The use of diatoms (*Amphora* sp.) as immunostimulant in Pacific White shrimp (*Litopenaeus vannamei*) diet

Miss Ekthida Thongdet

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Marine Science
Department of Marine Science
Faculty Of Science
Chulalongkorn University
Academic Year 2023

การใช้ไดอะตอม (*Amphora* sp.) เพื่อเป็นสารกระตุ้นภูมิคุ้มกันในอาหารกุ้งขาว (*Litopenaeus vannamei*)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

น.ส.เอกธิดา ทองเด็จ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางทะเล ภาควิชาวิทยาศาสตร์ทางทะเล
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2566

Thesis Title	The use of diatoms (*Amphora* sp.) as immunostimulant in Pacific White shrimp (*Litopenaeus vannamei*) diet
By	Miss Ekthida Thongdet
Field of Study	Marine Science
Thesis Advisor	Assistant Professor KORNRAWEE AIEMSOMBOOM, Ph.D.
Thesis Co Advisor	Narongsak Puanglarp, Ph.D. Assistant Professor Sumrarn Bunnajirakul, Ph.D., Vet.Med.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.... Dean of the FACULTY OF SCIENCE
(Professor PRANUT POTIYARAJ, Ph.D.)

DISSERTATION COMMITTEE

.... Chairman
(Professor SUCHANA CHAVANICH, Ph.D.)

.... Thesis Advisor
(Assistant Professor KORNRAWEE AIEMSOMBOOM, Ph.D.)

.... Thesis Co-Advisor
(Narongsak Puanglarp, Ph.D.)

.... Thesis Co-Advisor
(Assistant Professor Sumrarn Bunnajirakul, Ph.D., Vet.Med.)

.... Examiner
(Associate Professor SANIT PIYAPATTANAKORN, Ph.D.)

.... Examiner
(Sirinart Techa, Ph.D.)

.... External Examiner
(Associate Professor Somkiat Piyatiratitivorakul, Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

เอกธิดา ทองเด็จ : การใช้ไดอะตอม (*Amphora* sp.) เพื่อเป็นสารกระตุ้นภูมิคุ้มกันในอาหารกุ้งขาว (*Litopenaeus vannamei*). (The use of diatoms (*Amphora* sp.) as immunostimulant in Pacific White shrimp (*Litopenaeus vannamei*) diet) อ.ที่ปรึกษาหลัก : ผศ. ดร.กรณ์รวี เอี่ยมสมบูรณ์, อ.ที่ปรึกษาร่วม : ดร.ณรงค์ศักดิ์ พวงลาภ,ผศ. น.สพ.ดร.สําราญ บรรณจิรกุล

Amphora sp เป็นไดอะตอมกลุ่มหนึ่งที่น่าสนใจ เนื่องจากการเจริญเติบโตที่รวดเร็วและมีปริมาณไขมันสูง โดยมีปริมาณเฉลี่ยอยู่ระหว่าง 40 – 60% ของน้ำหนักแห้ง ประกอบด้วยกรดไขมันไม่อิ่มตัวที่สำคัญต่อสุขภาพของสัตว์น้ำ ทำให้ *Amphora* sp. เหมาะสมต่อการใช้เป็นอาหารสัตว์น้ำวัยอ่อน เช่น กุ้ง และหอยเป๋าฮื้อ ดังนั้นการศึกษานี้จึงมีจุดประสงค์เพื่อวิเคราะห์ศักยภาพของ *Amphora* sp ที่แยกได้จากเกาะสีชัง (ASC), เกาะแสมสาร (ASS), ปราณบุรี (APB), และแหลมใหญ่ (ALY) อ่าวไทย เพื่อใช้เป็นสารกระตุ้นภูมิคุ้มกันในอาหารของลูกกุ้งขาว (*Litopenaeus vannamei*) โดย *Amphora* sp. ที่แยกได้จากทั้ง 4 แห่ง ถูกนำมาเลี้ยงด้วยน้ำทะเลความเค็ม 30 ppt เติมอาหาร F/2 medium ในขวดแก้วขนาด 1 ลิตร และใช้แผ่นอะคริลิกใสเป็นวัสดุยึดเกาะ ผลการศึกษาพบว่า ASC และ ALY ให้ผลผลิตดีที่สุดเมื่อเลี้ยงเป็นเวลา 6 วัน โดยมีปริมาณไขมัน และองค์ประกอบของกรดไขมันสูงที่สุด โดยเฉพาะกรดไขมันไม่อิ่มตัว ในวันที่ 6, 9, และ 13 เมื่อเปรียบเทียบกับ *Amphora* sp. ที่แยกได้จากที่อื่นในสภาวะการเลี้ยงเดียวกัน ดังนั้น ASC และ ALY จึงถูกนำไปศึกษาสภาวะการเลี้ยงที่แตกต่างกัน 6 สภาวะ ได้แก่ 3 ระดับความเข้มแสง และ 2 ระดับความเข้มข้นของอาหาร F/2 medium ผลการศึกษาพบว่า ALY ที่เลี้ยงด้วยระดับความเข้มแสงระดับกลางและอาหาร F/20 ให้ผลผลิตปริมาณไขมัน และองค์ประกอบของกรดไขมันที่สูงที่สุด และสภาวะการเลี้ยงดังกล่าวถูกนำไปใช้เพื่อผลิต ALY และผสมลงในอาหารกุ้งสำหรับการทดสอบศักยภาพในการใช้ *Amphora* sp. เป็นสารกระตุ้นภูมิคุ้มกันในอาหารกุ้งต่อไป ผลการทดสอบพบว่าการผสม *Amphora* sp. ลงในอาหารกุ้ง 15% ช่วยให้อัตรารอด และน้ำหนักเฉลี่ยของกุ้งดีกว่าการผสมอาหารในระดับอื่น โดยกุ้งที่ได้รับอาหารผสม *Amphora* sp. เป็นเวลา 6 สัปดาห์ มีความต้านทานเชื้อ *Vibrio parahaemolyticus* ดีกว่า 3 สัปดาห์ และที่ 6 สัปดาห์กุ้งมีการแสดงออกของยีน LvALF B ใน hepatopancrease ซึ่งเป็นยีนที่ทำหน้าที่สร้าง Anti-lipopolysaccharide factors เพื่อกำจัดแบคทีเรีย ดังนั้นจึงอาจจะสรุปได้ว่าการใช้ *Amphora* sp. เป็นสารเสริมในอาหารกุ้งในอัตรา 15% เป็นระยะเวลา 6 สัปดาห์ อาจช่วยเพิ่มอัตรารอดของกุ้งเมื่อได้รับเชื้อแบคทีเรีย *V. parahaemolyticus* อย่างไรก็ตามควรมีการศึกษาเพิ่มเติมในพารามิเตอร์สำคัญ เช่น PO และ SOD ซึ่งเป็นพารามิเตอร์ทางภูมิคุ้มกันที่สำคัญ เพื่อทราบกลไกการเป็นสารกระตุ้นภูมิคุ้มกันของไดอะตอมที่ชัดเจนมากขึ้น

สาขาวิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต ....
ปีการศึกษา	2566	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5972883923 : MAJOR MARINE SCIENCE
KEYWORD: Diatom, Amphora sp., Pacific white shrimp, Litopenaeus vannamei, Aquaculture

Ekthida Thongdet : The use of diatoms (*Amphora* sp.) as immunostimulant in Pacific White shrimp (*Litopenaeus vannamei*) diet. Advisor: Asst. Prof. KORNRAWEE AIEMSOMBOOM, Ph.D. Co-advisor: Narongsak Puanglarp, Ph.D.,Asst. Prof. Sumrarn Bunnajirakul, Ph.D., Vet.Med.

Amphora sp. is one of the interesting microalgae due to its rapid growth, high lipid content, ranging from 40-60% of dry biomass, suitability as live food for commercial aquatic animal larvae such as shrimp and abalone. The objective of this study is to evaluate the potential of *Amphora* sp. isolated from four different locations in the Gulf of Thailand: Sichang Island (ASC), Samae Sarn Island (ASS), Pranburi (APB), and Laem Yai (ALY) as immunostimulant in Pacific white shrimp (*Litopenaeus vannamei*) diet. Four *Amphora* sp. isolations were cultured and compared productivity. The results showed that ASC and ALY had higher productivity, lipid content and polyunsaturated fatty acid composition compared to the other 2 strains. These ASC and ALY were chosen for optimum culture condition experiment, varied with 3 light intensities and 2 medium concentrations. The results showed that ALY cultured with medium light intensity with F/20 medium can produce higher biomass, saturated fatty acids, and important unsaturated fatty acids than the other culture condition. Shrimp fed with 15% of this *Amphora* sp. for 6 weeks demonstrated the higher survival rate from challenge test than 3 weeks. In addition, the expression of LvALF gene that response for Anti-lipopolysaccharide factors in hepatopancrease have been found. This finding indicated that adding 15% *Amphora* sp. in shrimp diet may enhance immunity of shrimp infected with *V. parahaemolyticus*. However further study in immune parameters such as PO and SOD must be included to clarify the mechanism of shrimp immune system.

Field of Study:	Marine Science	Student's Signature ....
Academic Year:	2023	Advisor's Signature ....
		Co-advisor's Signature ....
		Co-advisor's Signature ....

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ACKNOWLEDGEMENTS

First and foremost, I express gratitude to myself for the tolerance throughout this journey. I commend every fragment of my broken heart, marveling at its ability to continue beating. I commend my own bravery in willingly stepping into the fire and enduring until I emerged victorious. My appreciation extends to the comforting companionship of coffee, babo milk tea, Coca-Cola, food, and Netflix, particularly during challenging and exhausting moments.

Next, I extend my appreciation to my advisors, Asst. Prof. Dr. Kornrawee Aiumsomboon and Assoc. Prof. Dr. Sanit Piyapattanakorn. Their belief in me and the second chance they provided fueled my courage and strength to persevere. I am also thankful to the CEMB team for their crucial support during urgent times, generously offering their valuable time.

I am indebted to the researchers at the Aquatic Resources Research Institute (ARRI), Chulalongkorn University, for their invaluable time and support during the research. They not only warmed my heart but also played diverse roles – friends, brothers, and sisters.

Special thanks go to Asst. Prof. Dr. Weena Keaypudsa for her unwavering kindness, attentive listening, and sisterly warmth. I am grateful for the chocolate drinks and ice cream, symbols of her support. Prof. Dr. Gerald F. Plumley, your consistent encouragement has been instrumental in boosting my confidence. I am appreciated Asst. Prof. Dr. Ajcharaporn Piumsomboon for the beginning of this study.

I extend my gratitude to Mahanakorn University for 4 years of funding for my Ph.D. and would like to express my appreciation to my students for their invaluable assistance in the final experiment. Your support was crucial, and I couldn't have completed it without you.

A heartfelt acknowledgment goes to my family, especially my Mom, for her unyielding support in every aspect, including funding for this study.

To my friends, Khun Sarit, Khun Sarinee, Khun Pompam, Nong Noon-Chid, Nong Noon-Phat, Nong New, Nong Kate, and Nong Pat, and Nong Praewpraw your unwavering support has left an indelible mark on my Ph.D. journey.

In conclusion, I extend my sincere thanks to Khun Luo Xiao for his love, support, encouragement, and care.

P.S. A special mention to Durian Bababobo Thongdet Luo and Mungkud Kangkangkookoo Thongdet Luo for their comforting purrs. May they enjoy a wonderful life on Cat's planet. Until we meet again.

Ekthida Thongdet

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

TABLE OF CONTENTS

Page

.... iii

ABSTRACT (THAI) iii

.... iv

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES xii

LIST OF FIGURES xiii

Chapter I 1

General introduction 1

1. The important of *Amphora* sp. in aquaculture and nutrition 1

2. Biotechnology to culture *Amphora* sp. 1

   1. Physiological technique 2

   2. Genetic technique 5

3. Fatty acid and lipid biosynthetic process 5

4. Lipid production and Fatty acid composition in diatoms 7

   1. Saturated fatty acids (SFAs) 7

   2. Unsaturated fatty acids (UFAs) 7

5. Diatom as an immunostimulant application in crustacean 8

6. Objectives 10

Chapter II 11

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Lipid content screening of *Amphora* sp. isolated from the Gulf of Thailand for use in the *Litopenaeus vannamei* diet preparation 11

1. Introduction 11

2. Objective 12

3. Materials and methods 12

3.1 Amphora sp. culture medium preparation 12

3.2 Amphora sp. sampling 13

3.3 Amphora sp. isolation 14

3.4 Amphora sp. identification 14

3.5 Amphora sp. culture 14

3.6 Light intensity determination 15

3.7 Amphora sp. harvesting 15

3.8 Amphora sp. biomass determination 16

3.8.1 In vivo chlorophyll 16

3.8.2 Cell density and specific growth rate (SGR) 16

3.9 Total lipid content determination 17

3.10 Fatty acid composition determination 18

3.10.1 Preparation of FAMEs 18

3.10.2 Analysis of lipid composition (FAMEs analysis) 18

3.11 Diatom screening for highest growth, total lipid content and fatty acid composition 19

3.12 Data analysis 19

4. Results 20

4.1 Diatom identification 20

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.1.1 *Amphora* sp. isolated from Sichang Island (ASC), Chonburi province and *Amphora* sp. isolated from Laemyai (ALY), Rayong province. 20

4.1.2 *Amphora* sp. isolated from Samae Sarn (ASS) Island, Chonburi province. 21

4.1.3 *Amphora* sp. isolated from Pranburi (APB), Prachuap Khiri Khan province........ 22

4.2 Biomass 22

4.2.1 Chlorophyll 23

4.2.2 Cell density 24

4.2.3 Specific growth rate (SGR)........ 25

4.3 Total lipid content 26

4.4 Fatty acid composition 27

4.4.1 Saturated fatty acid (SFA) and unsaturated fatty acid (UFA) 27

4.4.2 Fatty acid composition 28

5. Discussion 29

5.1 Biomass and growth of Amphora sp. 29

5.2 Total lipid content 33

5.3 Fatty acid composition 35

6. Conclusion 38

Chapter III 40

The optimum light intensity and culture medium concentration for *Amphora* sp. cultivation........ 40

1. Introduction 40

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2. Objective 40

3. Materials and methods 41

3.1. Diatom screening for highest growth, total lipid content and fatty acid composition 41

3.2. Light intensity determination 41

3.3. Culture medium preparation 42

3.4. Amphora sp. harvesting 42

3.5. Biomass, lipid content and fatty acid composition determination 42

3.6. Data analysis 42

4. Results 43

4.1 Biomass 43

4.1.1 Chlorophyll 43

4.1.2 Cell density 44

4.1.3 Specific growth rate (SGR) 45

4.2 Total lipid content 46

4.2.1 Saturated fatty acid (SFA) 48

4.2.2 Unsaturated fatty acids (UFAs) 49

4.2.3 Fatty acid composition 50

5. Discussion 53

5.1 Biomass 53

5.2 Lipid content and fatty acid composition 54

6. Conclusion 54

Chapter IV 55

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The efficiency of diet with additional diatoms as immuno-stimulant for *Litopenaeus vannamei* 55
1. Introduction 55
2. Objective 56
3. Materials and methods 56
3.1 Diet preparation 56
3.2 Shrimp acclimatization 57
3.3 Water quality analysis 57
3.4 Feeding trial experiment 57
3.5 Vibrio parahemolyticus culture 58
3.6 The study of disease resistant by challenge test method 58
3.7 Growth performance of shrimp 58
3.7.1 Survival rate (SR; %) 58
3.7.2 Weight gain (WG; %) and specific growth rate (SGR; %) 58
3.7.3 Feed efficiency (FE; %) (Jana, et al. 2022) 59
3.8 Shrimp blood sampling 59
3.9 Total hemocyte count (THC) 59
3.10 Phagocytosis activity (PA) 59
3.11 ALF B gene analysis 59
3.12 Midgut histopathology 60
3.13 Data analysis 60
4. Results 60
4.1 Growth performance 60
4.1.1 Survival rate (SR) 60

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.1.2 Mean body weight (MBW) 61

4.1.3 Specific growth rate (SGR) 62

4.1.4 Feed efficiency (FE) 63

4.2 Disease resistance 63

4.2.1 Survival rate (SR) 64

4.2.2 Total hemocyte count (THC) 64

4.2.3 Phagocytic activity (PA) 65

4.2.4 Midgut intestinal epithelial area of shrimp 66

4.2.5 Expression of Anti-lipopolysaccharide factors: LvALF B gene 68

5. Discussion 68

5.1 Growth performance 68

5.2 Disease resistant of V. parahemolyticus 70

6. Conclusion 71

Chapter V 73

Conclusion 73

1. Lipid content screening of *Amphora* sp. isolated from the Gulf of Thailand for use in the *Litopenaeus vannamei* diet preparation 73

2. The optimum light intensity and culture medium concentration for *Amphora* sp. cultivation 73

3. The efficiency of diet with additional diatoms as immuno-stimulant for *Litopenaeus vannamei* 73

REFERENCES 75

VITA 90

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF TABLES

Page

Table 1 Stock solution and medium preparation. 12

Table 2 Fatty acid composition (mg/100 mg algal dry weight) determined from 2 strains of Amphora sp. at different time of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different. 28

Table 3 Growth and biomass of microalgae cultured in F/2 medium. 30

Table 4 Lipid and fatty acid content determined in diatom. 34

Table 5 The advantage characteristic of each Amphora sp. strain 38

Table 6 Fatty acid composition of ASC (mg/100 mg algal dry weight) determined from 2 strains, 3 light intensities, and 2 medium concentrations in each culture day. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each fatty acid. 51

Table 7 Fatty acid composition of ALY (mg/100 mg algal dry weight) determined from 2 strains, 3 light intensities, and 2 medium concentrations in each culture day. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each fatty acid. 52

Table 8 Concentration of V. parahemolyticus (cfu/ml) in culture water for challenge test experiment. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each testing day 63

Table 9 Midgut intestinal epithelial of shrimp after immersion with V. parahaemolyticus for 24 hrs 67

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF FIGURES

Page

Figure 1 Fatty acid biosynthetic process in diatoms: adapted from Zulu et al. (2018) 6

Figure 2 Sampling locations of 4 Amphora sp. strains in the Gulf of Thailand. 13

Figure 3 Amphora sp. growing bottle with 10 acrylic plates for diatom attachment. 15

Figure 4 Pendant temperature/Light data logger 64K, HOBO by Onset UA-002-64. ... 15

Figure 5 Amphora sp. attached on acrylic plate. 16

Figure 6 Hemocytometer (Avin, 2020). 17

Figure 7 Experimental unit of diatom culture for highest growth, total lipid content and fatty acid composition.. 19

Figure 8 Amphora sp. isolated from Sichang Island identified under light microscope and scanning electron microscope. 21

Figure 9 Amphora sp. isolated from Laemyai identified under light microscope and scanning electron microscope. 21

Figure 10 Amphora sp. isolated from Samae Sarn Island identified under light microscope and scanning electron microscope. 22

Figure 11 Amphora sp. isolated from Pranburi identified under light microscope and scanning electron microscope. 22

Figure 13 Amphora sp. attached to acrylic plate in glass bottle. 23

Figure 14 Relative fluorescence of 4 diatom strains determined on day 0, 2, 6, 9 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 24

Figure 15 Cell density of 4 diatom strains determined on day 0 – 2, 2 – 6, 6 – 9 and 9 – 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 25

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 16 SGR of 4 diatom strains determined on day 0 – 2, 2 – 6, 6 – 9 and 9 – 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 26

Figure 17 Total lipid content (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 27

Figure 18 SFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 28

Figure 19 UFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 28

Figure 20 Experimental unit 41

Figure 21 Relative fluorescence of 2 diatom strains determined on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 43

Figure 22 Cell density of 2 diatom strains determined on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 45

Figure 23 Levels of SGR in 2 diatom strains on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 46

Figure 24 Total lipid content (mg/100 mg Algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 47

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 25 SFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 49

Figure 26 Total levels of UFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day. 50

Figure 27 Survival rate of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day. 61

Figure 28 MBW of shrimp determined on week 0 to 6 of culture (Mean ± SE). 62

Figure 29 SGR of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day. 62

Figure 30 FE of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day. 63

Figure 31 Survival rate of shrimp determined after 24 hrs of V. parahemolyticus immersion. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day. 64

Figure 32 THC (cell/ml) of shrimp determined after 24 hrs of V. parahemolyticus immersion (Mean ± SE) between each culture day. 65

Figure 33 Phagocytic activity (%) of shrimp determined after 24 hrs of V. parahemolyticus immersion. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day. 66

Figure 34 Midgut intestinal epithelial area of shrimp determined after 24 hrs of V. parahemolyticus immersion (Mean ± SE) between each culture day. 67

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 35 Expression of LvALF B gene determined in hepatopancrease of 6 week feeding trial shrimp, after 24 hrs of V. parahemolyticus immersion, mean ± SE The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. 68

Chapter I

General introduction

1. The important of *Amphora* sp. in aquaculture and nutrition

Fatty acids are energy sources in marine food chain with polyunsaturated fatty acids (Paliwal et al.) as key substances in physiological process of innate immune (Gao et al.) in crustacean. Furthermore, fatty acids, especially eicosapentaenoic acid (Harel et al.) and docosahexaenoic acid (Harel et al.) are essential for growth, development and survival of juvenile larval crustacean and marine fish. In marine ecosystem, crustacean larvae and other marine larvae could directly achieve fatty acids (Paliwal et al.) by feeding on microalgae or phytoplankton which is dominated by diatoms in the water column. While in aquaculture system, live and supplementary feeds such as krill and artemia are major fatty acids sources for crustacean larvae. This supplementary diet contributes to major expense in crustacean culture. Therefore, using diatom with high PUFAs content can be a substitute of live diet and reduce expense at the same time since diatom has high ability of fatty acid synthesis from photosynthesis process. The content and composition of FAs in diatoms depends on growth phase and environmental factors which could be controlled by the culture conditions. In the perspective of animal health, mass mortality, disease resistance and environmental contamination are important issues in aquaculture. Using diatoms as diet not only provides PUFAs for growth but these PUFAs also act as immunostimulant due to their ability to produce anti-microbial compounds and biomolecules. In addition, diatoms could be cultured in low-cost photobioreactors, providing live microalgae as “green water supply” in the culture pond and suitable for storage and shipping by producing dried algae, frozen algal concentrates, and algal pastes.

2. Biotechnology to culture *Amphora* sp.

Diatoms can produce and accumulate lipids up to 70% of dry biomass in form of Triacylglycerols (Nomaguchi et al.) (Y.-C. Chen, 2012; Sayanova et al., 2017; Xue et al., 2017; Zulu et al., 2018). TAGs is storage lipids, the most abundant form of

neutral lipid stored in eukaryotic cells, commonly found within organelles and accounted higher than 60% of total lipids (Y.-C. Chen, 2012; Sajjadi et al., 2018; Sayanova et al., 2017; Xue et al., 2017; Zulu et al., 2018).

Sajjadi et al. (2018) and Zulu et al. (2018) stated that the storage of lipid in diatoms depends on the species, growth phase, and environmental conditions. Amounts of TAGs and other neutral lipids are higher during stationary growth phase and under stress conditions such as nutrient starvation, but often incorporated with low algal biomass. While low number of TAGs can be found during exponential phase and desired conditions (Y.-C. Chen, 2012; Sajjadi et al., 2018; Sayanova et al., 2017; Xue et al., 2017; Zulu et al., 2018). Zienkiewicz et al. (2016) indicated that under desired culture condition, microalgae produce small proportion of TAGs in lipid droplets. In contrast, under stress culture condition such as nitrogen ("<1.Genes group - เพิ่มเติม an immune-related ge.pdf>,") deprivation, microalgae produce large proportion of TAGs and rapidly transformation of lipid droplets due to temporary suspended of cell division and cell metabolisms in order to survive in the stress condition. But restoring of favorable conditions such as N resupply induces the restoring of cell divisions and cells metabolism, resulted in decreasing of TAGs accumulation. The total lipid content in *Amphora* sp., *Caloneis* sp., *Chaetoceros* sp., *Cocconeis* sp., *Cylindrotheca* sp., *Fistulifera* sp., *Melosira* sp., *Navicula* sp., *Nitzschia* sp., *Phaeodactylum* sp., *Seminavis* sp., *Skeletonema* sp. and *Thalassiosira* sp., can be found varies from 1.5 – 69 % of dry biomass (BenMoussa-Dahmen et al., 2016; Bertozzini et al., 2014; Boukhris et al., 2017; Y.-C. Chen, 2012; Cheng et al., 2014; El Arroussi et al., 2017; Jiang et al., 2016; Sajjadi et al., 2018; Sayanova et al., 2017; Wang et al., 2014)

The methods to enhance the maximum lipid and fatty acids production is provided by many researchers including physiological and genetic technique as followed.

1. Physiological technique

This technique involves the control of nutritional mode or introduced environmental stress such as culture mode (Young et al., 2022), carbon ("<25. Fish Oil Replacement and Alternative Lipid.pdf>,") sources (organic vs. inorganic) and

concentrations, nutrient limitation (nitrogen, carbon, phosphorus (P), or silicate (Si) deficiency), low or high light intensity, temperature, salinity, alkalinity, pH and dehydration (Zienkiewicz et al., 2016; Zulu et al., 2018). Among nutrient stress conditions, nutrient limitation is the most common technique used with the aim of enhancement the biomass and desired production. Sajjadi et al. (2018) explained that the manipulation of nutrient under limitations can be distinguished into three levels of limitation composed of:

1.1 Starvation condition diatoms are cultured in sufficient nutrient condition for the first step, then the cells are separated and transferred into the deficient nutrient condition of a particular nutrient, result in the biological shock and increasing of high-energy production.

1.2 Limitation condition diatoms are cultured in continuous system with low ratio of one particular nutrient in a media such as N limitation which is indicated by low ratio of N and P (N:P ratio) of about 5:1 and P limitation which is indicated by high ratio of N:P of about 30:1. In microbiology, this method called "law of minimum."

1.3 Depletion condition diatoms are cultured in batch cultures with sufficient and required nutrient condition, the biomass of diatoms are continually increased until achieve the nutrient depletion, results in physiological response by decreasing of growth rate and producing the high-energy storage production.

Zulu et al. (2018) explained that under C stress, C is necessary for both biomass and lipid production, could be both inorganic carbon in form of carbondioxide (CO_2) and organic carbon such as glycerol, glucose, starch, or acetate. Increasing CO_2 concentrations in photoautotrophic culture condition of diatom *Phaeodactylum tricornutum* could enhance biomass and lipid production. However, using glycerol as a C source in mixotrophic culture conditions are much more effective at enhancing lipid production than the normal photoautotrophic because resulted in 8-fold higher biomass production compared to photoautotrophic condition. This research also recorded that N, P and Si are the commonly stressed conditions, N limitation is the most commonly stress condition used for enhancement of lipid accumulation in diatoms. Both N and P limitation reduce or

even stop the growth and biomass production and increased 60% of TAGs, 14:0 and 16:0 fatty acids in *P. tricornutum*. N limitation can cause the shutting down of N pools and decrease the amount of cellular protein. In contrast, increasing total lipid levels can be induced by the activation of phospholipases and galactolipases. This leads to the degradation of phospholipids and galactolipids and the release of free fatty acids which is converted into acyl coenzyme A that are used for TAGs formation step. While P, an important composition of phospholipids and nucleic acids, plays a significant role in photosynthesis and respiration which are cellular metabolic processes. P limitation condition induces cell to performs an effective P conserving mechanism which is the accumulation of galactolipid (a type of glycolipids) due to losing of the phospholipids. This condition also reduces cell division rates due to slightly decreasing of photosynthetic rate but increasing of C accumulation in form of TAGs which are rich in saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) (Sajjadi et al., 2018). However, Zienkiewicz et al. (2016) informed that N deprivation is the best stress condition and widely used for TAGs accumulation induction compared to other factors which is only increased lipid synthesis. N deprivation leads to the ending of the cell division, increasing of TAGs synthesis up to 20 – 50% of dry weight of diatoms. The study of N deprivation by Yodsuwan et al. (2017) also found that an aged culture of *P. tricornutum* under high N concentration (32.09 and 64.29 mg/l $NaNO_3$) had increased PUFAs level. Si is one of the commonly used nutrients for environmental stress condition. Although there is no effect of Si limitation on lipid production of *P. tricornutum*, while lipid production in some other marine diatom such as *Chaetoceros gracilis* and *Thalassiosira pseudonana* were reported (Zulu et al., 2018).

