ฤทธิ์ต้านแบคทีเรียก่อโรควิบริโอซิสในหอยหวาน *Babylonia areolata* จากสาหร่ายเคลป์ *Ascophyllum nodosum*

นายภุมรินทร์ เตาวโรดม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

ANTIBACTERIAL ACTIVITY FOR VIBRIOSIS IN SPOTTED BABYLON *Babylonia areolata* FROM KELP *Ascophyllum nodosum*

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Academic Year 2007

Thesis Title	ANTIBACTERIAL ACTIVITY FOR VIBRIOSIS IN SPOTTED BABYLON *Babylonia areolata* FROM KELP *Ascophyllum nodosum*
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Field of Study	Biotechnology
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ภุมรินทร์ เตาวโรดม: ฤทธิ์ต้านแบคทีเรียก่อโรควิบริโอซิสในหอยหวาน *Babylonia areolata* จากสาหร่ายเคลป์ *Ascophyllum nodosum*. (ANTIBACTERIAL ACTIVITY FOR VIBRIOSIS IN SPOTTED BABYLON *Babylonia areolata* FROM KELP *Ascophyllum nodosum*) อ. ที่ปรึกษา: ผศ. ดร. วรินทร ชวศิริ, อ. ที่ปรึกษาร่วม: ผศ. ดร. วีณา เคยพุดซา, 105 หน้า

สารสกัดหยาบด้วยกรดไฮโดรคลอริกเข้มข้น 0.01 นอร์มอล จากสาหร่ายเคลป์ *Ascophyllum nodosum* สามารถยับยั้งการเจริญของเชื้อแบคทีเรียก่อโรควิบริโอซิสในหอยหวาน *Babylonia areolata* ได้แก่ *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. fluvialis* และ *V. cholerae* ได้ โดยมีค่า MIC เท่ากับ 16, 18, 16 และ 22 มิลลิกรัม ต่อมิลลิลิตร ตามลำดับ และมีค่า MBC เท่ากับ 46, 52, 50 และ 58 มิลลิกรัม ต่อมิลลิลิตร ตามลำดับ สาหร่ายเคลป์ยังมีผลต่อการทนต่อโรควิบริโอซิส และการเจริญเติบโตในหอยหวาน อีกด้วยโดยในการทดสอบการทนต่อโรควิบริโอซิสใช้หอยหวาน 120 ตัว แบ่งออกเป็นสี่กลุ่มๆ ละสามซ้ำ ให้อาหารที่เสริมสารสกัดร้อยละ 0, 1.5, 3.0 และ 4.5 โดยมวล แก่หอยหวานแต่ละกลุ่มเป็นเวลาเจ็ดวัน แล้วฉีดเชื้อ *V. alginolyticus* ที่ระดับความเข้มข้นเท่ากับค่า LD_{50} เข้าในหอยแต่ละตัว จากนั้นให้อาหารหอยหวานแต่ละกลุ่มต่อเนื่องไปอีกเจ็ดวัน โดยอัตราการรอดของหอยหวานแต่ละกลุ่มเท่ากับ 16.67±1.15, 46.67±5.77, 43.33 ± 5.77 และ 46.67±5.77 ตามลำดับ ซึ่งมีความแตกต่างอย่างมีนัยสำคัญ (P<0.05) และในการทดสอบผลต่อการเพิ่มการเจริญเติบโตใช้หอยหวาน 150 ตัว แบ่งออกเป็นห้ากลุ่มๆ ละสามซ้ำ ให้อาหารที่เสริมสาหร่ายเคลป์ป่นร้อยละ 0, 2.5, 5.0 และ 10.0 โดยมวล แก่หอยหวานแต่ละกลุ่มเป็นเวลา 30 วัน โดยความยาวเปลือกสุทธิของหอยวานแต่ละกลุ่มเท่ากับ 21.11±0.34, 21.23±0.20, 21.17±0.20, 21.11±0.16 และ 22.61±0.14 มิลลิเมตรตามลำดับ ซึ่งมีความแตกต่างอย่างมีนัยสำคัญ (P<0.05) และน้ำหนักเปียกทั้งตัวของหอยหวานแต่ละกลุ่มเท่ากับ 2.17±0.03, 2.17±0.02, 2.14±0.02, 2.10±0.06 และ 2.98±0.12 กรัม ตามลำดับ ซึ่งมีความแตกต่างอย่างมีนัยสำคัญ (P<0.05) การศึกษาครั้งนี้แสดงให้เห็นถึงศักยภาพของการใช้สาหร่ายเคลป์เป็นอาหารเสริม และควรมีการศึกษาอย่างต่อเนื่อง เพื่อประยุกต์ใช้ในการเลี้ยงหอยหวาน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: *Babylonia areolata/ Ascophyllum nodosum/ Vibrio* spp./ VIBRIOSIS

BHUMRINDRA TAUVAROTAMA: ANTIBACTERIAL AGENTS FOR VIBRIOSIS IN SPOTTED BABYLON *Babylonia areolata* FROM KELP *Ascophyllum nodosum*. THESIS ADVISOR: ASST. PROF. WARINTHON CHAVASIRI, Ph.D., THESIS COADVISOR: ASST. PROF. WEENA KOEYPUDSA, D.Tech.Sc., 105 pp.

The crude 0.01 N HCl extract of kelp *Ascophyllum nodosum* inhibited *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. fluvialis* and *V. cholerae* causing vibriosis disease in spotted babylon *Babylonia areolata*. The result on minimal inhibition concentrations MIC of four species were 16, 18, 16 and 22 mg mL^{-1}, respectively and minimal bactericidal concentrations MBC were 46, 52, 50 and 58 mg mL^{-1}, respectively. Kelp also presented effect on vibriosis resistance and growth of spotted babylon. On the vibriosis resistance experiment, 120 spotted babylon were divided into four groups with triplicates. Each group was fed on seven days with kelp extract supplement in diet 0%, 1.5%, 3.0% and 4.5% (w w^{-1}). Median lethal dose LD_{50} of *V. alginolyticus* was intramuscular injected in each spotted babylon. Then, each group was afterward raised on seven days. The result on percent survival rate of each group were 16.67 ± 11.55^{b}, 46.67 ± 5.77^{a}, 43.33 ± 5.77^{a} and 46.67 ± 5.77^{a}, respectively with significant difference ($P<0.05$). On the growth performance experiment, 150 spotted babylon were divided into five groups with triplicates. Each group was fed on 30 days with kelp meal supplement in diet 0%, 2.5%, 5.0% and 10.0% (w w^{-1}). The result on total shell length of each group were 21.11 ± 0.34^{c}, 21.23 ± 0.20^{b}, 21.17 ± 0.20^{bc}, 21.11 ± 0.16^{c} and 22.61 ± 0.14^{a} mm, respectively with significant difference ($P<0.05$). The result on wet body weight of each group were 2.17 ± 0.03^{b}, 2.17 ± 0.02^{b}, 2.14 ± 0.02^{c}, 2.10 ± 0.06^{d} and 2.98 ± 0.12^{a} g, respectively with significant difference ($P<0.05$). This study presents the potential of *A. nodosum* as diet supplement and should be continuously studied for application of *B. areolata* culture.

Field of study Biotechnology
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Student's signature B. Tauvarotama
Advisor's signature W. Chavasiri
Co-advisor's signature W. Koeypudsa

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LIST OF ABBREVIATION

Aero.	*Aeromonas*
CFU mL^{-1}	colonies forming unit per milliliter
CI	condition index
DO	dissolved oxygen
Far.	*Farfantepenaeus*
FCR	Feed conversion ratio
H	height
LD_{50}	median lethal dose
MBC	minimal bactericidal concentration
MIC	minimal inhibitory concentration
MHB	Muller Hinton Broth Media
Pad.	*Padina*
Plas.	*Plasmodium*
RGR	relative growth rate
RPS	relative percent survival
Sal.	*Salmonella*
Sar.	*Sargassum*
SGR	specific growth rate
SLI	shell length increase
Stap	*Staphylococcus*
TCBS	Thiosulfate Citrate Bile Salts
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
WG	weight gain
WWSV	White Spot Syndrome Virus
∅	diameter
‰	part per thousand unit

CHAPTER I

INTRODUCTION

1.1 Importance of the Study

The new of economical marine snails on genus *Babylonia* in Thailand has two species, *Babylonia areolata* and *B. spirata* but the consumption requirement and marketable price of *B. areolata* is higher than *B. spirata*

B. areolata, common name is spotted babylon; is the new economically important marine snail of Thailand and exportation to Taiwan, Hong-Kong, China and Japan. In the last period, its production came form catching fishery by babylon trap in the natural coastal sea areas including Trad, Chantaburi, Rayong, Phetchaburi, Surattani, Nakhon Si Thammarat, and Pattani. Spotted babylon fishery is an especial occupation of local fisheries because the production is less in quantity and no worth on reward.

At the present, *B. areolata* quantity is rapidly reducing to the critical level and its size is smaller than the past while the marketable requirement is rapidly increasing. So that, the study and research of the spotted babylon culture techniques are very importance for its production increment supporting the marketable requirement and natural stock enhancement.

In 1988, the study of *B. areolata* aquaculture was begun in Thailand by Department of Fishery. Now, cultured spotted babylon is being bacterial infected. Bacteria in genus *Vibrio* is cause of cultured spotted babylon disease. General therapy of bacterial disease in aquatic animal is use of antibiotic substances including Oxytetracycline, Sulfamethoxazole-trimethoprim, Erythromycin, Enrofloxacin and Florfenicol but antibiotic

substances can contaminate in tissue of animal causing many side effects for consumer.

Nowadays, there is an increasing interest in the identification of natural products for control of diseases in animal production and avoid the many side effects from excessive use of antibiotic substances and other chemicals.

Brown seaweed such as kelp contains a range of different polysaccharides including alginic acid or alginate, laminarins or larminarans and fucoidan or sulphated fucans. Algenic acid is used as thickeners, emulsifier, binder and gel forming agent in foods, cosmetics, textile and pharmaceutical or biomedical industries. Larminaran and fucoidan are currently subjects of much research interest to characterize their structure and understand their biological activities and potential applications.

Kelp is rich in polysaccharides that are potential therapeutic agents. Particularly interesting is the unique presence of fucoidan, a family of polysaccharides that have distinct antibacterial, anti-parasitic and antiviral properties.

Fucoidan, in particular shows much promise in diseases control and other feed applications. Among the algae, fucoidan is unique to brown seaweed which is present in intercellular junctions. In marine environment, fucoidan has been described from some invertebrate sources, in particular sea cucumbers, *Ludwigothurea grisea* and sea urchins (Hennequart *et al*. 2004).

This research is the basic knowledge of natural product application to prevent the impact of vibriosis and avoids use of antibiotic substances in spotted babylon culture. Kelp, *Ascophyllum nodosum* was tested for antibacterial, vibriosis resistance and growth performance in *B. areolata*.

1.2 Objectives

1. Effect of *A. nodosum* extract on *Vibrio* spp. causing vibriosis on *B. areolata* culture.

2. Effect of *A. nodosum* extract supplement in artificial diet on vibriosis resistance of *B. areolata*

3. Effect of dried *A. nodosum* meal supplement in artificial diet on growth performance of *B. areolata*

1.3 Characteristics of *B. areolata*

1.3.1 General Characters

B. areolata (Figure 1.1) is marine gastropod or snail having one pair of eyes and tentacle. Its shell has round ovate shape, white color and black brown spotted on shell whorl. The shell has a spiral pointed on the top and at the opened of shell has an operculum. The spiral groove around the shell is smooth unlike *B. spirata* (Figure 1.2) having intense brown color, more spotted and spiral groove around the shell is very ridge.

Figure 1.1 *B. areolata*

Figure 1.2 *B. spirata*

1.3.2 Distribution

B. areolata distribute generally in the sandy-mud bottom of coastal. In Thailand, spotted babylon can be find on both Gulf of Thailand including Rayong, Chantaburi, Trad, Petchaburi, Prajoubkirikant, Suratthani, Nakorn Sri Thammarat and Andaman Sea coastal including Krabi, Pung-nga, Trang, Ranong and Satun.

1.3.3 Feeding Behavior

Feedings are divided on developments in two stages. The first as planktonic larva or viliger lavae stage is filter feeder and the second is settlement stage is carnivorous or scavenger feeder. Feeding behavior of *B. areolata* is flock feeding (Figure 1.3). The fish or other mollusks meat is used as spotted babylon feed. Gastric juice is secreted from proboscis for external digestion. The digested feed is sucked and absorb in digestive tract. The proboscis can lengthy to 8 to 10 cm so that every spotted babylon can trough out lengthy to digests the feed. When the babylon have enough, they often move out from the feed and hide under the sand.

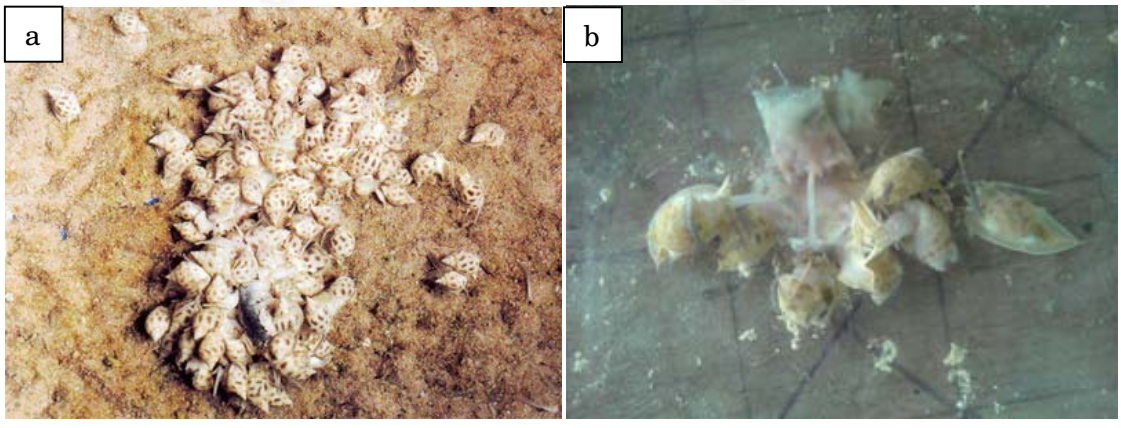

Figure 1.3 Feeding of *B. areolata* on sandy (a) and non sandy (b) floor

1.3.4 Sex and Reproduction

B. areolata is a dioeciously animal but its sexual cannot be identified by outer characters of the shell. Sex can be identified when its body emerges from the shell. Male has yellow leaflet shape organ as the penis at the right of tentacle base but this organ is no appear in same point of female. Female has an opening of egg sag under foot muscle. The reproductive system of female and male *B. areolata* is shown in Figure 1.4.

The first larva stage as trocophore larvae hatches in 24 h. after the fertilized egg is spawned. Larvae development is in the egg sac about 4 to 5 days. The second larvae stage as viliger larvae hatch out from egg sac. Larvae are zooplankton feeding phytoplankton in water mass about 14 to 16 days. Then, the larvae go to settle juvenile stage crawling on water floor and the characters are completely like adult. First maturity starts when the *B. areolata* has total length about 3.6 cm or 6 months old. The development of *B. areolata* is shown in Figure 1.5.

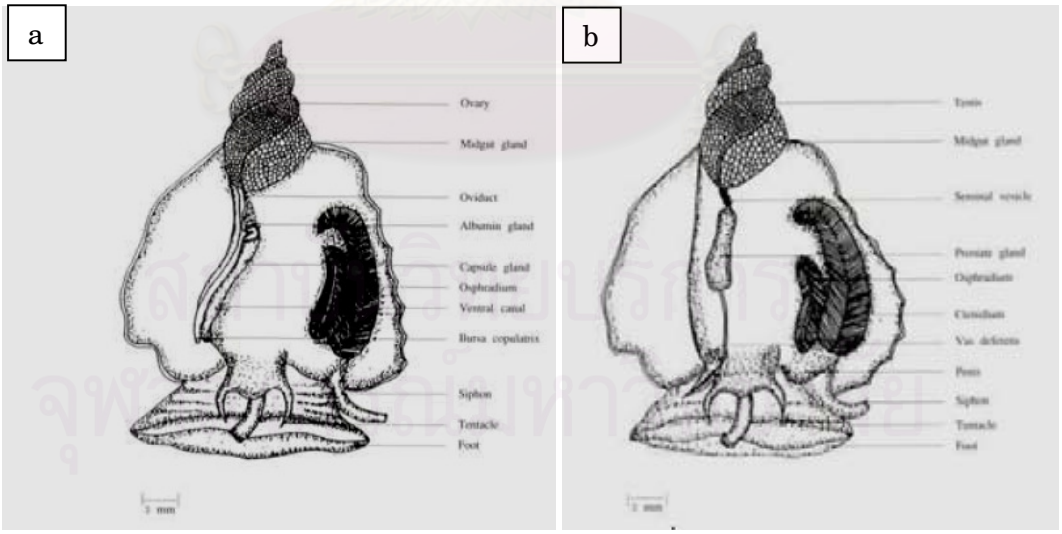

Figure 1.4 Reproductive system of female (a) and male (b) *B. areolat*

(Source: Chaitanawisuti and Kritsanapuntu, 2002)

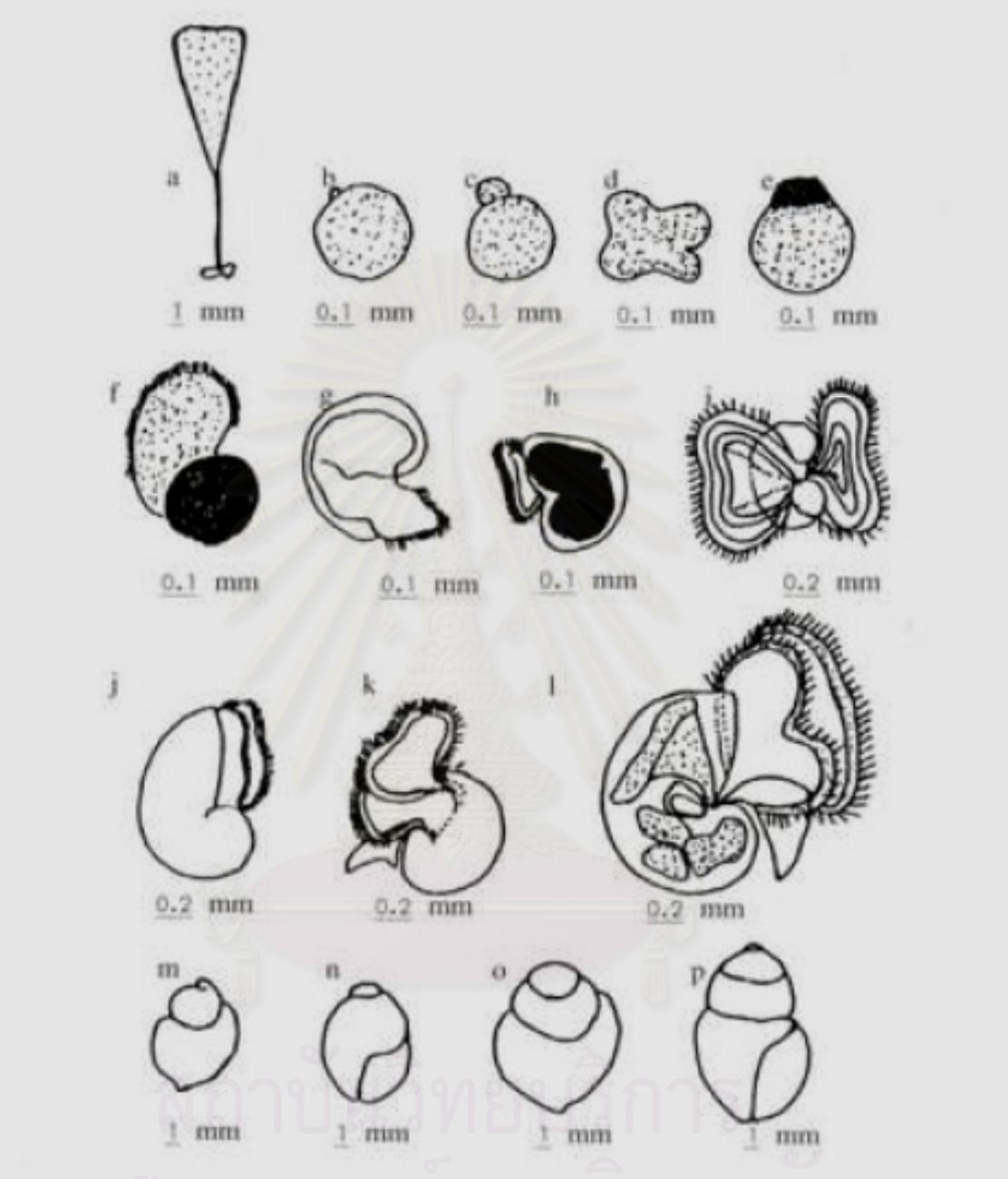

Figure 1.5 Development of *B. areolata*: egg capsule (a), fertilized egg (b), cleavage stages (c) (d) and (e), early embryonic stages (f) (g) (h) and (i), newly-hatched veliger larvae on 3 (j), 8 (k) and 15 (l) days old larvae, dorsal (m) and ventral (n) view of newly metamorphosed juvenile, dorsal (o) and ventral (p) view of 10 days old juvenile

(Source: Chaitanawisuti and Kritsanapuntu, 2002)

1.4 Artificial Diets Design for *B. areolata*

Originally, shrimps and other animals in aquaculture were fed with fresh or frozen fish and other mollusks. Commercial feeds are successfully used in semi intensive and intensive culture. Several commercial feeds contain high protein and vitamin premix. Due to *B. areolata* is new commercial animal, the artificial diet of is not wildly on animals feed market. The artificial diet of spotted Babylon should be continuously studied and developed.

Spotted baylon is benthic omnivore or scavenger, long period feeder and external digestion. The practical artificial feeds should be processed to sinking material. A dietary size is no problem because the *B. aerolata* can throw proboscis for digestion and feeding. The diets should be softness, attractive smell and enough stability in water supporting the long feeding period.

Various substances both natural and synthetic modified, have been used as binding agents for snail diets. Wheat gluten, high gluten wheat flour, tuber and cereal starches are the most commonly used as natural binder in aquatic pellets. Commercial binders, such as lignin sulfonate and bentonite, which are commonly used for fish feeds but not effective use for shrimp feeds.

B. aerolata require long duration of diets stability more than fish because of the feeding response of spotted babylon is mainly chemosensory. The attractants in the diets increase their feeding response. Various substances, such as amino acids, fatty acids and extracts of fish, shrimp, squid and mussel have been shown to stimulate feeding response in many aquatic species that may be applied in artificial diets of *B. aerolata* design.

1.5 Vibriosis

Vibrionaceae is a family of facultative, gram-negative, 0.3 to 1.0 × 1 to 3.5 μm, straight or slightly curved and non spore forming bacteria. Metabolism is chemo-organotroph and both oxidative and fermentative respiration. Most are oxidase positive. They are found primarily in water and in association with aquatic animals. Important genus is *Vibrio* and *Aeromonas* causing diseases of aquatic animals.

