable bacteria on stainless steel surfaces. This study confirmed that biosurfactants are effective to prevent microbial colonization of metallic surfaces (Meylheuc *et al.*, 2006).

OBJECTIVES

1. To investigate numbers of *V. parahaemolyticus* in clinical samples obtained from Hat Yai Hospital during 2006 to 2010.

2. To evaluate numbers and DNA profiles of *V. parahaemolyticus* isolates from environment.

3. To isolate biosurfactants producing bacteria from soil that possess inhibitory activity against *V. parahaemolyticus*.

4. To purify and identify biosurfactants derived from bacteria.

CHAPTER 2

RESEARCH METHODOLOGY

MATERIALS AND METHODS

1. Equipments

Equipments	**Company**
ABI 377 genetic analyzer	Applied Biosystems, Foster City
Autoclave	Tomy, Japan
Autopipette	Gilson, France
Balances	Denver Instrument Company,NY, USA
Bruker Data Analysis software version 3.3	Bruker Daltonics Inc., MA, USA
Centrifugation (H-103N)	Kokusan, Japan
CHEF-DRIII system	Bio-Rad Laboratories, MA, USA
Densimat	bioMerièux, Italy
Desicator	Kartell, Italy
Digital Dry Bath (Accublock)	Labnet International Inc., NJ, USA
Electrophoresis apparatus	Bio-Rad Laboratories, MA, USA
ESI-Q-TOF mass spectrometer	Bruker Daltonics Inc., MA, USA
Fraction collector Model 2110	Bio-Rad Laboratories, MA, USA
Freezer (4°C, -20°C and -70°C)	Sanyo, Japan
Gel Documentation	Syngene, MD, USA
Hot air sterilizer	BINDER, Germany
Hot plate-Stirrers	Fisher Scientific, PA, USA
HPLC, Agilent 1100 Series	Hewlett-Packard, Waldron, Germany
Hydraulic press	Specac, Kent, UK.
Incubator (MIR-262)	Sanyo, Japan

Equipments	Company
Laminar airflow cabinet (ABS 1200A)	ASTEC microflow, UK
MiniVE electrophoresis system	GE Healthcare Bio-Sciences, NJ, USA
Olympus CH40 light microscope	Olympus, Japan
Orbital shaker (TPM-2)	SARSTEDT, Germany
PCR GeneAmp (PCR system 2400)	Perkin Elmer, CT, USA
pH meter	Metrohm, Switzerland
Peristatic pump Model EP-1Econo	Bio-Rad Laboratories, MA,USA
Plastic column 1.6x25 cm	Sigma Aldrich, Inc., MO, USA
Power supply (PowerPac Basic)	Bio-Rad Laboratories, MA, USA
PowerWave XS2, Microplate Spectrophotometer	BioTek, USA
Program Temp Control system (PC-818)	Astec, Inc., Japan
Refrigerated Microcentrifuge	Hettich, Germany
Rotary vacuum evaporator R-124	BUCHI, Bern, Switzerland
Shaking incubator	Labline Instrument Co., IL, USA
Spectrophotometer (Lambda 25 UV/VIS)	Perkin Elmer, UK
Spectrum One Perkin-Elmer	Perkin Elmer Inc., MA, USA
Supelcosil ™ LC-18-HPLC column	Sigma Aldrich, Inc., MO, USA
Tensiomat (K6)	Kruss HB, Germany
Thermo Cycler (PTC-200)	MJ Research, USA
Ultrasonic cleaner (3200)	Branson, Germany
Vortex-Genie 2	Scientific Industries, Inc., NY, USA
Waterbath (1235)	Sheldon Manufacturing, Inc., OR, USA
0.45-µm Millipore filter	Millipore Filter Corp., MA, USA
96-well microtiter plates (Nunclon)	Sigma-Aldrich, Inc., MO, USA

2. Media

Microbiological media used in this study were purchased from Difco, MD, USA and Microbiology CHROMagar, Paris, France.

3. Enzymes, antibodies and other reagents

All enzymes, antibodies and other reagents used in this study are analytical grade

Enzymes, antibodies buffers and reagent kit used in this study

Enzymes	**Company**
Anti -K antibodies	Denka Seiken, Tokyo, Japan.
Anti -O antibodies	Denka Seiken, Tokyo, Japan.
Buffer A	Promega, USA
Buffer *Taq* Polymerase	Promega, USA
dNTPs	Takara Biochemicals, Tokyo, Japan
dNTPs	Promega, USA
Ex Taq	Takara Biochemicals, Tokyo, Japan
Ex Taq buffer	Takara Biochemicals, Tokyo, Japan
Gentra Puregene extraction kit	QIAGEN, Germany
Pfu DNA polymerase	Promega, USA
Proteinase K	Bio-Rad Laboratories, Hercules, CA, USA
*Not*I restriction enzyme	TOYOBO Co. Ltd., Osaka, Japan.
Taq DNA polymerase	Promega, USA

4. Primers

Oligonucleotide primers pair and expected amplification product sizes used in this study

Genes	Primer	Sequence of primer (5′ to 3′)	Amplicon size (bp)	References
toxR	T4	GTCTTCTG ACGCAATCGTTG	368	(Kim *et al.*, 1999)
	T7	ATACGAGTGGTTGCTGTCATG		
tdh	D3	GGT ACT AAA TGG CTG ACA TC	251	(Tada *et al.*, 1992)
	D5	CCA CTA CCA CTC TCA TAT GC		
trh	R2	GGC TCA AAA TGG TTA AGC G	250	(Tada *et al.*, 1992)
	R6	CAT TTC CGC TCT TCA TAT GC		
GS-PCR	GS-VP1	TAATGAGGTAGAAACA	651	(Matsumoto *et al.*, 2000)
	GS-VP2	ACGTAACGGGCCTACA		
AP-PCR	Primer 2	GTTTCGCTCC		(Matsumoto *et al.*, 2000)
16s rDNA	27F	AGAGTTTGATCMTGGCTCAG	1541	(Lane, 1991)
	1492R	TACGGYTACCTTGTTACGACTT		

M = A/C; Y = C/T

5. Other chemical substances

Chemical substances	**Company**
Absolute ethanol	Merck, Germany
Acetronitrile-HPLC grade	Merck, Germany
Agarose	Gibco, NY, USA
DHEF-DNA Size Marker 170-3605	Bio-Rad Laboratories, Hercules, CA, USA
Chloroform analytical grade	Merck, Germany
CTAB	Fluka, Buchs, Switzerland
Preswollen Whatman DE-52 cellulose	Sigma Aldrich, Inc., MO, USA
Lambda DNA/*Hin*dIII marker	Bio-Rad Laboratories, MA, USA
1 kb DNA Ladder	New England Biolabs, Inc., MA, USA
100 bp DNA Ladder	New England Biolabs, Inc., MA, USA
Methanol-HPLC grade	Merck, Germany
N-lauryl sarcosine	TOYOBO Co. Ltd., Osaka, Japan
Potassium bromine pellets	Merck, Germany
Pulsed-Field Certified agarose	Bio-Rad Laboratories, Hercules, CA, USA,
Sodium dodecyl sulphate	LAB-SCAN Asia Co. Ltd., Thailand
Sodium hydroxide	Promega, USA
Tris base	Sigma Aldrich, Inc., MO, USA
Tunemix solution	NH, USA
Trifluoroacetic acid	Sigma Aldrich, Inc., MO, USA

METHODS

This work was divided into two parts. Part I, Investigation and characterization of *V. parahaemolyticus* isolates in Hat Yai Hospital between 2006 to 2010. In addition, characterization of *V. parahaemolyticus* isolates from environments (shellfish samples) was carried out (Figure 12). Part II, Isolation of biosurfactant producing bacteria that possess inhibitory activity against *V. parahaemolyticus*. In addition, purification and identification of those biosurfactants were performed (Figure 13).

Part I. Investigation and characterization of *V. parahaemolyticus* isolates from clinical and environmental samples.

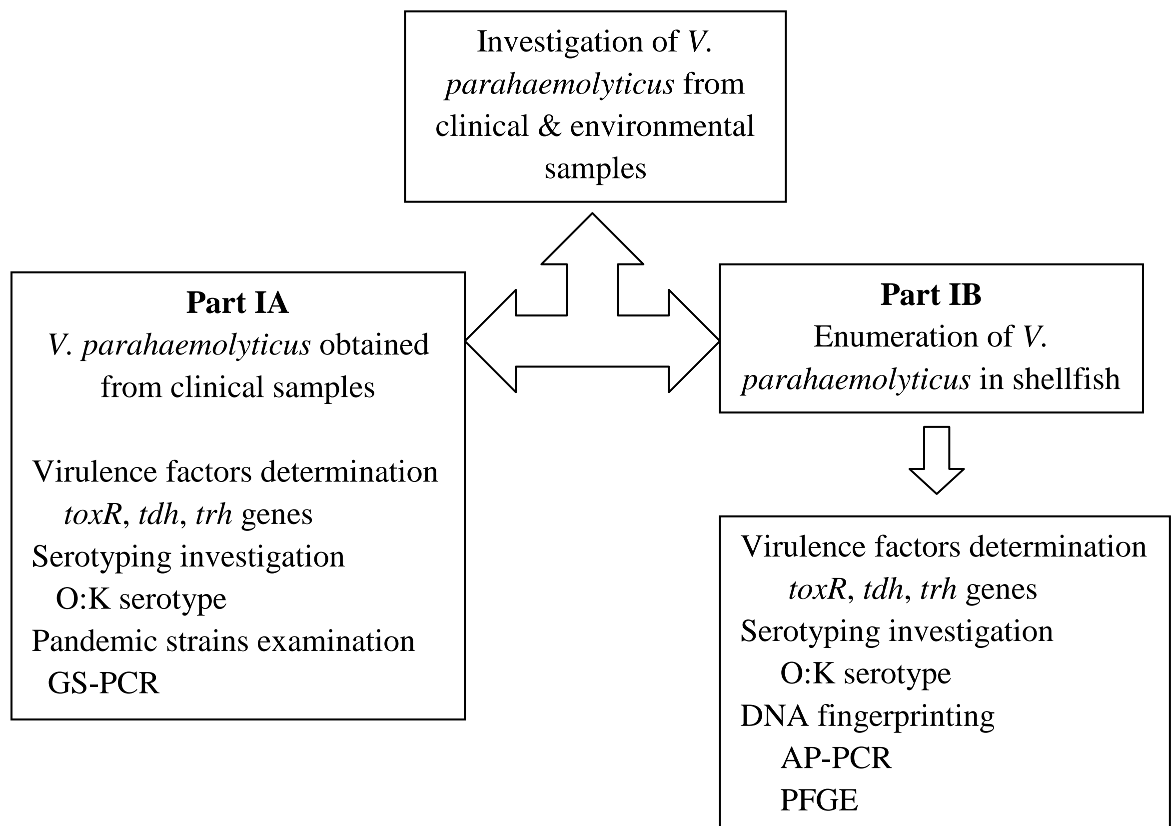

Figure 12 Illustrate diagram of Part I investigation.

Part IA Investigation and characterization of *V. parahaemolyticus* isolates from patients in Hat Yai Hospital

1. Sample collection and bacteriology

Rectal swab was obtained from diarrhea patient in Hat Yai Hospital, Songkhla Province, Thailand, between 2006 and 2010. Each sample was plated on MacConkey, Salmonella-Shigella and Thiosulfate Citrate Bilesalts Sucrose (TCBS) agars (Difco, MD., U.S.A.). After incubation at 37°C overnight, sucrose non-fermenter colonies on TCBS agar were selected and were identified as *V. parahaemolyticus* by standard biochemical tests. Confirmation of *V. parahaemolyticus* was performed by PCR targeted to the *toxR* gene.

2. Investigation of the *toxR* gene

The test isolate was grown in Luria-Bertani (LB) broth containing 1% NaCl with shaking (160 rpm) at 37°C overnight. One milliliter of the broth culture was centrifuged, and the bacterial cells were washed with sterile saline (0.85% NaCl) and then suspended in it. The cell suspension was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as the DNA template for PCR amplification. The PCR mixture consisted of 3 µL of the template, 5 µL of 10x buffer containing 20 mM $MgCl_2$ (*Taq* buffer; Promega, USA), 1.25 U of *Taq* polymerase (Promega, USA), 4 µL of 2.5 mM deoxynucleoside triphosphate, 2 µL of each primer (10 pmol/ml), and 33.75 µL of distilled water. The amplification conditions were 20 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1.5 min. The primer sequences were T4 (5′-GTCTTCTGACGCAATCGTTG-3′) (forward) and T7 (5′-ATACGAGTGGTTGCTGTCATG-3′) (reverse) (Kim *et al.*, 1999)**.** Amplification was performed in a Perkin-Elmer Thermal Cycler (GeneAmp PCR System 2400). Ten µL of PCR product was resolved by electrophoresis on a 1.5% agarose gel. After gel was stained with ethidium bromide, the amplicons were detected by a UV transilluminator.

3. Detection of *tdh* and *trh* genes

To determine the presence of the *tdh* and *trh* genes, the template was prepared as described above. PCR was carried out using the primers D3 (5′GGTACTAAATGGCTGACATC3′), D5 (5′CCACTACCACTCTCATATGC3′), R2 (5′GGCTCAAAATGGTTAAGCG3′), and R6 (5′CATTTCCGCTCTTCA TATGC3′), respectively (Tada *et al.*, 1992). PCR mixture consisted of 2 μL of DNA template, 1.6 μL of 2.5 mM dNTPs (Promega, USA), 5 μL of each primers (2 μM), 0.5 U of *Taq* polymerase (Promega, USA), 2 μL of 10x buffer (Promega, USA), 2 μL of 25 mM $MgCl_2$ (Promega, USA), and 2.3 μL of distilled water. The amplification conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 2 min, followed by 1 cycle of 72°C for 7 min (Tada *et al.*, 1992). Amplification was performed in a Perkin-Elmer Thermal Cycler (GeneAmp PCR System 2400). Ten μL of PCR product was resolved by electrophoresis on a 1.5% agarose gel. After gel was stained with ethidium bromide the amplicons were detected by a UV transilluminator.

4. Serotyping

Serotype of *V. parahaemolyticus* was investigated by slide-agglutination technique using anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan). Briefly, the test isolate was grown in trypticase soy broth containing 3% NaCl at 37°C for 18 h, and the bacterial cells were suspended in saline (3% NaCl). The bacterial cell suspension was subjected to agglutination with specific anti-K antibodies for determination of the K serotype. For determination of the O serotype, the bacterial cell suspension was autoclaved at 121°C for 30 min. Autoclaved bacterial cells were subjected to agglutination with specific anti-O antibodies.

5. GS-PCR

GS-PCR was carried out using the primers GS-VP1 (5′-TAATGA GGTAGAAACA-3′) and GS-VP2 (5′-ACGTAACGGGCCTACA-3′) (Matsumoto *et al.*, 2000). The 1:10 diluted supernatant of the boiled LB broth culture prepared as described above was used as the template solution. The 20 µL of PCR mixture consisted of 2 µL of *Taq* Polymerase 10x buffer (magnesium free, containing 100 mM Tris-HCl [pH 9.0], 500 mM KCl, and 1% Triton X-100; Promega, USA), 1.5 mM $MgCl_2$, 0.125 mM each deoxynucleoside triphosphate, 0.2 mM each primer, 2.5 µL of the template solution, and 0.5 U of *Taq* DNA polymerase in storage buffer A (Promega, USA). The amplification conditions were set at one cycle of 96°C for 5 min, followed by 25 cycles of amplification consisting of denaturation at 96°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 3 min, and then followed by one cycle of 72°C for 7 min. The PCR-amplified mixture was resolved by electrophoresis in a 1% agarose gel (Matsumoto *et al.*, 2000).

Part IB Isolation and characterization of *V. parahaemolyticus* isolates from shellfish

6. Enumeration of *V. parahaemolyticus* in shellfish

Shellfish samples were obtained from morning markets in Hat Yai City, Songkhla Province, Thailand between June and October 2010. They were examined within 1 h of collection. Their meat was removed and crushed in alkaline peptone water pH 8.6 supplemented with 1% NaCl at the ratio of 1:10. Then 0.1 ml of each sample was spread on CHROMagar Vibrio (CHROMagar Microbiology, Paris, France) in duplicate and incubated at 37°C for 24 h. The numbers of the total colonies and that of mauve colonies, presumed to be *V. parahaemolyticus*, were counted and their concentrations in the original shellfish in cfu/g were calculated. Five to ten mauve colonies from each sample were selected for confirmation of *V. parahaemolyticus* and detection of virulence genes.

7. Confirmation of *V. parahaemolyticus* and detection of virulence genes

The mauve colonies on CHROMagar Vibrio medium were presumed to be *V. parahaemolyticus*. To confirm this presumptive identification, the isolated mauve colonies were examined for *V. parahaemolyticus*-specific *toxR* gene sequence by a PCR method (Kim *et al.*, 1999). The test isolate was grown in Luria-Bertani (LB) broth containing 1% NaCl with shaking (160 rpm) at 37°C overnight. One ml of the broth culture was centrifuged, and the bacterial cells were washed and resuspended in sterile saline (0.85% NaCl). The cell suspension was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as the template for PCR amplification. Amplification of the *toxR* gene was performed using primers T4 and T7 as described previously (Kim *et al.*, 1999). Since the urease-positive phenotype of *V. parahaemolyticus* has been reported to be associated with the presence of the *trh* gene (Magalhaes *et al.*, 1992), all *toxR* positive isolates were screened for urease activity. Presence or absence of the *tdh* and *trh* genes was examined by PCR using the previously reported primers D3-D5 and R2-R6, respectively (Tada *et al.*, 1992).

8. Serotyping

Five isolates of *V. parahaemolyticus* from each of six selected shellfish were subjected to O:K serotyping. The O:K serotype of each isolate was determined by the slide-agglutination test using anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan).The method as described previously.

9. Arbitrarily primed polymerase chain reaction (AP-PCR)

In order to determine DNA profiles of *V. parahaemolyticus*, DNA of each test strain was extracted by the phenol-chloroform extraction method (Sambrook and Russell, 2001)**.** AP-PCR was performed using primer 2 (5′-GTTTCGCTCC-3′) as described previously (Bhoopong *et al.*, 2007). Briefly, amplification was performed in a 30 μL mixture composed of 0.33 mM dNTPs (TaKaRa Biochemicals, Tokyo,

Japan), 25 ng template DNA, 2.5 U *Ex Taq* (TaKaRa, Japan), 0.83 pmol primer, and 1x *Ex Taq* Buffer (TaKaRa, Japan). The PCR was performed in a Perkin-Elmer Thermal Cycler (Program Temp Control System PC-808, Astec Co., Tokyo, Japan). The thermo cycle was started with a cycle at 95°C for 4 min. This was followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min, in which a transition time of 5 min was set between the denaturation and annealing, annealing and extension, and extension and denaturation steps. The thermo cycle was finished with one cycle at 72°C for 7 min. The amplification products were analyzed by 1.5% agarose gel electrophoresis.

10. Pulsed Field Gel Electrophoresis (PFGE)

V. parahaemolyticus was grown in LB broth supplemented with 1% NaCl at 37°C overnight. An agarose plug was prepared by mixing equal volumes of bacterial suspension with melted agarose. Bacterial lysis in an agarose plug was achieved with 950 µL lysis solution bacterial lysis (containing 50 mM Tris-HCl [pH 8.0], 50 mM EDTA, 1% *N* lauryl sarcosine, and 1 mg proteinase K per ml), and the DNA was cleaved for 48 h and digested with 50 U of *Not*I restriction enzyme (TOYOBO Co. Ltd., Osaka, Japan). The digested DNA fragments and DNA markers were separated in 1% Pulsed-Field Certified agarose (Bio-Rad Laboratories, Hercules, CA, USA) using 0.5x TBE buffer on a CHEF-DRIII system (Bio-Rad). The running condition was set 6 V/cm at 14°C for 24 h at a field angle of 120°C and switch times were 1-18 sec for 12 h and 3-80 sec for 12 h. After completion of the electrophoresis, the gel was stained with ethidium bromide for 30 min, destained in distilled water for 1 h, and photographed under an UV transilluminator (Appendix C).

Part II Isolation and characterization of biosurfactants that possess inhibitory activity against *V. parahaemolyticus*

To determine bacteria that are able to decrease numbers of *V. parahaemolyticus* contaminated on surface areas, the effective biosurfactants produced bacteria were screened from soils collected from mangrove and coastal areas. Inhibitory activity against *V. parahaemolyticus* was investigated. Biosurfactant produced by selected bacterium was purified and characterized (Figure 13).