In addition, another physiological stress condition such as low and high light intensity, temperature and salinity or the combination of many stress factors in some species also improve the biomass, lipid and fatty acids production. According to the study by Cheng et al. (2014), diatom *Nitzschia* sp. was mutated by 60Co-g-ray irradiation and cultivated with high salinity to improve biomass and lipid production. The result shows that irradiation at 900 Gy biomass and lipid increased up to 53.8%

and 28.1%, respectively. While, under increasing of salinity up to 30 ppt, lipid content increased from 11.9% to 27.2%.

1.4 Multi-stage photobioreactors the study of Bertozzini et al. (2014), *Skeletonema marinoi* was cultured in 3 stages photobioreactor. The first stage, *S. marinoi* was cultured in enriched f/2 medium to achieve the maximum yield. The second stage is a semi-continueous regime, a slightly reduced concentration of nitrate to increase neutral lipid content. The third stage was a maturation phase, diatom was grown for an additional week without the addition of medium in order to increase more neutral lipid and biomass. Another type of photobioreactor is a 2 stages culture condition system by Ozkan andRorrer (2017) where the diatom *Cyclotella* sp. was first cultured in a silicon-starved state condition to achieve maximum biomass yield. At the second stage, the diatom cells were transferred to sufficient silicon condition aims to increase neutral lipid content.

2. Genetic technique

There are several genes involved in the enhancement of lipid production in diatom, for example in *P. tricornutum* target genes PtTE, PtME and PtDGAT2 can be overexpress and affects the increasing of total lipid content. While other target genes; UcTE (C12-TE), Δ5 desaturase, PtGPDH, ScDGA1, AtOLEO3, PtDGAT2 and PtAGPAT; have effects on TAGs content and target gene Δ5 elongase and Δ6 desaturase is responsible for an increase of Docosahexaenoic acid (DHA) level in TAGs content (Zulu et al., 2018). Some gene that involved in lipid production enhancement can be disrupted by using of small molecules, resulting in specific phenotype (Wase et al., 2018) such as Brefeldin A, an endoplasmic reticulum-stress inducer, using for TAGs accumulation in *Chaetoceros reinhardtii* and *Chlorella vulgaris* (Zienkiewicz et al., 2016).

3. Fatty acid and lipid biosynthetic process

The current model of fatty acids and lipid synthetic pathways in diatoms are derived mainly from flowering plants and green microalgae (Sayanova et al., 2017; Zulu et al., 2018). Biosynthesis of PUFAs occurs in plastids and endoplasmic reticulum. The formations of longer chain of polyunsaturated fatty acids (LC-PUFAs);

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

16 – 18 carbon atoms; and very long chain polyunsaturated fatty acids (VLC-PUFAs); 20 – 28 carbon atoms; in diatoms are catalyzed by fatty acid desaturases enzyme and elongases enzyme (Zulu et al., 2018). Both lipid and fatty acid synthesis have 2 pathways including a prokaryotic pathway and three-steps eukaryotic pathways (Sayanova et al., 2017; Zulu et al., 2018), controlled by endoplasmic reticulum, synthesized from glycerol and 3 fatty acids (Sayanova et al., 2017; Xue et al., 2017; Zienkiewicz et al., 2016; Zulu et al., 2018). The pathway as shown in Figure 1.

1. **The synthesis of TAGs** by endoplasmic reticulum. TAGs are synthesized from glycerol and fatty acids. The process occurs via acyl coenzyme A dependent pathway or the "Kennedy pathway" which is normally found in eukaryotic cells.
2. **The acylation of glycerol-3-phosphate** using acyl coenzyme A and glycerol-3-phosphate acyl transferase (GPAT) enzyme to form the lysophosphatidate.
3. **The acylation of lysophosphatidate** using acyl coenzyme A and lysophosphatidic acid acyltransferase (LPAAT) enzyme to produce phosphatidic acid.
4. **The de-phosphorylation of phosphatidic acid** using phosphatidic acid phosphatase (PAP) enzyme to produce diacylglycerol.
5. **The formation of triacylglycerols**, diacylglycerol is acylated by diacylglycerols acyltransferase (DGAT) and corporate with the third acyl coenzyme A.

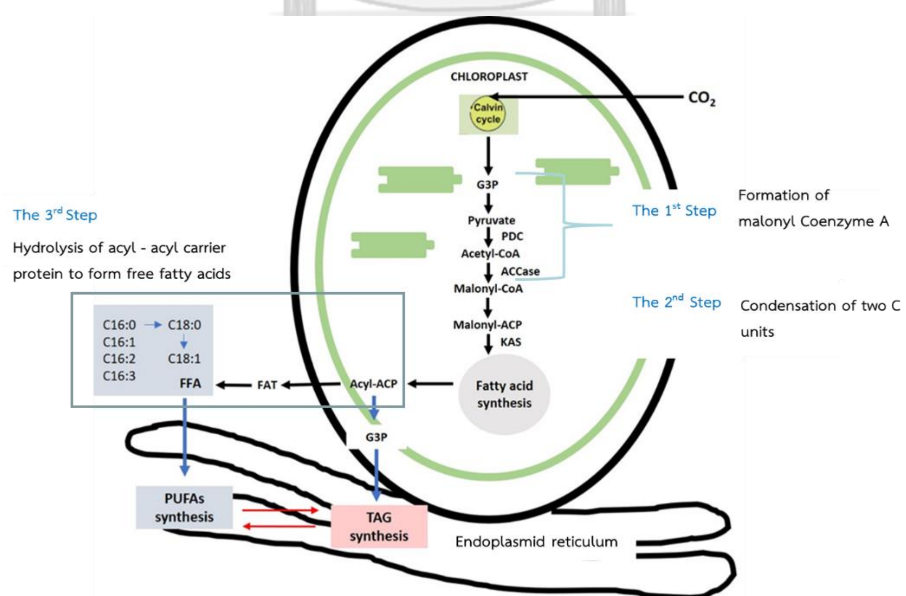


Figure 1 Fatty acid biosynthetic process in diatoms: adapted from Zulu et al. (2018)

4. Lipid production and Fatty acid composition in diatoms

In general, fatty acids composition in diatoms compose of 2 types including saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs)

1. **Saturated fatty acids (SFAs)**

SFAs content in diatoms varies from 6 – 73% of total lipid content and with number of carbon atoms ranging from 12 to 24 carbon atoms. These SFAs include dodecanoic acid or lauric acid (C12:0), tetradecanoic acid or myristic acid (C14:0), hexadecanoic acid or palmitic acid (C16:0), octadecanoic acid or stearic acid (C18:0), eicosanoic acid or arachidic acid (C20:0), docosanoic acid or behenic acid (C22:0), and tetracosanoic acid or linoceric acid with the formula C24:0 (Y.-C. Chen, 2012; Sayanova et al., 2017; Zulu et al., 2018). Besides, Y.-C. Chen (2012) and Sajjadi et al. (2018) informed that the most abundant SFAs found in diatoms are myristic acid (2 – 25% of total fatty acid) and palmitic acid (3 – 47% of total fatty acid).

2. **Unsaturated fatty acids (UFAs)**

UFAs in diatoms can be divided into 2 major groups depend on the number of double bonds that present in fatty acid chain including MUFAs which has one double bond and 16 – 18 carbon atoms and PUFAs which has two or more double bond and 20 – 28 carbon atoms. MUFAs content varies from 1 – 41% of total lipid content (BenMoussa-Dahmen et al., 2016; Boukhris et al., 2017; El Arroussi et al., 2017; Jiang et al., 2016; Sajjadi et al., 2018; Sayanova et al., 2017; Suh et al., 2015; Suroy et al., 2014; Widianingsih et al., 2013; Zulu et al., 2018)and consists of tetradecenoic acid or myristoleic acid (C14:1 n-5), hexadecenoic acid or palmitoleic acid (C16:1 n-7), octadecenoic acid or oleic acid (C18:1 n-9), eicosenoic acid or paullinic acid (C20:1 n-7), tetracosanoic acid or erucic acid (C22:1 n-9). While, PUFAs content varies from 0.2 – 51% of total lipid content (BenMoussa-Dahmen et al., 2016; Boukhris et al., 2017; El Arroussi et al., 2017; Jiang et al., 2016; Sajjadi et al., 2018; Sayanova et al., 2017; Suh et al., 2015; Suroy et al., 2014; Widianingsih et al., 2013; Zulu et al., 2018) and consists of octadecadienoic acid or linoleic acid (C18:2 n-6), octadecatrienoic acid or alpha (α)-linolenic acid (C18:3 n-3; ALA), and octadecatrienoic acid or alpha (α)-linolenic acid (C18:3 n-6; GLA) which is called long-

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

chain PUFAs (LC-PUFAs) and eicosadienoic acid (C20:2 n-6), eicosatrienoic acid (C20:3 n-3; ETE), dihomo-gamma-linolenic acid acid (C20:3 n-6; DGLA), eicosatetraenoic acid or arachidonic acid (C20:4 n-6;ARA), eicosapentaenoic acid (C20:5 n-3; EPA), docosadienoic acid (C22:2 n-6), docosahexaenoic acid (C22:6 n-3; DHA) which is called very long-chain PUFAs or VLC-PUFAs (Y.-C. Chen, 2012; Sayanova et al., 2017; Zulu et al., 2018). Besides, Y.-C. Chen (2012) informed that the most abundant UFAs found in diatoms are hexadecenoic acid, EPA and DHA while Sajjadi et al. (2018) stated that major PUFAs found in microalgae are palmitoleic acid, oleic acid, linoleic acid, and linolenic acid. Furthermore, the findings of numerous studies confirm that these PUFAs are the dominant PUFA molecules found in diatoms.

5. Diatom as an immunostimulant application in crustacean

The use of immunostimulant in shrimp aims to enhance the function of innate immunity in a period, lead to an increasing disease resistance and decreasing immune-suppression factors. It also emulates the process of infection in crustacean such as the increasing of total hemocyte count (THC), proPO and other antimicrobial substances. So, the continuous use of immunostimulant may stimulate the disease resistant in shrimp during the cultivation (Sonthi, 2016).

Sonthi (2016) explained that the immune defense responses mechanism in crustacean after the use of immunostimulant is the same with the infection of pathogen. Both cellular and humoral defense systems are activated and result in the higher resistance of the infection during the cultivation period.

There are many groups of immunostimulant which have been using in marine shrimp cultivation both in *Penaeus monodon* and *Litopenaeus vannamei* such as bacterial derivatives (live bacteria, bacterin, polysaccharide; lipopolysaccharide, peptidoglycan), yeast derivatives, nutritional factors (vitamins, antioxidant, astaxanthin), animal extracts (chitosan) and plant extracts (herbal extracts, seaweed extracts). Seaweed extracts is used as immunostimulant due to nutritional value and PUFAs (Gabriel et al., 2009; Mastan, 2015; Sonthi, 2016). The application of immunostimulant in marine shrimps reported by Sonthi (2016) that stated the variation in administration method (diet, injection and enriched Artemia),

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

concentration (4 μg/g meal - 5 mg/kg meal), and duration time (1 – 8 weeks). These applications resulted in increasing of immune parameters such as antimicrobial peptide, phagocytosis activity, superoxide dismutase activity and THC which enhance the resistance of shrimp to the pathogen such as bacteria *Vibrio harveyi* and *V. alginolyticus* and virus such as white spot syndrome virus (WSSV).

The digestibility and high nutritional value such as proteins, lipids and essential nutrients of microalgae could be health – promoting benefits as a nutritional supplement in feed meal. Some microalgae, their natural anti-microbial compounds and biomolecules such as PUFAs and pigments could be use as immunostimulants. The popular microalgae species used in aquaculture including *Chlorella* sp., *Tetraselmis* sp., *Isochrysis* sp., *Pavlova* sp., *Phaeodactylum* sp., *Chaetoceros* sp., *Nannochloropsis* sp., *Skeletonema* sp., and *Thalassiosira* sp. These microalgae can be used in form of live feed or dried algae as supplement to provide the balance of major and minor nutrition (Charoonnart et al., 2018). Desbois andLawlor (2013) reported that an antibacterial fatty acid such as EPA from diatom *P. tricornutum* 10–20 μM could affect activities of both Gram positive and Gram-negative bacteria, including *Staphylococcus aureus* (MRSA) and *Aeromonas hydrophila*. The study of *L.vannamei* cultured for 84 days with microalgae *Platymonas helgolandica*, green alga *C. vulgaris* and diatom *C. mulleri* at 0.02 mg chlorophyll_a /l, respectively had significantly lower *Vibrio* sp. counts in rearing water, shrimp stomach and intestines compared to the shrimp which cultured without microalgae.of (Ge et al., 2016).

The application of microalgae and diatoms as immunostimulant in crustacean health such as the use of microalgae *Spirulina elicits* (dried power) by 30 – 60 g/kg as supplementation for 4 weeks which was resulted in increasing of lipopolysaccharide and b-1,3-glucan binding protein transcript level, lysozyme activity and phagocytic activity of *L. vannamei* after the infection of *V. alginolyticus* (Y. Y. Chen et al., 2016). In addition, the study of Seraspe et al. (2014) showed that linolenic acid and EPA in diatom *Chaetoceros calcitrans* could increase the resistance of *V. haveyi,* proPO activity, plasma protein concentration and survival rate of juvenile of *P. monodon*. This study used freeze-dried *C. calcitran* as supplementation of 30 g/kg for 45 days.

6. Objectives

1. To isolate diatoms from the Gulf of Thailand which can be used as *Litopenaeus vannamei* diet.

2. To find the optimal conditions for the production of cultured diatoms.

3. To study the efficiency of diet with additional diatoms as immuno-stimulant for *Litopenaeus vannamei*.

Chapter II

Lipid content screening of *Amphora* sp. isolated from the Gulf of Thailand for use in the *Litopenaeus vannamei* diet preparation

1. Introduction

Fishmeal and fish oil contain an optimal mix of essential nutrients and have been used as major feed ingredients in aquaculture for many years. However, over the past few decades, supplies of fish-based materials in aquaculture feeds have decreased significantly. It is predicted that there will not be enough fish-based ingredients to match the expansion of aquaculture industries soon after. Therefore, searching for alternative feed ingredients have become necessity (Araújo et al., 2019).

Microalgae is one of the promising alternative fish meal ingredients due to the combination of essential amino acids, healthy triglycerides, rich in omega-3 fatty acids, carbohydrates, vitamins and pigments that could probably provide fish health (Sarker et al., 2020). Additionally, microalgae have the highest biomass productivity compared to another photosynthetic organism (Nagappan et al., 2021). Numbers of microalgae have been studied for fish oil replacement. These included the study of *Schizochytrium* sp. which the results showed that 75% of total fish oil were replaced successfully by the oil from *Schizochytrium* sp. with no apparent effect on digestibility, growth, and somatic parameter. Other microalgae such as *Isochrysis* sp., *Spirulina* sp., *Pavlova* sp., *Chlorella* sp., *Chlamydomonas* sp. and *Nannaochloropsis* sp. have also gained more attention as an alternative lipid source due to their potential enhancement of growth and the ability to synthesize essential fatty acid (Macias-Sancho et al., 2014; Nagappan et al., 2021).

Diatom, a member of microalgae, can produce and accumulate lipid up to 40 – 60% of dry biomass (Y.-C. Chen, 2012; Sayanova et al., 2017; Xue et al., 2017; Zulu et al., 2018). The main proportion is UFAs, especially LCFAs, which are necessary for the growth and health of aquatic animals. Among various species of diatom, *Amphora* sp. is one of the most interesting species due to its rapid growth

capabilities, the potential of high lipid content producing and its quality as food for abalone and sea urchin larvae because of its suitable size for grazing (de la Peña, 2007). Their lipid content, productivity and biosynthesis are species-specific and vary within different diatom species, growth stages and the environmental parameters (Eva Cointet et al., 2019; Upadhyay et al., 2017). Ideally, strains of microalgae suitable for being candidates as lipid sources in aquaculture should have the ability to grow rapidly in wide variety of growth conditions, well balance between cost and productivity, and industrial scale application.

2. Objective

To determined growth, total lipid content, and fatty acid composition of *Amphora* sp. isolated from different locations of The Gulf of Thailand as criteria to verify the potential use of these diatom as an alternative source of lipid in shrimp larviculture.

3. Materials and methods

3.1 *Amphora* sp. culture medium preparation

F/2 medium was used for *Amphora* sp. culture. 1 M NaOH or HCL was used to adjust the pH of natural 30 ppt and filtered seawater to 8.0. Seawater was sterilized by autoclaving for 15 minutes at 15 psi and allowed to cool to room temperature before use. Each stock solution was prepared, and the following medium amounts were added as follows Table 1.

จุฬาลงกรณ์มหาวิทยาลัย CHULALONGKORN UNIVERSITY

Table 1 Stock solution and medium preparation.

Stocks solution	Concentration
1. $NaNO_2$	75 g/l dH_2O
2. $NaH_2PO_4.2H_2O$	5.65 g/l dH_2O
3. $Na_2SiO_3.9H_2O$	30 g/L dH_2O
4. Trace elements (chelated)	
3.1 NA_2 EDTA	4.16 g/l dH_2O
3.2 $FeCl_3.6H_2O$	3.15 g/l dH_2O
3.3 $CuSO_4.5H_2O$	0.01 g/l dH_2O

3.4 $ZnSO_4.7H_2O$	0.022 g/l dH_2O
3.5 $CoCl_2.6H_2O$	0.01 g/l dH_2O
3.6 $MnCl_2.4H_2O$	0.18 g/l dH_2O
3.7 $Na_2MoO_4.2H_2O$	0.006 g/l dH_2O
5. Vitamin solution	
4.1 Cyanocobalamin (Vitamin B12)	0.0005 g/l dH_2O
4.2 Thiamine HCl (Vitamin B1)	0.1 g/l dH_2O
4.3 Biotin	0.0005 g/l dH_2O
Medium	
1. $NaNO_2$	1 ml
2. $NaH_2PO_4.2H_2O$	1 ml
3. $Na_2SiO_3.9H_2O$	1 ml
4. Trace elements (chelated)	1 ml
5. Vitamin solution	0.5 ml

3.2 *Amphora* sp. sampling

Four *Amphora* sp. strains were collected from Sichang island (ASC), Samaesarn island (ASS), Pranburi district (APB), and Laem Yai (ALY), Gulf of Thailand (Figure 2). Seawater was collected by 10-20 µm phytoplankton net, stored in plastic bottles, and ambient temperature. F/2 medium was added to maintain all collected diatom cells for isolation.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

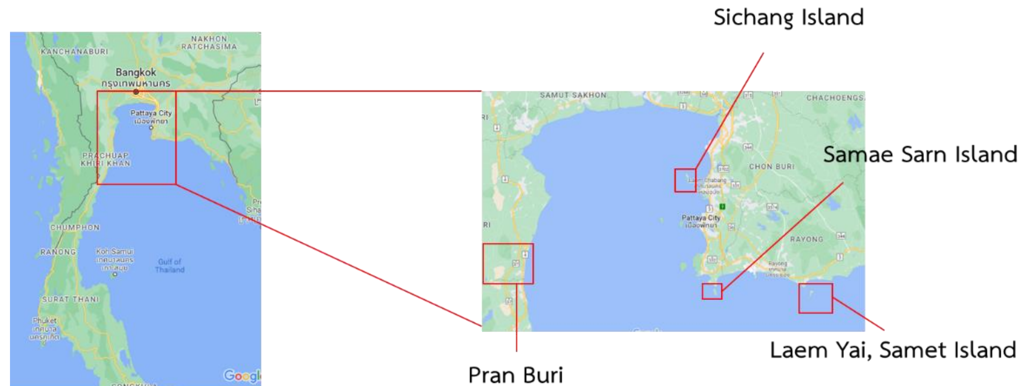


Figure 2 Sampling locations of *4 Amphora* sp. strains in the Gulf of Thailand.

3.3 *Amphora* sp. isolation

The *Amphora* sp. cells were isolated from seawater samples using pipette technique (Parvin et al., 2007). The isolated diatoms were separately cultured in the F/2 medium for the experiment (El Arroussi et al., 2017) and kept at Mangrove and coastal marine ecology Laboratory, Department of Marine Science, Faculty of Science and Aquatic Resources Research Institute (ARRI), Chulalongkorn University.

3.4 *Amphora* sp. identification

Light microscope identification, fresh *Amphora* sp. cells were placed on glass slide for light microscope, and identified under 40x magnification light microscope (Khumaidi et al., 2020).

Scanning electron microscope or SEM, Fresh *Amphora* sp. were cleaned by rinsing with distilled water and boiling in equal volume of H_2SO_4 for 3 mins. Then completely remove the acid by rinsing with distilled water. The removed frustule then were gradually dehydrated in ethanol series (30, 50, 70, 90, and 100%), each concentration was 30 min, mounted onto glass, and dried at room temperature over night for SEM (Wang et al., 2014).

3.5 *Amphora* sp. culture

Isolated *Amphora* sp. was grown in 1 L glass bottles containing 30 ppt sterilized seawater which enriched with F/2 medium at 25°C under 12:12 of the dark: light period and light intensity of 19 -35 μmol photon/m^2/s was provided by cool daylight LED. Ten acrylic plates (de la Vega et al.) were placed inside each culture chamber for diatom attachment (Figure 3).

Figure 3 Amphora sp. growing bottle with 10 acrylic plates for diatom attachment.

3.6 Light intensity determination

The duration of the diatom culture experiment, Pendant temperature/Light data logger 64K, HOBO by Onset UA-002-64 was used to detect light intensity (Figure 4)

Figure 4 Pendant temperature/Light data logger 64K, HOBO by Onset UA-002-64.

3.7 *Amphora* sp. harvesting

Amphora sp. was gently brushed from acrylic plate (Figure 5) into sterile seawater. Then harvested cells were rinsed with phosphate buffered saline solution (PBS) and used for biomass, lipid content, and fatty acid composition investigation.

Figure 5 Amphora sp. attached on acrylic plate.

3.8 *Amphora* sp. biomass determination

Biomass of *Amphora* sp. was determined by *in vivo* chlorophyll analysis, cell counting method (LeGresley & McDermott, 2010) and specific growth rate (SGR) according to Santos-Ballardo et al. (2015) and E. Cointet et al. (2019).

3.8.1 In vivo chlorophyll

In vivo chlorophyll analysis was conducted using handheld fluorometer (Aquafluor®, Turner Designs). Algal medium was used as blank. Chlorophyll extracted from Spinach was used as standard for calibration. Chlorophyll content was calculated according to Equation 1 and is presented in relative fluorescence chlorophyll (AquaFluor® User's Manual).

$$\textit{Relative fluorescence chlorophyll} = A - B \qquad (1)$$

Where A is Relative fluorescence chlorophyll measured from sample

B is relative fluorescence chlorophyll measured from culture medium.

3.8.2 Cell density and specific growth rate (SGR)

Cell counting method used hemocytometer (Figure 6) and optical light microscope to investigate cell density (E. Cointet et al., 2019). Algal cells were

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

calculated as Equation 2. Cell density was used for SGR calculation according to Santos-Ballardo et al. (2015) and E. Cointet et al. (2019) as Equation 3.

$$cells\ /\ cm^2 = \frac{(A)(B)}{C} \qquad (2)$$

Where *A* is cells counted in ml (cell/ml)

B is sampling volume (ml)

C is sampling area (cm^2)

$$\mu = \frac{\ln X_t - \ln X_0}{t} \qquad (3)$$

Where μ is SGR

X_0 is initial biomass

X_t is final biomass

t is time (days)

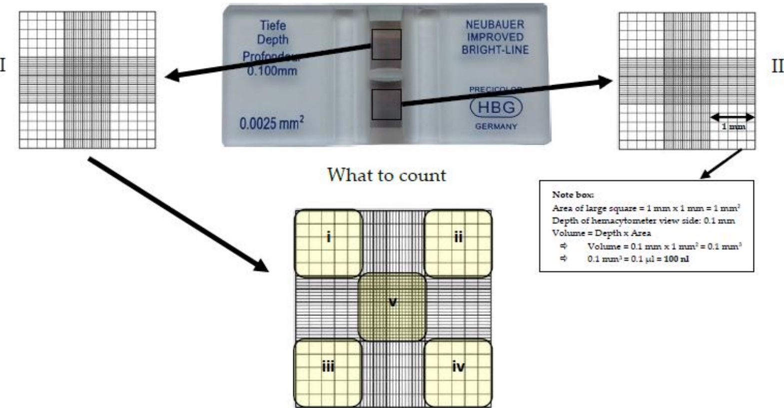


Figure 6 Hemocytometer (Avin, 2020).

3.9 Total lipid content determination

Total lipid extraction was conducted by two-step chloroform/methanol extraction (Kumar et al., 2019; L. Li et al., 2015; Nonwachai et al., 2010) with some modifications. The first step, 25 mg of each dried diatom (*W*) was mixed with 1.5 ml chloroform:methanol solution (2:1 V/V) and vortexed for 2 min. The second step, samples were stored at room temperature for 24 hr. The third step, mixtures were centrifuged at 12,000 rpm for 3 min. The final step, supernatants were transferred

into pre-weighed microcentrifuge tube (W_1). For the second step, the residual dried algae were re-extracted with 1.5 ml of chloroform:methanol (2:1 V/V) and followed by the method as described above. Thus, the supernatant from the second step were combined with the previous one and dried in an oven at 70°C to a constant weight (W_2). Total lipid content of each sample was calculated gravimetrically as Equation 4:

$$Total\ lipid\ content\ (mg./100\ mg.\ algal\ dried\ weight) = \frac{(W_2 - W_1)100}{W} \qquad (4)$$

Where W is dried algae, W_1 is pre-weight microcentrifuge tube weight.

W_2 is constantly weight of microcentrifuge tube weight after drying.

3.10 Fatty acid composition determination

Fatty acid composition determination composed of 2 steps including of preparation of fatty acid methyl esters (FAMEs) and analysis of FAMEs or fatty acid composition analysis (Y.-C. Chen, 2012).

3.10.1 Preparation of FAMEs

The 5 ml of extracted total and neutral lipid were trans-esterification by adding 1 ml of 2 M KOH in methanol, at 25 °C for 15 minutes and lower layer was used for FAMEs analysis (Xiaolong Li et al., 2018).

3.10.2 Analysis of lipid composition (FAMEs analysis)

The 5 µl of FAMEs were injected into gas chromatograph (GC) (Shimadzu GC-2010 Plus; Shimadzu, Kyoto, Japan) with split injection mode for analysis. The GC condition was Framewax (Crossband® Polyethylene glycol) column, 30 m. 0.32 mm I.D., 0.25 µm df and a flame ion detector (FID). Both the injector and detector temperatures were set at 250 °C. The initial column temperature was 140 °C and increased by 4 °C every 5 minutes to a final temperature of 240 °C. The running time was 30 minutes. Helium was used as the carrier gas at a flow rate of 1.73 ml/min. A chromatography data system was used to analyze the resulting peak areas and retention times. The standards of FAMEs are as follows: C10:0, C12:0, C13:0, C14:0, C16:0, C18:0, C18:1, C18:2, C20:0, C20:5 n-3 (EPA), C22:0, C22:6 n-3 (DHA), C24:0 and C26:0 (Sigma, St. Louis, MO); the standard fatty acid mixture was used GLC-461 (Nu-

Chek). All experiments were carried out with three replications (Xiaolong Li et al., 2018).

3.11 Diatom screening for highest growth, total lipid content and fatty acid composition

Four isolations of *Amphora* sp. were grown in 1,000 ml of 30 ppt sterile seawater enriched with F/2 medium at 25°C under 12:12 of dark: light period provided by cool daylight LED for 9 days. Ten pieces of acrylic plate were placed in each experimental unit for the attachment of diatom. Two plates were collected on day 2, 6, 9 and 13 of culture for biomass, total lipid content, and fatty acid composition investigation. The experiment was conducted with triplication (Figure 7).

