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นางสาววิชชุลดา ฉิมพิบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PREPARATION OF FISH OIL NANOSPHERES FOR SHRIMP FEEDS

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Thesis Title	PREPARATION OF FISH OIL NANOSPHERES FOR SHRIMP FEEDS
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วิชชุลดา ฉิมพิบูลย์ : การเตรียมน้ำมันปลารูปทรงกลมระดับนาโนเมตรสำหรับอาหารกุ้ง (PREPARATION OF FISH OIL NANOSPHERES FOR SHRIMP FEEDS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ศุภศร วนิชเวชารุ่งเรือง, 106 หน้า.

จุดมุ่งหมายของงานวิจัยนี้คือ กักเก็บน้ำมันปลาลงในอนุภาคขนาดไมโครเมตรสำหรับอาหารกุ้ง ด้วยวิธี solvent displacement ร่วมกับการใช้สารต้านอนุมูลอิสระ BHT (butylated hydroxytoluene) ได้ตรวจสอบรูปร่างของอนุภาคน้ำมันปลาด้วยภาพถ่ายอิเล็คตรอนแบบส่องกราด (SEM) และภาพถ่ายอิเล็คตรอนแบบส่องผ่าน (TEM) ขนาดของอนุภาคน้ำมันปลาที่ได้มีขนาด 1 ± 0.74 ไมโครเมตร อนุภาคสามารถกระจายตัวได้ดีในน้ำ อีกทั้งเมื่อนำอนุภาคน้ำมันปลาไปทดสอบเสถียรภาพต่อความร้อน ออกซิเจน และอายุการเก็บรักษา จากผลการทดลองพบว่า อนุภาคน้ำมันปลาร่วมกับการใช้สารต้านอนุมูลอิสระ มีเสถียรภาพต่อความร้อน ออกซิเจน และอายุการเก็บรักษาดีกว่าน้ำมันปลาที่ไม่ได้กักเก็บ นอกจากนี้ยังนำอนุภาคน้ำมันปลาดังกล่าวไปเตรียมอาหารกุ้งสำหรับเลี้ยงกุ้งขาวเพื่อเพิ่มกรดไขมันไม่อิ่มตัว (PUFAs) ในอาหารกุ้ง โดยเตรียมด้วย 2 วิธีคือ 1) นำอนุภาคน้ำมันปลาผสมรวมกับอาหารกุ้งแบบเปียกหลังจากนั้นนำไปขึ้นรูปเม็ดอาหารด้วยเครื่องอัดเม็ดอาหารกุ้ง และ 2) ฉีดพ่นสารแขวนลอยของอนุภาคน้ำมันปลาโดยตรงลงบนอาหารกุ้งแบบเม็ด หลังจากนั้นเลี้ยงกุ้งขาวด้วยอาหารกุ้งทั้งสองสูตร พบว่ากุ้งขาวที่เลี้ยงด้วยอาหารที่ผสมอนุภาคน้ำมันปลา มีอัตราการรอดชีวิต น้ำหนัก ความยาว เม็ดไขมันและสีของพูตับ ระบบภูมิคุ้มกัน และอัตราการรอดชีวิตในการต้านแบคทีเรียเรืองแสง (*Vibrio harveyi*) ได้ดีกว่ากุ้งขาวที่เลี้ยงด้วยอาหารปกติ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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The aim of this work is to fabricate the encapsulated fish oil particles for shrimp feed. The co-encapsuted fish oil with BHT (Butylated hydroxytoluene as the antioxidant) particles size is 1.00 ± 0.74 mm prepared solvent displacement method using ethylcellulose as the polymeric material. The morphology of these particles were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The encapsulated fish oil-BHT particles were tested to investigate the stability properties. The results indicate that encapsulated fish oil-BHT processes better thermal stability, oxidative stability and shelf life stability than unencapsulated fish oil. Moreover, encapsulated fish oil-BHT particles were added into the shrimp feed to increase the content of unsaturation of polyunsaturated fatty acids (PUFAs) in shrimp diet. The shrimp feeds were prepared with two different methods: 1) mixing encapsulated fish oil-BHT particle to shrimp feed paste for pelting machine before pelleting, 2) spraying encapsulated fish oil-BHT particle suspension onto the shrimp feed pellets. Both methods gave feed with minimal degradation of unsaturation functionality. In addition, *Litopenaeus vannamei* fed with feed containing the encapsulated fish oil-BHT particle shows better health such as, growth, weight, length and immunity index than those fed with unencapsulated fish oil containing feed.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
ml	Milliliter
µg	Microgram
µl	Microliter
µm	Micrometer
ppm	Parts per million
ppt	Parts per thousand
rpm	Revolution per minute
min	Minute
w/w	Weight by weight
Da	Dalton
MWCO	Molecular weight cut-off
SEM	Scanning electron microscope
TEM	Transmission electron microscope

EC	Ethyl cellulose
BHT	Butylated hydroxytoluene
%EE	Encapsulation efficiency
%loading	Loading capacity
Feed S	Feed prepared by spraying the encapsulated fish oil-BHT particle onto commercial shrimp feed and heating to 65°C for 1 h
Feed CS	Feed prepared by spraying distilled water onto commercial shrimp heating to 65°C for 1 h
Feed M	Feed prepared by mixing the encapsulated fish oil-BHT particles into the feed paste and passing to the pelleting machine at 95 °C for 10 min
Feed CM	Feed prepared by mixing the bulk fish oil into the feed paste and passing to the pelleting machine at 95 °C for 10 min

CHAPTER I

INTRODUCTION

Fish oil has many benefit both in human and animal. The important constituent in fish oil is polyunsaturated fatty acids (PUFAs) which are usually used for reduced inflammation [1], cancer prevention [2], joint pain improvement [3], glowing skin [4] and heart health [5], etc. Moreover, it has been used in aquaculture for high growth [6], reproduction [7], larval development [6] and increasing the immunity [8, 9]. For several years, the shrimp farmers popularly use *Artemia* as PUFAs-riched diet for aquaculture feeding because it is not only PUFAs-riched but it is also contain the essential nutrition such as, protein, lipid and carbohydrate [10, 11]. Because of pre-activation need and high cost of *Artemia*, marine oils are replaced as PUFAs source for shrimp farming.

Due to the susceptibility of unsaturation of PUFAs in fish oil, it is often prone to the oxidation under high humidity, high temperature, natural light exposure and longtime storage. Moreover, fish oil is water insoluble that is hard for mixing with water-based materials.

To stabilize PUFAs in fish oil, the polymeric matrixs are used and the mixture are subjected to several drying method such as, spray drying, extrusion, fluidized bed drying and melt injection. Although, the high loading and high encapsulation efficiency are obtained. The content of unsaturation of PUFAs remain after heating process is not reported. In this work, we use solvent displacement method using ethylcellulose as a polymer to encapsulate fish oil. This method is easy and no heating need with high loading of fish oil.

Litopenaeus vannamei is an important penaeid shrimp for Thailand exportation but it is very sensitive for pathogen infection during the shrimp rearing. Shrimp disease by *Vibrio* bacteria family is the serious problem for shrimp industry. The infected shrimp will immediately die after 1-2 days of infection. Many studies have reported the advantage of PUFAs to immunity and survival in aquamarine, including of leukotriene production, increasing the adhesive site of cell membrane for antibodies recognition, increasing the lipoprotein-β glucan biding protein, etc. Thereby, in this work, the basal shrimp diet added encapsulated fish oil-BHT was made and fed to *L. vannamei.* Studies were set up to compare those fed with basal shrimp diet. The immunity index, including of hemocyte and phenoloxidase were evaluated.

CHAPTER II

LITERATURE REVIEW

2.1 Fish oil

Fish oil is the oil extracted from muscle of deep ocean fish such as tuna, herring, sardine, anchovy, trout and salmon. The main important composition in fish oil is polyunsaturated fatty acid or PUFAs, especially those of the omega 3 family such as eicosapentaenoic acid (EPA) and docosahexanoic acid [12, 13].

2.2 Properties of fish oil

2.2.1 Physical property

Fish oil is usually liquid at room temperature but partially solidifies when the temperature is below 20°C. Similar to other oils, the solid part is known as stearin and liquid part is known as olein. Other physical properties of fish oil are present in Table 1.

Table 1 Physical characteristics of common fish oils.

Physical properties	Value
Specific heat (cal/g)	0.50-0.55
Heat of fusion (cal/g)	ca. 54
Calorific value (cal/g)	ca. 9,500
Slip melting point (°C)	10-15
Flash point (°C)	
as glycerides	ca. 360
as fatty acid	ca. 220
Boiling point (°C)	>250
Specific gravity at 15°C	ca. 0.92
30°C	ca. 0.91
45°C	ca. 0.90
Viscosity (cp) at 20°C	60-90
50°C	20-30
90°C	ca. 10
Refractive index	1.46- 1.48

Source [14]

2.2.2 Chemical property

Fish oils contain a unique diversity of polyunsaturated fatty acids (PUFAs). The major fatty acids are long chain omega-3 type with carbon chain lengths of 20 and/or 22 which generally make up one-fourth to one-third of all the fatty acids in fish oils.

2.2.2.1 Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. The common polyunsaturated fatty acids are methylene-interrupted polyenes. . Methylene-interrupted polyenes are fatty acids which have 2 or more *cis* double bonds in long chain and the double bonds are separated from each other by a single methylene bridge (CH_2- unit). This form is also sometimes called a divinylmethane pattern. The important fatty acids are all omega-3 and -6 methylene-interrupted fatty acids.

2.2.2.2 Omega 3 fatty acids

Omega-3 fatty acids are those that have the first double bond at the third carbon from the terminal methyl group of the carbon chain. The three types of omega-3 fatty acids involved in human physiology are alpha linoleic acid (ALA) (found in plant oils), Eicosapentaenoic acid (EPA) (Figure 1) and docosahexaenoic acid (DHA) (Figure 2) (both commonly found in marine oils). The omega 3 content in fish oil usually varies with the type of fish (Table 2).

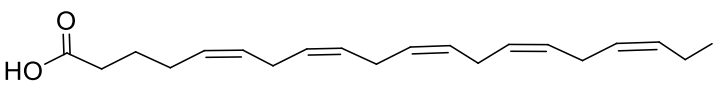


Figure 1 Structure of eicosapentaenoic acid (EPA) (20: 5ω-3).

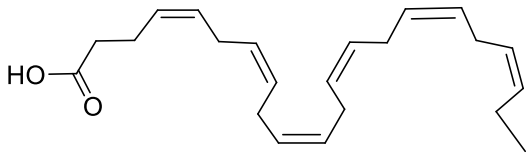


Figure 2 Structure of docosahexaenoic acid (DHA) (22: 6ω-3).

Table 2 Omega 3 content per 85 gram serving.

Common name of fish	Omega 3 content (gram)
Herring, sardines	1.3 - 2
Mackerel	1.1 – 1.7
Salmon	1.1 – 1.9
Tuna	0.21 – 1.1
Swordfish	0.91
Pollock	0.45
Cod	0.15 – 0.24

Source: [15]

2.2.3 Nutritional

The body oils of fish normally contain vitamins in small amounts. Therefore, people do not consume fish oil for the vitamin purpose, rather the essential fatty acids such as EPA and DHA are the main nutritional ingredients of the fish oils. Although some vitamins A and D can be found in the oil of most fish, many species store large amounts of vitamins A and D in their livers not in muscle (cod, halibut, and tuna).

2.2.4 Sensory

The odor of fish oil is the main limiting factor to its application in food. Furthermore, due to the susceptibility of fish oil to oxidation, the shelf life of the product is also reduced. The seafood industry has been reluctant to manufacture fish oil shortening due to the “fishy” flavor passed to the baked product and also due to the development of auto-oxidative rancidity.

2.3 Benefit of fish oil for human health

2.3.1 Benefit for the brain and eyes

The brain is one of the organs where the omega-3 PUFAs are essential. The tissue of this organ is particularly rich in DHA, showing a close correlation between the consumption of fatty acid and its deposition in the cellular membrane. DHA is mostly rich in the membranes of retinal photoreceptors and in neural tissue, especially in the grey matter of the brain, comprising %50-30 of the lipids in these tissues [16] and it has been found to be important for the development of the brain and retina [17]. DHA may accelerate the signal in brain region [16]. Thus, fish oil is suggested for mother who are pregnant for diet fish oil as source of omega 3 [17].

2.3.2 Effect on cancer

Polyunsaturated fatty acids were report on cancer development effect [18, 19]. Many studies have shown that fish oil has essential parts in inhibition of some types of cancer, including breast [18-20], colon [21, 22], renal [23, 24], pancreatic cell, prostate and liver [18, 23].

2.3.3 Cardiovascular benefit

Fish oil intake is associated with lower risk of cardiac arrhythmias, including sudden death, arrhythmic, coronary heart disease death, and atrial fibrillation [25, 26]. The almost indication for an effect of n-3 fatty acids and disease is the inverse relation between the amount of n-3 fatty acids in the diet, in blood, and in tissues, and the occurrence of coronary heart disease [27]. n-3 Fatty acids accomplish to inhibit heart disease over a variety of actions, by averting arrhythmias, creating prostanoids and leukotrienes with anti-inflammatory actions, and by hindering synthesis of cytokines and mitogens which augment the inflammation and promote plaque formation [27].

2.3.4 Human immune and inflammatory responses

It has been obvious for over 30 years that fatty acids can affect the immune system [28]. The phospholipids of immune cells (such as lymph node or splenic lymphocytes or peritoneal macrophages) taken from rats preserved on normal

laboratory contain 15–20% of fatty acids as arachidonic acid and contain very slight of the very long chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [29, 30]. Thus, it is achievable to enhance immune cells in polyunsaturated fatty acids by consuming a diet containing polyunsaturated fatty acids or in EPA and DHA by consuming a diet containing these fatty acids [31-33]. Saturated fatty acid increased macrophage adhesion to both tissue culture plastic and bacterial plastic when compared with polyunsaturated fatty acids. Macrophages enriched with the saturated fatty acids myristate or palmitate showed decreases of 28 % and 21 % respectively in their ability to phagocytose unopsonized zymosan particles. Those enriched with polyunsaturated fatty acids showed 25-55 % enhancement of phagocytic capacity [30].

Polyunsaturated fatty acids (PUFAs) which crowed in fish oil only have benefit in human but also have benefit in aquaculture. Twenty year ago, fish farmer normally used *Artemia* (see 2.3.1) as omega 3 rich source for feeding to aqua larval.

2.4 Polyunsaturated fatty acids for aquaculture

2.4.1 *Artemia* as polyunsaturated fatty acids source for aquaculture

Artemia is a genus of aquatic crustaceans known as brine shrimp (Figure 3). Its populations are found worldwide in inland saltwater lakes, but not in oceans [34]. The biochemical factor of *Artemia* is considered as an important nutrition for enhancing

larval nutrition (Table 3) for survival and growth of aquaculture species like finfish and shellfish [35]. The ability of the *Artemia* to produce dormant eggs, known as cysts, has led to extensive use of *Artemia* in aquaculture. The cysts may be stored for long periods and hatched on demand to provide a convenient form of live feed for larval fish and crustaceans [36].

Figure 3 *Artemia salina.*

Table 3 Average composition of *Artemia* nauplii and adult**.**

Nutrition	Nauplii	Adult
Protein	52.2 ± 8.8	56.4 ± 5.6
Lipid	18.9 ± 4.5	11.8 ± 5.0
Carbohydrate	14.8 ± 4.8	12.1 ± 4.4
Ash	9.7 ± 4.6	17.4 ± 6.3

2.4.1.1 Benefit of *Artemia* for aquaculture

Polyunsaturated fatty acids in *Artemia* have many advantages for aquaculture. Western rock lobster, *Panulirus cygnus*, phyllosoma were grown from hatching *Artemia*. Larvae fed with *Artemia* enriched with polyunsaturated fatty acids showed that the phyllosoma which fed with high content of polyunsaturated fatty acids were larger than phyllosoma which fed with normal shrimp food [6]. *Penaeus monodon* which fed with high content of PUFA showed the survival and growth rate related to the content of PUFA in *Artemia*[10]. Furthermore, the survival rate of *Penaeus marginatus* of postlarval stage were increased when fed with freshly hatched *Artemia* [37]. *Penaeus vannamei* were fed with *Artemia* replacement the marine polychaetes which are expensive and are of unpredictable supply indicated that Artemia may be useful as a supplement to or as a replacement for polychaetes in *Penaeus vannamai* maturation diets [38].

2.4.1.2 Problem of *Artemia*

The disadvantage of using *Artemia* is their larger size, which may be a problem for the early aqua larval stage. Furthermore, the nutrition of *Artemia* is not enough for aqua larval feeding. The process for increasing the nutritional value of *Artemia* are needed, such as feeding *Artemia* with highly unsaturated fatty acids (HUFAs) enrichment, improving the strain of *Artemia*.

On another hand, important problem of *Artemia* is the complicated production. Although using *Artemia* cysts appears to be easy, some factors are critical for incubating. The large quantities needed in larval fish production. These include cyst sterilization or decapsulation prior to incubation, and incubating under the following optimal conditions: constant temperature of 25–28 °C, 15–35 ppt salinity, minimum pH of 8.0, near saturated oxygen levels, maximum cyst densities of 2 g/l, and strong light of 2000 lx [39]. All these factors will affect the incubating rate and maximum output, and therefore, the production cost of the harvested *Artemia.* Due to the culturing *Artemia* in outdoor pond, the migratory birds usually visit that could be infected with cestode (tapeworm) parasites. The infected birds release tapeworm eggs into the environment in their excrement where the brine shrimp become infected with them. With the above shortcoming of *Artemia,* polyunsaturated oils such as fish oil are another candidate to help improving aquaculture health.