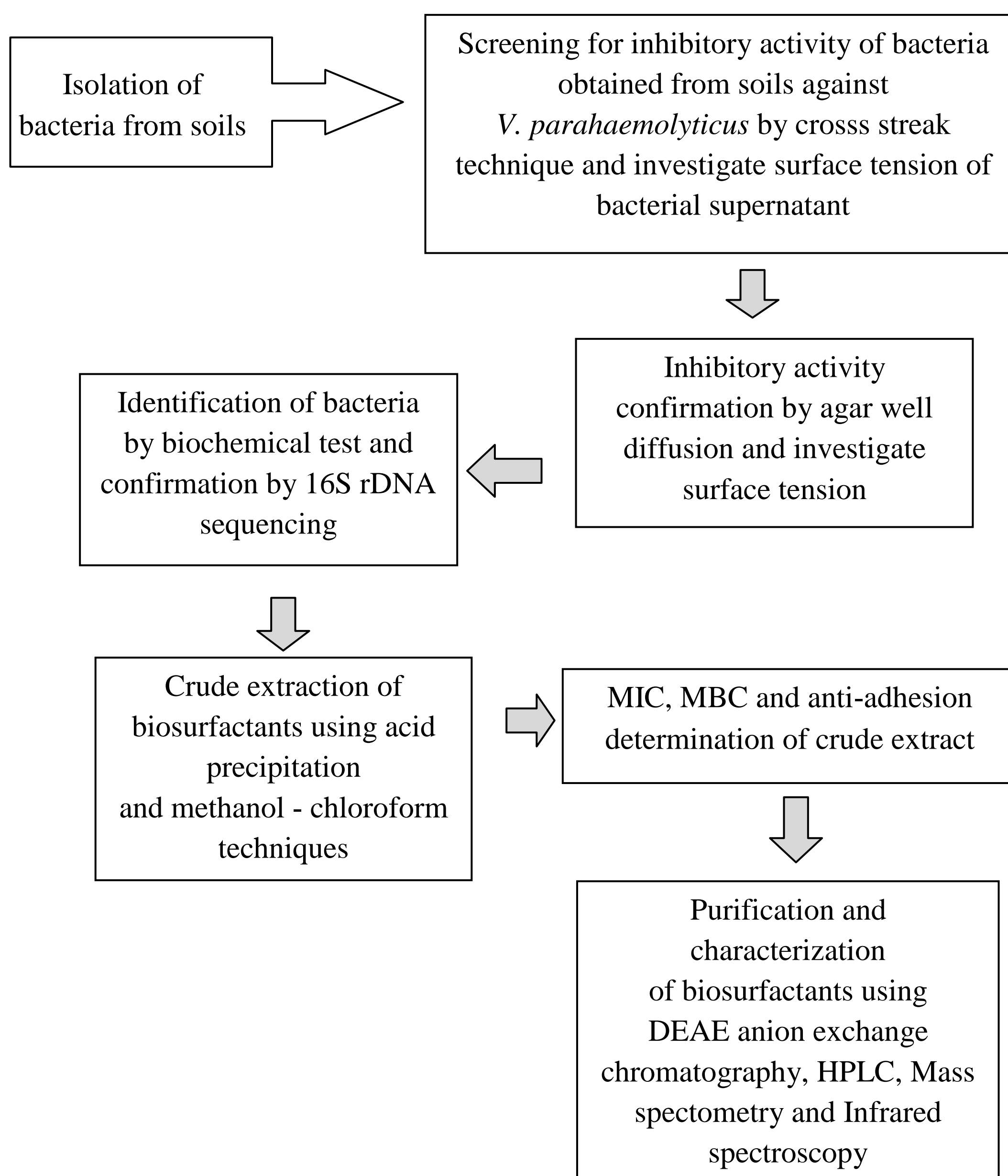

Figure 13 Illustrate diagram of Part II study

Part II

Bacterial strains

Standard strains of *V. parahaemolyticus* including *E. coli* ATCC25922 and *P. aeruginosa* used in this study were obtained from culture collection of Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand (Table 7).

Table 7 Bacterial strains used in this study

Bacterial strains	Serotypes	Virulence factors	
		tdh	*trh*
Escherichia coli ATCC25922	NT	NT	
Pseudomonas aeruginosa	NT	NT	
Vibrio parahaemolyticus PSU 2598	O4:K68	+	-
Vibrio parahaemolyticus PSU 4118	O1:K25	+	-
Vibrio parahaemolyticus PSU 4211	O3:K6	+	-
Vibrio parahaemolyticus PSU 4413	O1:KUT	+	+
Vibrio parahaemolyticus PSU 4886	O3:KUT	+	-
Vibrio parahaemolyticus PSU 5321	O2:KUT	-	-

NT = Not test

Soil samples

Soil samples were collected from mangrove and coastal areas of five provinces in southern Thailand (Table 8). Briefly, 50 g of soil at 1 cm. dept from the surface was collected in plastic bag and transfered to laboratory as soon as possible.

Table 8 Soil samples collected from mangrove and coastal areas in southern Thailand

Soil types	Number of collected locations	Provinces
mangrove soil	5	Songkhla
	2	Surat Thani
	3	Trang
	2	Satun
coastal soil	9	Songkhla
	2	Phatthalung
	2	Satun
	3	Surat Thani

11. Isolation of bacteria and screening for inhibitory activity to *V. parahaemolyticus*

A total of 28 soil samples were collected from various locations in five provinces of Thailand (Table 8). They were vigorously mixed and diluted with normal saline solution and spread on trypticase soy agar (TSA). After incubation overnight at room temperature, different characteristics of bacterial colonies were selected and inhibitory activity to *V. parahaemolyticus* was performed using cross streak technique (Lertcanawanichakul and Sawangnop, 2008). Briefly, *V. parahaemolyticus* was streaked on TSA containing 1% NaCl and a single cross streak of the tested bacteria was performed. Plate was kept at room temperature for 24 h and bacterial isolates that inhibited growth of *V. parahaemolyticus* were selected and their supernatant were partially purified by acid precipitation and confirmed their inhibitory activity by agar well diffusion technique.

12. Acid precipitation of biosurfactants and agar well diffusion technique

Biosurfactants were isolated by acid precipitation technique with slightly modification (Rivardo *et al.*, 2009). Briefly, the bacterial isolates were cultured in Modified Synthetic Sea Water medium (MSSW) by adding casein peptone, yeast extract and palm oil (Amano *et al.*, 1982; Button *et al.*, 1993). They were kept at room temperature for 48 h and the supernatant obtained by centrifugation was precipitated with 6 N HCl at 4°C for 6 h. The crude extract obtained was dissolved in distilled water and neutralized by NaOH before its inhibitory activity against *V. parahaemolyticus* was evaluated by agar well diffusion technique (Vuddhakul *et al.*, 2007). Briefly, 1.5 x 10^8 cfu/ml of *V. parahaemolyticus* was spread on Mueller Hinton agar supplemented with 1% NaCl. Wells were punched in the agar and 50 µL of dissolved crude extract was added in each well. The plates were incubated at room temperature for 24 h and diameter of inhibition zone was recorded. The experiment was done in triplicate.

13. Identification of biosurfactants producing bacterium

Biosurfactant producing bacterium was identified by the standard method described in Berkey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and was confirmed by 16S rDNA gene sequencing. Briefly, the bacterium was cultured in Luria-Bertani broth (Difco) and genomic DNA was purified using Gentra Puregene extraction kit (QIAGEN, Germany). PCR amplification of 16S rDNA gene was performed with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-′3) and 1492R (5′-TACGGYTACCTTGTTACGACTT-′3) (Lane, 1991). The 20 µL of PCR mixture containing 2 µL of 10x PCR buffer (Promega, USA); 10 ng of template DNA; 0.25 µmol/l each of the primers; 0.025 U *Pfu* DNA polymerase (Promega, USA); and 0.2 mmol/l dNTPs. PCR was performed in a Perkin Elmer GeneAmp 2400 PCR Thermal Cycler (Applied Biosystems, USA), and the thermal cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min; and an extension at 72°C for 5 min. Sequencing was performed in an ABI 377 genetic analyzer (Applied Biosystems). A search for sequence homology of the

16S rDNA region was performed using BLAST. A homology of >99% identity was the criterion used to identify an isolate to the species level (Appendix D).

14. Media and conditions of biosurfactants production

In order to evaluate a suitable medium for biosurfactants production, the test strain was grown in four different culture media, MSSW, trypticase soy broth, Landy media and Mckeen media (Amano *et al.*, 1982; Button *et al.*, 1993; Kumar *et al.*, 2009; Landy *et al.*, 1948; Suwansukho *et al.*, 2008). After 48 h of incubation at room temperature, the supernatant was harvested and tested against *V. parahaemolyticu*s using agar well diffusion technique. The medium that provides the highest antimicrobial activity was evaluated for suitable inoculum size of the bacterium and the best conditions for biosurfactants production including temperature (25 to 40°C), pH (4 to 8) and speed of shaker (100 to 180 rpm/min).

15. Kinetic production of biosurfactants

Bacterium was grown in 25 ml of TSB at room temperature for 15 h. Cells were resuspended in 0.85% NaCl and adjusted to 1.5x10^{8} cfu/ml, then 2% (v/v) of this inoculum was cultured in 100 ml of Mckeen medium at the optimal conditions for 48 h. Inhibitory activity against *V. parahaemolyticus* and surface tension of supernatant at various times of incubation were evaluated.

16. Surface tension measurement

Microbial surfactants obtained by acid precipitation of supernatant was determined by measuring surface tension of the crude extract. The extract was dissolved in distilled water and neutralized by NaOH. Its surface tension was determined via the Ring method using a Du Nouy tensiometer (K6; Kruss, Hamburg, Germany) (Cooper and Goldenberg, 1987). Briefly, 10 ml of each sample solution was loaded into a clean glass 30 ml miniplate and placed onto tensiometer platform. A platinum wire ring was slightly submerged on surface of solution and liquid was

slowly pulled through air interface to measure the surface tension (mN/m). Distilled water was used as positive control that exhibited surface tension at 72 mN/m at 25°C.

17. Chloroform and methanol extraction, MIC and MBC determination

Biosurfactants of the crude extract were subjected to be further purified with chloroform and methanol. Briefly the crude extract was mixed with chloroform and methanol at the ratio of 2:1 (v/v) (Kim *et al.*, 2010). After three times of extraction, solvent was removed by a rotary vacuum evaporator R-124 (BÛCHI, Bern, Switzerland). The obtained yellow-brown sediment was dissolved in sterile distilled water and filtered through 0.45 µm filters and was subjected to minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination. Briefly, the extract was diluted twofold in sterile Mueller Hinton broth supplemented with 1% NaCl and inoculated with 0.5 ml of the same medium containing 2.5 x 10^{5} cfu/ml of *V. parahaemolyticu*s. Tubes were incubated at 37°C for 24 h and highest dilution where no growth of *V. parahaemolyticu*s observed was defined as the MIC. For MBC, highest dilution of tube that exhibited no growth after culture on MHA+1% NaCl was recorded. *E. coli* ATCC25922 was used as a control.

18. Adhesion inhibition of crude biosurfactant against *V. parahaemolyticus*

Inhibition of *V. parahaemolyticus* to surface area was performed in 96-well flat bottom plate (Janek *et al.*, 2012). Briefly, crude extract of biosurfactants was dissolved in PBS pH 7.4 and filtered through 0.45 µm filters. 200 µL of 500-1000 µg/ml of crude biosurfactants was drop into 96-well plate and kept at room temperature for 4-20 h before removed. *V. parahaemolyticus* at the concentration of 1.0 x 10^{7} cfu/ml (Rivardo *et al.*, 2009) in PBS was added and the plate was kept at room temperature in a rotary shaker at 150 rpm for 2 h. Non-adherent cells were removed by triplicate washing with PBS. Then the plate was stained with 0.1% crystal-violet for 30 min and washed three times with PBS. The adherent bacterial cells were permeabilized and the dye was resolubilized with 150 µL of isopropanol-0.04 N HCl and 50 µL of 0.25% SDS per well. Optical density of each well was

determined with spectrophotometer at OD 590 nm (ODT). *V. parahaemolyticus* at the concentration of 1.0 x 10^7 cfu/ml in PBS without precoating of crude biosurfactants was used as control (ODC). All experiments were done in triplicate.The adhesion inhibition was calculated as follow;

$$\% \text{ adhesion inhibition} = [1 - (ODT/ODC)] \times 100$$

ODT - optical density of well with *V. parahaemolyticus* and crude biosurfactants
ODC - optical density of well with *V. parahaemolyticus* and PBS

19. Determination of ion of biosurfactants

Biosurfactants can be anion or cation, in this study ionic property of methanol-chloroform extract was determined using modified agar well diffusion technique (Saravanakumari and Mani, 2010). Briefly, wells were punched in the plate containing 1% agar and 30 μL of the extract was added in the well (Figure 23). Positive charge synthetic surfactant (2% of CTAB in distilled water) and negative charge of synthetic surfactant (2% of SDS in distilled water) were filled in the rest of wells. Distilled water was used as control. After incubation at room temperature for 24 h, precipitin line was investigated. The experiment was done in duplicate.

20. Purification and identification of biosurfactants

20.1 DEAE-cellulose anion exchange chromatography

The extract derived from chloroform and methanol extraction was further purified by DEAE-cellulose (DE 52; Sigma) anion exchange chromatography. Briefly, 0.5 g of extract was dissolved in alkaline distilled water pH 8.0 and loaded into the column (bed volume of column = 46 cm^3) saturated with 20 mM Tris HCl buffer pH 8.0. Then the column was washed with 2 bed volume of Tris-HCl pH 8.0 for removing the unbounded extract. Biosurfactants were eluted with the same buffer supplemented with NaCl 200 mM to 1000 mM (step-wise elution). Flow rate was adjusted at 0.7 ml/min at room temperature. Each fraction obtained was determined

for lipopeptide using spectrophotometer at OD 210 nm and 280 nm (Das *et al.*, 2008). Each fraction was pooled and determined for surface tension activity. In addition, inhibitory activity against *V. parahaemolyticus* of the pooled fractions was determined using agar well diffusion technique (Appendix E).

20.2 High-performance liquid chromatography (HPLC)

The pooled fraction derived from DEAE chromatography was further purified by reverse-phase HPLC (Supelcosil ™ LC-18- Sigma) with matrix of silica gel containing of octadecyl phase as active group. The mobile phase was 0.1% trifluoroacetic acid (TFA) (A) and acetonitrile (B). The gradient (A/B) was maintained at 90:10 in the first 5 min, then 40:60 in the next 10 min and 0:100 in the subsequently 35 min and finally at 40:60 for the last 5 min. Each fraction obtained was determined for lipopeptide by spectrophotometer at OD 210 nm (Das *et al.*, 2008).Fraction solvent was removed by a rotary vacuum evaporator R-124 (BÛCHI, Bern, Switzerland) and inhibitory activity against *V. parahaemolyticus* of each fraction was investigated.

20.3 Mass spectrometry

Active fractions obtained from HPLC were subjected to determine molecule masses of biosurfactants using electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) (Bruker Daltonics, Billerica, MA, USA). Each active fraction was dissolved in a mixture of acetonitrile and water at the ratio of 80:20 (v/v) and injected into ESI-Q-TOF mass spectrometer with electrical running of 4.5 kV, at 180°C. Tunemix solution (NH, USA) was used as standard electrospray ion (ESI) control. Data obtained was analysed by Bruker Data Analysis software version 3.3.

20.4 Infrared spectroscopy analysis

In order to determine functional groups and chemical compositions of biosurfactants, infrared absorption spectra of a biosurfactants were investigated using Fourier transform infrared (FTIR) spectrometer (Perkin- Elmer Inc., Boston, MA, USA). Briefly, fraction obtained from HPLC was mixed with 80 mg of KBr powder (Merck, Germany) and subjected to infrared spectrometer with absorbance range of 400 – 4000 wavenumbers (cm^{-1}).

CHAPTER 3

RESULTS

Part I Investigation and characterization of *V. parahaemolyticus* isolates from clinical and environmental samples

Part IA Investigation and characterization of *V. parahaemolyticus* isolates from patients in Hat Yai Hospital

In this study, during 2006 to 2010, the total number of *V. parahaemolyticus* isolated from patients in Hat Yai Hospital was 776 isolates (Table 9). Highest number of bacteria was obtained in 2006 and the lowest number was detected in 2009. The organism was classified into four groups: $tdh^{+}trh^{-}$, $tdh^{+}trh^{+}$, $tdh^{-}trh^{-}$ and $tdh^{-}trh^{+}$ based on the presence or absence *tdh* or *trh* toxin genes. Most of clinical *V. parahaemolyticus* isolates were in a $tdh^{+}trh^{-}$ group. This group was divided into pandemic (GS-PCR positive) and non-pandemic (GS-PCR negative) strains (Table 9). The predominant isolates of *V. parahaemolyticus* in each year were pandemic strains which highest number (73.6%) and the lowest number (51.3%) were detected in 2008 and 2010, respectively. The numbers of isolates detected in the rest of 3 groups ($tdh^{+}trh^{+}$, $tdh^{-}trh^{-}$ and $tdh^{-}trh^{+}$) during 2006 to 2010 were 34, 68 and 8, respectively. Two isolates of *V. parahaemolyticus* in $tdh^{-}trh^{-}$ group were GS-PCR positive (Table 9).

Comparisons to previous investigation of *V. parahaemolyticus* infection in Hat Yai Hospital between 2000 and 2005 which demonstrated that 55.5-69.7% from total of infections caused by pandemic strains (Figure 14) and infections by other pathogenic groups of *V. parahaemolyticus* were not significantly different from infection in 2006-2010 (Figure 15) (Wootipoom *et al.*, 2007). Thus, these

indicated that *V. parahaemolyticus* infections reported in this hospital does not decrease.

All isolates were subjected for serotype investigation and the most prevalent serotype detected in every year was O3:K6. This serotype was accounted for 46.8 % of the total isolates (Table 10). In addition, in the 5 years of investigation, O3:K6 was the predominant serotype of pandemic strains followed with O4:K8 and O1:K25 (Table 10 & Table 11). The serotypes of isolates that belong to non-pandemic strains were variable and predominant serotypes were O1:K56, O4:K8, O1:KUT and O4:K9 (Table 10).

Table 9 Characteristics of *V. parahaemolyticus* isolates from Hat Yai Hospital from 2006 to 2010.

Year	total	No. of isolates (%)				
		$tdh^{+}trh^{-}$		$tdh^{+}trh^{+}$	$tdh^{-}trh^{-}$	$tdh^{-}trh^{+}$
		GS-PCR positive	GS-PCR negative			
2006	214	138 (64.5)	52 (24.3)	7 (3.3)	16 (7.5)	1 (0.4)
2007	139	83 (59.7)	26 (18.7)	11 (7.9)	17 (12.2)	2 (1.5)
2008	193	142 (73.6)	32 (16.6)	6 (3.1)	11 (5.7)	2 (1.0)
2009	111	67 (60.4)	28 (25.2)	4 (3.6)	9*(8.1)	3 (2.7)
2010	119	61 (51.3)	37 (31.1)	6 (5.0)	15 (12.6)	0
total	776	491	175	34	68	8

* Two isolates were GS-PCR positive

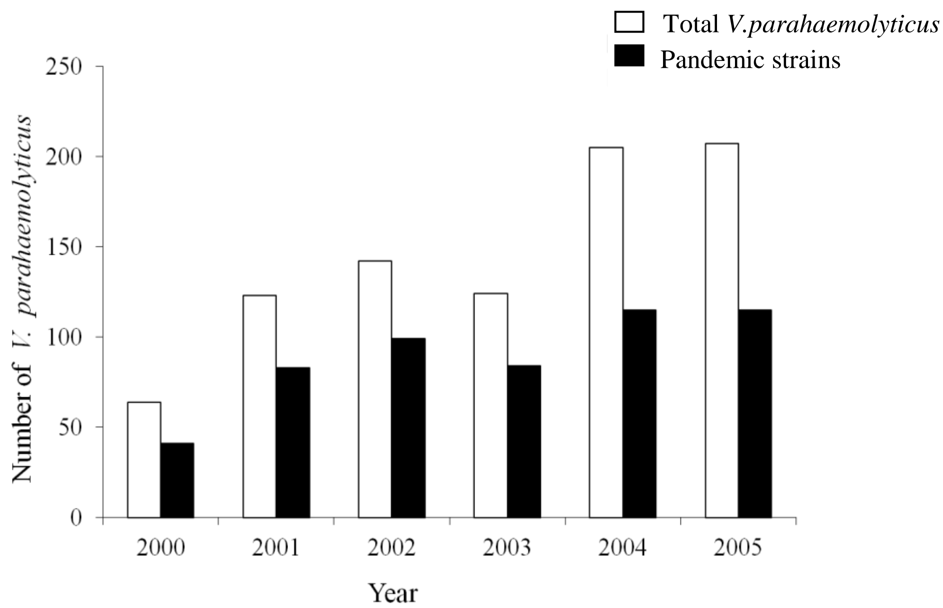

Figure 14 Numbers of total and pandemic strains of *V. parahaemolyitcus* isolated from patients in Hatyai Hospital from 2000 to 2005

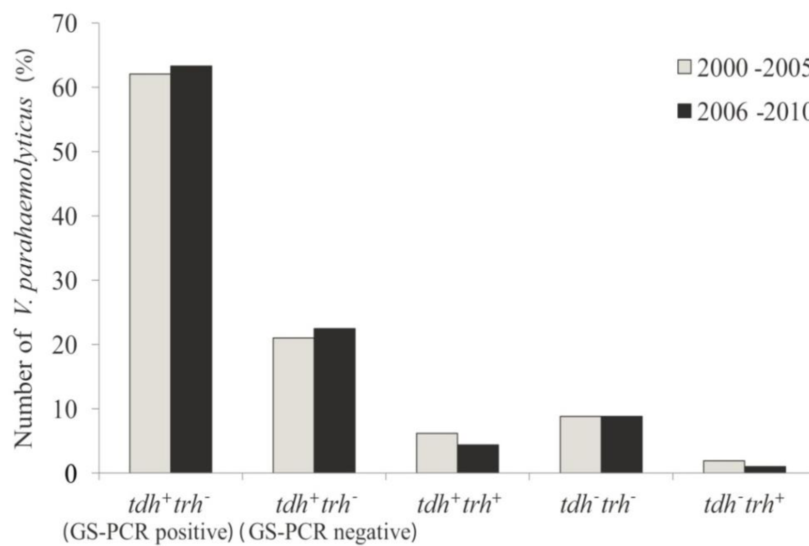

Figure 15 Comparison of number of *V. parahaemolyticus* isolates belong to 5 groups of toxin gene profile obtained from patients in Hat Yai Hospital between 2000-2005 and 2006-2010

Table 10 Serotypes of *V. parahaemolyticus* isolated between 2006 and 2010.

Year (total isolate)	No. of isolates*	Serotype	Year (total isolate)	No. of isolates*	Serotype
2006	107	O3:K6	2008	106	O3:K6
(214)	31	O4:K68	(193)	32	O1:K25
	19	O1:K56		9	O1:K56
	13	O4:K8		7	O1:KUT
	5	O1:KUT †, O4:K55		6	O4:K8
	4	O11:KUT, O12:KUT		3	O12:KUT
	3	O2:K3, O3:K29, O5:KUT,		2	O2:K3, O4:K13, O4:K53, O4:K55, O5:KUT, O8:K74, O9:K44
	2	O1:K9, O4:K9, OUT:KUT		1	O1:K9, O1:K58, O1:K69, O3:K5, O3:K29, O3:K45, O3:K75, O4:K12, O4:K68, O5:K17, O5:K61, O8:K38, O8:K41, O8:K44, O9:K44, O11:K22
	1	O1:K25, O3:K5, O3:K29,O3:K53 O4:K34, O4:K49 O4:K11, O5:K19 O5:K61, O8:KUT O11:K40,	2009	49	O3:K6
			(111)	18	O4:K68
				9	O4:K9
				6	O1:K56
				5	O1:KUT

* No. of isolates per serotype. †UT, Untypeable

Table 10 Serotypes of *V. parahaemolyticus* isolated between 2006 and 2010 (continued).