Figure 7 Experimental unit of diatom culture for highest growth, total lipid content and fatty acid composition

3.12 Data analysis

Biomass as relative fluorescence, cell density, SGR, total lipid content and fatty acids represent the mean ± standard error (mean ± SE) of three replicates. A one-way ANOVA ($p < 0.05$) and Duncan significance test for post hoc analysis was performed to determine the diatoms relative fluorescence, cell density, SGR, total

lipid content, saturated fatty acids and unsaturated fatty acid content of each culture day. Significant differences were indicated at the 0.05 level. IBM SPSS Statistic22 software was used.

4. Results

4.1 Diatom identification

All isolations were identified by light microscope and SEM. The identification results indicated that *Amphora* sp. isolated from four distinct locations likely represent different strains. However, pinpointing the specific species proves challenging due to the similarities in both the diatom valve outline and dimensions. Thus, phylogenic analysis is necessary for species identification. All isolation can be identified by morphological description as member of pennate diatom, Class Bacillariophyceae (raphid diatom). Cells are solitary, usually motile, and almost always lying in girdle view. (Khumaidi et al., 2020; Wang et al., 2014). Each isolation showed as the following.

4.1.1 *Amphora* sp. isolated from Sichang Island (ASC), Chonburi province and *Amphora* sp. isolated from Laemyai (ALY), Rayong province.

Picture from light microscope of these 2 isolations (Figure 8 and 9) showed the similarity of morphology with *Amphora marina*, identified by Wang et al. (2014), especially SEM of ASC that central nodule conspicuously showing (arrow). The valves are small, lunate, and have a convex dorsal margin. The ventral margin has rounded apices and is slightly concave. The median raphe is well-marked and slightly deflected. The marginal ridge is not noticeable. The ventral striae are made up of a single areola. The areolae grow larger as they approach the mid-valve and converge at the ends. Striae on the dorsal side are coarsely punctate and radiate throughout the dorsal side. Striae are broken up by the central area.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

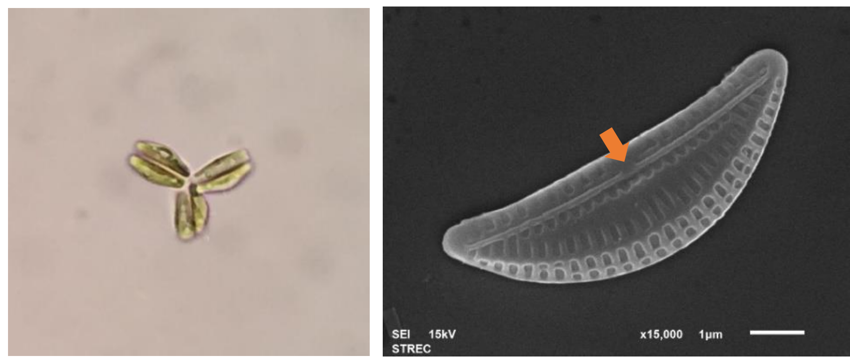


Figure 8 Amphora sp. isolated from Sichang Island identified under light microscope and scanning electron microscope.

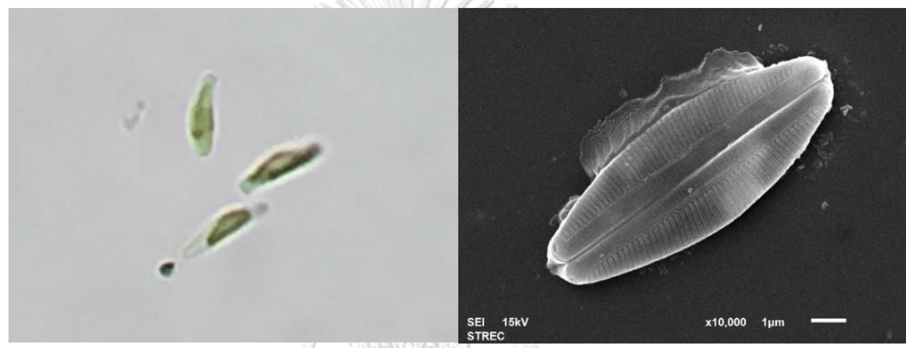


Figure 9 Amphora sp. isolated from Laemyai identified under light microscope and scanning electron microscope.

4.1.2 *Amphora* sp. isolated from Samae Sarn (ASS) Island, Chonburi province.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

This isolation showed the similarity of morphology with *Amphora proteus*, identified by Wang et al. (2014). The frustule is elliptical to lanceolate in shape, with truncated ends. Small and lunate valves with a very convex dorsal margin, a slightly concave ventral margin, and rounded apices. This study's photographed raphes, striae, and central nodule were clearly visible (Figure 10).

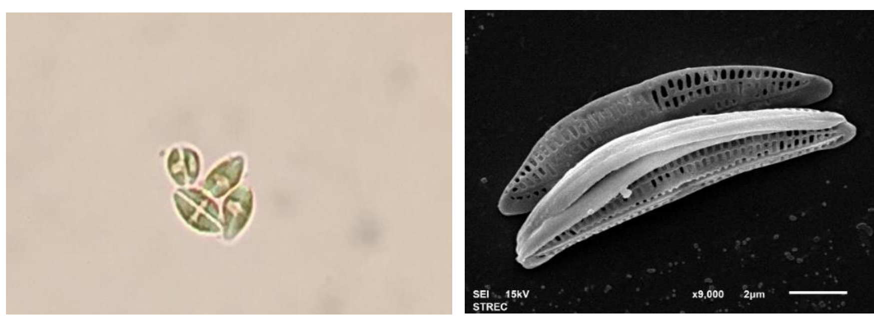


Figure 10 Amphora sp. isolated from Samae Sarn Island identified under light microscope and scanning electron microscope.

4.1.3 *Amphora* sp. isolated from Pranburi (APB), Prachuap Khiri Khan province.

This isolation showed the similarity of morphology with *Halamphora terroris*, identified by Wang et al. (2014). The valves are semi-lanceolate with a convex ventral margin and ventral deflected poles with protracted apices. Raphes are straight and perform along the ventral edge of the body. The raphe's central terminals are slightly bent to the dorsal side and then fold back. The central nodule stands out. Striae are parallel in the center and curved and divergent toward the ends (Figure 11).

Figure 11 Amphora sp. isolated from Pranburi identified under light microscope and scanning electron microscope.

4.2 Biomass

Amphora sp. is a benthic diatom. The growing of *Amphora* sp. can be observed optically as shown in Figure 13.

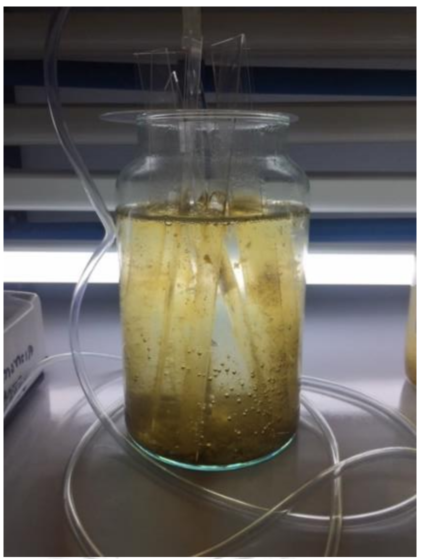


Figure 12 Amphora sp. attached to acrylic plate in glass bottle.

4.2.1 Chlorophyll

The differences of chlorophyll content between all 4 *Amphora* strains were obtained from day 0 to day 13 and present in relative fluorescence unit. Chlorophyll contents of ALY at day 6 (1,464.70 ± 137.94) and ASC at day 6 and day 9 (539.10 ± 69.35, 898.10 ± 231.02, respectively) were significantly higher than that of APB and ASS throughout the experimental period that were range from 0.44 – 197.93 ($p < 0.05$, ANOVA analysis, followed with Duncan). Hence, the chlorophyll peak of ALY was detected on day 6 while that of ASC was detected on day 9. Then, their chlorophyll contents were decreased and remained similar at the end of the experiment (day 13). The chlorophyll contents of APB and ASS slightly and constantly increased throughout the whole experiment but there is no significantly difference during this period, indicating that the highest point of their chlorophyll contents was probably beyond 13 days. These results indicated that ALY was the fastest and highest strain to reach the maximum chlorophyll content followed by ASC and both APB and ASS were the lowest (Figure 14).

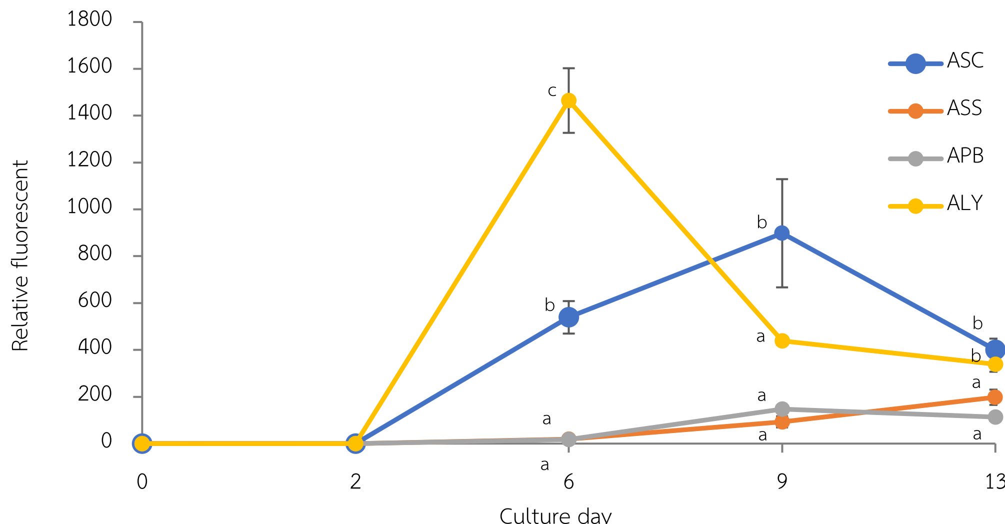


Figure 13 Relative fluorescence of 4 diatom strains determined on day 0, 2, 6, 9 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2.2 Cell density

The differences in cell densities of all 4 strains started to be detectable at day 2 of the experiment. At day 6, cell density of ALY was significantly higher than that of the other strains ($3.75 \times 10^5 \pm 5.43 \times 10^4$ cell/cm^2, $p < 0.05$, ANOVA analysis). After that, it slowly declined until the end of the experiment. Cell density of ASC gradually increased during day 2 ($4.19 \times 10^3 \pm 4.21 \times 10^2$ cell/cm^2) – day 9 ($1.95 \times 10^5 \pm 1.39 \times 10^4$ cell/cm^2) of the experiment and significantly surged at the end of the experiment (day 13: $1.01 \times 10^6 \pm 6.10 \times 10^4$ cell/cm^2). Cell density of APB (Day 2: $5.95 \times 10^3 \pm 1.26 \times 10^3$ – Day 13: $1.51 \times 10^5 \pm 5.13 \times 10^4$ cell/cm^2) and ASS (Day 2: $4.07 \times 10^3 \pm 7.62 \times 10^2$ – Day 13: $9.77 \times 10^4 \pm 2.32 \times 10^4$ cell/cm^2) slowly increased throughout the experiment period, as shown in (Figure 15).

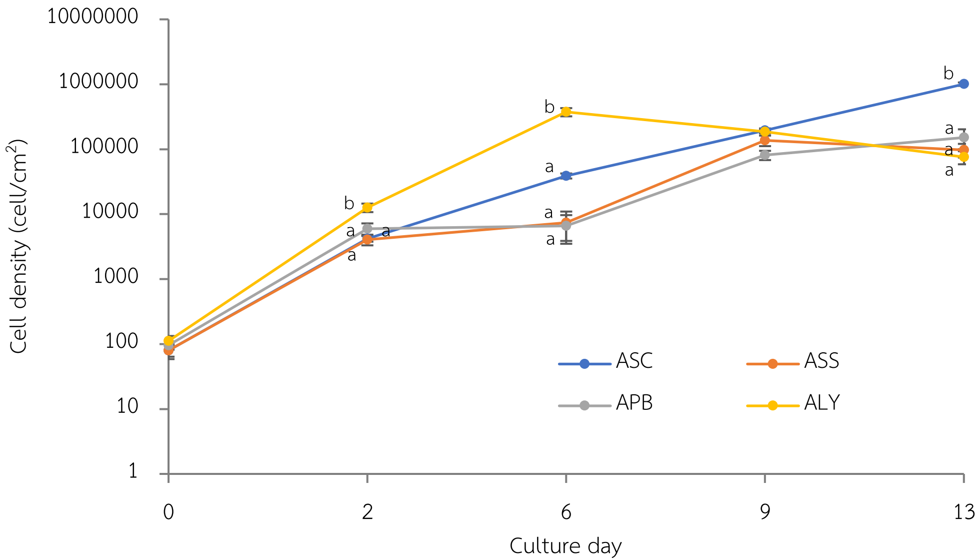


Figure 14 Cell density of 4 diatom strains determined on day 0 – 2, 2 – 6, 6 – 9 and 9 – 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2.3 Specific growth rate (SGR)

SGR was calculated during day 0-2, 2-6, 6-9 and 9–13 of culture period. Each strain appeared to have its own specific growth pattern. ASC entered the exponential phase only on day 0-2 (2.01 ± 0.18 μ/day) and remained in the stationary phase from day 2 to day 13 (0.56 ± 0.01 – 0.41 ± 0.02 μ/day). This strain had the longest stationary phase when compared to the other 3 strains (ASS, APB and ALY). ASS and APB entered the exponential phase on day 0-2, then entered and remained in stationary phase from day 2 to day 9 and reached the death phase on day 9-13. ALY had a shorter stationary phase than the other 3 strains. This strain entered the stationary phase on day 0-2, remained within day 2-6, then entered the death phase from day 6 to day 13 (Figure 16).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

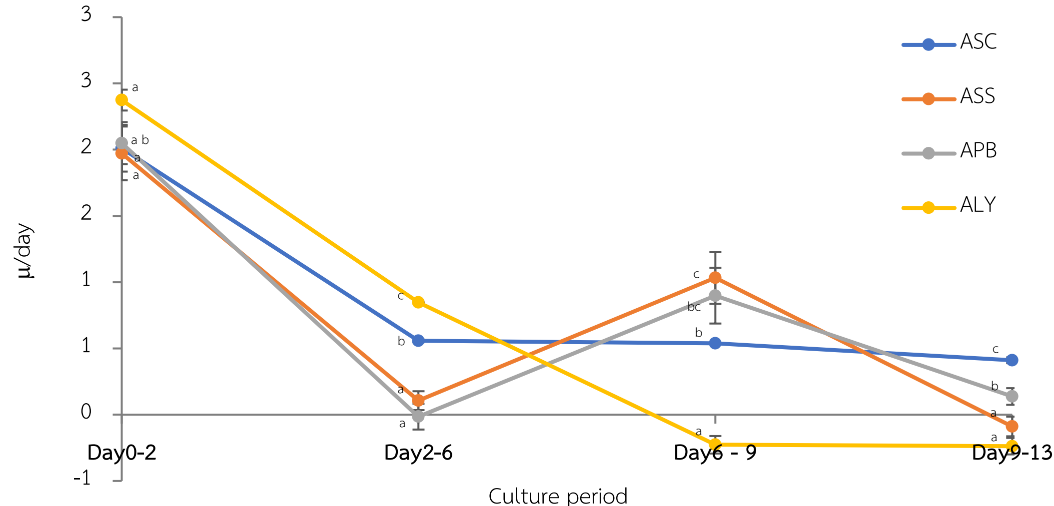


Figure 15 SGR of 4 diatom strains determined on day 0 – 2, 2 – 6, 6 – 9 and 9 – 13 of culture. Mean ± SE values with different lowercase superscripts are significantly (p < 0.05) different between Amphora sp. strain of each culture day.

According to the biomass and SGR results, ASC and ALY strains were chosen for further analysis, including total lipid contents, and fatty acid composition.

4.3 Total lipid content

Diatom samples were collected at the stationary and death phase which was day 6, 9 and 13. From day 6 to day 9, total lipid content of both strains increased slightly, but the total lipid content of ALY at day 6 (63.44 ± 3.36 mg/100 mg algal dried weight) was significantly higher than that of ASC (39.88 ± 4.35 mg/100 mg/algal dried weight) ($p<0.05$, ANOVA analysis). On day 9, when ALY entered death phase while ASC strain remained in the stationary phase, total lipid content of ALY (74.35 ± 2.46 mg/100 mg algal dried weight) was still significantly higher than that of ASC (45.38 ± 4.18 mg/100 mg/algal dried weight) ($p<0.05$, ANOVA analysis). At the last day of the culture (day13), total lipid content of both strains was similar (31.72 ± 4.71 and 36.24 ± 1.27 mg/100 mg algal dried weight, respectively and lower than the previous time (Figure 17).

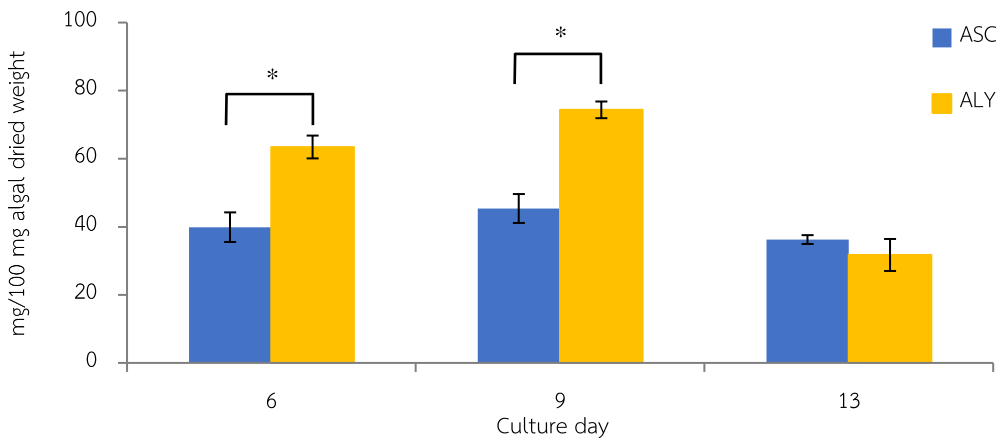


Figure 16 Total lipid content (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.4 Fatty acid composition

4.4.1 Saturated fatty acid (SFA) and unsaturated fatty acid (UFA)

The total amounts of SFAs in both ASC and ALY strains showed a similar pattern in which SFAs were increasing from day 6 to day 9 and declining afterward (Figure 18) while total amounts of UFAs showed the different pattern of alteration. UFAs of ASC strain remain in similar levels from day 6 to day 9 and day 13 while UFAs of ALY strain were increasing from day 6 to day 9 and declining from day 9 to day 13 (Figure 19).

CHULALONGKORN UNIVERSITY

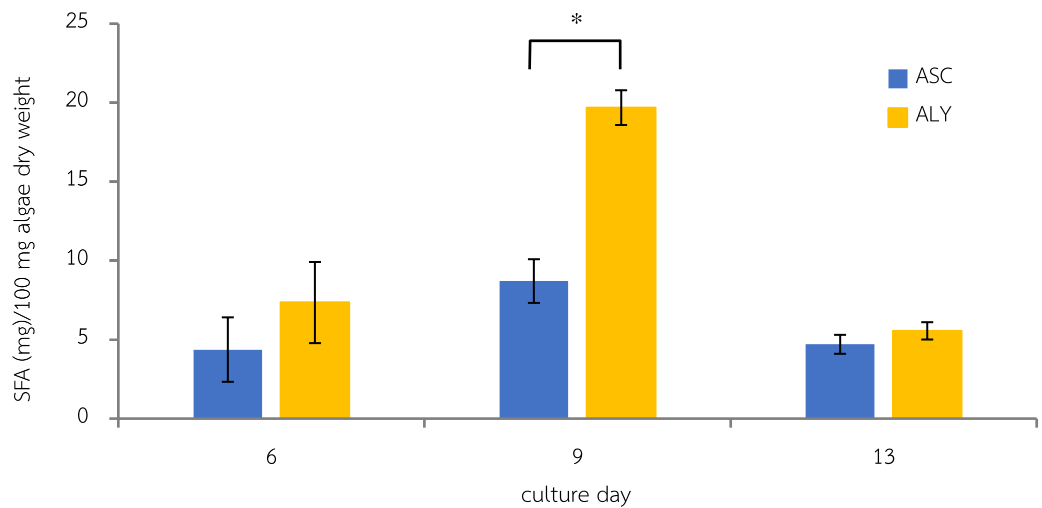


Figure 17 SFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

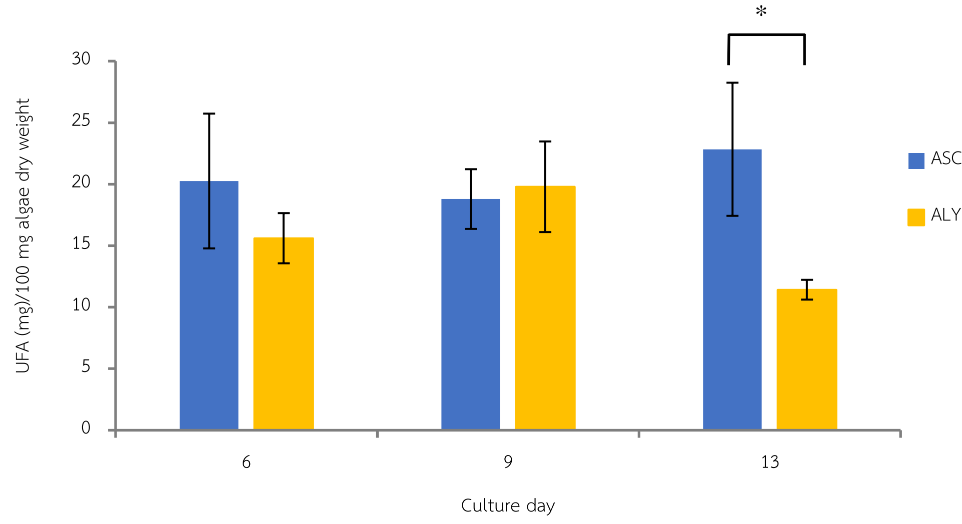


Figure 18 UFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 9 and day 13 of culture. Mean ± SE values with asterisk are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.4.2 Fatty acid composition

It shows that the main SFAs detected from the collected samples are Myristic acid (C14:0) and Palmitic acid (C16:0). Main UFAs detected from the collected samples are Palmitoleic acid (C16:1ω7), Arachidic acid (C20:1ω9), cis-11-docosenoic acid (C22:1ω11), Linoleic acid (C18:2ω6), Eicosapentaenoic acid EPA (C20:5ω3), and Docoxahexaenoic acid DHA (C22:6ω3) as showed in Table 2.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 2 Fatty acid composition (mg/100 mg algal dry weight) determined from 2 strains of Amphora sp. at different time of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different.

Fatty acid	ASC			ALY		
	Day6	Day9	Day13	Day6	Day9	Day13
SFAs						
Myristic acid (C14:0)	1.56 ± 0.56^{a}	4.07 ± 0.64^{b}	2.77 ± 0.62a^{b}	2.86 ± 3.36^{a}	10.58 ± 0.54^{b}	3.15 ± 0.15^{a}

Palmitic acid (C16:0)	5.38 ± 4.10^{a}	0.43 ± 0.10^{a}	0.14 ± 0.04^{a}	12.33 ± 1.58^{b}	1.28 ± 0.15^{a}	2.41 ± 0.64^{ab}
UFAs						
Palmitoleic acid (C16:1ω7)	0.48 ± 0.36^{a}	7.63 ± 0.68^{b}	7.74 ± 1.88^{b}	3.94 ± 5.41^{a}	6.03 ± 3.63^{a}	5.38 ± 0.41^{a}
Oleic acid (C18:1ω9)	1.87 ± 0.50^{a}	1.24 ± 0.34^{a}	1.52 ± 0.46^{a}	1.42 ± 2.88^{a}	1.89 ± 0.34^{a}	0.52 ± 0.21^{a}
Arachidic acid (C20:1ω9)	1.88 ± 0.66^{a}	2.21 ± 0.39^{a}	2.86 ± 0.57^{a}	4.28 ± 1.56^{b}	3.86 ± 1.13^{ab}	0.03 ± 0.03^{a}
Erucic acid (C22:1ω9)	0.36 ± 0.036^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
cis-11-docosenoic acid (C22:1ω11)	6.72 ± 5.36^{a}	0.56 ± 0.10^{a}	0.35 ± 0.07^{a}	7.11 ± 6.78^{a}	1.19 ± 0.29^{a}	1.22 ± 0.39^{a}
Linoleic acid (C18:2ω6)	3.76 ± 2.25^{a}	1.38 ± 0.12^{a}	1.08 ± 0.15^{a}	1.97 ± 1.02^{a}	2.20 ± 0.30^{a}	0.75 ± 0.08^{a}
Stearidonic acid (C18:4ω3)	0.28 ± 0.15^{a}	0.96 ± 0.96^{ab}	2.98 ± 0.66^{b}	0.37 ± 0.19^{a}	0.00 ± 0.00^{a}	0.01 ± 0.01^{a}
Eicosapentaenoic acid EPA (C20:5ω3)	1.37 ± 0.69^{a}	2.56 ± 0.39^{a}	4.11 ± 1.18^{a}	2.42 ± 0.43^{a}	2.38 ± 1.77^{a}	2.33 ± 0.30^{a}
Docosapentaenoic acid DPA (C22:5ω3)	2.91 ± 1.37^{a}	0.25 ± 0.25^{a}	0.18 ± 0.18^{a}	2.61 ± 1.50^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Docoxahexaenoic acid DHA (C22:6ω3)	16.14 ± 6.91^{b}	1.63 ± 0.61^{a}	1.00 ± 0.74^{a}	9.02 ± 5.53^{a}	1.76 ± 1.76^{a}	1.09 ± 0.84^{a}

5. Discussion

5.1 Biomass and growth of *Amphora* sp.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Maximizing biomass yield is always the main purpose of microalgae culture and choice of mediums is primarily considered the essential component. Presently, mediums such as F/2 medium (Guillard's), Diatom artificial medium, Walne's medium are increasingly acceptable and frequently used for *Amphora* sp. culture (Khwancharoen et al., 2021; Rani et al., 2022; Romero-Romero & Sánchez-Saavedra, 2017). Therefore, in this study, F/2 medium were used in the culture of all 4 tested strains of *Amphora* sp. The average cell density and generation time obtained from this study was in close proximity to the results of the previous reports (Khwancharoen et al., 2021; Rani et al., 2022; Romero-Romero & Sánchez-Saavedra, 2017). Therefore, the culture condition in this study was acceptable (Table 3).

Table 3 Growth and biomass of microalgae cultured in F/2 medium.

Diatom strain	SGR (μ/day)	Cell density (cell/cm^2)	Chlorophyll	Reach the stationary phase within (days)	Media
Amphora sp. ALY (This study)	2.37±0.08	$3.75 \times 10^5 \pm 5.41 \times 10^4$	1464.70±6.25	6	F/2 medium
Amphora sp. ASC (This study)	2.01±0.18	$1.01 \times 10^6 \pm 5.96 \times 10^4$	898.10±62.71	>13	F/2 medium
Amphora sp. APB (This study)	2.05±0.16	$1.51 \times 10^5 \pm 5.11 \times 10^4$	147.30±6.72	>13	F/2 medium
Amphora sp. ASS (This study)	1.97±0.20	$9.77 \times 10^4 \pm 2.32 \times 10^4$	197.93±32.63	>13	F/2 medium
Amphora sp. (Rani et al., 2022)		1.2×10^6			
Amphora sp. (Romero-Romero & Sánchez-Saavedra, 2017)	0.61 ± 0.02	$1.29 \pm 0.06 \times 10^6$		4-5	F/2 medium
A. coffeaeformis (Khwancharoen et al., 2021)				6	F/2 medium
Chaetoceros gracilis (Saxena et al., 2022)		$3.16 \times 10^7 \pm 0.4$			Modified F/2 medium, replaced silica form
Thalassiosira weissflogii (Saxena et al., 2022)		$3.24 \times 10^7 \pm 0.05$			Modified F/2 medium, replaced silica form
Navicula clavate (Rani et al., 2022)		1.3×10^6			F/2 medium
Cylindrotheca fusiformis (Rani et al., 2022)		6.91×10^6			F/2 medium
Green algae *Nannochloropsis salina* (Rani et al., 2022)		12.5×10^6			F/2 medium
Green algae *Chlorella* sp. (Rani et al., 2022)		11.28×10^6			F/2 medium

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

In order to assess the potential of this diatom for commercial culture, the reliability, biomass and lipid productivities of 4 strains of *Amphora* sp. are considered under typical conditions outdoor. The best performance in such criteria can be evaluated for further apply in large-scale economical commercial cultivation.