2.4.2 Fish oil as polyunsaturated fatty acids source for aquaculture

Fish oil have many benefit for aquaculture due to the PUFAs-riched in fish oil. In 1997, Kanazawa et al. founded that PUFAs-riched in larval red sea beam (*Pagrus major*) and juvenile marbled sole (*Limanda yokohamae*) diet showed highly resistance of larvae to difference stress such as the variation in temperature and salinity [40]. The survival of Asain seabass (*Lates calcarifer*) also increased during the larvae

metamorphosis [41]. PUFA acts an important role in prostaglandin regulation, leukotriene production [42], keep high adhesive site on cell membranes for antibodies to regulate immunological response and recognition [43]. Moreover, the presence of PUFA in shrimp diet is emphasized for shrimp immune system to increase the level of lipoprotein-beta glucan binding protein [8]. Non-specific cellular immune response in juvenile grouper fed with 1 or 2% PUFA was significantly increased [9]. The pathogen resistance and immune response of darkbarbel catfish fed with PUFA and vitamin E were higher under low ammonia conditions with high antibody level [44]. Erythrocyte cell wall strength of Atlantic salmon was observed with increasing level of omega 3 fatty acid in fish diet [45]. A greater respiratory burst and agglutination of hemocyte in *Litopenaeus stylirostris* was obtained after fed with a diet rich ω3 PUFA [46].

2.5 Problem of fish oil and polyunsaturated fatty acids

2.5.1 Lipid Oxidation

Unsaturated lipids are sensitive to oxidative processes which have catalytic systems such as light, heat, enzymes, metals and micro-organisms. It corresponds to autoxidation by photo-oxidation, thermal oxidation, and enzymatic oxidation under different conditions. Most of lipid oxidation is related with free radical or oxygen species [47].

Auto-oxidation is the main reaction leading to oxidative depreciation of lipid. It occurs when lipid was attacked with reactive oxygen species and the process is accelerated with high temperature (thermal oxidation) [48]. The unsaturated fatty acids are normally the reactant in this reaction. The autooxidation of polyunsaturated fatty acids occurs via 3 step free radical chain reaction; initiation, propagation and termination [49]. A simplified scheme explaining the mechanism of autoxidation is given below:

Initiation:

$$LH \xrightarrow{\text{initiator}} L\cdot$$

Propagation:

$$L\cdot + O_2 \rightarrow LOO\cdot$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot$$

Termination:

$$2LOO\cdot$$
$$LOO\cdot + L\cdot$$
$$L\cdot + L\cdot$$

} Nonradical products

*LH is lipid oxygen reacted with lipid

Lipid hydroperoxides (LOO·) have been identified as primary products of autoxidation. The decomposition of hydroperoxides can be aldehydes, ketones, alcohols, hydrocarbons, volatile, organic acids, and epoxy compounds, known as

secondary oxidation products leading to deterioration of lipids [50]. To prevent the auto-oxidation of lipid, the antioxidant is needed.

2.5.2 Antioxidant

Antioxidant is substance that delays or slows down the rate of oxidation reaction. The most common type of lipid soluble antioxidants are mono or polyhydric phenol with various ring substituents [47]. The mechanism of action of antioxidant is either inhibiting the formation of free radicals in the initiation step or interrupting propagation of the free radicals chains. A simplified scheme explaining the mechanism of antioxidation with antioxidant is given below:

$$LOO^{\cdot} + AH \leftrightarrow LOOH + A^{\cdot}$$

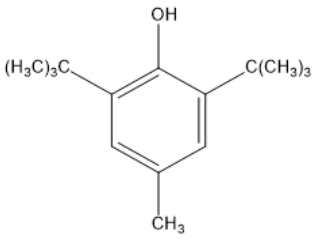
$$A^{\cdot} + LOO^{\cdot} \rightarrow$$

$$A^{\cdot} + A^{\cdot} \rightarrow$$

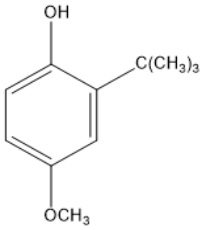
Nonradical product

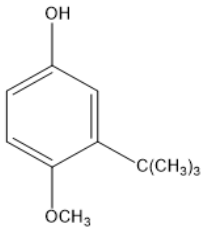
*AH is antioxidant.

The most widely used antioxidants in foods include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) (Figure 4)

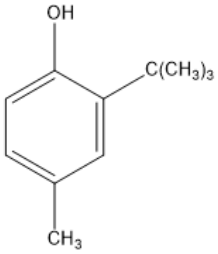


Butylated hydroxytoluene (BHT)

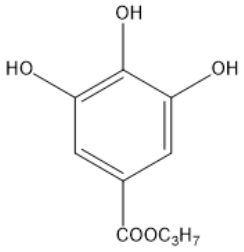




Butylated hydroxyanisole (BHA)



tert-Butylhydroquinone (TBHQ)



Propyl gallate (PG)

Figure 4 Antioxidant in food industry.

2.5.3 Prevention of Lipid Oxidation

To prevent or slow-down the lipid oxidation, the following can be done

- Prevent the initiation reaction from occurring (see 2.5.1)
- Reduce the oxygen content (see 2.5.1)
- Add anti-oxidants (2.5.2)
- Storage at low temperature
- Exclude light (see 2.5.2)

The rates of oxidation reaction depend on initial form of substance and the handling of raw material. For example, oxidation in an oil-in-water emulsion might

differ from that in an water-in-oil emulsion, the use of positively charged emulsifiers and thickeners may repel metal ions [51]. Alternatively, encapsulation also can reduce oxidation through limiting the exposure of substances to oxidative species (see 2.6).

2.6 Encapsulation technique

2.6.1 Encapsulation

Encapsulation technique is the process of covering the tiny particles of solid or droplet of liquid to protect them from external environment [52]. The product which harvested from this processes are called micro-nano particle, microcapsule, microsphere depending on the morphology and internal structure. Type of encapsulated particle showed in Figure 5.

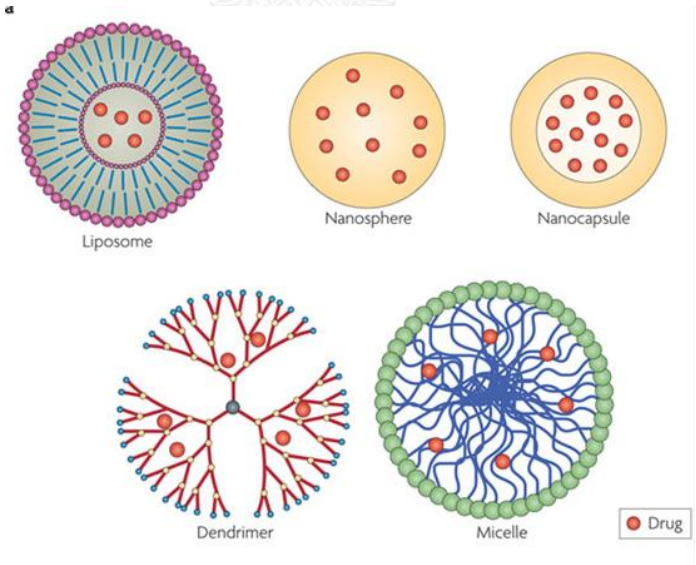


Figure 5 Various forms of particle.

Particle sizes from encapsulation technique are in ranking micrometer to nanometer [53]. The sphere can be made of natural and modified polysaccharides, gums, proteins, sugars, and synthetic polymers [54]. One of the main benefit of encapsulation is the stabilization of the encapsulated material. In food industry, the encapsulation technique is usually used to increase the storage time of active substance and minimize undesirable odor and color change in food [55] . For the essential oil, the encapsulation is used for various point such as for improving the solubility and volatility [56].

2.6.2 Properties of coating material

The suitable technique of encapsulation is based on the type of coating material. Coating materials which are normally film-forming materials, can be crated from a variety of synthetic or natural polymers, depending on the material to be covered and characteristics preferred in the final particle. The constituent of the coating material is the key factor of the useful properties of the microcapsule and might be used to adjust the performance of a particular ingredient. An ideal coating material should exhibit the following characteristics [57].

1. Suitable rheological properties at high concentration and comfortable workability during encapsulation.

2. Non-reactivity with the material to be encapsulated both during handling and on prolonged storage.

3. The ability to fasten and storage the active material within its structure during handling or storage.

4. The ability to totally release the solvent or other materials used during the process of encapsulation under drying or other desolventization conditions.

5. The ability to provide maximum defense to the active material against environmental conditions (e.g., humidity, heat, oxygen, light,).

6. Solubility in solvents satisfactory in the food industry (e.g., water, ethanol).

7. Chemical non reactivity with the active core materials.

8. Inexpensive, food-grade status.

Because no single coating material can be all ideal properties, therefore, the mixture of coating materials are need. The food grade and biocompatibility coating materials are listed in Table 4.

Table 4 Coating materials for microencapsulation of functional food additives

Category	Coating materials	Widely used methods
Carbohydrate	Starch, maltodextrins, chitosan, corn syrup solids, dextran, modified starch, cyclodextrins	Spray- drying, freeze-drying, extrusion, coacervation, inclusion complexation
Cellulose	Carboxymethylcellulose, methyl cellulose, ethylcellulose, celluloseacetate-phthalate, celluloseacetatebutylate-phthalate	Coacervation, spray-drying, edible films
Gum	Gum acacia, agar, sodium alginate, carrageenan	Spray-drying, Syringe method (gel beads)

Lipids	Wax, paraffin, beeswax, diacylglyerols, oils, fats	Emulsion, liposomes, film formation
Protein	Gluten, casein, gelatin, albumin, peptides	Emulsion, spray-drying

In this research, we focus on ethylcellulose (see 2.7) and alginate (see 2.8) as shell material for microencapsulation.

2.7 Ethyl cellulose as polymer for encapsulation

2.7.1 Ethyl cellulose

Various derivertive cellulose is used in the food and pharmaceutical industry, such as hydroxypropyl cellulose, cellulose acetate, hydroxypropyl methylcellulose, methylcellulose, hydroxypropyl methylcellulose phthalate and ethylcellulose. All are derived from, the polymeric backbone of cellulose which contains a basic repeating

structure of β-anhydroglucose units, each unit having three replaceable hydroxyl groups. The number of substituent groups of these hydroxyls can be designated either by a weight percentage or by the number of points where the groups are attached concept known as degree of substitution (DS). Ethylcellulose is cellulose derivative which substituted with ethyl ether groups (Figure 6).

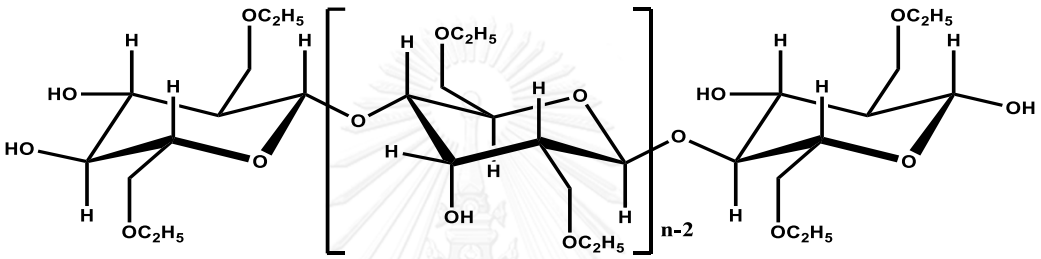


Figure 6 Structure of ethylcellulose.

2.7.2 Properties of ethylcellulose

2.7.2.1 Solubility

Ethylcellulose is soluble in a wide variety of solvents, therefore making it easy to use when solution application is desirable. Among the valuable solvents are the ketones, aromatic hydrocarbons, esters, chlorinated solvents and alcohols. Ethylcellulose is greatest soluble in solvents that have closely the same cohesive energy density or solubility parameter as the material itself [58].

2.7.2.2 Stability

Ethylcellulose is the high stable of the cellulose derivatives. It is partially dissolved in alkali, both concentrated and dilute, but is not dissolved in acids. It takes up very little water from moist air or during immersion, and this evaporates readily leaving ethylcellulose unchanged. Light, visible or ultraviolet has no discoloring action on ethylcellulose. Application of heat up to its softening point has little effect on ethylcellulose [58].

2.7.2.3 Softening (Glass Transition) Temperature

The high thermal softening points or glass transition temperatures of hydroxypropyl methylcellulose, hydroxypropylcellulose and ethylcellulose (180, 120 and 140^{o}C, respectively) create them ideal polymers. With ethylcellulose, the softening temperature is related to the degree of substitution, a minimum occurring at a DS of 2.55 (48.5% ethoxy content) [59].

2.7.3 Applications of ethylcellulose

2.6.3.1 Binder in tablets

Ethylcellulose may be mixed dry or by dissolving in a solvent such as alcohol. Nevertheless, tablets made with ethylcellulose as a binder tend to show poor dissolution and poor drug absorption. Diclofenac sodium which the drug has been

applied to reduce inflammation and as an analgesic was combined with ethylcellulose to construct the tablet [60]. Furthermore, microcapsules containing sodium phenobarbital cores in ethyl cellulose have been used to prepare tablets [61].

2.6.3.2 Slow drug release from films

Ethylcellulose was usually used for delaying drug release. Pure ethylcellulose films are badly permeable for many substances and very low release rates for certain drugs from coated dosage forms [62]. Release mechanisms of theophylline in pellets coated with ethylcellulose is dependent on the physical state of the swollen ethylcellulose and on the migration of the water soluble pore former [63]. Moreover, Amylose-ethylcellulose film coatings obtained from organic-based solvents to reduced drug release rate that was successfully resisted 5-aminosalicylic acid release in the upper gastrointestinal tract [64].

2.6.3.3 Encapsulation using ethylcellulose as core shell

The herbicide norflurazon was encapsulated in ethylcellulose (EC40) microspheres by the solvent evaporation technique to obtain controlled release formulations, which show large encapsulation efficiency for the herbicide [65]. Aspirin were encapsulated with ethylcellulose by oil-in-water emulsification/solvent evaporation technique, The release rate follows first-order kinetics during the first 12

hours, suggesting a monolithic system with aspirin uniformly distributed in the microcapsule [66]. Solvent evaporation method was used to prepare ibuprofen encapsulated ethylcellulose which evidenced the presence of a metastable molecular dispersion for intermediate loadings, coexisting with a solid solution and a crystalline dispersion of the drug in the polymer matrix [67].

2.8 Alginate as polymer for encapsulation

2.8.1 Structure of alginate

Alginates are unbranched polysaccharides consisting of 1⟶4 linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). It is a natural copolymer which extracted from brown algae, and is can also found in an exopolysaccharide of bacteria including *Pseudomonas aeruginosa* [68]. Alginates is composed of sequences of M (M blocks) and G (G-blocks) residues interspersed with MG sequences (MG-blocks) (Figure 7 and 8). The copolymer composition, sequence and molecular weights vary with the source and species that produce the copolymer [69].

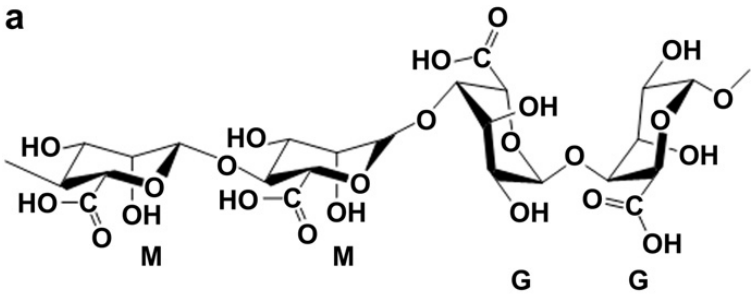


Figure 7 Chain formation of alginate

Figure 8 Block copolymer of alginate

2.8.2 Properties of alginate

2.8.2.1 Solubility

The solubility of alginates in water is controlled by three parameters, there are, presence of gelling ions in the solvent, ionic strength of the medium and pH of the solvent. To generate alginates soluble, it is important that the pH be above a certain critical value and the carboxylic acid groups were deprotonated. Changing the ionic strength of the medium affects solubility properties such as polymer conformation, chain extension and viscosit [70]. Alginates gel was governed of divalent cations such as Ca^{+2}, Sr^{+2} and Ba^{+2}. It is essential to have an aqueous solvent free of crosslinking ions to allow dissolution [71].

2.8.2.2 Ionic crosslinking

Alginate chelates with divalent cations to form hydrogels. Gel formation is driven by the interactions between G-blocks which associate to form tightly held junctions in the presence of divalent cations [72]. Alginates with high G contents yield stronger gels. The affinity of alginates towards divalent ions decreases in the following

order: Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn [73]. Ca^{+2} is the greatest usually used cation to persuade alginate gel formation (Figure 9).

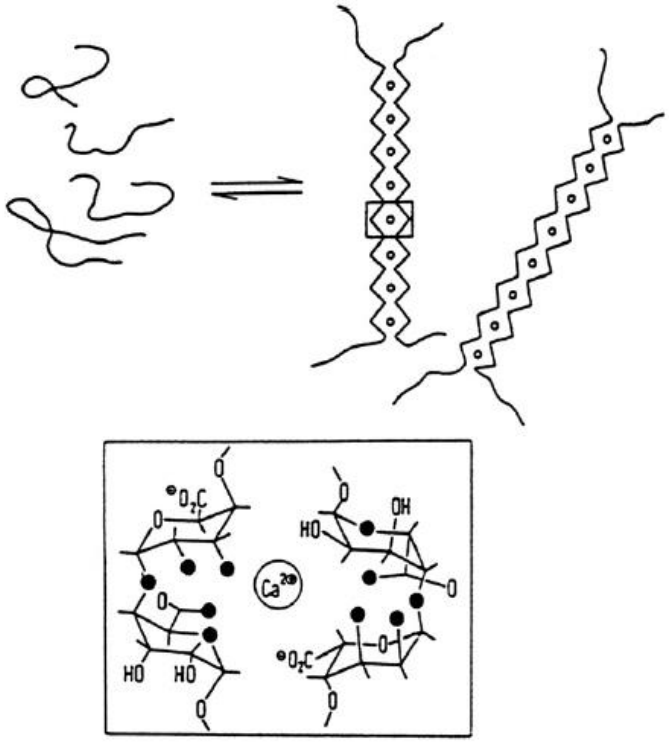


Figure 9 Schematic representation of the egg-box association of the poly-L-guluronate sequences of alginate crosslinked by calcium ions.

2.8.2.3 Chemical properties

Sodium alginate in the form of dry powder can be kept without degradation in a cool, dry place and away from sunlight for several months. The shelf life can be extended to several years by storing it in the freezer [74]. The degradation of alginate is followed when they are exposed to strongly alkaline environments. The rate of degradation rises quickly above pH 10.0 and below pH 5.0. Above a pH of 10.0,

the degradation arises mostly from the elimination mechanism, while below 5.0 the degradation is frequently because acid catalyzed hydrolysis [75].