The genus *Aeromonas* is important pathogenic bacteria in fresh water; they are ubiquitous, especially in the high organic loads areas. The genus *Vibrio* consists of gram-negative bacteria, straight or slightly curved rods. They are non spore forming and motile by monotrichous or multitrichus sheathed polar flagella. All are facultative anaerobes and chemo-organotrophs and most are oxidase positive. Most species grow well in sea water or salt based media and sodium ion stimulate the growth of all species. They are common in aquatic habitats, particularly in marine and estuarine environments and in association with marine animals. Several species are pathogenic for man as well as marine animals, eels, shrimp, frogs, other vertebrates and invertebrates and some *Vibrio* spp. are bioluminescent.

Many bacterial species are also isolated from Vibriosis shrimps such as *V. cholerae*, *V. harveyi*, *V. vulnificus*, *V. anguillarum*, *V. splendidus*, *V. alginolyticus*, *V. nereis*, *V. parahaemolyticus*, *V. damsela, V. tubiashii*, *V. fluvialis*, *V. fischeri* and *V. penaeicida*. Other bacteria such as *Psuedomonas* spp., *Aeromona*s spp. and *Flavobacterium* spp. *V. cholerae, V. alginolyticus*, *V. anguillarum*, *V. ordalii*, *V. salmonicida* and *V. vulnificus* are marine fish pathogens. *V. alginolyticus*, *V. carchariae*, *V. vulnificus* and *V. parahaemolyticus* are marine shell pathogens (Lee, *et al*., 2003 and Normanno *et al*., 2006).

All are associated with acute bacterial septicemias or chronic lesions in marine fish. Generally vibriosis in fish accompanies some other stress or physical trauma, but some strains, especially of *V. alginolyticus*, or *V. salmonicida*, appear to be highly infectious primary pathogens.

The name *Vibrio* derives from the Latin because these curved rods possess a single polar flagellum and appear “to vibrate”. *V. cholerae* was first isolated in pure culture in 1883 by Robert Koch. Most *Vibrio* has relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, since *Vibrio* is typical marine organisms, most species require 2% to 3% Sodium Chloride (NaCl) or a sea water base for optimal growth. In liquid media, *Vibrio* motion is controlled by polar flagella which are enclosed in a sheath continuous with the outer membrane of the cell wall. On solid media, they may synthesize numerous lateral flagella which are not sheathed.

The behavior of aquatic animals which are vibriosis infected, are change to abnormal. Some species and strains of *Vibrio* cause the fish and other to be luminescent. Mortality ranges from insignificant close to 100%, particularly in postlarvae and young juvenile shrimp.

Symptom of vibriosis is diagnosed by many methods. Observations, infected shrimp is observed on these symptoms such as black or brown lesions on skin, opacity of muscle, black lymphoid organ, and melanin forming of appendage tips. These symptoms are difference by the variety of type infection. On histology, significant necrosis and inflammation is frequently and especially appeared in the lymphoid organ, but usually less severe in the gills, heart, hepatopancreas and other tissues. Pathogenic bacteria having rod curved shape are often found within the tissues. Identification of *Vibrio* spp., all bacteria which are isolated from the sore tissues of moribund shrimp, fish and shell samples are cultured in the

selective media for *Vibrio* spp., Thiosulfate Citrate Bile Salts (TCBS) Agar. Small yellow or green (Figure 1.6) bacterial colonies forming on the TCBS can be estimated to *Vibrio* spp. but the correct identification of *Vibrio* spp. must use combination of other methods such as gram stain and biochemical identification.

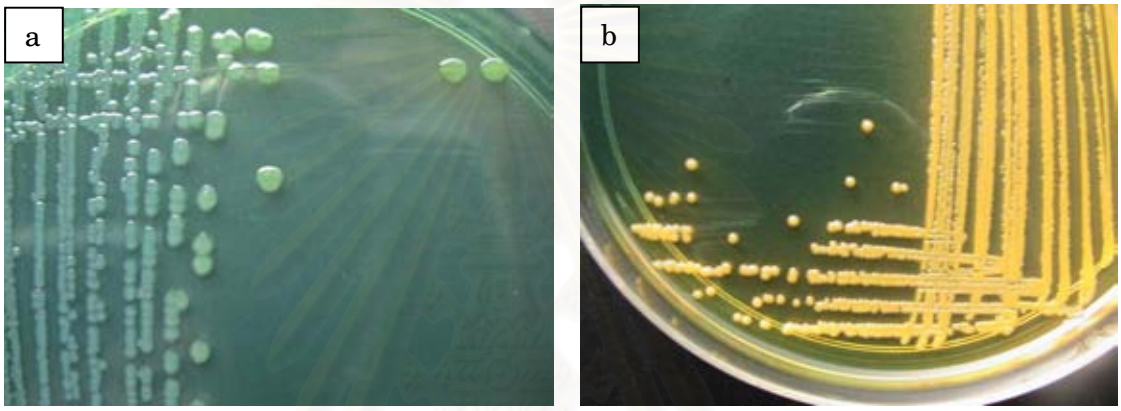

Figure 1.6 Green colonies of *V. parahaemolyticus* (a) and yellow colonies (b) of *V. alginolyticus* on green TCBS surface

Vibriosis preventable methods include with maintenance of water quality with low bacterial biomass, stability of phytoplankton bloom, systematical feeding program, sterilization or filter of rearing water, routine monitor of animals and pond for early diagnosis of a problem and avoidances in temperature extremes or rapid variation in temperature condition, handle without enwrap, over-crowd and other stressors.

General therapy of vibriosis and other bacterial diseases in aquatic animal is dissolution of antibiotic substances in pond. But antibiotic substances can contaminate in tissue of animal causing many side effects for consumer and not accept in food safety.

1.6 Brown Seaweed

1.6.1 Brown Seaweed Composition

Brown seaweed is variable on sizes, 10 cm to 100 m and grows out in the middle section of sea water. The large size brown seaweed is call kelp. The quality of brown seaweed is higher than other seaweed because the composition combines with the most quantity of amino acid vitamin and mineral such as *Fucus distichus*, *Pelvetia canaliculata*, *Sargassum polycystum* and *A. nodosum* (Figure 1.7).

Figure 1.7 Fresh *A. nodosum*

However, ratios of combinations in brown seaweed are influence by many factors. Seaweed which is collected from deep sea and non pollution seawater has higher qualities than wreck seaweed. Seaweed products which are produced from height temperature processes have lower qualities than cool temperature processes.

Brown seaweed has other nutrients (Table 1.1) that also need to be considered while formulating with the ingredient. The protein content of brown seaweed is lower than that of red or green seaweed. However, betaine levels are quite height, which may impart a certain attractant value to the ingredient.

Table 1.1 Nutritional Analysis of *A. nodosum*

Contents					
Macromolecule	**Value**	**Vitamin**	**Value**	**Mineral**	**Value**
Protein	5-15%	Bete-Carotine	35-80 ppm	Calcium	1-3%
Fat	1-5%	Vitamin C	140-1650 ppm	Cobalt	3.33 ppm
Carbohydrate	42-64%	Vitamin B1	1-5 ppm	Iodine	700-1200 ppm
Mannitol	4.2%	Vitamin B2	5-10 ppm	Iron	101-900 ppm
Alginic acid	26%	Vitamin B3	10-30 ppm	Magnesium	0.5-0.9%
Laminarans	10%	Vitamin B6	0.1-0.5 ppm	Manganese	10-15 ppm
Fucoidan	4-10%	Vitamin B12	0.8-3 ppm	Selenium	1.9 ppm
		Vitamin E	260-450 ppm	Sodium	3-4%
		Vitamin H	0.1-0.4 ppm	Zinc	70-240 ppm
		Vitamin K3	10 ppm		

Source: Morrissey *et al*., 2001

Fucoidan is one of the main anionic polysaccharides of marine algae. Various samples sea weeds which are collected from various regions present the difference extracts in the molecular weight, carbohydrate composition and number of sulfate groups. Their phase behavior in aqueous solutions, interactions with proteins and oppositely charged polysaccharides were studied.

The polysaccharides of *A. nodosum* contain L-fucose and sulfate as the only constituents. Combination of methylation analysis, Smith degradation, FTIR and NMR spectroscopy on the native and the de-sulfated polymers demonstrated that the fucoidan consisted of a highly branched core region with primarily α-(1→3)-linked fucosyl residues and a few α-(1→4) linkages. Branch points are at position 2 of the →3-linked internal residues. The side chains consisted of single and multi-unit fucosyl residues. The combined analytical data suggest also a complex sulfation pattern with substitution principally at position 2 and/or position 4. Such diversity in the structural features of this fucoidan may be importance for its various biological properties (Marais *et al*, 2001).

1.6.2 Bioactivities of Brown Seaweed Extract

Bioactive properties that have been indentified thus far for fucoidan include anti-angiogenesis activity, anti-complementary, anti-proliferative effects on tumor cells, anti-inflamentary, anticoagulant and antioxidant properties. Fucoidan fractions have demonstrated strong anticoagulant activity virtually equivalent to heparin. Heparin is a well-known highly sulphated polysaccharide traditionally used in medicine as anticoagulant but with many other important bioactive properties. Even though heparin is derived from animal sources and fucoidan are obtained from plant sources, there is close structural similarity (Figure 1.8) between both sulphated

polysaccharides which may explain their similar bioactive properties. The needs for safe, natural, non-animal heparin analogous as a precaution against are possible Bovine Spongiform Encephalopathy or BSE like incidences make fucoidan from algae as potential alternative to animal derived heparin (Hennequart *et al*., 2004).

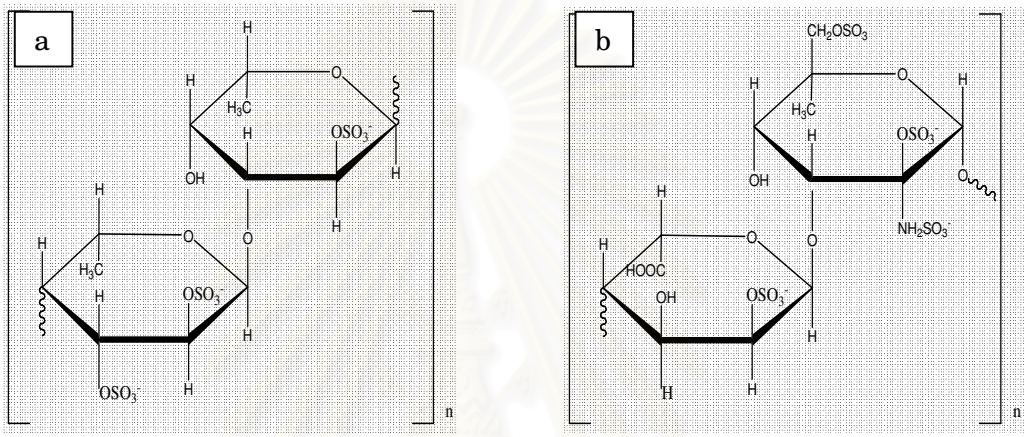

Figure 1.8 Complementary structure of (a) fucoidan from *A. nodosum* and (b) heparin

Fucoidan fractions of various origins and molecular weights show a marked anticancer effect in tested animals, whether they are oral administered or directly injected into the blood vessel or peritoneal cavity. Specifically, a significant reduction in development of cancer was observed in mice and rats after cancer cells had been implanted. This effect was seen in several cancers, including leukemia and breast cancer (Fitton, 2005).

1.6.2.1 Antibacterial Property

Some marine algae extracts have antibacterial properties. Fucoidan have been shown *in vitro* to inhibit the adhesion of staphylococcal bacteria to biomaterial mainly through hydrophobic interactions. They inhibited the biding of a wide range of enterococci and *Streptococcus bovis* strains to the extracellular matrix proteins of animal cells. A commercial product based on

macro-algal meal was recently shown to have positive effects on growth and the immune response of wearing pigs challenged by *Salmonella* infection. Extracts containing fucoidan from different brown algal species have been shown to inhibit the attachment of *Helicobacter pylori*, a bacterial pathogen of the gastric tract. The crude fucoidan from *Sar. polycystum* inhibited the growth of *V. harveyi*, *Staphylococcus aureus* and *Escherichia coli* in vitro (Chotigeat *et al*., 2004). The effectiveness depends on the type of fucoidan and the algal source. It is proposed that the inhibition occurs as a result of the coating at the bacterial surface by the polysaccharides charge.

1.6.2.2 Effects on Parasites

Commercial products based on seaweed meal have been recommended for limiting coccidial infections in poultry. The inhibition of host infection by *Plasmodium faliparum*, the causative agent of Malaria, has been documented (Ying *et al*., 1997). The effect is attributed to the prevention of the invasion of human erythrocytes by *Plas. merozoites* by the negative charge of fucoidan molecules. There are indications that the sulphate groups on fucoidan plat a major role in preventing the parasite infection. There are also a number of studies on the inhibition of other parasites by fucoidan.

1.6.2.3 Antiviral Property

The antiviral potential of sulphate fucans (polysaccharides comprised mainly of L-fucose) has been demonstrated *in vitro* for Human Immunodeficiency Virus (HIV), Herpes Simplex Virus (HSV) and human cytomegaloviruses. Tasmanian kelp *Undaria pinnatifida* extracts were found to inhibit in vitro mitogenic effects of HSV on Human T cells. Fucoidan from *F. esiculosus* inhibited HIV *in vitro*. The proposed mechanism suggests that fucoidan interacts with binding sites on the target cell surface, thus

preventing viral binding with the cell. However, all fuocidan fractions may not possess antiviral activity (Sugawara *et al*., 1989).

Brown seaweed is classified in Family Phaeophyceae. It produces families of sulfated fucoidan and other polysaccharide such as fucoidan which is located in the intercellular tissue and most strikingly in droplets (Doner and Whistler, 1973). Recently, one type of fucoidan, the complex sulfated polysaccharide from the algae *F. vesiculosus*, was found to inhibit human immunodeficiency virus or HIV *in vitro*. This activity presumably resulted from a direct interaction of the polysaccharide with the HIV binding site on the target cells.

1.7 Aquaculture Applications of Brown Seaweed

1.7.1 Brown Seaweed Extract in Fish and Shrimp

Several studies evaluating the effect of saccharide extracted from brown seaweed on the general health of fish have been published. The positive effect of *A. modosum* extracts on haemolytic and lysozymic activity in Salmon. Many researchers also noticed to improved resistance to bacteria including *Aero. salmonicida* in fish feeding seaweed extract supplement in diet that rich in polysaccharides. The positive effects were attributed to polysaccharides present in seaweed. The dietary pellets containing hyperborean *Laminaria* sp. extracts enhanced bacterial diseases resistance from *Aero. salmonicida* and *V. salmonicida* of Atlantic salmon. The effect of different seaweed (green, red and brown seaweed) extracts test showed an increment of the respiratory burst activity of the phagocytes on turbot's immune system. They concluded that most of the stimulatory capacity of the water soluble extract was associated with the presence of the polysaccharides.

There have been a few investigations on the effects of seaweed preparations on the shrimp immune system. Oral administration of crude extract from *Sar. polycystum* reduced the impact of White Spot Syndrome Virus or WSSV in black tiger shrimp *Penaeus monodon* (Chotigeat *et al*., 2004). Crude extract from Mexican kelp *Macrocystis pyrifera* reduced the impact of WSSV Pacific white shrimp *Litopenaeus vannamei* (Takahashi *et al*., 2000). Hot water extract from *Sar. duplicatum* increased immune ability as well as resistance to *V. alginolyticus* infection of *L. vannamei* (Yeh *et al*., 2006). *A. nodosum* meal supplement in diets also increased resistance of *P. monodon* against *V. harveyi* infection (Chungthanawong, 2004).

1.7.2 Brown Seaweed in Fish and Shrimp Feeds

The inclusion rate of *A. nodosum* meal in shrimp feed should be around 3.5 to 4%. The average level of fucoidan in *A. nodosum* is around 5% on dry weight basis. The inclusion rate of 3.5 to 4% means a fucoidan concentration of about 0.2% in the feed as dosage recommendation for effective antiviral activity (Takahashi *et. al*., 1998).

In the preparation of crude seaweed extracts for oral administration of fucoidan, the high variability of fucoidan levels should be taken into consideration. Significant variation exists in the amount and the composition of fucoidan depending on species, age or part of the plant, various climatic and environmental factors, as well as extraction procedures.

CHAPTER II

METHODOLOGY

2.1 *A. nodosum* Extraction

The dried *A. nodosum* meal (Figure 2.1) was sieved by handle sieve in order to remove fibers and other large particles. The fine kelp meal was consecutively extracted with acetone and EtOH by Soxhlet extraction to remove organic substances, pigment and fatty substances. The residual kelp meal was extracted three times with 0.01 N Hydrochloric acid (HCl) solution at 80°C in 3 h on the magnetic stirrer hotplate. An extract solution was concentrated, dialyzed against water to remove salt and then freeze dried. The brown solid extract was stored at 4°C until future use.

Figure 2.1 Sieved dried *A. nodosum* meal

2.2 Dietary Preparation

The experimental artificial diets formula ingredient is shown in Tables 2.1 and 2.2 (modified from Chungthanawong, 2004). The ingredient was mixed by blender and dried in oven at 60°C for 12 h. Kelp was not added in the control formula. On the vibriosis resistant experiment, various levels of

kelp extract were mixed in the control formula, while on the growth performance experiment the addition of various levels of kelp meal to control formula was performed. All experimental diets were stored at 4°C until future use. This dietary formulas study is primarily done under laboratory conditions. The composition of experimental artificial diets may not show high efficiency on vibriosis resistance and growth of spotted babylons.

The growth performance comparison between artificial diets and fish was studied. The fresh fish meat (short body mackerel *Rastrelliger neglectus*) as common feed of spotted babylon culture was used in this study.

The proximate analysis of all experimental diets was determined following methods of Association of Official Analytical Chemists (AOAC, 1996) and all procedures are shown in Appendix B. The stability of all dietary formulas and fish meat was also tested. The pieces of each diet were soaked in water until those completely decayed that duration was estimated to the stability period of the diets.

2.3 Experimental *B. areolata*

The juveniles of healthy *B. areolata* were obtained from Trad Coastal Aquaculture Station, Mueng District, Trad Province. The average of the total shell length and wet body weight of spotted babylon is between 0.8 to 1.3 cm and 1.10 to 1.20 g, respectively. The spotted babylon was fed with short body mackerel *Rastrelliger neglectus*, acclimatized to ambient laboratory culture condition more than seven days. *B. areolata* was fed once daily and uneaten food was removed after the feeding. When the experiment was started, the healthy spotted babylons were selected from the acclimatized stock and transferred to the experimental tanks. The initial average of total shell length and wet body weight of experimental spotted babylons were measured and recorded.

Table 2.1 Ingredient of artificial diets for the vibriosis resistant experiment

Ingredients	**% (w w^{-1})**			
	Control	**Kelp Extract 1.5%**	**Kelp Extract 3.0%**	**Kelp Extract 4.5%**
Fish Meal	47	47	47	47
Soybean Meal	5	5	5	5
Wheat Flour	25	23.5	22	20.5
Shrimp Head Meal	5	5	5	5
Wheat Gluten	16	16	16	16
Fish Oil	5	5	5	5
Vitamin Mixture	2	2	2	2
Mineral Mixture	2	2	2	2
0.01 N HCl Kelp Extract	**0**	**1.5**	**3.0**	**4.5**

Table 2.2 Ingredient of artificial diets for the growth performance experiment

Ingredients	**% (w w^{-1})**				**Fish Meat**
	Control	**Formula 1**	**Formula 2**	**Formula 3**	
Shot Body Mackerel	-	-	-	-	100%
Fish Meal	40	40	40	40	-
Soybean Meal	5	5	5	5	-
Wheat Flour	25	22.5	20	15	-
Shrimp Head Meal	5	5	5	5	-
Wheat Gluten	16	16	16	16	-
Fish Oil	5	5	5	5	-
Vitamin Mixture	2	2	2	2	-
Mineral Mixture	2	2	2	2	-
Kelp Meal	**0**	**2.5**	**5**	**10**	-

2.4 Experimental Bacteria

2.4.1 Bacterial Preparation

Four species of bacteria, *V. alginolyticus* as the most virulent species on *B. areolata* (Raveevong *et al*., 2005), *V. cholerea*, *V. parahaemolyticus* and *V. fluvialis* were isolated from spotted babylons and obtained from the Veterinary Medical Aquatic Animal Research Center (VMARC), Department of Veterinary Medicine, Faculty of Veterinary Science, Faculty of Veterinary Science, Chulalongkorn University. Each bacterial species was kept in the maintenance media until future use. The bacteria from the maintenance media were activated in nutrient media when the experiment was started.

The cultural media, Thiosulfate Citrate Bile Salts Media (TCBS Himedia®) as the *Vibrio* spp. selected media, Tryptone Soya Broth (TSB, Himedia®), Tryptone Soya Agar (TSA, Himedia®) and Muller Hinton Broth Media (MHB, Criterion®) were used on this bacterial experiment. The steriled 1%NaCl solution as the mineral requirement of *Vibrio* spp. was added in TSA, TSB and MHB.

2.4.2 Standard Curve of *V. alginolyticus*

V. alginolyticus was the high virulent vibriosis phathogenic species on *B. areolata* in high level of dissolved oxygen condition (Raveevong *et al*, 2005). The stocked *V. alginolyticus* from maintenance media was activated in 1%NaCl TSA and confirmed *Vibrio* sp. in TCBS and incubated at 30°C for 24 h. The activated single colonies of *V. alginolyticus* was transferred in tubes containing 1%NaCl TSB and incubated until bacterial cells increase is enough for serial dilution. Then, the tubes of *V. alginolyticus* mixture were centrifuged. The paste of bacterial cell were isolated from liquid media and applied into sterile 1%NaCl solution and serial diluted 1:10 to 10 series. Each series was measured at 600 nm. The absorbance of each series was recorded.

The 100 µL of each serial dilution was spread on TSA, incubated at 31°C in 24 h and the colonies forming of each serial dilution were counted. The plate appearing the range of 30 to 300 colonies were selected, counted and back calculated into colony forming unit per mL (CFU mL^{-1}) as the start concentration of each absorbance. The absorbance and CFU mL^{-1} were exponential plotted and converted to linear curve as the standard curve for *Vibrio* spp. calculation of this experiment (Appendix A, Figure A1).

2.5 The Median Lethal Dose (LD_{50}) of *V. alginolyticus* on *B. areolata* at 72 H

B. areolata was starved on 24 h before the experimental was started. *V. alginolyticus* in the maintenance media was activated in 0.1%NaCl TSA about 24 h, centrifuged, washed and adjusted concentration with sterile 0.1% NaCl solution to 10^7, 10^8, 10^9, 10^{10}, 10^{11} and $>10^{11}$ CFU mL^{-1} comparing with the standard curve. 210 healthy spotted babylons were divided into seven groups with triplicates. Six groups were intramuscular injected with 100 µL of *V. alginolyticus* suspension in foot muscle of each spotted babylon. The small disposable syringe (capacity 1 mL, needle size 0.45 mm Ø × 13 mm, REX®) was used in the intramuscular injection. The control group was injected with sterile 1%NaCl solution. Survivals spotted babylons were recorded daily for 72 h. LD_{50} of *V. alginolyticus* on *B. areolata* was calculated with equation 2.1 (modified from Pattanaargson, 1996).