Determination of biomass is a prerequisite for monitoring growth performance of microalgae. Several relevant parameters for assessing microalgal biomass have been used, however, cell density and chlorophyll quantity have increasingly been employed and proven to be ideal indicators for monitoring biomass of diatoms (E. Cointet et al., 2019; Eva Cointet et al., 2019). Therefore, in this study, cell density and chlorophyll content in all 4 tested strains of *Amphora* sp. were determined. The result clearly shows that ALY achieved the best performance with biomass production, followed by ASC, ASS and APB, respectively.

ALY reached the maximum cell density within 6 days when cell density of ALY was 10 times higher than that of ASC and about 51 and 57 times higher than that of ASS and APB, respectively. However, cell density of ALY dropped quickly to the death phase within 9 days while that of ASC increased in correlate with the culture time. Therefore, cell density of ASC at the end of experiment was significantly higher than that of ALY. On the other hand, it can be inferred that ASC requires longer period (13 days) of culture to achieve the same amounts of the biomass of ALY strain (6 days). Since high biomass and high productivity are ones of criteria that must be considered for the candidate species (E. Cointet et al., 2019; Reitan et al., 2021), ALY could be considered as the best productivity strain due to it spent 6 days of culture to reach the maximum growth rate and was the fastest growth strain compared to the other 3 strains.

In addition, chlorophyll content of ALY peaked at day 6 of culture and was significantly higher than the other three strains: the amounts in ALY are 2 times higher than that ASC and 10 and 7 folds higher than APB and ASS, respectively. When the maximum amounts of chlorophyll content and cell density are considered, it shows that peaks of chlorophyll levels and cell density occurred at the same period and found in ALY, ASS and APB *Amphora*. These results indicate that biomass of these 3 *Amphora* sp. strains was correlating with their chlorophyll contents. Chlorophyll

content of ASC, on the other hand, reached the maximum level within 9 days but decreased dramatically while its cell density remained unchanged (day 13). This is probably due to the longer period of culture causing the depletion of some essential nutrients which can be commonly found in various species of microalgae cultured in controlled condition. There were numbers of evidence reported earlier that, under various stress conditions, microalgae inevitably adjusted their metabolic activities to adapt to new environments. This process was also species specific and culture condition dependent (Carvalho et al., 2009; Encarnação et al., 2012). Therefore, it can explain why the performance in cell density and chlorophyll contents in ALY and ASC are different in which the former showed decline in both cell density and chlorophyll contents while the latter has dropped only in chlorophyll contents.

The chemical content of microalgae can vary with culture time and conditions (Carvalho et al., 2009). Chlorophyll is an important pigment for photosynthesis which is the most important source of energy for algae growth. From the literatures, chlorophyll concentrations often dropped from the exponential to the stationary growth phase in microalgae culture suffered from long culture causing nutrient starvation (Lim et al., 2017; Ruivo et al., 2011; Young et al., 2022). Abiotic stress factors such as temperature, CO_2, light, salinity can significantly affect the biochemical composition of algal cells including protein concentration, lipid production and photosynthetic components (C. Y. Chen et al., 2011; da Silva Ferreira & Sant'Anna, 2017; He et al., 2015). Growth under nutrient limitation also generates variations of effects on biochemical contents of microalgae. For example, nitrogen starvation affected on chlorophyll content of microalgae, *C. vulgaris*, *S. dimorphus* (Ferreira et al., 2016; Lv et al., 2010). Phosphorus and iron reduction affected on chlorophyll content of *C. pyrenoidosa* (Fan et al., 2014). Carbohydrate and protein contents of *Amphora* sp. were also influenced by environmental conditions and enrichment medium (de la Peña, 2007).

Microalgae accumulate large amounts of lipid bodies containing triacylglycerides under adverse conditions, such as during nutrient deprivation (Hu et al., 2008; K. K. Sharma et al., 2012). Under these circumstances, microalgae stop dividing but are still able to perform photosynthesis and the accumulation of

triacylglycerides which is considered a survival strategy to endure adverse conditions (Breuer et al., 2012; Liu & Benning, 2013; Schenk et al., 2008).

The result herein indicate that ASC had the longest stationary phase (>11 days) which can be cultured for a longer period of time and their biochemical contents (in this study, chlorophyll) can be processed with no significant impact on their growth rate for such period of time. The activity to tolerate nutrient exhaustion of the long-term culture and the ability to adjust biochemical composition with no obvious effect on the growth are considered the desirable characteristics of potential microalgae for commercial biomass production. The ability of microalgae to adjust their metabolic activity to different culture conditions provides a wide range of opportunities for biotechnological applications (Bumbak et al., 2011; Mohammad Mirzaie et al., 2016). However, the downside of ASC is that it is a slow-growing algae when compared to ALY. SGR of ALY was significantly better than that of ASC indicating a better growth rate of ALY over ASC. ALY can produce the maximum biomass in 6 days while it takes ASC almost twice as long to deliver the same amount of biomass that ALY can produce. Therefore, in this study, ALY is considered the best candidate for biomass production among 4 tested strains of Amphora sp. while ASC provides moderate growth rate but possesses a better cell viability which is a crucial property for maintaining culture.

จุฬาลงกรณ์มหาวิทยาลัย

5.2 Total lipid content

Chulalongkorn University

It was obvious that the maximum biomass of ALY and ASC was significantly higher than the other *Amphora* sp. strains in this study. Therefore, the determination of lipid composition was focused only on these 2 strains.

As observed from the result, total lipid contents in ALY in day-6 and day-9 culture were significantly higher (1.6 folds) than those in ASC, however the levels of lipids in ASC remained the same throughout the experiment. The specific increase of lipid content observed in ALY might be the result from response in the nutrient depletion.

There are increasing numbers of studies confirming that increase of lipid in microalgae during culture depended on depletion or removal of nutrients (Eva

Cointet et al., 2019; d'Ippolito et al., 2015; E. T. Yu et al., 2009). Study on diatom, *Phaeodactylum tricornutum*, grew in nitrate deprivation culture resulted in accumulation of lipid by 60% over 3 days (Burrows et al., 2012). Numbers of further studies also indicated that various diatoms were able to produce lipid up to 40 – 60% of dry biomass (Y.-C. Chen, 2012; Sayanova et al., 2017; Xue et al., 2017; Zulu et al., 2018).

In this study, the total lipid content in ALY found at its peak is greater than 70% of dried weight that was reported as the highest amounts in documents of diatoms, while that of ASC is about 45% of dried weight which is in close proximity to the results of other studies (Table 4)

Table 4 Lipid and fatty acid content determined in diatom.

Diatom strain	*Amphora sp.* ALY	*Amphora sp.* ASC	*Chaetoceros* sp. (Saxena et al., 2021)	*Thalassiosira* sp. (Saxena et al., 2021)	*C. gracilis* (Saxena et al., 2022)	*T. weissflogii* (Saxena et al., 2022)	Requirement for *L.vannamei* (Zhu et al., 2023)
Lipid content (mg/100 mg algal dried weight)	74.34±2.46	45.38±4.18	44.33±2.5	29.66±1.52	57.6±0.4	50.4±0.3	-
UFAs (mg/100 mg algal dried weight)	35.86±10.80	35.09± 8.00			-	-	-
ω3 content (mg/100 mg algal dried weight)	20.69±7.50	14.42±7.09	48.28	39.46	-	-	0.6%
ω6 content (mg/100 mg algal dried weight)	3.76±2.25	2.20±0.30	0	1.92	-	-	1.8
ω3/6 ratio	9.71±6.24	4.74 ± 1.25	48.28	20.06	-	-	
DHA (mg/100 mg algal dried weight)	9.02±5.53	16.14±6.91	12.47	5.82	-	-	0.5%
EPA (mg/100 mg algal dried weight)	2.42±0.43	3.76±2.25	13.07	3.47	-	-	0.1%
LA (mg/100 mg algal dried weight)	2.20 ± 0.30	4.85 ± 3.41	0	1.92	-	-	0.04%
DHA/EPA ratio	3.22±2.27	3.45±2.12			-	-	-

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

However, culturing for longer period results in the decrease of lipid content observed in ALY and similar result has been reported in *Amphora* sp. culture by (Paliwal et al.); Romero-Romero andSánchez-Saavedra (2017). This adverse response can be explained by the cell facing with the unavailable of nutrient. Decreasing total lipid content can happen in stressful environmental conditions such as continuous lack of nutrient or starvation condition. Under this stressful the storage lipids would be consumed, and detected in the reduction of chlorophyll, cell density and growth rate (D' Alessandro and Filho, 2016). In addition, lipid content as a storage

component in lipid droplets in diatom cells can be degraded during starvation as cell recovery (Leyland et al., 2020).

Since ALY and ASC possessed different characteristics of lipid production in which ALY requires 6 days to produce maximum levels of total lipid content while ASC needs 9 days of culture. ALY will be the best candidate for lipid production considered by higher yield and harvesting batches. Additionally, ALY also showed greater potential for lipid production when compared to other diatoms reported earlier such as *Chaetoceros* sp., *Chaetoceros* gracilis, *Thalassiosira* sp., and *Thalassiosira* weissflogii (Saxena et al., 2021; Saxena et al., 2022) (Table 3). On the contrary, ASC appeared to be inferior to the lipid production but the consistent production of lipid under long and stressful conditions e.g. nutrient deprivation renders ASC as optional candidate for culturing in extended culture period or specific purpose biomass production.

5.3 Fatty acid composition

In ALY and ASC, proportions of UFA are higher than those of SFA throughout the experiment. Similar to the result of total lipid content, both SFA and UFA levels of ALY increased, reached the peak, and decreased at the end of the experiment while those of ASC remained relatively the same level throughout the culture period. As expected, the increase of UFA and decrease of SFA by the age of the culture from both strains indicated the response of microalgae to cope with stress during nutrient depletion period which was the result of the negative correlation between UFAs and nitrogen starvation. Reduction of TAG synthesis in diatom cell and increase of polar lipid which mainly PUFAs (Paliwal et al., 2017). The major UFA mostly found in this study were n-3 PUFAs which were DHA, DPA, EPA and Stearidonic acid (C18:4ω3) while the minor UFA was Linoleic acid . UFA was higher than SFA in both strains and the same proportion was found throughout the experiment. Both UFA and SFA in ALY increased at the beginning and dropped at the end of the experiment while that of ASC remained constant throughout the experiment. Fatty acid profiles found in ALY and ASC were similar to the results of previous studies on diatoms. The major UFA mostly found in this study were n-3 PUFAs which were DHA, DPA, EPA, and

Stearidonic acid while the minor UFA was Linoleic acid (LA). Regardless of the increasing of EPA and decreasing of DHA found in both strains following the time of the culture, the contents of EPA and DHA from both strains were only slightly changed.

Most fatty acids in diatoms vary from C14:0 to C22:6. The most common fatty acids depend on strains and culturing day. SFAs show similar pattern in both ALY and ASC in which myristic acid (Ozogul et al.) peaks at day 9 of culture while palmitic acid (C16:0) reaches early at day 6. For the UFAs, fatty acid profiles ALY and ASC share similarity in which cis-11-docosenoic acid (C22:1ω11) and DHA are two major forms and peaks at day 6 of culture while EPA has been found in range of 1.37-4.11 mg throughout the experimental period. Levels of palmitoleic acid and arachidic acid in day 6 of ALY culture are higher than those in day 6 of ASC culture.

Also, the proportion of EPA in both strains increased according to the age of the culture while DHA were high at the beginning and declined to the lowest value at the end of the culture. Nonetheless, the contents of EPA and DHA from both strains were only slightly changed. The differences between the 2 strains shown in this study were the DPA and erucic acid contents. DPA from ASC started with high values and then declined in correlate with the culture period while that of ALY was undetectable after 6 days. Urecic acid was also found in very small amounts and undetectable from culture of both strains within 9 days. When compared to other diatoms, DHA content of ASC and ALY were superior to that of reported *Amphora* sp. and most other diatoms while EPA content of both ALY and ASC were inferior to that of other diatoms (Bélanger-Lamonde et al., 2018).

The high DHA content found in ASC could be useful for DHA enrichment in live feed such as copepods (Reitan et al., 2021) or an alternative source of DHA to some fish oils which contain high DHA content (e.g., Tuna oil) (Fard et al., 2020; Sprague et al., 2015). Omega 3 (ω-3) and omega 6 (ω-6) fatty acids are PUFAs essential for the body to function properly, establishing normal growth and development through all stages of life. Both ω-3 and ω-6 fatty acids cannot be synthesized in the body; therefore they are only supplied through food intake (An et

al., 2020; Araújo et al., 2019; González-Félix et al., 2002; Turchini et al., 2010; Zhang et al., 2022; Zhu et al., 2023).

When compared to other microalgae, ω-3 levels in both ALY and ASC were higher than the other previously reported in *Amphora* sp. but still lower than some other diatoms (*Chaetoceros* sp. and *Thalassiosira* sp). In contrast, ω-6 content of both strains was much lower than the other *Amphora* sp. but clearly surpassed the other diatoms. The activities of ω-3 and ω-6 carry out in opposite physiological functions, therefore imbalance in amounts and ratio of these fatty acids can cause detrimental health effects. For this reason, adequate amounts and optimal ratio of these fatty acids in the diet are crucial.

Moreover, the ω-6/ω-3 ratio of ALY and ASC are at 0.18 and 0.15, respectively, that are in similar range of ω-6/ω-3 ratio commonly found in most microalgae and marine organisms. These ratios are considered as ideal quality of lipid ingredient in aquaculture feed industry as such low ω-6/ω-3 ratios, it can be easily optimized into feed formulations which is desirable as fish meal or fish oil replacement. A number of studies in various animals, mainly in humans, indicated that the ideal ω-6/ω-3 ratio of the diet should be kept as low as 1:1 or 2:1 (AFSSA, 2010; Candela et al., 2011). It was reported by many studies that high ω-6/ω-3 ratio (too much ω-6 ratio and too little ω-3) was considered an unhealthy ratio and potentially caused high risk of many non-communicable diseases (Gayoso et al., 2019; Grootveld et al., 2018; Katiyar & Arora, 2020; L. Li et al., 2015; Zong et al., 2016).

In aquatic organisms, proper ω-6/ω-3 ratio of the diet varies depending on species and their environments. Therefore, lipid content of the diet is generally adjusted to the proximity of the one found in each target animal. It has been suggested that ω-6/ω-3 ratio was used as indicators to match the appropriate nutritional values of the diets with the cultivated animal (Katan et al., 2019; Piggott & Tucker, 1990; Simopoulos, 2003).

In marine animals, ω-6/ω-3 ratio varied vastly, ranging from 0.02 in European squid to 0.48 in striped piggy (DurmuŞ, 2019; Ozogul et al., 2009). In shrimp from

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

deep water (*Aristeus antennatus*, *Aristeomorpha foliacea*, *Plesionica martia*, *Parapenaeus longirostris*, and *Plesionica edwardsi*) and shallow water (*Metapenaeus monoceros*, *Penaeus semisulcatus*, *Penaeus kerathurus* and *Penaeus japonicus*), average ω-6/ω-3 ratio varied between 0.2 and 0.7 (Yerlikaya et al., 2013). In 4 strains of domesticated *L. vannamei*, it ranged between 1.14 and 2.34 (X. Li et al., 2021).

In microalgae, the ratio varied from 0.04 in Dinophyta to 0.40 in Cyanobacteria. The average ω-6/ω-3 ratio of diatoms was 0.06 (Jonasdottir, 2019). In this study, ω-6/ω-3 ratio of ALY and ASC were 0.18 and 0.15, respectively, which were within the range of ω-6/ω-3 ratio commonly found in most diatoms and marine organisms.

6. Conclusion

According to their fatty acid profiles, ALY and ASC would be considered as n-3 PUFAs producing diatoms. When their growth performances are considered, ALY and ASC are also showing the highest biomass production when compared to the other 2 strains. ALY is considered the best candidate for biomass production in shorter period while ASC requires longer time, but it is more tolerant to nutrient depletion than the other strains. In summary, this recent study provides the basic knowledge for biomass production of *Amphora* sp. that isolated from Gulf of Thailand that were cultured under the indoor condition. Moreover, two potential strains were analyzed for lipid contents and fatty acid profiles, and it reveals that n-3 PUFAs are highly produced in these diatoms, suggesting that ALY and ASC could be considered for further application (Table 5).

Table 5 The advantage characteristic of each Amphora sp. strain

Amphora sp. strain	Biomass	Shorter period of entering stationary phase	Total lipid content	SFA	USA	Important Fatty acid
ASC	+ +	+ +	+ + +	+ + +	+ + +	+ + +

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

ASS	+	+	+	+	+	+
APB	+	+	+	+	+	+
ALY	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Chapter III

The optimum light intensity and culture medium concentration for *Amphora* sp. cultivation

1. Introduction

Amphora sp. plays a crucial role in larviculture culture for species such as shrimp and abalone, as highlighted in studies by Khwancharoen et al. (2021). This diatom genus, *Amphora* sp., has demonstrated the ability to achieve a lipid content of up to 45% dry weight, as reported by El Arroussi et al. (2017), Boukhris et al. (2017), C. Y. Chen et al. (2011), Sayanova et al. (2017), and BenMoussa-Dahmen et al. (2016). The fatty acid composition of *Amphora* sp. includes saturated fatty acids (SFAs) ranging from 36% to 45% and unsaturated fatty acids (UFAs) ranging from 2% to 52%, as observed in studies by El Arroussi et al. (2017), Boukhris et al. (2017), C. Y. Chen et al. (2011), Sayanova et al. (2017), and BenMoussa-Dahmen et al. (2016).

In the realm of algal culture, physiological techniques have been employed to precisely regulate the desired biochemical composition of target algae. This approach, as discussed by Zienkiewicz et al. (2016); Zulu et al. (2018), involves manipulating various factors such as culture mode, carbon sources and concentrations, nutrient limitation, light intensity, temperature, salinity, alkalinity, pH, and dehydration. These methods contribute to the effective cultivation of *Amphora* sp. for optimal larviculture conditions.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2. Objective

This study is purposed to investigate the optimum condition including of 3 light intensity levels and 2 medium concentrations for chosen *Amphora* sp. which is ASC and ALY.

3. Materials and methods

3.1. Diatom screening for highest growth, total lipid content and fatty acid composition

Two selected *Amphora* sp. from previous experiment including ASC and ALY were grown in 1,000 ml of 30 ppt sterile seawater. Each strain was grown under 3 light intensities and 2 levels of medium concentrations. Ten pieces of acrylic plate were placed in each experimental unit for the attachment of diatom. Two plates were collected on day 0, 3, 6, 10 and 13 of culture for biomass, total lipid content, and fatty acid composition investigation. The experiment was conducted with triplication (Figure 20).

Figure 19 Experimental unit

3.2. Light intensity determination

The 3 levels of light intensity including high Light Intensity; HLI (124.48 ± 1.21 μmol photon/m^2/s), medium light intensity; MLI (77.09 ± 9.03 μmol photon/m^2/s), and low light intensity (12.87 ± 5.64 μmol photon/m^2/s) were conducted separately

in each culture shelf. The highest shelf, middle shelf, and lowest shelf were set for HLI, MLI, and LLI as shown in Figure 20. Light intensity was measured using Pendant temperature/Light data logger 64K, HOBO by Onset UA-002-64 was used to detect light intensity (Figure 4).

3.3. Culture medium preparation

The 2 levels of medium concentrations including F/2 and F/20 were prepared by the method mentioned above in Table 1 Chapter II. F/20 medium was prepared by 10x dilution of F/20 medium. The experimental unit was set up as shown in Figure 20, each light intensity level composed of 2 medium concentrations.

3.4. *Amphora* sp. harvesting

The harvesting of *Amphora* sp. was done as the method mentioned above in 3.7, Chapter II.

3.5. Biomass, lipid content and fatty acid composition determination

Biomass of ASC and ALY were determined by *in vivo* chlorophyll analysis, cell density and SGR follow as the method mentioned in 3.8.1 and 3.8.2, Chapter II. Total lipid content and fatty acid composition were measured followed as the method mentioned in 3.9 and 3.10, Chapter II.

3.6. Data analysis

Biomass as relative fluorescence, cell density, SGR, total lipid content and fatty acids represent the mean ± standard error (mean ± SE) of three replicates. A one-way ANOVA ($p < 0.05$) and Duncan significance test for post hoc analysis was performed to determine the diatoms relative fluorescence, cell density, SGR, total lipid content, saturated fatty acids and unsaturated fatty acid content of each culture day. Significant differences were indicated at the 0.05 level. IBM SPSS Statistic22 software was used.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4. Results

4.1 Biomass

4.1.1 Chlorophyll

The differences of chlorophyll content between culture condition were obtained from day 0 to day 13 and present in relative fluorescence unit. Chlorophyll contents of ASC cultured with HLI-F/2, MLI-F/2, and LLI-F/2 were significantly highest compared to other culture condition at day 6, 10, and 13, But not significantly different with ALY culture with LLI-F/2 at day 6. ALY cultured with LLI-F/2 and F/20 were the condition that provided the following high chlorophyll content compared to the rest culture conditions ($p < 0.05$, ANOVA analysis, followed with Duncan). In addition, results indicated that both ASC culture with MLI-F/2 and ALY culture with LLI-F/2 reached the maximum chlorophyll content at day 10 of culture. After day 10 of culture, chlorophyll of all culture conditions were decreased (Figure 21).

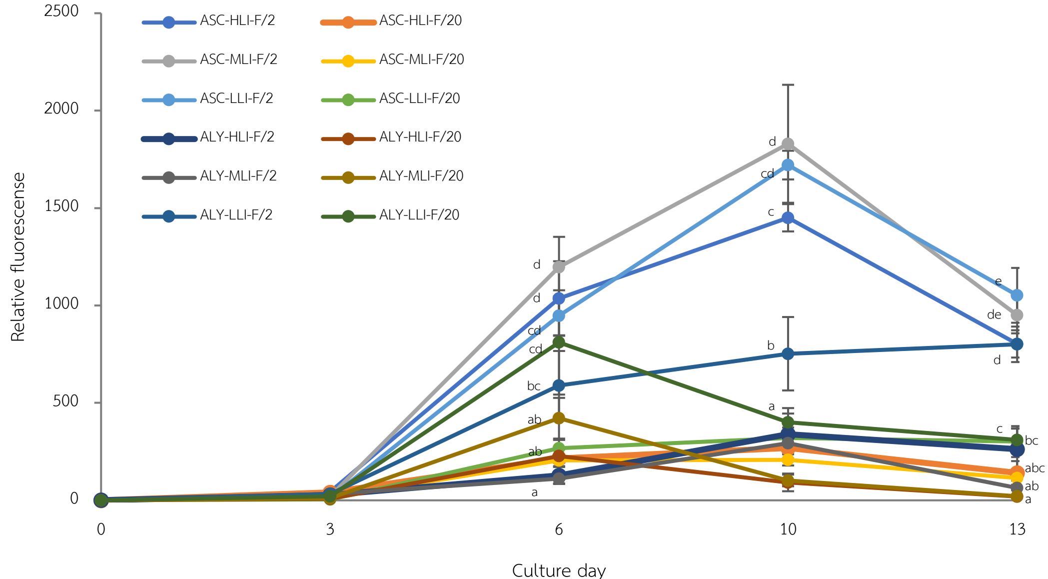


Figure 20 Relative fluorescence of 2 diatom strains determined on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.1.2 Cell density

The differences of cell density between culture condition were obtained from day 0 to day 13 as showed in Figure 22. The maximum cell density was found by ALY cultured with MLI-F/20 at day 6 ($2.66 \times 10^5 \pm 6.02 \times 10^4$ cell/cm^2). This cell density was significantly highest than other culture conditions in the same culture day ($p < 0.05$, ANOVA analysis, followed with Duncan).

The difference between culture conditions was found since day 3 of culture. On this day cell density of ALY cultured with LLI-F/2 ($9.61 \times 10^4 \pm 1.62 \times 10^4$ cell/cm^2) was significantly highest compared to other culture condition at day 3, followed by ALY cultured with HLI-F/2 ($3.55 \times 10^4 \pm 1.09 \times 10^4$ cell/cm^2) which was not significantly different with ALY cultured with MLI-F/2 ($3.31 \times 10^4 \pm 1.15 \times 10^4$ cell/cm^2), ASC cultured with HLI-F/2 ($2.30 \times 10^4 \pm 4.95 \times 10^3$ cell/cm^2) and ASC cultured with HLI-F/20 ($1.78 \times 10^4 \pm 6.22 \times 10^2$ cell/cm^2) ($p < 0.05$, ANOVA analysis, followed with Duncan).

At day 6 of culture, cell density of ALY cultured with MLI-F/20 ($2.66 \times 10^5 \pm 6.02 \times 10^4$ cell/cm^2) was significantly highest ($p < 0.05$, ANOVA analysis, followed with Duncan) compared to other culture condition, followed by ALY cultured with HLI-F/20 ($2.00 \times 10^5 \pm 1.15 \times 10^4$ cell/cm^2), ASC cultured with MLI-F/2 ($1.68 \times 10^5 \pm 2.92 \times 10^4$ cell/cm^2) and HLI-F/2 ($1.53 \times 10^5 \pm 1.24 \times 10^4$ cell/cm^2).

At day 10 of culture, cell density of ASC cultured with HLI, MLI, and LLI in F/2 and HLI and MLI culture in F/20 were higher than that of ALY. Cell density of ASC cultured with HLI-F/2 ($1.33 \times 10^5 \pm 1.65 \times 10^4$ cell/cm^2) and LLI-F/2 ($1.23 \times 10^5 \pm 5.08 \times 10^3$ cell/cm^2) were significantly highest than other culture condition, followed by ASC cultured with MLI-F/2 ($7.85 \times 10^4 \pm 1.59 \times 10^4$ cell/cm^2) and ACS cultured with HLI-F/20 ($6.41 \times 10^4 \pm 4.14 \times 10^3$ cell/cm^2), and MLI-F/20 ($4.66 \times 10^4 \pm 4.15 \times 10^3$ cell/cm^2).

At day 13 of culture, cell density of all culture condition was decreased except ASC cultured with MLI-F/2 that increased from $7.85 \times 10^4 \pm 1.59 \times 10^4$ cell/cm^2 at day 10 to be $1.06 \times 10^5 \pm 8.28 \times 10^3$ cell/cm^2 at day 13.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

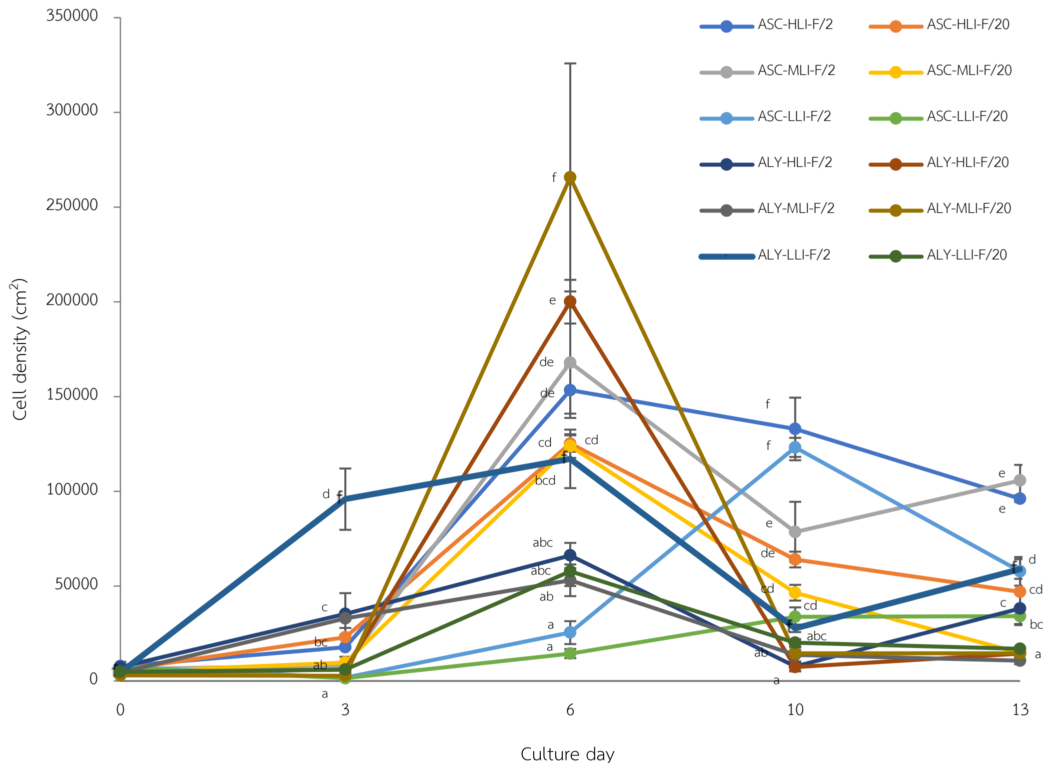


Figure 21 Cell density of 2 diatom strains determined on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.1.3 Specific growth rate (SGR)

SGR investigated during day 0 – 3, 3 – 6, 6 – 10, and 10 – 13 showed in Figure 23. ALY cultured in F/2 medium with every level of light intensity entered exponential phase during day 10 – 13 which is later that ALY cultured in F/20 with every light intensity and all culture condition for ASC. For ALY cultured in F/20 with every light intensity and all culture condition of ASC culture, these entered exponential phase during day 0 – 3, stationary phase during day 3 – 6, slightly entering death during day 6 – 10, and completely entering death phase during day 10 – 13.