2.8.3 Application of alginate

2.8.3.1 Food industry

The thickening property of alginate is beneficial in sauces and in toppings and syrups for ice cream. By thickening pie fillings with alginate, softening of the pastry by liquid from the filling is reduced. Addition of alginate can make icings non-sticky and allow the baked goods to be enclosed with plastic wrap [54]. Alginate recovers the texture, body and sheen of yoghurt [57]. Alginates have several applications that are not related to either their gel or viscosity properties. They act as stabilizers in ice cream; addition of alginate reduces the formation of ice crystals during freezing, giving a smooth product [76].

2.8.3.2 Immobilization and encapsulation

Beads created with calcium alginate were one of the first materials to be used for immobilization for cell and enzyme production. Various commercial chemical syntheses and conversions are best carried out using biocatalysts such as active whole cells or enzymes [77]. To complete these procedures on intermediate, to large scale, the biocatalysts must be in a concentrated form and be recoverable from the process

for re-use [78]. The whole cells are suspended in a solution of sodium alginate and this is added dropwise to a calcium chloride solution [79]. *Lactobacillus plantarum* were immobilized and lyophilized in Ca-alginate bead, The resultant higher alginate concentration per unit volume in the beads with glycerol led to the prospective higher mechanical strength [80]. Moreover, *Zymomonas mobilis* cells were immobilized into small 1 mm diameter beads of Ca-alginate in order to minimize mass transfer limitations and maximize immobilized cell activity [81]. Furthermore, *Saccharomyces cerevisiae* cells grown in alginate reveal ethanol production rates 1.5 times greater than cells grown in suspension [82].

For this research, we focus on the encapsulation technique using ethylcellulose as coating material for encapsulating fish oil.

2.9 Microencapsulation Technologies of Fish Oil

Fish oil can be encapsulated to avoid off-flavor by inhibiting interaction between fish oil and oxygen, inhibiting interaction between fish oil and metal ions, inhibiting direct contact to light, and catching off-flavor. In Addition, another benefit of encapsulation is the change of a liquid fish oil into a powder form that eases the handling during supply chain or incorporation into food powder products. The process which used to encapsulate fish oil is divided into 2 method, based on solubility of

encapsulated fish oil particle. There are water-soluble and inwater-soluble microencapsulation.

2.9.1 Water-Soluble Microencapsulates

2.9.1.1 Cyclodextrins

Cyclodextrins containing six, seven, or eight glucose molecules are mentioned to as α-, β-, and γ-cyclodextrin, respectively. Cyclodextrin may reduce the odor by either forming of off-flavors or developing a complex with polyunsaturated fatty acids [83]. Fish oil did not oxidize for more than 24 h upon keep at 100°C under air when using γ-cyclodextrin. Loading capacity of fish oil when using γ-cyclodextrin was high (15-40%) and higher loads resulted in more oxidation. The particle size is 100 μm max and this product is now marketed as OmegaDry® by Wacker Chemical Corporation.

2.9.1.2 Spray-Drying

Spray-drying is one of the oldest and popular procedure of encapsulation in the food industry and it is consumed most often to make fish oil encapsulated particle. Spray-drying of active agent is usually attained by dispersing, dissolving or emulsifying in an aqueous solution of carrier material, followed by atomization and spraying of the mixture into a hot chamber. Film is produced at the droplet surface, thus delaying the larger active molecules whereas the tiny water molecules are evaporated. Fairly low

spray-drying temperatures are beneficial to reduce the lipid oxidation [84]. The application of nitrogen in place of air to dry the scattering can also be applied, but it increase the costs. Carrier material in spray-drying process can be proteins, sugars, glucose syrup, gums maltodextrin and modified cellulose [85, 86]. Even though the spray-drying technology is frequently used, it has some weaknesses. Spray-drying procedure might be activated oxidation and the fish oil may have a restricted shelf life because the porous construction of the obtained spray-dried powder, and high inlet of air to the oil [87-89]. Loading capacity of fish oil in particle which were made from spray drying differs from 1 to approximately 60 wt%. The particle size of these microcapsules differs from 10 to 400 μm. Fish oil encapsulates with a bigger particle size may be more stowage steady than those with a tiny size [90]. Not only extreme moisture content, but also the temperature influences the shelf life of spray-dried fish oil [84, 90, 91].

2.8.2 Water-Insoluble Microencapsulates

2.8.2.1 Coacervation

Complex coacervation involved two or more polymers, one of polymer is gelation or whey protein and other one is an oppositely charged polymer such as gum arabic, sodium polyphosphate or methylcellulose. Coacervation is started by making emulsion of fish oil droplet in aqueous polymer solution. Phase separation of two

polymers solution is obtained by decreasing pH, the poly precipitate on the interface of oil droplet. Loading capacity of fish oil from coacervation process is 40–90 wt% and a particle size between 10 and 800 μm, relying on the process conditions used and materials [92].

2.8.2.2 Calcium Carbonate Capsules

Calcium carbonate capsules is based on the electrostatic adsorption of oil surface of a fish oil-in-water emulsion during stirring with negatively charged of calcium carbonate particles, antioxidant in the oil is also added. The benefit of calcium carbonate shells is only dissolves at low pH, in this condition can be attained in the human body only when the food matrix, containing the calcium carbonate encapsulates, and is present in the stomach. Loading capacity of fish oil from calcium carbonate capsules is about 30–40 wt%, is effective when compared with other types of fish oil encapsulates. The particle size of fish oil encapsulated is about 20 μm which is quite tiny compared to other types of encapsulates [93].

2.8.2.3 Emulsions with protein or polymer shell

In fact, type of charge of emulsifier may effect to level of oxidation in fish oil [51]. Anionic emulsifier attract positively charge metal ion which passed them closer to the lipid phase and increase the oxidation level. Therefore, positively charge

emulsifier may resist and reduce oxidation such as sodium caseinate. Moreover, protein layer may protect fish oil from free radical scavenging[94]. For example tuna oil-in-water emulsion droplets (particle size 0.2–1 μm) which can be prepared by first emulsification with a food-grade anionic surfactant such as lecithin or fatty acid salts incubated with a cationic bio-polymer such as chitosan. This method may increase the stability of fish oil [51]. However, applications of this technology in food matrixes remain difficult because chitosan is not a food grade ingredient in the USA and Europe and is only allowed in food supplements and pharmaceutical applications. Furthermore, chitosan has no Kosher or Halal status because it is obtained from crustaceans.

2.8.3 Solvent displacement method

In this research, fish oil was encapsulated with ethylcellulose with solvent displacement method which is a new method for encapsulation fish oil. Solvent displacement implicates the precipitation of an operated and the diffusion of the organic solvent in the aqueous medium in the absence or presence of a surfactant and polymer from an organic solution. The polymer is dissolved in a water-based solvent of middle polarity, leading to the precipitation of spheres, this phase is added into a stirred aqueous solution. Polymer accumulation on the interface between the organic solvent and water, produced by fast diffusion of the solvent, leads to the

sudden formation of a colloidal suspension. The solvent displacement technique permits the formulation of nanocapsules when a slight volume of oil is combined in the organic phase (Figure 10).

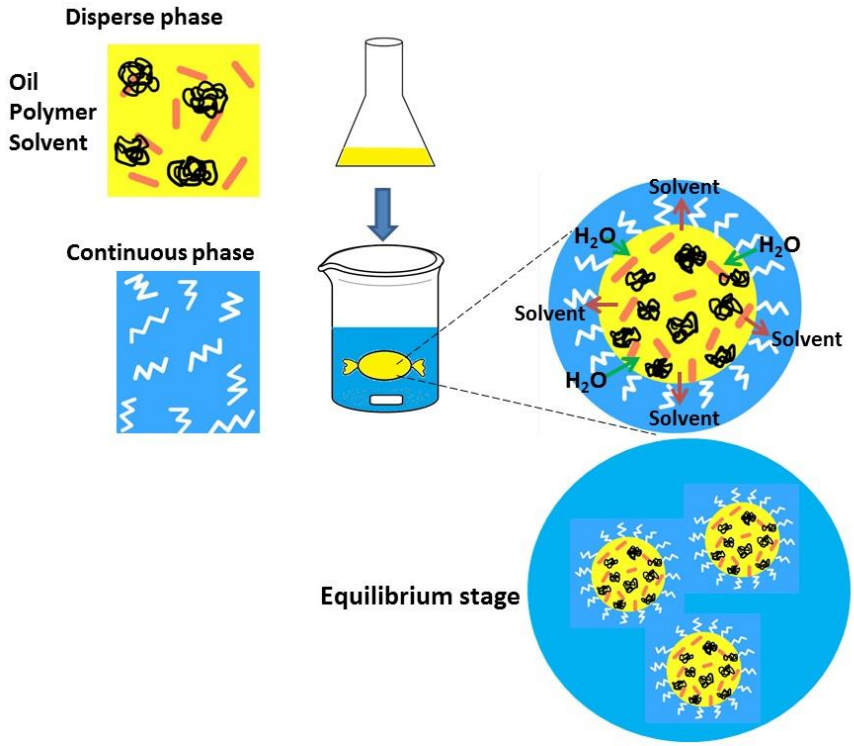


Figure 10 Solvent displacement method

2.10 White shrimp or *Litopenaeus vananmei*

White shrimp or *Litopenaeus vananmei* is the same family as black tiger shrimp *(Penaeus monodon*) and originated in Central and South America. It grows to a maximum length of 230 mm, with a carapace length of 90 mm, the moderately long rostrum with 7–10 teeth on the dorsal side and 2–4 teeth on the ventral side (Figure 11). The body is separated into 2 parts, the head which merged with the chest called

the cephalothorax and body section. Body and the abdomen consists of 6 segments, each segment has a pair of swimming feet are also segmented (6th pairs). White shrimp is the carnivorous animal that are naturally nocturnal animals and active at night to find food, whereas during the day hiding in the substrate or mud.

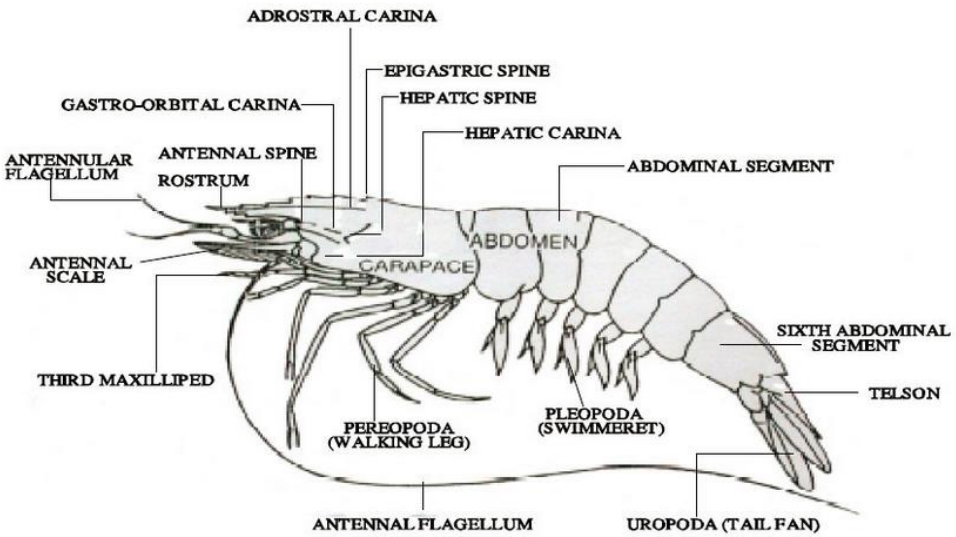


Figure 11 Morphology of white shrimp

In 1990, Thailand arise as the world's leading farmed shrimp producer and exporter. Therefore, white shrimp become economically important in Thailand. Nowadays, Thai shrimp farming and industry have a strong market focus with many processing and exporting companies to distribute Thai product worldwide.

2.11 Shrimp cycles

The penaeid shrimp cycle is composed of 5 stages. The first stage is egg shrimp's development. The laid eggs are sink in the deep water. Survived eggs are undergo to the nauplius phase. The unsegmented body which is pyriform in shape with three

pairs of appendages. The nauplius shrimp is molted within 50 hours into a protozoa. In this stage, the body is elongated with an obvious cephalothorax. After 4–6 days, the protozoea undergoes to a mysis stage. At this stage, mysis larva supposes the form of a juvenile shrimp which the which the swimming legs are developed. Next stage, shrimp change from Mysis to Postlarva. At the postlarva stage, the walking legs are seen on the ventral side of the abdominal segments. The body size of postlarva shrimp is 0.8-1 cm in body length. l. The shrimp grow very fast and are able to swim freely. The last stage is Juveniles which shrimp continues to grow on the sea bottom. Juveniles mature into sub-adults and adults which lay eggs before migrating back into the deep water to spawn (Figure 12).

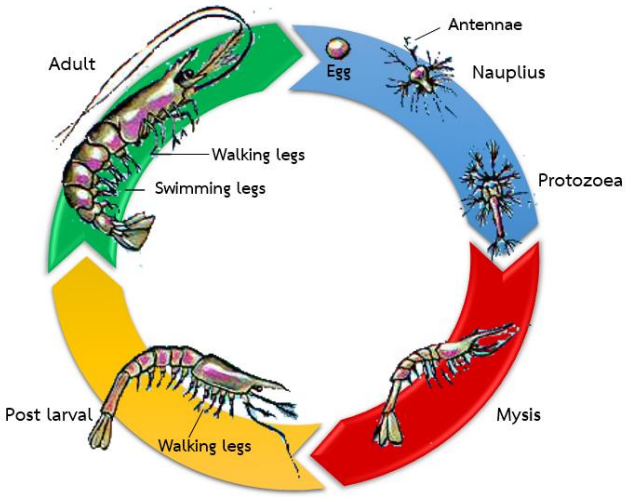


Figure 12 Shrimp lift cycle.

2.12 Post larvae evaluation

The postlarva shrimp quality is evaluated in term of physical and biological. In term of physical, the method is simple by using a microscope which muscle gut ratio (MGR), hepatopancreas, appendage and back muscle are assessed. For MGR ratio, the ventral muscle in the 6th abdominal segment under the intestine is investigated. The satisfied ratio between width of this muscles and the width of the gut is in a ratio 4:1 (Figure 13A). In addition, the condition of shrimp hepatopancreas gives a good indication of condition of the postlarva shrimp and feeding state. In fertile shrimp, the full and intensive colored hepatopancreas is observed (Figure 13B) due to the ingested feed whereas starved shrimp, the hepatopancreas takes on a pale and shrunken appearance. The back muscle (abdominal segment 1-5th) is evaluated based on their clarity. If discolored color appearance, the shrimp is considered as unhealthy whereas colorless appearance, shrimp is considered as healthy condition (Figure 13C). The rostrum and appendages of the healthy postlarva shrimp should be of normal shape without black discoloration (Figure 13D).

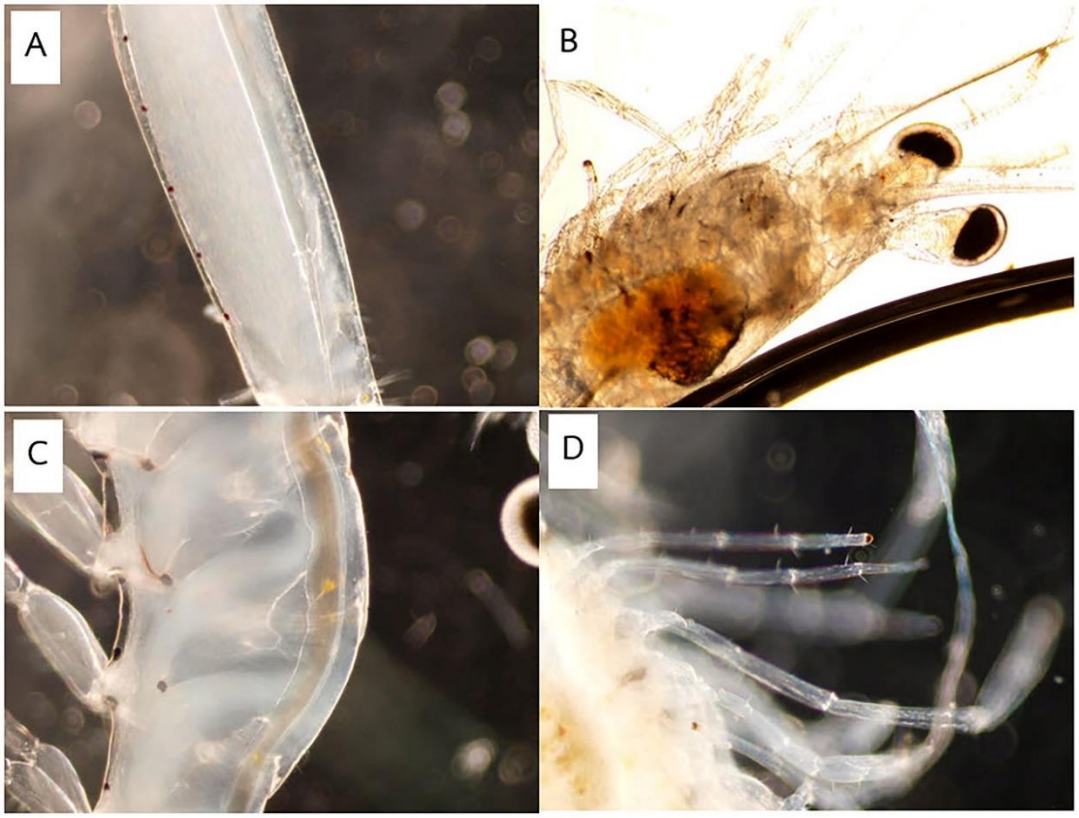


Figure 13 Muscle gut ratio (A), hepatopancreas (B), back muscle (C) and appendages (D) of healthy shrimp.