The Median Lethal Dose, LD_{50}

$$LD_{50} = \ln CB\ 50\% + \frac{(50 - MB\ 50\%) \times (\ln CA\ 50\% - \ln CB\ 50\%)}{(MA\ 50\% - MB\ 50\%)} \quad \textbf{...2.1}$$

ln CA 50% = ln(concentration above 50% mortality)

ln CB 50% = ln(concentration below 50% mortality)

MA 50% = mortality above 50%

MB 50% = mortality below 50%

2.6 Experimental Cultural Unit and Facilities

The experimental cultural unit was set at Bang Krachao Sub-district, Meung District, Samut Sakhon Province. The high concentrate sea water of the salt farm was used as this rearing water. The salt water was diluted to 30‰ and stocked for three days before use. The culture water qualities were monitored along this experimental period. The rearing water was removed about 60% daily. Clearly circular plastic tanks (20 cm Ø × 30 cm H) (Figure 2.2) were used in this experiment. Sandy material was not filled into the tank. The air supply was set and opened in every cultural tank.

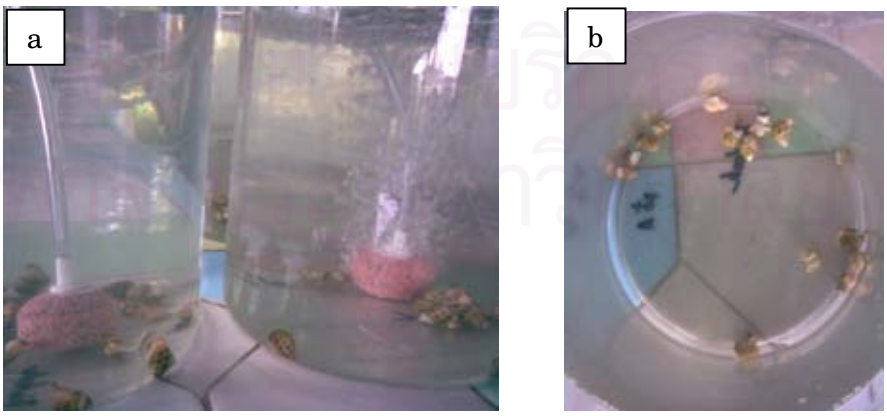

Figure 2.2 The side view (a) and top view (b) of cultural tanks

2.7 The Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC) of the Extracts on *Vibrio* spp.

Macro-broth dilution was used in this test. Four species of *Vibrio* spp. suspensions, *V. alginolyticus*, *V. cholerea*, *V. parahaemolyticus* and *V. fluvialis* were adjusted to 10^7 CFU mL^{-1} in sterile 1%NaCl Mueller Hinton Broth (1%NaCl MHB). The kelp extract was adjusted to 10, 12, 14 continuously 60 mg mL^{-1} in sterile 1%NaCl MHB. The 1%NaCl MHB was used as control group. The 100 µL of each bacterial species suspension in 1%NaCl MHB was added in each 1%NaCl MHB kelp extract mixture groups. All treatments were performed in triplicates. All tubes of each group were incubated at 31°C for 24 h. The view of turbidity in media as growth of bacteria was checked. The clear 1%NaCl MHB mixture containing the lowest concentration of kelp extract should represent the MIC of *A. nodosum* extract on *Vibrio* spp.

The MBC test is continuance from the MIC test. The 100 µL of all clear 1%NaCl MHB mixtures from MIC test was dropped into each new sterile 1%NaCl MHB and incubated at 31°C for 24 h and turbidity was checked. The clear 1%NaCl MHB mixture containing the lowest concentration from MIC should represent the MBC of *A. nodosum* extract on *Vibrio* spp.

2.8 Vibriosis Resistance Experiment of *B. areolata*

Healthy *B. areolata* was reared in opened system, 30‰ saltwater and ten spotted babylons per tank. 120 spotted babylons were divided into four groups with triplicates. Each group was fed for seven days with kelp extract supplement in diet 0%, 1.5%, 3.0% and 4.5% (w w^{-1}). They were fed once daily, uneaten food and fecal matters were removed by siphon every day after feeding and the rearing water was removed about 60% daily. After feeding for seven days, the LD_{50} of *V. alginolyticus* was intramuscular injected in pedal

area of each spotted babylon while the control group was injected with 1%NaCl solution. Then, each group was afterward raised on seven days. The survival of *B. areolata* was recorded at the end of this experiment. The relative percent survival was used on efficiency of kelp extract assessment calculating with equation 2.2 (Baulny *et al*., 1996 and Chansue *et al*., 2007).

The relative percent survival, RPS (%)

$$RPS = \frac{(\text{nontreat lethal} - \text{treat lethal}) \times 100}{\text{nontreat lethal}} \quad \text{.....2.2}$$

2.9 Growth Performance Experiment of *B. areolata*

Healthy *B. areolata* was reared in opened system, 30‰ saltwater and ten spotted babylons per tank. 150 spotted babylons were divided into five groups with triplicates. Each group was fed on 30 days with kelp meal supplement in diet 0%, 2.5%, 5.0% and 10.0% (w w^{-1}) and short body mekeral. They were fed once daily, uneaten food and fecal matter were removed by siphon every day after feeding and the rearing water was removed about 60% daily. The total shell length and wet body weight were measured at the end of this experiment. The relative growth rate (Equation 2.3) and specific growth rate (Equation 2.4) were estimated from total shell length and wet body weight, the feed conversion ratio (Equation 2.5), percentage of shell length increase (Equation 2.6) and weight gain (Equation 2.7) (Zhou *et al*., 2007) and the condition index (Equation 2.8) were used on growth assessment (Brett, 1979, Sang-ngam, 1998 and Immanuel, *et a1*, 2003).

Relative growth rate, RGR (%/day)

$$RGR = \frac{(\text{final size} - \text{initial size}) \times 100}{\text{initial size} \times \text{number of feeding day}} \quad \text{.....2.3}$$

Specific growth rate, SGR (%/day)

$$SGR = \frac{(\ln \text{final size} - \ln \text{initial size}) \times 100}{\text{number of feeding day}} \quad \text{.....2.4}$$

Feed conversion ratio, FCR

$$FCR = \frac{\text{weight of feed}}{(\text{final weght} - \text{initial weght})} \quad \text{.....2.5}$$

Percent Shell length increase, SLI

$$SLI = \frac{(\text{final shell length} - \text{initial shell lelngth}) \times 100}{\text{initial shell elngth}} \quad \text{.....2.6}$$

Percent Weight gain, WG

$$WG = \frac{\text{final weight} \times 100}{\text{initial weight}} \quad \text{.....2.7}$$

Condition index, CI

$$CI = \frac{\text{final weight} \times 100}{(\text{final length})^3} \quad \text{.....2.8}$$

2.10 Water Qualities Determination

The major qualities of the rearing water were measured every day along the experimental period. Salinity was measured by Hand-Held Refractometer (Atago®), temperature was measured by handle thermometer, pH was measured by indicator paper, dissolve oxygen (DO), total ammonia and total alkalinity were measured by test kits (AQUA–VBC®)

2.11 Statistical Analyses

All of the experiments in this study were set on completely randomized design (CRD). All parameters were statistical evaluated using analysis of variance at level of significance 0.05, homogeneity of variance and multiple comparisons by Duncan's New Multiple Range Tests at confidence interval 95% (Sang-ngam, 1998 and Sokal and Rohlf, 1981).

2.12 Study Sites

1. General experimental preparations were prepared at Biotechnology laboratory, Program in Biotechnology, Faculty of Science, Chulalongkorn University.

2. Extraction and chemical preparations were prepared at Biochemical Nutrition Lab, Department of Marine Science and Natural Products Research Unit (NPRU), Department of Chemistry, Faculty of Science, Chulalongkorn University.

3. Bacterial tests were prepared at Aquatic Animal Veterinary Medical Research Center (AVMRC), Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University.

4. Aquaculture tests were prepared at Bang Krachao Sub-district, Meung District, Samut Sakhon Province.

2.13 Study Period

August 2006 to December 2007

CHAPTER III

RESULTS AND DISCUSSION

3.1 Crude Extraction from *A. nodosum*

The crude extract was obtained from three times extraction from 100 g of *A. nodosum*. The average yield of EtOH extract is 5.33±0.97% (Mean±SD, n=10) dry weight. The EtOH extract is oily dark-green. The average yield of 0.01 N HCl extract is 24.46±4.51% dry weight. The 0.01 N HCl extract as the target compound is brown and tea smell-like (Figure 3.1). The final extract was stored at 4°C until future use.

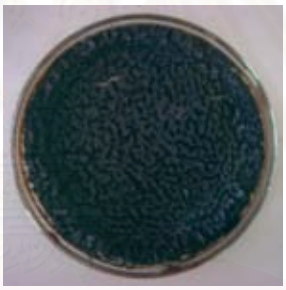

Figure 3.1 *A. nodosum* extract

The yield of seaweed extract was varied in the wide range depending on the method and the seaweed species used. The inexpensive and convenient method which has been developed for the large scale preparation using HCl was used in this experiment.

In this study, %yield of the extract of *A. nodosum* dried weight was close to that of the *Sar. polycystum* extract, 22.3±4.51% (Chotigeat *et al*., 2004), but more than that of *Pelvetica canaliculata* 15.3% (Colliec *et al*., 1994) by the same HCl extraction method.

Diverse extraction methods were adapted to extract fucoidan from different species using different solvents, such as *Dictyota mertensis*, *Padina gymnospora* and *Sar. vulgare* by Maxatase enzyme (Dietrich *et al*., 1995), *Sar. horneri* by 10% trichloroacetic acid (TCA) (Hoshino *et al*., 1998), *Sar. duplicatum* by hot-water and other reported researches.

3.2 Experimental Artificial Diets

In the vibriosis resistant experiment, the result of proximate analysis of experimental artificial diets is shown in Table 3.2. The kelp extract 1.5% and 4.5% formulas are the highest average of protein. The kelp extract 1.5% formula is the highest average of lipid. The control formula is the highest average ash and moisture percentage. The kelp extract 3.0% formula is the highest average of fiber percentage. All composition of each formula is no significant difference (P>0.05) with other formulas. The stability of all experimental diets is approximately 2.0 h.

In the growth performance experiment, the result of proximate analysis of experimental artificial diets is shown in Table 3.3. The control formula is the highest average of protein percentage and significant difference (P>0.05) with other formulas. The average of lipid percentage is no significant difference (P>0.05) between four dietary formulas. The kelp 10% formula is the highest average ash, moisture and fiber percentage. The stability of all experimental diets is approximately 2.0 h.

In the proximate analysis of the experimental artificial dietary formulas, both vibriosis resistant and growth performance experiments, are high level of protein content. The high protein formula showed good effect on survival and growth of healthy *B. areolata* than low protein formula (Sang-ngam, 1997).

The high kelp level formula showed high ash and fiber content that may be affected on kelp content (Chungthanawong, 2004). The stability of the experimental diets for 2 h is long enough for babylon feeding.

3.3 MIC and MBC of *A. nodosum* Extract on *Vibrio* spp.

The kelp, *A. nodosum* extract revealed the inhibitory effect of some species of vibriosis bacteria. The MIC of the extract on four species of vibriosis bacteria, *V. alginolyticus*, *V. parahaemolyticus*, *V fluvialis* and *V. cholerae* are 16, 18, 16 and 22 mg mL^{-1}, respectively. The MBC on four species are 46, 52, 50 and 58 mg mL^{-1}, respectively (Table 3.1). The effect of kelp extract on *V. alginolyticus* displayed the lowest concentration and on *V. cholera* showed the highest concentration both MIC and MBC.

Table 3.1 MIC and MBC of *A. nodosum* extract on *Vibrio* spp.

***Vibrio* spp.**	**MIC (mg mL^{-1})**	**MBC (mg mL^{-1})**
V. alginolyticus	16	46
V. parahaemolyticus	18	52
V fluvialis	16	50
V. cholerae	22	58

The *A. nodosum* extract in this experiment inhibited Gram negative bacteria, *Vibrio* spp. and was reported according to the MIC and MBC of the extract. The MBC of all species in this experiment were higher than 100% of MIC. There are also reported that certain seaweed extracts have great antibacterial activity against Gram positive and Gram negative bacteria including *Pad. gymnospora* and *D. dichotoma* extracts while the *Hypnea musciformis* extracts exhibited activity against *Salmonella typhosa ParaA* (Rao and Parekh, 1981). MIC of the crude extract from *Sar. polycystum* also

inhibited the growth of *V. harveyi*, *Staphylococcus aureus* and *E. coli* at 12, 12 and 6 mg mL^{-1}, respectively of (Chotikiat *et al*., 2004) and MIC of crude fucoidan from leafy bladderwort *Utricularia aurea* against *V. harveyi* and *Escherichia coli* was 20 and 10 mg mL^{-1} (Choosawad *et al*., 2005).

Both water soluble and organic crude extracts of many species of brown seaweed such as *A. nodosum*, *Sar. Muticum* and *Larminaria* spp. inhibited both marine Gram positive and negative bacteria but the organic extract presented toxicity in invertebrates as oyster and sea urchin larvae (Hellio *et al*., 2001). The specific substance in the extracts having antibacterial activity was not clearly identified. In this study used the water soluble extract only because the next studies were tested in spotted babylons that prevented the toxicity in invertebrate species. The mechanism of the extract on antibacterial activity may be the coating of bacterial surface by the charged polysaccharides. However, the specific mechanism was required to be future studied.

3.4 Efficiency of *A. nodosum* Extract Supplement in Diet on Vibriosis Resistance of *B. areolata*

The symptoms of vibriosis spotted babylon were no feeding and motion, limp of proboscis and foot muscle, red swell of soft body and die (Figure 3.2). *B. areolata* was fed with four *A. nodosum* extract dietary formulas, 0% as control, 1.5%, 3.0% and 4.5% of kelp extract in diet (w w^{-1}) before and after vibriosis infected. The percent survival rates of vibriosis spotted babylons were 16.67 ± 1.15^{b}, 46.67 ± 5.77^{a}, 43.33 ± 5.77^{a} and 46.67 ± 5.77^{a}, respectively with significant difference ($P<0.05$) (Table 3.4 and Figure 3.3).

Table 3.2 Dietary proximate analysis of the vibriosis resistant experiment

Diet Formulas	Contents					Stability
	Protein (%)	**Lipid (%)**	**Ash (%)**	**Moisture (%)**	**Fiber (%)**	**(h)**
Kelp Extract 0% (Control)	44.30 ± 0.03[a]	10.48 ± 0.17[a]	12.84 ± 0.14[a]	7.52 ± 0.01[a]	2.62 ± 0.02[a]	2
Kelp Extract 1.5%	44.50± 0.16[a]	10.61 ± 0.26[a]	12.55 ± 0.19[a]	7.34 ± 0.18[a]	2.73 ± 0.13[a]	2
Kelp Extract 3.0%	44.46 ± 0.08[a]	10.34 ± 0.17[a]	12.43± 0.50[a]	7.42± 0.05[a]	2.74 ± 0.09[a]	2
Kelp Extract 4.5%	44.50 ± 0.15[a]	10.53 ± 0.07[a]	12.48 ± 0.14[a]	7.48 ± 0.29[a]	2.62 ± 0.31[a]	2

Mean ± SD

Note: the same superscript indicates no significant difference (P>0.05)

Table 3.3 Dietary proximate analysis of the growth performance experiment

Diet Formulas	Contents					Stability
	Protein (%)	**Lipid (%)**	**Ash (%)**	**Moisture (%)**	**Fiber (%)**	**(h)**
Kelp 0% (Control)	44.30 ± 0.03^{a}	10.48 ± 0.17^{b}	12.84 ± 0.14^{d}	7.52 ± 0.01^{a}	2.62 ± 0.02^{c}	2
Kelp 2.5%	43.92 ± 0.07^{ab}	10.48 ± 0.09^{b}	13.60 ± 0.17^{c}	7.28 ± 0.06^{a}	2.73 ± 0.13^{bc}	2
Kelp 5.0%	43.78 ± 0.01^{ab}	10.34 ± 0.11^{b}	15.37 ± 0.05^{b}	7.23 ± 0.05^{a}	2.84 ± 0.06^{b}	2
Kelp 10.0%	43.56 ± 0.03^{b}	10.31 ± 0.04^{b}	16.21 ± 0.13^{a}	7.18 ± 0.02^{a}	3.11 ± 0.12^{a}	2
Fish Meat	19.07 ± 0.53^{c}	12.63 ± 0.57^{a}	1.39 ± 0.13^{e}	67.04 ± 1.64^{b}	$>0.01 \pm 0.00^{d}$	>2

Mean ± SD

Note: the same superscript indicates no significant difference (P>0.05)

Vibriosis *B. areolata* which were not fed with kelp extract supplement showed lower survival rate than the spotted babylons which were fed kelp extract formulas on seven days. *A. nodosum* extract supplement 1.5% and 4.5% in diet showed the highest in survival of vibriosis infected *B. areolata*. The statistical analysis of this experiment is shown in Appendix C.

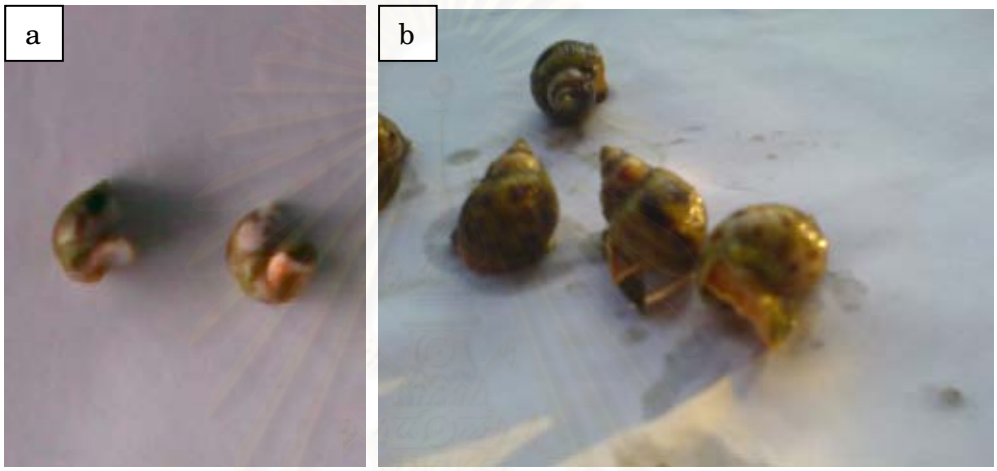

Figure 3.2 Vibriosis *B. areolata*

The RPS of vibriosis infected *B. areolata* which fed with four kelp extract dietary formulas were 0, 36%, 32% and 36%, respectively (Table 3.4). *A. nodosum* extract supplement 1.5% and 4.5% in diet presented the best RPS and the 0% supplement presented 0% RPS of vibriosis *B. areolata*.

Table 3.4 Survival rate of *B. areolata* and RPS with different diets

Treatments	**Survival Rate (%)**	**RPS (%)**
Kelp Extract 0.0%	16.67 ± 1.15^{b}	0
Kelp Extract 1.5%	46.67 ± 5.77^{a}	36
Kelp Extract 3.0%	43.33 ± 5.77^{a}	32
Kelp Extract 4.5%	46.67 ± 5.77^{a}	36

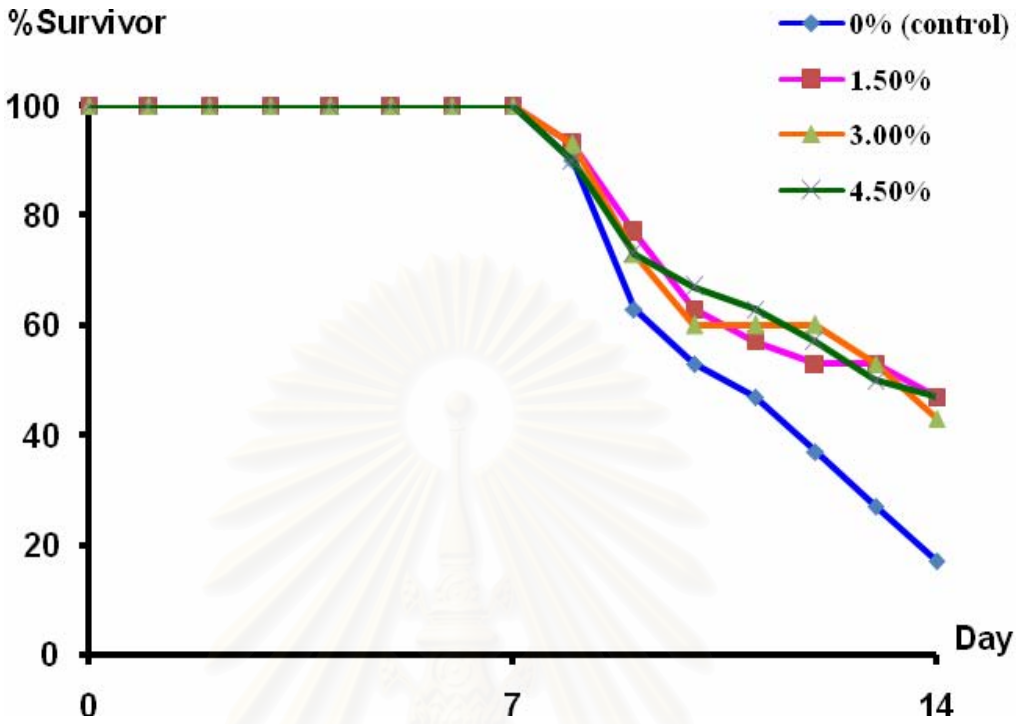

Figure 3.3 Percentages of vibriosis *B. areolata* survior with different diets

Several extracts from green, red and brown algae have been reported to increase the resistance of several fish and shrimp species against virus and bacterial infections (Fujiki *et al*., 1992, Cheng *et al*., 2004, Cheng *et al*., 2005 and Fujiki *et al*., 1994). Oral administration of fucoidan extracted from brown seaweed *Sar. polycystum* has been reported to reduce the impact of the WSSV infection in *P. monodon* (Chotikiat *et al*., 2004). In this study, all *B. areolata* which were fed with 0.01 N HCl extract of *A. nodosum* increased vibriosis resistance from *V. alginolyticus*. This may be because fucoidan as polysaccharides from kelp displayed positive effects on vibriosis resistance of spotted babylons.

Several extracts from marine algae increased the non-specific immune system in both fish and shrimps. In addition, an intraperitoneal injection with sodium alginate extracted from brown alga *M. pyrifera* increased the migration of head kidney phagocytes to the peritoneal cavity (Fujiki and Yano,. 1997). *In vitro* study, laminaran extracted from brown algae *Larminaria digitata* was reported to increase activity of the prophenoloxidase (ProPO) system in brown shrimp *Farfantepenaeus californiensis* (Hernández *et al*., 1996), São Paulo shrimp *Far. paulensis* (Perazzolo and Barracco, 1997), and *P. monodon* (Sritunyalucksana *et al*., 1999). The immune-stimulant mechanism of *B. areolata* was clearly required on future study.

Although the extract can be used as the immune-stimulant to control fish, shrimp and mollusk disease in aquaculture, it may not be effective against all diseases and for aquatic animals. Therefore, the timing, dosages and methods of administration as well as the side effects are needed to be investigated. Thus, plant or seaweed extracts may be effectively used as a dietary source to enhance the disease resistance as well as to have better survival rate and production of aquaculture systems.