Year (total isolate)	No. of isolates*	Serotype	Year (total isolate)	No. of isolates*	Serotype
2007	73	O3:K6	2009	4	O2:K3
(139)	9	O1:KUT		3	O4:K8
	7	O1:K25		2	O1:K25,O3:KUT, O8:K22, O11:K36
	5	O4:K8, O4:K68		1	O1:K9, O3:K29, O4:K13, O4:K42, O4:K55, O5:K15, O8:K32, O9:K44, 10:KUT
	4	O1:K56			
	3	O13:K5, O4:K11, O5:KUT,O8:KUT, O11:KUT	2010 (119)	47	O3:K6
	2	O1:K69, O3:K5, OUT:KUT		24	O1:KUT
	1	O1:K9, O1:K32, O1:K64, O2:K3, O2:K28, O3:K75, O3:KUT, O4:K13, O4:KUT, O9:KUT, O10:K19, O10:KUT, O12:KUT, O13:K75, OUT:K6		15	O4:K9
				7	O1:K56, O4:K8
				4	O3:KUT
				3	O10:KUT, O11:KUT
				2	O4:K4, O4:KUT, O5:KUT
				1	O4:K3, O8:KUT, O8:K70

* No. of isolates per serotype. [†]UT, Untypeable

Table 11 Predominant serotypes of GS-PCR positive strains**.**

Year	Serotypes			
	O3:K6	O4:K68	O1:K25	Others
2006	105	30	1	2
2007	70	5	7	1
2008	106	1	31	4
2009	47	16	2	2
2010	47	0	0	14

Part IB Investigation and characterization of *V. parahaemolyticus* isolates from shellfish

Enumeration of *V. parahaemolyticus* in shellfish

A total of 52 shellfish samples including shrimps, crabs, mussels, hard clams, bloody clams and squids were investigated. The average total number of bacteria was the highest for bloody clam at 7.7 x 10^6 cfu/g (ranged from 3.0 x 10^3 to 7.5 x 10^7cfu/g) (Table 12). The average lowest number of bacteria was observed for squid at 1.6 x 10^4 cfu/g (ranged from 1.2 x 10^3 to 4.0 x 10^4cfu/g). Five to ten mauve colonies selected from CHROMagar Vibrio were confirmed to be *V. parahaemolyticus* by PCR for each shellfish sample. All the isolates were *toxR* positive but they harbored neither the *tdh* nor *trh* virulence genes. It was of interest that two isolates are urease positive. Highest number of *V. parahaemolyticus* was detected in mussel (4.7 x 10^4 cfu/g average) and the lowest number was detected in crab (1.0 x 10^2 cfu/g average). Interestingly, proportion of *V. parahaemolyticus* relative to the total number of bacteria was very high for some molluscan bivalves (10.9% for mussel, 10.6% for hard clams) and very low for some molluscan bivalves (0.07% for bloody clam) (Table 12).

Diversity of serotype and DNA fingerprints

Five mauve colonies were selected from a culture plate for each of six selected single shellfish samples and their O:K serotypes were determined (Table 13). Three to five different serotypes of *V. parahaemolyticus* were demonstrated in each of those six single shellfish samples (Table 13). Two to three identical serotypes of *V. parahaemolyticus* were observed in shrimp (O8:KUT), crab (O4:K4), mussel (O1:KUT) and hard clam (O8:KUT) (Table 13). DNA fingerprints of those isolates, including *V. parahaemolyticus* isolates that exhibited the same O antigen but different K antigens (one possessed a known K antigen and another was KUT) were examined next. AP-PCR showed that strains with identical serotypes, O8:KUT (strains PSU 3185 and PSU 3192) and O4:K4 (PSU 3242 and PSU 3244) isolated from shrimp and crab, respectively, produced different DNA fingerprints (Table 13 & Figure 16). In addition, the *V. parahaemolyticus* strains isolated from a single shellfish with the same O antigen but with different K antigens (O4:K4, O4:KUT isolated from crab; O4:K63 and O4:KUT, O3:K48 and O3:KUT isolated from bloody clam; O4:KUT and O4:K8, O10:K52 and O10:KUT isolated from squid) also exhibited different DNA profiles (Figure 16). However, DNA profiles of two of three identical serotypes, O1:KUT (PSU 5312 and PSU 5313) and O8:KUT (PSU 5310 and PSU 5311) of *V. parahaemolyticus* isolated from mussel and hard clam, respectively, were identical. Confirmation by PFGE revealed that both isolates of each serotype were indistinguishable (Figure 17).

Table 12 Meat weight, total number of bacteria and *V. parahaemolyticus* detected on CHROMagar Vibrio from single shellfish samples[a].

Seafood samples (no. of tested samples)	Average meat weight (g/single seafood)	No. of total bacteria (cfu/g)		No. of *V. parahaemolyticus* (cfu/g)		%*V. parahaemolyticus* in total no. of bacteria[b]
		Average	Min-Max	Average	Min-Max	
Shrimp (10)	8.5 ± 0.2[c]	1.0×10^5	4.0×10^2– 4.9×10^5	5.8×10^3	1.0×10^2– 4.6×10^4	5.8
Crab (2)	34.3 ± 1.4	1.1×10^5	8.0×10^4–1.4×10^5	1.0×10^2	0–2.0×10^2	0.1
Mussel (10)	7.6 ± 0.7	4.3×10^5	8.0×10^3–1.5×10^6	4.7×10^4	1.0×10^3–2.1×10^5	10.9
Hard clam (10)	3.8 ± 0.2	3.0×10^4	4.0×10^2–1.2×10^5	3.2×10^3	4.0×10^2–1.6×10^4	10.6
Bloody clam (10)	4.1 ± 0.1	7.7×10^6	3.0×10^3–7.5×10^7	5.1×10^3	4.4×10^2–3.6×10^4	0.07
Squid (10)	8.6 ± 0.2	1.6×10^4	1.2×10^3– 4.0×10^4	6.6×10^2	1.0×10^2–2.0×10^3	4.1

[a] Mauve colonies on CHROMagar Vibrio was presumed as that of *V. parahaemolyticus* and this assumption was confirmed as described in the text.

[b] The value obtained from $\frac{\text{average no. of } V.\ parahaemolyticus}{\text{average no. of total bacteria}} \times 100$

[c] Mean ± SD

Table 13 Serotypes of *V. parahaemolyticus* detected in single shellfish samples.

Shellfishes	O:K serotypes				
Shrimp	O1:KUT	O4:K34	O10:K24	O8:KUT	O8:KUT
Crab	O3:K6	O4:K4	O4:K4	O4:KUT	O5:K17
Mussel	O1:KUT	O1:KUT	O1:KUT	O2:KUT	O11:KUT
Hard clam	O3:KUT	O4:KUT	O8:KUT	O8:KUT	O8:KUT
Bloody clam	O3:K48	O3:KUT	O4:K63	O4:KUT	ND[a]
Squid	O3:KUT	O4:K8	O4:KUT	O10:K52	O10:KUT

Five isolates of *V. parahaemolyticus* were determined for each shellfish sample.

The O:K serotype of each isolate was determined by agglutination with anti-O and anti-K antibodies.

[a]Not determine.

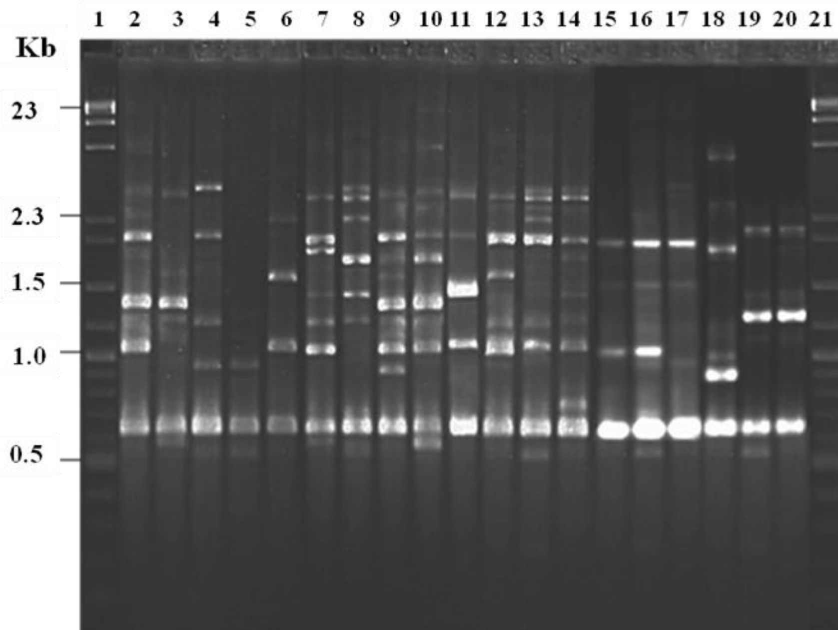

Figure 16 AP-PCR profile of *V. parahaemolyticus* isolates from shellfish.
DNA fingerprints of identical or nearly identical serotypes of *V. parahaemolyticus* isolates from shellfish were determined. The results were obtained with primer 2.

Lane 1 and 21 λ/ Hind III + 100 bp ladders
Lane 2 VP PSU 3185, O8:KUT, shrimp[a]
Lane 3 VP PSU 3192, O8:KUT, shrimp
Lane 4 VP PSU 3242, O4:K4, crab
Lane 5 VP PSU 3244, O4:K4, crab
Lane 6 VP PSU 3245, O4:KUT, crab
Lane 7 VP PSU 3098, O4:K63, bloody clam
Lane 8 VP PSU 3103, O4:KUT, bloody clam
Lane 9 VP PSU 3100, O3:K48, bloody clam
Lane 10 VP PSU 3106, O3:KUT, bloody clam
Lane 11 VP PSU 3195, O4:KUT, squid
Lane 12 VP PSU 3200, O4:K8, squid
Lane 13 VP PSU 3201, O10:K52, squid
Lane 14 VP PSU 3202, O10:KUT, squid
Lane 15 VP PSU 5312, O1:KUT, mussel
Lane 16 VP PSU 5313, O1:KUT, mussel
Lane 17 VP PSU 5314, O1:KUT, mussel
Lane 18 VP PSU 5309, O8:KUT, hard clam
Lane 19 VP PSU 5310, O8:KUT, hard clam
Lane 20 VP PSU 5311, O8:KUT, hard clam

VP = *V. parahaemolyticus*

[a] Sample which *V. parahaemolyticus* was isolated.

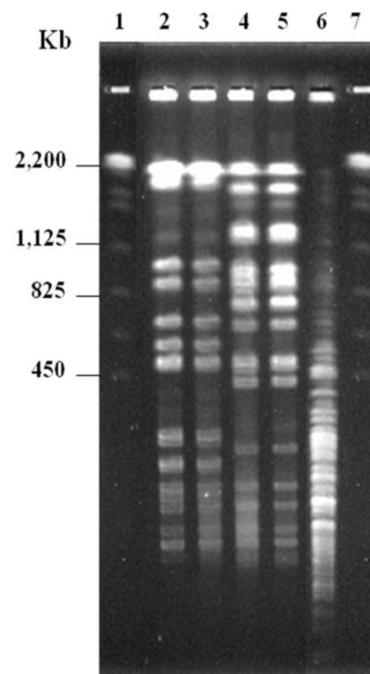

Figure 17 PFGE confirmation of *V. parahaemolyticus* isolates from mussel and hard clam. Both isolates of each serotype (O1:KUT and O8:KUT) of *V. parahaemolyticus* obtained from mussel and hard clam were investigated.

Lane 1 and 7 *Saccharomyces cerevisiae* PFGE marker
Lane 2 VP PSU 5312 O1:KUT, mussel
Lane 3 VP PSU 5313 O1:KUT, mussel
Lane 4 VP PSU 5310 O8:KUT, hard clam
Lane 5 VP PSU 5311 O8:KUT, hard clam
Lane 6 *Pseudomonas aeruginosa* control strain
VP = *V. parahaemolyticus*

Part II. Isolation and characterization of biosurfactants that possess inhibitory activity against *V. parahaemolyticus*.

Isolation and identification of bacterium producing biosurfactants

In this study, 12 and 16 of soil samples were collected from mangrove and coastal area, respectively. A total of 462 bacterial isolates were obtained, using cross streak technique, 11 and 15 isolates (5.6% of total isolates) from mangrove and coastal area, respectively exhibited antimicrobial activity against *V. parahaemolyticus*. Crude extract derived from acid precipitation revealed that seven isolates exhibited inhibitory activity against *V. parahaemolyticus* with inhibition zone between 11.8-14.3 mm (Table 14). Surface tension of those isolates was evaluated and it was between 33.2 and 38.3 mN/m. The isolate designated as SM11 that exhibited the lowest surface tension property was selected and designated as SM11 was identified by morphological colony and biochemical test (Table 15 & Figure 18). The isolated strain SM11 was further classified in Genus *Bacillus* by the standard method described in Berkey's Manual of Determinative Bacteriology, it was Gram positive, aerobic, ellipsoidal endospore-forming rod with swollen sporangium and motile with peritrichous flagella. Confirmation of this bacterium by 16S rDNA sequencing by using neighbour-joining phylogenetic tree of *Bacillus* sp. SM11 was constructed by Mega 4.0.2. SM11 sequences were showed and bar (0.01) represented 10% sequence divergence (Figure 19). *Stenotrophomonas maltophilia* and *Staphylococcus lentus* were used as outgroup comparison. *Bacillus* sp. SM11 was closed to *B. amyloliquefaciens* dhs-28 (GQ903336) and *B. amyloliquefaciens* ES-2 (DQ177319) at 99.8 % similarity, respectively. Thus, it is concluded that *Bacillus* sp. SM11 is *Bacillus amyloliquefaciens*.

Table 14 Inhibitory activity against *V. parahaemolyticus* and surface tension of bacterial isolates from soil.

Isolates	Sources	Inhibition zone (mm) [a]	Surface tension [c] (mN/m)
S15	coastal area	12.4 ± 0.2 [b]	38.3 ± 0.1 [b]
YS24	mangrove	11.8 ± 0.1	38.0 ± 0.0
KS43	coastal area	12.4 ± 0.1	36.1 ± 0.1
KS21	coastal area	13.3 ± 0.1	35.6 ± 0.1
SM6	mangrove	12.1 ± 0.1	34.6 ± 0.0
KS33	coastal area	14.3 ± 0.1	34.3 ± 0.1
SM11	mangrove	13.2 ± 0.1	33.2 ± 0.0

[a]Agar well diffusion method

[b]Mean ± SD

[c] Initial surface tension of medium = 45.2 ± 0.2 mN/m

Table 15 Biochemical test of *Bacillus* sp. SM11

Characteristics	Results of reactions
Motility	+
Growth in 7% NaCl	-
Acid from: Glucose	+
Acid from: Arabinose	+
Acid from: Xylose	+
Acid from: Mannitol	-
Gas from glucose	-
Hydrolysis of starch	+
Utilization of citrate	-
Production of indole	-
Voges-Proskauer reaction	-
Nitrate to nitrite	+
Oxidase	+
H_2S production	-
Urease	+
Esculin hydrolysis	-
Gelatin hydrolysis	+

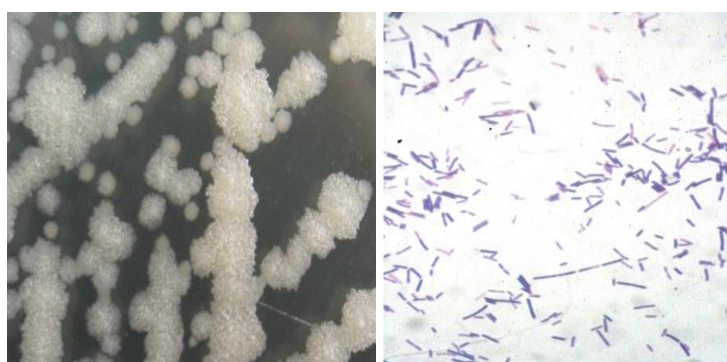

Figure 18 Colony characteristic and Gram staining of *B. amyloliquefaciens* SM11 on TSA agar

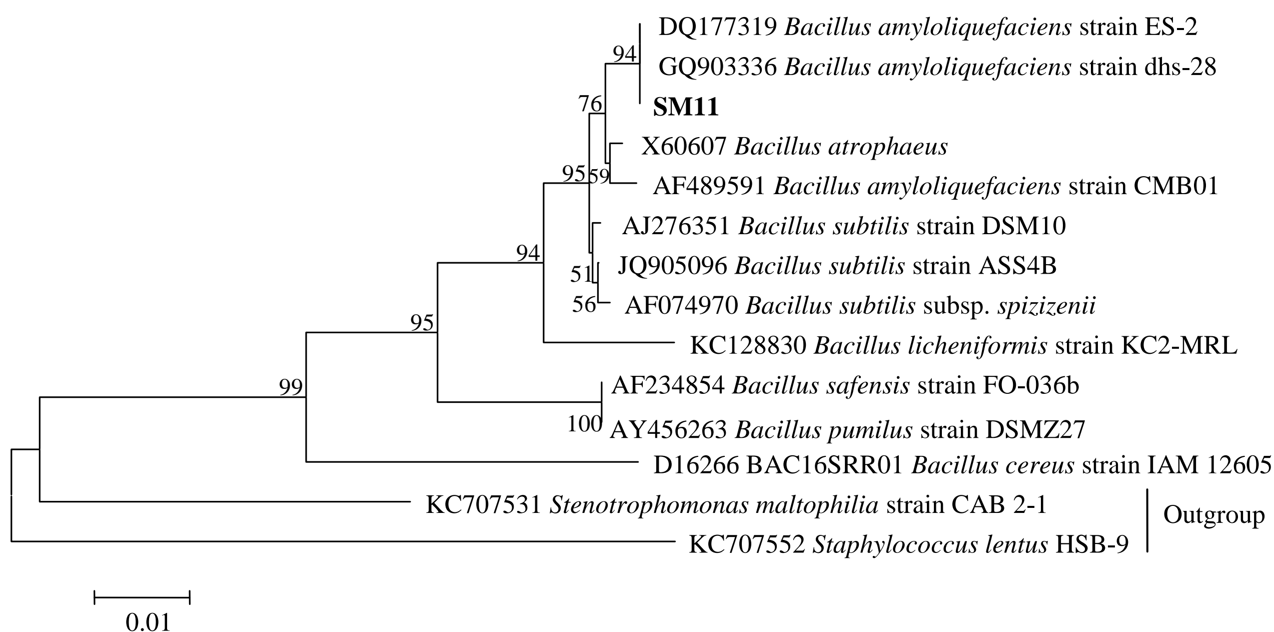

Figure 19 Neighbour-joining phylogenetic tree of *B. amyloliquefaciens* SM11 constructed by Mega 4.0.2.

Optimization of biosurfactants production.

A suitable medium for biosurfactants production of *B. amyloliquefaciens* was evaluated. It was found that inhibitory activity and surface tension of supernatant obtained from Mckeen medium were superior to MSSW, TSB and Landy media because diameter of inhibition zone against *V. parahaemolyticus* of crude extract obtained from this medium was the highest (16.9 ± 0.3 mm) and surface tension was 31.3 ± 0.2 mN/m (Table 16). Therefore, this isolate was cultured in Mckeen medium and it was found that the suitable inoculum size for producing biosurfactants was 10^8 cfu/ml with conditions of 30°C, pH 7.0 and shaker speed of 150 rpm/min (Figure 20).

Table 16 Comparison of 4 different media for biosurfactants producing by *B.amyloliquefaciens*.

Media	Inhibition zone (mm) [a]	Surface tension [c] (mN/m)
MSSW	12.6 ± 0.1[b]	33.9 ± 0.2[b]
TSB	12.7 ± 0.2	33.2 ± 0.2
Landy	13.8 ± 0.2	32.1 ± 0.2
Mckeen	16.9 ± 0.3	31.3 ± 0.2

[a]Agar well diffusion method

[b]Mean ± SD

[c]Initial surface tension of medium = 44.3 ± 0.2 mN/m

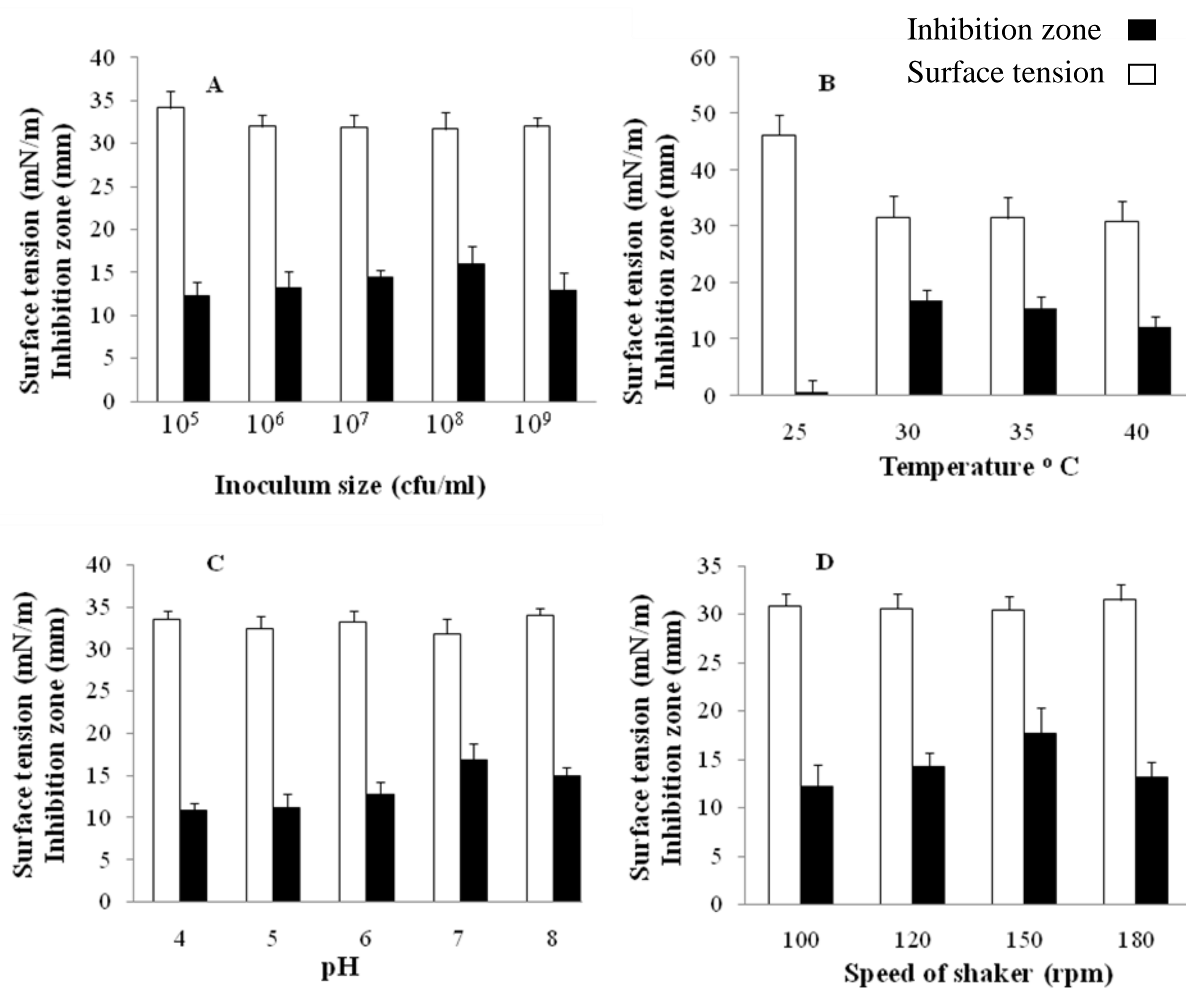

Figure 20 Conditions of biosurfactants production in Mckeen medium by *B. amyloliquefaciens*; A: inoculums size; B: temperature; C: pH and D: speed of shaker.