During stationary phase, day 3 – 6 of culture. Aly cultured with MLI-F/20 performed the significantly highest (1.51 ± 0.07) compared to other culture condition, followed by ALY cultured with HLI-F/20 (1.20 ± 0.13) and ASC cultured with MLI-F/2

(1.00 ± 0.06) and MLI-F/20 (0.90 ± 0.15). These results indicated that ALY is related with alteration of light intensity, but ASC related with nutrient concentration. Afterward, ASC and ALY cultured with these of culture condition slightly entered death phase, even though the result of chlorophyll content and cell density was still high.

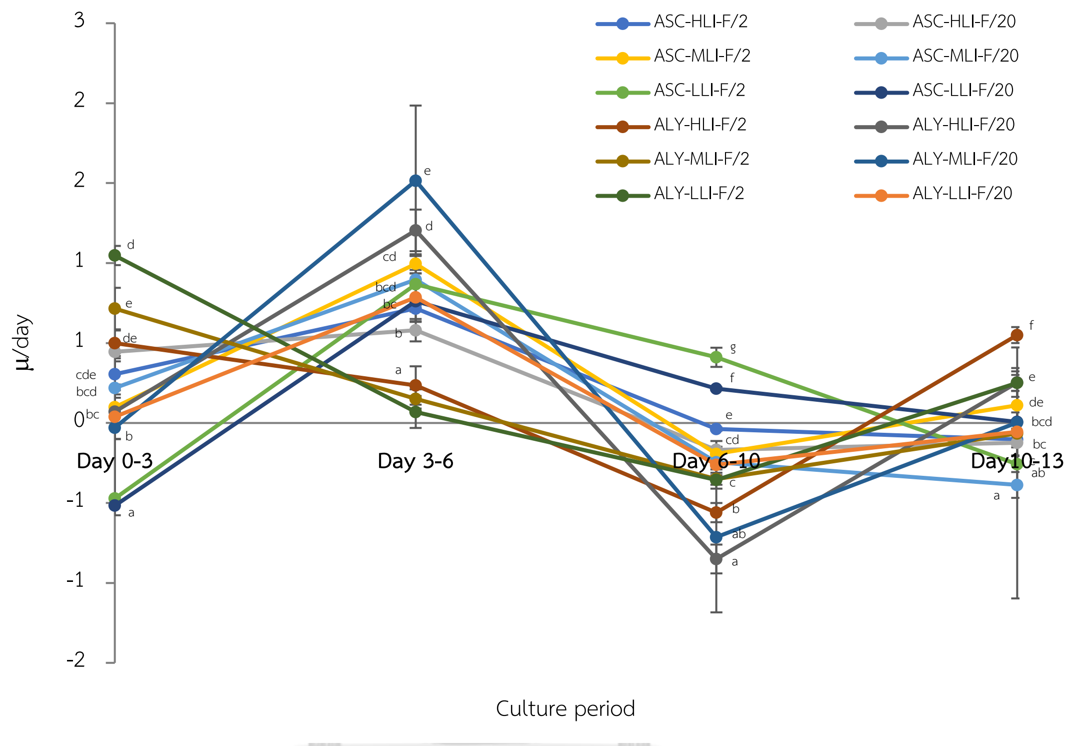


Figure 22 Levels of SGR in 2 diatom strains on day 0, 3, 6, 10 and 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2 Total lipid content

Total lipid content was investigated on day 6, 10, and 13 of culture (Figure 24). The result showed that Total lipid content of ALY cultured with MLI-F/20 was significantly highest at day 6 and 13 of culture compared with other culture conditions but was not significantly different with ASC cultured with MLI-F/20 and ASC cultured with MLI-F/2 which showed the highest result at day 10 of culture. The highest total lipid content of each culture day varied between 40 – 45 mg/100 mg algal dried weight.

Day 6 ALY culture with MLI-F/20, ASC cultured with MLI-F/20 and LLI-F/2 provide significantly highest total lipid content compared to other culture condition ($p < 0.05$, ANOVA analysis, followed with Duncan). Total lipid content investigated from ALY cultured with MLI-F/20, ASC cultured with MLI-F/20 and LLI-F/2 were 55.71 ± 2.19, 50.87 ± 1.77, and 50.80 ± 6.23 mg/100 mg algal dried weight.

At day 10, ASC cultured in F/20 with HLI and MLI was significantly highest total lipid content compared to ALY cultured in F/20 with all light intensity and ALY cultured in f/2 with HLI and LLI. But total lipid content of these 2 culture conditions was not significantly different with ASC cultured in F/20 with LLI and those cultured in F/2 with all light intensity and ALY cultured with MLI-F/2. ASC cultured in F/20 with HLI and MLI had 41.98 ± 8.52 and 41.79 ± 2.91 mg total lipid content/100 mg algal dried weight, respectively.

At day 13, Total lipid content of ASL cultured with LLI-F/20 and ALY cultured with MLI-F/20 were significantly highest than ALY cultured with HLI-F/2, MLI-F/2 and LLI-F/20 but were not significantly different with the rest culture conditions. Total lipid content of ASL cultured with LLI-F/20 and ALY cultured with MLI-F/20 were 54.23 ± 11.61 and 54.22 ± 11.68 mg /100 mg algal dried weight, respectively.

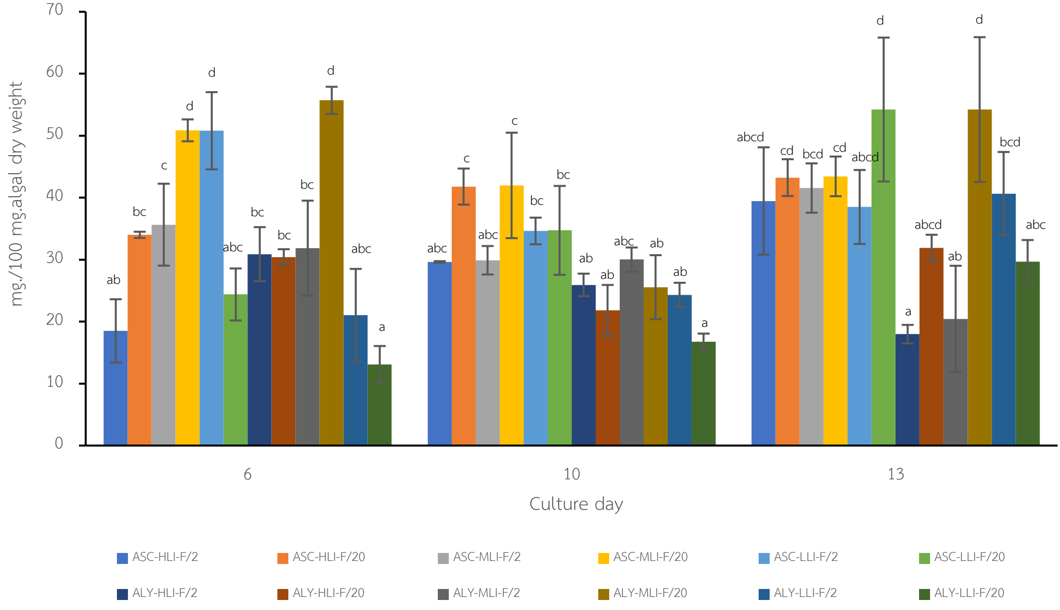


Figure 23 Total lipid content (mg/100 mg Algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different

lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2.1 Saturated fatty acid (SFA)

SFA was investigated at day 6, 10, and 13 of culture. The results are shown in Figure 25. There was a variation on SFA between culture condition and culture time. At day 6 of culture, SFA of AlY cultured with MLI-F/20 and ASC cultured with HLI-F/20 and MLI-F/20 were significantly highest than ASC cultured with HLI-F/2, LLI-F/2, LLI-F/20 and ALY cultured with MLI-F/2, LLI-F/2, and LLI-F/20 but were not significantly different with ASC cultured with MLI-F/2 and ALY cultured with HLI-F/2 and HLI-F/20. The highest SFA were 29.67 ± 10.47 mg/100 mg algal dry weight (ALY-MLI-F/20), 27.88 ± 1.29 mg/100 mg algal dry weight (ASC-HLI-F/20), and 27.38 ± 9.09 mg/100 mg algal dry weight (ASC-MLI-F/20).

At day 10 of culture, there was not significantly different of SFA among the culture condition. The highest SFA was found in ASC cultured with LLI-F/20 (29.55 ± 9.93 mg/100 mg algal dry weight), HLI-F/20 (29.05 ± 12.21 mg/100 mg algal dry weight), and HLI-F/2 (27.04 ± 0.99 mg/100 mg algal dry weight)

The significant difference between SFA were found again at day 13 of culture. SFA of ASC cultured with HLI-F/2 was significantly highest than all culture condition, except ASC cultured with HLI-F/20 and MLI-F/2. The highest SFA was 38.38 ± 9.15 mg/100 mg algal dry weight.

Chulalongkorn University

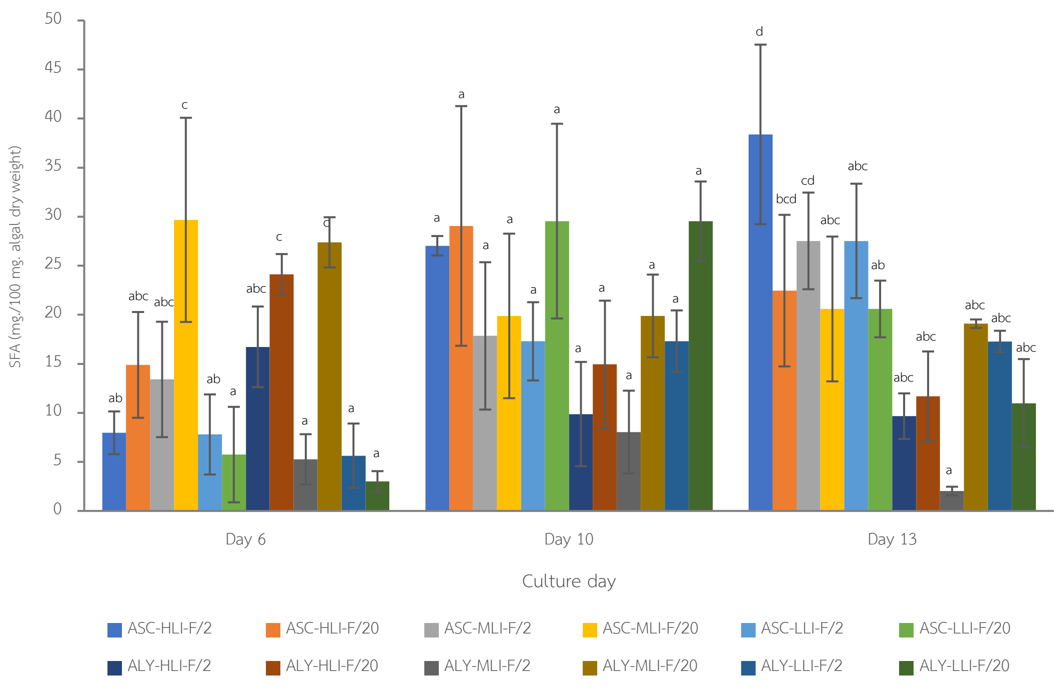


Figure 24 SFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2.2 Unsaturated fatty acids (UFAs)

Total UFA was investigated at day 6, 10, and 13 of culture and showed a variation similar with SFA in which the differences relate with culture condition and culture time as shown in Fig.26. UFA investigated from ASC cultured with LLI-F/2 was significantly highest than those other culture condition on day 6 and 10 but was not significantly highest at day 13 which belong to ALY cultured with MLI-F/20. The highest UFA at day 6 and 10 of culture were 34.29 ± 10.83 and 16.89 ± 2.34 mg/100 mg algal dry weight, respectively and day 13 was 28.06 ± 10.78 mg/100 mg algal dry weight.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

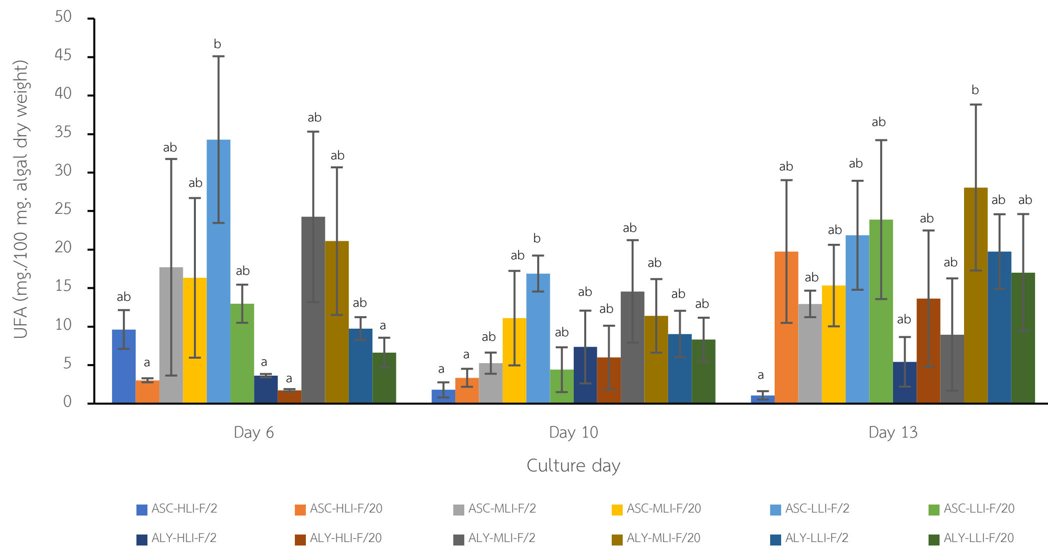


Figure 25 Total levels of UFA (mg/100 mg algal dry weight) of 2 diatom strains determined on day 6, 10, and day 13 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between Amphora sp. strain of each culture day.

4.2.3 Fatty acid composition

Each fatty acid composition was determined from the same culture day with total lipid content, SFA, and UFA of ASC (Table 6) and ALY (Table 7). The result showed that the major SFA was Myristic acid (C14:0) and Palmitic acid (C16:0). The Palmitic acid showed a larger proportion than Myristic acid, this pattern was indicated in all sampling day. The major UFA was Palmitoleic acid (C16:1ω7), Arachidic acid (C20:1ω9), Linoleic acid (C18:2ω6), Eicosapentaenoic acid EPA (C20:5ω3), and Docoxahexaenoic acid DHA (C22:6ω3). ASC cultured with MLI-F/20 showed significantly highest result of SFA which was myristic acid at day 6 of culture (10.45 ± 1.20 mg/100 mg algal dried weight) and the major UFA including linoleic acid at day 10 (3.69 ± 1.92 mg/100 mg algal dried weight) and day 13 (7.64 ± 2.12 mg/100 mg algal dried weight) of culture, EPA at day 13 of culture (2.07 ± 0.37 mg/100 mg algal dried weight), and DHA at day 6 of culture (16.48 ± 9.51 mg./100 mg. Algal dried weight). While ALY cultured with MLI-F/20 showed significantly highest result of SFA including myristic acid at day 6 (12.09 ± 0.84 mg/100 mg algal dried weight) and day

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

13 of culture (11.46 ± 2.10 mg./100 mg. Algal dried weight) and the major UFA including palmitoleic acid at day 13 (8.04 ± 1.70 mg/100 mg algal dried weight), arachidic acid at day 6 (5.86 ± 2.87 mg/100 mg algal dried weight) and day 10 (1.15 ± 0.42 mg/100 mg algal dried weight) of culture, linoleic acid at day 6 (5.65 ± 2.11 mg/100 mg algal dried weight) and day 13 (7.11 ± 3.17 mg/100 mg algal dried weight) of culture, EPA at day 6 (3.00 ± 0.89 mg/100 mg algal dried weight) and day 10 (2.84 ± 1.20 mg/100 mg algal dried weight) of culture, and DHA at day 10 (3.39 ± 1.89 mg/100 mg algal dried weight) and day 13 of culture (3.22 ± 1.27 mg/100 mg algal dried weight).

Table 6 Fatty acid composition of ASC (mg/100 mg algal dry weight) determined from 2 strains, 3 light intensities, and 2 medium concentrations in each culture day. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each fatty acid.

Fatty acid	Culture day	ASC-HLI-F/2	ASC-HLI-F/20	ASC-MLI-F/2	ASC-MLI-F/20	ASC-LLI-F/2	ASC-LLI-F/20
SFAs							
Myristic acid (C14:0)	6	1.41±0.21^{a}	5.15±0.09^{ab}	3.86±0.87^{ab}	10.45±1.20^{c}	2.00±0.63^{ab}	4.93±2.76^{ab}
	10	10.61±1.70^{ab}	11.16±1.88^{b}	7.81±1.44^{ab}	7.78±1.22^{ab}	7.31±0.79^{ab}	6.76±1.84^{ab}
	13	10.11±1.20^{cde}	8.33±1.28^{bcde}	14.86±0.98^{e}	9.04±1.54^{cde}	7.87±0.96^{bcd}	1.85±0.76^{ab}
Palmitic acid (C16:0)	6	15.25±0.10^{a}	54.85±2.35^{c}	21.35±6.24^{ab}	27.56±8.05^{ab}	3.53±1.94^{a}	1.14±0.45^{a}
	10	50.13±2.34^{bc}	37.37±11.02^{abc}	24.70±7.13^{abc}	19.35±8.24^{abc}	15.84±4.93^{abc}	52.15±9.65^{c}
	13	67.76±3.48^{a}	24.55±7.19^{a}	19.85±2.99^{a}	14.38±4.61^{a}	9.73±4.69^{a}	2.15±1.08^{a}
UFAs							
Palmitoleic acid (C16:1ω7)	6	5.41±0.79^{c}	2.38±0.14^{ab}	0.92±0.29^{ab}	3.24±1.19^{bc}	0.52±0.24^{a}	0.35±0.09^{a}
	10	1.11±0.62^{a}	1.73±0.45^{a}	0.28±0.16^{a}	2.10±0.46^{a}	4.38±1.89^{a}	0.32±0.09^{a}
	13	0.74±0.29^{ab}	3.26±0.99^{abc}	2.84±1.11^{abc}	1.66±0.67^{ab}	6.27±1.79^{bc}	0.74±0.33^{ab}
Oleic acid (C18:1ω9)	6	0.81±0.08^{ab}	0.13±0.00^{a}	0.19±0.17^{a}	0.01±0.01^{a}	0.72±0.25^{ab}	0.96±0.43^{ab}
	10	0.25±0.06^{a}	0.11±0.05^{a}	2.64±1.01^{a}	1.62±0.65^{a}	0.26±0.12^{a}	0.62±0.20^{a}
	13	0.05±0.02^{a}	0.59±0.17^{a}	0.94±0.25^{a}	1.84±0.67^{a}	1.75±0.91^{a}	0.07±0.04^{a}
Arachidic acid (C20:1ω9)	6	0.07±0.02^{a}	0.01±0.00^{a}	0.19±0.08^{a}	0.04±0.01^{a}	2.47±0.26^{a}	8.40±4.84^{a}
	10	0.00±0.00^{a}	0.00±0.00^{a}	0.16±0.08^{a}	0.31±0.17^{a}	0.74±0.28^{ab}	0.24±0.14^{a}
	13	0.00±0.00^{a}	6.49±3.67^{a}	2.36±0.73^{a}	0.83±0.13^{a}	1.05±0.37^{a}	8.26±3.78^{a}
Erucic acid (C22:1ω9)	6	0.00±0.00^{a}	0.00±0.00^{a}	1.80±1.04^{a}	0.00±0.00^{a}	0.15±0.09^{a}	0.08±0.05^{a}
	10	0.00±0.00^{a}	0.00±0.00^{a}	0.05±0.02^{a}	0.14±0.06^{a}	0.26±0.15^{a}	0.33±0.19^{a}
	13	0.00±0.00^{a}	0.01±0.01^{a}	0.29±0.09^{a}	0.26±0.06^{a}	0.82±0.27^{a}	3.63±1.47^{a}
cis-11-docosenoic acid (C22:1ω11)	6	0.00±0.00^{a}	0.00±0.00^{a}	0.21±0.11^{a}	0.15±0.05^{a}	0.20±0.06^{a}	0.05±0.03^{a}
	10	0.24±0.12^{a}	0.04±0.02^{a}	0.04±0.01^{a}	0.64±0.31^{a}	0.07±0.03^{a}	0.08±0.03^{a}
	13	0.00±0.00^{a}	0.16±0.05^{a}	0.39±0.06^{a}	1.42±0.36^{b}	0.36±0.10^{a}	0.02±0.01^{a}
Linoleic acid (C18:2ω6)	6	0.00±0.00^{a}	0.01±0.00^{a}	0.98±0.36^{a}	0.74±0.26^{a}	6.31±2.48^{a}	1.68±0.78^{a}
	10	0.57±0.29^{a}	0.12± 0.07a	0.11±0.03^{a}	3.69±1.92^{a}	0.28±0.10^{a}	0.50±0.15^{a}
	13	0.01±0.00^{a}	1.05±0.43^{a}	.07±0.14^{a}	7.64±2.12^{a}	1.92±0.51^{a}	4.95±2.53^{a}
Stearidonic acid (C18:4ω3)	6	0.73±0.11^{a}	0.08±0.01^{a}	2.24±1.18^{a}	0.09±0.05^{a}	0.26±0.08^{a}	0.85±0.40^{a}
	10	0.07±0.01^{a}	0.13±0.02^{a}	0.40±0.00^{a}	0.25±0.06^{a}	0.94±0.13^{a}	1.41±0.76^{a}
	13	0.01±0.00^{a}	4.75±2.48^{a}	0.86±0.22^{a}	0.91±0.25^{a}	1.75±0.65^{a}	2.56±0.07^{a}
Eicosapentaenoic acid EPA (C20:5ω3)	6	4.77±0.21^{c}	0.29±0.08^{a}	1.43±0.43^{ab}	0.67±0.18^{a}	0.61±0.26^{a}	0.73±0.30^{a}
	10	0.71±0.22^{a}	0.68±0.38^{a}	2.28±1.07^{a}	1.58±0.46^{a}	1.85±0.31^{a}	0.20±0.09^{a}
	13	0.07±0.02^{a}	0.45±0.26^{ab}	1.06±0.16^{ab}	2.07±0.37^{bc}	0.78±0.27^{ab}	0.65±0.23^{ab}
Docosapentaenoic	6	0.01±0.00^{a}	0.03±0.02^{a}	0.22±0.06^{a}	0.28±0.11^{a}	0.27±0.06^{a}	0.75±0.34^{a}

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

acid DPA	10	0.05±0.02^{a}	0.13±0.07^{a}	0.48±0.27^{ab}	0.72±0.21^{ab}	2.69±0.78^{c}	0.15±0.05^{a}
(C22:5ω3)	13	0.07±0.02^{a}	0.07±0.02^{a}	0.64±0.27^{a}	0.70±0.24^{a}	1.00±0.09^{ab}	0.24±0.10^{a}
Docoxahexaenoic	6	0.49±0.03^{a}	0.03±0.00^{a}	3.01±1.73^{a}	16.48±9.51^{ab}	30.15±9.93^{b}	0.08±0.05^{a}
acid DHA	10	0.01±0.00^{a}	0.13±0.07^{a}	0.40±0.21^{a}	1.21±0.69^{a}	0.74±0.21^{a}	1.55±0.85^{a}
(C22:6ω3)	13	0.03±0.02^{a}	5.12±1.48^{ab}	1.32±0.41^{ab}	1.05±0.33^{ab}	0.36±0.18^{ab}	11.27±5.93^{ab}

Table 7 Fatty acid composition of ALY (mg/100 mg algal dry weight) determined from 2 strains, 3 light intensities, and 2 medium concentrations in each culture day. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each fatty acid.