3.5 Efficiency of *A. nodosum* Meal Supplement in Diet on the Growth of *B. areolata*

The efficiency of *A. nodosum* meal supplement in diet on the growth of *B. areolata* is shown in Tables 3.5 and 3.6. The period of this experiment is 30 days. The means of total shell length and wet body weight of *B. areolata* are significant difference (P<0.05) for five levels of kelp meal supplement, 0% as control, 2.5%, 5.0% and 10.0% in diet (w w^{-1}) and fish meat. The results on the averages of total shell length were 21.11 ± 0.34^{c}, 21.23 ± 0.20^{b}, 21.17 ± 0.20^{bc}, 21.11 ± 0.16^{c} and 22.61 ± 0.14^{a} mm, respectively with significant different (P<0.05). The averages of wet body weight of the spotted babylon fed with

different kelp levels were 2.17±0.03^{b}, 2.17±0.02^{b}, 2.14±0.02^{c}, 2.10±0.06^{d} and 2.98±0.12^{a} g, respectively with significant difference (P<0.05).

Five dietary treatments exhibited the RGR estimating from total shell length that were 0.56±0.06%^{bc}, 0.58±0.04%^{b}, 0.56±0.04%^{bc}, 0.55±0.03%^{c} and 0.83±0.03%^{a}, respectively with significant difference (P<0.05). The relative growth rates estimating from wet body wet weight were 2.80±0.09%^{b}, 2.79±0.05%^{b}, 2.72±0.05%^{c}, 2.61±0.04%^{d} and 5.10±0.03%^{a} with significant difference (P<0.05).

Five dietary treatments expressed the SGR estimating from total shell length that were 0.51±0.05^{bc}, 0.53±0.03%^{b}, 0.52±0.03%^{bc}, 0.51±0.02%^{c} and 0.74±0.02%^{a}, respectively with significant difference (P<0.05). The SGR estimating from wet body wet weight were 2.03±0.05%^{b}, 2.03±0.03%^{b}, 1.99±0.03%^{c}, 1.92±0.02%^{d} and 3.10±0.01%^{a} with significant difference (P<0.05).

Five dietary treatments showed the SLI were 16.63±1.89%^{c}, 17.31±1.01%^{bc}, 16.96±1.01%^{c}, 16.59±0.89%^{c} and 24.90±0.77%^{a}, respectively with significant difference (P<0.05).

The FCR of five dietary formulas were 2.03±0.06^{e}, 2.06±0.04^{d}, 2.09±0.04^{c}, 2.18±0.03^{b} and 3.86±0.03^{a} respectively with significant difference (P<0.05).

Five dietary treatments showed the WG were 184.02±2.71%^{b}, 183.67±1.59%^{b}, 181.50±1.45%^{c}, 178.14±1.16%^{d} and 252.91±1.04%^{a}, respectively with significant difference (P<0.05).

The CI of five dietary treatments were 0.0231±0.0008^{b}, 0.0226±0.0005^{c}, 0.0225±0.0005^{c}, 0.0223±0.0004^{c} and 0.0258±0.0004^{a}, respectively with significant difference (P<0.05).

Fish meat showed the highest average of total length, wet body weight, RGR, SGR, SLI, FCR, WA and CI. Kelp meal supplement 2.5% in diet exhibited high of all parameters than other formulas. Survival rate of *B. areolata* was 100% in all dietary treatments which not showed significant effect on survival rate. The statistical analysis of this experiment is shown in Appendix D.

The effect of seaweed on growth performance has been studied in pig (Turner *et al*., 2002), calve (Evans *et al*., 2002), fish (Valente *et al*., 2006 and Davies *et al*., 1997) and shrimp (Chungthanawong, 2004), but no on mollusk species.

In this study, kelp supplement showed the effect on total shell length and wet weight of healthy *B. areolata*. In terms of total shell length, the spotted babylons fed with kelp 2.5% and 5.0% dietary formulas were significantly longer than those from control and 10.0% formulas. In terms of wet weight, *B. areolata* which were fed with kelp control and 2.5% dietary formulas were significantly heavier than those from 5.0% and 10.0% formulas. However, kelp meal supplement 0% and 2.5% were no significant difference on total shell length and wet body weight. In particular, there was no importance for kelp meal supplement in diet because kelp exhibited unclear effect on growth performance of *B. areolata*.

These results showed that the growth gain of juvenile spotted babylons increased with high protein of artificial diets. These results are similar to those in other mollusk species (Uki *et al*., 1986, Mai *et al*., 1995). On the basis of this study, 45% protein in artificial diet may be recommended for growth of *B. areolata*. The optimal protein level for abalone was between 20% and 35% (Uki and Watanabe 1992). These differences might be that *B. areolata* was a carnivorous or scavenger mollusk whereas abalone was omnivorous or herbivorous.

Table 3.5 Growth on total shell length and wet body weight of *B. areolata* with different diets

Diet Formulas	Total Length (mm)		Wet Weight (g)		Survival Rate (%)
	Initial	Final	Initial	Final	
Kelp 0%	18.10 ± 0.24	21.11 ± 0.34^{c}	1.18 ± 0.13	2.17 ± 0.03^{b}	100
Kelp 2.5%	18.10 ± 0.24	21.23 ± 0.20^{b}	1.18 ± 0.13	2.17 ± 0.02^{b}	100
Kelp 5.0%	18.10 ± 0.24	21.17 ± 0.20^{bc}	1.18 ± 0.13	2.14 ± 0.02^{c}	100
Kelp 10.0%	18.10 ± 0.24	21.11 ± 0.16^{c}	1.18 ± 0.13	2.10 ± 0.06^{d}	100
Fish Meat	18.10 ± 0.24	22.61 ± 0.14^{a}	1.18 ± 0.13	2.98 ± 0.12^{a}	100

Mean ± SD (n=30, triplicate)

Note: the same superscript indicates non significant difference (P>0.05)

Table 3.6 Growth indexes of *B. areolata* with different diets

Formulas	RGR (%/day)		SGR (%/day)	
	Total Length	Wet Weight	Total Length	Wet Weight
Kelp 0%	0.56 ± 0.06^{bc}	2.80 ± 0.09^{b}	0.51 ± 0.05^{bc}	2.03 ± 0.05^{b}
Kelp 2.5%	0.58 ± 0.04^{b}	2.79 ± 0.05^{b}	0.53 ± 0.03^{b}	2.03 ± 0.03^{b}
Kelp 5.0%	0.56 ± 0.04^{bc}	2.72 ± 0.05^{c}	0.52 ± 0.03^{bc}	1.99 ± 0.03^{c}
Kelp 10.0%	0.55 ± 0.03^{c}	2.61 ± 0.04^{d}	0.51 ± 0.02^{c}	1.92 ± 0.02^{d}
Fish Meat	0.83 ± 0.03^{a}	5.10 ± 0.03^{a}	0.74 ± 0.02^{a}	3.10 ± 0.01^{a}

Mean ± SD (n=30, triplicate)

Note: the same superscript indicates non significant difference (P>0.05). Initial weight was about 1.18 ± 0.13 g and initial shell length was 18.10 ± 0.24 cm.

RGR : relative growth rate and SGR : specific growth rate

Table 3.6 (cont) Growth indexes of *B. areolata* with different diets

Formulas	FCR	SLI (%)	WG (%)	CI
Kelp 0%	2.03 ± 0.06^{e}	16.63 ± 1.89^{c}	184.02 ± 2.71^{b}	0.0231 ± 0.0008^{b}
Kelp 2.5%	2.06 ± 0.04^{d}	17.31 ± 1.01^{bc}	183.67 ± 1.59^{b}	0.0226 ± 0.0005^{c}
Kelp 5.0%	2.09 ± 0.04^{c}	16.96 ± 1.01^{c}	181.50 ± 1.45^{c}	0.0225 ± 0.0005^{c}
Kelp 10.0%	2.18 ± 0.03^{b}	16.59 ± 0.89^{c}	178.14 ± 1.16^{d}	0.0223 ± 0.0004^{c}
Fish Meat	3.86 ± 0.03^{a}	24.90 ± 0.77^{a}	252.91 ± 1.04^{a}	0.0258 ± 0.0004^{a}

Mean ± SD (n=30, triplicate)

Note: the same superscript indicates non significant difference (P>0.05). Initial weight was about 1.18 ± 0.13 g and initial shell length was 18.10 ± 0.24 cm.

FCR : feed conversion ratio, SLI : shell length increase, WG : weight gain and CI : condition index

The spotted babylons fed with fish meat showed the highest growth than other formulas since fish meat was an appropriately feed. The artificial diet is important for intensive aquaculture in long term due to the production of fish being not stable on market supply. The artificial diet can be designed to many requirements. Thus the research on artificial diet of spotted babylons should continuously study and developed.

The potential of brown seaweed as a valuable resource of bioactive compounds, particularly fucoidan based on previous research is high. In an aquaculture, the antiviral and antibacterial properties of fucoidan extract was likely high benefit. The shrimp and other farming industries lose many profits due to viral and bacterial disease problems. The prevention and therapy of microbial infections with a natural product such as algal product, brown seaweed extract or meal would certainly be the most advantage. In conclusion, brown seaweed should be the efficacious matter for aquaculture application.

3.6 Water Qualities

The major qualities of the rearing water were determined everyday. The range of temperature was 27 to 28°C, salinity was 27 to 30‰, pH was 8.0 to 8.5, dissolved oxygen (DO) is 6 to 8 mg L^{-1}, total ammonia was 0.00 to 0.2 and alkalinity was 150 to157 mg L^{-1}

All water parameters along the experimental period were rather stable because the water was exchanged 60% daily after feeding. The dissolved oxygen, DO was in high level because the air supply was opened throughout during all the experimental periods. The qualities of water were in the standard range for aquaculture and the culture density was suitable for the growth of juvenile *B. areolata* (Liu and Xiao, 1998 and Chaitanawisuti and Kritsanapuntu 2002).

CHAPTER IV

CONCLUSIONS AND RECOMENDATIONS

4.1 Conclusions

4.1.1 Effect of *A. nodosum* extract on *Vibrio* spp.

The 0.01 N HCl extract from kelp *A. nodosum* inhibits the growth of *V. alginolyticus, V. parahaemolyticus, V fluvialis* and *V. cholera in vitro* that present in MIC and MBC.

4.1.2 Effect of *A. nodosum* extract on vibriosis resistance of *B. areolata*

The vibriosis *B. areolata* feeding *A. nodosum* extract supplement in artificial diet show higher survival rate than those do not feed *A. nodosum* extract supplement.

4.1.3 Effect of dried *A. nodosum* meal supplement in artificial diet on growth performance of *B. areolata*

The *B. areolata* feeding high level of *A. nodosum* meal supplement in artificial diet exhibited bad significant effect on growth of *B. areolata* than those feed low level or none of *A. nodosum* meal supplement. *A. nodosum* meal may not be an effective diet material on growth performance of *B. areolata*.

4.2 Recommendations

This research is the pioneer study on the potential utilization of kelp, *A. nodosum* application in *B. areolata* culture. The related fields of this study should be continued studying.

4.2.1 Chemistry and Biochemistry of *A. nodosum*

4.2.1.1 The stability of the extract during long period storage.

4.2.1.2 The purification of crude extract is necessary to investigate because the crude extract contains many compositions. The bioactivity may be different from each fraction in crude extract.

4.2.1.3 The use of selective enzymes as tools to modify fucoidan fractions to correlate structure with biological activity.

4.2.1.4 The interaction and possible synergy between fucoidan and other biomolecules present in brown seaweed extracts also need to be investigated.

4.2.2 Bioactivity of *A. nodosum*

4.2.2.1 The potential of brown seaweed extracts as antifungal agents also requires for investigation. Fungal infections are notoriously difficult to treat and often develop into chronic infections. For example, black gill disease caused by *Fusarium* spp. in fish and crustaceans may result in significant pre-harvest mortality.

4.2.2.2 The mechanism of *A. nodosum* extracts on antiviral, antibacterial, growth performance and immune-stimulus activities in spotted babylon, shrimp or other animals.

4.2.2.3 The kelp applications in commercial feed of other animals.

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Appendices

Appendix A

Bacterial Preparation

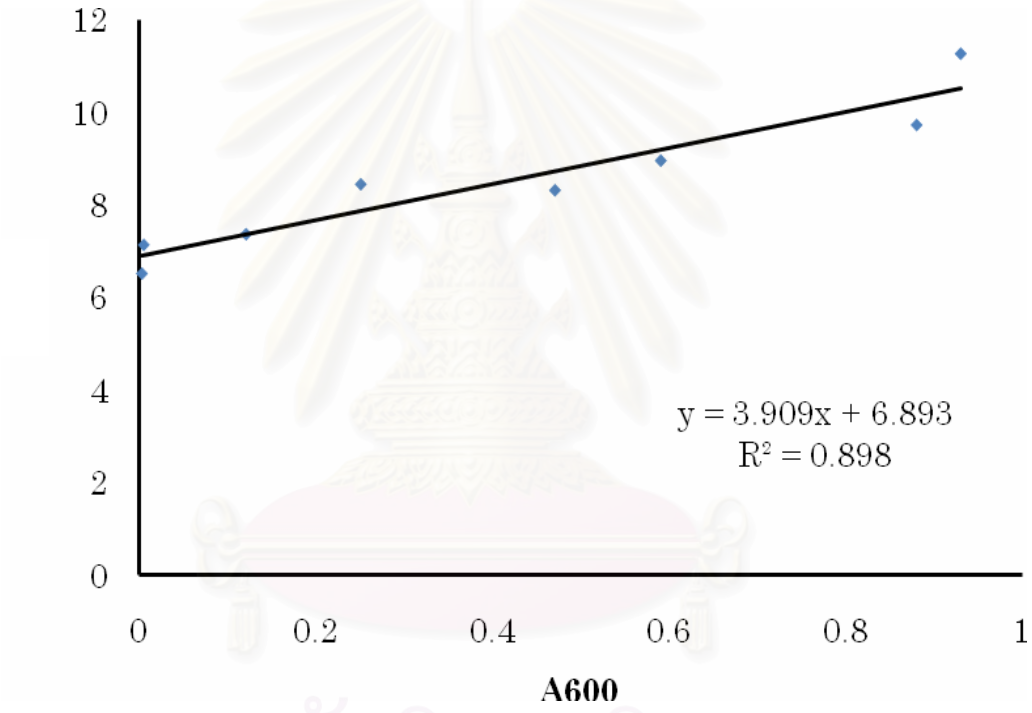

Figure A1 Standard curve of *V. alginolyticus*

Table A1 Mortality of Vibriosis Infected *B. areolata*

Concentration of *V. alginolyticus* (CFU·mL^{-1})	**24 Hours**				**48 Hours**				**72 Hours**				**96 Hours**			
	Replicate				**Replicate**				**Replicate**				**Replicate**			
	n_1	**n_2**	**n_3**	**%**	**n_1**	**n_2**	**n_3**	**%**	**n_1**	**n_2**	**n_3**	**%**	**n_1**	**n_2**	**n_3**	**%**
Control	0	1	1	7	0	1	1	7	0	1	1	7	0	1	1	7
10^7	1	2	1	13	3	4	3	33	4	4	5	43	5	6	6	57
10^8	2	1	2	17	4	5	3	40	5	4	4	53	7	7	6	67
10^9	2	1	2	17	4	5	3	40	5	4	4	53	7	7	6	67
10^{10}	4	3	3	33	5	5	6	53	7	8	10	83	7	8	10	83
10^{11}	4	4	5	43	9	8	7	80	9	9	8	87	9	10	9	93
10^{12}	4	5	5	47	10	10	9	97	10	10	10	100	10	10	10	100

Table A2 LD_{50} of *V. alginolyticus* in *B. areolata* calculation

C	ln(C)	n	nD	nA	∑D	∑A	T	M
1×10^{7}	16.1181	30	13	17	13	57	70	18.57
1×10^{8}	18.42068	30	13	17	26	40	66	39.39
1×10^{9}	20.72327	30	16	14	42	23	65	64.62
1×10^{10}	23.02585	30	25	5	67	9	76	88.16
1×10^{11}	25.32844	30	26	4	93	4	97	95.88
1×10^{12}	27.63102	30	30	0	123	0	123	100.00

C = concentration of *V. alginolyticus* (CFU mL^{-1})

n = number of *B. areolata* per concentration

nD = number of death *B. areolata*

nA = number of alive *B. areolata*

∑D = summation of death *B. areolata*

∑A = summation of alive *B. areolata*

T = ∑D + ∑A per conc.

M = (∑D/T) × 100

ln CA 50% = ln(concentration above 50% mortality)

ln CB 50% = ln(concentration below 50% mortality)

MA 50% = mortality above 50%

MB 50% = mortality below 50%

LD_{50} Calculation

$$LD_{50} = \ln CB\ 50\% + \frac{(50 - MB\ 50\%) \times (\ln CA\ 50\% - \ln CB\ 50\%)}{(MA\ 50\% - MB\ 50\%)}$$

$$LD_{50} = 18.42 + \frac{(50 - 39.39) \times (20.72 - 18.42)}{(64.62 - 39.39)}$$

$LD_{50} = 19.39$

$Exp(LD_{50}) = 2.63 \times 10^{8}$ CFU mL^{-1}

Appendix B

Proximate Analysis of Experimental Diets

1. Proximate Analysis of Crude Protein

1.1 Digestion

1.1.1 Pour 2 g of dried diet into digestion tube

1.1.2 Add 10.01 g of catalyst and 25 mL of conc. H_2SO_4

1.1.3 Transfer the digestion tube to rack of Kjeldatherm digestion block, set the vacuum smoke suction digest until solution is almost colorless or blackly green

1.1.4 Set the Kjeldatherm digestion block at 100°C and increase 20°C every 20 minutes until 380°C.

1.1.5 Digest until the solution is almost clear blue as complete digestion

1.1.6 Cool at room temperature

1.1.7 Add 200 mL of distilled water into the digestion tube

1.2 Distillation

1.2.1 Open the Vapodest 1 Machine; push the arm to fill position for fill water to 1/6 of boiler flask and push back to stand by position.

1.2.2 Add 100 mL of 4% boric acid into 500 mL of Erlenmeyer flask and drop 5 or 6 drops (about 5-6 drops) of Tashiro Indicator until the solution becomes violet.

1.3.3 Put the flask of 1.2.2 under the drainage tube of Vapodest 1 Machine and the open end of drainage tube must dip into in solution all time.

1.3.4 Put the digestion tube containing digested solution on the clamp of Vapodest 1 Machine and snuggle seal up to cone-shape rubber stopper.

1.3.5 When water boiled and steam vapor, push "added NaOH" button for fill 50%NaOH into digestion tube and the bubble appear. Add continuously 50%NaOH until bubble disappeared and add more 10 mL of 50%NaOH and the violet solution will be change to be green. In this step, the water is flowed into condenser all time for condense the NH_3 gas into Erlenmeyer flask containing 4% boric acid.

1.3.6 Push the arm to distillation position for open steam into digestion tube, distill to 300 mL of Erlenmeyer flask containing 4% boric acid and push the arm to stand by position.

1.3.7 Transfer the Erlenmeyer flask containing 300 mL of the green solution to titrate with 0.5 N H_2SO_4 solution until endpoint, finally, the green solution will change to be light violet.

1.3 Calculation

$$\%\text{Protein} = \frac{V_s \times N_s \times 1.4007}{\text{weight (g) of sample}} \times 100 \times 6.25 \quad \textbf{B1}$$

Where: V_s = volume of H_2SO_4 titration

N_s = concentration of H_2SO_4 titration (Normal)

6.25 = the protein-nitrogen conversion factor for fish and fish by-products

2. Proximate Analysis of Crude Lipid

2.1 Extraction

2.1.1 Place the extractor bottle in drying oven at 130°C about 2-3 h, cool in desiccator and record the constant weight of the extractor bottle.

2.1.2 Weigh the dried sample about 2 g, pack with the filter paper Whatman No 1, put in paper thimble, put the thimble in the extractor bottle and fill 90 mL of petroleum ether in the extractor bottle (no soak to thimble).

2.1.3 Set the extractor bottle to the Soxhterm machine, turn on switch and set 150°C of oil bath, turn on the pressure control pump and open the condenser supply.

2.1.4 Push down the arm of Soxhterm machine to reflux mode, extract on 6 hr and remove the thimble from the extractor bottle.

2.1.5 Vapor the petroleum ether and Place the extractor bottle in drying oven at 100°C on 3 h, cool in desiccator and record the constant weight of the extractor bottle.

Note: the residue left in the thimble may be used to determine crude fiber.

2.2 Calculation

$$\%\text{Crude fat (wet)} = \frac{(\text{Wres} - \text{Wta})}{\text{weight of sample}} \times 100 \quad \text{.....B2}$$

Where: Wta = tare weight of extractor bottle

Wres = weight of extractor bottle and fat residue

3. Proximate Analysis of Ash

3.1 Procedure

3.1. Place crucible in drying oven at 130°C on 12 h, cool in desiccator and record the weight of the crucible.

3.1.2 Place 2 g of sample in crucible, transfer the crucible to cool muffle furnace and increase the temperature step wise to 600°C on 6 hr or until a white ash is obtained.

3.1.3 Cool in desiccator and record the constant weight of crucible.

3.2 Calculation

$$\% \text{ Ash (wet)} = \frac{(\text{wt. crucible with ash - wt. crucible})}{\text{weight of sample}} \times 100 \quad \text{.....B3}$$

4. Proximate Analysis of Moisture

4.1 Procedure

4.1.1 Place aluminum cup in drying oven at 130°C on 2-3 h, cool in desiccator and record the weight of the crucible.

4.1.2 Place 2 g of sample in aluminum cup, transfer the sample cup to oven and increase the temperature step wise to 100°C on 6 h.

4.1.3 Cool in desiccator and record the constant weight of the sample cup

4.2 **Calculation**

$$\% \text{ Moisture} = \frac{100\,(\text{weight of sample - weight of dried sample})}{\text{weight of sample}} \quad \text{.....B}$$

5. Proximate analysis of Crude Fiber

5.1 Procedure

5.1.1 Place the filter paper and crusible in an oven at 105°C to constant weight. Cool in a desiccator and record the constant weight.

5.1.2 Mix the residual sample of fat analysis in beaker with 200 mL of 0.225 N H_2SO_4, set the condenser, Boil mixture on hot plate on 30 minutes.

5.1.3 Filter the mixture with filter paper Whatman No 41 and wash the sediment three times with 30 mL of hot distilled water.

5.1.4 Add the residual sediment in beaker, fill 200 mL of 0.131 N NaOH, set the condenser on beaker, Boil mixture on hot plate on 30 minutes.

5.1.5 Filter the mixture with filter paper Whatman No 41 and wash the sediment three times with 30 mL of 95% ethanol until.

5.1.6 Place the sediment on filter paper in an oven at 100°C to about 2 h. Cool in a desiccator and record the constant weight.