Kinetic production of biosurfactants by *B. amyloliquefaciens*

Biosurfactants were clearly detected at the 12 h of culture because surface tension of supernatant was decreased from 46.2 ± 0.2 to 31.1 ± 0.1 mN/m (Figure 21). At that time, diameter of inhibition zone of supernatant against *V. parahaemolyticus* was observed and increased within 24 h of incubation (17.3 ± 0.2 mm) with surface tension at 30.0 ± 0.1 mN/m. Biosurfactants that correlates to reduction of surface tension was detected continuously until the end of 48 h of culture.

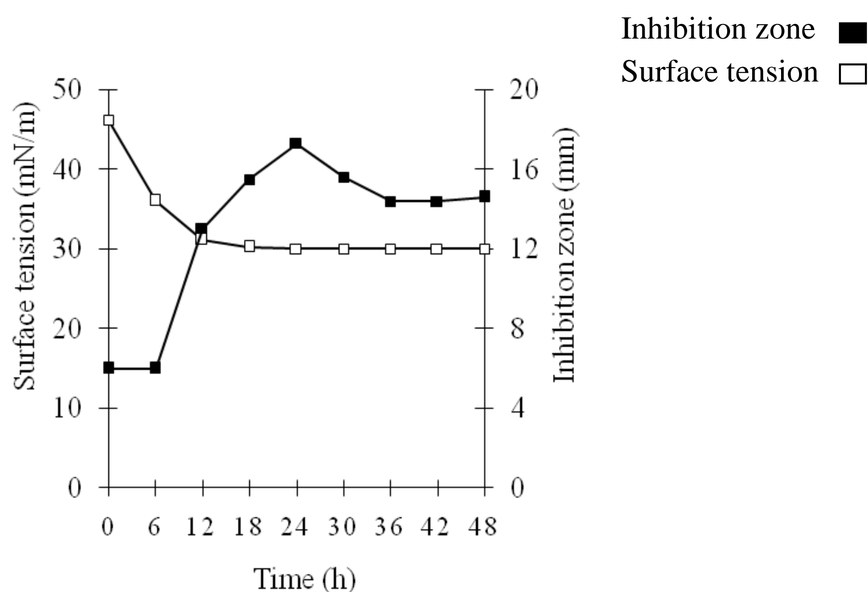

Figure 21 Kinetic production of biosurfactants produced by *B. amyloliquefaciens*.

Chloroform and methanol extraction, MIC and MBC

The crude extract obtained from *B. amyloliquefaciens* after acid precipitation was partially purified by chloroform and methanol. It was found that diameter of inhibition zone of purified extract at the concentrations of 900, 1800 and 2600 µg/ml was 16.9 ± 0.2, 18.4 ± 0.5 and 25.0 ± 0.1 mm, respectively. MIC and MBC of the extract were evaluated with three clinical and five environmental *V. parahaemolyticus* strains (Table 17). *E. coli* ATCC25922 was used as a control. MIC of the extract against *V. parahaemolyticus* was between 256 and 512 µg/ml whereas MBC was between 512 and 1024 µg/ml. There was no different in MIC and MBC among clinical and environmental isolates.

Table 17 MIC and MBC of chloroform and methanol extract of *B. amyloliquefaciens*.

V. parahaemolyticus	Sources	Serotypes	Virulence factors		MIC (µg/ml)	MBC (µg/ml)
			tdh	*trh*		
PSU4118	C	O1:K25	+	-	256	512
PSU4211	C	O3:K6	+	-	256	512
PSU2598	C	O4:K68	+	-	256	512
PSU5313	E	O1:KUT	-	-	256	512
PSU4413	E	O1:KUT	+	+	512	1024
PSU5321	E	O2:KUT	-	-	256	512
PSU4886	E	O3:K6	+	-	512	1024
PSU5310	E	O8:KUT	-	-	256	512

C: Clinical strain

E: Environmental strain

MIC and MBC to tetracycline of *E. coli* ATCC25922 were 32 µg/ml and 128 µg/ml, respectively (control).

Adhesion inhibition of crude biosurfactants against *V. parahaemolyticus*

In order to determine the effect of biosurfactants in decrease cross contamination of *V. parahaemolyticus* on surface area, adhesion inhibition assay was performed. It was found that wells coated with chloroform and methanol purified biosurfactants at the concentration of 500 µg/ml for 4 h could inhibit more than 60% of *V. parahaemolyticus* adherence, further incubation for 20 h showed no significant difference (data not shown). In addition, there was no significant difference in adhesion inhibition if the concentration of crude biosurfactant was increased to 750 and 1000 µg/ml (Figure 22) and *V. parahaemolyticus* at the concentration of 1.0 x 10^7 cfu/ml in PBS without precoating of crude biosurfactants was used as control.

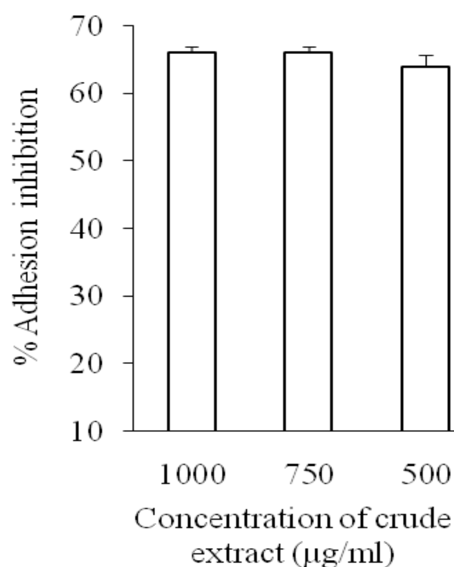

Figure 22 Adhesion inhibition of biosurfactants derived from *B. amyloliquefaciens* to *V. parahaemolyticus*.

Ionic property of biosurfactants

Ionic property of biosurfactants was determined by modified agar well diffusion technique. After 24 h of incubation, no precipitin line between test sample and SDS was detected. However, precipitin line between test sample and CTAB was observed whereas Mckeen medium showed no precipitin line with both CTAB and SDS (data not shown). This indicated that biosurfactants derived from *B. amyloliquefaciens* possess negative charge. (Figure 23).

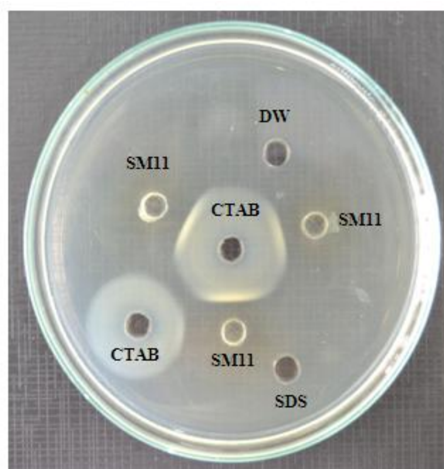

Figure 23 Ionic charge of biosurfactant derived from *B. amyloliquefaciens*; CTAB: cetyltrimethylammonium (cationic surfactant); SDS: sodium dodecyl sulfate (anionic surfactant); DW: distilled water.

DEAE-cellulose anion exchange chromatography

Chloroform and methanol extract was purified by DEAE-cellulose chromatography**.** After elution, 6 different fractions were obtained (Figure 24). Three fractions (fouth to sixth fractions) exhibited antimicrobial activity against *V. parahaemolyticus*. However, fifth fraction possessed the highest inhibitory activity against *V. parahaemolyticus* with diameter of clear zone of 16.1 ± 0.4 mm. In addition, surface tension of this fraction was lowest (30.8 ± 0.4 mN/m).

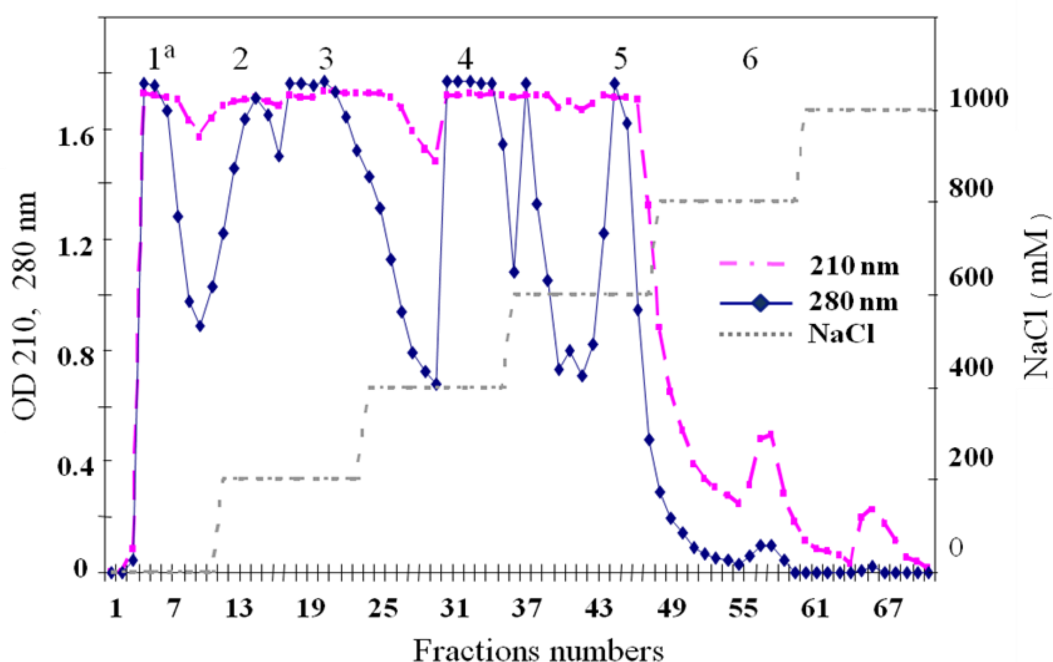

Figure 24 Biosurfactants purification by DEAE cellulose anion exchange chromatography.

[a] Pooled fraction; 1 = first fraction (Fraction number 3-7); 2 = second fraction (Fraction number 10-15); 3 = third fraction (Fraction number 16-25); 4 = fourth fraction (Fraction number 30-39); 5 = fifth fraction (Fraction number 42-48); and 6 = sixth fraction (Fraction number 55-60)

Biosurfactants purification by HPLC

The fifth fraction obtained from DEAE column chromatography was further purified by RP-HPLC. At retention time of 19.9, 27.1, 37.3, 42.2 and 44.1 min, 5 active fractions designated as PSM 1-5 were obtained (Table 18 & Figure 25). All of them possessed inhibitory activity against *V. parahaemoltyicus* with diameter of clear zone between 20.1-32.9 mm (Table 18). The highest activity was observed in PSM4 fraction. In addition, one major peak at retention time of 32.4 min was found, but no antimicrobial activity was presented.

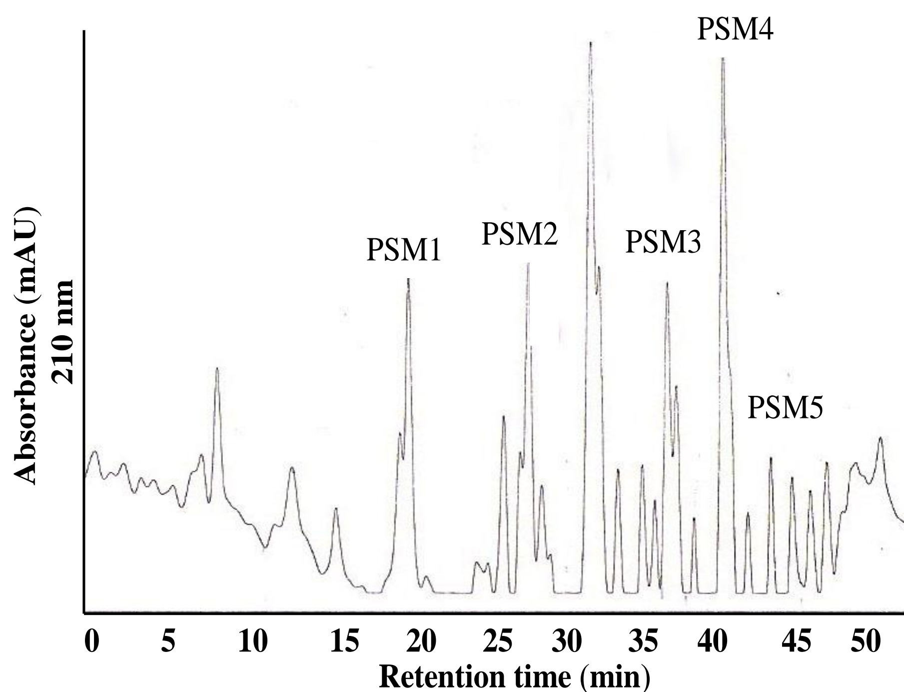

Figure 25 Active fractions (PSM1-PSM5) of biosurfactants detected by RP-HPLC.

Table 18 Antimicrobial activity against *V. parahaemolyticus* of PSM1-5 fractions obtained from RP-HPLC.

Fractions	Retention time (min)	Inhibition zone (mm) [a]
PSM1	19.9	24.7 ± 0.1 [b]
PSM2	27.1	20.1 ± 0.3
PSM3	37.3	24.9 ± 0.1
PSM4	42.2	32.9 ± 0.1
PSM5	44.1	29.9 ± 0.3

[a]Agar well diffusion method

[b]Mean ± SD

Characteristics of biosurfactants

Mass spectrometry analysis

PSM1, PSM2, PSM3, PSM4 and PSM5 were analysed by ESI-Q-TOF MS. PSM3 showed a cluster of molecular mass (*m/z* 1002.5, 1016.6, 1030.6, 1044.6, 1060.5, 1074.5 and 1088.5), whereas PSM4 showed only one mass (*m/z* 1044.6) (Table 19 & Figure 26). The mass spectra analysis indicated that those peaks were lipopeptides and belonged to surfactins (Table 19), one of the biosurfactant families. Diameter of inhibition zone of PSM3 and PSM4 against *V. parahaemolyticus* was 24.9 ± 0.1 and 32.9 ± 0.1 mm, respectively. It is possible that PSM3 contained 6 isoforms of surfactin (C11-C16) with low intensity. Thus, PSM4 showed greater inhibition zone against *V. parahaemolyticus*.

PSM1 and PSM2 also showed a cluster of molecular mass at *m/z* 1079.7, 1093.5 and 1093.5, 1107.5, respectively (Figure 27A and 27B). The mass spectra analysis indicated that all peaks were lipopeptides as mycosubtilin and belonged to iturin family (Table 19). Whereas, PSM5 showed major peaks of surfactin and iturin A with molecular mass at *m/z* 1102.6 and 1120.6, respectively (Table 19 & Figure 27C). In addition, PSM5 was superior to PSM 1 and PSM 2 against *V. parahaemolyticus* with diameter of clear zone of 29.9 ± 0.3 mm (Table 18).

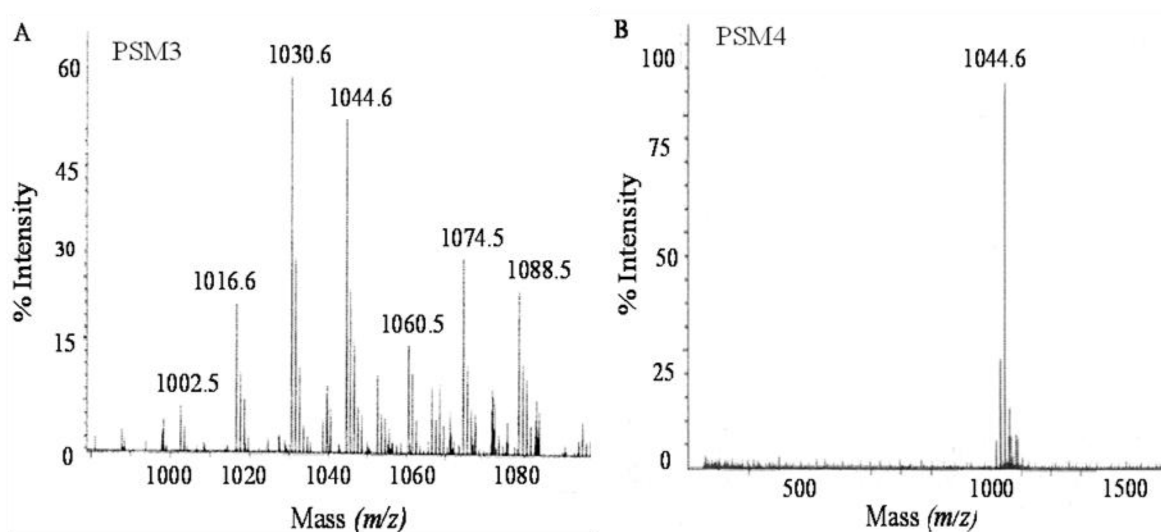

Figure 26 ESI-Q-TOF MS spectrometric characteristic of PSM3 (A) and PSM4 (B) fractions.

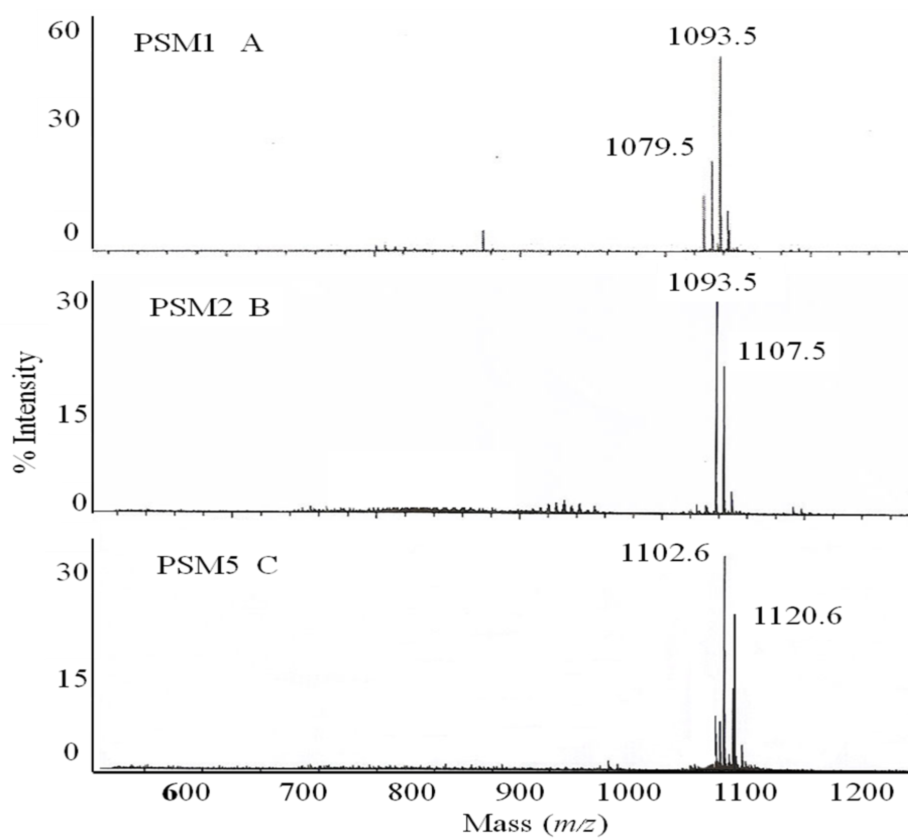

Figure 27 ESI-Q-TOF MS spectrometric characteristic of PSM1 (A), PSM2 (B), and PSM5 (C) fractions.

Table 19 Mass spectrometric characteristic of PSM1-5 fractions after eluted from RP-HPLC

Fractions	Retention time (min)	Mass peak (*m/z*)	Assignment	References
PSM1	19.9	1079.5	Mycosubtilin C15, $[M+Na]^+$	(Leclère *et al.*, 2005)
		1093.5	Mycosubtilin C16, $[M+Na]^+$	(Leclère *et al.*, 2005)
PSM2	27.1	1093.5	Mycosubtilin C16, $[M+Na]^+$	(Leclère *et al.*, 2005)
		1107.5	Mycosubtilin C17, $[M+Na]^+$	(Leclère *et al.*, 2005)
PSM3	37.3	1002.5	Surfactin C11, $[M+Na]^+$	(Pathak and Keharia, 2013)
		1016.6	Surfactin C12, $[M+Na]^+$	(Pathak and Keharia, 2013)
		1030.6	Surfactin C13, $[M+Na]^+$	(Vater *et al.*, 2002)
		1044.6	Surfactin C14, $[M+Na]^+$	(Vater *et al.*, 2002)
		1060.5	Surfactin C14, $[M+K]^+$	(Vater *et al.*, 2002)
		1074.5	Surfactin C15, $[M+K]^+$	(Pathak and Keharia, 2013)
		1088.5	Surfactin C16, $[M+K]^+$	(Hofemeister *et al.*, 2004)
PSM4	42.2	1044.6	Surfactin C14, $[M+Na]^+$	(Vater *et al.*, 2002)
PSM5	44.1	1102.6	Surfactin C17, $[M+K]^+$	(Hofemeister *et al.*, 2004)
		1120.6	Iturin A C18, $[M+Na]^+$	(Koumoutsi *et al.*, 2004)

Infrared spectroscopy analysis

Surfactins of PSM4 and PSM5 were confirmed by infrared spectroscopy analysis (Figure 28 & Figure 29). It was found that absorption bands of C-H or N-H bonds located at 3407 cm^{-1} , C-CH_3 functional group located at 2959 and 2855 cm^{-1}, peptide group located at 1666 cm^{-1}, C=O bond located at 1553 cm^{-1}, C-H bond located at 1408 cm^{-1} and CH_2 functional group located at 838 cm^{-1} were detected

(Figure 28). Comparison of those bonds in functional groups of surfactin (Sigma) previously reported in literature (Das *et al.*, 2008), it is concluded that PSM4 biosurfactant (Figure 28) is lipopeptide that belongs to surfactins family. In addition, infrared spectrum pattern of PSM5 biosurfactant (Figure 29) that possessed 2 major peaks of mass molecule belonged to surfactin and iturin A (Figure 27C) also showed IR pattern similar to surfactin.