In term of biological, immunity and infection of pathogen are evaluate. For the immunity index; hemolymph, phenoloxidase are assessed. In shrimp, hemolymph is composed of plasma and hemocytes [95]. Coagulation of hemocyte (clot formation) forms a physical barrier to shield the loss of hemolymph and the penetration of pathogen into the hemolymph after an injury or infection [96]. The efficiency and velocity of coagulation are important for the survival of shrimp that confide on innate immunity. In addition, the prophenoloxidase (proPO) activation system is important immune systems in shrimp. The system is controlled by phenoloxidase (PO) which is the key enzyme and catalyses the early steps in the melanin synthesis [97]. PO

catalyzes hydroxylation of monophenols into o-diphenols, and following the oxidation of **O**-diphenols into **O**-quinones, which nonspecifically crosslinked with neighboring molecules to form the insoluble melanin. The production of **O**-quinones that are intermediates for cuticle sclerotization, and the sclerotized cuticle turned to be a barrier for infection. The highly toxic quinone substances and melanization around pathogens help in killing the pathogens directly [96].

2.13 Shrimp diet nutrition

The essential nutrients in shrimp diet are protein, lipid, carbohydrate, vitamin and mineral. Protein supplements are obtained from plant, animal and yeast such as; soybean meal, fishmeal and wheat flour [98]. Moreover, the raw material have been used as the protein source in shrimp diet including of shrimp meal, fishmeal, squid and soybean meal [99]. Commercial shrimp feed usually contains approximately 25% fishmeal [100] which is a good source of protein, high-energy content and high essential minerals. However, fishmeal is highly contained of lipid results in rancidity from lipid oxidation [101]. Fish oil usually used as the lipid source in shrimp diet (see 2.2.3) and contained approximately 2% in shrimp feed [102]. Wheat is a most ingredient which uses as carbohydrate source and binder to improve water stability in pellet feed (≈ 20%) [103]. Due to high gluten in wheat, it is required as binders for shrimp feed pelleting [104]. Moreover, other additives such as growth promoters, enzymes and

health additives and toxin absorber (probiotics, immunostimulants, vaccines), are added. Commercial synthetic antioxidants can be added for protection of lipid oxidation in shrimp diet, e.g., butylated hydroxyanisole (BHA) (250 to 500 ppm), butylated hydroxytoluene (BHT) (250 to 500 ppm), and ethoxyquin (125 to 150 ppm) [105].

2.14 Shrimp disease

For several years, the shrimp farmers concern about shrimp disease because it is the main cause to economic loss for shrimp industry. The death of shrimp by old age is normally fate, but the death by infestation, starvation and infection are much more considered. The death may be occurred by general environment or some agent. For example, the attraction in general environment is low temperature [106], lack of oxygen [107], and salinity extreme [108]. Moreover, shrimp disease by viral or bacterial infection is the crucial problem for shrimp culture industry. The example of disease by bacterial and viral etiologies in penaeid shrimp are shrimp early mortality syndrome (EMS), vibriosis, white spot syndrome, hepatopancreatic parvovirus (HPV), etc. Vibrio is the gram negative bacteria which frequently infects in penaeid shrimp. *Vibrio* bacteria species includes *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. penaeicida* and *V. alginolyticus*. *Vibrio* is the one chitinoclastic bacteria which associated with shell disease [109]. The grill of *Vibrio* infected shrimp is sensitive to bacteria penetration

because it has the thin skeleton [110]. *Vibrio harveyi* is the cause of luminescent bacterial disease. The syndrome of infected shrimp by *V. harveyi* is luminous, gastro-enteritis, eye- lesion and vasculitis and cloudy hepatopancreas [111]. The infected penaeid shrimp suffered mortalities within 48 h after challenge with *V. harveyi* [112].

CHAPTER III

EXPERIMENTAL

3.1 Materials, chemicals, reagents

All chemicals and reagents were analytical grade. Ethyl cellulose (EC; viscosity 250-300 cP; ethoxy content 48%), butylated hydroxytoluene (BHT), sodium thiosulfate ($Na_2S_2O_3$), starch powder, ninhydrin and sodium alginate, L-3,4-dihydroxyphenylalanine and trypsin were purchased from Sigma Aldrich (St. Louis, USA). Ethanol, sodium chloride (NaCl) and acetic acid ($C_2H_4O_2$) were purchased from Merck (Darmstadt, Germany). Fish oil (26% docosahexaenoic acid, 6% eicosapentaenoic acid) was purchased from TC Unoin Agrotech (Samut Sakorn, Thailand). Vegetable oil was purchased from Lam soon company (Bangkok, Thailand). Calcium chloride ($CaCl_2$) was purchased from Carlo Erba Reagent (Ronado, Italy). Tryotophan was purchased from Yuanshi Hongsheng Chemical Co., Ltd. (Hebei, China). Dialysis cellulose membranes (MWCO 12,400 Da) tubing, size 76mm×49mm was purchased from Sigma-Aldrich (St. Louis, USA).

3.2 Encapsulation of fish oil

The fish oil (26% docosahexaenoic acid, 6% eicosapentaenoic acid) was encapsulated by solvent displacement method [113]. At a fish oil: EC ratio 1:1, 5 g EC,

5.0 g fish oil and 0.5 g BHT were dissolved in 200 mL ethanol. Amount of fish oil and EC for the others ratio are shown in Table 5. Then, the distilled water was added under stirring with the flow rate of 1.0 mL/min, to obtain the final volume of 1.0 L. The encapsulated fish oil suspension was dialyzed against distilled water before being drying by freeze drying method.

Table 5 Various weight ratios of fish oil: EC for making encapsulated fish oil particle**.**

Weight ratio of fish oil: EC	Weight (g)		
	Fish oil	EC	BHT
1: 1	5	5	0.5
1: 2	5	10	0.5
1: 3	5	15	0.5

3.3 Characterization of fish oil particles

3.3.1 Morphology and size of microparticles

Morphology of encapsulated fish oil particles was characterized using scanning electron microscope (SEM, JSM-6400, JEOL, Tokyo, Japan) and transmission electron microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan).

3.3.1.1 Scanning electron microscopic analysis (SEM)

SEM was performed by the Center for Analytical Service, Faculty of Science, Chulalongkorn University, Thailand. A drop of the particle suspension was placed on a glass slide and dried overnight. The sample was coated with a gold layer under vacuum at 15 kV for 90 s. The coated sample was then observed by SEM with the accelerating voltage of 20 kV.

3.3.1.2 Transmission electron microscopic analysis (TEM)

TEM was performed by the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand. TEM photographs were obtained using an accelerating voltage of 100-120 kV together with selected area electron diffraction (SAED). To prepare the sample, a glass slide was dipped into the obtained suspension to obtain the particle film of nanoparticles on the glass slide surface.

3.4 Determination of loading capacity and encapsulation efficiency of fish oil particles

The amounts of fish oil incorporated into the polymeric particles were determined using NMR technique.

3.4.1 NMR spectrometry

Fish oil-loaded particle powder was dissolved in $CDCl_3$ and the obtained solution was subjected to 400 MHz NMR analysis (Varian Mercury NMR spectrometer, Agilent). The ratio of fish oil and EC were varied to prepare the calibration standard curve and shown in Table 6. The peak area of unsaturation proton (CH=CH, 5.2-5.4 ppm) and ethoxy protons ($-OCH_2CH_3$, 2.8-4.0 ppm) were compared the ratio and plotted versus the loading fish oil.

Table 6 Various weight ratios of fish oil: EC for making calibration standard curve.

Weight ratio of fish oil: EC (w/w)	Weight (mg)	
	Fish oil	EC
1 :1	10	10
2 :3	10	15
1 :2	10	20
1 :3	10	30

3.4.2 Encapsulation efficiency and loading

Encapsulation efficiency and loading capacity were then obtained as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Weight of encapsulated fish oil}}{\text{Total fish oil}} \times 100 \qquad (1)$$

$$\text{Loading (\%)} = \frac{\text{Weight of encapsulated fish oil}}{\text{Weight of fish oil and polymer}} \times 100 \qquad (2)$$

After plotting the ratio between proton of ethoxy (2.8-4.0 ppm) and unsaturation proton (5.2-5.3 ppm) versus the loading fish oil, the encapsulated fish oil was determined according to equation (1) and (2), respectively.

3.5 Oxidative stability

Oxidative stability of bulk fish oil, bulk fish oil added with BHT, encapsulated fish oil, and encapsulated fish oil-BHT were evaluated. All samples were kept under 4 different conditions: 1) under light exposure at room temperature, 2) in the dark at room temperature, 3) under light exposure at 4 °C, and 4) in the dark at 4 °C, and were evaluated for the peroxide value (PV) . Assessment was observed every four day during the 2 months storage using ISO 3960 (1998) method which is an iodometric titration. Briefly, sample 10 mg of bulk fish oil or 25 mg of encapsulated fish oil was dissolved in 3.0 mL of the acetic: chloroform (3:2 v/v) mixture. Then potassium iodide (50 µL, 0.005M) and water (3.0 mL) were added, and the titration was started. The

liberated I_2 was titrated with 0.01 N $Na_2S_2O_3$ using a starch solution as an indicator. The PV was calculated as follow:

$$PV = \frac{\text{Normality of } Na_2S_2O_3 \text{ (ml)} \times \text{consumption volume of } Na_2S_2O_3 \text{ (ml)}}{\text{Weight of oil (g)}} \times 1000$$

3.6 Thermal stability

Thermal stability of bulk fish oil, bulk fish oil mixed with BHT, encapsulated fish oil, encapsulated fish oil-BHT were heated up to the temperatures of 120 °C (mimicking the temperature during spray drying) and 95 °C (mimicking the temperature during shrimp feed pelleting). The samples were heated for 1, 2, 3, 4, 6, 8, 10 min. Then, it was immediately chilled on ice. The chilled samples were dissolved in $CDCl_3$ and subjected to ^{1}H NMR analysis. All samples before heating were also evaluated the unsaturation by ^{1}H NMR analysis.

3.7 Shelf life stability

Both encapsulated fish oil-BHT and bulk fish oil-BHT were kept at room temperature in brown bottles for 12 months. To determine the unsaturation content, 10 mg of bulk fish oil-BHT or 25 mg of encapsulated fish oil-BHT were dissolved with 1 mL in $CDCl_3$ and subjected to ^{1}H NMR analysis.

3.8 Micro gel bead fabrication

Fabrication of microbead using sodium alginate was proceeded by emulsification/ internal gelation method. Fifty milligrams of sodium alginate was dissolved in distilled water (25 mL) with gentle heating. After cooling, tryptophan (50 mg) and encapsulated fish oil-BHT (50 mg) were mixed in alginate suspension. The weight ratio of alginate, tryptophan and encapsulated fish oil-BHT experiment included 1: 1: 1, 1 :1 :2, 1 :1 :3, 1 :2 :1, 1 :2 :2, 1 :2 :3, 1 :3 :1, 2 :1 :1 and 2: 1: 3. Each mixture of alginate suspension containing of tryptophan and encapsulated fish oil was homoginized in 80 mL vegetable oil using homogenizer (IKA T-25 ULTRA-TURRAX Digital Homogenizer, Germany) by 8000 rpm for 1 hour. Then, the 50 mM $CaCl_2$ (25 mL) was gentle dropped into the mixture and left for 1 hour to allow phase separation. The microgel beads were collected from water phase and the filtered on a Whatman paper. Then, the microgel bead was washed with ethanol and distilled water. After that, the microbeads were dried by freeze drying.

3.9 Microgel bead characterization

Morphology and size of the dry microbead was characterized by SEM and TEM. The dried microbead was immersed in 50 mM pH 7 for 4 h and the size of wet microbead was estimated by light microscope. The encapsulation efficiency and loading of tryptophan were determined by 1) submerging 100 mg of microgel beads in

10 ml of 50 mM NaCl solution for 24 h and 2) the quantifying for tryptophan by ninhydrin method. Briefly, 1 ml of the ninhydrin solution was added to 5 ml of sample solution. Then, the test tubes were closed with paraffin film to avoid evaporation of solvent. Next, the mixture was kept at 100 °C for 8-10 min by submerging in water bath. After cooling in a cold water bath, the solution was made up to 10 ml with 60 % ethanol, and the absorbance at 570 nm was determined. The encapsulation efficiency (EE) and loading capacity were calculated as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Weight of encapsulated tryptophan}}{\text{Weight of originally used tryptophan}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{Weight of encapsulated tryptophan}}{\text{Weight of the microgel bead}} \times 100$$

Encapsulation efficiency and loading of encapsulated fish oil were determined by submerging of 100 mg of microgel beads in NaCl solution (25 mM, 10 mL) for 24 h. Here, 2 ml of the suspension were extracted with 2×2 ml of hexane for 24 h to extract the fish oil. The extract was evaporated under nitrogen flow to remove hexane. Then, the dried residue was dissolved in 1 mL $CDCl_3$ and 4 mg benzene was added as internal standard for ^1H NMR analysis. The peak area of unsaturation proton (CH=CH, 5.2-5.3ppm) and peak area of benzene (7.6 ppm) were used to calculate the fish oil content in the microgel bead.

The encapsulation efficiency (EE) and loading capacity were calculated as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Weight of encapsulated fish oil}}{\text{Weight of fish oil used}} \times 100$$

$$\text{Loading (\%)} = \frac{\text{Weight of encapsulated fish oil}}{\text{Weight of microgel bead}} \times 100$$

3.10 Confocal laser scanning fluorescence microscopy

Here we detect the presence of tryptophan through its auto fluorescence at 308-355 nm with the excitation at 348 nm, under the confocal fluorescence microscope. A drop of the microbead suspension was placed on a glass slice and covered with glass cover slice for observation. The confocal laser scanning fluorescence microscope used was the Nikon Digital Eclipse C1-Si (Tokyo, Japan) equipped with Plan Apochromat VC 100x, BDLaser (405 nm (Melles Griot, Carlsbad, CA, USA)), Nikon TE2000-U microscope, 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software.

3.11 Release of microgel bead

In vitro release studies were performed in various release mediums: 1) 427 M (25 ppt for mimicking the NaCl concentration of sea water), 12.5, 25, 50 mM NaCl solution in distilled water pH 7, to study the effect of salt, 2) 0.1N HCl pH 1.2, acetate buffer pH 4 and distilled water pH 7.0 in 50 mM NaCl solution to study the effect of pH. Fifty grams of microbead (the ratio of alginate: tryptophan: encapsulated fish oil was 1: 2: 2) was placed in conical flasks containing 25 ml of the release medium under gentle mixing. At predetermined time, the medium was placed in conical flasks containing 25 ml of the release medium under gentle mixing medium aliquots (5 ml) were collected and the fresh medium of similar volume was put into the flask to replace the withdrawn medium. The content of tryptophan was determined by ninhydrin reaction (see 3.9). The content of released fish oil from microgel bead was determined by taking 2 mL of release medium and twice extraction with 2 ml of hexane for 24 h. The extract was evaporated under nitrogen flow, the extracted microgel bead dissolved in 1 mL $CDCl_3$ and added 4 mg benzene as internal standard to ^1H NMR analysis. The peak area of unsaturation proton (CH=CH, 5.2-5.3ppm) and peak area of benzene (7.6 ppm) were used to calculate the fish oil content in the microgel bead.

3.12 The tryptophan and fish oil in microbead stability study

Microgel bead was kept for 12 months at room temperature in brown bottle. After 12 months keeping, 20 mg of the microbeads were collected and twice extracted by soaking in hexane for 24 h and then evaporated under nitrogen flow. The extract was subjected to ^{1}H NMR analysis. The peak area of unsaturation proton (CH=CH, 5.2-5.3ppm) and peak area of benzene (7.6 ppm) were used to calculate the fish oil content in the microgel bead. In other hand, 20 mg of microbeads were collected to determine the remained tryptophan in the microgel bead by ninhydrin reaction (see 3.9).

3.13 Preparation of shrimp feeds

3.13.1 Mixing before pelleting

The basal shrimp feed paste used was form Vet Superior Aquaculture co., ltd, Thailand. The pasts contains protein ≈ 35%, lipid ≈ 5%, fiber ≈ 4%; made from fish meal, wheat flour, soy meal, tuna fish oil, mineral, vitamin mixture, cholesterol, lecithin, vitamin C & E, astaxanthin and cellulose) were mixed with the fish oil sample. The shrimp feeds were prepared by two different methods: 1) the 2 kg basal shrimp paste mixing with 50g unencapsulated fish oil and 5g BHT (called as Feed CM) and 2) the 2 kg basal shrimp paste mixing with 125g encapsulated fish oil (containing of EC 75g, fish oil 50g and BHT 5g) (called as Feed M). All feed formulation was passed

through the pelleting machine for constructing the shrimp feed pellet at 85 °C for 10 min. The shrimp feed pellets were sieved through the mesh to obtain the pellet size 100-200 μm as shrimp food no.2 and 200-300 μm as shrimp food no.3. Four hundred milligrams of obtained feeds were soaked in hexane 1 ml, 2 times for 24h. The dry extracted feeds were dissolved in $CDCl_3$ and determined the unsaturation in feed before and after pelleting machine by ^1H NMR analysis. The peak area of methyl end protons ($-CH_3$, 0.8-0.9 ppm) and protons at the unsaturation (CH=CH, 5.30-5.35 ppm) were compared the ratio.

3.13.2 Spraying onto the pellet

One kilogram of commercial shrimp feed pellets (basal feed pellet, containing protein ≥ 38%, lipid ≥ 8%, fiber < 3%; including of fish meal, squid meal, fish oil, wheat flour, soy meal, wheat gluten, fish hydrolysate, lecithin, vitamin, mineral salt and food preservative, shrimp food no.3, 1-2 mm (Charoen Pokphand Foods Public Company Limited, Thailand) were made with two difference methods: 1) one kilogram of commercial shrimp feed pellets spraying by 400 mL encapsulated fish oil-BHT (2.5 g fish oil, 2.5 g EC, 0.25 g BHT) (called as Feed S) and 2) one kilogram of commercial shrimp feed pellets spraying by 400 mL distilled water. Both Feed S and Feed CS were heated at 65°C for 1h to discard the excess moisture. Four hundred milligrams of obtained feeds were soaked in hexane 1 ml, 2 times for 24h. The dry extracted feeds

were dissolved in $CDCl_3$ and determined the unsaturation in feed before and after heating by ^1H NMR analysis. The peak area of methyl end protons ($-CH_3$, 0.8-0.9 ppm) and protons at the unsaturation (CH=CH, 5.30-5.35 ppm) were compared the ratio.