Fatty acid		**ALY-HLI-F/2**	**ALY-HLI-F/20**	**ALY-MLI-F/2**	**ALY-MLI-F/20**	**ALY-LLI-F/2**	**ALY-LLI-F/20**
SFAs							
Myristic acid	6	3.30±0.42^{ab}	7.38±0.53^{bc}	0.34±0.44^{a}	12.09±0.84^{c}	0.73±0.13^{a}	1.76±0.30ab
(C14:0)	10	5.09±1.42^{ab}	8.28±1.74^{ab}	2.04±0.82^{a}	8.44±2.39^{ab}	6.60±0.95^{ab}	4.29±0.70ab
	13	5.17±0.18^{abcd}	5.22±0.80^{abcd}	1.00±0.10^{a}	11.46±2.10^{de}	4.28±1.34^{abc}	6.56±1.33abcd
Palmitic acid	6	22.92±3.19^{ab}	44.38±4.66^{bc}	7.55±4.14^{a}	20.57±9.68^{ab}	6.09±1.75^{a}	3.09±0.91a
(C16:0)	10	16.20±8.84^{abc}	19.98±11.07^{abc}	7.62±3.36^{ab}	5.39±1.73^{a}	16.53±6.00^{abc}	15.35±7.53abc
	13	20.75±8.38^{a}	12.47±5.11^{a}	9.99±5.67^{a}	9.98±5.10^{a}	14.49±1.48^{a}	9.76±2.88a
UFAs							
Palmitoleic acid	6	0.57±0.17^{a}	1.22±0.15^{ab}	0.28±0.16^{a}	2.13±0.43^{ab}	0.98±0.29^{ab}	0.10±0.06^{a}
(C16:1ω7)	10	1.11±0.32^{a}	0.72±0.29^{a}	2.85±1.70^{a}	0.77±0.18^{a}	0.29±0.08^{a}	1.14±0.24^{a}
	13	1.51±0.33^{ab}	3.58±1.04^{abc}	0.26±0.08^{a}	8.04±1.70^{c}	3.00±1.08^{abc}	0.55±0.16^{b}
Oleic acid	6	0.33±0.09^{a}	0.04±0.03^{a}	0.11±0.04^{a}	0.30±0.15^{a}	2.41±0.82^{b}	1.62±0.41^{ab}
(C18:1ω9)	10	0.37±0.13^{a}	0.39±0.07^{a}	0.66±0.08^{a}	2.66±1.37^{a}	0.48±0.20^{a}	0.15±0.06^{a}
	13	0.27±0.13^{a}	0.41±0.09^{a}	0.32±0.09^{a}	0.39±0.07^{a}	1.00±0.34^{a}	1.48±0.67^{a}
Arachidic acid	6	0.31±0.08^{a}	0.02±0.01^{a}	3.83±1.26^{a}	5.86±2.87^{a}	3.86±1.13^{a}	1.16±0.46^{a}
(C20:1ω9)	10	0.23±0.07^{a}	0.87±0.48^{ab}	0.56±0.16^{ab}	1.15±0.42^{ab}	0.25±0.10^{a}	5.23±2.87^{b}
	13	0.23±0.11^{a}	0.29±0.14^{a}	1.98±0.64^{a}	3.21±1.30^{a}	0.54±0.29^{a}	3.95±1.40^{a}
Erucic acid	6	0.00±0.00^{a}	0.00±0.00^{a}	2.07±1.20^{a}	0.96±0.32^{a}	0.00±0.00^{a}	0.14±0.08^{a}
(C22:1ω9)	10	0.30±0.16^{a}	1.13±0.65^{a}	0.35±0.18^{a}	0.18±0.10^{a}	0.10±0.04^{a}	0.20±0.06^{a}
	13	0.12±0.07^{a}	2.18±1.24^{a}	0.17±0.10^{a}	3.56±1.55^{a}	1.39±0.46^{a}	1.17±0.43^{a}
cis-11-docosenoic	6	0.02±0.00^{a}	0.06±0.02^{a}	0.03±0.01^{a}	1.34±0.53^{a}	0.05±0.03^{a}	0.10±0.04^{a}
acid (C22:1ω11)	10	0.05±0.01^{a}	0.10±0.05^{a}	0.03±0.01^{a}	0.15±0.04^{a}	0.40±0.20^{a}	0.06±0.01^{a}
	13	0.19±0.10^{a}	0.05±0.01^{a}	0.03±0.01^{a}	0.56±0.21^{a}	0.19±0.08^{a}	0.37±0.13^{a}
Linoleic acid	6	0.18±0.01^{a}	0.23±0.08^{a}	0.84±0.21^{a}	5.63±2.11^{a}	2.06±1.09^{a}	1.92±1.01^{a}
(C18:2ω6)	10	3.23±1.82^{a}	0.47±0.26^{a}	0.26±0.07^{a}	0.62±0.23^{a}	2.74±1.49^{a}	0.27±0.05^{a}
	13	0.70±0.36^{a}	0.41±0.09^{a}	0.87±0.25^{a}	7.11±3.71^{a}	3.64±0.98^{a}	1.69±0.41^{a}
Stearidonic acid	6	0.25±0.14^{a}	0.01±0.01^{a}	1.63±0.94^{a}	1.22±0.51^{a}	0.15±0.05^{a}	0.18±0.04^{a}
(C18:4ω3)	10	0.01±0.00^{a}	0.57±0.20^{a}	0.11±0.03^{a}	0.56±0.27^{a}	0.73±0.03^{a}	1.52±0.65^{a}
	13	0.16±0.05^{a}	0.83±0.47^{a}	0.38±0.19^{a}	0.15±0.07^{a}	0.32±0.11^{a}	4.04±1.76^{a}
Eicosapentaenoic	6	1.14±0.16^{a}	0.29±003^{a}	0.78±0.14^{a}	3.00±0.89^{b}	0.94±0.20^{a}	1.12±0.22^{a}
acid EPA	10	0.31±0.07^{a}	0.50±0.16^{a}	0.70±0.21^{a}	2.84±1.20^{a}	0.57±0.25^{a}	0.77±0.23^{a}
(C20:5ω3)	13	0.86±0.40^{ab}	1.03±0.45^{ab}	0.97±0.25^{ab}	0.77±0.29^{ab}	2.83±0.65^{c}	0.44±0.08^{ab}
Docosapentaenoic	6	0.02±0.00^{a}	0.00±0.00^{a}	0.34±0.20^{a}	0.51±0.21^{a}	0.10±0.01^{a}	0.19±0.07^{a}
acid DPA	10	0.19±0.09^{a}	0.26±0.08^{ab}	0.88±0.39^{ab}	0.72±0.24^{ab}	2.17±0.52^{bc}	0.77±0.36^{ab}
(C22:5ω3)	13	0.52±0.28^{a}	0.06±0.02^{a}	0.14±0.05^{a}	0.64±0.32^{a}	1.82±0.33^{b}	0.77±0.21^{ab}
Docoxahexaenoic	6	0.37±0.16^{a}	0.01±0.00^{a}	10.92±3.14^{ab}	2.08±1.14^{a}	7.91±3.25^{ab}	3.90±1.36^{a}
acid DHA	10	6.17±3.54^{a}	1.11±0.63^{a}	5.50±3.00^{a}	3.39±1.89^{a}	2.30±0.89^{a}	0.54±0.05^{a}
(C22:6ω3)	13	0.69±0.29^{ab}	1.11±0.49^{ab}	2.07±1.11^{ab}	3.02±1.27^{ab}	3.03±0.80^{ab}	1.70±0.28^{b}

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5. Discussion

5.1 Biomass

The highest chlorophyll content was found at day 10 of culture in both chosen *Amphora* sp. and there was some difference between them. The chlorophyll content of ASC seems related to medium concentration. This was indicated by the different chlorophyll content between ASC cultured in F/2 medium which was significantly higher than those that cultured in F/20 medium. While chlorophyl content of ALY related to light intensity. The finding indicated that chlorophyll content investigated from ALY cultured with LLI had higher than MLI and HLI. This can be inferred that chlorophyll content of ALY may be inhibited by higher light intensity.

The highest cell density was found in ALY cultured with MLI-F/20 at day 6 of culture. This culture day showed the highest cell density of both chosen *Amphora* sp. strains. The finding from cell density indicates the similarity finding with chlorophyll content which is each strain has specie – specifically responsible for culture conditions. ASC cultured in F/2 medium produced higher cell density than ASC cultured in F/20 medium while ALY cultured with MLI produced higher cell density than ALY cultured with HLI, and with LLI which maybe the deficiency light intensity. comparison between 2 chosen *Amphora* sp. strain, the results showed that ALY can produced higher cell density than ASC.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Growth pattern evaluated from SGR indicated that ASC cultured in all conditions and ALY cultured in F/20 with all light intensity showed growth rate than ALY cultured in F/2 with all light intensity. This finding can be explained that medium concentration probably a minor factor for growth of ALY. In addition, the period that diatom enter stationary phase (day 3 – 6) especially ASL cultured with MLI-F/2 and ALY cultured with MLI-F/20, the condition that provided the highest biomass, is importance finding for diatom harvesting.

The subsequent decrease in chlorophyll content, cell density, and SGR across all culture conditions after day 10 could be attributed to factors such as nutrient depletion affecting diatom growth.

5.2 Lipid content and fatty acid composition

The results indicated that ASC cultured with MLI-F/20 and ALY cultured with MLI-F/20 showed significantly highest result in many fatty acid compared with other conditions which showed significantly highest result in just some fatty acid and some culture day. Comparison between ASC cultured with MLI-F/20 and ALY cultured with MLI-F/20, the result showed that ALY cultured with MLI-F/20 had provided significantly highest result in all SFA, while ASC cultured with MLI-F/20 showed significantly result in only myristic acid. Comparison the result between UFA, the finding also indicated that ALY cultured with MLI-F/20 showed larger number of UFA that was significantly highest.

6. Conclusion

ASC cultured with MLI-F/20 and ALY cultured with MLI-F/20 can provide maximum biomass on day 6 of culture. ALY cultured with MLI-F/20 can produce the higher cell density than ASC cultured with MLI-F/20. Evaluation total lipid content and fatty acid composition, the result showed that ALY cultured with MLI-F/20 had provided significantly highest result in all SFA and higher result of many UFA compared to ASC cultured with MLI-F/20 especially important UFA. Therefore, ALY cultured with MLI-F/20 was chosen for further experiment as shrimp supplementary diet.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chapter IV

The efficiency of diet with additional diatoms as immuno-stimulant for *Litopenaeus vannamei*

1. Introduction

In recent times, there has been a notable surge in interest regarding the use of algal cells, including microalgae and seaweeds, as feed additives. This is primarily attributed to their rich concentration of natural bioactive compounds. These additions to animal diets have demonstrated enhancements in growth performance, feed utilization, and immunity stimulation among cultured animal species. Additionally, the utilization of algal cells as feed additives has shown positive effects on improving water quality in aquaculture settings (Mansour et al., 2022).

Examining the influence of *Amphora* sp., a noteworthy microalga incorporated into *L. vannamei's* diet, is essential in assessing its effects on shrimp health across multiple aspects like growth, disease resilience, immune system, and digestive structure. This research holds significant importance in the realm of aquaculture as it paves the way for enhancing shrimp diets and refining aquaculture methodologies for better farming outcomes. The potential to enhance growth, disease resistance, and reduce the need for antibiotics can provide sustainable aquaculture and reduce environmental impact from shrimp farming and significantly promote economic implications, and develope strategies to mitigate disease risks. Javahery et al. (2019) stated that culture shrimp in pathogenic, infectious disease, stress and pollution free system is very difficult. *Vibrios*, such as *Vibrio parahaemolyticus*, *V. harveyi*, and *V. alginolyticus*, are implicated in causing illnesses in both shrimp and human gastroenteritis following the ingestion of seafood tainted with these bacteria (Liang et al., 2020). Employing nutritional supplements with beneficial feed additives is crucial to enhance the immune response and safeguard shrimp from potential pathogenic outbreaks. Furthermore, those beneficial feed additives must promote growth, reduce infectious disease, and less environmental impact.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

To establish the *Amphora* sp. as supplementary diet for shrimp, impact and potential of *Amphora* sp. is imperative to conduct a comprehensive investigation encompassing various parameters such as growth performance, health indicators including total hemocyte count (THC) and phagocytic activity (PA), digestive morphology, and the expression of crucial immune-related genes like *LvALF B*. This knowledge holds the potential for practical applications in biotechnology, offering avenues to enhance disease resistance in aquaculture species. Molina-Cárdenas andSánchez-Saavedra (2017) demonstrated that diatoms have the ability to generate inhibitory compounds against bacteria, providing an alternative to the utilization of chemicals for the regulation of pathogenic bacterial growth. In addition, this research suggests that the tested diatom species, likely attributed to the production of antibacterial compounds. These results advocate for considering diatom cultures as a nutritional source that effectively regulate the concentration of pathogenic vibrios and in aquaculture systems.

Studies have shown that incorporating low to moderate amounts of microalgae (up to 15%) in fish diets can result in comparable or even reduced feed conversion ratio (FCR), contributing to more efficient and sustainable aquaculture practices. However, it is crucial to optimize the type and concentration of microalgae in the feed to achieve the desired outcomes, as excessive algal content may lead to issues such as starvation in certain species (Nagappan et al., 2021).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2. Objective

The objective of this study was to assess the efficacy of diet supplemented with *Amphora* sp. for *L. vannamei*.

3. Materials and methods

3.1 Diet preparation

During the current 6 weeks of feeding trial. Four dietary supplementation treatments were implemented. The first diet, 0% *Amphora* sp. served as the control and involved feeding the shrimp a commercial diet. The remaining three diets, 5% *Amphora* sp., 10% *Amphora* sp., and 15% *Amphora* sp. incorporated varying levels of Amphora sp. powder (50, 100 and 150 g/kg diet, respectively). To prepare the diets,

the control diet was commercial post-larvae shrimp diet, first powdered and divided into four equal portions. Subsequently, specific quantities of *Amphora* sp. powder were thoroughly mixed with the three powdered diets. Then air-dried at 60 °C in the oven for 30 mins, cooled down at room temperature, and stored in aluminum bags at 4°C until utilization (Mansour et al., 2022).

3.2 Shrimp acclimatization

A total of 1,200 healthy *L. vannamei*, at the post-larval stage 12 (PL12), the stage that immunity is not completely develop and cause of mass mortality by *V. haemolyticus* infection, were acclimated for of 7 days. Shrimp were cultured in 200 L fiberglass tanks containing seawater with a salinity of 15 parts per thousand (Jana et al., 2022), fed twice a day, with an amount equivalent to 3% of the total biomass, aeration, and conducted at ambient temperature the whole acclimatization period. Water qualities were maintained approximately 80% water exchanging and daily aeration (Chuchird et al., 2015; Luo et al., 2023; Mansour et al., 2022).

3.3 Water quality analysis

Water quality including Total Ammonia nitrogen (TAN), Nitrite (NO2), Dissolved Oxygen (DO), Alkalinity, and pH were tested using TestKit (Tetra®Test Kit) every 3 days. Temperature was measured using thermometer, and salinity was measured using refractometer weekly.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.4 Feeding trial experiment

After acclimatization for 7 days, fifty shrimp with initial average weight ranging from 0.78 ± 0.07 g/individual to 0.88 ± 0.04 g/individual were randomly allocated to each 5 L seawater experimental unit and fed with designated dietary supplementation treatments. The feeding regimen included twice-daily feedings at a rate of 3% of the total biomass, sustained over a period of 6 weeks. Water qualities were maintained approximately 80% water exchanging and daily aeration (Chuchird et al., 2015; Luo et al., 2023; Mansour et al., 2022). Growth performance was assessed using survival rate (%SR), mean body weight (MBW), specific growth rate (SGR), and feed efficiency ratio (FE). %SR was measured weekly, while MBW, SGR, and FE were recorded at the beginning, 3rd week, and 6th week of the culture period.

Shrimp health was evaluated in the study of disease resistant through a challenge test method conducted at the 3rd and 6th weeks of the feeding trial (Chuchird et al., 2015; Luo et al., 2023; Mansour et al., 2022).

3.5 *Vibrio parahemolyticus* culture

V. parahemolyticus was provided by Songkla aquatic animal health research and development center, Department of Fisheries. Ministry of Agriculture and Cooperatives. Bacteria was culture in Tryptic Soy Broth (TSB) + 0.15% NaCl, incubated at 37°C for 24 hrs. Total plate count was conducted by Thiosulfate-Citrate-Bile-Salt Sucrose Agar, or TCBS Agar at 37°C, and 24 hrs of incubation.

3.6 The study of disease resistant by challenge test method

Ten shrimps from each treatment in the feeding trial were immersed in a bacteria *V. parahemolyticus* solution at a final concentration of 10^5 cfu/ml after 3 and 6 weeks of culture (Chuchird et al., 2015; Luo et al., 2023). After 24 hrs of immersion, %SR were recorded. Two shrimp were preserved in Davison's solution for midgut intestinal epithelium investigation. The rest of the shrimp were sampling for hepatopancrease and hemolymph for THC, phagocytic activity, and immune – related genes (Luo et al., 2023; Mansour et al., 2022).

3.7 Growth performance of shrimp

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.7.1 Survival rate (SR; %)

The number of shrimps in each experimental unit was observed and calculated according to the study of Jana et al. (2022) and Mansour et al. (2022) as follows.

SR (%) = 100 × final number of shrimp / initial number of shrimp

3.7.2 Weight gain (WG; %) and specific growth rate (SGR; %)

All shrimps in each experimental unit were weight. Weight gain and specific growth rate were calculated according to the study of Jana et al. (2022) as follows.

WG (%) = 100 × (final weight - initial weight)/ initial weight

SGR (%) = 100 × (ln(final weight) - ln (initial weight))

3.7.3 Feed efficiency (FE; %) (Jana, et al. 2022)

Amount of feed and final weight gain were used for FE calculation according to the study of Jana et al. (2022) and Mansour et al. (2022) as follows.

FE (%) = 100 × (wet weight gain/ dry feed intake)

3.8 Shrimp blood sampling

Hemolymph was collected from the ventral sinus cavity using 25G x 1”, and transferred into 1.5 mL microtubes with 10% sodium citrate as anticoagulant in ratio 1:1 for total hemocyte count and phagocytic activity investigation (Luo et al., 2023; Setyawan et al., 2018; Wei et al., 2022).

3.9 Total hemocyte count (THC)

Fresh 20 μl of hemocyte were counted using a hemocytometer. The results were present in unit of cell/ml (Avin, 2020).

3.10 Phagocytosis activity (PA)

Twenty microliters of hemolymph were pipetted into a 96-well microplate and mixed with an equal volume of *Stapphylococcus aurius*. After a 20-minute incubation at 30°C, each 5 μL sample was spread on a glass slide, air-dried for 20 minutes. Subsequently, the slides were rinsed with 0.85% NaCl, air-dried once more, and stained with 10% Wright stain for 20 minutes. The phagocytic activity was determined through microscopic observation at 400X magnification, with PA calculated based on 100 phagocytes per slide. (Chuchird et al., 2015; Setyawan et al., 2021; Y. Yu et al., 2020)

3.11 ALF B gene analysis

Shrimp hepatopancrease triplicate samples from each replicate were precisely excised using sterile dissecting tools in a cold environment. The excised samples were promptly preserved in RNA*later*® (Labreuche et al., 2009; Shakweer et al., 2023) and keep at −20°C until gene expression analysis. Total RNA extraction from the samples was carried out using the TRIzol method (Maisons-Alfort, 2010).

RT-qPCR amplifications were performed in the CFX Real-time PCR System (BIO-RAD). PCR amplification in a final volume of 10 μl containing 2 μM of each primer, 5 μl of reaction mix (Maxima SYBR Green/Rox qPCR Master Mix 2x; BIO-RAD)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

and 1 μl of cDNA (diluted 1:10). Primer sequences for elongation factor 1-alpha (LvEF1a) as reference genes are TGGCTGTGAACAAGATGGACA (FW 5'-3') and TCAGAGAGAGTGCGACCATC (Rv 5'-3'). Primer sequences for anti-lipopolysaccharide factors (LvALF B) as target gene are GTGTCTCCGTGTTGACAAGC (FW 5'-3') and ACAGCCCAACGATCTTGCTG (Rv 5'-3'). The thermal profile of PCR consisted of an initial denaturation step of 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 55 °C for 1 min. Melt curve were 65-95 °C at a temperature transition rate of 0.05 °C/s. (Silveira et al., 2018; Yocawibun, 2011; Y. Yu et al., 2023).

3.12 Midgut histopathology

Shrimp was preserved in Davison solution for midgut intestinal area and histopathology investigation. The mid-gut segments were dehydrated in a gradient of ethanol solutions. Subsequently, the cleaned mid-gut segments were treated with toluene and embedded in paraffin. Cross sections, approximately 5 μm thick, were then cut from the embedded samples using a rotary microtome. These sections were further stained with hematoxylin and eosin (H&E) and examined under a light microscope (ZEISS Primostar 3, Germany) and Labscope version 3.3.1 software for imaging purposes (Liang et al., 2020).

3.13 Data analysis

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The results of growth performance and shrimp health were presented as mean ± SE. Statistic analysis was performed by SPSS software. A two-way ANOVA ($p<0.05$) was performed to determine the difference of growth performance and shrimp health in the different Amphora sp. supplementation concentration. The significant differences of the results were indicated at the 0.05 level. Post hoc analysis was done by Duncan.

4. Results

4.1 Growth performance

4.1.1 Survival rate (SR)

The results showed that there was a trend of decreasing SR of *L. vannamei* over the weeks. After six weeks of cultivation, SR of shrimp exhibited a range

between 26.00% ± 4.00% and 36.67% ± 3.53%. Shrimp fed with a 5% *Amphora* sp. diet consistently demonstrated the significantly lowest survival rate from the first week to the sixth week of cultivation. (Figure 27)

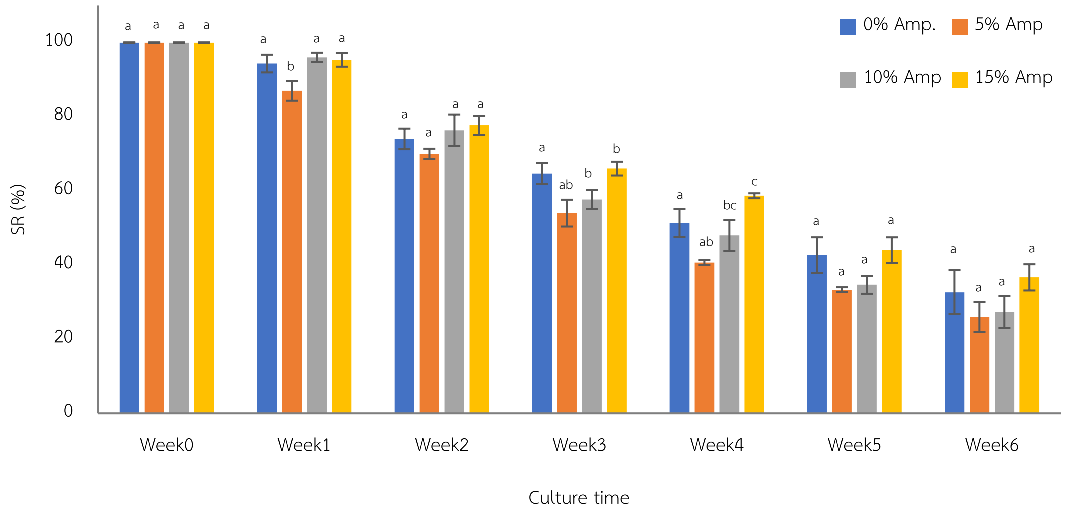


Figure 26 Survival rate of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day.

4.1.2 Mean body weight (MBW)

Post-larvae of *L. vannamei*, aged 12 days, were initially used with individual MBW ranging from 0.78 ± 0.07 to 0.88 ± 0.04 g. Following six-week feeding trial, the MBW increased to a range of 1.97 ± 0.59 g./individual to 3.21 ± 0.65 g./individual without significantly different between culture groups in each culture day. Shrimps that were provided with a diet containing 15% *Amphora* sp. exhibited the highest MBW among the tested conditions (Figure 28).

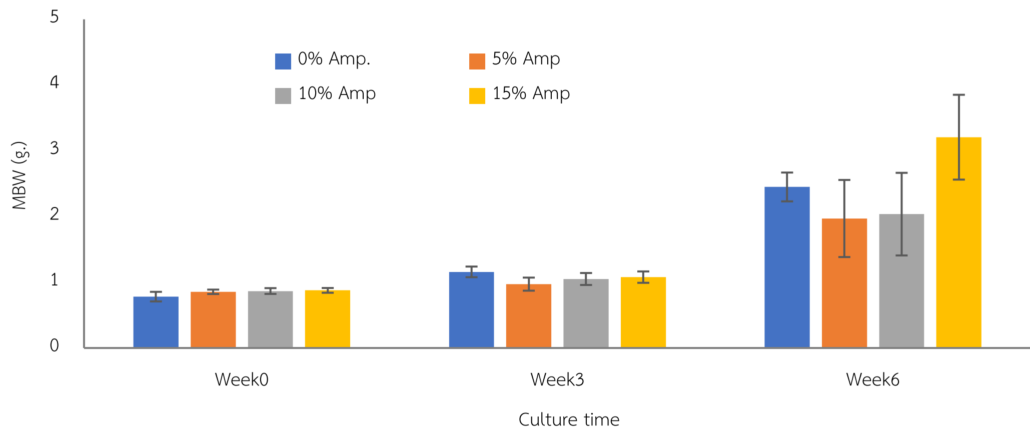


Figure 27 MBW of shrimp determined on week 0 to 6 of culture (Mean ± SE).

4.1.3 Specific growth rate (SGR)

The results indicated that from the initial week to the third week of feeding, SGR of shrimp ranged between 6.04 ± 3.05% and 13.50 ± 3.44%. Notably, the control group exhibited the highest SGR during this period. However, from the third to the sixth week of feeding, the SGR increased to a range of 9.67 ± 2.90% to 31.96 ± 4.77%. Among the *Amphora* sp. supplemented diet, Shrimp received 15% *Amphora* sp. diet demonstrated the highest SGR during this later phase of the feeding trial while no difference was found between this condition and the control one (Figure 29).

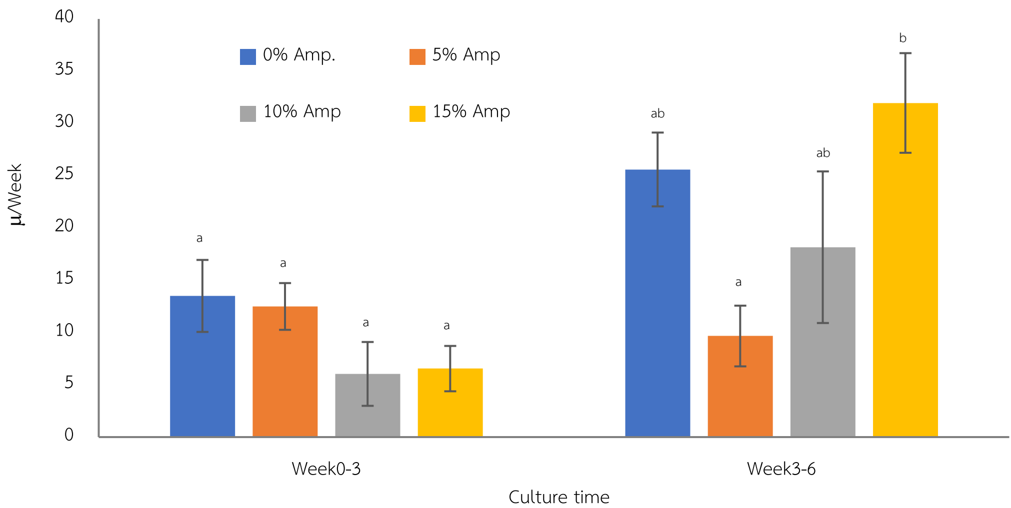


Figure 28 SGR of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day.

4.1.4 Feed efficiency (FE)

During the initial three weeks of culture, the FE was lower compared to the last three weeks. The first three weeks, FE ranged from 21.66 to 76.49. However, in the subsequent three weeks, it was increased to a range of 149.16 to 312.81, with the highest observed in shrimp that were fed with a 15% *Amphora* sp. diet (Figure 30).

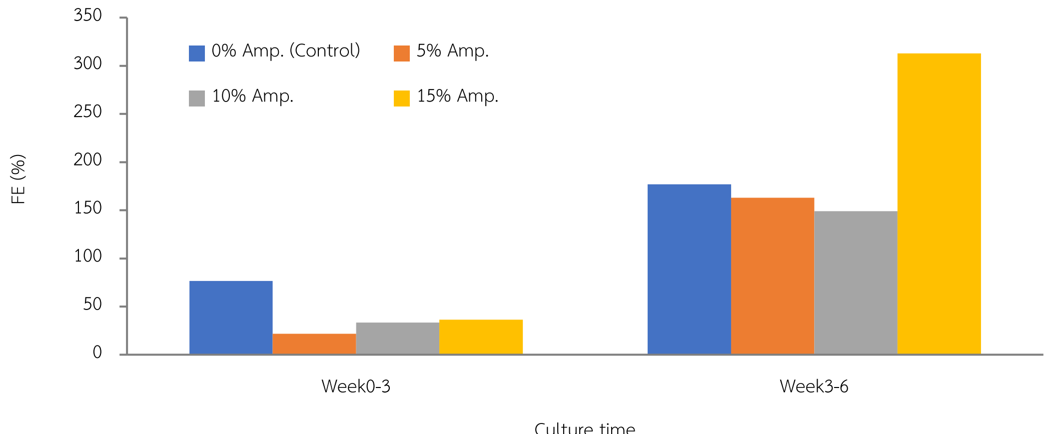


Figure 29 FE of shrimp determined on week 0 to 6 of culture. Mean ± SE values with different lowercase superscripts are significantly (p < 0.05) different between each culture day.

4.2 Disease resistance

The investigation into disease resistance included immersing the shrimp, which had been fed diet contained different *Amphora* sp. concentration for 3 and 6 weeks, in sea water containing the pathogenic bacteria *V. parahemolyticus* at a concentration of 10^5 cfu/ml for 24 hrs and there were no significantly difference between experimental group, except control group and other groups at week 6 (Table 8)

Table 8 Concentration of *V. parahemolyticus* (cfu/ml) in culture water for challenge test experiment. *Mean ± SE values with different lowercase superscripts are significantly (p < 0.05) different between each testing day*

Treatment	Week3	Week6
0% *Amphora* sp. (Control)	$5.37 \times 10^5 \pm 8.99 \times 10^{4a}$	$1.87 \times 10^5 \pm 2.60 \times 10^{4a}$

5% *Amphora* sp.	$7.57 \times 10^{5} \pm 1.09 \times 10^{5a}$	$4.97 \times 10^{5} \pm 7.06 \times 10^{4b}$
10% *Amphora* sp.	$6.73 \times 10^{5} \pm 8.84 \times 10^{4a}$	$6.07 \times 10^{5} \pm 8.09 \times 10^{4b}$
15% *Amphora* sp.	$6.70 \times 10^{5} \pm 1.00 \times 10^{5a}$	$6.43 \; 10^{5} \pm 3.76 \times 10^{4b}$

4.2.1 Survival rate (SR)

The results indicated that SR of shrimp fed a diet containing *Amphora* sp. for 3 weeks and immersed in *V. parahemolyticus*, was lower compared to those fed for 6 weeks. Specifically, at the 3-week mark, the group of shrimp fed with 15% *Amphora* sp. demonstrated significantly higher survival rates than those fed with 10% *Amphora* sp. However, the survival rates were not significantly higher than those of shrimp not fed with a diet containing *Amphora* sp. and shrimp fed with a diet containing 5% *Amphora* sp. In contrast, at 6 weeks, there were no significant differences in SR between the experimental groups (Figure31).