In this study, two peaks obtained from PSM1 and PSM2 were classified as mycosubtilins which belong to iturin family (Table 19 & Figure 27A and 27B).

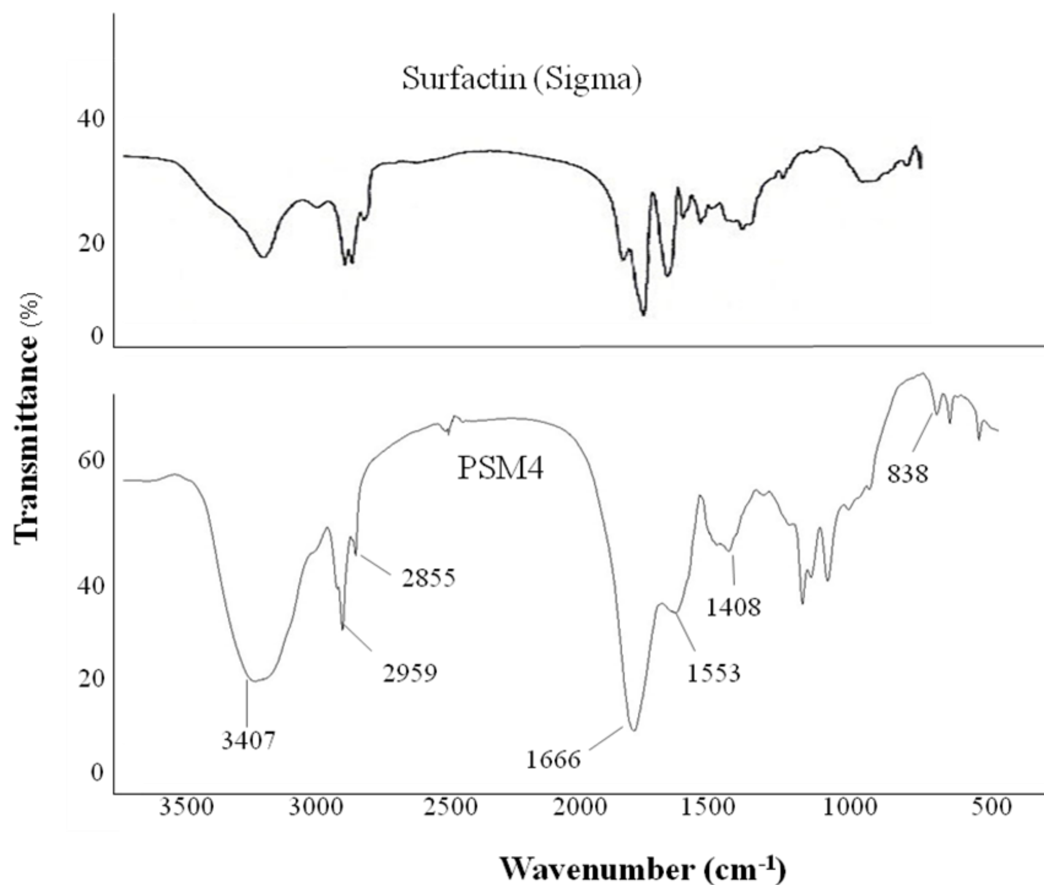

Figure 28 Infrared spectrum analysis of RP-HPLC-purified bioactive PSM4 fraction to compare with those bonds in functional groups of surfactin (Sigma) (Das *et al.*, 2008).

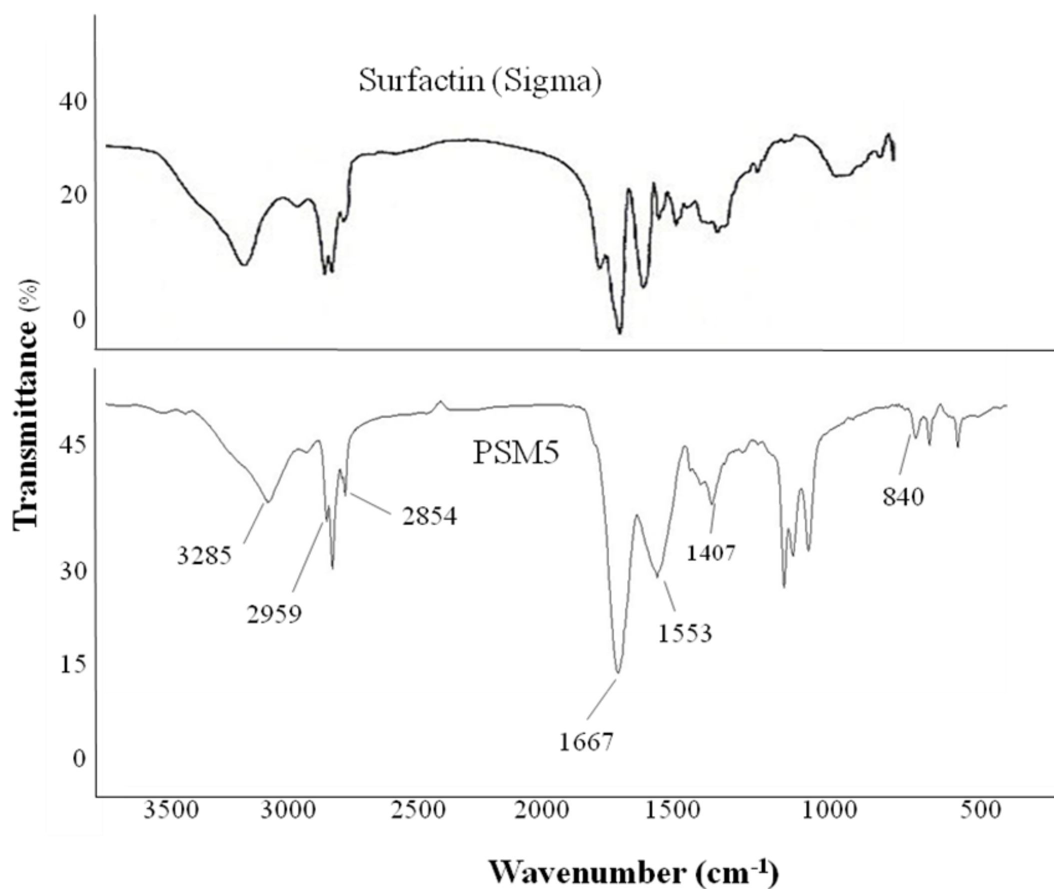

Figure 29 Infrared spectrum analysis of RP-HPLC-purified bioactive PSM5 fraction to compare with those bonds in functional groups of surfactin (Sigma) (Das *et al.*, 2008).

CHAPER 4

DISCUSSIONS

V. parahaemolyticus has been implicated in a number of illness linked to consumption of seafood. In this study, infection due to *V. parahaemolyticus* was investigated for 5 years between 2006 and 2010. It was found that most of the isolates detected were pandemic strains ($tdh^{+}trh^{-}$), and belonged to O3:K6 serotype (Table 9 and Table 11). The result is consistent in this hospital since 2000 in which O3:K6 pandemic strains were the main isolates from the patients (Figure 14) (Wootipoom *et al.*, 2007). This pandemic clone probably constantly exists in this area till the present time. In this study, the percentages of O3:K6 pandemic strains between 2006 and 2010 were alternatively up and down (Table 10), but the total percentage median of pandemic strains detected in this study (63.3%) was not significantly different from those (62.1%) detected in 2000-2005 (Figure 15) (Wootipoom *et al.*, 2007). This indicates that incidence of *V. parahaemolyticus* infection by the pandemic strains in this southern part of Thailand did not increase.

Clonal diversity of pandemic strains detected in this study was similar to those observed in the previous investigation in 2000-2005 (Table 9 & Figure 15) (Wootipoom *et al.*, 2007), except in the year 2010 in which serotype O4:K68 and O1:K25 (Table 11) were not detected as pandemic strains. However, three other serotypes (14 isolates) including O1:KUT (11), O4:K9 (2) and O3:KUT (1) were identified as pandemic strains (Table 10) in the same year.

Investigation of number of *V. parahaemolyticus* in other toxin gene profiles revealed that the numbers of isolates belonging to $tdh^{-}trh^{-}$ group was higher than the numbers of isolates belonging to $tdh^{+}trh^{+}$, and $tdh^{-}trh^{+}$ groups, and this trait was not different from the incidence previously detected in the 2000-2005 year period (Figure 15) (Wootipoom et al., 2007).

It has been reported that in Dhaka, Bangladesh, *tdh*$^{-}$*trh*$^{-}$ *V. parahaemolyticus* belonged to O5:KUT and GS-PCR negative were isolated from patients with acute diarrhea (Bhuiyan *et al.*, 2002). In this study, evidence of *tdh*$^{-}$*trh*$^{-}$ *V. parahaemolyticus* in causing disease has not been clearly understood but it has been demonstrated that a clinical *V. parahaemolyticus* strain lacking *tdh* gene was resulted from IS-mediated *tdh* gene deletion of a *tdh*$^{+}$ strain (Bhoopong *et al.*, 2007). In this study, we also detected two isolates of *tdh*$^{-}$*trh*$^{-}$ *V. parahaemolyticus* in 2009 that belonged to O3:K6 serotype and GS-PCR positive pandemic group (Table 9). Therefore, it is possible that *tdh* gene deletion was occurred in those strains. However, we cannot eliminate the possibility of another virulence factor of *V. parahaemolyticus*, type three secretion systems (T3SS). *V. parahaemolyticus* possesses T3SS that is a complex needle like structure. Its fuction is sending effector proteins from bacteria cytosol into the host cell (Tampakaki *et al.*, 2004). Two T3SS, T3SS1 and T3SS2, have been reported in *V. parahaemolyticus* (Makino *et al.*, 2003). It has been demonstrated that T3SS2 of *V. parahaemolyticus* involved in enterotoxicity in the rabbit ileal loop model (Park *et al.*, 2004b). Thus, this may be one factor involved in pathogenesis of *tdh*$^{-}$*trh*$^{-}$ strains.

In this 5 years investigation, 103 out of 776 isolates of *V. parahaemolyticus* possessed serotype KUT (untypeable K antigen). The highest number of this serotype (39 isolates) was detected in 2010. They were O1:KUT (24), O3:KUT (4), O11:KUT (3), O10:KUT (3), O4:KUT (2), O5:KUT (2) and O8:KUT (1) (Table 10). Different DNA profiles of these K serotype strains with identical O antigen have been investigated (P.M. submitted manuscript). Thus, molecular typing of those isolates needed to be performed in epidemiological investigation.

It is known that shellfish especially mollusk is one of the important sources of *V. parahaemolyticus* because it is a filter feeder (Pereira *et al.*, 2007). In Thailand, consumption of shellfish especially cockle contaminated with *V. parahaemolyticus*, is probably a major cause of acute gastroenteritis because the popular way to prepare this seafood is semi-cooking.

In this study, shellfish, especially three kinds of mollusk, were selected to evaluate the total number of bacteria and that of *V. parahaemolyticus*. CHROMagar Vibrio was used as a selective isolation medium as it is suitable for detection of *V.*

parahaemolyticus (Hara-Kudo *et al*., 2001). This agar also supports growth of bacteria belonging to most species of the genus *Vibrio* (Hara-Kudo *et al.*, 2001). These include enteropathogenic *Vibrio* species such as *Vibrio cholerae*, *Vibrio mimicus* and *Vibrio vulnificus*. If the number of total bacterial colonies on CHROMagar Vibrio well reflects the number of colonies belonging to enteropathogenic *Vibrio* species in general including *V. parahaemolyticus*, the number of total bacteria on CHROMagar Vibrio may be used as an indicator for enteropathogenic *Vibrio* species including *V. parahaemolyticus*. If so, the total bacterial count on CHROMagar Vibrio can be an alternative to quantitation of enteropathogenic *Vibrio* species by the Most Probable Number (MPN) estimation based method. The former method is easier than the latter. However, in this study, correlation does not seem to exist between the relative frequencies of total bacterial count and *V. parahaemolyticus* because the percentage of *V. parahaemolyticus* in total number of bacteria ranged from sample to sample and varied greatly among various seafood species (Table 12).

Bloody clam, mussel and hard clam which are filter feeding shellfish seem to harbor greater numbers of *V. parahaemolyticus* than other shellfish except for shrimp (Table 12). The O3:K6 pandemic strains are accumulated in these molluscan bivalves (Vuddhakul *et al.*, 2000; Vuddhakul *et al.*, 2006). *Vibrio* spp. have been demonstrated in various tissues of molluscan bivalves such as clams, oysters and mussels to levels sometimes 100 times that of the overlying water (Tantillo *et al.*, 2004). Wang *et.al* (2010) demonstrated that *V. parahaemolyticus* was accumulated in digestive glands, gills, adductor muscle and mantle cilia of oysters after artificial inoculation. In this study, the percentage of *V. parahaemolyticus* in total number of bacteria detected in bloody clam was lowest (0.07%) but the average number of this bacterium in this shellfish was not much different from those detected in hard clam and shrimp (Table 12).

It has been demonstrated that around 3-6 % of *V. parahaemolyticus* isolated from the environment carry the virulence genes (*tdh* or *trh*) (Deepanjali *et al.*, 2005; Vuddhakul *et al.*, 2006). Thus, it is not easy to isolate *tdh* and/or *trh* positive strains from seafood. In this study, we did not obtained any virulence strains of *V. parahaemolyticus* although we usually detect the presence of *tdh*$^{+}$ and/or *trh*$^{+}$ strains

by examining the boiled supernatant of the enrichment culture in our similar studies (unpublished observations). This could be due to the small number of colonies examined per sample in this study. However, two isolates of *V. parahaemolyticus* (PSU 3103 and PSU 3200) obtained from bloody clam and squid, respectively, exhibited urease activity. It has been demonstrated that the presence of *trh* gene is close to the *ure* gene on the chromosome of *V. parahaemolyticus* (Park *et al.*, 1998). Park *et al*. (2000) demonstrated an insertion sequence-like element present at the end of the DNA region containing the *trh* and *ure* genes. Thus, it seems possible that the absence of *trh* gene in these isolates may be associated with an insertion sequence mediated deletion mechanism**.**

Serotype diversity of this bacterium was demonstrated in all six single shellfish samples examined (Table 13). Two to three identical serotypes of *V. parahaemolyticus* were detected in shrimp, crab, mussel and hard clam. However, DNA fingerprinting revealed those exhibiting identical serotypes were not identical except both isolates of each serotype obtained from mussel (O1:KUT) and hard clam (O8:KUT) (Figure 16 & Figure 17). DNA profiles of those isolates obtained from a single shellfish and possessing the same O but different K antigens were also different. In addition, the DNA profiles of the isolates that possessed the same serotypes (O4:KUT) but were obtained from different kinds of shellfish (PSU 3103 from bloody clam; PSU 3195 from squid; and PSU 3245 from crab) were different (Figure 16). These indicate that most of the single shellfish harbor heterogeneous population of *V. parahaemolyticus*. The shellfish responsible for *V. parahaemolyticus* infection must contain a pathogenic strain-bearing heterogeneous population of *V. parahaemolyticus*.

V. parahaemolyticus can attach to abiotic surfaces such as stainless steel and glass surfaces during food processing and food keeping. This evidence caused by many factors such as pili (Craig *et al.*, 2004; Wong *et al.*, 2002). Decrease or elimination number of *V. parahaemolyticus* on surface may be an alternative choice to reduce cross-contamination of this bacterium to other foods. In addition, reduction of microorganisms adhere to the surface is an essential step of food safety (Nitschke *et al.*, 2009). Thus, biosurfactants that exhibit anti-adhesion activity of bacteria can resolve this problem.

Biosurfactants are a complex structure group of surface active molecules. Microbial surfactants have been characterized as (i) glycolipids, (ii) lipopeptides, (iii) fatty acids, neutral lipids, and phospholipids, (iv) polymeric surfactants, and (v) particulate biosurfactants (Vater *et al.*, 2002). Effective biosurfactants possess surface tension property ranging from 27 to 35 mN/m (Kim *et al.*, 2000; Lee *et al.*, 2002). *Bacillus* spp. has been reported to produce many kinds of surfactants (Kim *et al.*, 2009; Vater *et al.*, 2002). Some of them are cyclic lipopeptides such as surfactins, iturins, and fengycins. They may play important roles in development and survival of *Bacillus* spp. in environments such as heavy metal binding, bacterial pathogenesis and biofilm formation (Mulligan, 2005; Ron and Rosenberg, 2001). They have been involved in biotechnology and biopharmaceutical applications due to their surface active properties (Banat *et al.*, 2000; Lee *et al.*, 2006).

In this study, *B. amyloliquefaciens* SM11 derived from a mangrove area exhibited highest antimicrobial activity toward *V. parahaemolyticus* when cultured in modified synthetic sea water medium (MSSW). This medium was modified by adding peptone and yeast extract as nitrogen sources and palm oil as a carbon source for support growth of bacteria derived from brackish environments as well as for biosurfactants production (Amano *et al.*, 1982; Button *et al.*, 1993). However, we found that Mckeen was superior to MSSW, TSB and Landy media for biosurfactant production; thus, Mckeen was used throughout this study. Composition of this medium is similar to Landy medium which has been reported to produce high yield and variety of biosurfactants among of *Bacillus* spp. (Arguelles *et al.*, 2009; Gordillo *et al.*, 2009; Kinsinger *et al.*, 2005). Investigation of biosurfactants producing by this bacterium revealed that it should be a secondary metabolite because surface tension and inhibitory activity were highest at 24 h of culture (stationary phase) (Table 16 & Figure 21).

For partially purify of biosurfactants, acid precipitation followed by chloroform: methanol extraction are standard and low cost techniques. Crude biosurfactants obtained can be screened for their activities. Agar double diffusion technique (Figure 23), revealed precipitin lines between crude extract of SM11 and cationic surfactant (CTAB). This technique demonstrated the anionic character of

biosurfactants derived from *B. amyloliquefaciens* SM11 (Meylheuc *et al.*, 2001). However, to identify biosurfactants, ion-exchange column chromatography, RP-HPLC, mass spectrometry and infrared spectroscopy are needed.

Purification of supernatant derived from this *B. amyloliquefaciens* isolate and analysis by mass spectrometry revealed peaks of PSM3, PSM4 and PSM5. PSM3 contained six isoforms of surfactins (C11-C16) (Table 19 & Figure 26A) which intensity was lower than PSM4 surfactin (C14) (Figure 26B). This correlated to its inhibitory activity against *V. parahaemolyticus* in which diameter of clear zone of PSM3 was 24.9 ± 0.1 mm whereas diameter of clear zone of PSM4 was 32.9 ± 0.1 mm (Table 18). PSM5 contained 2 major peaks of high intensity of C17-surfactin and medium intensity of C18-iturin A (Table 19 & Figure 27C), although, it also possessed intensity lower than PSM4.

Infrared spectroscopy is a technique used to identify chemical structures of compounds because their functional groups provide peaks both in terms of intensity and position (cm^{-1}). Infrared spectra in term of shape and intensity of peaks obtained from PSM4 and PSM5 were compared with known pattern of surfactins previously reported or standard surfactin (Sigma). In this study, PSM4 and PSM5 were classified as surfactin because their spectra were similar to infrared spectrum of surfactin derived from *B. circulans* and standard surfactin from Sigma (Das *et al.*, 2008) (Figure 28 & Figure 29). Using mass spectrometry and infrared spectroscopy, molecule structure of PSM4 (1044.6 Da) at N-terminus should be L-Glu_1-L-Leu_2-D-Leu_3-L-Ala_4-L-Asp_5-D-Leu_6-L-Leu_7-C14 (Romano *et al.*, 2011; Vater *et al.*, 2002) and molecule structure of surfactin of PSM5 (1102.6 Da) at N-terminus should be L-Glu_1-L-Leu_2-D-Leu_3-L-Ala_4-L-Asp_5-D-Leu_6-L-Leu_7-C17.

Surfactin isoforms comprised of short and long carbon chains may influence antimicrobial activity because it has been demonstrated that long carbon chain surfactin analogs increase antimicrobial activity compare to the shortest chain (Dufour *et al.*, 2005; Vollenbroich *et al.*, 1997a). In addition, it has been reported that C14-C15 carbon atom of surfactin showed higher antiviral activity than C13 isoform. Therefore, hydrophobic of long chain carbon atom may be important determination for application of antimicrobial activity. This is the first report of seven surfactin isoforms produced by *B. amyloliquefaciens* in one condition of culture.

Surfactin is a cyclic lipoheptapeptides which contains a hydroxyl fatty acid. It decreases surface tension of two phases and has been demonstrated to increase surface area for anti-adhesion activity to prevent biofilm formation of human bacterial pathogens (Rivardo *et al.*, 2009). In addition, surfactin is able to induce formation of ion channels in lipid bilayer membrane (Deleu *et al.*, 2003; Vollenbroich *et al.*, 1997b). Surfactin has been reported to inactivated herpes and retroviruses. In addition, it has been demonstrated to prevent virus and mycoplasma in biotechnological product (Vollenbroich *et al.*, 1997a; Vollenbroich *et al.*, 1997b).

Other two peaks including PSM1 and PSM2 belonged to iturin family and classified as mycosubtilins (C15-C17) (Table 19 & Figure 27A and 27B). They exhibited antimicrobial activity against *V. parahaemolyticus* with clear zone of 24.7 ± 0.1 and 20.1 ± 0.3 mm, respectively (Table 18). Generally, antimicrobial activity of iturin family in bacteria was limited (Ongena and Jacques, 2007). However, in this study, mycosubtilins inhibited *V. parahaemolyticus* in the same manner as surfactin.