3.14 Rearing of *Litopenaeus vannamei*

3.14.1 The raring shrimp by Feed M and Feed CM

Post-larva (PL4) of *Litopenaeus vannamei* were obtained from a shrimp farm in Chachoengsao province, Thailand. Shrimp were raised in 150-L flat bottom glass tanks with a closed recirculating system for 12 days. During this period they were fed four times daily with shrimp feed no.2 (see 3.13.1). The cultured water salinity level was initially at 25 ppt. Shrimp were placed randomly in test tanks at stocking density of 1000 shrimp/tank. The initial post-larva shrimp (PL4) were divided into control and treatment group. The control group was fed with Feed CM, whereas the treatment group was fed with Feed M. Triplicate trials per treatment were performed. Lengths and weights of 100 randomly collected shrimp from each individual tank (three tanks from treatment and three tanks from control) were determined after 3, 7 and 12 days. At the same time, living shrimp were counted for their survival record.

3.14.2 The raring shrimp by Feed S and Feed CS

Litopenaeus vannamei were obtained from a shrimp farm in Chachoengsao Province, Thailand and grew to 6-7 g. Shrimp were raised in 40-L flat bottom glass tanks with a closed recirculating system for 7 days. Shrimp were fed two times daily at 4% body weight per day with commercial shrimp feed no.3 (size 1-2 mm). The cultured water salinity level was initially at 25ppt. Shrimp were placed randomly in test tanks at the stocking density of 10shrimps/tank. The initial shrimp were divided into control and treatment group. The control group was fed with Feed CS, whereas the treatment group was fed with Feed S. Triplicate trials per treatment were performed. The hemolymph of shrimp were collected 2 shrimps for each tanks after 0, 3 and 7 days feeding.

3.15 Hemolymph collection

Hemolymph from each shrimp was collected from the ventral-sinus cavity using a 26-gauge needle and 1-ml syringe containing anticoagulant solution (13 mM EDTA, 50 mM NaOH, 0.45 M NaCl, 34 mM tri-sodium citrate, 29 mM citric acid and 0.1 M D-glucose).

3.16 Total hemocyte count

Hemolymph 0.2 mL was collected from two randomly collected shrimp per treatment using and was immediately mixed with 0.2 mL anticoagulant solution. Then, 0.1 μL hemolymph were taken to count and calculated the hemocyte (cells ml^{-1}) using a hemocytometer with light microscope at 10x magnification.

3.17 Hemocyte lysate supernatant preparation

The remained hemolymph (from 3.16) was centrifuged at 6500 rpm for 5 min at 4 °C. Hemocytes were washed and kept in ice-cold cacodylate CAC buffer (300 of μL, 0.01 M sodium cacodylate, 26 mM $MgCl_2$, 0.45 M NaCl, 10 mM $CaCl_2$, pH 7.0). The suspension was chilled at -30 °C for 1 h for breaking hemocyte cell and then centrifuged at 9700 rpm for 20 min at 4 °C. The obtained hemocyte lysate supernatant (HLS) was used immediately as an enzyme source.

3.18 Phenoloxidase activity assay

Phenoloxidase activity in HLS was evaluated by spectrophotometry using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate and trypsin as an elicitor [114]. Briefly, 50 μL of HLS was incubated with 50 μL of 1% trypsin in CAC buffer at room temperature for 20 min, and then 50 mL of L-DOPA 0.3% in CAC buffer were added

and incubated at room temperature for 10 min. The optical density was measured at a 490 nm against a blank consisting of CAC buffer, L-DOPA and elicitor. One unit of enzyme activity was determined. Protein content in HLS was measured via the Bradford method [115], using bovine serum albumin as a standard protein.

3.19 Challenge tests

After 7 days raring, shrimp (from 3.14.2) were challenged with *V. harveyi* which had been cultured and maintained using Tryptic soy broth and agar with 2% NaCl (w/v). Shrimp were divided randomly into control (2 tanks) and treated groups (2 tanks) 10 shrimp per tanks and each group was separately cultured in 3-L plastic tank with flat bottom. The cultured water salinity level was initially at 25 ppt. *V. harveyi* suspension was added in each tank 10^4 CFU mL^{-1}. The survival rate was observed.

3.20 Lipid in shrimp hepatopancreas

Two shrimp were randomly collected from each cultured tank at the end of the feeding trial (after 12 days culture for 3.14.1 and after 7 days cultured for 3.14.2). The living shrimp and glass slide were washed with distilled water. Then, the shrimp hepatopancreas was cut, fixed and covered with glass slide. The hepatopancreas investigation was performed under light microscope (Olympus CX21 for 3.14.1 and Olympus CX41 for 3.14.2).

3.21 Statistical analysis

Mann Whitney U test was used to analyze the differences in Peroxide value (p <0.05) of different fish oil samples. Differences (p <0.05) of unsaturation in shrimp feed, growth, length, weight and survival rate between the control shrimp and the treated shrimp groups were analyzed using paired t-test. Difference (p <0.05) in phenoloxidase and total hemolymph with same condition were analyzed using one way ANOVA Duncan.

CHAPTER IV

Results and discussion

4.1. Encapsulation of fish oil

This study prepared encapsulated fish oil microparticles using solvent displacement method. Fish oil was encapsulated into polymeric particles via self-assembly process. Here, ethylcellulose (EC) was used as a polymeric carrier. The weight ratio of fish oil: EC was varied to 1: 1, 1: 2 and 1: 3. During solvent displacement process, ethanol was slowly displaced by water. Polymer chains of ethylcellulose can self-assemble into particles. Most hydroxyl groups direct themselves toward outer surface of particles to interact with water molecules and stabilize the particles suspension, while most of ethoxy groups of sugar units orientate inside the particles to escape themselves from water and interact with fish oil which hydrophobic molecules within particles.

4.2 Characterizations of encapsulated fish oil

4.2.1 Morphology of encapsulated fish oil

The milky white suspension of encapsulated fish oil was dried by freeze drying. Morphology of encapsulated fish oil particle was investigated using TEM and SEM. SEM and TEM photographs showed that the encapsulated fish oil microparticles were

spherical shape (Figure 14). The particle size of encapsulated fish oil, determined from SEM photograph (Figure 14A), showed the mean size 1 ± 0.74 μm. Furthermore, the milky white suspension of encapsulated fish oil shows the good dispersion in water. Therefore, fish oil in the form of microparticles might be a new approach to overcome the water insolubility of fish oil.

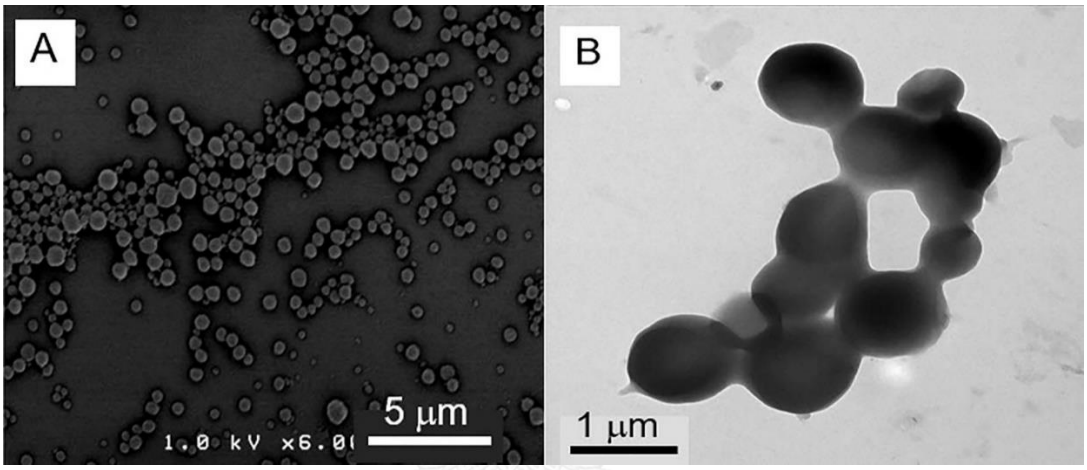


Figure 14 SEM (A) and TEM (B) images of fish oil-loaded ethylcellulose particles.

4.2.2 Encapsulation efficiency and loading capacity of encapsulated fish oil

Amount of fish oil in the particles was determined by ^{1}H NMR analysis. The ^{1}H NMR spectra of bulk fish oil (Figure 15A) is absence the resonance of proton of ethoxy in EC (2.8-4.0 ppm) (Figure 15B). The ratio of peak area between proton ethoxy of EC (2.8-4.0 ppm) and unsaturated carbon (CH=CH, 5.35 ppm) of the fish oil were compared to those of the standard mixtures at various mixing ratios (Figure 16). Assuming that no degradation of unsaturation took place during encapsulation, the analysis shows the

fish oil loading of 41%. This corresponds to the encapsulation efficiency of 81% for the encapsulation process at a fish oil: EC ratio, 1:1. The encapsulation efficiency and loading of encapsulated fish oil with difference ratio are shown in Table 7.

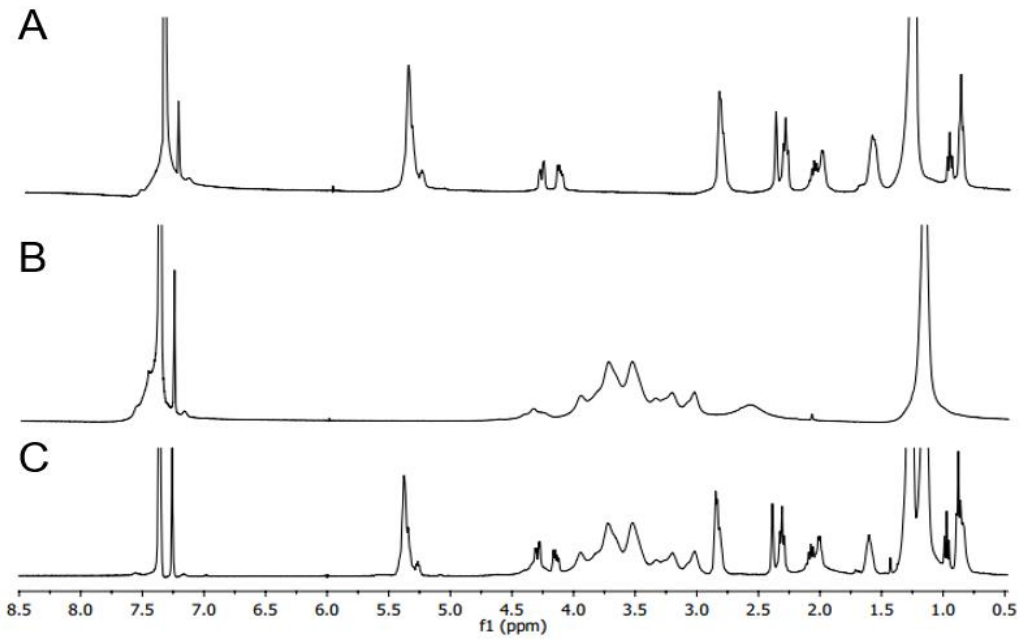


Figure 15 ^{1}H NMR spectra of fish oil (A), ethylcellulose (B) and encapsulated fish oil (C).

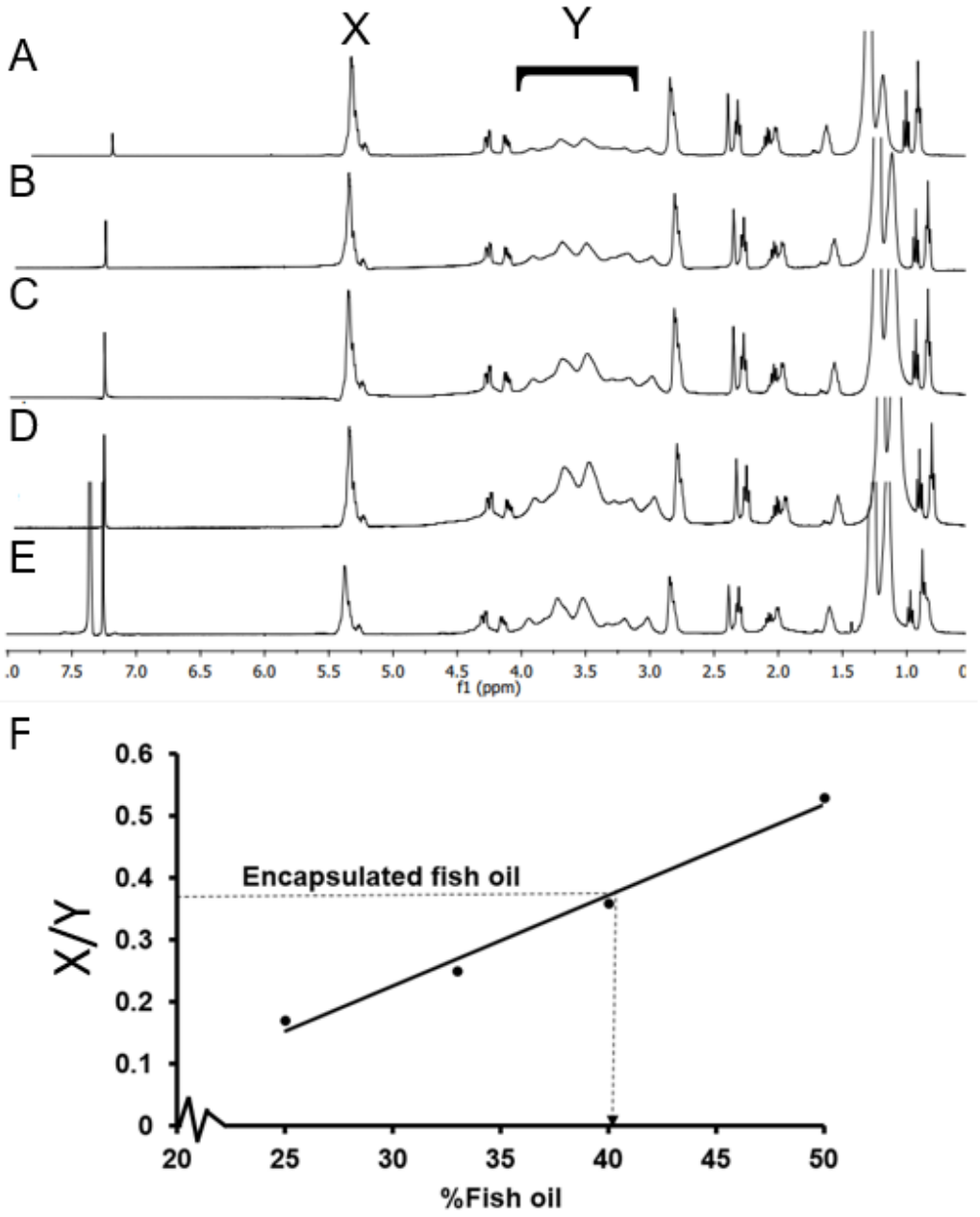


Figure 16 ^{1}H NMR spectra of fish oil/ethylcellulose mixtures at 1:1 (A), 2:3 (B), 1:2 (C), 1:3 (D) ratio (w/w), and of the encapsulated fish oil sample (E). The plot between ratio of peak area representing protons from unsaturation (5.30-5.35 ppm) and protons from ethylcellulose (2.8-4.0 ppm) versus fish oil percentage in the fish oil-EC standard mixtures, is shown in (F). The ratios of peak area representing unsaturation protons and ethylcellulose protons of the encapsulated fish oil sample from spectrum E, is used

to estimate the loading content of fish oil in the sample as shown with the dotted line in (F).

Table 7 Encapsulation efficiency and loading of encapsulated fish oil with difference fish oil: EC ratio.

Weight ratio of fish oil: EC	% Loading	%Encapsulation efficiency
1: 1	40.56	81.12
1 :2	28.23	84.61
1 :3	22.75	91.01

4.3 Oxidative stability

The peroxide value (PV) is the index to evidence the rancidity in unsaturated fats and oils. The double bonds found in oils and fats play an important role in autoxidation which is a free radical reaction related with oxygen that leads to deterioration of fats and oils which form off-flavors and off-odors. The stability of bulk fish oil, bulk fish oil-BHT, encapsulated fish oil, encapsulated fish oil-BHT under various storage conditions were evaluated for the PV by simple iodometric method. PV of all samples show the same pattern of gradually increase upon storage. At room temperature, the increasing of PV in bulk fish oil samples are higher than those the encapsulated fish oil samples (Figure 17). In addition, the PV of sample which kept at room temperature is higher than those keeping in fridge at 4 ^{o}C. It suggests that the heat by normal environment is important cause for fat degradation. Interestingly, bulk

fish oil at 4 °C shows the highest PV under light exposure. Although, it was kept under light-off condition, the PV of bulk fish oil was still high. While, the encapsulated fish oil and encapsulated fish oil-BHT at 4 °C show no significantly difference between light and light-off condition (Figure 17B). The results indicate that the BHT as antioxidant is no effect for encapsulated fish oil samples for protecting the photo-oxidation. In contrast, BHT have an important role when kept all sample at room temperature as shown in Figure 17B. The PV of all samples at room temperature which added with BHT show the lower PV than those samples without BHT. Fish oil encapsulated EC is more stable than unencapsulated fish oil. Moreover, fish oil- BHT encapsulated EC is more stable than fish oil encapsulated EC. The results suggest that fish oil should be co-encapsulated with BHT to EC particle for highest stability.

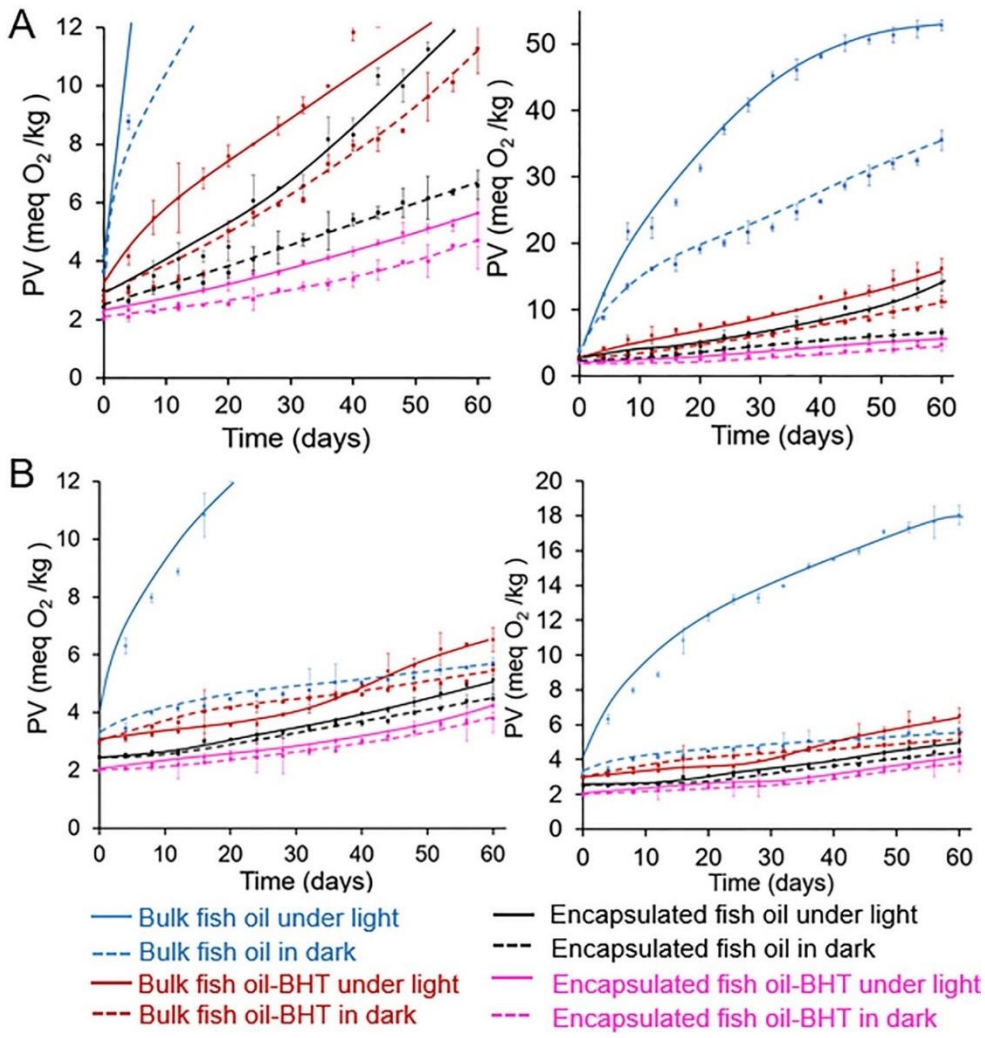


Figure 17 Peroxide values (PV) of the bulk fish oil, bulk fish oil-BHT, encapsulated fish oil and encapsulated fish oil-BHT when kept at room temperature (A), and at 4 °C (B) for 2 months. Graphs on the left are expansions of those on the right.

4.4. Thermal stability

The all oil samples were evaluated for stability when being expose to 95 °C (mimicking the temperature in feed manufacturing process) and 120 °C (mimicking the temperature used during spray drying) for 10 minutes. The decreasing of unsaturation

at 0, 1, 2, 4, 6, 8, 10 min were evaluated by ^{1}H NMR analysis. The ratio of the peak area between methyl end protons (CH_3, 0.8-0.9 ppm) and unsaturation carbon (5.2-5.5 ppm) was compared (Figure 18).

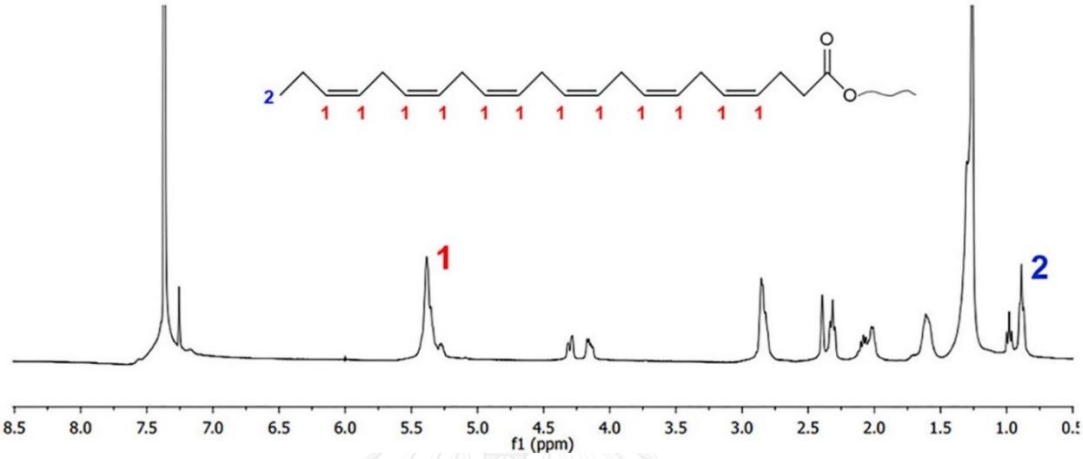


Figure 18 ^{1}H-NMR spectrum of fish oil in $CDCl_3$. The ratio between peak integration of protons on the unsaturation (1) and peak integration of protons at the terminal methyl groups (2) are used to estimate the amount of unsaturation in the fish oil sample. Structure of docosahexaenoyl moieties is shown as an example of unsaturated fatty acid constituents in the fish oil sample.

After heating at 95 °C for 10 min, the unsaturation lose from the encapsulated fish oil with and without 10% BHT was approximately 24 ± 3% and 70 ± 3% of their unsaturation, respectively, whereas the two bulk fish oil (with and without BHT) lose over 90% of the unsaturation at only 3 min of heating (Figure 19B). After heating at 120 °C for 10 min, the encapsulated fish oil with and without BHT lose 36 ± 3% and 74 ± 3% of their unsaturation, whereas the bulk oils lose over 90% of the unsaturation after

2 min heating (Figure 19A). The results indicate that the encapsulated samples show the higher thermal stability than those the unencapsulated. Moreover, in BHT adding samples shows thermal stability better than without BHT samples. The results clearly show that encapsulation process with antioxidant is more effective for protection fish oil from shrimp feed preparation machine and spray dryer.

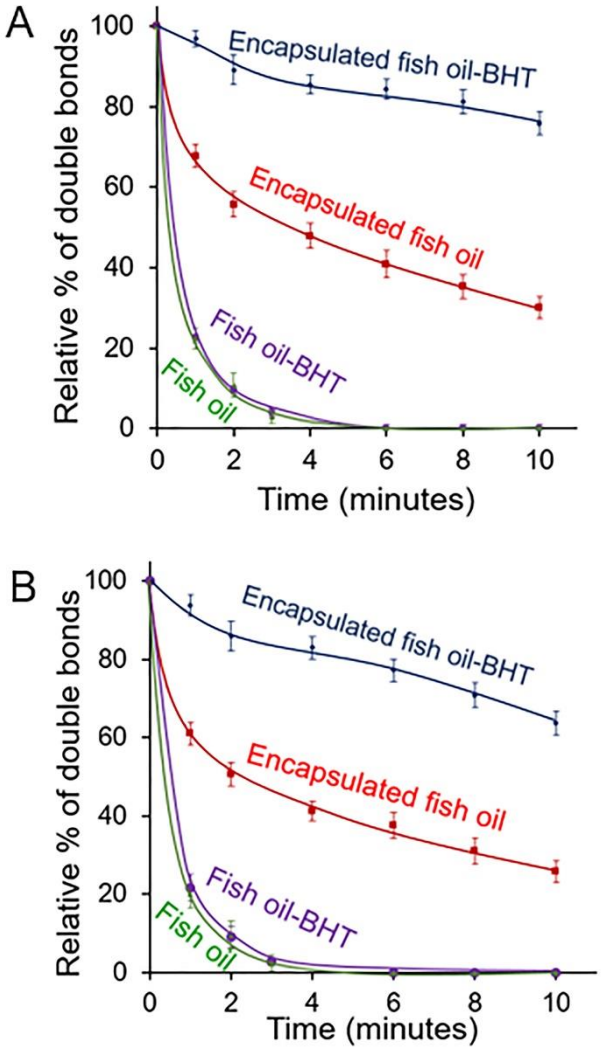


Figure 19 Thermal degradation of unsaturation functionality in the bulk fish oil, bulk fish oil-BHT, encapsulated fish oil and encapsulated fish oil-BHT, when being kept at 95 °C (A) and 120 °C (B) for various times.

4.5. Shelf life stability

The all oil samples were kept for 12 months at room temperature in brown bottle to observe the shelf life stability. The peak area between methyl end protons (CH_3, 0.8-0.9 ppm) and unsaturation carbon was compared to determine the decreasing of unsaturation in oil sample. After 12 months storage, the loss of unsaturation in encapsulated fish oil-BHT is only 15%, whereas loss of unsaturation in bulk fish oil-BHT raised to 90%. Moreover, the bulk fish oil and encapsulated fish oil without BHT were evaluated the loss unsaturation which showed that the unsaturation in bulk fish oil is only 8.71%, whereas the unsaturation in encapsulated fish oil is 52.82% (Figure 20). The results indicate that long shelf life was observed for the encapsulated fish oil-BHT

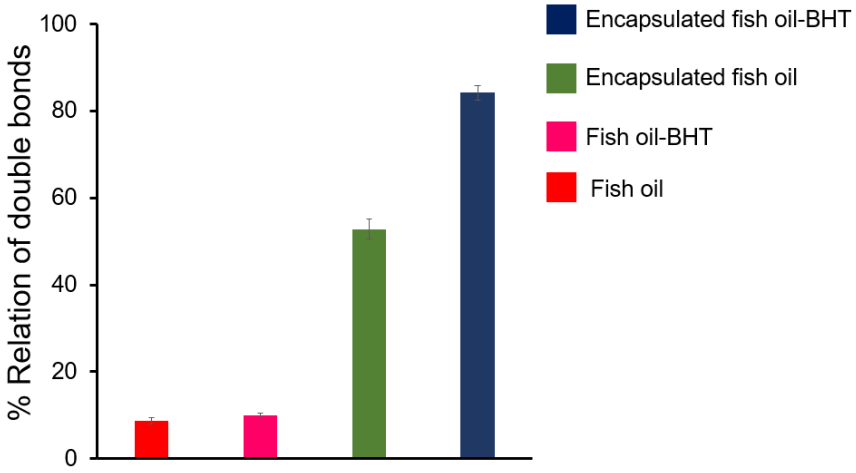


Figure 20 Time degradation of unsaturation functionality in the bulk fish oil (■), bulk fish oil-BHT (■), encapsulated fish oil (■) and encapsulated fish oil-BHT (■) for 12 months in brown bottle at room temperature.

All parameter using for determination the stability of bulk fish oil, bulk fish oil-BHT, encapsulated fish oil and encapsulated fish oil-BHT are shown in Table 8.

Table 8 The summarized parameters for measurement the stability of bulk fish oil, bulk fish oil-BHT, encapsulated fish oil and encapsulated fish oil-BHT.

Samples	Parameters						
	PV after 2 months (meq O2/kg)				%Unsaturation after heating for 10 min		%Unsaturation after 2 month storage
	At room temperature		At 4 °C				
	Light	Light off	Light	Light off	95 °C	120 °C	
Fish oil	52.0 ± 0.7	34.2 ± 1.5	18.7±0.5	5.5 ± 0.2	-	-	8.7 ± 1.2
Fish-BHT	15.1 ± 1.5	11.4 ± 0.7	6.3 ± 0.2	5.2 ± 0.2	-	-	9.9 ± 1.5
Encapsulated fish oil	12.0 ± 1.2	6.5 ± 0.5	5.1 ± 0.5	4.4 ± 0.1	30.1 ± 2.7	25.87± 2.7	52.8 ± 2.8
Encapsulated fish oil-BHT	5.5 ± 0.7	4.5 ± 0.5	4.2 ± 0.5	3.4 ± 0.2	75.8 ± 2.9	63.7 ± 3.1	84.2 ± 2.7

4.6 Shrimp feed containing encapsulated fish oil

4.6.1 Shrimp feed for pelleting machine

The bulk fish oil-BHT and encapsulated fish oil-BHT were mixed with commercial shrimp paste and subjected the paste to pelleting machine at 85-95 °C for 10 min to construct the shrimp feed pellet. The unsaturation functionality part in each shrimp feed was evaluated. Feed M (preparing by adding the encapsulated fish oil-BHT particles and passing to the pelleting machine) shows the remained

unsaturation of 84.75%, whereas the Feed CM (preparing by adding the fish oil-BHT and passing to the pelleting machine) only obtained unsaturation is 22.56% (Figure 21) unsaturation content as related to the unsaturation content before heating. The results clearly indicated that the encapsulated fish oil-BHT was stable than those the bulk fish oil-BHT. Therefore, we conclude that the unsaturated fatty acid components in the fish oils is well protected during the shrimp feed production that requires high temperature exposure.

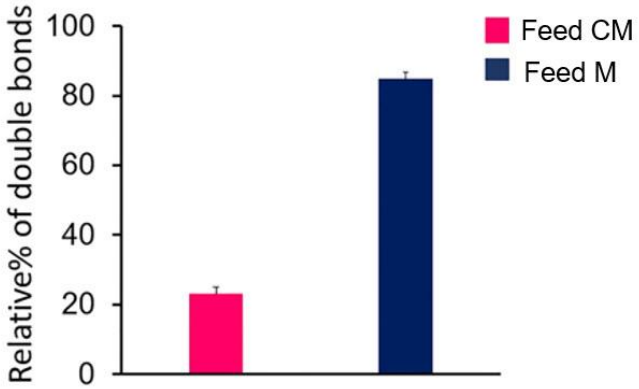


Figure 21 Thermal degradation of unsaturation functionality in Feed CM (■) and Feed M (■), after passing pelleting machine at 85 °C for 10 min.

4.6.2 Shrimp feed by spraying the encapsulation fish oil-BHT

The encapsulated fish oil-BHT suspension was sprayed on the commercial shrimp feed pellet (Feed S). The control shrimp feed (Feed CS) was sprayed the distilled water replacing the encapsulated fish oil-BHT suspension on the commercial

shrimp feed pellet. Two formulation shrimp feeds were heated at 65 °C for 1h to discard the excess moisture. Then, the unsaturation in each feed was evaluated by ^{1}H NMR analysis. The remained unsaturation in Feed S after heating at 65 °C for 1 h was 95.33% (Figure 22). The result agrees with the above experiment (4.6.1) which indicates that the encapsulated fish oil-BHT is stable at high temperature.

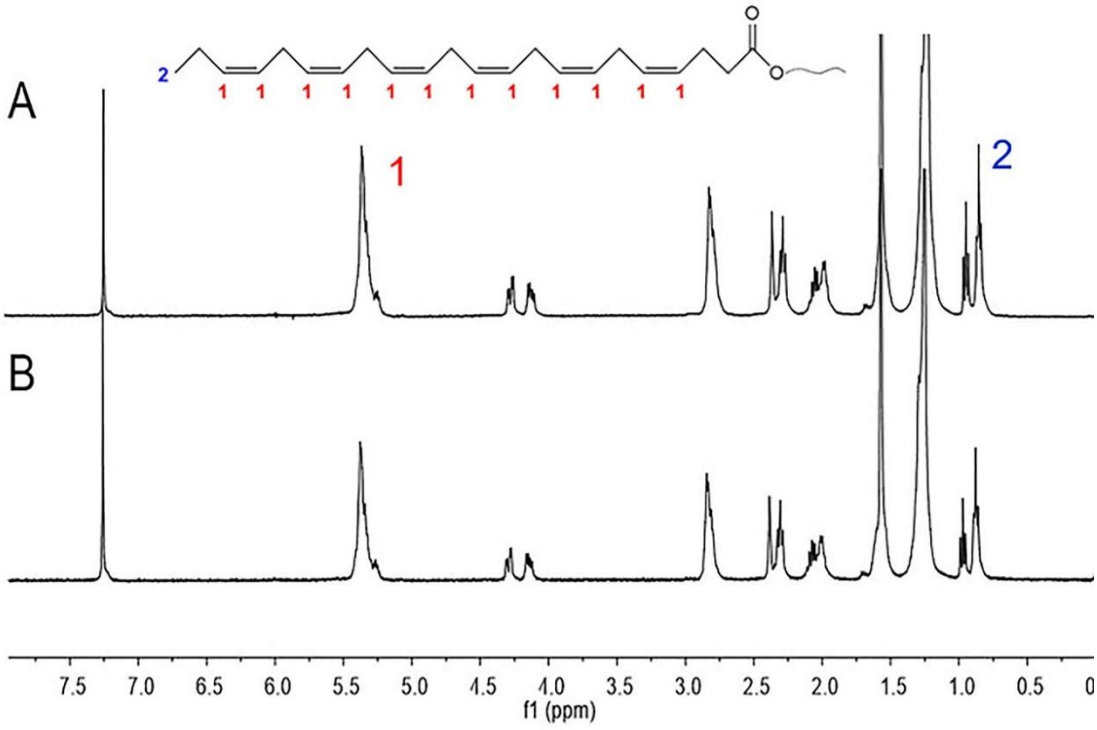


Figure 22 ^{1}H NMR spectrum of the extract of the freshly spray Feed S (in $CDCl_3$) (A) and Feed S that has been dried at 65 °C for 1h (B).

4.7 Effect of encapsulated BHT-fish oil on growth and survival of *Litopenaeus vannamei*

To investigate the shrimp growth, two experiment were carried out, first is the shrimp feeding with Feed M and Feed CM and second is the raring with Feed S and Feed CS.

4.7.1 The shrimp raring with Feed M and Feed CM

The postlarva (PL4) *Litopenaeus vannamei* 1000 shrimp/ tank were cultured and fed with Feed M (preparing by adding encapsulated fish oil-BHT particle and passing the pelleting machine) as treatment and Feed CM (preparing by adding fish oil-BHT and passing the pelleting machine) (control) for 12 days. The survival, weight and length were measured. After 7 days culturing, mean weight of the treat shrimp was significantly better than that of the control shrimp (p <0.05) (Figure 23A). However, after 12 days, no significant difference in the weight between these two groups was observed. Mean length of the control shrimp and the treatment groups is not significantly different from those of the control groups (p >0.05) (Figure 23B). After 12 feeding, the survival of control and treat shrimp show the significantly different (p <0.05) (Figure 4.9C). The results indicate that treat shrimp which fed Feed M shows the positive growth effect to increased survival rate, comparing with control shrimp which fed with Feed CM. This result is correlated with the contained unsaturation in Feed M

which show the high remained unsaturation content in fed than those compare the remained unsaturation in Feed CM (see 4.6.1).

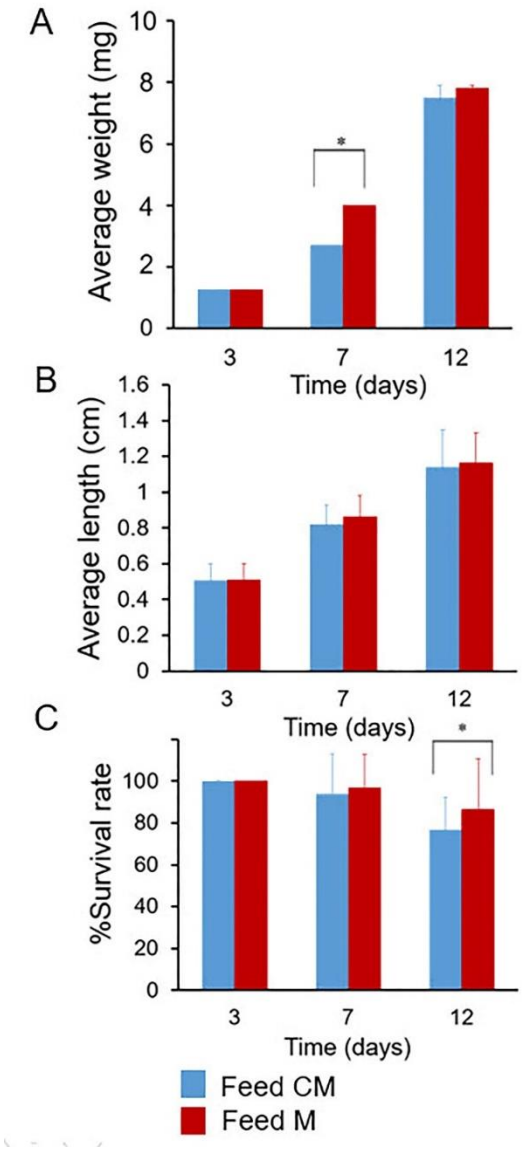


Figure 23 Weight (A), length (B) and survival rate (C) during the 12 day observation of the postlarva *Litopenaeus vannamei* fed with Feed CM (control) and Feed M

(treatment). Statistical significant differences were determined by paired *t*-test at $p < 0.05$.

4.7.2 The shrimp raring with Feed S and Feed CS

Litopenaeus vannamei (6-7 g) were fed with Feed S (preparing by spraying encapsulated fish oil-BHT particle) as treatment and Feed CS (preparing by spraying distilled water) as control shrimp for 7 days. After 7 days feeding, the survival, length and weight were measured. After 7 days culturing, mean shrimp weights were 6.7 $\pm$ 0.5 and 7.8 $\pm$ 0.1 g for the control and the treatment shrimp, respectively. Shrimp lengths were 9.59 $\pm$ 0.17 and 9.72 $\pm$ 0.21 cm, and shrimp survivals were 86.7 $\pm$ 5.8% and 93.3 $\pm$ 11.5% (Table 9), for the control and the treat shrimp, respectively. All parameters were significantly different between the control group and the treatment group ($p < 0.05$). The results indicate that *L. vannamei* fed with Feed S grow and survive better than those Feed CS (using distilled water instead of encapsulated fish oil-BHT). This suggests the effect of fish oil in encapsulated fish oil-BHT particle in shrimp feed.

Table 9 Weight, length and %survival of *L. vannamei* fed with Feed S (treatment) and Feed CS (control).

Parameters	Treated shrimp	Control shrimp
Weight (g)	7.8 ± 0.1	6.7 ± 0.5
Length (cm)	9.72 ± 0.2	9.59 ± 0.2
Survival (%)	93.3 ± 11.5	86.7 ± 5.8

4.8 Hepatopancreas *of Litopenaeus vannamei*

Hepatopancreas of shrimp is the one cause to show the shrimp health. Shrimp with good condition shows full and intense yellow-orange color of hepatopancreas, whereas starved shrimp shows shrunken and pale color of hepatopancreas. It is an organ in shrimp for digestive feed like the liver and pancreas in mammals. In this work, the hepatopancreas of shrimp feeding with Feed M, Feed CM, Feed S and Feed CS were investigated under light microscope. After 7 days feeding, shrimp were collected and cut the hepatopancreas. Treat shrimp which fed with Feed S shows full and dark orange color of hepatopancreas. The lipid droplets in hepatopancreas give more droplets and larger size than those the control shrimp (feeding with Feed CS) (Figure 24). Moreover, shrimp fed with Feed M (treatment) and Feed CM (control) were observed the hepatopancreas. The results show the same pattern that the treat shrimp

hepatopancreas condition is better than those control shrimp (Figure 25). The condition of hepatopancreas both control and treat shrimp were correlated with the unsaturation in the shrimp feed which shows the high remained content unsaturation of unsaturated fatty acid in Feed M and Feed S.

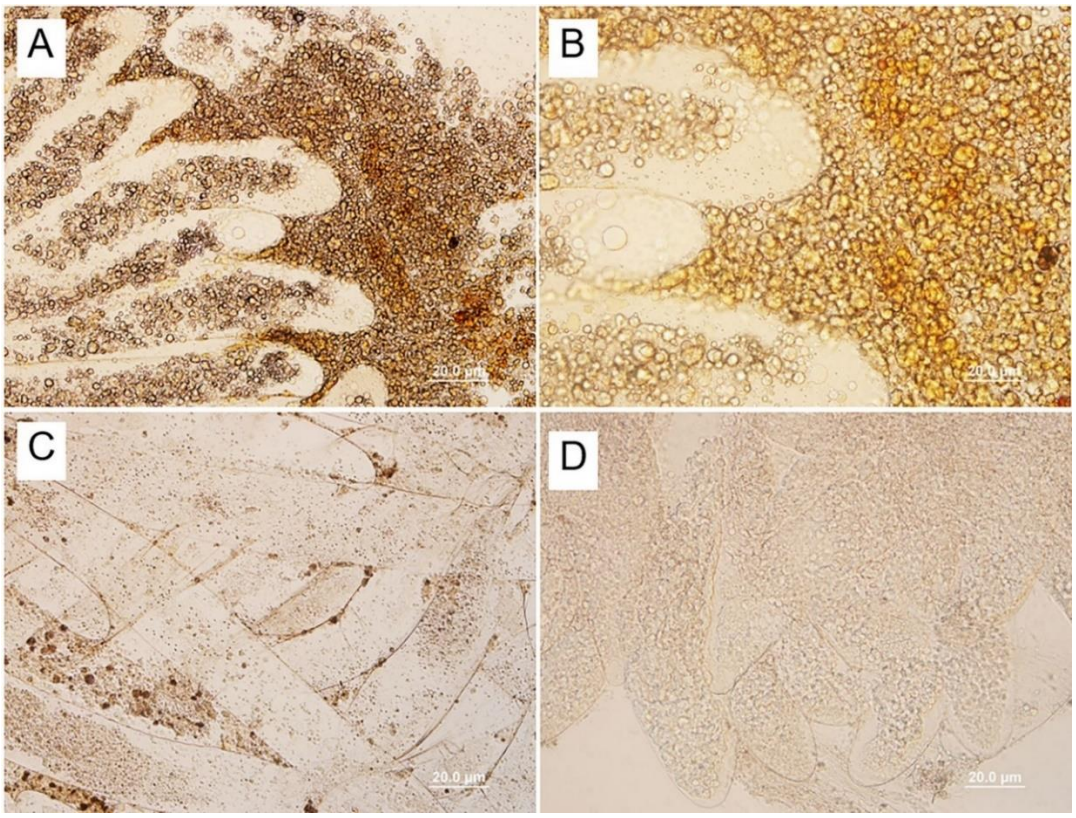


Figure 24 Hepatopancreas of Litopenaeus vannamei fed with Feed S (A, B) and Feed CS (C, D). The whole view of hepatopancreas is shown in the 10x magnified picture (A and C) and oil droplets can be viewed in the 20x magnified pictures (B and D).

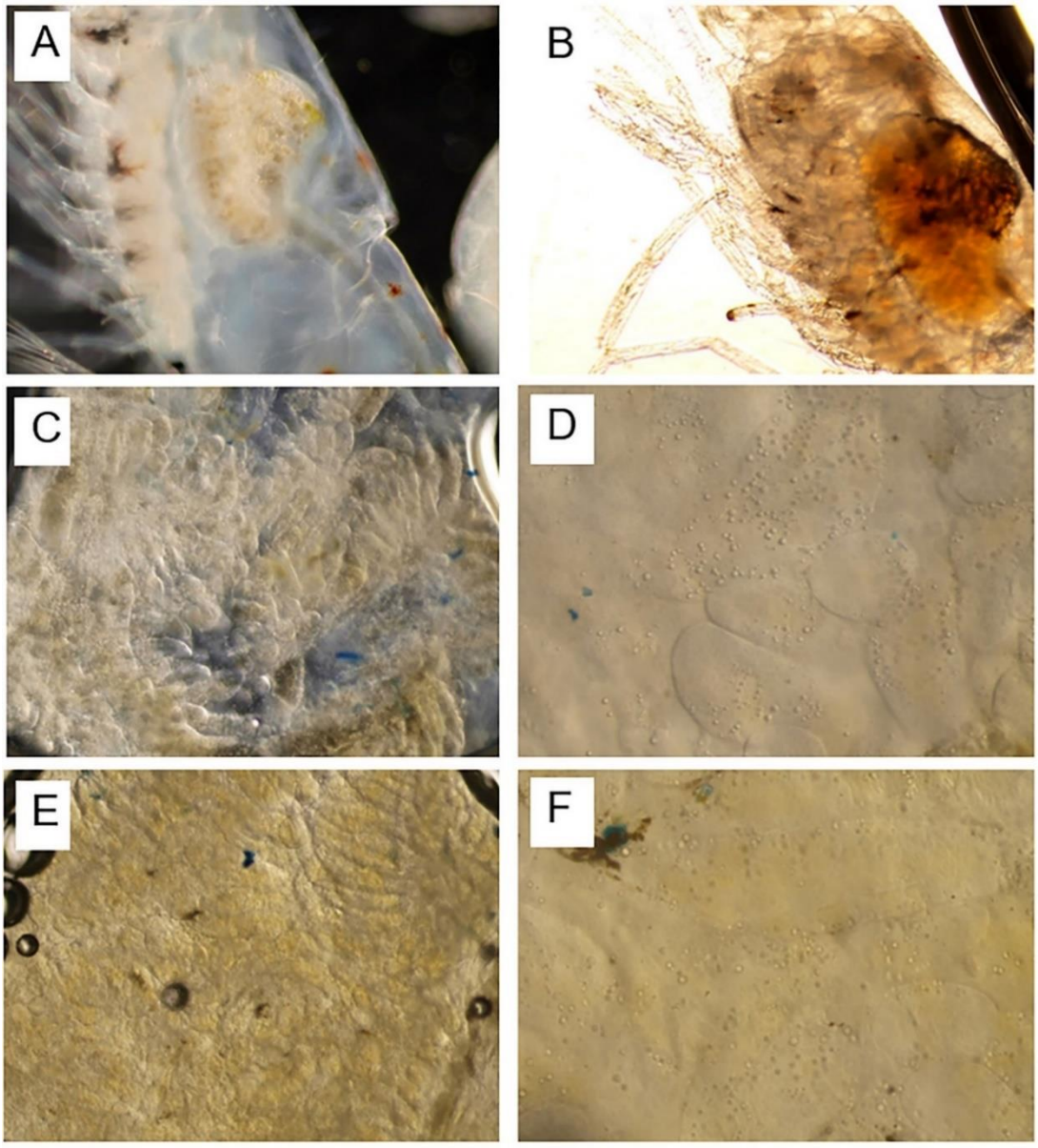


Figure 25 Hepatopancreas of postlarva *Litopenaeus vannamei* fed with Feed CM (A, C, E) and Feed M (B, D, F): The whole view of hepatopancreas (A and B), magnification at 4x (C and D) and 25x (E and F) showing oil droplet in the hepatopancreas.

4.9 Immunity indexes in shrimp

Shrimp immune response system is divided into cellular and humeral immune response. The cellular defense related to the hemocyte-mediated immune response. The hemocytes recognize to the receptor on the surface of foreigners and result to the nodulation, phagocytosis and encapsulation [96]. In contrast, the humeral defense refers to create antimicrobial peptide, nitrogen intermediated and reactive oxygen which toxic to the foreigner and related with the phenoloxidase activity [116]. Shrimp (feeding with Feed S and Feed CS) were collected the hemolymph after 0, 3 and 7 days feeding and the hemocyte and phenoloxidase were evaluated. Both control and treated shrimp, total hemocyte means showed the same pattern. No significant differences ($p > 0.05$) occurred between control and treatment shrimp means after 7 days feeding. It suggests that the cellular defense of control shrimp is not difference when compared to those of treated shrimp. In the other hand, all treated shrimp showed significantly greater phenoloxidase than those of the controls ($p < 0.05$). After 7 day feeding, treat shrimp showed high phenoloxidase of 885.9 $\pm$ 100.3 unit/ mg protein (Table 10). The results indicate that treated shrimp shows the higher humeral defense than control shrimp.

Table 10 Mean immunity index value of control and treat- L. vannamei during 7 days culture.

Immunity index	Duration of shrimp culture (day)	Mean ± S.D.	
		Control	Treatment
Total hemocytes (1×10^6 cell ml^{-1})	0	1.24 ±0.24 [a]	1.37 ±0.26 [a]
	3	1.51 ±0.79 [a]	1.53 ±0.64 [a]
	7	1.53 ±0.56 [a]	1.53 ±0.29 [a]
Phenoloxidase (unit /mg protein)	0	137.5 ±19.6 [a]	128.6 ±15.1 [a]
	3	282.4 ±50.2 [b]	316.4 ±58.5 [b]
	7	310 ± 63.1 [b]	885.9 ±100.3 [c*]

Mean not sharing a common superscript letter between column value for each index differ significantly ($p< 0.05$)

[*] Indicates significantly difference ($p< 0.05$) between controls and treat shrimp means for a given row.

4.10 Shrimp challenging

After 7 days feeding with Feed S and Feed CS, both treat (feeding with Feed S) and control (feeding with Feed CS) shrimp were challenged with *V. harveyi* and continued raring for 3 days without feeding. After 3 days challenging, the survival of shrimp were evaluated. The result showed that all control shrimp (feeding with Feed CS) were death after 2 days, whereas the survival of treat shrimp (feeding with Feed S) after 3 days challenging was 70% (Table 11). It suggests that shrimp feed with high content of unsaturated fatty acid shows the good resistance for the infected pathogen.

Table 11 The survival shrimp after challenging with *V. harveyi* after 3 days.

Challenging day (days)	Survival shrimp (shrimps)			
	Control		Treatment	
	Tank 1	Tank 2	Tank 1	Tank 2
1	10	10	10	10
2	-	-	10	10
3	-	-	7	7

4.11 Microgel bead

4.11.1 Encapsulation efficiency and loading of encapsulated fish oil and tryptophan

This study prepared the microgel bead containing both hydrophilic and hydrophobic oil by emulsification- internal gelation method. The microgel beads were made with different ratio of alginate: tryptophan: encapsulated fish oil-BHT, 1: 1: 1, 1: 1: 2, 1: 1: 3, 1: 2: 1, 1: 2: 2, 1: 2: 3, 1: 3: 1, 2: 1: 1, 2: 1: 2 and 2: 2: 2.

The microgel bead was fabricated by mixing tryptophan, encapsulated fish oil-BHT with sodium alginate solution and dispersed the obtained mixture in vegetable oil. The droplets were gelled by the addition of $CaCl_2$ aqueous solution into the suspension. The ninhydrin reaction was used to determine the encapsulation efficiency and loading of tryptophan which show 92.76% encapsulation efficiency and 37.11% loading at alginate: tryptophan: encapsulated fish oil-BHT ratio of 1: 2: 2 (w/w). The ^{1}H NMR analysis was used to carry out the encapsulation efficiency and loading of fish oil in microgel bead which show 83.81% encapsulation efficiency and 13.40% loading at alginate: tryptophan: encapsulated fish oil-BHT ratio of 1: 2: 2 (w/w) (Table 12).

Table 12 Encapsulation efficiency (EE) and % loading of tryptophan and fish oil in microbead with different weight ratios.

Weight ratio of alginate: tryptophan :encapsulated fish oil	Tryptophan		Fish oil	
	%EE	%loading	%EE	%loading
1 :1 :1	91.54	30.51	83.58	11.14
1 :1 :2	90.13	22.53	84.11	16.82
1 :1 :3	*	*	*	*
1 :2 :1	77.68	38.84	84.35	8.43
1 :2 :2	92.76	37.11	83.81	13.40
1 :2 :3	*	*	*	*
1 :3 :1	*	*	*	*
2 :1 :1	96.13	24.03	89.22	8.92
2 :1 :2	94.88	18.98	88.05	14.08
2 :2 :2	94.12	37.65	88.97	7.12

* The microbead cannot be formed.

However, at the ratio of 1 :1 :3, 1 :2 :3 and 1 :3 :1 the microbead were not fabricated due to high proportion powder of encapsulated fish oil and tryptophan result to non-homogenous solution in alginate mixture and non-gelling by $CaCl_2$.

4.11.2 Morphology of microgel bead

The morphology of microgel bead was analyzed by SEM and TEM. SEM and TEM photographs showed that the microgel beads were bumpy particle due to the confinement of encapsulated fish oil-BHT particle in microgel bead (Figure 26). TEM photograph clearly show the presence of encapsulated fish oil-BHT particles in microgel beads. The particle size of microgel bead, estimated by SEM and light microscope were 10-65 μm and 25-160 μm, respectively.

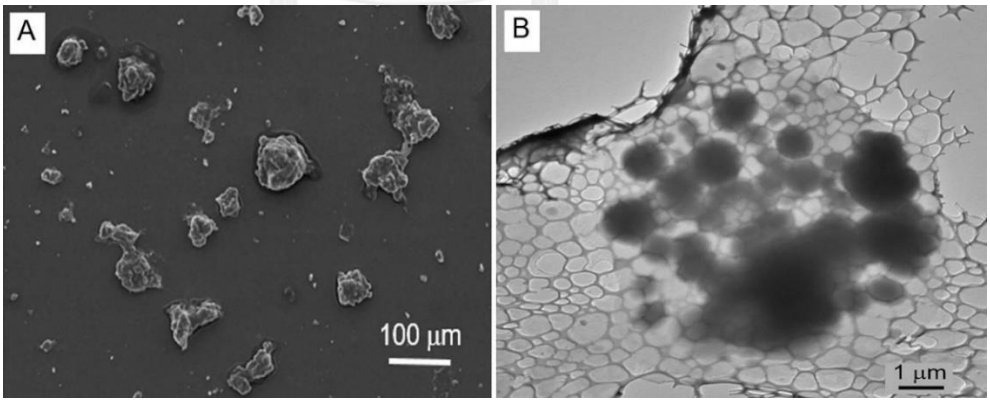


Figure 26 SEM (A) and TEM (B) images of microgel bead containing of tryptophan and encapsulated fish oil-BHT.

The microgel bead fabrication via emulsification/ internal gelation method usually gives irregular size. In this method, the droplets of aqueous phase are made

and surrounded with oil phase and suddenly formed the microgel bead by divalent ion (Ca^{2+}) crossed linking.

The size distribution of dried microgel bead is between 10-65 μm, whereas the wet is 25-160 μm, indicating big swelling (Figure 27).

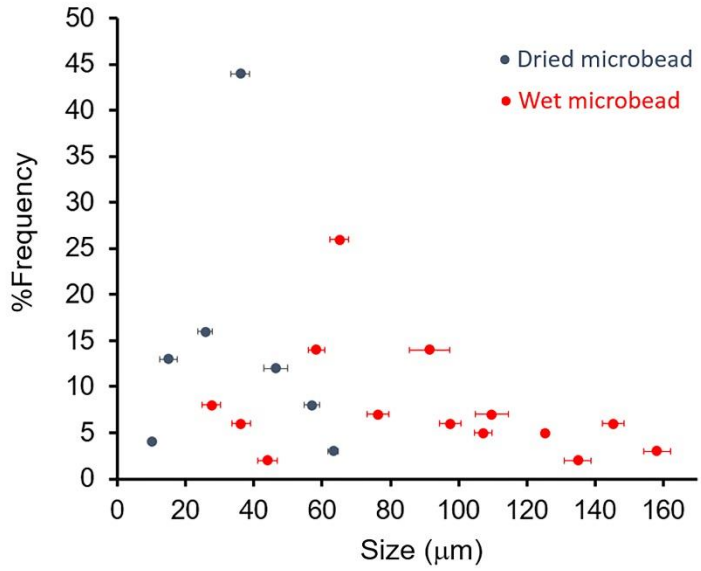


Figure 27 Size distribution of dried (●) and wet (●) microgel bead. The % frequency of microgel bead size is measured and calculated by counting from 100 microgel beads.

Moreover, the presence of tryptophan in microbeads was investigated by confocal laser scanning microscope using tryptophan as fluorescence tracker. Generally, tryptophan has a maximum absorption wavelength 280 nm and the emission wavelength ranges from 300 to 350 nm. This depending on the polarity of the local environment. The glow of tryptophan is evidently present in microgel bead (Figure 28). The result clearly showed that tryptophan was confined in the microbead.

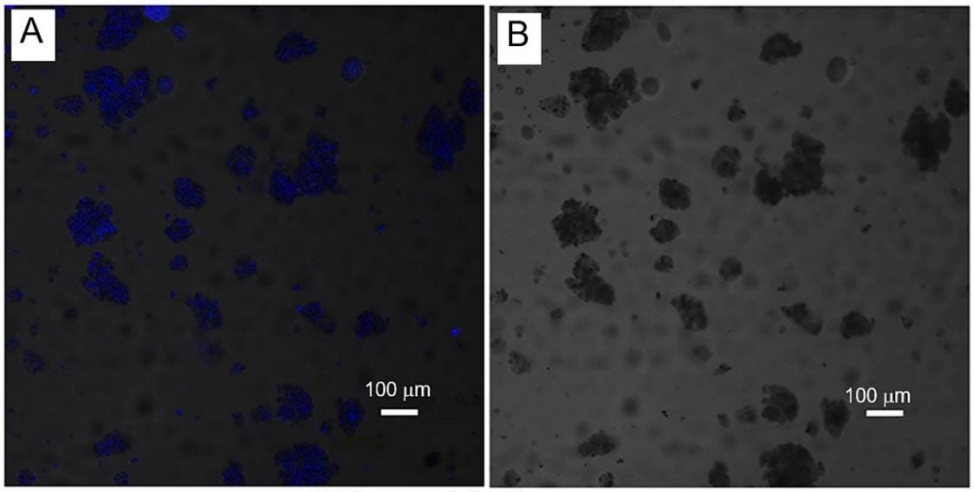


Figure 28 Confocal laser scanning image of microbead, fluorescent mode (A), differential interference contrast mode (B).

4.12 The tryptophan and encapsulated fish oil-BHT release study

The *in vitro* release of encapsulated fish oil-BHT and tryptophan from microgel bead was investigated under various salt and pH condition. Although, the alginate is hydrophilic and water soluble polysaccharide, but the Ca^{2+} ions persuades crosslinked microgel bead of alginate and stable in aqueous medium. The ion exchange between Ca^{2+} and Na^{+} ions is the cause of the microgel bead swelling and results in the deformation of the bead.

In order to investigate the effect of NaCl on the release of encapsulated fish oil and tryptophan, the concentration of NaCl was varied to 12.5, 25, 50 mM. The high concentration of NaCl at 25 ppt (427 M) mimicked the sea water condition. The release

profile of tryptophan from microgel bead in various concentration of NaCl are shown in Figure 29A. The tryptophan release from alginate was effect by concentration of NaCl release medium. Tryptophan release in 25 ppt NaCl release medium was rapid and complete release within 30-60 min. In others NaCl release medium, the release profile of tryptophan are fast in 50 mM NaCl, following the 25 mM and 12.5 mM NaCl release medium, respectively (Figure 29A). The release profile of fish oil from microgel bead in various concentration of NaCl are shown in Figure 4.16A. Fish oil release in 25 ppt NaCl release medium are faster than those of 50 mM, 25 mM and 12.5 mM NaCl release medium, respectively. However, only 35% of fish oil was left from the microbead in 25 ppt NaCl release medium after 4h (Figure 30A). It can explain that the slow release profile of fish oil is cause of double encapsulation by ethylcellulose and alginate. The encapsulated fish oil-BHT particles are released in NaCl medium and then, the fish oil is gradually penetrated out of encapsulated fish oil-BHT particle.

In order to investigate the influence of pH of the medium, the 0.1 HCl (pH 1.2), acetate buffer (pH) and distilled water (pH 7) were used for tryptophan and fish oil release from microgel bead. It can be seen that the tryptophan and fish oil release in distilled water pH 7 are faster than those of 0.1 HCl pH 1.2 and acetate buffer pH 4 (Figure 29B, 30B). Tryptophan was completely released in distilled water pH 7 release medium after 4 h, while only 20% fish oil was released after 4 h. It can explain that calcium alginate tend to shrink at low pH due to the loss of negative charge on their

molecule when the carboxyl group (-COOH) was protonated [117]. In contrast, the microbead tend to swell when placed in high pH medium because the alginate molecules become highly charged and drived each other [118].

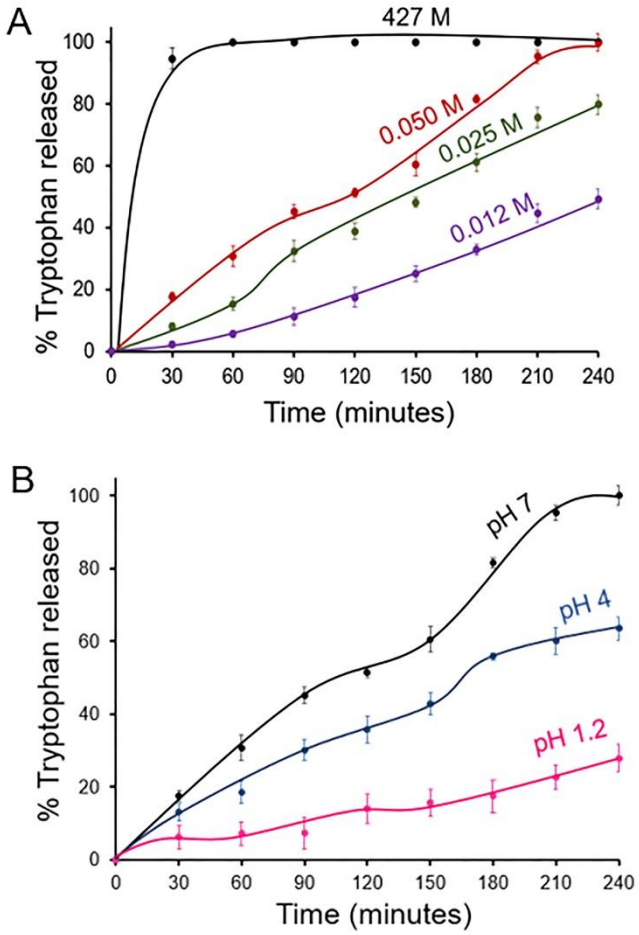


Figure 29 The tryptophan release profile after 4h. The effect of salt (NaCl) of released tryptophan from microbead in 0.012M, 0.025M, 0.050M and 427 M (25 ppt) solution of NaCl at pH 7.0 (A). The effect of pH of released tryptophan from microbead in solution pH 1.2, 4, 7 in 50 mM NaCl solution (B).Values are mean ± standard deviation (S.D.) of three experiments.

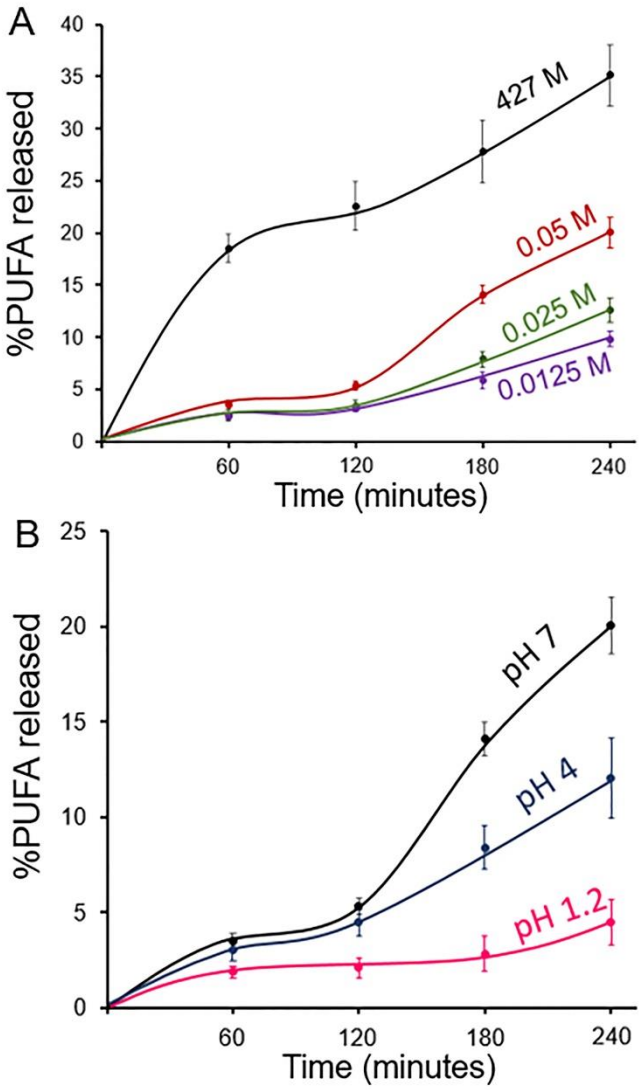


Figure 30 The fish oil release profile after 4h. The effect of salt (NaCl) of release fish oil from microbead in 0.012M, 0.025M, 0.050M and 427 M (25 ppt) NaCl medium at pH 7.0 (A). The effect of pH of released fish oil from microbead in pH 1.2, 4, 7 medium in 50 mM NaCl solution (B).Values are mean ± standard deviation (S.D.) of three experiments.

4.13 Stability of microgel bead

The 12 months shelf life stability of dried microgel bead was evaluated at room temperature in brown bottle. After 12 months storage, the dried microgel beads were determined the remained fish oil using ^{1}H NMR analysis. The peak area between methyl end proton (-CH_3, 0.8-0.9 ppm) and unsaturation carbon (CH=CH, 5.2-5.3 ppm) before and after storage were compared. The result shows that relative percentage of double bond in encapsulated fish oil-BHT and amount of tryptophan in microbead after 12 months were not changed, approximately 90.44% and 78.97%, respectively (Figure 31A). Interestingly, after 12 months relative percentage of double bond in encapsulated fish oil (not in microbead) (84.84%) was lower than those in the microgel bead (90.44%) (Figure 31B). It is likely that the double bond in encapsulated fish oil was double protected by two polymeric matrix, EC and alginate. At the same time, the amount of tryptophan in microbead and tryptophan not in microgel bead were observed the shelf life stability. After 12 months storage, the amount of tryptophan in microgel bead was 82% (Figure 31A), whereas the amount of tryptophan not in microgel bead was 65% **(**Figure 31B**)**.

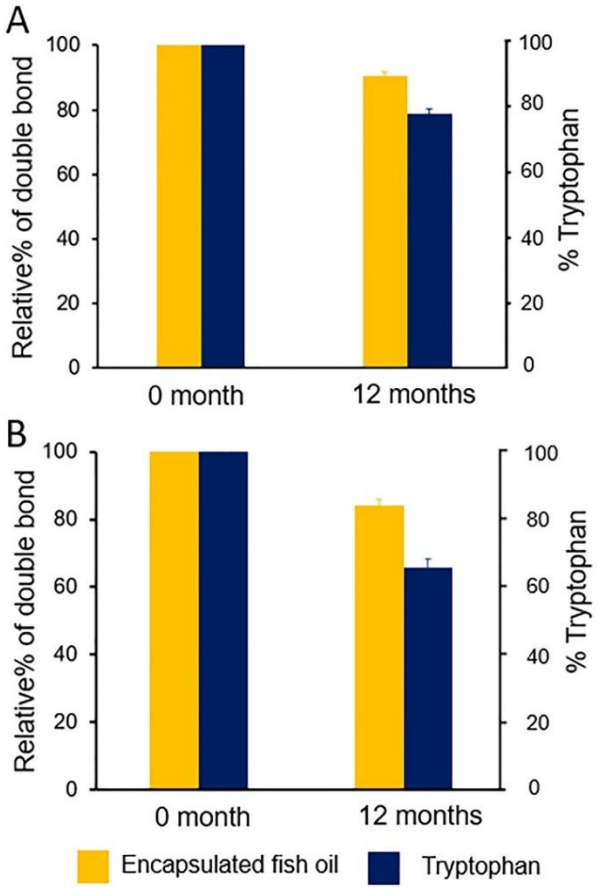


Figure 31 The shelf life stability of tryptophan and encapsulated fish oil-BHT in microbead (A), tryptophan and encapsulated fish oil without microgel bead (B) after 12 months. Values are mean ± standard deviation (S.D.) of three experiments.

CHAPTER V

CONCLUSION

Here, the co-encapsulated fish oil-BHT particles were fabricated using ethylcellulose as the polymeric matrix by solvent displacement method. The obtained particles contain fish oil content of 41% and the encapsulation efficiency is 81% into microsized. The encapsulated fish oil particles show better, thermal stability, oxidative stability and shelf life stability than unencapsulated fish oil.

In addition, the encapsulated fish oil particles were added into the commercial shrimp feed and the content of unsaturation in the shrimp feed before and after was acquired processing. The shrimp feeds were prepared by two methods: 1) encapsulated fish oil-BHT particles mixing with shrimp feed paste and passing through the pelleting machine, 2) encapsulated fish oil-BHT particles suspension spraying on commercial shrimp feed pellet. All shrimp feeds formulation which contained the encapsulated fish oil-BHT particles give the high remained unsaturation content after shrimp feed preparation process, comparing with shrimp feed mixing with unencapsulated fish oil.

L. vannamei were fed with difference shrimp formulation feeds both appearance and unappearence of encapsulated fish oil-BHT particles. The results show that all factor such as, survival rate, weight, length, hepatopancreas quality and

immunity index in shrimp which fed with the appearance of encapsulated fish oil-BHT in feed are better than those fed with unencapsulated fish oil in feed.

In conclusion, encapsulated fish oil-BHT particles were successfully prepared. It have efficiency protecting the unsaturation fatty acids in fish oil from external environment, mixing well with the shrimp feed and raising the shrimp health.

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APPENDIX

VITA

Miss Wichchulada Chimpibul was born on June 27, 1983 in Samut Songkhram, Thailand. She received a Bachelor's degree of Science in Biotechnology from Silpakorn University 2004. In 2005-2006, she was an assistance researcher in Program of biotechnology, Faculty of Science, Mahidol University. After that she received a Master's degree in Program of Biotechnology, Faculty of Science, Chulalongkorn University in 2010. She started doctoral degree in Program of Biotechnology, Faculty of Science, Chulalongkorn University under the supervision of Professor Supason Wanichwecharungruang. After that she has started Dual Doctoral degree between Chulalongkorn and Japan Advanced Institute of Science and Technology (JAIST) in 2013. She has started Doctoral degree in School of Materials Science (JAIST) under supervision of Accosicate Professor Matsumura Kazuaki.

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