5.2 Calculation

$$\% \text{ Crude fiber} = \frac{(W2 - W1)}{W1} \times 100 \quad \text{.....B5}$$

Table B1 Descriptive of proximate analysis of diet in vibriosis resistant experiment

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Protein	Control	3	44.2967	.03215	.01856	44.2168	44.3765	44.26	44.32
	Kelp Extract 1.5%	3	44.5033	.16442	.09493	44.0949	44.9118	44.38	44.69
	Kelp Extract 3.0%	3	44.4567	.08021	.04631	44.2574	44.6559	44.38	44.54
	Kelp Extract 4.5%	3	44.5000	.14526	.08386	44.1392	44.8608	44.36	44.65
	Total	12	44.4392	.13365	.03858	44.3542	44.5241	44.26	44.69
Lipid	Control	3	10.4767	.16563	.09563	10.0652	10.8881	10.32	10.65
	Kelp Extract 1.5%	3	10.6100	.26000	.15011	9.9641	11.2559	10.35	10.87
	Kelp Extract 3.0%	3	10.2900	.16703	.09644	9.8751	10.7049	10.11	10.44
	Kelp Extract 4.5%	3	10.5333	.06506	.03756	10.3717	10.6950	10.47	10.60
	Total	12	10.4775	.19583	.05653	10.3531	10.6019	10.11	10.87
Ash	Control	3	12.8367	.14295	.08253	12.4816	13.1918	12.68	12.96
	Kelp Extract 1.5%	3	12.5500	.19000	.10970	12.0780	13.0220	12.36	12.74
	Kelp Extract 3.0%	3	12.4267	.50213	.28990	11.1793	13.6740	12.00	12.98
	Kelp Extract 4.5%	3	12.4833	.14468	.08353	12.1239	12.8427	12.39	12.65
	Total	12	12.5742	.29506	.08518	12.3867	12.7616	12.00	12.98

TableB1 (cont.) Descriptive of proximate analysis of diet in vibriosis resistant experiment

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Moisture	Control	3	7.5200	.01000	.00577	7.4952	7.5448	7.51	7.53
	Kelp Extract 1.5%	3	7.3433	.17926	.10349	6.8980	7.7886	7.23	7.55
	Kelp Extract 3.0%	3	7.4267	.04509	.02603	7.3147	7.5387	7.38	7.47
	Kelp Extract 4.5%	3	7.4800	.29052	.16773	6.7583	8.2017	7.20	7.78
	Total	12	7.4425	.16232	.04686	7.3394	7.5456	7.20	7.78
Fiber	Control	3	2.6233	.01528	.00882	2.5854	2.6613	2.61	2.64
	Kelp Extract 1.5%	3	2.7267	.12503	.07219	2.4161	3.0373	2.64	2.87
	Kelp Extract 3.0%	3	2.7367	.08963	.05175	2.5140	2.9593	2.68	2.84
	Kelp Extract 4.5%	3	2.6200	.31193	.18009	1.8451	3.3949	2.43	2.98
	Total	12	2.6767	.15922	.04596	2.5755	2.7778	2.43	2.98

Table B2 Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Protein	2.026	3	8	.189
Lipid	.895	3	8	.484
Ash	2.943	3	8	.099
Moisture	3.450	3	8	.072
Fiber	8.240	3	8	.008

Table B3 ANOVA table

		Sum of Squares	df	Mean Square	F	Sig.
Protein	Between Groups	.085	3	.028	2.045	.186
	Within Groups	.111	8	.014		
	Total	.196	11			
Lipid	Between Groups	.167	3	.056	1.756	.233
	Within Groups	.254	8	.032		
	Total	.422	11			
Ash	Between Groups	.298	3	.099	1.207	.368
	Within Groups	.659	8	.082		
	Total	.958	11			
Moisture	Between Groups	.052	3	.017	.590	.639
	Within Groups	.237	8	.030		
	Total	.290	11			
Fiber	Between Groups	.036	3	.012	.401	.756
	Within Groups	.242	8	.030		
	Total	.279	11			

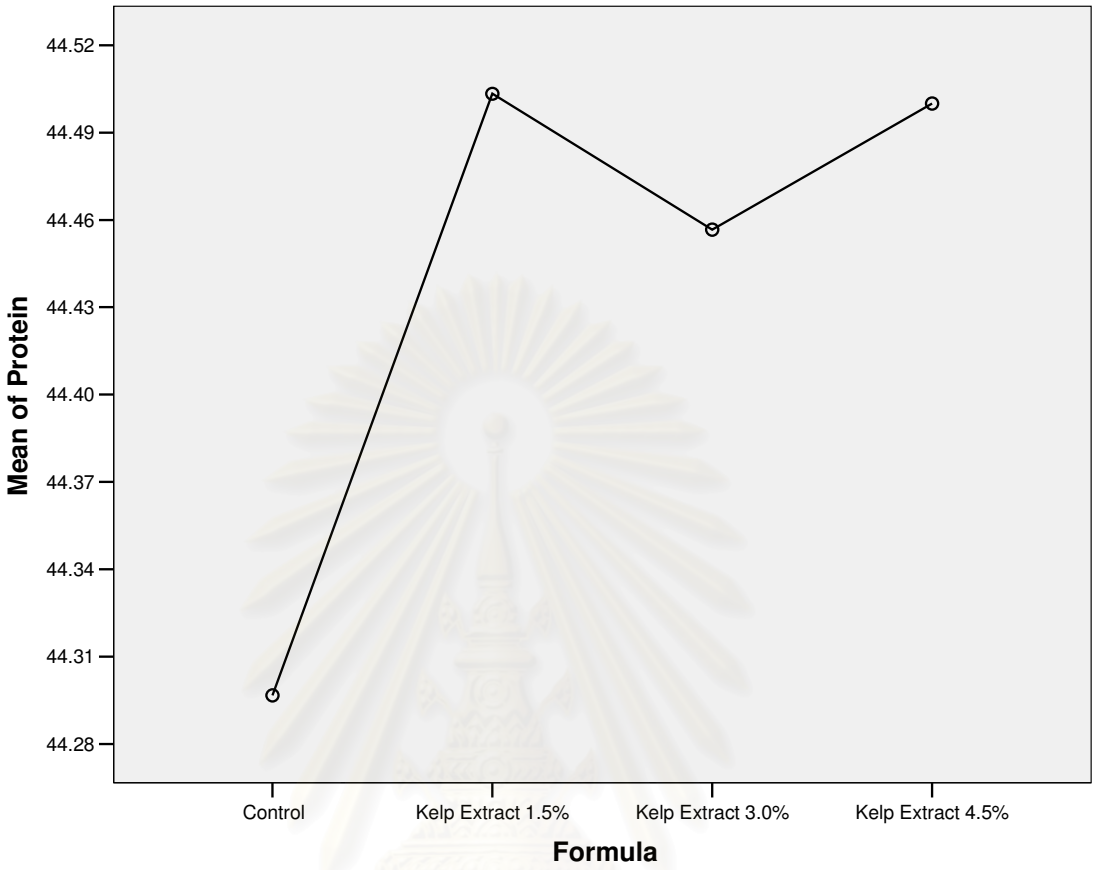

Figure B1 Mean plot of protein

Table B4 Duncan's New Multiple Range Tests of protein

Formula	N	Subset for alpha = .05
		1
Control	3	44.2967
Kelp Extract 3.0%	3	44.4567
Kelp Extract 4.5%	3	44.5000
Kelp Extract 1.5%	3	44.5033
Sig.		.079

Means for groups in homogeneous subsets are displayed

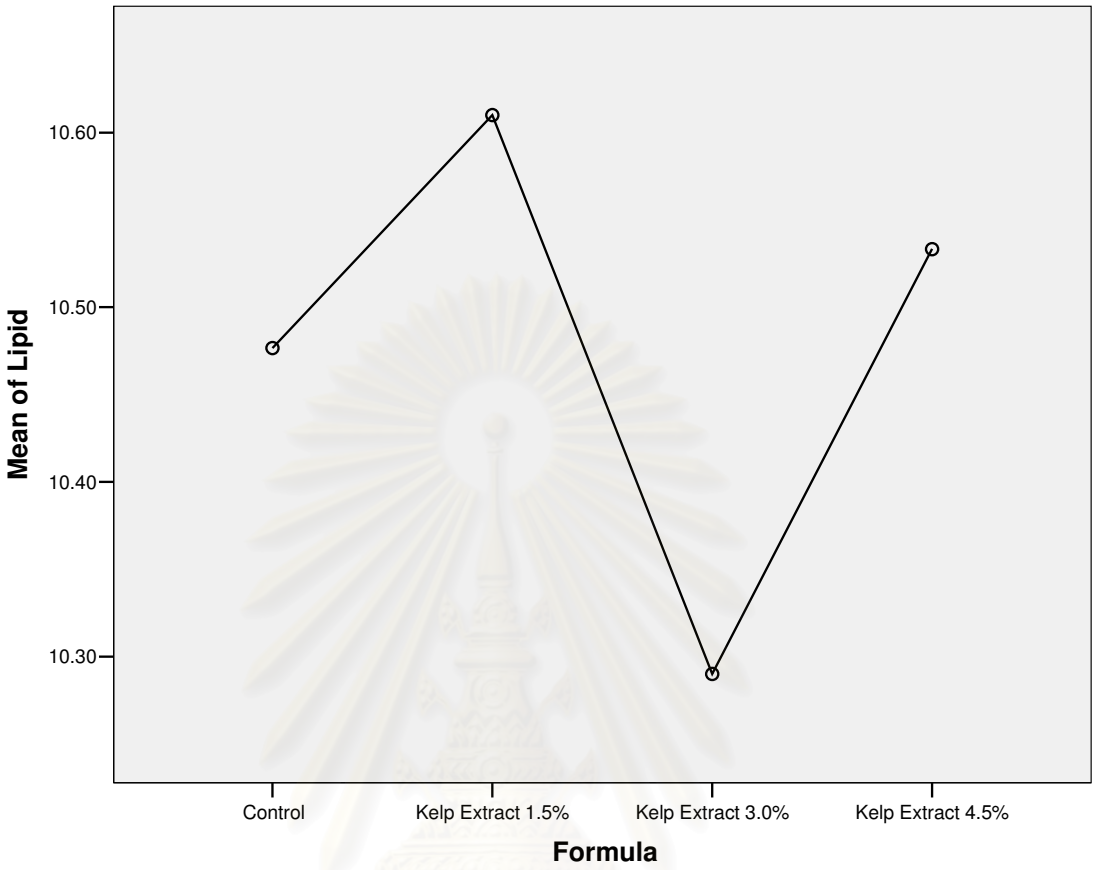

Figure B2 Mean plot of lipid

Table B5 Duncan's New Multiple Range Tests of lipid

Formula	N	Subset for alpha = .05
		1
Kelp Extract 3.0%	3	10.2900
Control	3	10.4767
Kelp Extract 4.5%	3	10.5333
Kelp Extract 1.5%	3	10.6100
Sig.		.073

Means for groups in homogeneous subsets are displayed.

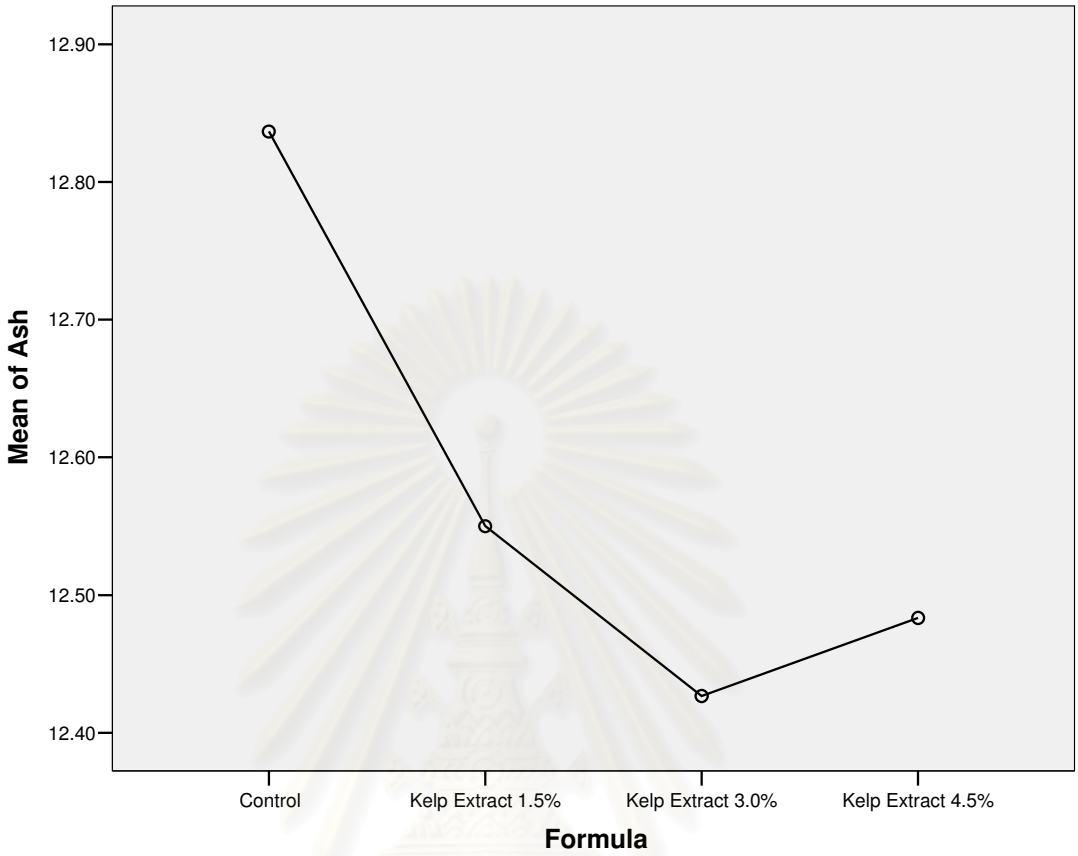

Figure B3 Mean plot of ash

Table B6 Duncan's New Multiple Range Tests of ash

Formula	N	Subset for alpha = .05
		1
Kelp Extract 3.0%	3	12.4267
Kelp Extract 4.5%	3	12.4833
Kelp Extract 1.5%	3	12.5500
Control	3	12.8367
Sig.		.139

Means for groups in homogeneous subsets are displayed.

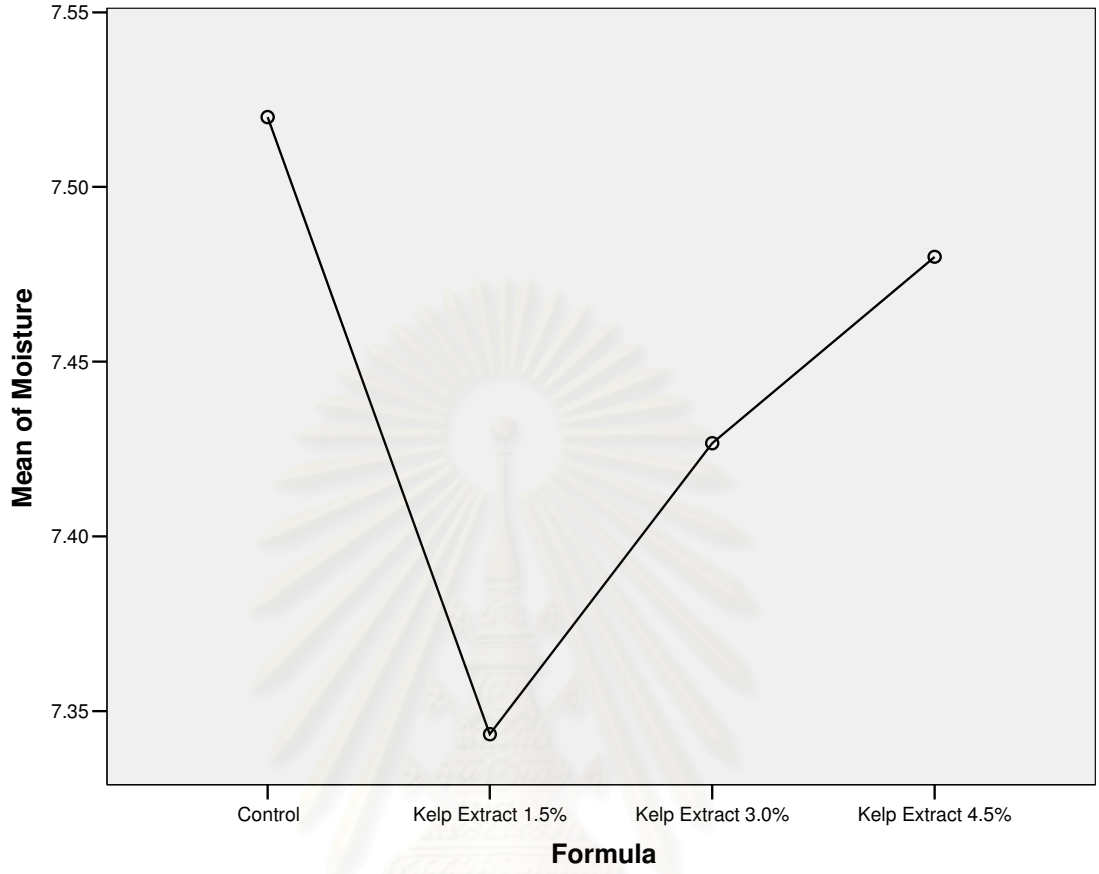

Figure B4 Mean plot of moisture

Table B7 Duncan's New Multiple Range Tests of moisture

Formula	N	Subset for alpha = .05
		1
Kelp Extract 1.5%	3	7.3433
Kelp Extract 3.0%	3	7.4267
Kelp Extract 4.5%	3	7.4800
Control	3	7.5200
Sig.		.271

Means for groups in homogeneous subsets are displayed

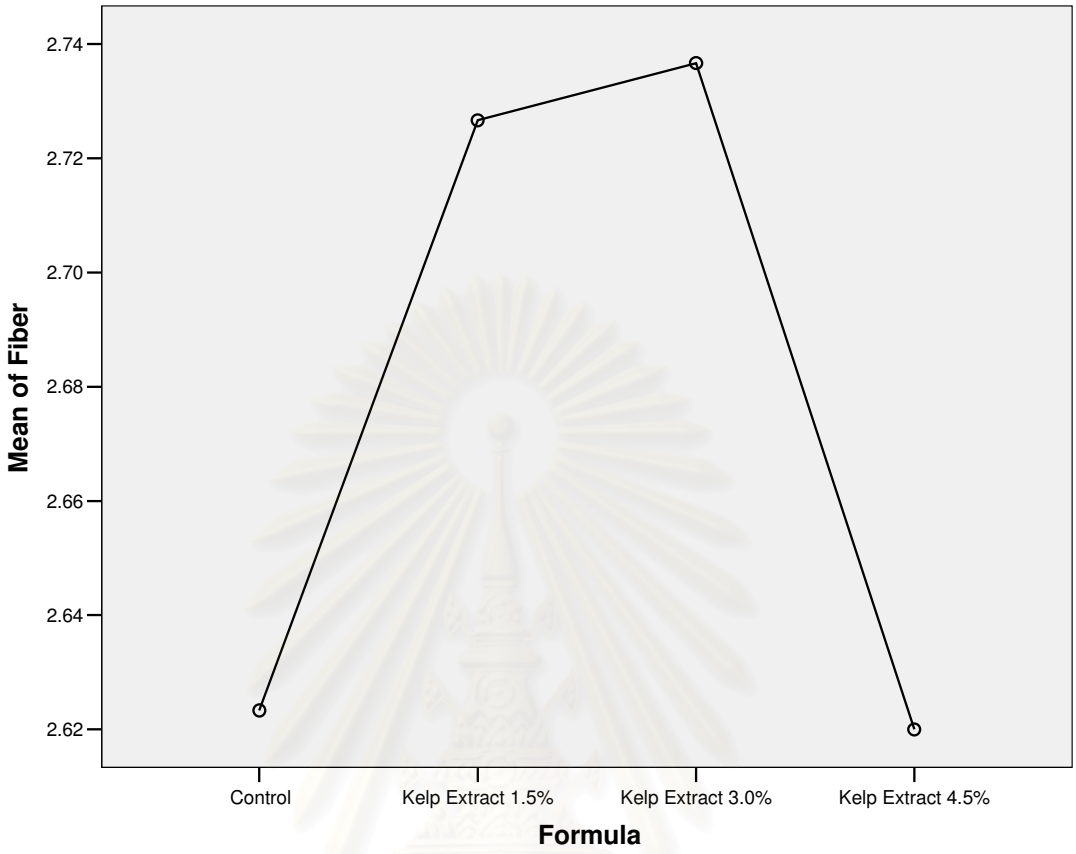

Figure B5 Mean plot of fiber

Table B8 Duncan's New Multiple Range Tests of fiber

Formula	N	Subset for alpha = .05
		1
Kelp Extract 4.5%	3	2.6200
Control	3	2.6233
Kelp Extract 1.5%	3	2.7267
Kelp Extract 3.0%	3	2.7367
Sig.		.461

Means for groups in homogeneous subsets are displayed

Table B9 Descriptive of proximate analysis of diet in growth performance experiment

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Protein	Control	3	44.2967	.03215	.01856	44.2168	44.3765	44.26	44.32
	Kelp 2.5%	3	43.9167	.06658	.03844	43.7513	44.0821	43.84	43.96
	Kelp 5.0%	3	43.7733	.01155	.00667	43.7446	43.8020	43.76	43.78
	Kelp 10.0%	3	43.5533	.03055	.01764	43.4774	43.6292	43.52	43.58
	Fish	9	19.0656	.53320	.17773	18.6557	19.4754	18.32	19.64
	Total	21	33.2481	12.59204	2.74781	27.5163	38.9799	18.32	44.32
Lipid	Control	3	10.4767	.16563	.09563	10.0652	10.8881	10.32	10.65
	Kelp 2.5%	3	10.4833	.08505	.04910	10.2721	10.6946	10.42	10.58
	Kelp 5.0%	3	10.3433	.10693	.06173	10.0777	10.6090	10.25	10.46
	Kelp 10.0%	3	10.3067	.04163	.02404	10.2032	10.4101	10.26	10.34
	Fish	9	12.6344	.56697	.18899	12.1986	13.0703	11.77	13.24
	Total	21	11.3590	1.19082	.25986	10.8170	11.9011	10.25	13.24

Table B9 (cont.) Descriptive of proximate analysis of diet in growth performance experiment

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Ash	Control	3	12.8367	.14295	.08253	12.4816	13.1918	12.68	12.96
	Kelp 2.5%	3	13.5933	.16653	.09615	13.1796	14.0070	13.46	13.78
	Kelp 5.0%	3	15.3667	.04509	.02603	15.2547	15.4787	15.32	15.41
	Kelp 10.0%	3	16.2100	.13229	.07638	15.8814	16.5386	16.06	16.31
	Fish	9	1.3856	.13417	.04472	1.2824	1.4887	1.11	1.51
	Total	21	8.8805	6.73353	1.46938	5.8154	11.9455	1.11	16.31
Moisture	Control	3	7.5200	.01000	.00577	7.4952	7.5448	7.51	7.53
	Kelp 2.5%	3	7.2767	.06429	.03712	7.1170	7.4364	7.23	7.35
	Kelp 5.0%	3	7.2267	.04509	.02603	7.1147	7.3387	7.18	7.27
	Kelp 10.0%	3	7.1800	.02000	.01155	7.1303	7.2297	7.16	7.20
	Fish	9	67.0389	1.64096	.54699	65.7775	68.3002	64.25	69.53
	Total	21	32.9029	30.31068	6.61433	19.1056	46.7001	7.16	69.53
Fiber	Control	3	2.6233	.01528	.00882	2.5854	2.6613	2.61	2.64
	Kelp 2.5%	3	2.7267	.12503	.07219	2.4161	3.0373	2.64	2.87
	Kelp 5.0%	3	2.8367	.05508	.03180	2.6999	2.9735	2.78	2.89
	Kelp 10.0%	3	3.1133	.11930	.06888	2.8170	3.4097	2.98	3.21
	Fish	9	.0100	.00000	.00000	.0100	.0100	.01	.01
	Total	21	1.6186	1.43562	.31328	.9651	2.2721	.01	3.21