Biosurfactant in the family iturin represented by iturin A, mycosubtilin and bacillomycin are most commonly studied for strong antifungal activity (Leclère *et al.*, 2005; Maget-Dana and Peypoux, 1994).

Iturin A possesses antibiotic and antifungal activities. Effect of iturin A on *B. subtilis* surface hydrophobicity has been documented (Ahimou *et al.*, 2000). Furthermore, previous studies showed that iturin A lysed protoplasts of *Micrococcus luteus* and formed channels in artificial lipid membranes (Maget-Dana *et al.*, 1985a). Investigation of antifungal activity of iturin A on *Saccharomyces cerevisiae* revealed that its activity was on membrane permeability which permitted nucleotides, proteins, polysaccharides and lipids to escape from cells (Latoud *et al.*, 1987). In addition, hemolytic activity of iturin A has been demonstrated. iturin A released intracellular K^+ ions and hemoglobin from erythrocytes (Latoud *et al.*, 1986). This evidence indicated that target of iturin A is the cytoplasmic membrane of cells.

Mycosubtilin exhibited strong antifungal activities toward fungal cytoplasmic membrane (Fickers *et al.*, 2009; Leclère *et al.*, 2005; Nasir *et al.*, 2010). Binding to artificial phospholipid monolayers (DMPC) at a ratio of a mycosubtilin/DMPC (1:2) increased ion permeability of DMPC by forming ion conducting pores. Mode of action of mycosubtilins was similar to other members in

iturin family which depended on amount of lipid in membrane (Fickers *et al.*, 2009; Maget-Dana and Ptak, 1990; Nasir and Besson, 2011). In this study, mycosubtilin isoforms were detected identically in terms of peptide sequence but they were different in length of aliphatic side chain. Their structures from N terminal should be L-Asn_1-D-Tyr_2-D-Asn_3-L-Gln_4-L-Pro_5-D-Ser_6-L-Asn_7-C15-17, respectively (Fickers *et al.*, 2009; Ongena and Jacques, 2008).

In this study, we evaluated chloroform and methanol extract of biosurfactant derived from *B. amyloliquefaciens* to reduce interfacial tension between *V. parahaemolyticus* and surface area in order to reduce cross contamination of food during preparation. It was found that partial purified biosurfactant that contained surfactins, iturin A and mycosubtilins at the concentration of 500 μg/ml could inhibit more than 60% of *V. parahaemolyticus* adhesion levels through the previous coating of biosurfactants on polystylene surfaces (Figure 22). Synergistic effect of surfactin and iturin A have been demonstrated on monolayer membrane traget and increase hemolysis (Maget-Dana *et al.*, 1992; Razafindralambo *et al.*, 1997). Thus, crude extract biosurfactants obtained in this study may be more useful than purification form. Most of biosurfactants have been reported to be low toxicity for plants and animals as well as low irritancy and compatibility with human skin (Cameotra and Makkar, 2004). Thus, biosurfactants obtained in this study may be applied in food processing to reduce cross-contamination of *V. parahaemolyticus* on surface area of cooking.

CHAPTER 5

CONCLUSIONS

Investigation of *V. parahaemolyticus* isolates from Hat yai Hospital, southern Thailand during 2006-2010 revealed that the predominant isolates were pandemic O3:K6 strains. The numbers of isolates belonging to each toxin gene profile were not significant different from the numbers of those isolates obtained from the same hospital in 2000-2005. This indicates that people in this area are constantly exposed to pathogenic *V. parahaemolyticus* in the same level. Therefore, this area is probably endemic area of *V. parahaemolyticus*. Moreover, gastroenteritis of *V. parahaemolyticus* infection in 2010 was related to KUT serotype that mostly isolated from seafood samples in the same area. Therefore, to reduce number of *V. parahaemolyticus* in this area is important to decrease incidence of infections by this organism.

Determination of *V. parahaemolyticus* in single shellfish using CHROMagar Vibrio isolation medium, revealed that highest numbers of total vibrios and *V. parahaemolyticus* were detected in bloody clam and mussel, respectively**.** There was no correlation between the number of total vibrios and that of *V. parahaemolyticus***.** Virulence genes (*tdh* and *trh*) could be detected in none of the isolated strains. Diversities in the serotype and DNA fingerprints indicated that most of the single shellfish harbors heterogeneous population of *V. parahaemolyticus* and the concentrations of pathogenic strains are low.

B. amyloliquefaciens SM11 was isolated from a mangrove area and exhibited biosurfactant property. Biosurfactants derived from this bacterium were identified as surfactins, iturin A and mycosubtilins. Crude extract of biosurfactants from this bacterium inhibited *V. parahaemolyticus* adherence. Thus, these biosurfactants may be useful to decrease cross-contamination of food due to this bacterium. Biosurfactants derived from this bacterium may be developed as surface cleaning solution for food safety in the future.

REFERENCES

Aarestrup, F. M., and Wegener, H. C. 1999. The effects of antibiotic usage in food animals on the development of antimicrobial resistance of importance for humans in *Campylobacter* and *Escherichia coli*. Microbes and Infection 1:639-644.

Ahimou, F., Jacques, P., and Deleu, M. 2000. Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enzyme and Microbial Technology 27:749-754.

Amano, M., Hara, S., and Taga, N. 1982. Utilization of dissolved amino acids in seawater by marine bacteria. Marine Biology 68:31-36.

Aranda, F. J., Teruel, J. A., and Ortiz, A. 2005. Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. Biochimica et Biophysica Acta (BBA)-Biomembranes 1713:51-56.

Arguelles, A. A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B., and Fickers, P. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microbial Cell Factories 8:1-12.

Arima, K., Kakinuma, A., and Tamura, G. 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochemical and Biophysical Research Communications 31:488-494.

Ashida, H., Ogawa, M., Kim, M., Mimuro, H., and Sasakawa, C. 2011. Bacteria and host interactions in the gut epithelial barrier. Nature Chemical Biology 8:36-45.

Baffone, W., Casaroli, A., Campana, R., Citterio, B., Vittoria, E., Pierfelici, L., and Donelli, G. 2005. In vivo:studies on the pathophysiological mechanism of *Vibrio parahaemolyticus* TDH (+)-induced secretion. Microbial Pathogenesis 38:133-137.

Baker-Austin, C., McArthur, J. V., Tuckfield, R. C., Najarro, M., Lindell, A. H., Gooch, J., and Stepanauskas, R. 2008. Antibiotic resistance in the shellfish

pathogen *Vibrio parahaemolyticus* isolated from the coastal water and sediment of Georgia and South Carolina, USA. Journal of Food Protection 71:2552-2558.

Banat, I. M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Bioresource Technology 51:1-12.

Banat, I. M., Makkar, R. S., and Cameotra, S. S. 2000. Potential commercial applications of microbial surfactants. Applied Microbiology and Biotechnology 53:495-508.

Banerjee, S., and Hansen, J. N. 1988. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. Journal of Biological Chemistry 263:9508-9514.

Bernheimer, A. W., and Avigad, L. S. 1970. Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. Journal of General Microbiology 61:361-369.

Bhoopong, P., Palittapongarnpim, P., Pomwised, R., Kiatkittipong, A., Kamruzzaman, M., Nakaguchi, Y., Nishibuchi, M., Ishibashi, M., and Vuddhakul, V. 2007. Variability of properties of *Vibrio parahaemolyticus* strains isolated from individual patients. Journal of Clinical Microbiology 45:1544-1550.

Bhuiyan, N. A., Ansaruzzaman, M., Kamruzzaman, M., Alam, K., Chowdhury, N. R., Nishibuchi, M., Faruque, S. M., Sack, D. A., Takeda, Y., and Nair, G. B. 2002. Prevalence of the pandemic genotype of *Vibrio parahaemolyticus* in Dhaka, Bangladesh, and significance of its distribution across different serotypes. Journal of Clinical Microbiology 40:284-286.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T., and Prinsep, M. R. 2006. Marine natural products. Natural Product Reports 21:1-49.

Bodour, A. A., Wang, J. M., Brusseau, M. L., and Maier, R. M. 2003. Temporal change in culturable phenanthrene degraders in response to long-term exposure to phenanthrene in a soil column system. Environmental Microbiology 5:888-895.

Bouchotroch, S., Quesada, E., del Moral, A., Llamas, I., and Bajar, V. 2001. *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-

producing bacterium. International Journal of Systematic and Evolutionary Microbiology 51:1625-1632.

Butt, A. A., Aldridge, K. E., and Sanders, C. V. 2004. Infections related to the ingestion of seafood part I: viral and bacterial infections. The Lancet Infectious Diseases 4:201-212.

Button, D. K., Schut, F., Quang, P., Martin, R., and Robertson, B. R. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Applied and Environmental Microbiology 59:881-891.

Caldeira, A. T., Santos Arteiro, J. M., Coelho, A. V., and Roseiro, J. C. 2011. Combined use of LC-ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochemistry 46:1738-1746.

Cameotra, S. S., and Makkar, R. S. 2004. Recent applications of biosurfactants as biological and immunological molecules. Current Opinion in Microbiology 7:262-266.

Cao, X., Wang, A. H., Jiao, R. Z., Wang, C. L., Mao, D. Z., Yan, L., and Zeng, B. 2009. Surfactin induces apoptosis and G2/M arrest in human breast cancer MCF-7 cells through cell cycle factor regulation. Cell Biochemistry and Biophysics 55:163-171.

Chen, Y., Stine, O. C., Badger, J. H., Gil, A. I., Nair, G. B., Nishibuchi, M., and Fouts, D. E. 2011. Comparative genomic analysis of *Vibrio parahaemolyticus*: serotype conversion and virulence. BMC Genomics 12:294-305.

Commission, Joint FAO/WHO Codex Alimentarius, and Programme, Joint FAO/WHO Food Standards. 2003. *Codex alimentarius: food hygiene basic texts*: Food & Agriculture Organization.

Cook, D. W., Bowers, J. C., and DePaola, A. 2002. Density of total and pathogenic (*tdh+*) *Vibrio parahaemolyticus* in Atlantic and Gulf Coast molluscan shellfish at harvest. Journal of Food Protection 65:1873-1880.

Cooper, D. G., and Goldenberg, B. G. 1987. Surface-active agents from two *Bacillus* species. Applied and Environmental Microbiology 53:224-229.

Cooper, D. G., and Zajic, J. E. 1980. Surface-active compounds from microorganisms. Advances in Applied Microbiology 26:229-253.

Craig, L., Pique, M. E., and Tainer, J. A. 2004. Type IV pilus structure and bacterial pathogenicity. Nature Reviews Microbiology 2:363-378.

Cutler, A. J., and Light, R. J. 1979. Regulation of hydroxydocosanoic acid sophoroside production in *Candida bogoriensis* by the levels of glucose and yeast extract in the growth medium. Journal of Biological Chemistry 254:1944-1950.

Dadisman, T. A., Nelson, R., Molenda, J. R., and Garber, H. J. 1972. *Vibrio parahaemolyticus* gastroenteritis in Maryland I. clinical and epidemiologic aspects. American Journal of Epidemiology 96:414-426.

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., Thompson, S., Wilson, S., Bean, N. H., and Griffin, P. M. 2000a. *Vibrio parahaemolyticus* infections in the United States, 1973-1998. Journal of Infectious Diseases 181:1661-1666.

Daniels, N. A., Ray, B., Easton, A., Marano, N., Kahn, E., McShan II, A. L., Del Rosario, L., Baldwin, T., Kingsley, M. A., and Puhr, N. D. 2000b. Emergence of a new *Vibrio parahaemolyticus* serotype in raw oysters. The Journal of the American Medical Association 284:1541-1545.

Das, P., Mukherjee, S., and Sen, R. 2008. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. Journal of Applied Microbiology 104:1675-1684.

Das, P., Mukherjee, S., and Sen, R. 2009. Biosurfactant of marine origin exhibiting heavy metal remediation properties. Bioresource Technology 100:4887-4890.

De Araujo, L. V., Abreu, F., Lins, U., Anna, L. M. de M. S., Nitschke, M., and Freire, D. M. G. 2011. Rhamnolipid and surfactin inhibit *Listeria monocytogenes* adhesion. Food Research International 44:481-488.

de Faria, A. F., Stéfani, D., Vaz, B. G., Silva, S., Garcia, J. S., Eberlin, M. N., Grossman, M. J., Alves, O. L., and Durrant, L. R. 2011. Purification and structural characterization of fengycin homologues produced by *Bacillus subtilis* LSFM-05 grown on raw glycerol. Journal of Industrial Microbiology & Biotechnology 38:863-871.

Deepanjali, A., Kumar, H. S., and Karunasagar, I. 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters

along the southwest coast of India. Applied and Environmental Microbiology 71:3575-3580.

Deleu, M., Bouffioux, O., Razafindralambo, H., Paquot, M., Hbid, C., Thonart, P., Jacques, P., and Brasseur, R. 2003. Interaction of surfactin with membranes: a computational approach. Langmuir 19:3377-3385.

DePaola, A., Kaysner, C. A., Bowers, J., and Cook, D. W. 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). Applied and Environmental Microbiology 66:4649-4654.

Desai, J. D., and Banat, I. M. 1997. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology reviews 61:47-64.

DiRita, V. J. 1992. Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Molecular Microbiology 6:451-458.

Drake, S. L., DePaola, A., and Jaykus, L. A. 2007. An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Comprehensive Reviews in Food Science and Food Safety 6:120-144.

Dubey, K. V., Juwarkar, A. A., and Singh, S. K. 2005. Adsorption desorption process using wood based activated carbon for recovery of biosurfactant from fermented distillery wastewater. Biotechnology Progress 21:860-867.

Dufour, S., Deleu, M., Nott, K., Wathelet, B., Thonart, P., and Paquot, M. 2005. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. Biochimica Biophysica Acta 1726:87-95.

Falagas, M. E., and Makris, G. C. 2009. Probiotic bacteria and biosurfactants for nosocomial infection control: a hypothesis. Journal of Hospital Infection 71:301-306.

Fickers, P., Guez, J. S., Damblon, C., Leclère, V., Béchet, M., Jacques, P., and Joris, B. 2009. High-level biosynthesis of the anteiso-C17 isoform of the antibiotic mycosubtilin in *Bacillus subtilis* and characterization of Its candidacidal activity. Applied and Environmental Microbiology 75:4636-4640.

Geneste, C., Dab, W., Cabanes, P. A., Vaillant, V., Quilici, M. L., and Fournier, J. M. 2000 Les vibrioses noncholériques en France: cas identifiés de 1995 à 1998

par le Centre National de Référence. Bulletin Epidémiologie Hebdomadaire 9:38-40.

Gordillo, M. A., Navarro, A. R., Benitez, L. M., Torres de Plaza, M., and Maldonado, M. C. 2009. Preliminary study and improve the production of metabolites with antifungal activity by a *Bacillus* sp. strain IBA 33. Microbiology Insights 2:15-24.

Grau, A., Gómez-Fernández, J. C., Peypoux, F., and Ortiz, A. 2001. Aggregational behavior of aqueous dispersions of the antifungal lipopeptide iturin A. Peptides 22:1-5.

Gudina, E. J., Rocha, V., Teixeira, J. A., and Rodrigues, L. R. 2010. Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20. Letters in Applied Microbiology 50:419-424.

Gurjar, M., Khire, J. M., and Khan, M. 1995. Bioemulsifier production by *Bacillus stearothermophilus* VR8 isolate. Letters in Applied Microbiology 21:83-86.

Hall, T. 2004. BioEdit 6.0. 7. Department of Microbiology, North Carolina State University.

Han, Y., Huang, X., Cao, M., and Wang, Y. 2008. Micellization of surfactin and its effect on the aggregate conformation of amyloid Aβ(1-40). The journal of Physical Chemistry B 112:15195-15201.

Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J., and Kumagai, S. 2001. Improved method for detection of *Vibrio parahaemolyticus* in seafood. Applied and Anvironmental Microbiology 67:5819-5823.

Hewald, S., Josephs, K., and Bulker, M. 2005. Genetic analysis of biosurfactant production in *Ustilago maydis*. Applied and Environmental Microbiology 71:3033-3040.

Hofemeister, J., Conrad, B., Adler, B., Hofemeister, B., Feesche, J., Kucheryava, N., Steinborn, G., Franke, P., Grammel, N., and Zwintscher, A. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptide-and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Molecular Genetics and Genomics 272:363-378.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. 1994. Bergey's manual of determinative bacteriology (9th edn.), Baltimore,

Philadelphia, Hong Kong. London, Munich, Sydney, Tokyo: Williams & Wilkins.

Honda, T., Ni, Y. X., and Miwatani, T. 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infection and Immunity 56:961-965.

Hood, S. K., and Zottola, E. A. 1995. Biofilms in Food Processing. Food control 6:9-18.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiology and Molecular Biology Reviews 62:379-433.

Hurley, C. C., Quirke, A. M., Reen, F. J., and Boyd, E. F. 2006. Four genomic islands that mark post-1995 pandemic *Vibrio parahaemolyticus* isolates. BMC Genomics (1):104-118.

Hutchison, M. L., Tester, M. A., and Gross, D. C. 1995. Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction. Molecular Plant-Microbe Interactions 8:610-620.

Iida, T., and Yamamoto, K. 1990. Cloning and expression of two genes encoding highly homologous hemolysins from a Kanagawa-phenomenon-positive *Vibrio parahaemolyticus* T4750 strain. Gene 93:9-15.

Ito, T., and Ogawa, H. 1959. Chemical studies on the antibiotic esperin part III. on the structure of esperin. Bulletin Agricultural Chemical Society of Japan 23:536.

Janek, T., Lukaszewicz, M., and Krasowska, A. 2012. Antiadhesive activity of the biosurfactant pseudofactin II secreted by the Arctic bacterium *Pseudomonas fluorescens* BD5. BMC Microbiology 12:24-32.

Jatapai, A., Moungthong, B., Thunyaharn, S., Huttayananont, S., and Rangsin, R. 2010. An acute gastroenteritis outbreak of *Vibrio parahaemolyticus* O4: K55 in Nursing College, Thailand. Tropical Biomedicine 27:265-274.

Johnson, C. N., Flowers, A. R., Young, V. C., Gonzalez-Escalona, N., DePaola, A., Noriea III, N. F., and Grimes, D. J. 2009. Genetic relatedness among *tdh*+ and *trh*+ *Vibrio parahaemolyticus* cultured from Gulf of Mexico oysters

(*Crassostrea virginica*) and surrounding water and sediment. Microbial Ecology 57:437-443.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T. L. 2008. NCBI BLAST: a better web interface. Nucleic Acids Research 36:W5-W9.

Joseph, B., Otta, S. K., Karunasagar, I., and Karunasagar, I. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. International journal of Food Microbiology 64:367-372.

Joseph, S. W., Colwell, R. R., and Kaper, J. B. 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. Critical Reviews in Microbiology 10:77-124.

Kelly, M. T., and Stroh, E. M. 1989. Urease-positive, Kanagawa-negative *Vibrio parahaemolyticus* from patients and the environment in the Pacific Northwest. Journal of Clinical Microbiology 27:2820-2822.

Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L., and Kokare, C. 2012. Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4. Desalination 285:198-204.

Kim, K. M., Lee, J. Y., Kim, C. K., and Kang, J. S. 2009. Isolation and characterization of surfactin produced by *Bacillus polyfermenticus* KJS-2. Archives of Pharmacal Research 32:711-715.

Kim, P. I., Jaewon, R., Kim, Y. H., and Chi, Y. T. 2010. Production of biosurfactant lipopeptides iturin A, fengycin, and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. Journal of Microbiology and Biotechnology 20:138-145.

Kim, S. H., Lim, E. J., Lee, S. O., Lee, J. D., and Lee, T. H. 2000. Purification and characterization of biosurfactants from *Nocardia* sp. L-417. Biotechnology and Applied Biochemistry 31:249-253.

Kim, S. Y., Li, T., Heo, J. Y., Bae, Y. M., Hwang, I. K., Lee, S. Y., and Moon, B. 2012. Efficacies of cleaning methods for decontaminating *Vibrio parahaemolyticus* on the surfaces of cutting boards cross-contaminated from grated fish fillet. Journal of food Safety 32:459-466.

Kim, Y. B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., and Nishibuchi, M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level

by PCR targeted to the *toxR* gene. Journal of Clinical Microbiology 37:1173-1177.

Kinsinger, R. F., Kearns, D. B., Hale, M., and Fall, R. 2005. Genetic requirements for potassium ion-dependent colony spreading in *Bacillus subtilis*. Journal of Bacteriology 187:8462-8469.

Kiran, G. S., Hema, T. A., Gandhimathi, R., Selvin, J., Thomas, T. A., Rajeetha, R. T., and Natarajaseenivasan, K. 2009. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. Colloids and Surfaces B: Biointerfaces 73:250-256.

Kishishita, M., Matsuoka, N., Kumagai, K., Yamasaki, S., Takeda, Y., and Nishibuchi, M. 1992. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. Applied and Environmental Microbiology 58:2449-2457.

Koumoutsi, A., Chen, X. H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., and Borriss, R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology 186:1084-1096.

Kracht, M., Rokos, H., Ozel, M., Kowall, M., Pauli, G., and Vater, J. 1999. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. The Journal of Antibiotics 52:613.

Kumar, A. S., Mody, K., and Jha, B. 2007. Evaluation of biosurfactant/bioemulsifier production by a marine bacterium. Bulletin of Environmental Contamination and Toxicology 79:617-621.

Kumar, A., Saini, P., and Shrivastava, J. N. 2009. Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian Journal of Experimental Biology 47:57-62.

Landy, M., Warren, G. H., Rosenman, S. B., and Colio, L. G. 1948. Bacillomycin: an antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proceedings of the Society for Experimental Biology and Medicine 67:539-541.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In *Nucleic acid Techniques in Bacterial Systematics*, edited by E. Stackebrandt and M. Goodfellow: John Wiley & Sons, New York:115-175.

Latoud, C., Peypoux, F., and Michel, G. 1987. Action of iturin A, an antifungal antibiotic from *Bacillus subtilis*, on the yeast *Saccharomyces cerevisiae*: modifications of membrane permeability and lipid composition. The Journal of Antibiotics 40:1588-1595.

Latoud, C., Peypoux, F., Michel, G., Genet, R., and Morgat, J. L. 1986. Interactions of antibiotics of the iturin group with human erythrocytes. Biochimica et Biophysica Acta (BBA)-Biomembranes 856:526-535.

Lawrence, D. N., Blake, P. A., Yashuk, J. C., Wells, J. G., Creech, W. B., and Hughes, J. H. 1979. *Vibrio parahaemolyticus* gastroenteritis outbreaks aboard two cruise ships. American Journal of Eidemiology 109:71-80.

Leclère, V., Béchet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M., and Jacques, P. 2005. Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Applied and Environmental Microbiology 71:4577-4584.

Lee, S. C., Jung, Y. J., Yoo, J. S., Cho, Y. S., Cha, I. H., and Choi, Y. L. 2002. Characteristic of biosurfactants produced by *Bacillus* sp. LSC11. Korean Journal of life Science 12:745-751.

Lee, S. C., Yoo, J. S., Kim, S. H., Chung, S. Y., Hwang, C. W., Joo, W. H., and Choi, Y. L. 2006. Production and characterization of lipopeptide biosurfactant from *Bacillus subtilis* A8-8. Journal of Microbiology and Biotechnology 16:716-723.

Lertcanawanichakul, M., and Sawangnop, S. 2008. A comparison of two methods used for measuring the antagonistic activity of *Bacillus* species. Walailak Journal of Science and Technology (WJST) 5:161-171.

Levine, W. C., and Griffin, P. M. 1993. *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. The Journal of Infectious Diseases 167:479-483.

Li, H., Tanikawa, T., Sato, Y., Nakagawa, Y., and Matsuyama, T. 2005. *Serratia marcescens* gene required for surfactant serrawettin W1 production encodes putative aminolipid synthetase belonging to nonribosomal peptide synthetase family. Microbiology and Immunology 49:303-310.

Lin, Z., Kumagai, K., Baba, K., Mekalanos, J. J., and Nishibuchi, M. 1993. *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. Journal of Bacteriology 175:3844-3855.

Ma, L., and Su, Y. C. 2011. Validation of high pressure processing for inactivating *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*). International Journal of Food Microbiology 144:469-474.

Magalhaes, M., Takeda, Y., Magalhaes, V., and Tateno, S. 1992. Brazilian urease-positive strains of *Vibrio parahaemolyticus* carry genetic potential to produce the TDH-related hemolysin. Memórias do Instituto Oswaldo Cruz 87:167-168.

Maget-Dana, R., and Peypoux, F. 1994. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology 87:151-174.

Maget-Dana, R., and Ptak, M. 1990. Iturin lipopeptides: interactions of mycosubtilin with lipids in planar membranes and mixed monolayers. Biochimica et Biophysica Acta (BBA)-Biomembranes 1023:34-40.

Maget-Dana, R., Ptak, M., Peypoux, F., and Michel, G. 1985a. Pore-forming properties of iturin A, a lipopeptide antibiotic. Biochimica et Biophysica Acta (BBA)-Biomembranes 815:405-409.

Maget-Dana, R., Thimon, L., Peypoux, F., and Ptak, M. 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie 74:1047-1051.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., and Yamashita, A. 2003. Genome

sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *Vibrio cholerae*. The Lancet 361:743-749.

Marques, A. M., Pinazo, A., Farfan, M., Aranda, F. J., Teruel, J. A., Ortiz, A., Manresa, A., and Espuny, M. J. 2009. The physicochemical properties and chemical composition of trehalose lipids produced by *Rhodococcus erythropolis* 51T7. Chemistry and Physics of Lipids 158:110-117.

Martinez, U. J., Blanco, A. V., Rodriguez, C. A., Ansede, B. J., Miranda, A., and Rodriguez, A. M. X. 2012. Ecological determinants of the occurrence and dynamics of *Vibrio parahaemolyticus* in offshore areas. The ISME Journal 6:994-1006.

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong, H. C., Depaola, A., Kim, Y. B., and Albert, M. J. 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. Journal of Clinical Microbiology 38:578-585.

McLaughlin, J. B., DePaola, A., Bopp, C. A., Martinek, K. A., Napolilli, N. P., Allison, C. G, Murray, S. L., Thompson, E. C., Bird, M. M., and Middaugh, J. P. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. New England Journal of Medicine 353:1463-1470.

Meylheuc, T., Methivier, C., Renault, M., Herry, J. M., Pradier, C. M., and Bellon-Fontaine, M. N. 2006. Adsorption on stainless steel surfaces of biosurfactants produced by Gram-negative and Gram-positive bacteria: consequence on the bioadhesive behavior of *Listeria monocytogenes*. Colloids and Surfaces B: Bbiointerfaces 52:128-137.

Meylheuc, T., Van Oss, C. J., and Bellon-Fontaine, M. N. 2001. Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes* LO28. Journal of Applied Microbiology 91:822-832.

Mittraparp-arthorn, P., Saetang, J., Bhoopong, P., and Vuddhakul, V. 2013, Discrimination between isolates of pandemic O1:KUT *Vibrio parahaemolyticus* recovered from gastroenteritis patients. Current Microbiology (being submitted)

Morikawa, M., Hirata, Y., and Imanaka, T. 2000. A study on the structure function relationship of lipopeptide biosurfactants. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1488:211-218.

Moyne, A. L., Shelby, R., Cleveland, T. E., and Tuzun, S. 2001. Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. Journal of Applied Microbiology 90:622-629.

Mukherjee, A. K., and Das, K. 2010. Microbial surfactants and their potential applications: an overview. In Biosurfactants. New York: Springer:54-64.

Mulligan, C. N. 2005. Environmental applications for biosurfactants. Environmental Pollution 133:183-198.

Naruse, N., Tenmyo, O., Kobaru, S., Kamei, H., Miyaki, T., Konishi, M., and Oki, T. 1990. Pumilacidin, a complex of new antiviral antibiotics. production, isolation, chemical properties, structure and biological activity. The Journal of Antibiotics 43:267-280.

Nasir, M. N., and Besson, F. 2011. Specific interactions of mycosubtilin with cholesterol-containing artificial membranes. Langmuir 27:10785-10792.

Nasir, M. N., Thawani, A., Kouzayha, A., and Besson, F. 2010. Interactions of the natural antimicrobial mycosubtilin with phospholipid membrane models. Colloids and Surfaces B: Biointerfaces 78:17-23.

Navon-Venezia, S., Zosim, Z., Gottlieb, A., Legmann, R., Carmeli, S., Ron, E. Z., and Rosenberg, E. 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Applied and Environmental Microbiology 61:3240-3244.

Neu, T. R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiological Reviews 60:151-166.

Neu, T. R., and Poralla, K. 1990. Emulsifying agents from bacteria isolated during screening for cells with hydrophobic surfaces. Applied Microbiology and Biotechnology 32:521-525.

Nishibuchi, M., Fasano, A., Russell, R. G., and Kaper, J. B. 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. Infection and Immunity 60:3539-3545.

Nishibuchi, M., and Kaper, J. B. 1985. Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus*. Journal of Bacteriology 162:558-564.

Nishibuchi, M., and Kaper, J. B. 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infection and Immunity 63:2093-2099.

Nishibuchi, M., Kumagai, K., and Kaper, J. B. 1991. Contribution of the *tdh* 1 gene of Kanagawa phenomenon-positive *Vibrio parahaemolyticus* to production of extracellular thermostable direct hemolysin. Microbial Pathogenesis 11:453-460.

Nishibuchi, M., Taniguchi, T, Misawa, T., Khaeomanee-Iam, V., Honda, T., and Miwatani, T. 1989. Cloning and nucleotide sequence of the gene (*trh*) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infection and Immunity 57:2691-2697.

Nithya, C., Aravindraja, C., and Pandian, S. K. 2010. *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. Research in Microbiology 161:293-304.

Nitschke, M., Arajo, L. V., Costa, S. G. V. A. O., Pires, R. C., Zeraik, A. E., Fernandes, A. C. L. B., Freire, D. M. G., and Contiero, J. 2009. Surfactin reduces the adhesion of foodborne pathogenic bacteria to solid surfaces. Letters in Applied Microbiology 49:241-247.

Nitschke, M., and Pastore, G. M. 2006. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresource Technology 97:336-341.

Noriea Iii, N., Johnson, C. N., Griffitt, K. J., and Grimes, D. J. 2010. Distribution of type III secretion systems in *Vibrio parahaemolyticus* from the northern Gulf of Mexico. Journal of Applied Microbiology 109:953-962.

Okada, N., Iida, T., Park, K.S., Goto, N., Yasunaga, T., Hiyoshi, H., Matsuda, S., Kodama, T., and Honda, T. 2009. Identification and characterization of a novel type III secretion system in *trh*-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity of pathogenic machinery beyond the species level. Infection and Immunity 77:904-913.

Okuda, J., Ishibashi, M., Abbott, S. L., Janda, J. M., and Nishibuchi, M. 1997. Analysis of the thermostable direct hemolysin (*tdh*) gene and the *tdh*-related hemolysin (*trh*) genes in urease-positive strains of *Vibrio parahaemolyticus* isolated on the west coast of the United States. Journal of Clinical Microbiology 35:1965-1971.

Okura, M., Osawa, R., Iguchi, A., Takagi, M., Arakawa, E., Terajima, J., and Watanabe, H. 2004. PCR-based identification of pandemic group *Vibrio parahaemolyticus* with a novel group-specific primer pair. Microbiology and Immunology 48:787-792.

Olano, C., Lombra, F., Mendez, C., and Salas, J. A. 2008. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metabolic Engineering 10:281-292.

Ongena, M., and Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16:115-125.

Park, K. S., Ono, T., Rokuda, M., Jang, M. H., Okada, K., Iida, T., and Honda, T. 2004b. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. Infection and Immunity 72:6659-6665.

Park, K. S., Suthienkul, O., Kozawa, J., Yamaichi, Y., Yamamoto, K., and Honda, T. 1998. Close proximity of the *tdh*, *trh* and ure genes on the chromosome of *Vibrio parahaemolyticus*. Microbiology 144:2517-2523.

Pathak, K. V., and Keharia, H. 2013. Characterization of fungal antagonistic bacilli isolated from aerial roots of banyan (*Ficus benghalensis*) using intact-cell MALDI-TOF mass spectrometry (ICMS). Journal of Applied Microbiology 114:1300-1310.

Penland, R. L., Boniuk, M., and Wilhelmus, K. R. 2000. *Vibrio* ocular infections on the US Gulf Coast. Cornea 19:26-29.

Pereira, C. S., Possas, C. A., Viana, C. M., and Rodrigues, D. P. 2007. Characteristics of *Vibrio parahaemolyticus* isolated from mussels (*Perna perna*) commercialized at Niterói, Rio de Janeiro. Revista da Sociedade Brasileira de Medicina Tropical 40:56-59.

Razafindralambo, H., Popineau, Y., Deleu, M., Hbid, C., Jacques, P., Thonart, P., and Paquot, M. 1997. Surface-active properties of surfactin/iturin A mixtures produced by *Bacillus subtilis*. Langmuir 13:6026-6031.

Reich, K.A., and Schoolnik, G.K. 1994. The light organ symbiont *Vibrio fischeri* possesses a homolog of the *Vibrio cholerae* transmembrane transcriptional activator ToxR. Journal of Bacteriology 176:3085-3088.

Reiling, H. E., Thanei-Wyss, U., Guerra-Santos, L. H., Hirt, R., Kappeli, O., and Fiechter, A. 1986. Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 51:985-989.

Rivardo, F., Turner, R. J., Allegrone, G., Ceri, H., and Martinotti, M. G. 2009. Anti-adhesion activity of two biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens. Applied Microbiology and Biotechnology 83:541-553.

Romano, A., Vitullo, D., Di Pietro, A., Lima, G., and Lanzotti, V. 2011. Antifungal lipopeptides from *Bacillus amyloliquefaciens* strain BO7. Journal of Natural Products 74:145-151.

Ron, E. Z., and Rosenberg, E. 2001. Natural roles of biosurfactants. Environmental Microbiology 3:229-236.

Rooney, R. M., Cramer, E. H., Mantha, S., Nichols, G., Bartram, J. K., Farber, J. M., and Benembarek, P. K. 2004. A review of outbreaks of foodborne disease associated with passenger ships: evidence for risk management. Public Health Reports 119:427-434.

Rosenberg, E. 1993. Microbial diversity as a source of useful biopolymers. Journal of Industrial Microbiology 11:131-137.

Rosenberg, E., and Ron, E. Z. 1999. High-and low-molecular-mass microbial surfactants. Applied Microbiology and Biotechnology 52:154-162.

Ruangwong, O. U., Chang, C. I., Lamine, S. A., and Liang, W. J. 2012. Identification of antifungal compound produced by *Bacillus subtilis* LB5 with ability to control anthracnose disease caused by *Colletotrichum gloeosporioides*. African journal of Microbiology Research 6:3732-3738.

Sambrook, J., and Russell, D.W. 2003. Molecular cloning; a laboratory manual (3rd ed.). Edited by. Vol. 2: Cold spring harbor laboratory press, New York, pp.4.55-4.57.

Sanyal, S. C., and Sen, P. C. 1974. Human volunteer study on the pathogenicity of *Vibrio parahaemolyticus*. In International symposium on *Vibrio parahaemolyticus*, Saikon Publishing, Tokyo, pp.227-230.

Saravanakumari, P., and Mani, K. 2010. Structural characterization of a novel xylolipid biosurfactant from *Lactococcus lactis* and analysis of antibacterial activity against multi-drug resistant pathogens. Bioresource Technology 101:8851-8854.

Seghal Kiran, G., Anto Thomas, T., Selvin, J., Sabarathnam, B., and Lipton, A.P. 2010. Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresource Technology 101:2389-2396.

Sen, R., and Swaminathan, T. 2004. Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. Biochemical Engineering Journal 21:141-148.

Shen, X., Cai, Y., Liu, C., Liu, W., Hui, Y., and Su, Y. C. 2009. Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (*Crassostrea plicatula*). International Journal of Food Microbiology 136:129-132.

Shepherd, R., Rockey, J., Sutherland, I. W., and Roller, S. 1995. Novel bioemulsifiers from microorganisms for use in foods. Journal of Biotechnology 40:207-217.

Shete, A. M., Wadhawa, G., Banat, I. M., and Chopade, B. A. 2006. Mapping of patents on bioemulsifier and biosurfactant: A review. Journal of Scientific and Industrial Research 65:91-115.

Shirai, H., Ito, H., Hirayama, T., Nakamoto, Y., Nakabayashi, N., Kumagai, K., Takeda, Y., and Nishibuchi, M. 1990. Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. Infection and Immunity 58:3568-3573.

Snook, M. E., Mitchell, T., Hinton, D. M., and Bacon, C. W. 2009. Isolation and characterization of Leu7-surfactin from the endophytic bacterium *Bacillus*

mojavensis RRC 101, a biocontrol agent for *Fusarium verticillioides*. Journal of Agricultural and Food Chemistry 57:4287-4292.

Stewart, B. J., and McCarter, L. L. 2003. Lateral flagellar gene system of *Vibrio parahaemolyticus*. Journal of Bacteriology 185:4508-4518.

Su, Y. C., and Liu, C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiology 24:549-558.

Su, Y. C., Yang, Q., and Hase, C. 2010. Refrigerated seawater depuration for reducing *Vibrio parahaemolyticus* contamination in Pacific oyster (*Crassostrea gigas*). Journal of Food Protection 73:1111-1115.

Suwansukho, P., Rukachisirikul, V., Kawai, F., and H-Kittikun, A. 2008. Production and applications of biosurfactant from *Bacillus subtilis* MUV4. Sonklanakarin Journal of Science and Technology 30:87-93.

Tabatabaee, A., Assadi, M. M., Noohi, A. A., and Sajadian, V. A. 2005. Isolation of biosurfactant producing bacteria from oil reservoirs. Iranian Journal of Environmental Health Science & Engineering 2:6-12.

Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, H., Fukushima, S., Takano, J., Nishibuchi, M., and Takeda, Y. 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Molecular and Cellular Probes 6:477-487.

Takahashi, A., Kenjyo, N., Imura, K., Myonsun, Y., and Honda, T. 2000a. Cl^- secretion in colonic epithelial cells induced by the *Vibrio parahaemolyticus* hemolytic toxin related to thermostable direct hemolysin. Infection and Immunity 68:5435-5438.

Takeda, Y. 1982. Thermostable direct hemolysin of *Vibrio parahaemolyticus*. Pharmacology & Therapeutics 19:123-146.

Tampakaki, A. P., Fadouloglou, V. E., Gazi, A. D., Panopoulos, N. J., and Kokkinidis, M. 2004. Conserved features of type III secretion. Cellular Microbiology 6:805-816.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood,

evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731-2739.

Tantillo, G. M., Fontanarosa, M., Di Pinto, A., and Musti, M. 2004. Updated perspectives on emerging vibrios associated with human infections. Letters in Applied Microbiology 39:117-126.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Tugrul, T., and Cansunar, E. 2005. Detecting surfactant-producing microorganisms by the drop-collapse test. World Journal of Microbiology and Biotechnology 21:851-853.

Vanittanakom, N., Loeffler, W., Koch, U., and Jung, G. N. 1986. Fengycin--a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. The Journal of Antibiotics 39:888-901.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N., and Cameotra, S. S. 2002. Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Applied and Environmental Microbiology 68:6210-6219.

Vollenbroich, D., Ozel, M., Vater, J., Kamp, R. M., and Pauli, G. 1997a. Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals 25:289-297.

Vollenbroich, D., Pauli, G., Ozel, M., and Vater, J. 1997b. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. Applied and Environmental Microbiology 63:44-49.

Vuddhakul, V., Bhoopong, P., Hayeebilan, F., and Subhadhirasakul, S. 2007. Inhibitory activity of Thai condiments on pandemic strain of *Vibrio parahaemolyticus*. Food Microbiology 24:413-418.

Vuddhakul, V., Chowdhury, A., Laohaprertthisan, V., Pungrasamee, P., Patararungrong, N., Thianmontri, P., Ishibashi, M., Matsumoto, C., and Nishibuchi, M. 2000. Isolation of a pandemic O3: K6 clone of a *Vibrio*

parahaemolyticus strain from environmental and clinical sources in Thailand. Applied and Environmental Microbiology 66:2685-2689.

Vuddhakul, V., Soboon, S., Sunghiran, W., Kaewpiboon, S., Chowdhury, A., Ishibashi, M., Nakaguchi, Y., and Nishibuchi, M. 2006. Distribution of virulent and pandemic strains of *Vibrio parahaemolyticus* in three molluscan shellfish species (*Meretrix meretrix*, *Perna viridis*, and *Anadara granosa*) and their association with foodborne disease in southern Thailand. Journal of Food Protection 69:2615-2620.

West, C. K. G., Klein, S. L., and Lovell, C. R. 2013. High frequency of virulence factor genes *tdh*, *trh*, and *tlh* in *Vibrio parahaemolyticus* strains isolated from a Pristine Estuary. Applied and Environmental Microbiology 79:2247-2252.

Wong, H. C., Chen, C. H., Chung, Y. J., Liu, S. H., Wang, T. K., Lee, C. L., Chiou, C. S., Nishibuchi, M., and Lee, B. K. 2005. Characterization of new O3: K6 strains and phylogenetically related strains of *Vibrio parahaemolyticus* isolated in Taiwan and other countries. Journal of Applied Microbiology 98:572-580.

Wong, H. C., Chung, Y. C., and Yu, J. A. 2002. Attachment and inactivation of *Vibrio parahaemolyticus* on stainless steel and glass surface. Food Microbiology 19:341-350.

Wootipoom, N., Bhoopong, P., Pomwised, R., Nishibuchi, M., Ishibashi, M., and Vuddhakul, V. 2007. A decrease in the proportion of infections by pandemic *Vibrio parahaemolyticus* in Hat Yai Hospital, southern Thailand. Journal of Medical Microbiology 56:1630-1638.

Xu, M., Yamamoto, K., and Honda, T. 1994. Construction and characterization of an isogenic mutant of *Vibrio parahaemolyticus* having a deletion in the thermostable direct hemolysin-related hemolysin gene (*trh*). Journal of Bacteriology 176:4757-4760.

Yakimov, M. M., Timmis, K. N., Wray, V., and Fredrickson, H. L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Applied and Environmental Microbiology 61:1706-1713.

Yano, Y., Kaneniwa, M., Satomi, M., Oikawa, H., and Chen, S. 2006. Occurrence and density of *Vibrio parahaemolyticus* in live edible crustaceans from markets in China. Journal of Food Protection 69:2742-2746.

Yeung, P. S. M., and Boor, K. J. 2004. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. Foodborne Pathogens & Disease 1:74-88.

Yoshida, S., Hiradate, S., Tsukamoto, T., Hatakeda, K., and Shirata, A. 2001. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 91:181-187.

Zeraik, A. E., and Nitschke, M. 2010. Biosurfactants as agents to reduce adhesion of pathogenic bacteria to polystyrene surfaces: effect of temperature and hydrophobicity. Current Microbiology 61:554-559.

Zeriouh, H., Romero, D., Garcia-Gutierrez, L., Cazorla, F. M., de Vicente, A., and Perez-Garcia, A. 2011. The iturin-like lipopeptides are essential components in the biological control arsenal of *Bacillus subtilis* against bacterial diseases of cucurbits. Molecular Plant-Microbe Interactions 24:1540-1552.

Zhang, Y., and Miller, R. M. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Applied and Environmental Microbiology 58:3276-3282.

Zheng, L., Han, X., Chen, H., Lin, W., and Yan, X. 2005. Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Annals Microbiology 55:119-124.

APPENDIX

APPENDIX A

Media preparation

1. CHROMagar Vibrio (Microbiology CHROMagar, France)

Composition of media in g/liter;

Peptone and yeast extract	8	g
Salts	51.4	g
Chromogenic mixture	0.3	g
Agar	15	g
dH_2O	1000	ml

Suspend 74.7 g/liter in dH_2O. Heat and bring to a boil (100°C) while swirling or stirring regularly until complete fusion of the agar (do not autoclave). Cool in a water bath to 45-50°C, stirring gently, mixes well and pour into sterile plates (pH 9.0 ± 0.2 at 25°C).

2. Landy medium

Composition of media in g/liter;

Glucose	20	g
L-glutamic acid	5.0	g
$MgSO_4$	0.5	g
KCl	0.5	g
KH_2PO_4	1	g
$Fe_2(SO_4)_3$	0.0012	g
$MnSO_4$	0.0014	g
$CuSO_4$	0.0016	g
dH_2O	1000	ml

Suspend all ingredients in dH_2O and heat while swirling and stirring. Bring to volume to 1000 ml with dH_2O and adjust pH to 7.0. Media sterile by autoclaving at 121°C 15 min.

3. Luria–Bertani broth (Difco, USA)

Composition of media in g/liter;

Yeast extract	5	g
Tryptone	10	g
Sodium chloride	10	g
dH_2O	1000	ml

Suspend 25 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.0 ± 0.2 at 25°C).

4. MacConkey agar (Difco, USA)

Composition of media in g/liter;

Peptone from gelatin	17	g
Proteose peptone	3	g
Sodium chloride	5.0	g
Lactose	10	g
Bile salt mixture	1.5	g
Neutral red	0.03	g
Crystal violet	0.001	g
Agar	13.5	g
dH_2O	1000	ml

Suspend 50 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.1 ± 0.2 at 25°C).

5. Mckeen medium

Composition of media in g/liter;

Glucose	20	g
L-glutamic acid	5.0	g
Yeast extract	1	g
$MgSO_4$	1.02	g
KCl	0.5	g

K_2HPO_4	1	g
dH_2O	1000	ml

Suspend all ingredients in dH_2O. Bring to boil to dissolve the medium completely and supplemented with 1 ml of trace elements solution. Sterilize by autoclaving at $121^{o}C$ 15 minutes (pH 7.1 ± 0.2 at 25°C).

Trace elements solution composition g/liter;

$CuSO_4.5H_2O$	0.16	g
$FeSO_4.7H_2O$	0.015	g
$MnSO_4.7H_2O$	0.5	g

6. Mueller Hinton agar (Difco, USA)

Composition of media in g/liter;

Beef extract powder	2	g
Soluble starch	1.5	g
Casein hydrolysate	17.5	g
Agar	15	g
dH_2O	1000	ml

Suspend 38 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at $121^{o}C$ 15 minutes (pH 7.3 ± 0.1 at 25°C).

7. Mueller Hinton broth (Difco, USA)

Composition of media in g/liter;

Beef extract powder	2	g
Soluble starch	1.5	g
Casein hydrolysate	17.5	g
dH_2O	1000	ml

Suspend 21 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at $121^{o}C$ 15 minutes (pH 7.3 ± 0.1 at 25°C).

8. Nutrient agar (Difco, USA)

Composition of media in g/liter;

Beef extract	3	g
Peptone	5	g
Agar	15	g
dH_2O	1000	ml

Suspend 23 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 6.8 ± 0.2 at 25°C).

9. Nutrient broth (Difco, USA)

Composition of media in g/liter;

Beef extract	3	g
Peptone	5	g
dH_2O	1000	ml

Suspend 8 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 6.8 ± 0.2 at 25°C).

10. SSW medium

Composition of media in g/liter;

Palm oil	1	%
Peptone from casein	3	g
Yeast extract	1	g
Synthetic sea water	10	g
dH_2O	1000	ml

Suspend all ingredients in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.0 ± 0.2 at 25°C).

11. Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Difco, USA)

Composition of media in g/liter;

Proteose peptone no.3	10	g
Yeast extract	5	g
Sodium citrate	10	g
Sodium thiosulfate	10	g
Ox bile (Oxgall)	8	g
Sucrose	20	g
Sodium chloride	10	g
Iron ammonium citrate	1	g
Thymol blue	0.04	g
Bromothymol blue	0.04	g
Agar	15	g
dH_2O	1000	ml

Suspend 89 g/liter in dH_2O. Bring to boiling (do not autoclave) while swirling and stirring. Cool in a water bath to 45-50°C, stirring gently, pouring into sterilized plates (pH: 8.6 ± 0.2 at 25 °C).

12. Trypticase soy agar (TSA) (Difco, USA)

Composition of media in g/liter;

Peptone from casein	15	g
Peptone from soymeal	5	g
Sodium chloride	5	g
Agar	15	g
dH_2O	1000	ml

Suspend 40 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.3 ± 0.2 at 25°C).

13. Trypticase soy broth (TSB) (Difco, USA)

Composition of media in g/liter;

Glucose	2.5	g
Peptone from casein	15	g
Peptone from soymeal	5	g
Sodium chloride	5	g
K_2HPO_4	2.5	g
dH_2O	1000	ml

Suspend 30 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.3 ± 0.2 at 25°C).

14. Salmonella-Shigella (Difco, USA)

Composition of media in g/liter;

Beef Extract	5	g
Casein hydrolysate	2.5	g
Animal Tissue hydrolysate	2.5	g
Lactose	10	g
Bile Salts	8.5	g
Sodium Citrate	8.5	g
Sodium Thiosulfate	8.5	g
Ferric Citrate	1	g
Brilliant Green	0.00033	g
Neutral Red	0.025	g
Agar	13.5	g
dH_2O	1000	ml

Suspend 60 g/liter in dH_2O. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes (pH 7.0 ± 0.2 at 25°C).

APPENDIX B

1. EDTA, 0.5 M pH 8.0

Dissolve 186.12 g of Na_2EDTA in 800-900 ml of dH_2O. Adjust pH to 8.0 with 10 N NaOH. Add dH_2O to make 1 liter. Autoclave 15 min at 121°C.

2. Phenol:Chloroform

Mix an equal volume of phenol and chloroform (1:1). Extract 3-4 times with 0.1 M Tris-HCl pH 7.6. Store under 0.01 M Tris-HCl pH 7.6 at 4°C in the dark.

3. Phosphate-Buffer Saline (PBS), pH 7.4

NaCl (Lab-Scan)	7.650	g
Na_2HPO_4, anhydrous (Merck)	0.724	g
KH_2PO_4 (Merck)	0.210	g
dH_2O	1,000	ml

Dissolve ingredients in dH_2O Adjust pH to 7.4 (with 1 N NaOH) and autoclave 15 min at 121°C.

4. RNase (DNase free), 10 mg/ml

Mix 100 mg of RNase A with 10 ml of 10 mM Tris pH 7.5, 15 mM NaCl. Heat to 100°C for 15 min. Allow to cool to room temperature (overnight). Store at -20°C.

5. Sodium acetate, 3 M pH 5.2-7.0

Mix 4.08 g of sodium acetate-$3H_2O$ with 8 ml of dH_2O. Adjust pH to 5.2-7.0 with glacial acetic acid. Add dH_2O to 10 ml. Autoclave 15 min at 121°C.

6. Sodium dodecyl sulfate (SDS); 10%

Mix 1 g of SDS with 9 ml of dH_2O. Heat to 68°C. Adjust pH to 7.2 with HCl. Add dH_2O to 10 ml.

7. Tris-EDTA (TE) buffer pH 8.0

1 M Tris-HCl pH 8.0	1	ml
0.5 mM EDTA pH 8.0	200	μL
dH_2O	98.8	ml

Mix Tris-HCl and EDTA. Add dH_2O to 100 ml. Autoclave 15 min at 121°C.

8. Ethidium bromide, 10 mg/ml

Mix 1 g of ethidium bromide with 100 ml of dH_2O. Stir overnight. Store in the dark at room temperature

9. Gel loading buffer

(0.25% bromophenol blue and 4.0% (w/v) sucrose); 6x

Weigh out 25 mg bromophenol blue and 4 g sucrose. Make up volume to 10 ml with dH_2O. Store at 4°C.

10. Mini-scale extraction of total genomic DNA from *Vibrio* spp.

10.1 Grow the organism in 5 ml of LB broth at 37°C for 6-8 h with shaking (150 rpm).

10.2 Inoculate 10 μL of bacterial culture into fresh 5 ml of LB broth and incubate with shaking at 37°C overnight.

10.3 Harvest the cells by centrifugation at 10,000 x *g* for 3 min.

10.4 Suspend the cell pellet in 1 ml of PBS, pH 7.4 and transfer to a 1.5 ml microcentrifuge tube (chloroform-resistant) (Eppendorf).

10.5 Harvest the cells by centrifugation for 3 min.

10.6 Suspend the cell pellet in 300 μL of PBS-EDTA (240 μL PBS and 60 μL of 0.5 M EDTA)

10.7 Add 150 μL of 10% SDS, mix and then incubate at room temperature for 10 min. Confirm that all cells are lysed.

10.8 Add 450 μL of phenol-chloroform solution (1:1) and mix vigorously, then centrifuge for 2 min at room temperature.

10.9 The upper aqueous phase was carefully collected to a 1.5-ml microtube.

10.10 Add 40 µL of 3 M NaOAc and 1 ml of cold absolute ethanol and mix carefully. DNA will aggregate as white flocculation.

10.11 The precipitated DNA was obtained by centrifugation for 5 min, wash twice with 70% cold EtOH and allow to air-dried for 15 min.

10.12 Dissolve DNA in a 0.3 ml of distilled water.

10.13 Add 60 µL of 50 µg/ml RNase and incubate at 37°C for 30 min.

10.14 Repeat step 10.8 to 10.11 (phenol-chloroform extraction) and dissolve dried DNA in TE buffer.

10.15 Measure the concentration of DNA at OD 260 nm (1 OD_{260nm} = 50 µg/ml of DNA).

10.16 Store the stock DNA solution at -20°C until used.

APPENDIX C

1. Salt –EDTA (SE) buffer

[75 mM NaCl, 25 mM EDTA pH 8.0]

NaCl	43.875	g
0.5 M EDTA pH 8.0	5	ml
dH_2O	10	ml

Mix all ingredients and keep at 4°C before use.

2. Low melting-agarose (LMA) buffer for PFGE method

[10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 10 mM $MgCl_2$]

1 M Tris-HCl pH 7.5	1	ml
0.5 M EDTA	0.2	ml
1M $MgCl_2$	1	ml
dH_2O	10	ml

Mix all ingredients and keep at 4°C before use.

3. Lysis solution for PFGE method

[50 mM Tris-HCl, 50 mM EDTA, 1% N-lauryl sarcosine, 1 mg/ml proteinase K]

1 M Tris-HCl pH 8.0	0.5	ml
0.5 M EDTA	1	ml
N-lauryl sarcosine	0.1	g
proteinase K	0.01	g
Distilled water	10	ml

Mix all ingredients and keep at 4°C before use.

4. Tris- Borate- EDTA (TBE) buffer; 10x

Tris-base	54	g
Boric acid	27.5	g
$Na_2EDTA, 2H_2O$	3.72	g
Distilled water	1000	ml

Suspend all ingredients in distilled water. Bring to boil to dissolve the medium completely and sterilize by autoclaving at 121° C 15 minutes. When required, dilute 10x TBE buffer to a final concentration of 0.5x TBE buffer before used.

5. Pulsed-Field Gel Electrophoresis (PFGE) method

5.1 Cells are grown on LB agar supplement with 1% NaCl at 37°C

5.2 Pick up one colony and inoculate into 5 ml fresh LB broth supplement with 1% NaCl at 37°C overnight with continuous shaking at 160 rpm.

5.3 Collect one ml of culture sample and centrifuge at 10,000 x g for 10 min and then remove supernatant.

5.4 Add 150 µL of SE buffer and mix by pipetting.

5.5 Add 150 µL of low-melting agarose (LMA) in a buffer (50°C), mix by pipetting.

5.6 Charge into disposable plug mold (Bio-Rad Laboratories Hercules, USA) harden the agarose at room temperature (can place it at 4°C).

5.7 Push the gel and put it into 2.0 ml tube (round type is better) and gel should be pushed from top, not from bottom.

5.8 Treat the plug by adding 0.95 ml of lysis solution.

5.9. Incubate in water bath at 50°C overnight (about 22 h), the gel become transparency.

5.10 Wash plug each with 0.5 to 1 ml of TE buffer sixth times for 15 min, shake gently at room temperature (can store the gel at 4°C on this step).

5.11 Equilibrate plug with 1 ml of 1x enzyme buffer approximately 1 h at room temperature.

5.12 Add 180 µL of fresh enzyme buffer containing 50 U of *Not*I.

5.13 Keep at 37°C for 24 h in water bath.

5.14 Plug can be stored in TE buffer at 4°C for few weeks.

5.15 Make 1% plus-Field Certified agarose gel in 0.5x TBE buffer.

5.16 Incubate the DHEF-DNA size marker is at 50°C for 10 min before loading into the gel.

5.17 Cut a small piece of plug and load it into the gel very carefully.

5.18 Fill up the rest of the space with 1% LMA.

5.19 Run PFGE power pack at 14°C, 80 rpm flow rate, plus time 1- 18 s linear, 6 V/cm, 120° angle in a CHEF-DRIII system for 36 h.

5.20 Stain the gel in ethidium bromide for 30 min and destain for 20 min.

5.21 Observe under UV light with a UV transilluminator.

APPENDIX D

Identification of *Bacillus* spp. by 16S rRNA gene sequence analysis

1. Isolation of *Bacillus* spp. chromosomal DNA

Genomic DNA was purified using a protocol from Gentra Puregene Yeast/Bact. Kit (QIAGEN) with slightly modifications as follow:

1.1 Grow the organism in 0.5 ml of LB broth overnight at 30°C with shaking (150 rpm).

1.2 Harvest the cells by centrifugation at 15,000 x *g* for 5 min.

1.3 Suspend the cell pellet in 500 µL of PBS, pH 7.4 and transfer to a 1.5 ml microcentrifuge tube.

1.4 Vortex and harvest the cells by centrifugation for 4 min.

1.5 Carefully discard the supernatant by pipetting or pouring.

1.6 Suspend the cell pellet in 300 µL of PBS, and pipet up and down.

1.7 Add 50 µL of Proteinase K (20 mg/ml) and 20 µL of Lysozyme (10 mg/ml), mix by inverting 25 times. Incubate for 30 min at 55°C. Centrifuge at 15,000 x *g* for 3 min and discard the supernatant.

1.8 Add 300 µL of Cell Lysis Solution and pipet up and down to lyse the cells. Then incubate at 80°C for 10 min. Confirm that all cells are lysed.

1.9 Add 1.5 µL RNase A solution, and mix by inverting 25 times. Incubate for 45 min at 37°C.

1.10 Incubate for 1 min on ice to quickly cool the sample.

1.11 Add 100 µL of Protein Precipitation Solution, and vortex vigorously 15,000 x *g* for 20 s. Centrifuge at 15,000 x *g* for 3 min.

1.12 Pipet 300 µL of isopropanol into a clean 1.5 microcentrifuge tube and add the supernatant from the previous step by pouring carefully.

1.13 Mix by inverting gently 50 times.

1.14 Centrifuge for 10 min at 15,000 x *g* and carefully discard the supernatant.

1.15 Add 300 µL of cold 70% ethanol and invert several times to wash the DNA pellet. Centrifuge for 1 min at 15,000 x *g* and carefully discard the supernatant. Allow to air dried for up to 15 min.

1.16 Add 100 µL of DNA Hydration Solution and vortex 5 s to mix. Incubate at 65°C for 15 min to dissolve DNA. Incubate at room temperature overnight. Store the stock DNA solution at -20°C until used.

2. DNA sequence analysis

Sequences of 16S rDNA gene were tested accuracy sequences by BioEdit program (Hall, 2004) and compared with those sequences available in the NCBI using BLAST software (Basic Local Alignment Search Tool) (Johnson *et al.*, 2008), and then, related sequence that similar to sequence of *Bacillus* sp. SM11 and out group sequence were selected for multiple alignment by Clustal W program (Thompson *et al.*, 1994). Analyzed sequences were construced phylogenetic tree by Mega 4.0.2 (Tamura *et al.*, 2011) and Neighbor-joining (bootstrap 1,000 times). Finally, % 16S rDNA sequence identity was performed.

APPENDIX E

1. Tris-HCl buffer pH 8.0; 20 mM

Mix 24.228 g of tris (hydroxymethyl) aminomethane in 800 ml of distilled water. Bring to boil to dissolve the buffer completely and adjust pH 8.0 by 0.5 N HCl. Adjust volumes to 1000 ml into volumetric flask (10x Tris-HCl buffer). Sterilize by autoclaving at 121°C 15 minutes. When required, dilute 10x Tris-HCl buffer to a final concentration of 1x Tris-HCl buffer before used.

2. Tris HCl buffer pH 8.0 (20 mM) + NaCl

Mix 24.228 g of tris (hydroxymethyl) aminomethane (supplemented with 200 mM-1000 Mm NaCl) (Table 22) in 800 ml of distilled water. Bring to boil to dissolve the buffer completely and adjust pH 8.0 by 0.5 N HCl. Adjust volumes to 1000 ml into volumetric flask (10x Tris-HCl buffer+NaCl). Sterilize by autoclaving at 121°C 15 minutes. When required, dilute 10x Tris-HCl buffer+NaCl to a final concentration of 1xTris-HCl buffer+NaCl before used.

Table 22 Tris-HCl buffer pH 8.0 (20 mM) supplemented with NaCl concentration

NaCl concentration (mM)	NaCl (g)
200	11.69
400	23.38
600	35.06
800	46.75
1000	58.44

3. HCl; 0.5 N

Mix conc. HCl 4.2 ml in 96 ml of dH_2O into 100 ml of volumetric flask

4. NaOH; 0.5 N

Mix 20 g of sodium hydroxide pellets in 800 ml of distilled water into 1000 ml of volumetric flask and vigourous shaking till it should be clear solution and distilled water to volume at 1000 ml.

5. Slurry of pre-swollen DE 52 celluloses preparation

5.1 Stir the ion exchanger into a volume of the buffer as in 20 mM Tris HCl buffer pH 8.0.

5.2 Leave for ten minutes and decant off or filter Tris HCl buffer.

5.3 Repeat the treatment until the filtrate or the supernatant has exactly pH 8.0 and conductivity as used buffer. This step may require many changes of buffer and be time consuming when buffers of low concentrationns are used.

5.4 Fine celluloses removal and equilibrium of slurry celluloses will be packed into column.

6. Column packing and equilibration

6.1 Set up the suitable column vertically in a suitable area.

6.2 Pour the stirred slurry into the column in one time.

6.3 Allow the eluent from the column to run to waste.

6.4 Pump or run the buffer through the column at a flow-rate of at least 45 ml/hour/cm of the internal cross-sectional area of the column until the column bed height is constant.

6.5 Check pH and conductivity from eluent of buffer must be identical to starting buffer (pH 8.0).

6.6 Pass the starting buffer through the column until the conductivity and pH of the eluent are exactly the same as the starting buffer.

6.7 When pH and conductivity are constant, adjust flow rate at 0.7 ml/min and continually pass 2 bed volumes of buffer (bed volume of column= 46 cm^3).

6.8 Stop the flow rate of buffer into and out of the column.

7. Sample loading and elution

7.1 0.5 g of extract was dissolved in alkaline distilled water pH 8.0.

7.2 Stop flow rate and load sample into the center of the end of column.

7.3 Open flow rate of column and wash with 2 bed volumes of Tris HCl pH 8.0 for removing unbounded extract. Each fraction collects 6 ml of sample volume.

7.4 Elute sample with the same buffer supplemented with NaCl 200 mM to 1000 mM (step-wise elution). Each elution step uses 2 bed volumes of Tris HCl pH 8.0.

7.5 Each fraction obtained was determined for lipopeptide using spectrophotometer at OD 210 nm and 280 nm.

8. Degenerate of used DE 52 cellulose

8.1 Stir the used DE 52 into 15 volumes. (w/v) of first treatment with 0.5 N HCl.

8.2 Leave for at least 30 min for equilibrium of microgranular.

8.3 Filter or decant off supernatant and wash until the filtrate is stable at pH 4.0.

8.4 Stir ion exchanger into 15 volumes. of second treatment' with 0.5 N NaOH and leave for a further 30 minutes.

8.5 Repeat second treatment (4) and follow by washing until the filtrate is near neutral.

APPENDIX F

1. Alkaline peptone water (APW); 1%

Peptone from meat	10	g
NaCl	10	g
dH_2O	1000	ml

Suspend peptone and NaCl dH_2O 1000 ml. Bring to boil to dissolve the medium completely. Adjust pH 8.6 by 0.2 N NaOH and sterilize by autoclaving at 121°C 15 min.

2. Crystal-violet solution; 0.1%

Suspend 0.1 g of crystal- violet in 100 ml of distilled water and stir to completely dissolve. This solution prepare overnight before use.

3. Isopropanol-0.04 N HCl

To prepare 150 ml, add 500 µL of conc. HCl into 149.5 ml of isopropyl alcohol. This solution fresh prepare before use.

4. SDS; 0.25 %

Suspend 0.25 g of SDS in 100 ml of dH_2O and stir to completely dissolve.

6. Cetyltrimethylammonium bromide (CTAB); 2%

Suspend 2 g of CTAB in 100 ml of dH_2O and stir to completely dissolve

7. Trifluoroacetic acid (TFA); 0.1%

Suspend 1 ml of trifluoroacetic acid in 1000 ml of dH_2O and stir to completely dissolve. Filter through 0.45 µm of membrane before use.

VITAE

Name Miss Junthip Thongjun

Student ID 5110230003

Educational Attainment

Degree	Name of Institution	Year of Graduation
M.Sc. (Applied Microbiology)	King Mongkut's University of Technology Thonburi	2003
B.Sc. (Microbiology)	King Mongkut's University of Technology Thonburi	1999

Scholarship Awards during Enrolment

Scholarship of CHE-PhD-SW-INDV program by Commission on Higher Education, Bangkok, Thailand

List of Publications

Suntornsuk, W., Thongjun, J., Onnim, P., Oyama, H., Ratanakanokchai, K., Kusamran, T., and Oda, K. 2005. Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. World Journal of Microbiology and Biotechnology 21:1111-1117.

Thongjun, J., Bhoopong, P., Yingkajorn, M., Nishibuchi, M., and Vuddhakul, V. 2013. Total number, virulence genes, and heterogeneity of *Vibrio parahaemolyticus* in a single shellfish. ScienceAsia (Accepted).

Thongjun, J., Yingkajorn, M., Kongreung, J., Nishibuchi, M., and Vuddhakul, V. 2013. Trend of *Vibrio parahaemolyticus* infections in years 2006-2010 in southern Thailand. Tropical Medicine and Health (Revised).

Thongjun, J., Tansila, N., Panthong, K., and Vuddhakul, V. 2013. Biosurfactant derived from *Bacillus amyloliquefaciens* against *Vibrio parahaemolyticus.* Food Control (being submitted).