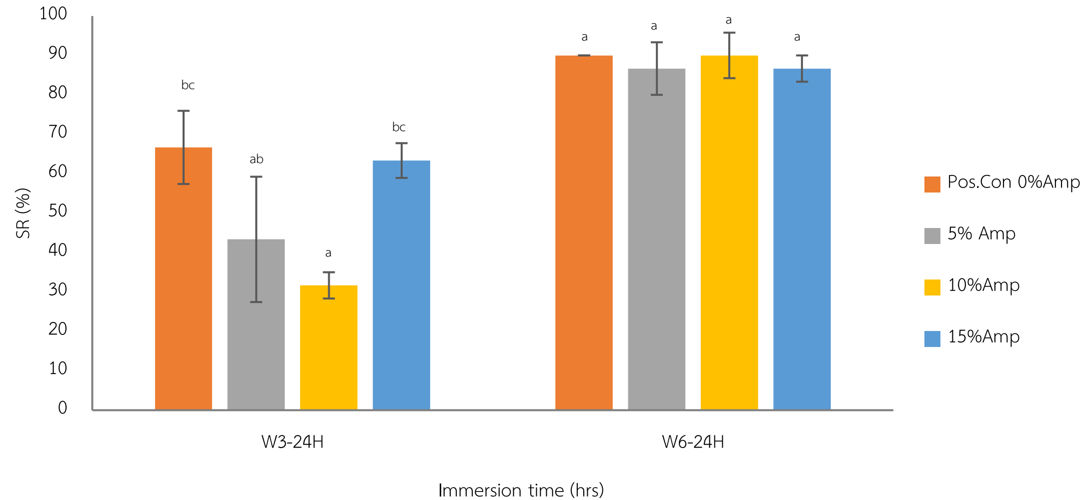


Figure 30 Survival rate of shrimp determined after 24 hrs of V. parahemolyticus immersion. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day.

4.2.2 Total hemocyte count (THC)

The results indicated that there were no significant differences in THC between the treatment groups at both the 3-week and 6-week intervals of the feeding trial. At week 3, THC ranged from $9.33 \times 10^{3} \pm 1.76 \times 10^{3}$ cell/ml to $6.20 \times 10^{4} \pm 4.64 \times 10^{4}$ cell/ml by week 6. At 3 weeks, shrimp fed with 15% *Amphora* sp.

exhibited the lowest THC, while at 6 weeks, shrimp fed with 15% *Amphora* sp. showed the highest THC. This shift in THC levels over time may indicate variations in the immune response or physiological changes in the shrimp as the feeding trial progressed (Figure 32).

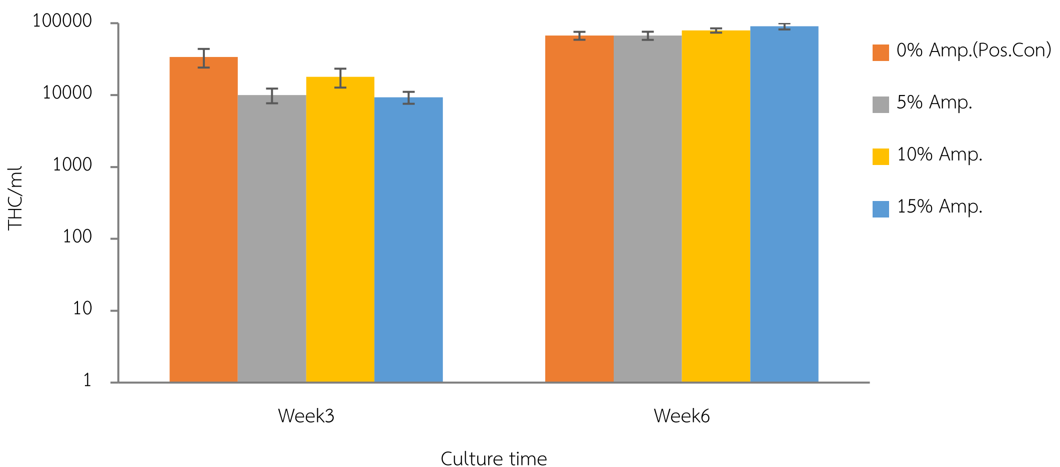


Figure 31 THC (cell/ml) of shrimp determined after 24 hrs of V. parahemolyticus immersion (Mean ± SE) between each culture day.

4.2.3 Phagocytic activity (PA)

The results showed that significant differences in PA were not observed among the experimental groups at week 3. However, at week 6, there was a significant difference in PA between different concentrations of *Amphora* sp. Interestingly, the trends in PA at these two time points were opposite.

At week 3, shrimp in control group demonstrated the highest PA compared to another group. While at week 6, those consuming a diet with 15% *Amphora* sp. exhibited significantly greater observed than another group (Figure 33). This suggests that the concentration of *Amphora* sp. has a notable impact on PA in shrimp, with distinct patterns emerging over the course of the experiment.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

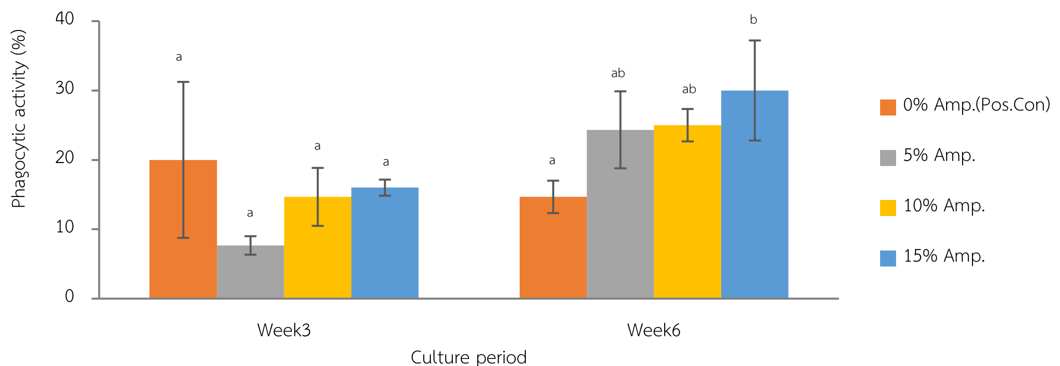


Figure 32 Phagocytic activity (%) of shrimp determined after 24 hrs of V. parahemolyticus immersion. Mean ± SE values with different lowercase superscripts are significantly ($p < 0.05$) different between each culture day.

4.2.4 Midgut intestinal epithelial area of shrimp

The results indicated variations in the intestinal epithelium area of shrimp fed a diet containing *Amphora* sp. but there were not significantly different among experimental groups. The 3-week feeding trial revealed larger intestinal epithelium areas for all experimental groups compared to the 3-week feeding trial, except for the control and 10% *Amphora* sp. group, where the intestinal epithelium area remained similar. Throughout the 3-week feeding trial, the intestinal epithelium area varied within each experimental group, ranging from 2,691.54 μm^2 to 4,608.95 μm^2. In the 6-week feeding trial, the intestinal epithelium area for each experiment group exhibited a range of 2,639.40 μm^2 to 4,296.58 μm^2 (Figure 34).

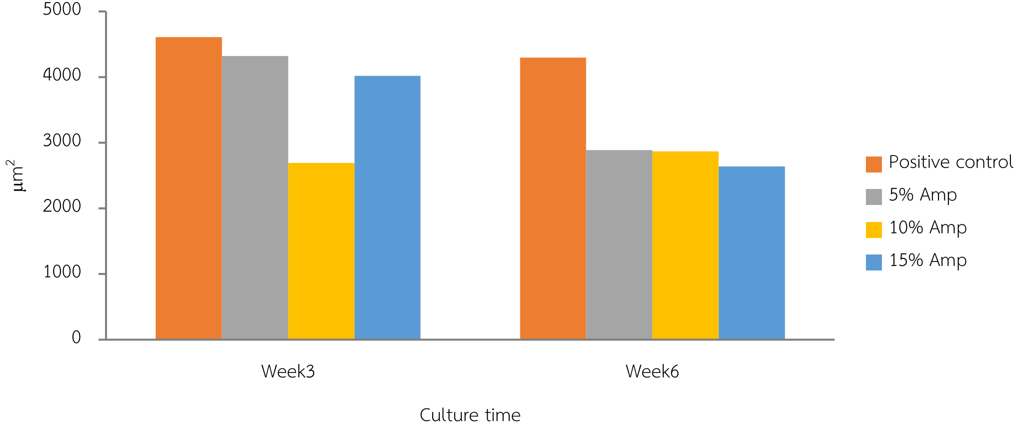
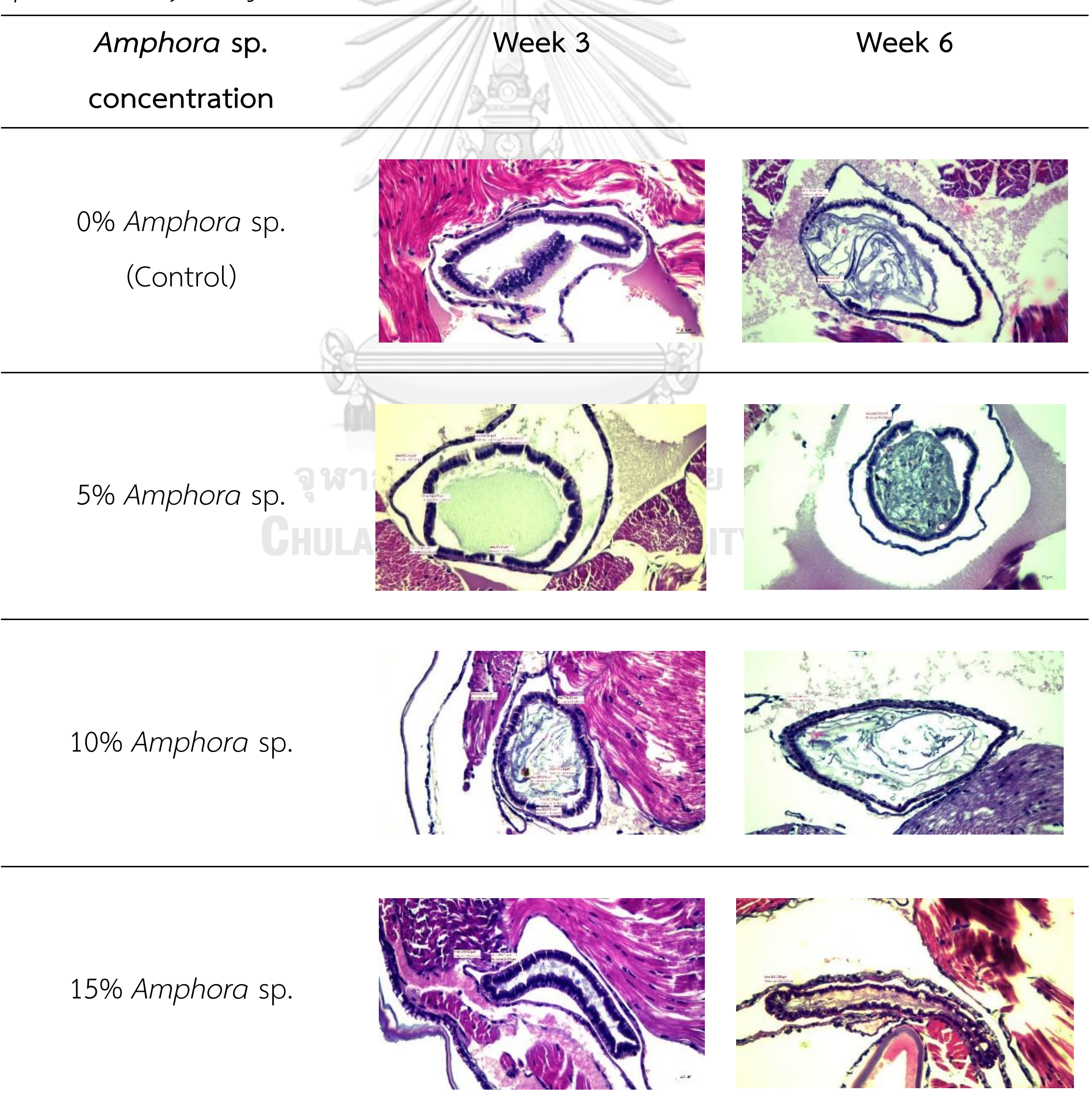


Figure 33 Midgut intestinal epithelial area of shrimp determined after 24 hrs of V. parahemolyticus immersion (Mean ± SE) between each culture day.

Midgut intestinal epithelium was observed by staining with hematoxylin and eosin (H&E). The findings indicated that there were no histopathological alterations in the midgut of shrimp intestines following the consumption of a diet containing *Amphora* sp. However, epithelial hyperplasia was observed in shrimp fed both the *Amphora* sp.-containing diet and the non-*Amphora* sp. diet. Furthermore, no changes were detected in the other structures of the intestinal tissue (Table 9).

Table 9 Midgut intestinal epithelial of shrimp after immersion with V. parahaemolyticus for 24 hrs



***Amphora* sp. concentration**	**Week 3**	**Week 6**
0% *Amphora* sp. (Control)		
5% *Amphora* sp.		
10% *Amphora* sp.		
15% *Amphora* sp.		

4.2.5 Expression of Anti-lipopolysaccharide factors: LvALF B gene

The findings revealed that the expression of the LvALF B gene, responsible for Anti-lipopolysaccharide factors, was significantly highest in shrimp fed with 10% and 15% *Amphora* sp. compared the 0% and 5% *Amphora* sp. (Figure 35). This result related to SR after fed with diatom and after immersion with bacteria.

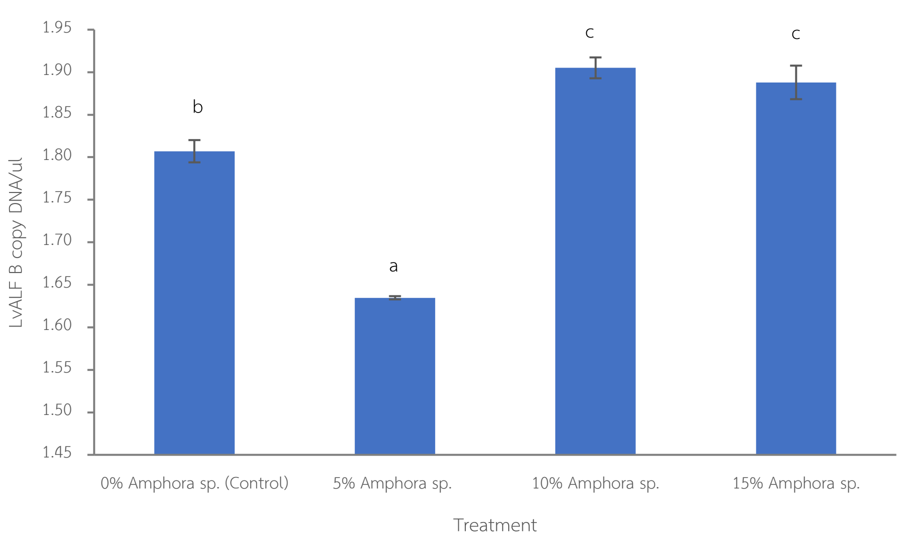


Figure 34 Expression of LvALF B gene determined in hepatopancrease of 6 week feeding trial shrimp, after 24 hrs of V. parahemolyticus immersion, mean ± SE The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5. Discussion

5.1 Growth performance

The results of the study revealed a noticeable trend of decreasing of SR of *L. vannamei* over the six-week cultivation period. Interestingly, shrimp fed with a 15% *Amphora* sp. diet consistently exhibited the highest SR throughout the entire six weeks. The percentage of SR observed in this study was found to be lower compared to a similar study involving shrimp of the same age. Hamidoghli et al. (2019) reported a %SR of 79.3 ± 2.31% at the conclusion of their experiment in outdoor biofloc system. While the study by Ju et al. (2009), shrimp that were fed

diets containing whole diatoms, specifically *Nannochloropsis* and *Thalassiosira weissflogii*, demonstrated SR ranging from 93.8% to 100% after the eight-week feeding trial. This experimental setup involved an indoor shrimp culture facility equipped with 36 glass aquaria (52 L; 76 cm × 31 cm × 31 cm). Initial shrimp weighing between 0.94 g and 0.97 g were stocked in each aquarium at a density of 12 shrimp per aquarium (50 shrimp/m^2) which is the same culture condition, except culture water management. Our study used water exchanging while the study by Ju et al. (2009) used a flow-through of seawater (1 L/min). This factor could potentially influence the lower SR observed in the shrimp. The decreasing trend in overall SR is an intriguing observation. Despite this decline, shrimp fed with a 15% *Amphora* sp. diet consistently exhibited the highest SR throughout the entire six weeks. This highlights the potential positive impact of *Amphora* sp. on shrimp survival, indicating a promising aspect for further investigation and optimization.

In terms of MBW and SGR after 6 week or 42 days of feeding trial, the post-larvae of *L. vannamei* that were provided with a diet containing 15% *Amphora* sp. displayed the highest MBW among the tested conditions. Final MBW of shrimp fed with diet contain 15% *Amphora* sp. was 3.21 ± 0.65 g/shrimp and SGR was 31.96 ± 4.77 g/week. In addition, SGR of shrimp during the initial week to the third week, the control group exhibited the highest SGR during this period. Notably, shrimp fed with a diet containing 15% *Amphora* sp. demonstrated the significantly highest SGR during the third to the sixth week. Our study was consistent with the study of Nonwachai et al. (2010) that PL12 *L. vannamei* reared under research conditions. After 70 days of culture the average body weight of shrimp ranged from 7.57 to 7.64 g for the various treatments. Ju et al. (2009) determined that the inclusion of whole algae led to increased fatty acid and astaxanthin levels in shrimp tail muscles. The enhanced growth rates suggest potential benefits associated with incorporating dried algae as a feed additive, highlighting the advantages of cultivating phytoplankton in shrimp ponds.

The FE exhibited a distinct pattern over the course of the culture period. In the initial three weeks, FE was comparatively lower, ranging from 21.66 to 76.49. Notably, during this phase, the control group appeared to have a more favorable

FER. However, a noticeable shift occurred in the subsequent three weeks, with FE increasing to a higher range of 149.16 to 312.81. The highest FE was observed in shrimp that were fed with a 15% *Amphora* sp. diet. In our study, the FE observed from week 0 to 3 was lower than that reported by Hamidoghli et al. (2019), but it was higher than the observed FE during weeks 3 to 6 in our study. Hamidoghli et al. (2019) demonstrated FE ranging from 87.7 ± 4.51 to 94.0 ± 1.54 at the end of an 8-week experiment. They found that the growth performance and feed utilization of juvenile white leg shrimp increased with higher dietary lipid levels, with the optimal results observed at 60–120 g/kg of lipids. However, when dietary lipids were below 60 g/kg or above 120 g/kg, there was a decline in performance metrics such as mean final body weight (FBW), weight gain (WG), SGR, FE, and protein efficiency ratio (PER). This implies that extremely low or high lipid contents may have contributed to suboptimal growth and protein utilization. FE patterns reveal a distinct change over the culture period. The lower FE in the initial three weeks, particularly favoring the control group, may be attributed to adaptation phases and efficient feed utilization.

5.2 Disease resistant of *V. parahemolyticus*

The findings suggest that a longer feeding period with *Amphora* sp. positively influences the shrimp's survival rates when exposed to *V. parahemolyticus*.

In terms of immune response, THC and PA. The variations in THC levels and PA suggest that the concentration of *Amphora* sp. in the diet influences the shrimp's immune response. The shift in THC levels over time implies dynamic changes in immune function during the feeding trial. While immune related gene which is the expression of *LvALF B*. The differential expression of the LvALF B gene among groups indicates that *Amphora* sp. concentration influences the expression of genes related to anti-lipopolysaccharide factors. The upregulation of the LvALF B gene appears to be associated with enhanced growth performance and disease resistance in shrimp. Shrimp exposed to higher concentrations of *Amphora* sp. exhibited elevated expression of this gene, correlating with improved growth and heightened resistance to diseases.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Pacific white shrimp rely on innate immune responses for detecting and eliminating pathogens (Liang et al, 2020). THC has been identified as a potential indicator of immune status in crustaceans, and a diminished count of circulating hemocytes in crustaceans is well-associated with decreased resistance to pathogens (S. R. Sharma et al., 2010). Utomo et al. (2023) demonstrated that after coinfection of myonecrosis virus (IMNV) and *V. parahaemolyticus*, there were notable increases in THC and lipopolysaccharide gene. Tinwongger et al. (2019) stated that anti-lipopolysaccharide factor (ALF) is a compact polypeptide (comprising 114–124 amino acid residues) belonging to the antimicrobial peptide family. ALFs have been subsequently found in various crustaceans, including crabs, crayfish, lobsters, and shrimp. In penaeid shrimp, ALFs play diverse roles in immunity, participating in antibacterial, antifungal, and antiviral activities. The expression of LvALF AV-R suggested the inducing of the hepatopancreas of shrimp in response to the presence of the Vp_PirABlike toxin, carried by pathogenic *V. parahaemolyticus* with a plasmid encoding Vp_PirAB-like toxins.

In addition, digestive morphology, the variations in the intestinal epithelium area indicate that the effects of *Amphora* sp. on the shrimp's intestinal morphology are complex. The consistency in the area for the control and 10% *Amphora* sp. group over the six weeks may suggest stability in these groups. The intestine in vertebrates plays a critical role in metabolism, nutrient absorption, and immune function (Liang et al, 2020).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

6. Conclusion

In conclusion, the concentration of *Amphora* sp. in the shrimp diet has multifaceted effects on survival rates, immune responses, intestinal morphology, and gene expression related to disease resistance. The findings underscore the importance of considering both concentration and duration of *Amphora* sp. supplementation in shrimp diets for optimal disease resistance and overall health.

The observed trends collectively suggest that the inclusion of 15% *Amphora* sp. in the diet positively influences survival rates, mean body weight, specific growth rate, and feed efficiency in *L. vannamei*. Further investigation into the specific

nutritional components of *Amphora* sp. responsible for these effects, as well as potential long-term impacts on shrimp health and productivity, would be valuable for optimizing aquaculture practices. Additionally, considerations for economic feasibility and sustainable sourcing of *Amphora* sp. should be explored for practical implementation in shrimp farming.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chapter V

Conclusion

1. Lipid content screening of *Amphora* sp. isolated from the Gulf of Thailand for use in the *Litopenaeus vannamei* diet preparation

According to their fatty acid profiles, ALY and ASC would be considered as n-3 PUFAs producing diatoms. When their growth performances are considered, ALY and ASC are also showing the highest biomass production when compared to the other 2 strains. ALY is considered the best candidate for biomass production in shorter period while ASC requires longer time, but it is more tolerant to nutrient depletion than the other strains. In this chapter, the result provides the basic knowledge for biomass production of *Amphora* sp. that isolated from Gulf of Thailand that were cultured under the indoor condition. ALY and ASC could be considered for further application as potential strains for high productivity and n-3 PUFAs production.

2. The optimum light intensity and culture medium concentration for *Amphora* sp. cultivation

ASC cultured with MLI-F/20 and ALY cultured with MLI-F/20 can provide maximum biomass on day 6 of culture. ALY cultured with MLI-F/20 can produce the higher cell density than ASC cultured with MLI-F/20. Evaluation total lipid content and fatty acid composition, this culture condition also provided significantly highest result in all SFAs, and many important UFAs content compared to ASC cultured with MLI-F/20. Therefore, ALY cultured with MLI-F/20 was chosen for further experiment as shrimp supplementary diet.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. The efficiency of diet with additional diatoms as immuno-stimulant for *Litopenaeus vannamei*

The concentration of *Amphora* sp. within shrimp diets significantly impacts survival rates, disease resistance, and immune-related gene expression LvALF B. This highlights the need to carefully consider both the concentration and duration of *Amphora* sp. supplementation for maximizing disease resistance and overall shrimp health.

Observations indicate that a 15% inclusion of *Amphora* sp. positively affects survival rates, mean body weight, specific growth rate, and feed efficiency in *L. vannamei*. Exploring the specific nutritional components responsible for these effects and assessing potential long-term impacts on shrimp health and productivity would greatly enhance aquaculture practices.

REFERENCES

AFSSA. (2010). Opinion of the French Food Safety Agency on the request for an assessment of the initial assessment report by the Dutch authorities concerning the placing on the market of a novel food ingredient: bovine lactoferrin. *Maisons-Alfort, 3 June 2010 AFSSA Request No 2010-SA-0112,* , 1-16.

An, W., He, H., Dong, X., Tan, B., Yang, Q., Chi, S., Zhang, S., Liu, H., & Yang, Y. (2020). Regulation of growth, fatty acid profiles, hematological characteristics and hepatopancreatic histology by different dietary n-3 highly unsaturated fatty acids levels in the first stages of juvenile Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture Reports, 17*.

Araújo, B. C., Mata-Sotres, J. A., Viana, M. T., Tinajero, A., & Braga, A. (2019). Fish oil-free diets for Pacific white shrimp *Litopenaeus vannamei*: The effects of DHA-EPA supplementation on juvenile growth performance and muscle fatty acid profile. *Aquaculture, 511*.

Avin, F. A. (2020). *Easy way to count spores and prepare spore suspension by Hemocytometer*: Otis L. Floyd Nursery Research Center Tennessee State University.

Bélanger-Lamonde, A., Sarker, P. K., Ayotte, P., Bailey, J. L., Bureau, D. P., Chouinard, P. Y., Dewailly, É., Leblanc, A., Weber, J.-P., & Vandenberg, G. W. (2018). Algal and Vegetable Oils as Sustainable Fish Oil Substitutes in Rainbow Trout Diets: An Approach to Reduce Contaminant Exposure. *Journal of Food Quality, 2018*, 1-12.

BenMoussa-Dahmen, I., Chtourou, H., Rezgui, F., Sayadi, S., & Dhouib, A. (2016). Salinity stress increases lipid, secondary metabolites and enzyme activity in Amphora subtropica and Dunaliella sp. for biodiesel production. *Bioresour Technol, 218*, 816-825.

Bertozzini, E., Galluzzi, L., Penna, A., & Magnani, M. (2014). Enhancing neutral lipid content in Skeletonema marinoi through multiple phase growth in a bench photobioreactor. *Algal Research, 5*, 32-36.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Boukhris, S., Athmouni, k., Hamza-Mnif, I., Siala-Elleuch, R., Ayadi, H., Nasri, M., & Sellami-Kamoun, A. (2017). The Potential of a Brown Microalga Cultivated in High Salt Medium for the Production of High-Value Compounds. *Hindawi BioMed Research International*, 1 - 10.

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., & Wijffels, R. H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresour Technol, 124*, 217-226.

Bumbak, F., Cook, S., Zachleder, V., Hauser, S., & Kovar, K. (2011). Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. *Appl Microbiol Biotechnol, 91*(1), 31-46.

Burrows, E. H., Bennette, N. B., Carrieri, D., Dixon, J. L., Brinker, A., Frada, M., Baldassano, S. N., Falkowski, P. G., & Dismukes, G. C. (2012). Dynamics of Lipid Biosynthesis and Redistribution in the Marine Diatom *Phaeodactylum tricornutum* Under Nitrate Deprivation. *BioEnergy Research, 5*(4), 876-885.

Candela, C. G., López, L. M. B., & Kohen, V. L. (2011). Importance of a balanced omega 6/omega 3 ratio for the maintenance of health. Nutritional recommendations. *Nutr Hosp., 26*(2), 323-329.

Carvalho, A. P., Monteiro, C. M., & Malcata, F. X. (2009). Simultaneous effect of irradiance and temperature on biochemical composition of the microalga *Pavlova lutheri. Journal of Applied Phycology, 21*(5), 543-552.