Table B10 Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Protein	16.141	4	16	.000
Lipid	8.070	4	16	.001
Ash	.940	4	16	.466
Moisture	5.501	4	16	.006
Fiber	12.327	4	16	.000

Table B11 ANOVA table

		Sum of Squares	df	Mean Square	F	Sig.
Protein	Between Groups	3168.904	4	792.226	5541.279	.000
	Within Groups	2.287	16	.143		
	Total	3171.191	20			
Lipid	Between Groups	25.694	4	6.423	38.532	.000
	Within Groups	2.667	16	.167		
	Total	28.361	20			
Ash	Between Groups	906.530	4	226.632	12977.202	.000
	Within Groups	.279	16	.017		
	Total	906.809	20			
Moisture	Between Groups	18353.188	4	4588.297	3405.767	.000
	Within Groups	21.555	16	1.347		
	Total	18374.743	20			
Fiber	Between Groups	41.154	4	10.288	2484.144	.000
	Within Groups	.066	16	.004		
	Total	41.220	20			

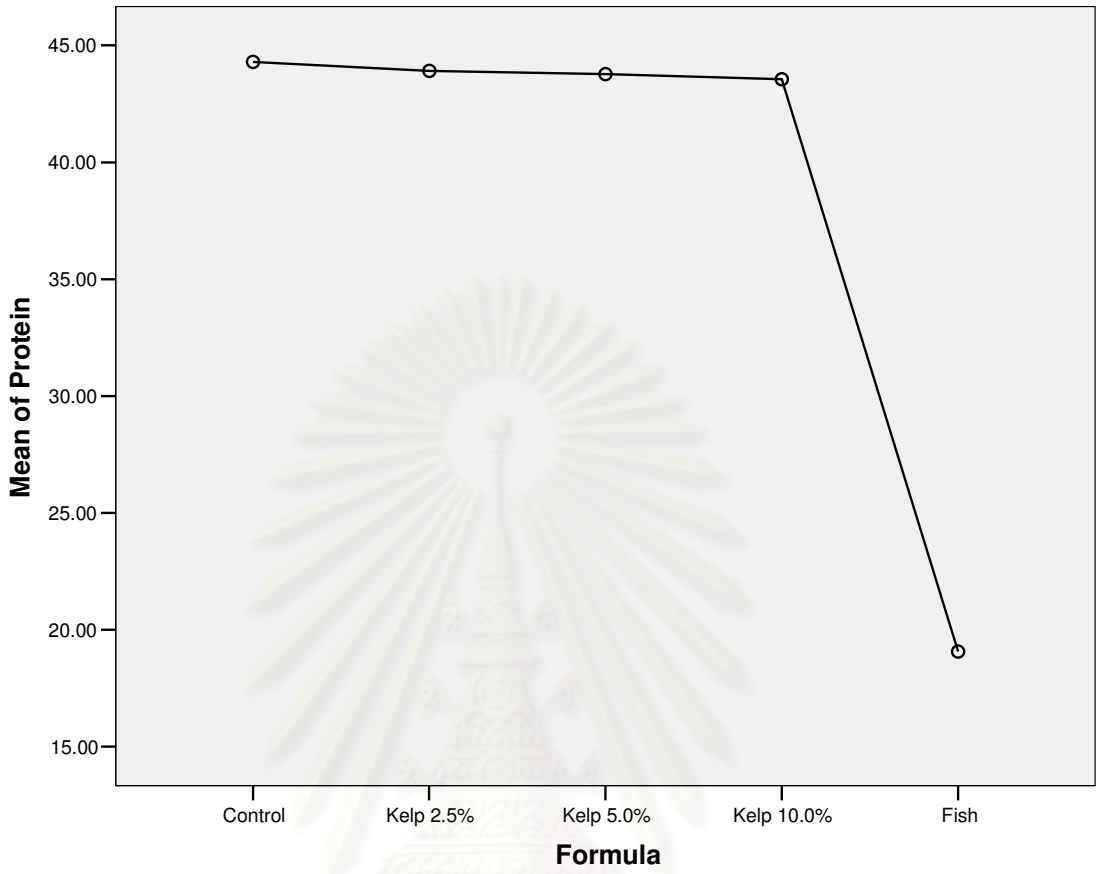

Figure B6 Mean plot of protein

Table B12 Duncan's New Multiple Range Tests of protein

Formula	N	Subset for alpha = .05		
		1	2	3
Fish	9	19.0656		
Kelp 10.0%	3		43.5533	
Kelp 5.0%	3		43.7733	43.7733
Kelp 2.5%	3		43.9167	43.9167
Control	3			44.2967
Sig.		1.000	.248	.103

Means for groups in homogeneous subsets are displayed

.

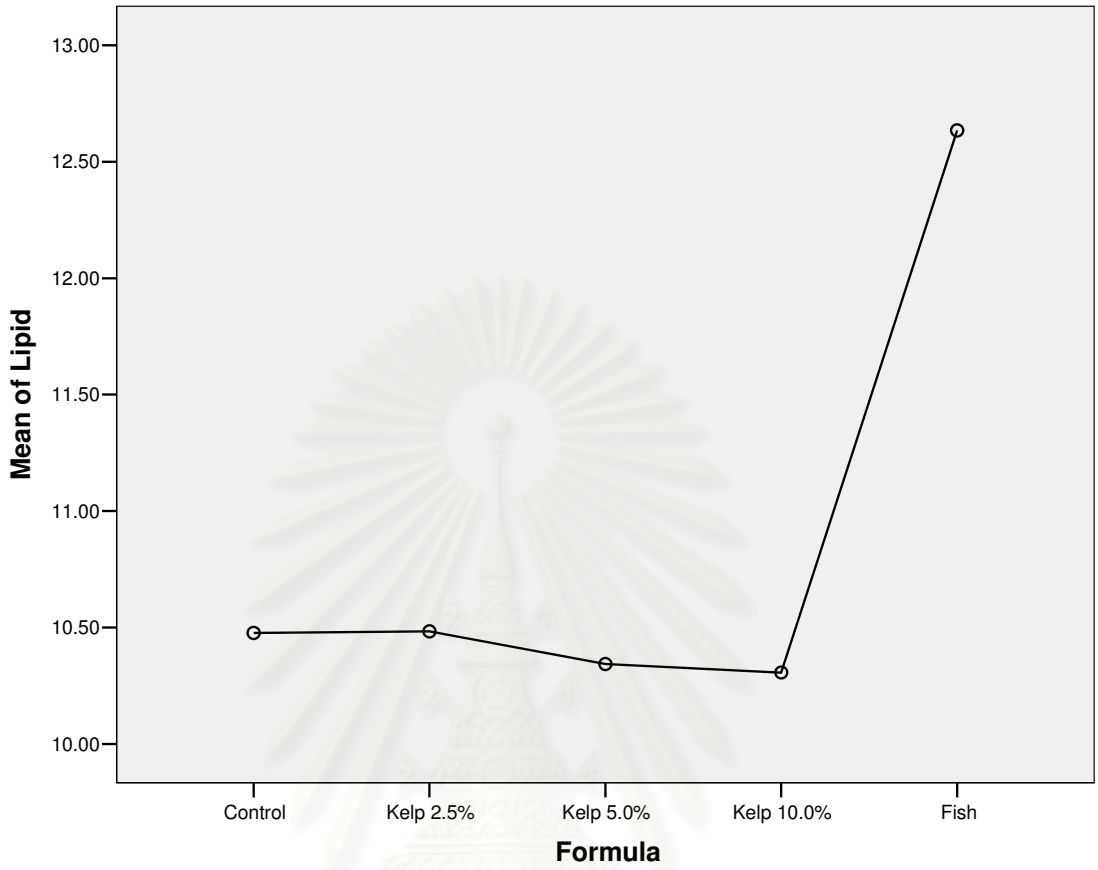

Figure B7 Mean plot of lipid

Table B13 Duncan's New Multiple Range Tests of lipid

Formula	N	Subset for alpha = .05	
		1	2
Kelp 10.0%	3	10.3067	
Kelp 5.0%	3	10.3433	
Control	3	10.4767	
Kelp 2.5%	3	10.4833	
Fish	9		12.6344
Sig.		.608	1.000

Means for groups in homogeneous subsets are displayed

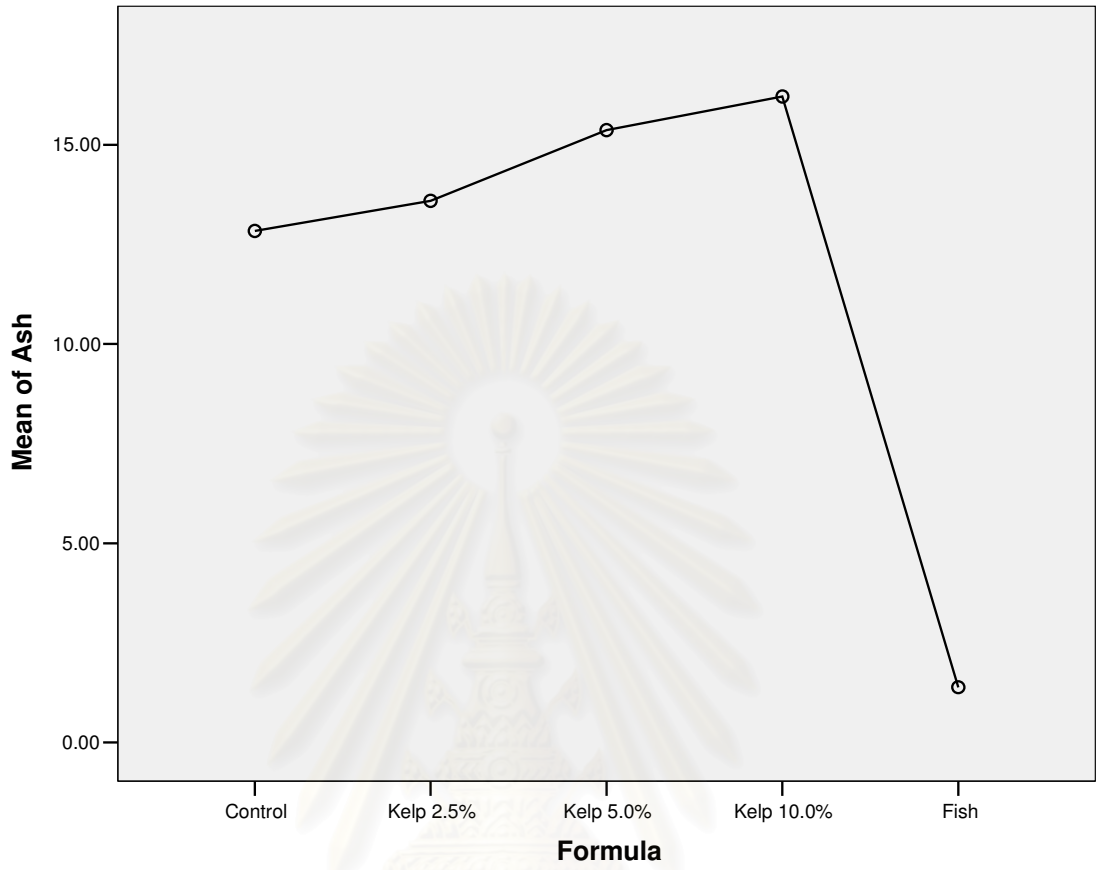

Figure B8 Mean plot of ash

Table B14 Duncan's New Multiple Range Tests of ash

Formula	N	Subset for alpha = .05				
		1	2	3	4	5
Fish	9	1.3856				
Control	3		12.8367			
Kelp 2.5%	3			13.5933		
Kelp 5.0%	3				15.3667	
Kelp 10.0%	3					16.2100
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed

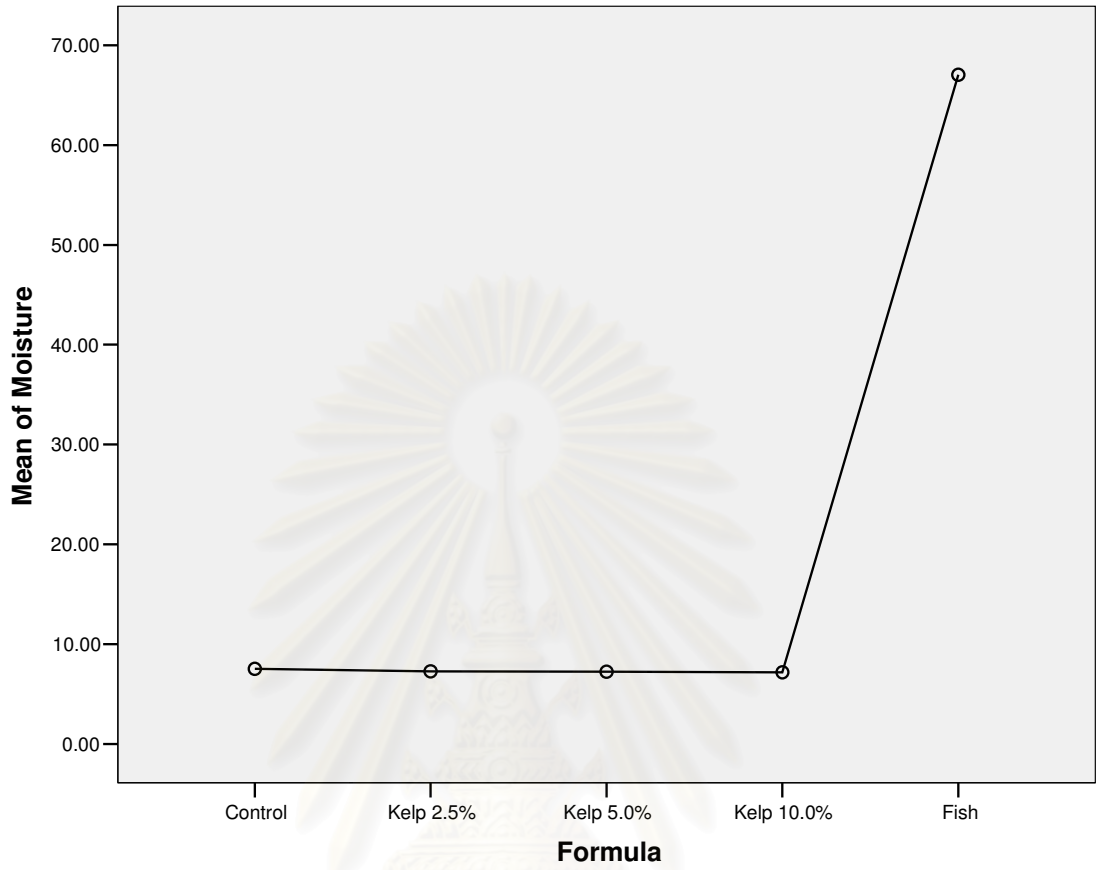

Figure B9 Mean plot of protein

Table B15 Duncan's New Multiple Range Tests of protein

Formula	N	Subset for alpha = .05	
		1	2
Kelp 10.0%	3	7.1800	
Kelp 5.0%	3	7.2267	
Kelp 2.5%	3	7.2767	
Control	3	7.5200	
Fish	9		67.0389
Sig.		.728	1.000

Means for groups in homogeneous subsets are displayed

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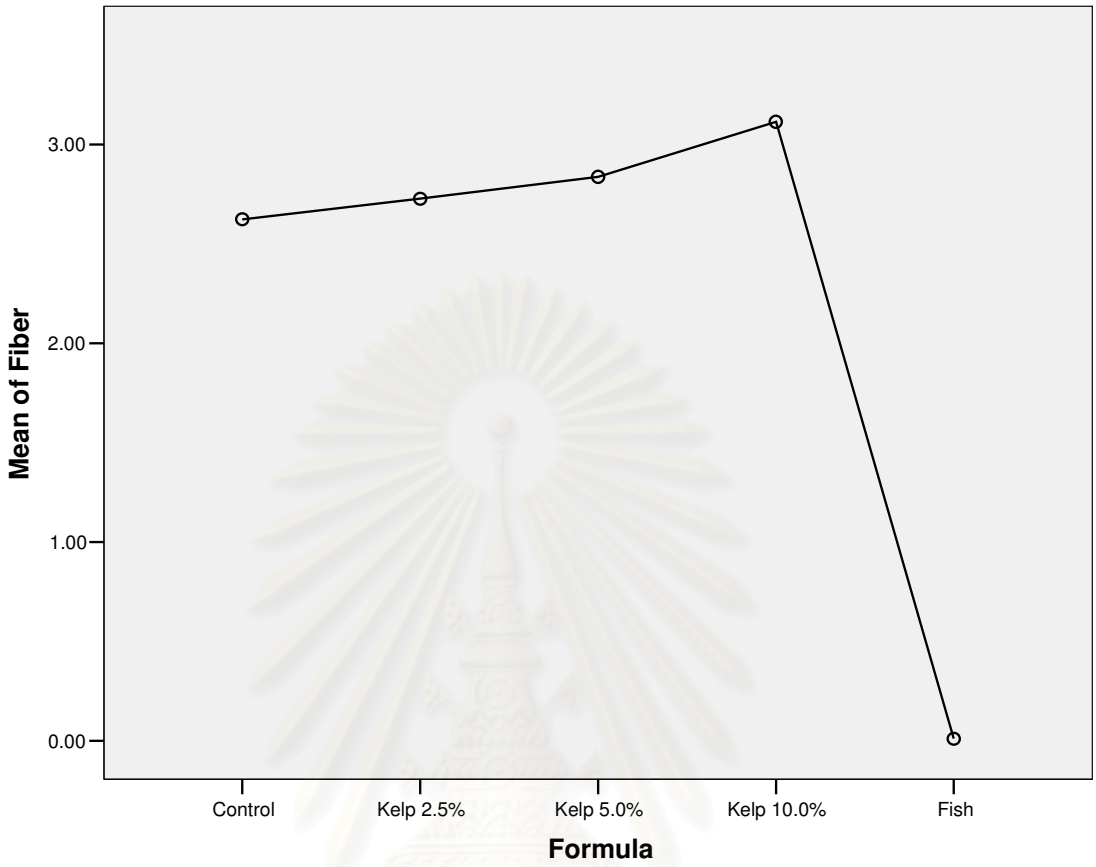

Figure B10 Mean plot of fiber

Table B16 Duncan's New Multiple Range Tests of fiber

Formula	N	Subset for alpha = .05			
		1	2	3	4
Fish	9	.0100			
Control	3		2.6233		
Kelp 2.5%	3		2.7267		
Kelp 5.0%	3			2.8367	
Kelp 10.0%	3				3.1133
Sig.		1.000	.051	1.000	1.000

Means for groups in homogeneous subsets are displayed

.

Appendix C

Vibriosis Resistance Experiment

Table C1 Survival Rate of Vibriosis Infected *B. areolata* Experiment

Day	Survivor of Vibriosis *B. areolata*															
	Kelp Extract 0%				Kelp Extract 1.5%				Kelp Extract 3.0%				Kelp Extract 4.5%			
	Replicate				Replicate				Replicate				Replicate			
	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%
1	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
2	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
3	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
4	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
5	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
6	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
7	10	10	10	100	10	10	10	100	10	10	10	100	10	10	10	100
8	9	10	8	90	10	10	8	93	9	10	9	93	9	9	9	90
9	7	6	6	63	8	8	7	77	8	7	7	73	7	8	7	73
10	5	5	6	53	7	7	6	63	6	6	6	60	6	7	7	67
11	4	4	6	47	6	6	5	53	6	6	6	60	7	6	6	63
12	3	3	5	37	6	5	5	53	6	6	6	60	6	5	6	57

Table C1 (Cont.) Survival Rate of Vibriosis *B. areolata* Experiment

Day	Survivor of Vibriosis *B. areolata*															
	Kelp Extract 0%				Kelp Extract 1.5%				Kelp Extract 3.0%				Kelp Extract 4.5%			
	Replicate				Replicate				Replicate				Replicate			
	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%
13	2	2	4	27	6	5	5	57	5	5	6	53	5	5	5	50
14	1	1	3	17	5	4	5	47	4	4	5	43	5	5	4	47
15	0	1	2	13	4	3	4	37	3	3	4	33	4	4	3	37
16	0	1	1	7	4	3	3	33	2	2	3	23	3	3	2	27
17	0	1	1	7	3	2	2	23	2	2	3	23	2	3	2	23
18	0	0	1	3	2	2	2	20	2	2	3	23	2	3	2	23
19	0	0	1	3	2	2	2	20	2	2	3	23	2	2	2	20
20	0	0	1	3	2	2	2	20	1	2	3	20	2	2	2	20
21	0	0	0	0	2	2	2	20	1	2	3	20	2	2	2	20
22	0	0	0	0	1	2	2	17	1	2	3	20	1	2	2	17
23	0	0	0	0	1	2	2	17	1	2	3	20	1	2	2	17
24	0	0	0	0	1	2	2	17	1	2	3	20	1	2	2	17

Table C1 (Cont.) Survival Rate of Vibriosis *B. areolata* Experiment

Day	Survivor of Vibriosis *B. areolata*															
	Kelp Extract 0%				Kelp Extract 1.5%				Kelp Extract 3.0%				Kelp Extract 4.5%			
	Replicate				Replicate				Replicate				Replicate			
	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%	n_1	n_2	n_3	%
25	0	0	0	0	1	2	2	17	1	2	3	20	1	2	2	17
26	0	0	0	0	1	2	2	17	1	2	3	20	1	2	2	17
27	0	0	0	0	1	2	2	17	1	2	2	17	1	2	2	17
28	0	0	0	0	1	2	2	17	1	2	2	17	1	1	2	13
29	0	0	0	0	1	2	2	17	1	2	2	17	1	1	2	13
30	0	0	0	0	1	2	2	17	1	2	1	13	1	1	2	13
31	0	0	0	0	1	2	2	17	1	2	2	17	1	1	2	13
32	0	0	0	0	1	2	2	17	1	2	2	17	1	1	2	13
33	0	0	0	0	1	2	2	17	1	2	2	17	1	1	2	13
34	0	0	0	0	1	2	2	17	1	2	1	13	1	1	2	13
35	0	0	0	0	1	2	2	17	1	2	1	13	1	1	2	13
36	0	0	0	0	1	2	2	17	1	2	1	13	1	1	2	13

Table C2 Descriptive of survival rate of Vibriosis *B. areolata* Experiment at the 14th Day

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Kelp Extract 0%	3	16.6667	11.54701	6.66667	-12.0177	45.3510	10.00	30.00
Kelp Extract 1.5%	3	46.6667	5.77350	3.33333	32.3245	61.0088	40.00	50.00
Kelp Extract 3.0%	3	43.3333	5.77350	3.33333	28.9912	57.6755	40.00	50.00
Kelp Extract 4.5%	3	46.6667	5.77350	3.33333	32.3245	61.0088	40.00	50.00
Total	12	38.3333	14.66804	4.23430	29.0137	47.6530	10.00	50.00

Table B3 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.286	3	8	.156

Table C4 ANOVA Table

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1900.000	3	633.333	10.857	.003
Within Groups	466.667	8	58.333		
Total	2366.667	11			

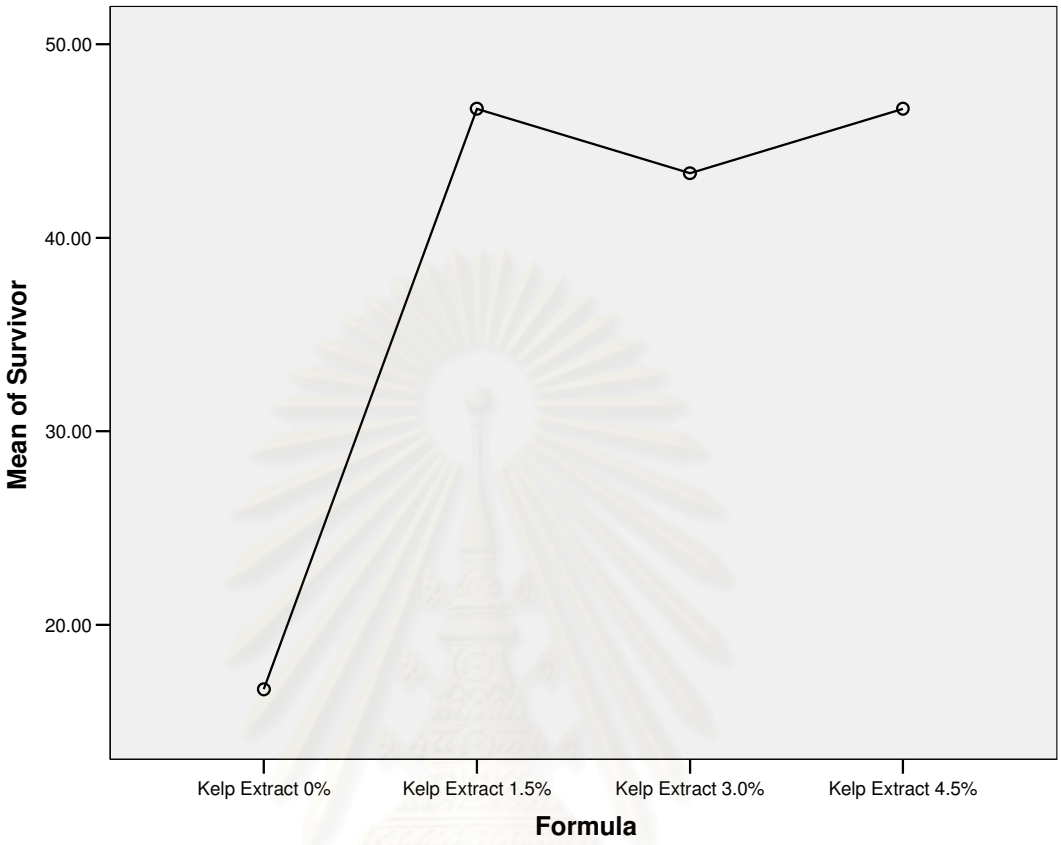

Figure C1 Mean Plot of Survivor

Table C5 Duncan's New Multiple Range Tests of Survivor

Formula	N	Subset for alpha = .05	
		1	2
Kelp Extract 0%	3	16.6667	
Kelp Extract 3.0%	3		43.3333
Kelp Extract 1.5%	3		46.6667
Kelp Extract 4.5%	3		46.6667
Sig.		1.000	.622

Means for groups in homogeneous subsets are displayed

Appendix D

Table D1 Comparing on Total Shell Length of *B. areolata*

Initial length (mm)	Final length (mm)			
	kelp 2.5%	kelp 5%	kelp 10%	Fish Meat
18.1	20.5	21.3	21.2	20.9
18.1	20.6	21.3	21.3	20.9
18.1	20.7	21.4	21.2	21.0
18.1	20.7	21.3	21.2	20.9
18.1	20.7	21.4	21.2	21.1
18.1	20.8	21.3	21.2	20.9
18.1	20.8	21.4	21.3	21.1
18.1	20.7	21.4	21.2	21.4
18.1	20.9	21.3	21.3	21.5
18.1	20.9	21.2	21.4	21.1
18.1	21.0	21.2	21.3	21.2
18.1	21.0	21.1	21.2	21.1
18.1	21.0	21.1	21.1	21.1
18.1	21.2	21.2	21.2	21.0
18.1	21.2	21.2	21.4	21.2
18.1	21.3	21.2	20.6	21.1
18.1	21.3	21.3	21.2	20.9
18.1	21.4	21.5	21.1	21.0
18.1	21.2	21.5	21.0	21.0
18.1	21.3	21.4	21.1	21.0
18.1	21.2	21.2	20.6	21.2
18.1	21.2	21.1	20.8	21.1
18.1	21.4	21.3	21.2	21.1
18.1	21.5	20.5	21.2	21.2
18.1	21.4	21.2	21.2	21.2
18.1	21.4	21.3	21.2	21.2
18.1	21.4	21.1	21.2	21.2
18.1	21.5	21.1	21.4	21.0
18.1	22.0	20.8	21.4	21.5
		21.4		21.0

TableD2 Comparing on Wet Body Weigh of *B. areolata*

Initial weight (g)	Final weight (g)			
	kelp 2.5%	kelp 5%	kelp 10%	Fish Meat
1.18	2.12	2.18	2.14	2.08
1.18	2.12	2.18	2.15	2.10
1.18	2.13	2.19	2.14	2.10
1.18	2.14	2.17	2.15	2.10
1.18	2.13	2.18	2.14	2.10
1.18	2.14	2.16	2.14	2.08
1.18	2.14	2.19	2.14	2.08
1.18	2.14	2.18	2.14	2.12
1.18	2.15	2.18	2.15	2.13
1.18	2.15	2.16	2.16	2.1.0
1.18	2.15	2.17	2.15	2.11
1.18	2.16	2.16	2.15	2.10
1.18	2.16	2.16	2.14	2.10
1.18	2.17	2.17	2.15	2.10
1.18	2.18	2.17	2.16	2.12
1.18	2.19	2.16	2.09	2.10
1.18	2.19	2.18	2.15	2.08
1.18	2.20	2.19	2.14	2.09
1.18	2.20	2.20	2.13	2.09
1.18	2.20	2.18	2.14	2.10
1.18	2.19	2.16	2.10	2.11
1.18	2.19	2.15	2.10	2.11
1.18	2.18	2.16	2.15	2.12
1.18	2.20	2.12	2.15	2.12
1.18	2.20	2.15	2.15	2.10
1.18	2.20	2.18	2.15	2.10
1.18	2.19	2.14	2.15	2.10
1.18	2.21	2.14	2.16	2.10
1.18	2.25	2.13	2.15	2.13
		2.18		2.09

Appendix E

Statistical Analyses

Table E1 Descriptive on Final Total Shell Length and Wet Body Weight of *B. areolata*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Length	Control	29	21.1103	.34262	.06362	20.9800	21.2407	20.50	22.00
	Kelp 2.5%	30	21.2333	.20057	.03662	21.1584	21.3082	20.50	21.50
	Kelp 5%	29	21.1690	.20018	.03717	21.0928	21.2451	20.60	21.40
	Kelp 10%	30	21.1033	.16078	.02935	21.0433	21.1634	20.90	21.50
	Fish	30	22.6067	.13880	.02534	22.5548	22.6585	22.40	22.90
	Total	148	21.4486	.62635	.05149	21.3469	21.5504	20.50	22.90
Weight	Control	29	2.1714	.03204	.00595	2.1592	2.1836	2.12	2.25
	Kelp 2.5%	30	2.1673	.01874	.00342	2.1603	2.1743	2.12	2.20
	Kelp 5%	29	2.1417	.01713	.00318	2.1352	2.1482	2.09	2.16
	Kelp 10%	30	2.1020	.01375	.00251	2.0969	2.1071	2.08	2.13
	Fish	30	2.9843	.01223	.00223	2.9798	2.9889	2.96	3.00
	Total	148	2.3155	.33988	.02794	2.2603	2.3707	2.08	3.00

Table D2 Descriptive on Relative Growth Rate on Length and Weight of *B. areolata*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
RGRL	Control	29	.5552	.06367	.01182	.5310	.5794	.44	.72
	Kelp 2.5%	30	.5773	.03814	.00696	.5631	.5916	.44	.63
	Kelp 5%	29	.5648	.03738	.00694	.5506	.5790	.46	.61
	Kelp 10%	30	.5523	.03014	.00550	.5411	.5636	.52	.63
	Fish	30	.8310	.02708	.00494	.8209	.8411	.79	.88
	Total	148	.6169	.11602	.00954	.5980	.6357	.44	.88
RGRW	Control	29	2.7993	.08968	.01665	2.7652	2.8334	2.66	3.02
	Kelp 2.5%	30	2.7880	.05149	.00940	2.7688	2.8072	2.66	2.88
	Kelp 5%	29	2.7162	.04858	.00902	2.6977	2.7347	2.57	2.77
	Kelp 10%	30	2.6053	.03989	.00728	2.5904	2.6202	2.54	2.68
	Fish	30	5.0950	.03381	.00617	5.0824	5.1076	5.03	5.14
	Total	148	3.2068	.95944	.07887	3.0509	3.3626	2.54	5.14

Table D3 Descriptive on Specific Growth Rate on Length and Weight of *B. areolata*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
SGRL	Control	29	.5134	.05334	.00990	.4932	.5337	.42	.65
	Kelp 2.5%	30	.5320	.03123	.00570	.5203	.5437	.42	.57
	Kelp 5%	29	.5231	.03253	.00604	.5107	.5355	.43	.56
	Kelp 10%	30	.5130	.02452	.00448	.5038	.5222	.48	.57
	Fish	30	.7417	.01877	.00343	.7347	.7487	.71	.78
	Total	148	.5653	.09559	.00786	.5497	.5808	.42	.78
SGRW	Control	29	2.0328	.05035	.00935	2.0136	2.0519	1.95	2.15
	Kelp 2.5%	30	2.0283	.03007	.00549	2.0171	2.0396	1.95	2.08
	Kelp 5%	29	1.9862	.02744	.00510	1.9758	1.9966	1.91	2.02
	Kelp 10%	30	1.9243	.02128	.00389	1.9164	1.9323	1.89	1.97
	Fish	30	3.0943	.01223	.00223	3.0898	3.0989	3.07	3.11
	Total	148	2.2159	.44716	.03676	2.1433	2.2886	1.89	3.11

Table D4 Descriptive on Feed Conversion Ratio and Wet Gain of *B. areolata*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
FCR	Control	29	2.0272	.06408	.01190	2.0029	2.0516	1.87	2.13
	Kelp 2.5%	30	2.0643	.03884	.00709	2.0498	2.0788	2.00	2.16
	Kelp 5%	29	2.0883	.03846	.00714	2.0736	2.1029	2.05	2.21
	Kelp 10%	30	2.1783	.03075	.00561	2.1669	2.1898	2.12	2.23
	Fish	30	3.8620	.02592	.00473	3.8523	3.8717	3.83	3.92
	Total	148	2.4493	.71767	.05899	2.3327	2.5658	1.87	3.92
WG	Control	29	1.8402E2	2.71485	.50414	182.9825	185.0478	179.66	190.68
	Kelp 2.5%	30	1.8367E2	1.58866	.29005	183.0801	184.2665	179.66	186.44
	Kelp 5%	29	1.8150E2	1.44963	.26919	180.9507	182.0535	177.12	183.05
	Kelp 10%	30	1.7814E2	1.16464	.21263	177.7021	178.5719	176.27	180.51
	Fish	30	2.5291E2	1.03807	.18952	252.5214	253.2966	250.85	254.24
	Total	148	1.9623E2	28.80291	2.36758	191.5480	200.9058	176.27	254.24

Table D5 Descriptive on Shell Length Increase and Condition index of *B. areolata*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
SLI	Control	29	16.6310	1.89346	.35161	15.9108	17.3513	13.26	21.55
	Kelp 2.5%	30	17.3107	1.10757	.20221	16.8971	17.7242	13.26	18.78
	Kelp 5%	29	16.9562	1.10641	.20545	16.5354	17.3771	13.81	18.23
	Kelp 10%	30	16.5917	.88809	.16214	16.2600	16.9233	15.47	18.78
	Fish	30	24.8987	.76651	.13995	24.6124	25.1849	23.76	26.52
	Total	148	18.5004	3.46070	.28447	17.9382	19.0626	13.26	26.52
CI	Control	29	.023100	.0007935	.0001473	.022799	.023402	.0211	.0246
	Kelp 2.5%	30	.022647	.0004912	.0000897	.022463	.022830	.0220	.0246
	Kelp 5%	29	.022584	.0004853	.0000901	.022400	.022769	.0219	.0240
	Kelp 10%	30	.022370	.0004012	.0000732	.022221	.022520	.0214	.0230
	Fish	30	.025835	.0003956	.0000722	.025687	.025983	.0250	.0265
	Total	148	.023314	.0013995	.0001150	.023086	.023541	.0211	.0265

Table D6 Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
WG	12.403	4	143	.000
FCR	9.845	4	143	.000
Length	8.181	4	143	.000
Weight	12.401	4	143	.000
RGRL	7.691	4	143	.000
RGRW	11.627	4	143	.000
SGRL	8.881	4	143	.000
SGRW	15.400	4	143	.000
SLI	8.198	4	143	.000
CD	5.547	4	143	.000

Table D7 ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
Length	Between Groups	50.786	4	12.696	263.742	.000
	Within Groups	6.884	143	.048		
	Total	57.670	147			
Weight	Between Groups	16.924	4	4.231	1.062E4	.000
	Within Groups	.057	143	.000		
	Total	16.981	147			
RGRL	Between Groups	1.736	4	.434	256.036	.000
	Within Groups	.242	143	.002		
	Total	1.979	147			

Table D7 (Cont.) ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
RGRW	Between Groups	134.869	4	33.717	1.078E4	.000
	Within Groups	.447	143	.003		
	Total	135.316	147			
SGRL	Between Groups	1.178	4	.295	254.939	.000
	Within Groups	.165	143	.001		
	Total	1.343	147			
SGRW	Between Groups	29.258	4	7.314	7.705E3	.000
	Within Groups	.136	143	.001		
	Total	29.394	147			
FCR	Between Groups	75.466	4	18.866	1.092E4	.000
	Within Groups	.247	143	.002		
	Total	75.713	147			
WG	Between Groups	121543.371	4	30385.843	1.062E4	.000
	Within Groups	408.988	143	2.860		
	Total	121952.359	147			
SLI	Between Groups	1550.389	4	387.597	263.750	.000
	Within Groups	210.147	143	1.470		
	Total	1760.536	147			
CD	Between Groups	.000	4	.000	218.873	.000
	Within Groups	.000	143	.000		
	Total	.000	147			

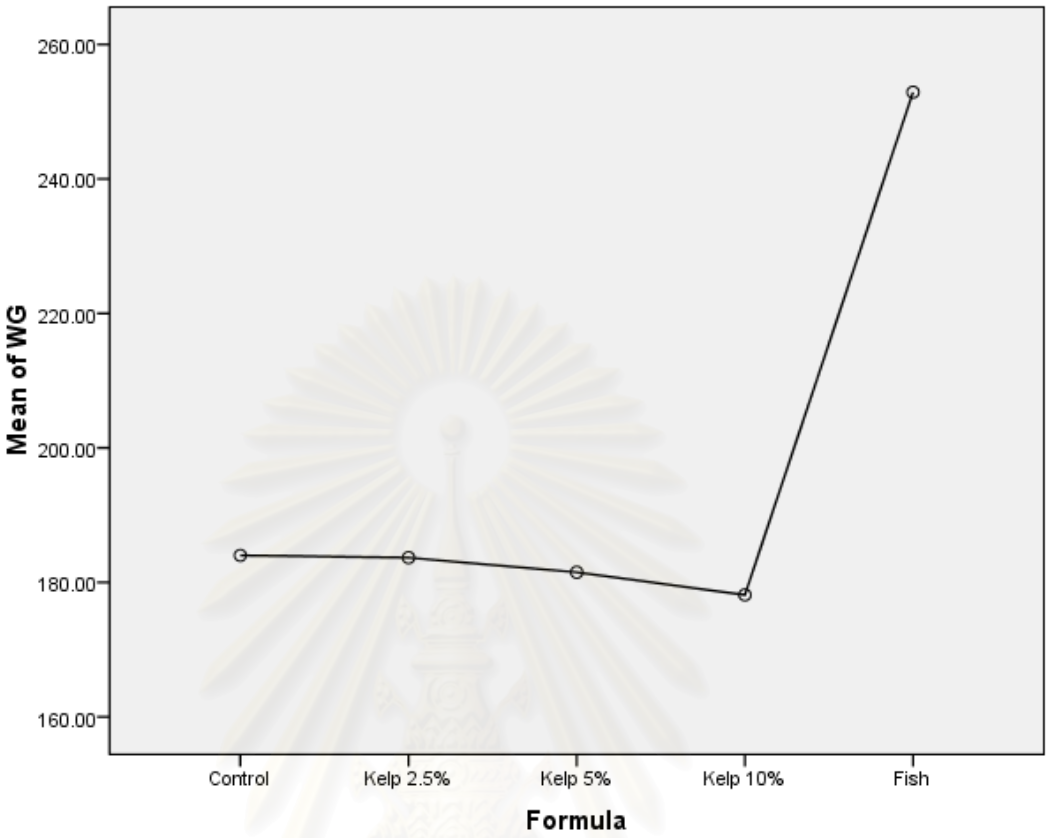

Figure D1 Mean plot of weigh gain

Table D8 Duncan's New Multiple Range Tests of weigh gain

Formula	N	Subset for alpha = 0.05			
		1	2	3	4
Kelp 10%	30	1.7814E2			
Kelp 5%	29		1.8150E2		
Kelp 2.5%	30			1.8367E2	
Control	29			1.8402E2	
Fish	30				2.5291E2
Sig.		1.000	1.000	.438	1.000

Means for groups in homogeneous subsets are displayed.

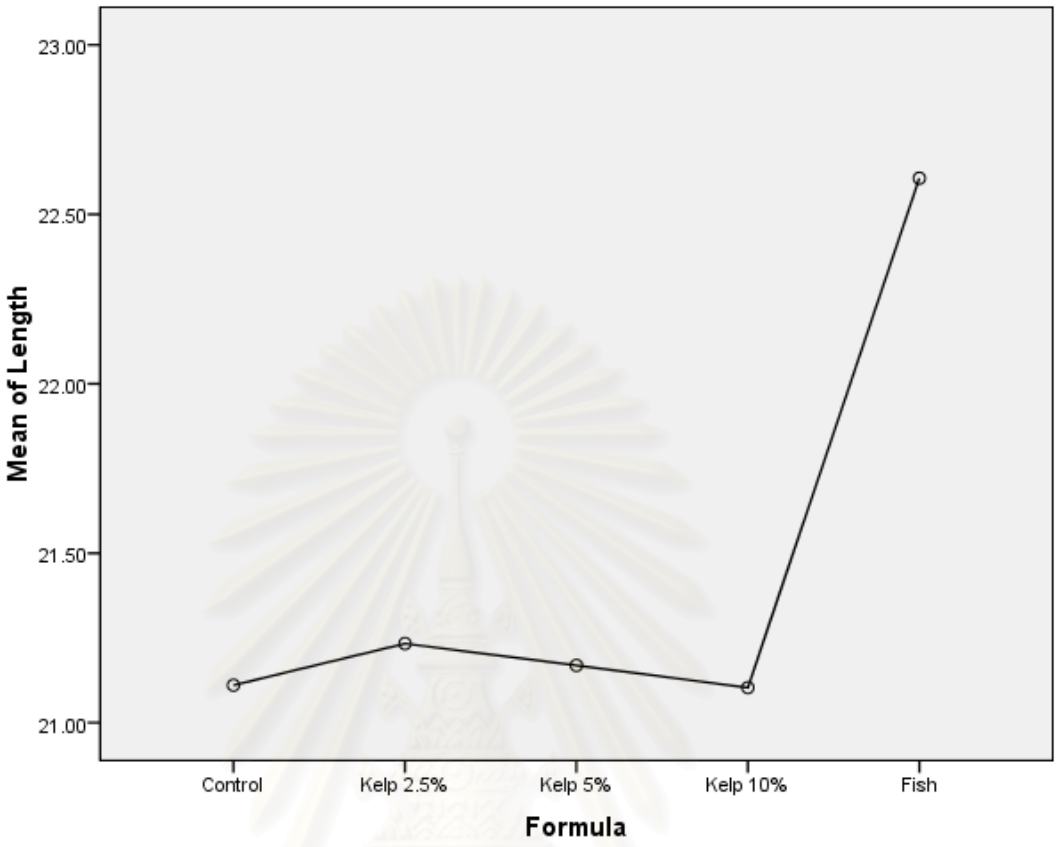

Figure D2 Mean plot of length

Table D9 Duncan's New Multiple Range Tests of length

Formula	N	Subset for alpha = 0.05		
		1	2	3
Kelp 10%	30	21.1033		
Control	29	21.1103		
Kelp 5%	29	21.1690	21.1690	
Kelp 2.5%	30		21.2333	
Fish	30			22.6067
Sig.		.282	.261	1.000

Means for groups in homogeneous subsets are displayed.

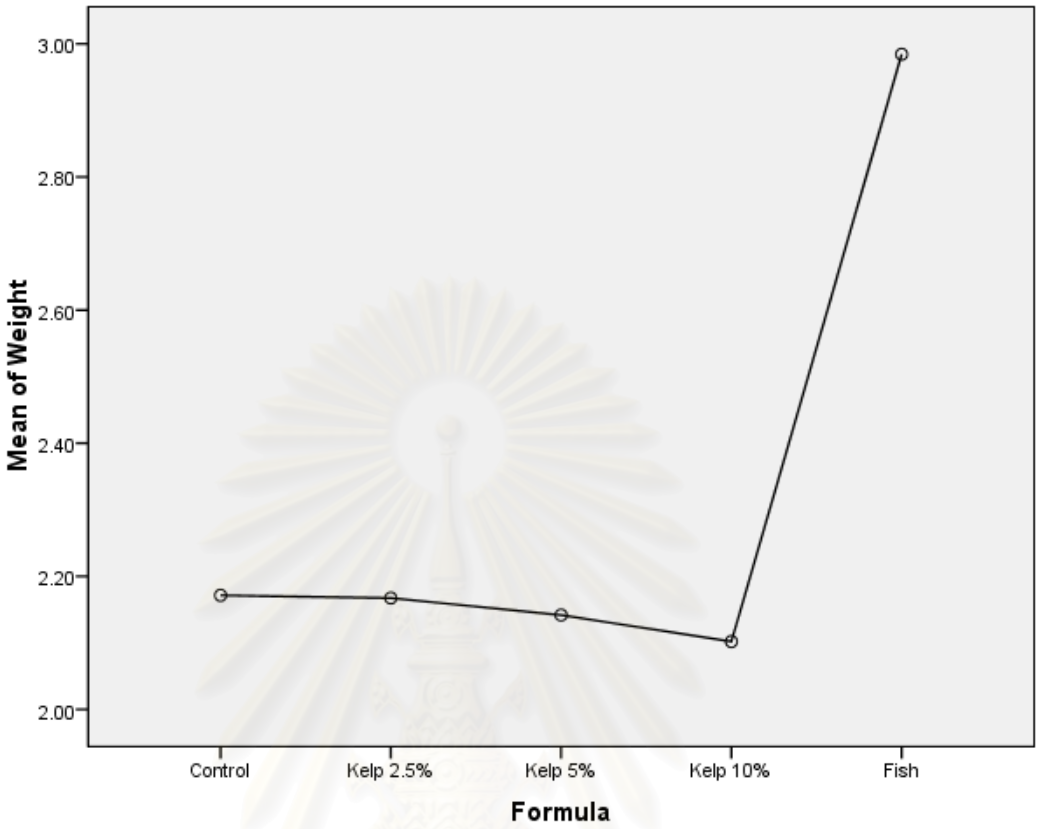

Figure D3 Mean plot of weight

Table D10 Duncan's New Multiple Range Tests of weight

Formula	N	Subset for alpha = 0.05			
		1	2	3	4
Kelp 10%	30	2.1020			
Kelp 5%	29		2.1417		
Kelp 2.5%	30			2.1673	
Control	29			2.1714	
Fish	30				2.9843
Sig.		1.000	1.000	.437	1.000

Means for groups in homogeneous subsets are displayed.

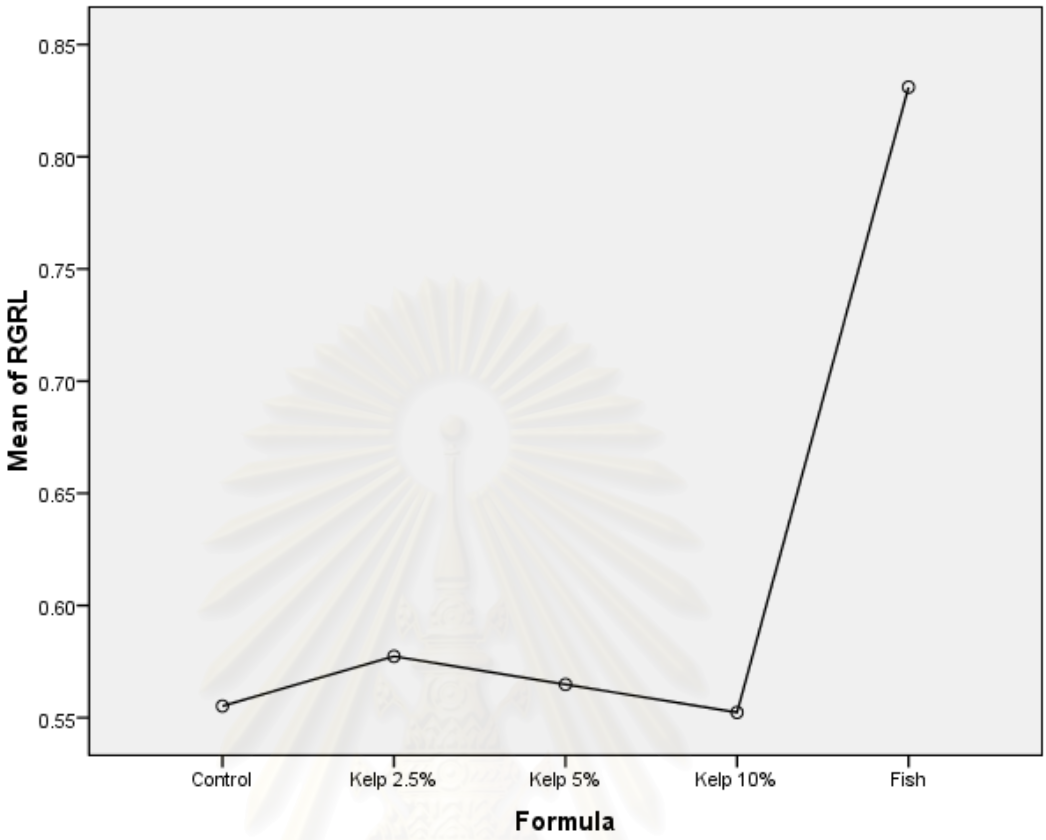

Figure D4 Mean plot of relative growth rate in length

Table D11 Duncan's New Multiple Range Tests of relative growth rate in length

Formula	N	Subset for alpha = 0.05		
		1	2	3
Kelp 10%	30	.5523		
Control	29	.5552	.5552	
Kelp 5%	29	.5648	.5648	
Kelp 2.5%	30		.5773	
Fish	30			.8310
Sig.		.275	.051	1.000

Means for groups in homogeneous subsets are displayed.

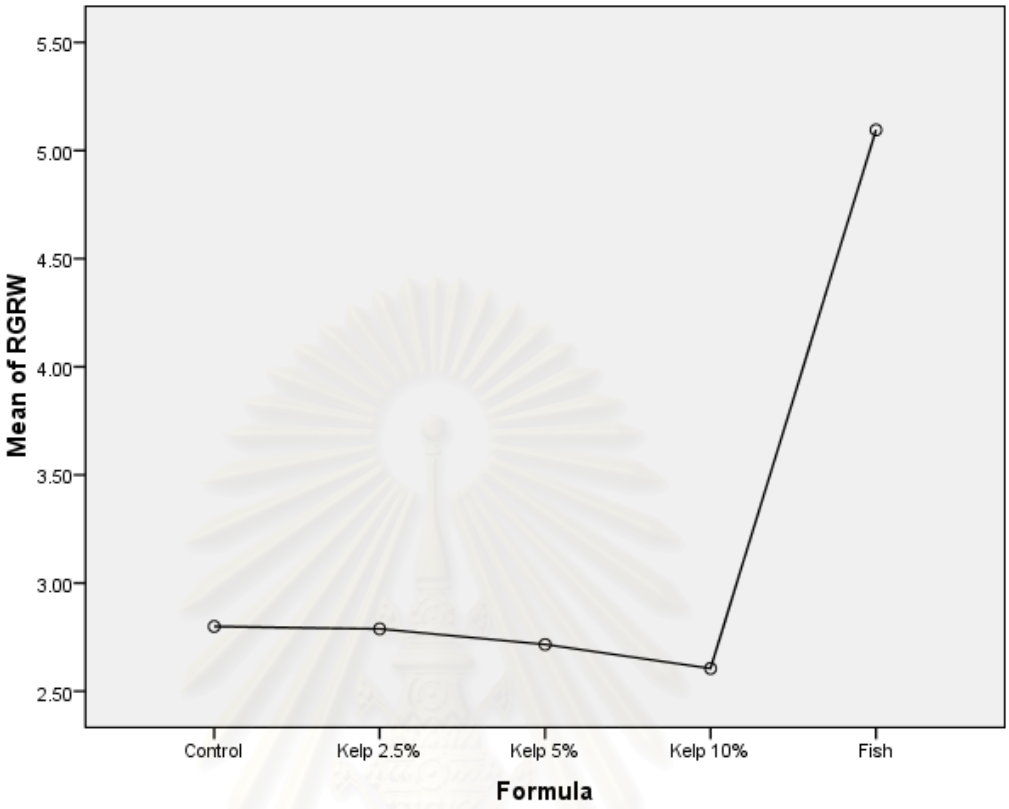

Figure D5 Mean plot of relative growth rate in weight

Table D12 Duncan's New Multiple Range Tests of relative growth rate in weight

Formula	N	Subset for alpha = 0.05			
		1	2	3	4
Kelp 10%	30	2.6053			
Kelp 5%	29		2.7162		
Kelp 2.5%	30			2.7880	
Control	29			2.7993	
Fish	30				5.0950
Sig.		1.000	1.000	.438	1.000

Means for groups in homogeneous subsets are displayed.

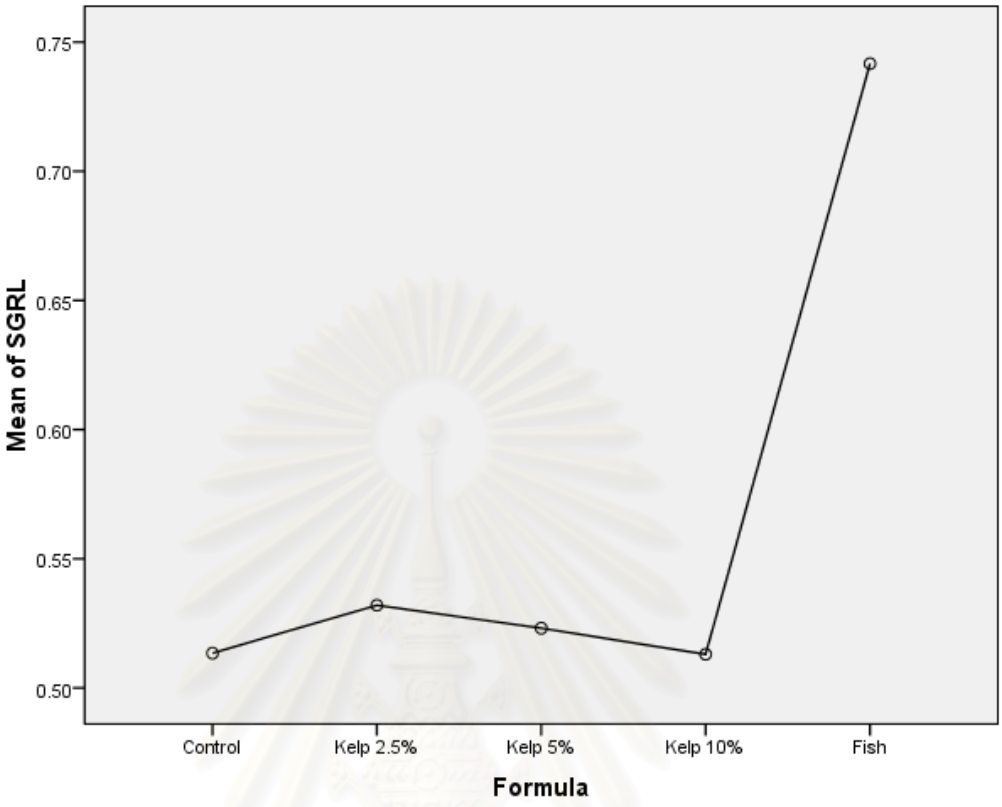

Figure D6 Mean plot of specific growth rate in length

Table D13 Duncan's New Multiple Range Tests of specific growth rate in length

Formula	N	Subset for alpha = 0.05		
		1	2	3
Kelp 10%	30	.5130		
Control	29	.5134		
Kelp 5%	29	.5231	.5231	
Kelp 2.5%	30		.5320	
Fish	30			.7417
Sig.		.285	.316	1.000

Means for groups in homogeneous subsets are displayed.

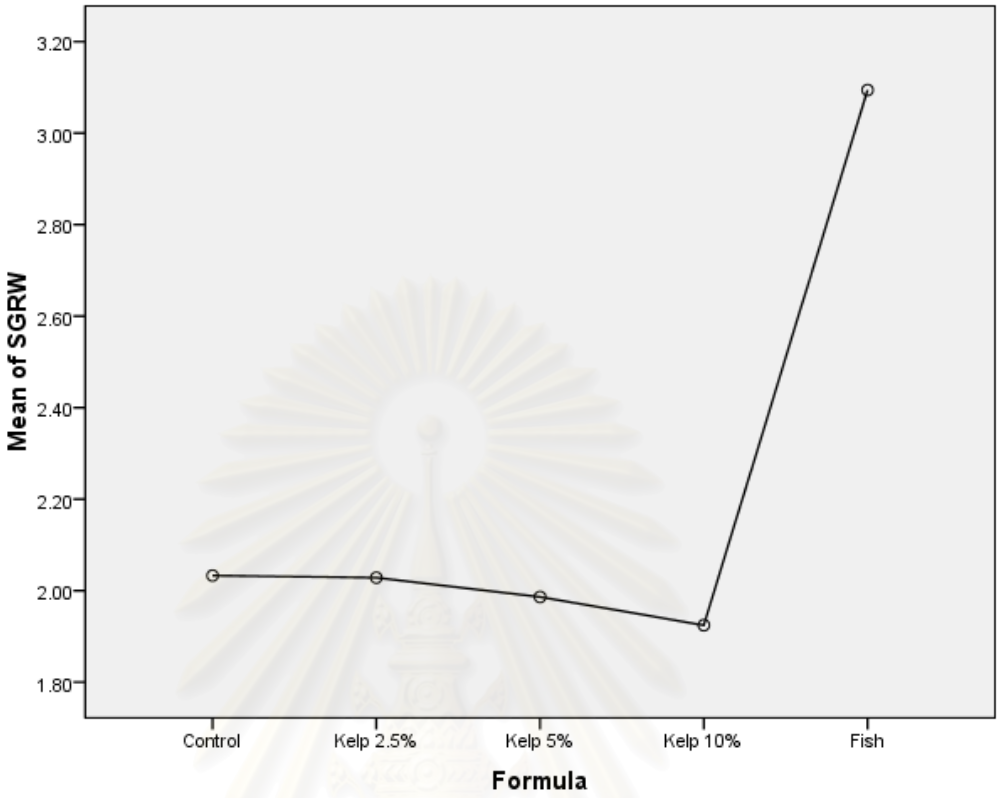

Figure D7 Mean plot of specific growth rate in weight

Table D14 Duncan's New Multiple Range Tests of specific growth rate in weight

Formula	N	Subset for alpha = 0.05			
		1	2	3	4
Kelp 10%	30	1.9243			
Kelp 5%	29		1.9862		
Kelp 2.5%	30			2.0283	
Control	29			2.0328	
Fish	30				3.0943
Sig.		1.000	1.000	.581	1.000

Means for groups in homogeneous subsets are displayed.

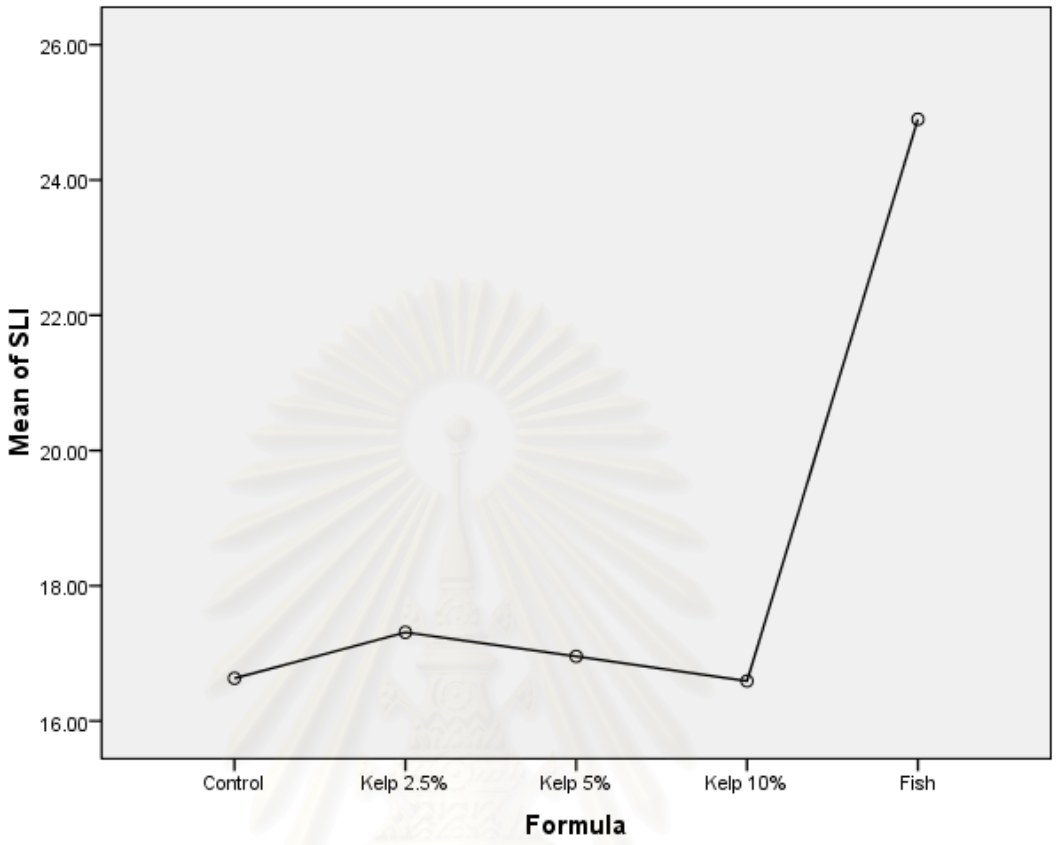

Figure D8 Mean plot of shell length increase

Table D15 Duncan's New Multiple Range Tests of shell length increase

Formula	N	Subset for alpha = 0.05		
		1	2	3
Kelp 10%	30	16.5917		
Control	29	16.6310		
Kelp 5%	29	16.9562	16.9562	
Kelp 2.5%	30		17.3107	
Fish	30			24.8987
Sig.		.280	.263	1.000

Means for groups in homogeneous subsets are displayed.

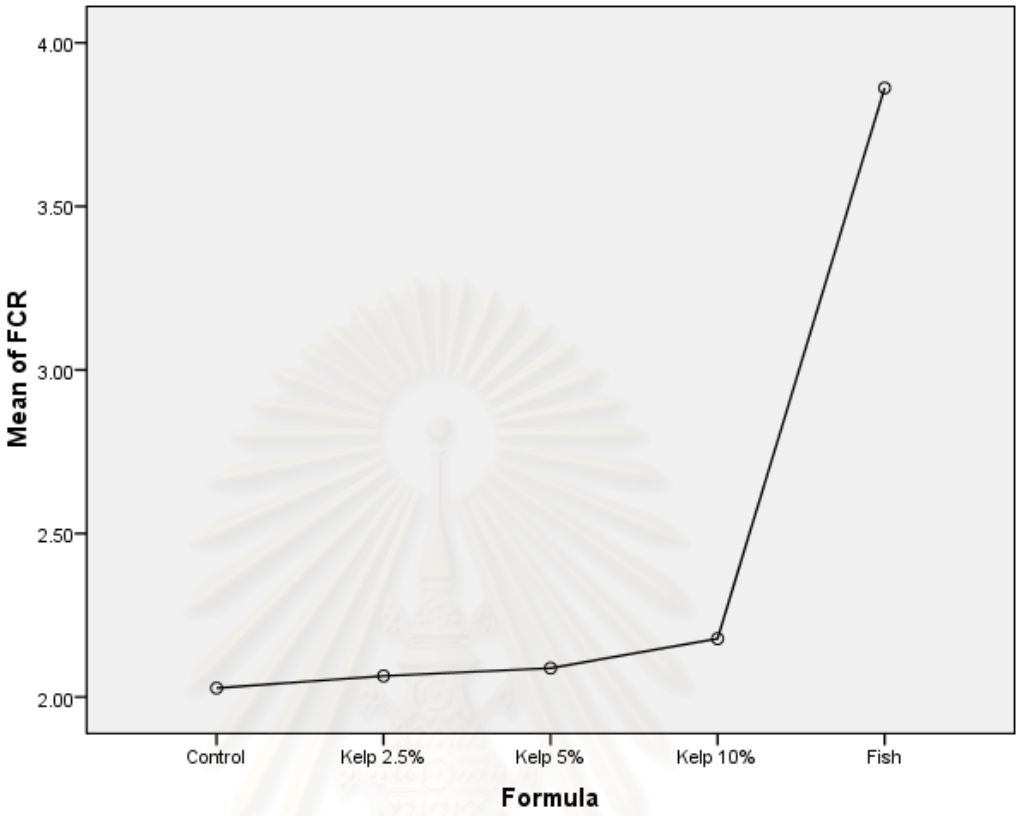

Figure D9 Mean plot of feed conversion ratio

Table D16 Duncan's New Multiple Range Tests of feed conversion ratio

		Subset for alpha = 0.05				
Formula	N	1	2	3	4	5
Control	29	2.0272				
Kelp 2.5%	30		2.0643			
Kelp 5%	29			2.0883		
Kelp 10%	30				2.1783	
Fish	30					3.8620
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

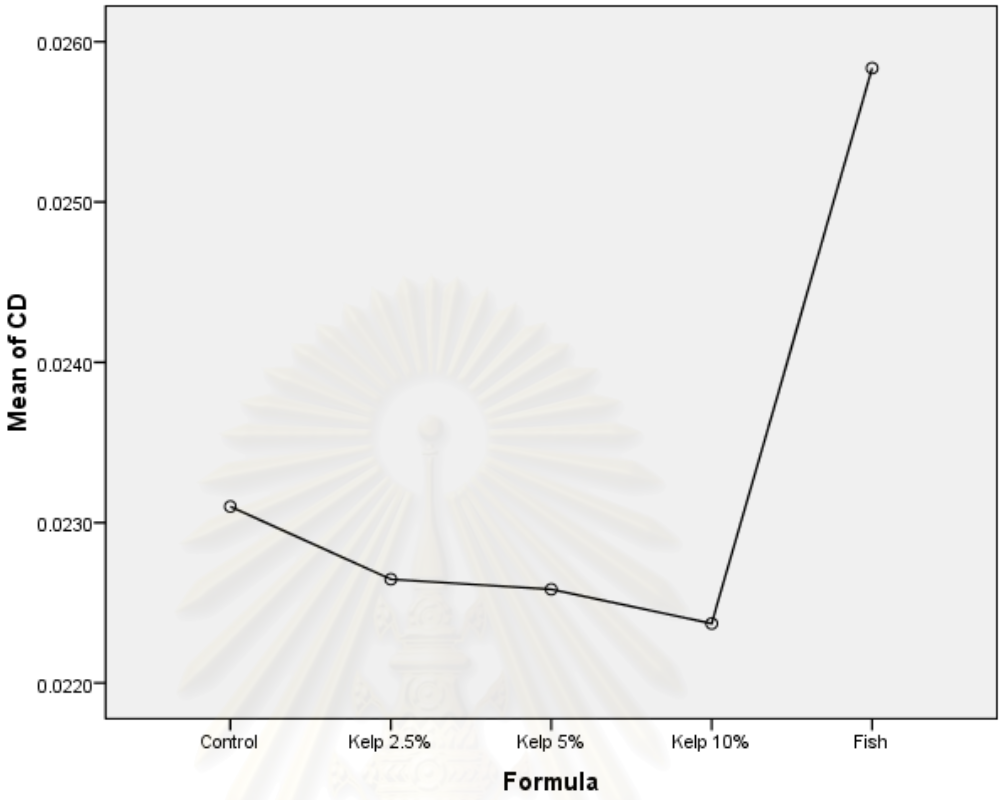

Figure D10 Mean plot of condition index

Table D17 Duncan's New Multiple Range Tests of condition index

Formula	N	Subset for alpha = 0.05		
		1	2	3
Kelp 10%	30	.022370		
Kelp 5%	29	.022584		
Kelp 2.5%	30	.022647		
Control	29		.023100	
Fish	30			.025835
Sig.		.060	1.000	1.000

Means for groups in homogeneous subsets are displayed.

BIOGRAPHY

Mr. Bhumrindra Tauvarotama was born on Tuesday 3rd August, 1982 in Samut Sakorn Province.

Graduated in Bachelor Degree of Science (Marine Science) from Faculty of Science, Chulalongkorn University and also Bachelor Degree of Arts (Information Science) from Sukhothai Thammatirat Opened University, both in the second semester, academic year 2004.

Continuous study in Master Degree of Science, Program in Biotechnology, Faculty of Science, Chulalongkorn University in the first semester, academic year 2005.