Charoonnart, P., Purton, S., & Saksmerprome, V. (2018). Applications of Microalgal Biotechnology for Disease Control in Aquaculture. *Biology (Basel), 7*(2).

Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., & Chang, J. S. (2011). Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. *Bioresour Technol, 102*(1), 71-81.

Chen, Y.-C. (2012). The biomass and total lipid content and composition of twelve species of marine diatoms cultured under various environments. *Food Chemistry, 131*(1), 211-219.

Chen, Y. Y., Chen, J. C., Tayag, C. M., Li, H. F., Putra, D. F., Kuo, Y. H., Bai, J. C., & Chang, Y. H. (2016). *Spirulina elicits* the activation of innate immunity and increases resistance against *Vibrio alginolyticus* in shrimp. *Fish Shellfish Immunol, 55*, 690-

698.

Cheng, J., Feng, J., Sun, J., Huang, Y., Zhou, J., & Cen, K. (2014). Enhancing the lipid content of the diatom *Nitzschia* sp. by 60 Co-γ irradiation mutation and high-salinity domestication. *Energy, 78*, 9-15.

Chuchird, N., Rorkwiree, P., & Rairat, T. (2015). Effect of dietary formic acid and astaxanthin on the survival and growth of Pacific white shrimp (*Litopenaeus vannamei*) and their resistance to *Vibrio parahaemolyticus. SpringerPlus, 4*, 440.

Cointet, E., Wielgosz-Collin, G., Bougaran, G., Rabesaotra, V., Goncalves, O., & Meleder, V. (2019). Effects of light and nitrogen availability on photosynthetic efficiency and fatty acid content of three original benthic diatom strains. *PLoS One, 14*(11), e0224701.

Cointet, E., Wielgosz-Collin, G., Méléder, V., & Gonçalves, O. (2019). Lipids in benthic diatoms: A new suitable screening procedure. *Algal Research, 39*.

d'Ippolito, G., Sardo, A., Paris, D., Vella, F. M., Adelfi, M. G., Botte, P., Gallo, C., & Fontana, A. (2015). Potential of lipid metabolism in marine diatoms for biofuel production. *Biotechnol Biofuels, 8*, 28.

da Silva Ferreira, V., & Sant'Anna, C. (2017). Impact of culture conditions on the chlorophyll content of microalgae for biotechnological applications. *World J Microbiol Biotechnol, 33*(1), 20.

de la Peña, M. R. (2007). Cell growth and nutritive value of the tropical benthic diatom, *Amphora* sp., at varying levels of nutrients and light intensity, and different culture locations. *Journal of Applied Phycology, 19*(6), 647-655.

de la Vega, E., O'Leary, N. A., Shockey, J. E., Robalino, J., Payne, C., Browdy, C. L., Warr, G. W., & Gross, P. S. (2008). Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol Immunol, 45*(7), 1916-1925.

Desbois, A. P., & Lawlor, K. C. (2013). Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. *Mar Drugs, 11*(11), 4544-4557.

DurmuŞ, M. (2019). Fish oil for human health: omega-3 fatty acid profiles of marine

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

seafood species. *Food Science and Technology, 39*(suppl 2), 454-461.

El Arroussi, H., Benhima, R., El Mernissi, N., Bouhfid, R., Tilsaghani, C., Bennis, I., & Wahby, I. (2017). Screening of marine microalgae strains from Moroccan coasts for biodiesel production. *Renewable Energy, 113*, 1515-1522.

Encarnação, T., Burrows, H. D., Pais, A. C., Campos, M. G., & Kremer, A. (2012). Effect of N and P on the Uptake of Magnesium and Iron and on the Production of Carotenoids and Chlorophyll by the Microalgae *Nannochloropsis* sp. *Journal of Agricultural Science and Technology, A2*, 824-832.

Fan, J., Cui, Y., Wan, M., Wang, W., & Li, Y. (2014). Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. *Biotechnology for Biofuels 7*(17), 1-14.

Fard, M. B., Mirbagheri, S. A., Pendashteh, A., & Alavi, J. (2020). Estimation of effluent parameters of slaughterhouse wastewater treatment with artificial neural network and B-spline quasi interpolation. *International Journal of Environmental Research, 14*, 527-539.

Ferreira, V. S., Pinto, R. F., & Sant'Anna, C. (2016). Low light intensity and nitrogen starvation modulate the chlorophyll content of *Scenedesmus dimorphus*. *J Appl Microbiol, 120*(3), 661-670.

Gabriel, A.-G., Jesus, G. S.-M., Angel, I. C.-C., Antonio, L.-G., & Felipe, A. (2009). Penaeid Shrimp Immune System. *Thai Journal of Veterinary Medicine, 39*(3), 205-215.

Gao, Y., Zheng, S. C., Zheng, C. Q., Shi, Y. C., Xie, X. L., Wang, K. J., & Liu, H. P. (2018). The immune-related fatty acids are responsive to CO(2) driven seawater acidification in a crustacean brine shrimp Artemia sinica. *Dev Comp Immunol, 81*, 342-347.

Gayoso, L., Ansorena, D., & Astiasaran, I. (2019). DHA rich algae oil delivered by O/W or gelled emulsions: strategies to increase its bioaccessibility. *J Sci Food Agric, 99*(5), 2251-2258.

Ge, H., Li, J., Chang, Z., Chen, P., Shen, M., & Zhao, F. (2016). Effect of microalgae with semicontinuous harvesting on water quality and zootechnical performance of white shrimp reared in the zero water exchange system. *Aquacultural*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Engineering, 72-73, 70-76.

González-Félix, M. L., Gatlin, D. M., Lawrence, A. L., & Perez-Velazquez, M. (2002). Effect of Various Dietary Lipid Levels on Quantitative Essential Fatty Acid Requirements of Juvenile Pacific White Shrimp *Litopenaeus vannamei. Journal of the World Aquaculture Society, 33*(3), 330-340.

Grootveld, M., Percival, B. C., & Grootveld, K. L. (2018). Chronic non-communicable disease risks presented by lipid oxidation products in fried foods. *Hepatobiliary Surg Nutr, 7*(4), 305-312.

Hamidoghli, A., Won, S., Aya, F. A., Yun, H., Bae, J., Jang, I. K., & Bai, S. C. (2019). Dietary lipid requirement of whiteleg shrimp *Litopenaeus vannamei*
juveniles cultured in biofloc system. *Aquaculture Nutrition, 26*(3), 603-612.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zohar, Y., & Place, A. R. (2002). Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. *Aquaculture, 213*(1-4), 347-362.

He, Q., Yang, H., Wu, L., & Hu, C. (2015). Effect of light intensity on physiological changes, carbon allocation and neutral lipid accumulation in oleaginous microalgae. *Bioresour Technol, 191*, 219-228.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J, 54*(4), 621-639.

Jana, P., Sahu, N. P., Sardar, P., Varghese, T., Deo, A. D., Shamna, N., Harikrishna, V., Paul, M., Chuphal, N., & Krishna, G. (2022). Dietary lipid requirement of juvenile white-leg shrimp, *Penaeus vannamei* (Boone, 1931) reared in inland ground saline water of 15 ppt. *Aquaculture Research, 53*(15), 5270-5286.

Javahery, S., Noori, A., & Hoseinifar, S. H. (2019). Growth performance, immune response, and digestive enzyme activity in Pacific white shrimp, *Penaeus vannamei* Boone, 1931, fed dietary microbial lysozyme. *Fish Shellfish Immunol, 92*, 528-535.

Jiang, X., Han, Q., Gao, X., & Gao, G. (2016). Conditions optimising on the yield of biomass, total lipid, and valuable fatty acids in two strains of *Skeletonema*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

menzelii. Food Chem, 194, 723-732.

Jonasdottir, S. H. (2019). Fatty Acid Profiles and Production in Marine Phytoplankton. *Mar Drugs, 17*(3).

Ju, Z. Y., Forster, I. P., & Dominy, W. G. (2009). Effects of supplementing two species of marine algae or their fractions to a formulated diet on growth, survival and composition of shrimp (*Litopenaeus vannamei*). *Aquaculture, 292*(3-4), 237-243.

Katan, T., Caballero-Solares, A., Taylor, R. G., Rise, M. L., & Parrish, C. C. (2019). Effect of plant-based diets with varying ratios of omega6 to omega3 fatty acids on growth performance, tissue composition, fatty acid biosynthesis and lipid-related gene expression in Atlantic salmon (Salmo salar). *Comp Biochem Physiol Part D Genomics Proteomics, 30*, 290-304.

Katiyar, R., & Arora, A. (2020). Health promoting functional lipids from microalgae pool: A review. *Algal Research, 46*.

Khumaidi, A., Iranawati, F., Kilawati, Y., Yanuhar, U., & Fadjar, M. (2020). Morphology, molecular, and nutritional value of *Amphora* sp. from coastal water of the grouper cultivation center (Situbondo, Indonesia). *Ecology, Environment and Conservation Journal, 26*(2), 943-949.

Khwancharoen, C., Chotipan, N., Nawatmai, T., & Direkbussarakom, S. (2021). Effects of Nitrogen Sources on the Growth and Biochemical Composition of Diatom (*Amphora coffeaeformis*) Used for Shrimp Larviculture. *Walailak Journal of Science and Technology (WJST), 18*(4).

Kumar, B. R., Deviram, G., Mathimani, T., Duc, P. A., & Pugazhendhi, A. (2019). Microalgae as rich source of polyunsaturated fatty acids. *Biocatalysis and Agricultural Biotechnology, 17*, 583-588.

Labreuche, Y., O'Leary, N. A., de la Vega, E., Veloso, A., Gross, P. S., Chapman, R. W., Browdy, C. L., & Warr, G. W. (2009). Lack of evidence for *Litopenaeus vannamei* Toll receptor (lToll) involvement in activation of sequence-independent antiviral immunity in shrimp. *Dev Comp Immunol, 33*(7), 806-810.

LeGresley, M., & McDermott, G. (2010). Counting chamber methods for quantitative phytoplankton analysis - haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell. In B. Karlson, C. Cusack, & E. Bresnan (Eds.), *Microscopic and*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Molecular Methods for Quantitative Phytoplankton Analysis (pp. 25 - 30). Paris: UNESCO.

Leyland, B., Boussiba, S., & Khozin-Goldberg, I. (2020). A Review of Diatom Lipid Droplets. *Biology (Basel), 9*(2).

Li, L., Cui, J., Liu, Q., Ding, Y., & Liu, J. (2015). Screening and phylogenetic analysis of lipid-rich microalgae. *Algal Research, 11*, 381-386.

Li, X., Pan, Y., & Hu, H. (2018). Identification of the triacylglycerol lipase in the chloroplast envelope of the diatom *Phaeodactylum tricornutum*. *Algal Research, 33*, 440-447.

Li, X., Wang, Y., Li, H., Jiang, X., Ji, L., Liu, T., & Sun, Y. (2021). Chemical and quality evaluation of Pacific white shrimp *Litopenaeus vannamei:* Influence of strains on flesh nutrition. *Food Sci Nutr, 9*(10), 5352-5360.

Liang, F., Li, C., Hou, T., Wen, C., Kong, S., Ma, D., Sun, C., & Li, S. (2020). Effects of Chitosan–Gentamicin Conjugate Supplement on Non-Specific Immunity, Aquaculture Water, Intestinal Histology and Microbiota of Pacific White Shrimp (*Litopenaeus vannamei*). *Marine drugs, 18*(149).

Lim, Y.-K., Phang, S.-M., Sturges, W. T., Malin, G., & Rahman, N. B. A. (2017). Emission of short-lived halocarbons by three common tropical marine microalgae during batch culture. *Journal of Applied Phycology, 30*(1), 341-353.

Liu, B., & Benning, C. (2013). Lipid metabolism in microalgae distinguishes itself. *Curr Opin Biotechnol, 24*(2), 300-309.

Luo, K., Liu, Y., Qin, G., Wang, S., Wei, C., Pan, M., Guo, Z., Liu, Q., and, & Tian, X. (2023). A comparative study on effects of dietary three strains of lactic acid bacteria on the growth performance, immune responses, disease resistance and intestinal microbiota of Pacific white shrimp, *Penaeus vannamei. Fish and Shellfish Immunology, 136*(108707), 12.

Lv, J. M., Cheng, L. H., Xu, X. H., Zhang, L., & Chen, H. L. (2010). Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. *Bioresour Technol, 101*(17), 6797-6804.

Macias-Sancho, J., Poersch, L. H., Bauer, W., Romano, L. A., Wasielesky, W., & Tesser, M. B. (2014). Fishmeal substitution with Arthrospira (*Spirulina platensis*) in a

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

practical diet for *Litopenaeus vannamei*: Effects on growth and immunological parameters. *Aquaculture, 426-427*, 120-125.

Maisons-Alfort. (2010). *Opinion of the French Food Safety Agency on the request for an assessment of the initial assessment report by the Dutch authorities concerning the placing on the market of a novel food ingredient: bovine lactoferrin*.

Mansour, A. T., Ashour, M., Abbas, E. M., Alsaqufi, A. S., Kelany, M. S., El-Sawy, M. A., & Sharawy, Z. Z. (2022). Growth Performance, Immune-Related and Antioxidant Genes Expression, and Gut Bacterial Abundance of Pacific White Leg Shrimp, *Litopenaeus vannamei,* Dietary Supplemented With Natural Astaxanthin. *Front Physiol, 13*, 874172.

Mastan, S. A. (2015). Use of Immunostimulants in aquaculture disease management. *International Journal of Fisheries and Aquatic Studies, 2*(4), 277 - 280.

Mohammad Mirzaie, M. A., Kalbasi, M., Mousavi, S. M., & Ghobadian, B. (2016). Investigation of mixotrophic, heterotrophic, and autotrophic growth of *Chlorella vulgaris* under agricultural waste medium. *Prep Biochem Biotechnol, 46*(2), 150-156.

Molina-Cárdenas, C. A., & Sánchez-Saavedra, M. d. P. (2017). Inhibitory effect of benthic diatom species on three aquaculture pathogenic vibrios. *Algal Research, 27*, 131-139.

Nagappan, S., Das, P., AbdulQuadir, M., Thaher, M., Khan, S., Mahata, C., Al-Jabri, H., Vatland, A. K., & Kumar, G. (2021). Potential of microalgae as a sustainable feed ingredient for aquaculture. *Journal of Biotechnology, 341*, 1-20.

Nomaguchi, T., Maeda, Y., Liang, Y., Yoshino, T., Asahi, T., & Tanaka, T. (2018). Comprehensive analysis of triacylglycerol lipases in the oleaginous diatom *Fistulifera solaris* JPCC DA0580 with transcriptomics under lipid degradation. *J Biosci Bioeng, 126*(2), 258-265.

Nonwachai, T., Purivirojkul, W., Limsuwan, C., Chuchird, N., Velasco, M., & Dhar, A. K. (2010). Growth, nonspecific immune characteristics, and survival upon challenge with *Vibrio harveyi* in Pacific white shrimp (*Litopenaeus vannamei*) raised on diets containing algal meal. *Fish Shellfish Immunol, 29*(2), 298-304.

Ozkan, A., & Rorrer, G. L. (2017). Effects of light intensity on the selectivity of lipid and

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

chitin nanofiber production during photobioreactor cultivation of the marine diatom Cyclotella sp. *Algal Research, 25*, 216-227.

Ozogul, Y., Ozogul, F., Cicek, E., Polat, A., & Kuley, E. (2009). Fat content and fatty acid compositions of 34 marine water fish species from the Mediterranean Sea. *Int J Food Sci Nutr, 60*(6), 464-475.

Paliwal, C., Mitra, M., Bhayani, K., Bharadwaj, S. V. V., Ghosh, T., Dubey, S., & Mishra, S. (2017). Abiotic stresses as tools for metabolites in microalgae. *Bioresour Technol, 244*(Pt 2), 1216-1226.

Parvin, M., Zannat, M. N., & Habib, M. A. B. (2007). Two Important Techniques for Isolation of Microalgae. *Asian Fisheries Science, 20*(1).

Piggott, G. M., & Tucker, B. W. (1990). *Seafood: Effects of Technology on Nutrition*.

Rani, V., Deepika, S., Abrna, K., & Uma, A. (2022). Isolation, identification, and optimization of growth conditions for the marine microalgae isolated from the Gulf of Mannar, South-east coast of India. *Regional Studies in Marine Science, 51*.

Reitan, K. I., Øie, G., Jørgensen, H., & Wang, X. (2021). Chemical composition of selected marine microalgae, with emphasis on lipid and carbohydrate production for potential use as feed resources. *Journal of Applied Phycology, 33*(6), 3831-3842.

Romero-Romero, C. C., & Sánchez-Saavedra, M. d. P. (2017). Effect of light quality on the growth and proximal composition of *Amphora* sp. *Journal of Applied Phycology, 29*(3), 1203-1211.

Ruivo, M., Amorim, A., & Cartaxana, P. (2011). Effects of growth phase and irradiance on phytoplankton pigment ratios: implications for chemotaxonomy in coastal waters. *Journal of Plankton Research, 33*(7), 1012-1022.

Sajjadi, B., Chen, W.-Y., Raman, A. A. A., & Ibrahim, S. (2018). Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition. *Renewable and Sustainable Energy Reviews, 97*, 200-232.

Santos-Ballardo, D. U., Rossi, S., Hernández, V., Gómez, R. V., del Carmen Rendón-Unceta, M., Caro-Corrales, J., & Valdez-Ortiz, A. (2015). A simple spectrophotometric method for biomass measurement of important microalgae

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

species in aquaculture. *Aquaculture, 448*, 87-92.

Sarker, P. K., Kapuscinski, A. R., McKuin, B., Fitzgerald, D. S., Nash, H. M., & Greenwood, C. (2020). Microalgae-blend tilapia feed eliminates fishmeal and fish oil, improves growth, and is cost viable. *Sci Rep, 10*(1), 19328.

Saxena, A., Marella, T. K., Singh, P. K., & Tiwari, A. (2021). Indoor mass cultivation of marine diatoms for biodiesel production using induction plasma synthesized nanosilica. *Bioresour Technol, 332*, 125098.

Saxena, A., Mishra, B., & Tiwari, A. (2022). Mass cultivation of marine diatoms using local salts and its impact on growth and productivity. *Bioresour Technol, 352*, 127128.

Sayanova, O., Mimouni, V., Ulmann, L., Morant-Manceau, A., Pasquet, V., Schoefs, B., & Napier, J. A. (2017). Modulation of lipid biosynthesis by stress in diatoms. *Philos Trans R Soc Lond B Biol Sci, 372*(1728).

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O., & Hankamer, B. (2008). Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *BioEnergy Research, 1*(1), 20-43.

Seraspe, E. B., Gabotero, S., De la Peña, M. R., Pahila, I. G., & Amar, E. C. (2014). Evaluation of dietary freeze-dried Chaetoceros calcitrans supplementation to control Vibrio harveyi infection on Penaeus monodon juvenile. *Aquaculture, 432*, 212-216.

Setyawan, A., Isnansetyo, A., Murwantoko, Indarjulianto, S., & Handayani, C. R. (2018). Comparative immune response of dietary fucoidan from three Indonesian brown algae in white shrimp *Litopenaeus vannamei. AACL Bioflux, 11*(6), 1107 - 1723.

Setyawan, A., Riana, Supono, Hudaidah, S., & Fidyandini, H. P. (2021). Non-specific immune response of Pacific white shrimp *Litopenaeus vannamei* by supplementation of sodium alginate of Sargassum collected from Lampung Indonesia. *IOP Conference Series: Earth and Environmental Science, 890* (012015), 1-8.

Shakweer, M. S., Elshopakey, G. E., Abdelwarith, A. A., Younis, E. M., Davies, S. J., & Elbahnaswy, S. (2023). Comparison of Immune Response of *Litopenaeus vannamei* Shrimp Naturally Infected with Vibrio Species, and after Being Fed

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

with Florfenicol. *Fishes, 8*(3).

Sharma, K. K., Schuhmann, H., & Schenk, P. M. (2012). High Lipid Induction in Microalgae for Biodiesel Production. *Energies, 5*(5), 1532-1553.

Sharma, S. R., Shankar, K. M., Sathyanarayana, M. L., Sahoo, A. K., Patil, R., Narayanaswamy, H. D., & Rao, S. (2010). Evaluation of immune response and resistance to diseases in tiger shrimp, *Penaeus monodon* fed with biofilm of Vibrio alginolyticus. *Fish Shellfish Immunol, 29*(5), 724-732.

Silveira, A. S., Matos, G. M., Falchetti, M., Ribeiro, F. S., Bressan, A., Bachere, E., Perazzolo, L. M., & Rosa, R. D. (2018). An immune-related gene expression atlas of the shrimp digestive system in response to two major pathogens brings insights into the involvement of hemocytes in gut immunity. *Dev Comp Immunol, 79*, 44-50.

Simopoulos, A. (2003). Importance of the Ratio of Omega-6/Omega-3 Essential Fatty Acids: Evolutionary Aspects. *World Rev Nutr Diet., 92*, 1-22.

Sonthi, M. (2016). Use of immunostimulants in marine shrimps. *Khon Kaen Agriculture Journal 44*(2), 373 - 382.

Sprague, M., Walton, J., Campbell, P. J., Strachan, F., Dick, J. R., & Bell, J. G. (2015). Replacement of fish oil with a DHA-rich algal meal derived from Schizochytrium sp. on the fatty acid and persistent organic pollutant levels in diets and flesh of Atlantic salmon (*Salmo salar*, L.) post-smolts. *Food Chem, 185*, 413-421.

Suh, S. S., Kim, S. J., Hwang, J., Park, M., Lee, T. K., Kil, E. J., & Lee, S. (2015). Fatty acid methyl ester profiles and nutritive values of 20 marine microalgae in Korea. *Asian Pac J Trop Med, 8*(3), 191-196.

Suroy, M., Moriceau, B., Boutorh, J., & Goutx, M. (2014). Fatty acids associated with the frustules of diatoms and their fate during degradation—A case study in Thalassiosira weissflogii. *Deep Sea Research Part I: Oceanographic Research Papers, 86*, 21-31.

Sutthangkul, J., Amparyup, P., Eum, J. H., Strand, M. R., & Tassanakajon, A. (2017). Anti-melanization mechanism of the white spot syndrome viral protein, WSSV453, via interaction with shrimp proPO-activating enzyme, PmproPPAE2. *J Gen Virol, 98*(4), 769-778.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Tinwongger, S., Thawonsuwan, J., Kondo, H., & Hirono, I. (2019). Identification of an anti-lipopolysaccharide factor AV-R isoform (LvALF AV-R) related to Vp_PirAB-like toxin resistance in *Litopenaeus vannamei. Fish Shellfish Immunol, 84,* 178-188.

Turchini, G. M., Ng, W. K., & Tocher, D. R. (2010). *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feed*. New York: CRC press.

Upadhyay, S., Lawrence, K., Shekhar, S. K., & Anantharaman, P. (2017). Lipid Screening, Extraction and Production of Biodiesel from Marine Diatom *Odontella aurita. International Journal of Current Microbiology and Applied Sciences, 6*(3), 1259-1269.

Utomo, A. S., Yuhana, M., Widanarni, W., & Afiff, U. (2023). Immune response, gene expression, and intestinal microbial composition of Pacific white shrimp fed with multispecies synbiotic for the prevention of coinfection disease. *Aquac Int, 31*(1), 53-64.

Wang, P., Park, B. S., Kim, J. H., Kim, J.-H., Lee, H.-O., & Han, M.-S. (2014). Phylogenetic position of eight *Amphora sensu* lato (Bacillariophyceae) species and comparative analysis of morphological characteristics. *Algae, 29*(2), 57-73.

Wase, N., Black, P., & DiRusso, C. (2018). Innovations in improving lipid production: Algal chemical genetics. *Prog Lipid Res, 71*, 101-123.

Wei, Y., Wang, X., Xie, F., Shen, H., Gao, W., Zhang, W., & Mai, K. (2022). Influences of replacing dietary fish meal by Antarctic krill meal on growth performance, immunity and muscle quality of white shrimp *Litopenaeus vannamei. Aquaculture Reports, 25*.

Widianingsih, Hartati, R., Endrawati, H., & Mamuaja, J. (2013). Fatty acid composition of marine microalgae in Indonesia. *Journal of tropical biology and conservation, 10*, 75 - 82.

Xue, L. L., Chen, H. H., & Jiang, J. G. (2017). Implications of glycerol metabolism for lipid production. *Prog Lipid Res, 68*, 12-25.

Yerlikaya, P., Topuz, O. K., Buyukbenli, H. A., & Gokoglu, N. (2013). Fatty Acid Profiles of Different Shrimp Species: Effects of Depth of Catching. *J. Aquat. Food Prod. Technol, 22*(3), 290-297.

Yocawibun, P. (2011). *Cloning and expression analysis of genes in the G protein*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

pathway in the black tiger shrimp Penaeus monodon. (Master of Science), Chulalongkorn university, Thailand.

Yodsuwan, N., Sawayama, S., & Sirisansaneeyakul, S. (2017). Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom *Phaeodactylum tricornutum*. *Agriculture and Natural Resources, 51*(3), 190-197.

Young, E. B., Reed, L., & Berges, J. A. (2022). Growth parameters and responses of green algae across a gradient of phototrophic, mixotrophic and heterotrophic conditions. *PeerJ, 10*, e13776.

Yu, E. T., Zendejas, F. J., Lane, P. D., Gaucher, S., Simmons, B. A., & Lane, T. W. (2009). Triacylglycerol accumulation and profiling in the model diatoms Thalassiosira pseudonana and *Phaeodactylum tricornutum* (Baccilariophyceae) during starvation. *Journal of Applied Phycology, 21*(6), 669-681.

Yu, Y., Liu, Y., Yin, P., Zhou, W., Tian, L., Liu, Y., Xu, D., & Niu, J. (2020). Astaxanthin Attenuates Fish Oil-Related Hepatotoxicity and Oxidative Insult in Juvenile Pacific White Shrimp (*Litopenaeus vannamei*). *Mar Drugs, 18*(4).

Yu, Y., Tang, M., Wang, Y., Liao, M., Wang, C., Rong, X., Li, X., Ge, J., Gao, Y., Dong, X., & Zhang, Z. (2023). Virulence and antimicrobial resistance characteristics assessment of Vibrio isolated from shrimp (*Penaeus vannamei*) breeding system in south China. *Ecotoxicology and Environmental Safety, 252*(114615), 1-11.

Zhang, H., Liu, M., Shao, R., Zhang, J., Zuo, R., Tan, B., Ai, Q., Mai, K., & Wan, M. (2022). The effects of different lipid sources on the growth, intestinal health, and lipid metabolism of the pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture, 548*.

Zhu, W., Dong, R., Ge, L., Yang, Q., Lu, N., Li, H., & Feng, Z. (2023). Effects of dietary n-6 polyunsaturated fatty acids (PUFA) composition on growth performances and non-specific immunity in pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture Reports, 28*.

Zienkiewicz, K., Du, Z. Y., Ma, W., Vollheyde, K., & Benning, C. (2016). Stress-induced neutral lipid biosynthesis in microalgae - Molecular, cellular and physiological insights. *Biochim Biophys Acta, 1861*(9 Pt B), 1269-1281.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Zong, G., Li, Y., Wanders, A. J., Alssema, M., Zock, P. L., Willett, W. C., Hu, F. B., & Sun, Q. (2016). Intake of individual saturated fatty acids and risk of coronary heart disease in US men and women: two prospective longitudinal cohort studies. *BMJ, 355*, i5796.

Zulu, N. N., Zienkiewicz, K., Vollheyde, K., & Feussner, I. (2018). Current trends to comprehend lipid metabolism in diatoms. *Prog Lipid Res, 70*, 1-16.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

VITA

NAME	Ekthida Thongdet
DATE OF BIRTH	23 August 1983
PLACE OF BIRTH	Ranong
INSTITUTIONS ATTENDED	B.Sc. (Aquatic Science) from Faculty of Natural Resources, Prince of Songkla University. M.Sc. (Marine Science) From Faculty of Science, Chulalongkorn University Lecturer in Aquatic animal clinic, Faculty of Veterinary Medicine. Mahanakorn University.
HOME ADDRESS	Kapoe, Ranong, Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY