การห่อหุ้ม *Haematoccus pluvialis* โดยใช้ไคโตซานเพื่อปรับปรุงเสถียรภาพของแอสตาแซนติน

นาย ประชานาถ กิตติไกวัล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

ISBN 974-14-2558-9

ENCAPSULATION OF *Haematococcus pluvialis* USING CHITOSAN FOR ASTAXANTHIN STABILITY ENHANCEMENT

Mr. Prachanart Kittikaiwan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2006
ISBN 974-14-2558-9

Thesis Title	ENCAPSULATION OF *Haematococcus pluvialis* USING CHITOSAN FOR STABILITY ENHANCEMENT
By	Mr. Prachanart Kittikaiwan
Field of Study	Chemical Engineering
Thesis Advisor	Assistant Professor Artiwan Shotipruk, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Engineering
(Professor Direk Lavansiri, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor Seeroong Prichanont, Ph.D.)

..... Thesis Advisor
(Assistant Professor Artiwan Shotipruk, Ph.D.)

..... Member
(Associate Professor Prasert Pavasant, Ph.D.)

..... Member
(Assistant Professor Joongjai Panpranot, Ph.D.)

..... Member
(Sorada Kanokpanont, Ph.D.)

ประชานาถ กิตติไกวัล : การห่อหุ้ม *Haematococcus pluvialis* โดยใช้ไคโตซานเพื่อปรับปรุงเสถียรภาพของแอสตาแซนติน. (ENCAPSULATION OF *Haematococcus pluvialis* USING CHITOSAN FOR ASTAXANTHIN STABILITY ENHANCEMENT) อ. ที่ปรึกษา: ผศ. ดร. อาทิวรรณ โชติพฤกษ์, หน้า 70. ISBN 974-14-2558-9.

แอสตาแซนตินเป็นที่ยอมรับในเชิงการค้าเนื่องจากสามารถนำไปใช้ผสมกับอาหารสัตว์ในอุตสาหกรรมการเลี้ยงปลาเพื่อเป็นการเพิ่มสีสันให้กับสัตว์น้ำ นอกจากนี้ยังมีฤทธิ์ในการต่อต้านอนุมูลอิสระที่สูงกว่าเบต้าโตโคฟีรอล (β-tocopherol) 100 เท่า และยังมีความสามารถในการต่อต้านการเกิดโรคมะเร็ง แต่โดยทั่วไปแล้วแอสตาแซนตินสามารถที่จะสลายตัวได้เมื่อสัมผัสกับความร้อนหรือสภาวะออกซิเดชั่นระหว่างการผลิตและการเก็บรักษา งานวิจัยนี้จึงเสนอวิธีการป้องกันเซลล์สาหร่ายจากจากสภาวะออกซิเดชั่นแวดล้อมต่างๆ เพื่อปรับปรุงเสถียรภาพของแอสตาแซนตินโดยการห่อหุ้มสาหร่ายด้วยฟิล์มของไคโตซาน เนื่องจากไคโตซานเป็นพอลิเมอร์ที่สามารถสลายตัวได้เองตามธรรมชาติ และไม่มีความเป็นพิษต่อมนุษย์และสิ่งแวดล้อมในการห่อหุ้มสาหร่ายด้วยไคโตซาน สาหร่าย *H.pluvialis* จะถูกปั้นเป็นเม็ดกลมและเคลือบด้วยชั้นของฟิล์มไคโตซานจำนวน 5 ชั้น ซึ่งวิธีการนี้พบว่าให้ประสิทธิภาพการแคปซูลของสาหร่ายถึง 99% โดยแคปซูลที่ได้มีขนาดโดยเฉลี่ยของแคปซูลเท่ากับ 0.43 เซนติเมตร และชั้นฟิล์มไคโตซานมีความหนารวมของทั้งหมดประมาณ 100 ไมโครเมตร นอกจากนี้ยังพบว่าการแคปซูลสาหร่ายด้วยวิธีนี้ไม่ทำให้เกิดการสูญเสียของปริมาณของแอสตาแซนตินอย่างมีนัยสำคัญ อย่างไรก็ตามพบว่าความสามารถในการต่อต้านอนุมูลอิสระของแอสตาแซนตินจะลดลงประมาณ 3% เมื่อทำการศึกษาเสถียรภาพของสาหร่ายแห้งในสภาวะการเก็บรักษาต่างๆกัน พบว่าสภาวะการเก็บรักษาที่เหมาะสมที่สุด คือ ที่อุณหภูมิ -18 องศาเซลเซียส ภายใต้บรรยากาศของไนโตรเจนในที่มืด และเมื่อเปรียบเทียบเสถียรภาพของสารหร่ายแห้งและสาหร่ายที่เคลือบด้วยฟิล์มของไคโตซาน พบว่าสาหร่ายที่เคลือบด้วยฟิล์มไคโตซานมีปริมาณแอสตาแซนตินและความสามารถในการต่อต้านอนุมูลอิสระสูงกว่าสาหร่ายแห้งที่ไม่ถูกเคลือบด้วยไคโตซาน จากผลการทดลองแสดงให้เห็นว่าแม้ว่ากระบวนการเคลือบสาหร่ายทำให้ความสามารถในการต่อต้านอนุมูลอิสระลดลงประมาณ 3% แต่การเคลือบสาหร่ายด้วยไคโตซานเพิ่มเสถียรภาพให้กับในสาหร่ายสำหรับการเก็บรักษาในระยะยาว

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต.....
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2549	

46700365221 : MAJOR CHEMICAL ENGINEERING

KEY WORD: ENCAPSULATION / *Haematococcus pluvialis* / CHITOSAN / ASTAXANTHIN / STABILITY

PRACHANART KITTIKAIWAN: ENCAPSULATION OF *Haematococcus pluvialis* USING CHITOSAN FOR ASTAXANTHIN STABILITY ENHANCEMENT: THESIS ADVISOR: ASST. PROF. ARTIWAN SHOTIPRUK, Ph.D., pp 70. ISBN 974-14-2558-9.

Astaxanthin is receiving commercial interest due to its use as a preferred pigment in aquaculture feeds. Its anti-oxidant activity is approximately 100 times higher than that of β-tocopherol, and can be used as a potential agent against cancer. Astaxanthin can easily be degraded by thermal or oxidative processes during the manufacture and storage. In this study, astaxanthin and its biological activity were protected against oxidative environmental conditions by encapsulating the homogenized cells in chitosan, a nontoxic and biodegradable natural polymer. *H.pluvialis* was formed into beads, which were then coated with 5 layers of chitosan film. The efficiency of this technique of encapsulation was as high as 99% and the resulting chitosan capsules have the mean diameter of 0.43 cm and the total film thickness of approximately 100 μm. No significant loss in the amount of astaxanthin content in *H.pluvialis* was found due to the process of encapsulation. However, approximately 3% loss of antioxidant activity of the *H.pluvialis* was observed after encapsulation. For the study of stability under different storage conditions, the best storage condition was found to be at -18 ^{O}C under N_2 atmosphere in the dark. Results from this study showed that although encapusulation caused 3% loss of antioxidant activity, the longer term stability study of the dried algae biomass, beads, and capules under different storage conditions indicated that encapsulation of *H.pluvailis* in chitosan film was capable of protecting the algae cells from oxidative stresses.

Department Chemical Engineering Student's Signature.....

Field of Study Chemical Engineering Advisor's Signature.....

Academic Year 2006

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Assist. Prof. Artiwan Shotipruk for her encouragement and guidance throughout my work.

Thanks to all my thesis committee, Assist. Prof. Seeroong Prichanont, Assoc. Prof. Prasert Pavasant, Dr. Sorada Kanokpanont, and Assist. Prof. Joongjai Panpranot, for their kind advice on my work.

Special thanks for Prof. Motonobu Goto for the *Haematococcus pluvialis* algae supports on my work.

Many thanks also go to the staff of Analytical Laboratory of Chemical Engineering, Chulalongkorn University, for assistance in analytical work.

Thanks to Faculty of Engineering, Chulalongkorn University for financial support.

Sincere thanks are given to all members of the Biochemical Engineering Research Laboratory, especially, Mr. Thamrong Ampornrat, Ms. Pongpen Accasevorn, Ms. Kamonpan Kaewpintong and Ms. Praiya Tana for their any assistance and warm collaborations.

Finally, I would like to express the highest gratitude to my parents, all family members, and all my friends for their help, their unfailing understanding, and affectionate encouragements.

CONTENTS

Page

CONTENTS (Cont.)

Page

CONTENTS (Cont.)

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF FIGURES

Page

Chapter I

Introduction

1.1 Rationale

Astaxanthin (3,3'-dihydroxy-β-β'-carotene-4-4'-dione) is receiving commercial interest due to its use as a preferred pigment in aquaculture feeds. Its anti-oxidant activity is approximately 100 times higher than that of β-tocopherol, and can be used as a potential prophylactic agent against skin cancer and as a possible chemopreventive agent for bladder carcinogenesis. Despite the availability of synthetic astaxanthin, astaxanthin from natural sources still received more interest due to its greater antioxidant activity and stability (Zhang et al., 1999). The compound can be produced by a number of microorganisms, such as green algae *Haematococcus pluvialis* and *Chlorella zofingiensis*, red yeast *Phaffia rhodozyma*, and marine bacterium *Agrobecterium aurantiacum* (Yaun and Chen, 2000). Of these microorganisms, micro-alga *H.puvialis* is believed to be the world's richest source of astaxanthin, thus it is of particular interest in this study.

Despite the great deal of activity, astaxanthin is a highly unsaturated molecule, which can be degraded easily by high temperature, light, and oxidative conditions.

In *H.pluvialis,* the production and accumulation of intracellular astaxanthin take place as the vegetative cells transform into cyst cells, during which thick cell walls are formed around them. This thick and quite impermeable cell walls help protect astaxanthin and other carotenoids within the cells against degradation even under oxidative conditions such as high light, temperature, and oxygen exposure, as was reported by Gouveia and Empis (2003). On the other hand, these cell walls make the carotenoids unavailable when the whole cells are taken due to the low product release (Mendes-Pinto et al., 2001). Therefore in manufacturing the agal cells for aquaculture or for human consumption, algal cells are generally homogenized to enhance the product bioavailabilty. This in turns lower the product stability.

Generally, when handling stubstances with high instability, encapsulation process is generally performed by forming a polymeric matrix or coating layer around the substances in order to protect its biological activity from environmental factors. Natural polymers are preferable to synthetic polymer due to the safety and the biodegradability these polymers offer. One of the natural polymers that can potentially be used for this purpose is chitosan, which is a biodegradable polymer derived from exoskeleton of arthropod and crustacean. The purpose of this study is therefore to determine the feasibility of enhancing the physicochemical stability of astaxanthin in the *H.pluvialis* by encapsulating the homogenized cells in rigid polymeric matrix of chitosan.

1.2 Objectives

1.2.1 To prepare encapsulated cells in chitosan shell and to determine the characteristics of these capsules such as efficiency of encapsulation, size and size distribution, membrane thickness, and the amount of astaxanthin and antioxidant activity lost during process.

1.2.3 To determine the stability of the encapsulated cells as compared with unencapsulated controls.

1.3 Expected benefit

To Enhance stability of astaxanthin from *H.pluvialis* while maintain bioavailability.

1.4 Working scopes

1.4.1 Investigation of cell stability was be conducted under the following range storage conditions: the temperature at -18, 30 and 80 ^{O}C, pure $N_{2,}$ and atmospheric condition, and in the dark or under high light whose intensity is 6,000 lux

1.4.2 Fabrication and determination of the characteristics of algae capsules was be performed to find the efficiency of encapsulation, size/weight and size/weight distribution, encapsulation efficiency, membrane thickness, and the amount of astaxanthin and antioxidant activity lost during process.

1.4.3 Analysis of Astaxanthin content was performed spectrophotometically. The analysis of antioxidant activity will be conducted using ABTS method.

Chapter II

Backgrounds and Literature Reviews

2.1 Astaxanthin

Chemical name : 3,3’-dihydroxy-β,β-carotene-4,4’-dione

Molecular formula : $C_{40}H_{52}O_4$

Molecular weight : 596.82

Astaxanthin (3,3’-dihydroxy-β,β-carotene-4,4’-dione), a member of the carotenoid family. This red-orange pigment is closely related to other well-known carotenoids such as β-carotene, lutein, zeaxanthin, canthaxanthin whose chemical structures are shown in Fig. 2.1.

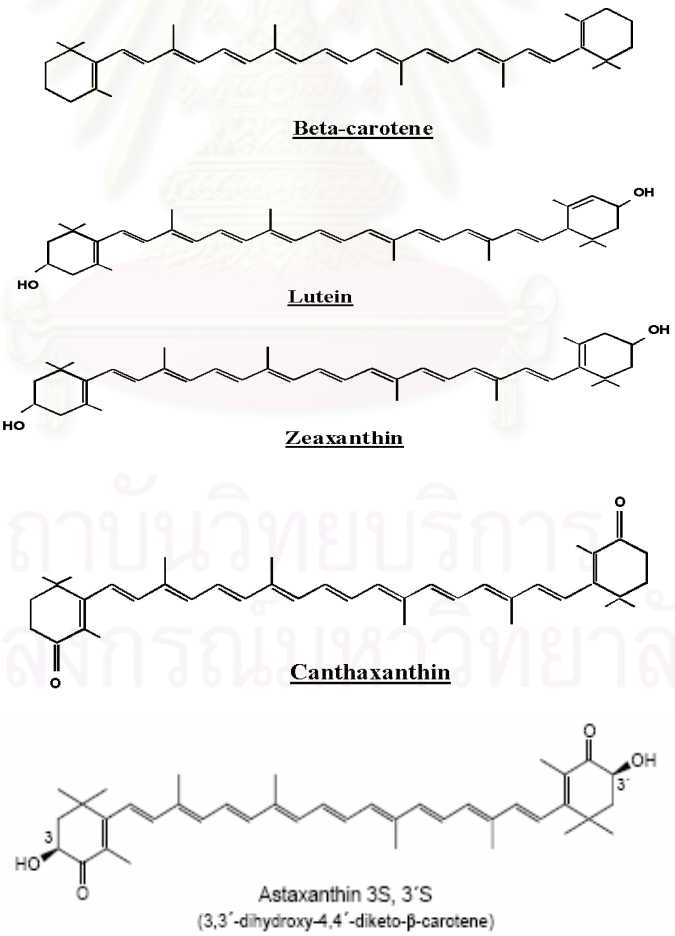


Figure. 2.1. Structure of selected carotenoids

In nature, astaxanthin can be obtained from microalgae, such as *Haematococcus pluvialis* and *Chlorella zofingiensis*, from red yeast *Phaffia rhodozyma* or from marine bacterium *agrobecterium aurantiacum* (Yaun and Chen, 2000). Of these microorganisms, the micro-alga *H. pluvialis* is the richest source of astaxanthin, containing more than 3% astaxathin on dry biomass basis.

Astaxanthin is used as a pigmentation source in marine fish aquaculture. Moreover, the compound has been found to be more effective antioxidant agents than any other agents. It is 10 times more effective than beta-carotene and 4 times more than lutein, and is 100 to 500 times more effective in inhibiting lipid peroxidation as than Vitamin E. It provides superior protection against UVA light-induced oxidative stress as well as immune protection against initiation and promotion of tumors (Richard, 1999).

Production of astaxanthin from alga *Haematococcus pluvialis* is associated with large changes in the morphology, physiology and photosynthetic characteristics of the alga. Generally, the life cycle of *Haematococcus pluvialis* can be divided into four cell stages, as illustrated in Fig. 2.2: I, vegetative cell growth; II, encystment (transformation of vegetative to immature cystcells); III, maturation (transformation of immature to mature cyst cells); IV, germination (transformation of mature cyst to vegetative cells) (Hata et al., 2001).Under good growth conditions, most cells remain in the vegetative form (zoospore) and accumulate little or no astaxanthin. However, under stress conditions such as high light and/or nutrient deficiency, the cells change to thick-walled immotile spores (cyst).

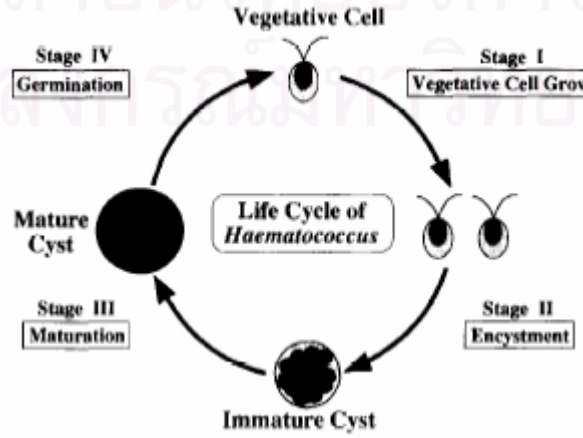


Figure. 2.2. Schematic diagram of the model life cycle of *H. pluviulis.*

Astaxanthin in nature have 2 structures: *trans*-astaxanthin and *cis*-astaxanthin. *Trans*-astaxanthin has higher antioxidant activity than *cis*-astaxanthin. Generally, *H. pluvialis* astaxanthin from is found in the *trans* form, and the compound is usually protected against the transformation by the thick cell walls. Nevertheless, these thick cell walls do not allow astaxanthin intracellularly produced to be easily released out of the cells, thus the compound becomes bio-unavailable. Algal cells must therefore be broken before use or before extraction by organic solvent (Yaun and Chen, 2000). Cell disruption increases the exposure to thermal, light and oxidative conditions which unfavorably promotes the isomerization to the *cis* form. To improve the stability of highly antioxidative compounds, encapsulation of the compound in polymer matrix is often used. of the natural polymers available for encapsulation purposes, chitosan is a widely used natural polymer due to its nontoxicity and biodegradability.

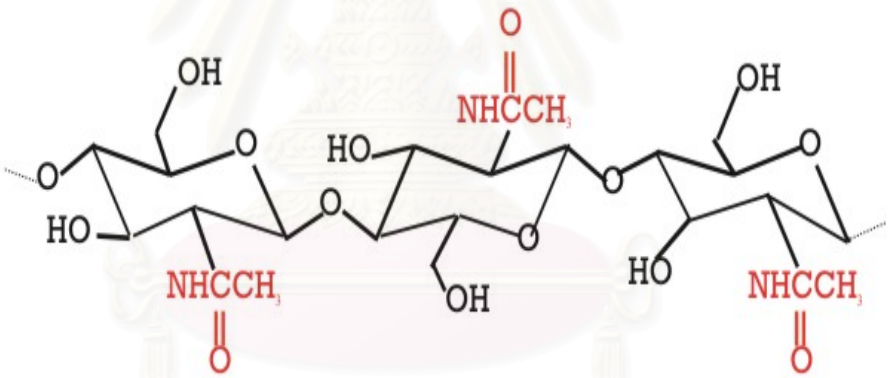
2.2 Chitosan

Chitosan, poly(β-(1→ 4)-2-amino-2-deoxy-D-glucose, is a natural linear biopolyaminosaccharide. It is generally obtained by alkaline deacetylation of chitin, straight homopolymer composed of β-(1,4)-linked *N*-acetyl-glucosamine units, and the second most abundant polysaccharide next to cellulose, which is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as *aspergillus* and *mucor* (Sinha et al., 2004). The different percentages of chitin in natural materials are summarized in Table 2.1. While chitosan is a homopolymer, chitosan comprises of copolymers of glucosamine and N acetyl- glucosamine, whose composition depends on the degree of deacetylation. Chitosan is a weak base which is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH < 6.5). Typically, chitosan has an average molecular weight ranging between 3800 and 2,000,000 and is from 66 to 95% deacetylated (Sinha et al., 2004).

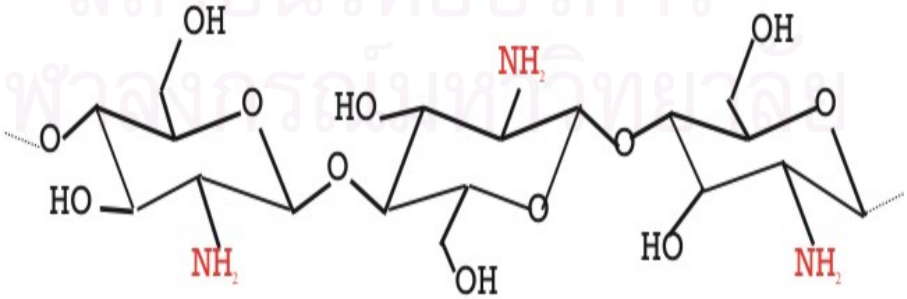
Table. 2.1. Percentages of chitin contained in different sources (Muzzarelli, M., 2000)

Type	Percentages (%)
Fungi	5-20
Worms	20-38
Squids/Octopus	3-20
Scorpions	30
Spiders	38
Cockroaches	35
Water Beetle	37
Silk Worm	44
Hermit Crab	69
Edible Crab	70

Chitosan is a weak base and is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH < 6.5). It has an average molecular weight ranging between 3800 and 2,000,000 and is from 66 to 95% deacetylated (Sinha et al., 2004).



Chitin



Chitosan

Figure. 2.3. Structure of chitin and chitosan

2.2.1 Preparation of chitosan

2.2.1.1 Purification of chitin

Chitin derived from natural sources is closely associated with lipids, proteins, inorganic material which is mainly $CaCO_3$, and pigments. Various procedures have been adopted to remove these impurities. Demineralisation is first carried out and is most frequently done by treatment with HCl. Deproteinisation is then performed by treatment with NaOH. Then the pigments can be removed by extraction with ethanol or acetone or by bleaching with use of $KMnO_4$ or H_2O_2.

2.2.1.2 Deacetylation of chitin

One of the main reactions carried out of in chiotsan production from chitin is deacetylation, which is most commonly carried out by using aqueous alkali. The most frequently used alkali is NaOH. The extent of deacetylation is governed by the alkali concentration, temperature, reaction time, and particle size and density. While treatment with 50 wt-% NaOH at 100 ^{0}C for 1 hour gives a product having 82% deacetylation, extending the reaction time to 48 hours enables almost 100% deacetylation to be achieved. However this is at the expense of a considerable decrease in solution viscosity, indicating chain degradation. Other variations on the standard aqueous alkali treatment to avoid chain degradation have been directed towards reducing the quantity of alkali required in the treatment. In this study we use chitosan from Wako Pure Chemical Industries, Ltd. The properties of chitosan following: deacetylation rate (after drying) min. 80.0 mol/mol%, viscosity (5g/l, 20 ^{O}C) 50-150 cP, loss on drying at 105 ^{O}C max. 10.0%, pH (10 g/l aqueous slurry, 25 ^{O}C) ~ 7-5.

2.2.2 Application of chitosan

Chitosan has been shown to be of potential use in many different fields including the following :

2.2.2.1 Bio-medicine

Due to its biocompatibility with human body tissue, the cicatrizant properties of chitin and chitosan have demonstrated their effectiveness for all forms of dressings - artificial skin, corneal bandages and suture thread in surgery - as well as for implants or gum cicatrization in bone repair or dental surgery.

Moreover, chitosan is an excellent medium for carrying and slow release of medicinal active principles in plants, animals and human. Since it is undigested by the stomach, it is, for example, a good means of retarding the release of encapsulated products that must reach the intestine without undergoing any transformation (http://cecon.com/res/microencap_2.html).

2.2.2.2 Dietetics

Chitosan is already part of our everyday diet: we absorb it in its natural state in shellfish, crustaceans and mushrooms. Since chitosan is not digested by the human body, it behaves like a fibre, which is an important element of diet. Above all, it is an excellent fat trap. It precipitates lipids when they arrive in the intestine, reducing the human body's rate of cholesterol absorption by 20 to 30%. It has become the leading natural slimming product (Kanauchi *et al.*, 1995).

2.2.2.3 Cosmetics

Chitosan forms a protective tensor film on the skin's surface that can fix other active principles for the skin. Thus other hydrating agents, solar filters, organic acids or other active principles can be combined with the derivatives of chitin. Chitin facilitates their effects. Chitin and its derivatives allow active principles to be placed in close contact with the skin by means of a medium that is not only a film-forming tensor but is especially hydrating. This is a new double advantage that makes chitosan of great interest in cosmetics. Now, chitin and chitosan are therefore used in skin creams, shampoos, lacquers, varnishes, etc (Lang *et al.*, 1985; Amerchol, 1998).

2.2.2.4 Water treatment

Chitin and its derivatives have two major properties that are of interest for industry and for nature conservation: they are remarkable chelation agents and heavy metal traps. Employed as a chelation agent, chitin and its derivatives are used for treating drinking water by separating organic compounds and heavy metals, and for treating sewage by precipitating certain anionic wastes and capturing pollutants such as DDT and PCBs (polychlorobenzene) (Hirano, 1996).

2.2.2.5 Paper manufacture

Other application of chitosan involves the manufacturing of paper. Due to the chitosan's close molecular resemblance with cellulose, the main constituent of plant walls, it replaces amine substitutes such as guar gum and polysynthetic polysaccharides used in paper industry, thus saving chemical additives. Furthermore, the paper produced with chitosan has a smoother surface and is more resistant to moisture. Among other things, chitosan is of great value in the production of toilet paper and for wrapping paper and cardboard (Lertsutthiwong et al., 2002)

2.2.2.6 Agriculture

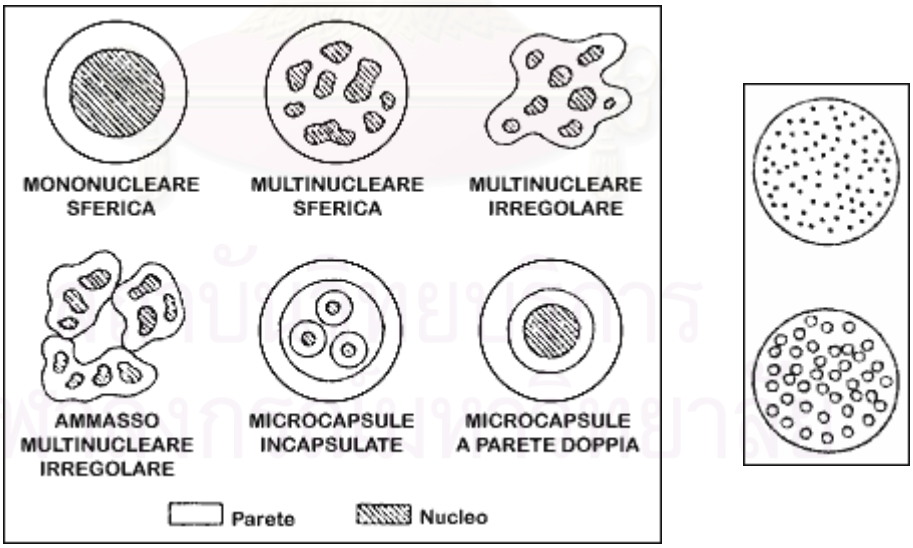
Chitosan and its derivatives have plant protecting and antifungal properties. They can trigger defensive mechanisms in plants against infections and parasite attacks, in very low concentrations in the order of a few milligrams per cubic meter of water. They can be used in solution, in powder form or as coatings of seeds.

Chitosan acts on several levels. Apart from its specifically antifungal action, it strengthens the root system and thickens the stem. Some studies also show that chitosan stimulates the plant's synthesis of protective agents. In related fields, chitosan behaves like a fertilizer by accelerating the germination and growth of plants (Hirano, 1996; *Anon.*, 1999)

2.3 Encapsulation

Encapsulation is a process by which small particles or droplets are surrounded by a coating to produce encapsulated spheres or capsules. If the small particles are dispersed uniformly within the polymer matrix either the macroscopic (particulates) or molecular (dissolution) levels, these are called encapsulated spheres. If the capsules or spheres are in the micrometer to millimeter range, they are known as microcapsules or microspheres, or if the particles are smaller than 1 micron, they are called nanocapsules or nanospheres. In the capsules, the material inside the capsule is referred to as the core, internal phase or fill, whereas the wall is sometimes called a shell, coating or membrane. The configuration of the core can be a spherical or irregular particle, liquid-phase suspended solid, solid matrix, dispersed solid and aggregates of solids or liquid forms. Some examples of capsules and spheres are shown in Figure 2.3.

MICROPARTICLES/MICROSPHERE



A. Microparticles B. Microsphere

Figure. 2.4 Various forms of encapsulated particles

Typically, reasons of encapsulation of substances are to protect the reactive substances from the environment, to convert liquid active components into a dry solid system, to mask undesired properties of the active components and to control release of the active components for delayed (timed) release or long-acting (sustained) release. For any specific purposes, an appropriate polymer or polymer blend well as the methods of encapsulation must be selected to ensure reproducibility, high encapsulation efficiency, and perseveration of the activity of the encapsulation efficiency.

2.3.1 Methods of encapsulation

2.3.1.1 Emulsion-solvent evaporation/extraction methods

Single emulsion method

This method has been primarily used to encapsulate hydrophobic substances through oil-in-water (o/w) emulsification process. The polymer is dissolved in a water-immiscible, volatile organic solvent such as dichloromethane, and the substances are dissolved or suspended into the polymer solution. The resulting mixture is emulsified in a large volume of water in the presence of an emulsifier. The solvent in the emulsion is removed by either evaporation at elevated temperatures or extraction in a large amount of water, resulting in formation of compact microparticles. This method, however, is only available for the hydrophobic substrances because the hydrophilic drugs may diffuse out or partition from the dispersed oil phase into the aqueous phase, leading to poor encapsulation efficiencies .

In an attempt to encapsulate hydrophilic substances (e.g., peptides and proteins), an oil-in-oil (o/o) emulsification method has recently received considerable attention. In this method, the water miscible organic solvents are employed to dissolve the substances and polymer, whereas hydrophobic oils are used as a continuous phase of the o/o emulsion. The microparticles are obtained by removing the organic solvents through evaporation or extraction process (Park et al.,2005).

Double emulsion method

Most water-soluble substances have been encapsulated by water-in-oil-in-water (w/o/w) methods. The aqueous solution of the water-soluble substances are emulsified with polymer-dissolved organic solution to form the water-in-oil (w/o) emulsion. The emulsification is carried out using either high speed homogenizers or sonicators. This primary emulsion is then transferred into an excess amount of water containing an emulsifier under vigorous stirring, thus forming a w/o/w emulsion. In the subsequent procedure, the solvent is removed by either evaporation or extraction process (Park et al.,2005).

2.3.1.2 Phase separation

This method involves phase separation of a polymer solution by adding an organic nonsolvent. Substances are first dispersed or dissolved in a polymer solution. To this mixture solution is added an organic nonsolvent (e.g., silicon oil) under continuous stirring, by which the polymer solvent is gradually extracted and soft coacervate droplets containing the drug are generated. The rate of adding nonsolvent affects the extraction rate of the solvent, the size of microparticles and encapsulation efficiency of the substances. The commonly used nonsolvents include silicone oil, vegetable oil, light liquid paraffin, and low-molecular-weight polybutadiene. The coacervate phase is then hardened by exposing it into an excess amount of another nonsolvent such as hexane, heptane, and diethyl ether. The characteristics of the final microspheres are determined by the molecular weight of the polymer, viscosity of the nonsolvent, and polymer concentration (Park et al.,2005).

2.3.1.3 Spray drying

The substances are dissolved or dispersed in the polymer solution, in which volatile solvents (e.g., dichloromethane and acetone) are preferred. The resulting solution or suspension is sprayed in a stream of heated air to produce microparticles. The size of the microparticles is determined depending on the atomizing conditions (Park et al.,2005).

2.3.1.4 Droplet extrusion

The substances are dispersed in a polymer solution. This mixture is contained into a needle which is extruded by the force through a needle. When droplets from needle drop into a cross-linking agent, the droplets will form gel beads. For example, drops of alginate solution from needle into $CaCl_2$ are shown in Figure 2.3. (Poncelet et al., 1988)

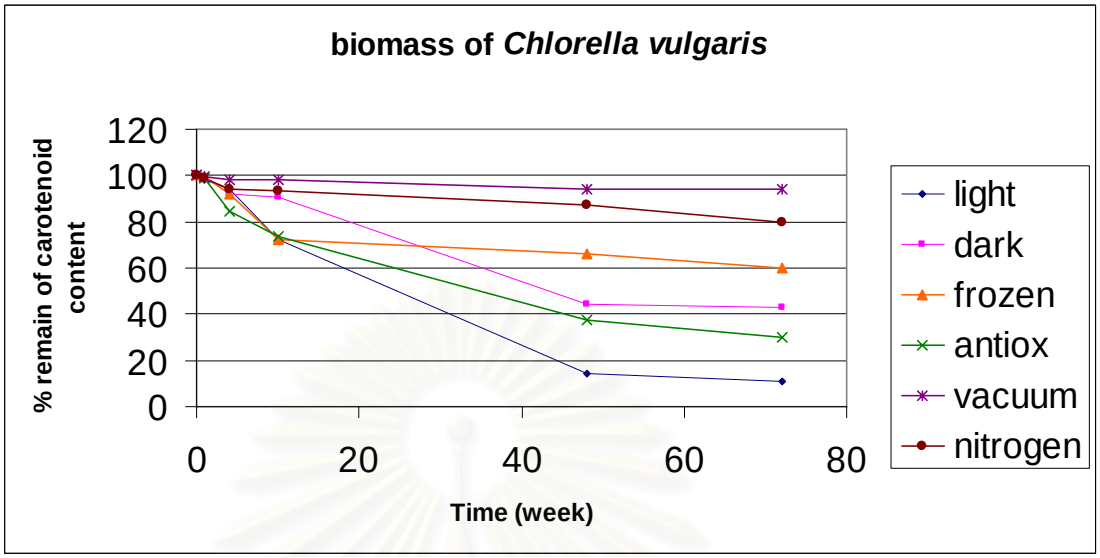
Figure. 2.5 Dropping of alginate

Literature review

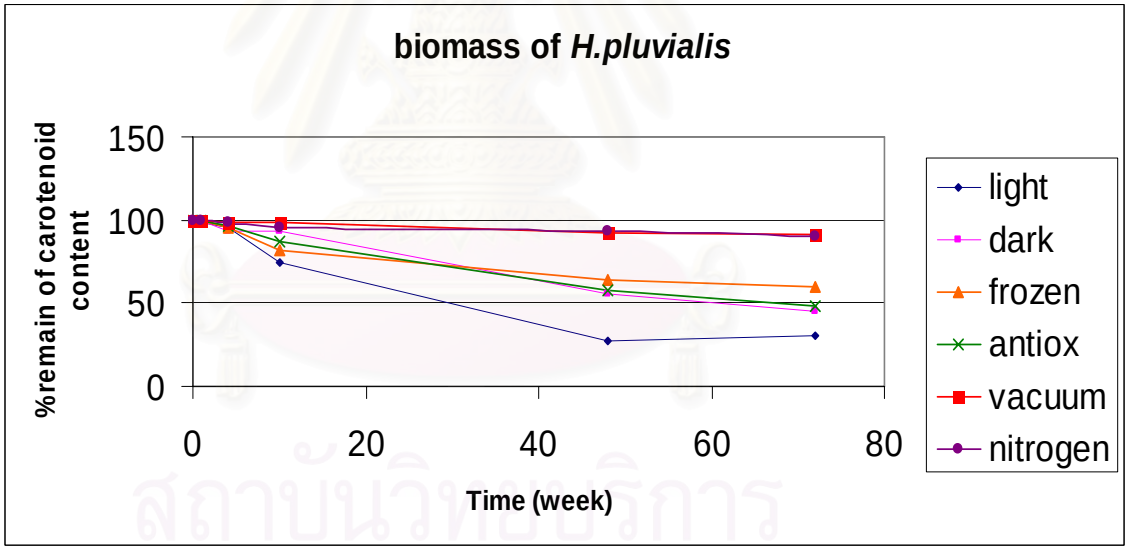
Today encapsulation and microencapsualtion have a wide range of applications in biotechnology, biomedical engineering, food technology, and agriculture. For example, in biotechnology, encapsulation of certain microalgae, such as *Haematococcus pluvialis*, *Chorella minutissima*, *Dunaliella bardawil*, *Pavlova lutheri* in Ca-alginate (Joo et al., 2001) has been shown to increase the algal growth for certain type of algae due to the protection of the cells against shear stress. Another example for biotechnology application involves the use of encapsulated bacterial cells for waste water management (Kaya and Picard, 1996). In biomedical application, encapsulation of drugs is utilized to control the rate of delivery of a bioactive substance to a target organism or reaction site and to protect the bioactive compound from the oxidative environments such as high light and oxygen exposure. For the application of control drug release, microencapsulation technique is typically employed (Sriamornsak et al., 1998; Jameera et al., 1998; Sinha et al., 2004; Sezer and Akbuga., 1995). In agriculture, an example involves the development of controlled release fertilizer formulations containing semipermeable barriers. Such formulations allow fertilizers to be leached out over an extended period of time reducing the number of applications, improving crop yields, and reducing environmental problems due to the fertilizer runoffs.

Of particular interest in this study is the application of encapusulation for protection of bioactive material from environmental conditions, particularly, the antioxidative compound, astaxanthin in *H. pluvilis* cells. Information regarding the stability of carotenoids from *H. pluvialis* and *C. vulgaris* algae at various storage conditions has previously been reported both in biomass and in acetone extract solution (Gouveia and Empis, 2003). Their results are summarized graphically here in Figure 2.6. From their study, it can be concluded that the stability of the carotenoid in biomass and in the extracts of both algae is predominantly affected by light, and in second order by oxygen content, whereas the storage temperature is only important to a lesser extent. Moreover, the results indicated that when carotenoids are contained within the cell biomass in both types of algae, the compound stability is better maintained than if they are extracted in acetone. The acetone extract shows the complete loss of carotenoid pigments after 15 and 30 days, respectively, for *C. vulgaris* and *H. pluvialis*, whereas the depletion of carotenoid content of the algae biomass, even at room temperature and in presence of light, occurred only after approximately 1 month. Only approximately 7-10 % loss was observed during the first 2.5 months for biomass maintained in the dark at room temperature. And when compared with *C. vulgaris, H. pluvialis* however revealed a greater stability, probably due to the thick cyst walls surrounding the cells in the carotenogenic stage.

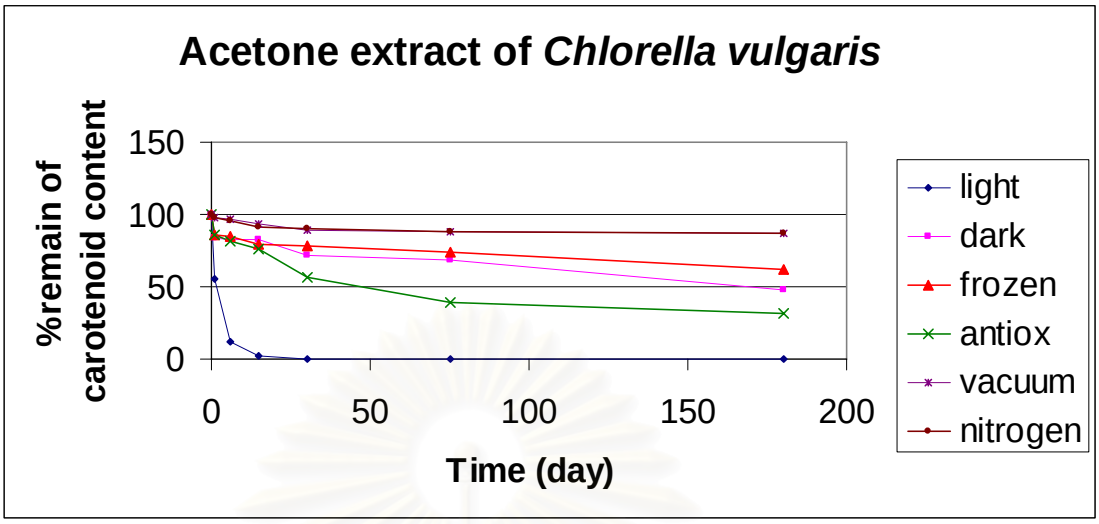
Despite their advantage described above, the thick cell walls however has been shown to prevent the product from releasing extracellularly, thus making it unavailable to aquaculture animals or impossible for carotenoid extraction (Mendes-Pinto et al., 2001). Thus crushing the cells is typically a final necessary step in manufacturing of *H.pluvialis* algae. As a result, the stability is compromised. A study on encapsulation of astaxanthin in chitosan has been conducted and reported by Higuera-Ciapara et al. (2004). The authors determined the stability of the astaxanthin standard in the microcapsules produced via multiple emulsion/solvent evaporation technique under storage conditions at 25, 35, and 45 ^{o}C for eight weeks by measuring isomerization and loss of concentration. The results suggested that the microencapsulated pigment did not suffer isomerization nor chemical degradation under the investigated storage conditions.



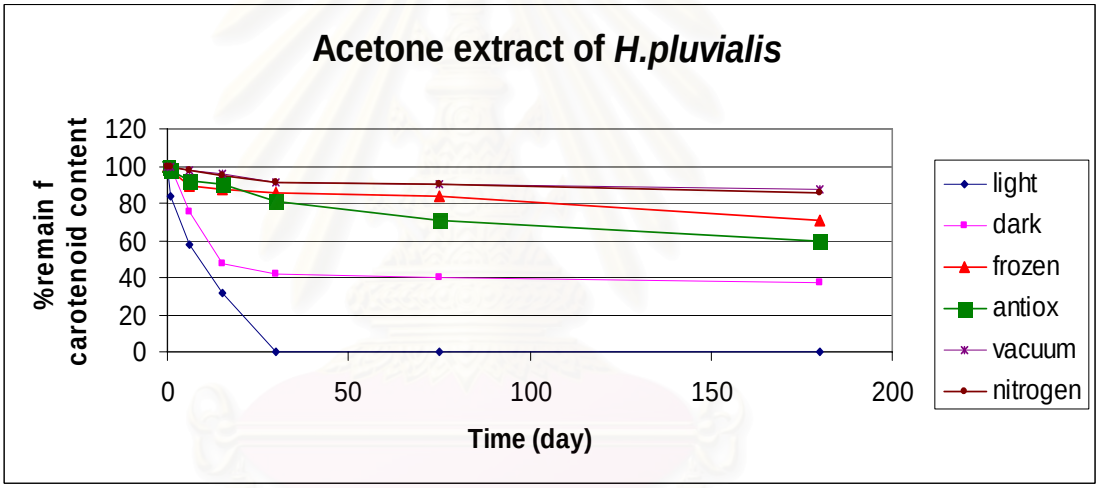
(A)



(B)



(C)



(D)

Figure 2.6 Percentage of carotenoid content remained. (A) Dry biomass of *Chlorella vuigans*, (B) Dry biomass of *Haematococus pluvialis*, (C) Acetone extract of *Chlorella vulgaris* and (D) Acetone extract of *Haematococus pluvialis*.

This study was the first reported study on astaxanthin microencapsulation in a chitosan matrix. However, to our knowledge, the encapsulation study of whole *H. pluvialis* crushed cells is nonexistent. It is therefore the purpose of our study to determine the feasibility of using chitosan matrix to prolong the stability of these crushed cells. In the next chapter, detailed experimental procedures are described.

Chapter III

Materials and Methods

3.1 Material

Astaxanthin standard, chitosan, and all organic solvents used such as acetone and ethanol were obtained from Wako Chemicals, Japan. ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and potassium persulfate (di-potassium peroxdisulfate) were obtained from Sigma-Aldrich (Poole, Dorset, UK). The *Haematococcus pluvialis* powder samples used in this study were the commercial algae powder (NatuRose®), manufactured by Cyanotech, USA. The algae samples were stored at 4°C until use.

3.2 Encapsulation of *H. pluvialis* with chitosan

The Encapsulation method employed in this study was coating the algal beads with chitosan film. To obtain these chitosan coated beads, *H. pluvialis* powder was formed into bead. These beads were then coated repeatedly with multiple layers of chitosan film. To form spherical beads, some water was added to dry *Haematococcus pluvialis* powder (1 ml of water per g dry algae powder). The mixture was then rolled into long cylindrical rods which were sliced into small pellets and molded into spherical beads using a traditional Chinese pill making apparatus. Each bead contains approximately 0.05 g dry biomass. The pellets were then immersed into chitosan solution (0.15 g chitosan in 10 ml of 2 % v/v acetic acid). The chitosan coated beads were then immersed into 1.5 % (w/v) sodium-tri-polyphosphate ($Na_5P_3O_{10}$) solution at pH 5.5. The coated pellets were allowed to set and the process was repeated 4 times to obtain capsules with thicker gel layers. As the final step of encapsulation, the capsules were immersed in 1.5 % (w/v) sodium-tri-polyphosphate ($Na_5P_3O_{10}$) at pH 8.5 for 3 hours to improve the gel strength. The capsules were then washed 3 times with distilled water and air dried. The method of capsule preparation is shown in Figure 3.1. The prepared capsules were then characterized by measuring size and size distribution, and membrane thickness. Any loss in the amount of astaxanthin and antioxidant activity as a result of encapsulation process was also determined.

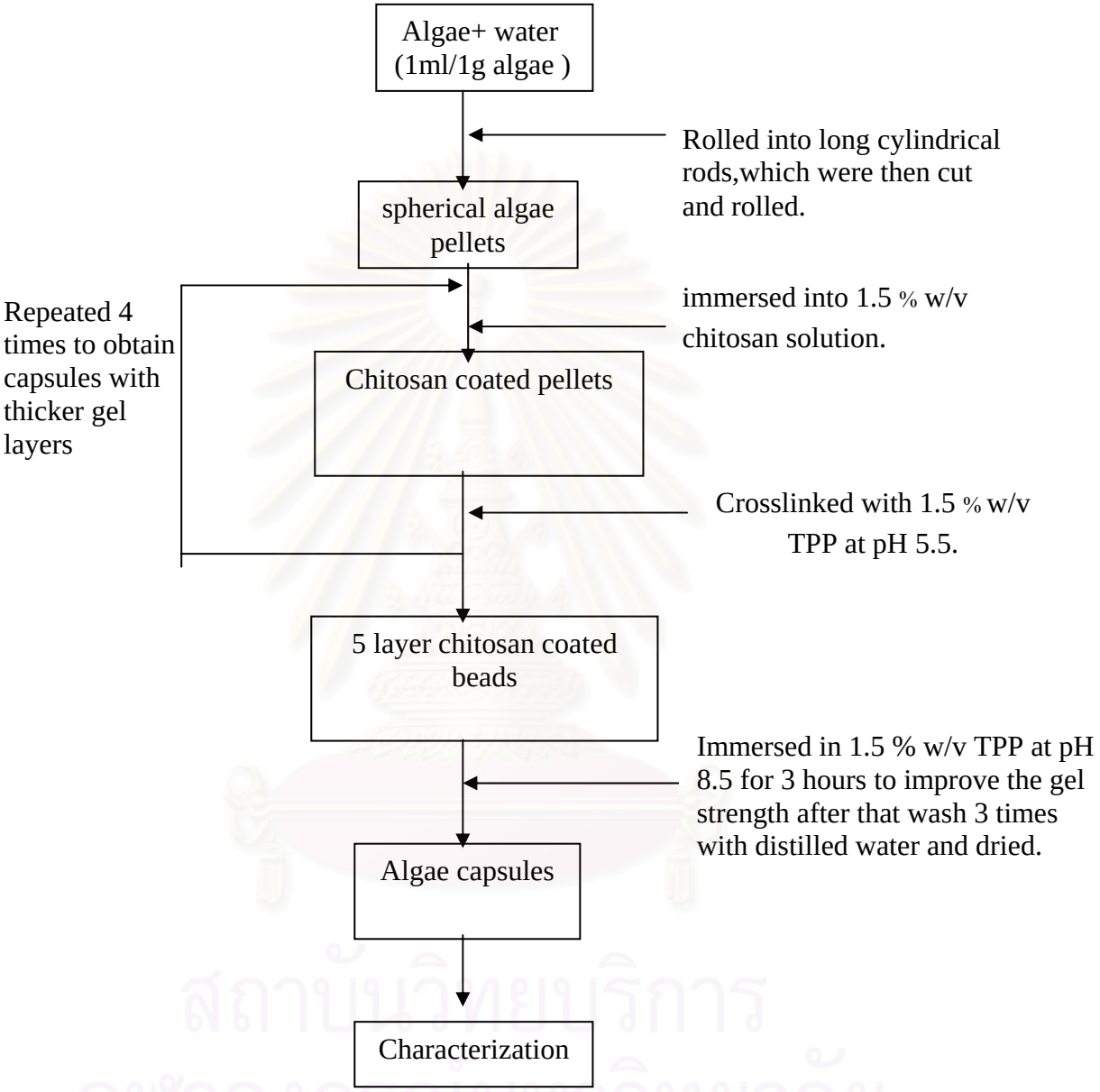


Figure 3.1 Method of capsule preparation

3.3 Characterization of chitsan coated beads

The efficiency of encapsulation is defined as the dry weight of *H. pluvialis* algae in the encapsulated beads divided by that of the starting biomass measured prior to encapsulation process according to the following expression.

$$\text{efficiency of encapsulation} = \frac{\textit{H. pluvialis} \text{ algae in the encapsulated beads}}{\textit{H. pluvialis} \text{ algae starting biomass}}$$

For the size and weight measurements, 150 of the chitosan coated beads were sampled from the coated beads prepared. In measuring the size, the diameter of the sampled capsules was measured with a vernier ruler. The morphology of the beads and chitosan films were examined under a scanning electron microscope (SEM, JSM-5400, USA) at an acceleration voltage of 10 kV and the thickness of the chitosan films were determined. The the morphology study under SEM, the samples were coated with thin film of gold using JEOL model JFC-1100E ion sputtering device before obtaining the micrograph.

Other than the size and weight of the encapsulated beads, the effectiveness of the encapsulation method was evaluated by measuring the content of astaxanthin in the capsules and the antioxidant activity of the encapsulated algal cells and compare with those of the starting algal powder.

3.4 Evaluation of stability of astanxanthin under different storage conditions

The stability of the astaxanthin in the *H.pluvialis* was examined under different storage conditions. Since *H.pluvialis* is not only used as whole cells for aquaculture feeds, the extract form is also suitable for human consumption. Therefore in this study, the stability of astaxanthin both in the algae powder and in the acetone extract of *H.pluvialis* was determined under the conditions as listed in Table 3.1. For the acetone extracts, only conditions 2-7 were tested, and measurements of stability was continued for 9 weeks. In the stability study of the acetone extract, 10 ml of acetone extract was contained in a glass bottle and stored under various storage

conditions, and the analysis of astaxanthin content and the antioxidant activity of the extract were conducted every week. All experiments were performed in triplicates.

Table 3.1 Storage conditions tested for the stability of the algae powder, beads, and capsules

Run #	Temperature	Exposure to air	Light condition	Symbol
1	80 ^{O}C	Air	Dark	DaAir80
2	RT (30 ^{O}C)	N_2	Dark	DaN_2
3	RT (30 ^{O}C)	N_2	Light	LiN_2
4	RT (30 ^{O}C)	Air	Dark	DaAir
5	RT (30 ^{O}C)	Air	Light	LiAir
6	Frozen (-18 ^{O}C)	N_2	Dark	FroN_2
7	Frozen (-18 ^{O}C)	Air	Dark	FroAir

After determining the stability of the astaxanthin in the dry algal powder, the stability of the unencapsulated beads, and chitosan encapsulated beads were examined during a specified period and the results were compared with those of the algal powder. First, for a quick and preliminary test of stability of the algae samples, the samples were exposed to accelerated condition for which the temperature was 80 ^{O}C (Run# 1). An oven was used to provide this condition. For normal storage conditions, Run # 2-7 were set up for experiment. In setting up the experiment, the algal powder, beads, and capsules were contained in small glass bottles placed under different storage conditions. For the samples that were exposed to light, the bottles were placed on acrylic surface. Lighting (6,000 lux) was provided by fluorescent lamps illuminated from the bottom side of the acrylic surface (width of 35 cm., length of 31 cm. and the fluorescent lamps were placed approximately 20 cm from the response surface. For the samples that were subjected to air exposure, the bottles were uncapped and air was flown over the bottles to provide the air exposures. Those that were subjected to N_2 atmosphere, N_2 was purged into the bottles which were then

capped and secured tightly. For each of the conditions in Table 3.1, 10 beads or capsules were kept in the bottle. For the dry biomass, 0.5 g of the powder was placed in each of these bottles. During the storage under different conditions, dry biomass, beads, and capsules were sampled and extracted periodically every 2 weeks for astaxanthin content and the antioxidant activity of the extract.

3.5 Analytical determination of astaxanthin content

3.5.1 Extraction of astaxanthin from *H. pluvialis* and encapsulated cells

20 mg dry biomass can be extracted repeatedly with 5 ml of acetone. The process may be terminated when the supernatant from final extract has absorbance at 475 nm less than 0.05. For the encapsulated cells and bead, the capsules and bead are first crushed and 20 mg of the algae content was separated from the beads. The sample was then extracted and the analysis of astaxanthin content was performed using a spectrophotometer.

3.5.2 Determination of astaxanthin content

The amount of astaxanthin in the algae acetone extract was determined by measuring the absorbance under 475 nm. The content of astaxanthin in the solution was determined according to the following expression.

$$\text{Astaxanthin content (mg/g dry algae)} = \frac{0.0074 * Abs * \text{ml of extract} * M.W * \text{dilution} * 0.8}{1000 * \text{mg of sample}}$$

where M.W is the molecular weight and Abs is Absorbance of the solution, respective.

3.6 Antioxidant analysis of *H. pluvialis* extracts

The antioxidant activity of the *Haematococcus pluvialis* extract was measured using a ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) method, modified from that described in previous research (Re et al., 1999). The extract was diluted in series by acetone and each diluted solutions were added into ABTS solution with the volume ratio 1:2 (extract:ABTS). The solutions were mixed using a vortex and the mixture were incubated in the dark at room temperature for 10 min, after which the absorbance was measured at the wavelength of 734 nm using acetone to ABTS (1:2) as a reference.

For comparing the antioxidant activity in various extracts, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index**.** The value of IC_{50} can found from the plot of percent inhibition (PI) versus the corresponding concentration of astaxanthin, in which the values of PI can be calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100 \qquad (3.1)$$

When A_t and A_r are absorbance of test sample and absorbance of the reference, respectively.

CHAPTER IV
RESULTS AND DISCUSSION

For the purpose of increasing the stability of active compounds by encapsulation in polymer matrix, the polymer or polymer blend as well as the methods of encapsulation must be selected to ensure reproducibility, high encapsulation efficiency, and perseveration of the activity of the encapsulated compound. Furthermore, the encapsulation process itself should not significantly result in the loss of the compound activity. In this chapter, the results will be presented on the effect of encapsulation of *H. pluvialis* with chitosan on the stability astaxanthin under different storage conditions. First, the physical characteristics of the capsules such as encapsulation efficiency, size and size distribution, and chitosan membrane thickness were determined. Then the astaxanthin content and antioxidant activity of the encapsulated algae measured during the time course of the study were compared with those of the unecapsulated cells to determine the functional characteristics of the capsules.

4.1 Physical characteristics of chitosan coated *H. pluvialis* beads

Chitosan coated beads have the average size of 0.431±0.028 cm in diameter. The size distribution of the chitosan capsules is shown in Figure 4.1. All capsules are below 0.600 cm in diameter. Out of these, 8.23% are between 0.351-0.400 cm, 74.05% were between 0.401-0.450 cm, 16.46% were between 0.451-0.500 cm, and 1.27% were between 0.551-0.600 cm. The average weights of the chitosan coated beads were found to be 0.056±0.007 g. The weight distribution of the chitosan capsules is shown in Figure 4.2. All capsules are below 0.08 g. Out of these, 0.65% are between 0.0301-0.0400 g, 20.65% are between 0.0401-0.0500 g, 55.48% are between 0.0501-0.0600 g, 18.06% are between 0.0601-0.0700 g and 5.16% are between 0.0701-0.0800 g. Approximately 93% of the beads have the weight between 0.04 and 0.06 g. These results indicated that the methods of encapsulation used could produce fairly uniform capsules.

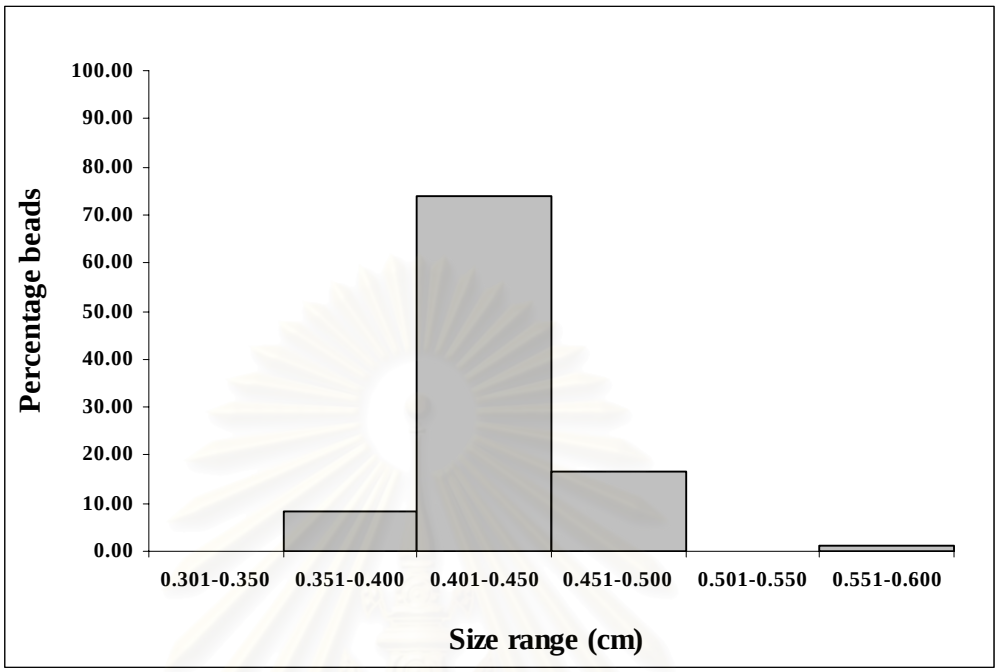


Figure 4.1 Size distribution of encapusulated beads

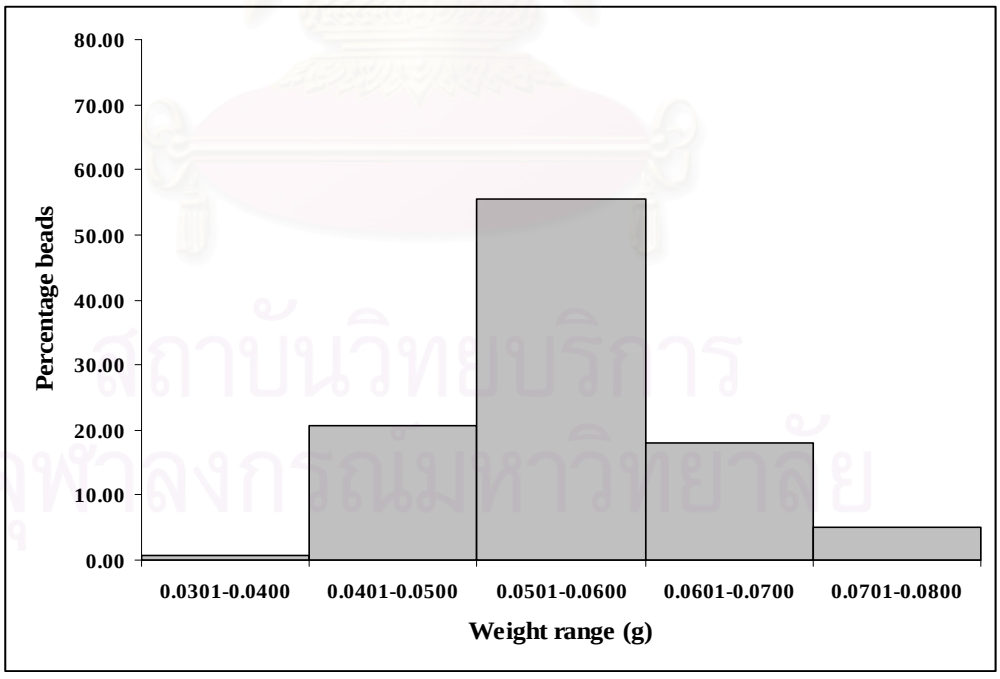


Figure 4.2 Weight distribution of encapsulated beads

The other characteristics of chitosan beads that is important for the purpose of maintaining astaxanthin stability would be the ability of the chitosan film protect the active compound from the oxidative stress such as oxygen and light. The film should have adequate thickness to insure partial impermeability and mechanical strength. In preparing the capsules in this study, the algal beads were coated repeatedly with chitosan films. Figure 4.3 shows the scanning electron micrograph of a chitosan encapsulated *H.pluvialis* bead coated with 5 layers of chitosan films. Each layer has the thickness of approximately 20 μm. These films provide reasonable mechanical stability and partial impermeability to the oxidative stresses. The optimal thickness however has not been determined in this study and could be a subject of the future work.

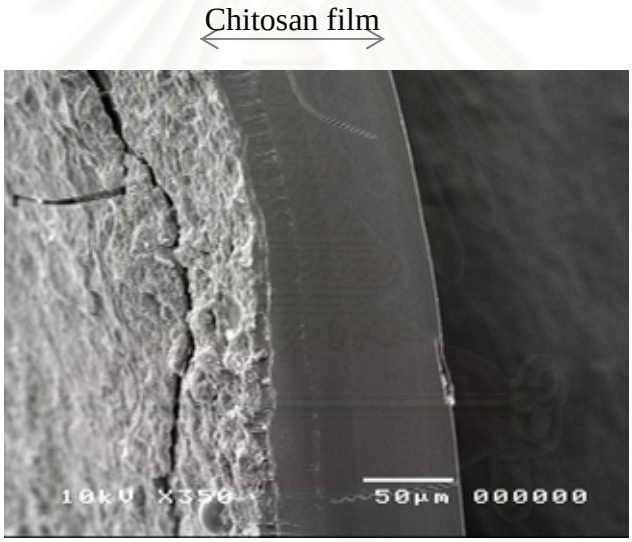


Figure 4.3 Scanning electron micrograph (SEM) of encapsulated beads

.

4.2 Evaluation of encapusulation method

4.2.1 Encapsulation efficiency

The efficiency of encapsulation is defined as the dry weight of *H. pluvialis* algae in the encapsulated beads divided by that of the starting biomass measured prior to encapsulation process. For encapsulation technique employed, the spherical beads of chitosan were first formed, dried, and then coated by dipping in the chitosan solution. With this method, very small loss of algae was resulted during the encapsulation process. The encapsulation efficiency was determined to be higher than 99%.

4.2.2 Loss of astaxanthin content and antioxidant activity during encapsulation

As was previously mentioned, an effective encapsulation process should not result in the loss of astaxanthin. The amount of astaxanthin in *H.pluvialis* was determined before and after encapsulation and the results are shown in Figure 4.4. As shown in the figure, the amounts of astaxanthin per unit mass of algae in the dry biomass, beads, and capsule were the same. This means that the encapsulation procedure employed did not cause a significant loss in the content of this compound. In addition, the antioxidant activity of the extracts of dried algae, algae beads, and encapsulated algae beads was determined and the results are shown in Figure 4.5. The antioxidant activity was measured in terms of IC_{50}, a concentration of the extract that give a 50% reduction in the absorbance of free radical ABTS. Therefore the higher value of IC_{50} means the lower antioxidant activity. Comparison of means by Duncan's new multiple range test at 95% confidence interval indicated that no difference in the activity was found for dried biomass and the algae beads. However, the antioxidant activity of astaxanthin after encapsulation was found to decrease by approximately 3% compare with that of the dried algae biomass. This means that the encapsulation procedure employed did cause a slight loss in the antioxidant activity.

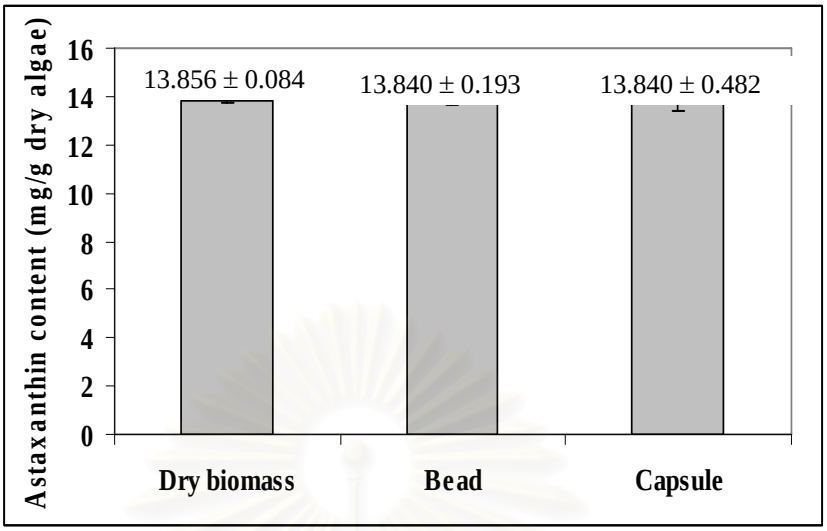


Figure 4.4 Astaxanthin content in *Haematococcus pluvialis* before and after encapsulation

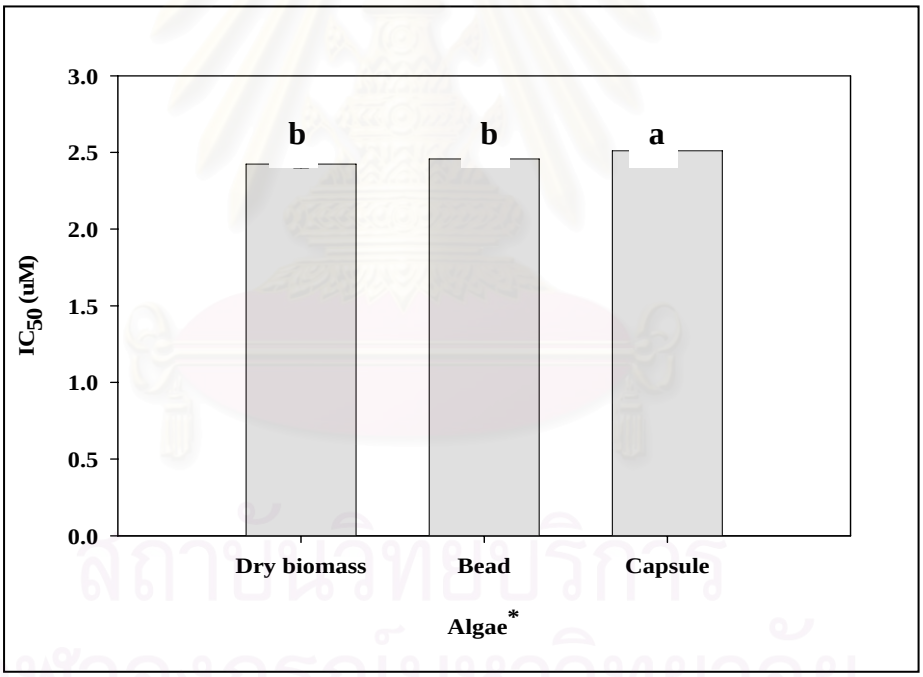


Figure 4.5 Antioxidant activity of astaxanthin in *Haematococcus pluvialis* biomass, beads, and capsules. * Different alphabets a,b indicates statistically significant difference ($P \leq 0.05$).

4.3 Evaluation of astaxanthin stability under different storage conditions

When applied as food color, the content and quality of astaxanthin in *H. pluvialis* microalgae not only affect the attractive color of foods but also their nutritive value and flavor. Thus, the enhanced stability of astaxanthin during storage is a very important criteria for the encapsulated algae beads to be attractive and acceptable as (Cinar, 2004). In this study, the comparison will be made on the stability of astaxanthin in dry biomass, unencapsulated beads and chitosan-algae capsules. Prior to this comparison, the stability of astaxanthin under different storage conditions was evaluated to determine the effects of different oxidative stresses such as light, exposure to oxygen in air, and temperature. Since the *H.pluvialis* is used in the form of dried biomass for aquaculture feeds or in extract form for human use, the stability of astaxanthin in both dry algal biomass and acetone extract under different storage conditions were investigated.

The result of astaxanthin content in *H.pluvialis* biomass measured under different storage conditions, during the 24 weeks of the study are shown in Figure 4.5. Minimal changes of the astaxanthin contents were observed in the first two week of storage at all conditions. Loss of astaxanthin content started to occur after the second week at different rates depending on the storage conditions. The best storage condition was under nitrogen atmosphere at -18 ^{O}C in the dark. The minimum loss of astaxanthin of only 8% was observed at this condition after 24 weeks. This was followed by the storage condition under air atmosphere at -18 ^{O}C in the dark, where an approximate loss of 16% of astaxanthin was observed after 24 weeks.

The astaxantin content in the acetone extract of *H.pluvialis* stored under different conditions are shown in Figure 4.6. The results show that astaxanthin disappeared by the third weeks when stored in the presence of light at 30 ^{O}C both under nitrogen atmosphere and air atmosphere. The rate of astaxanthin content loss under the other storage conditions was similar to those of the dried algae biomass, possibly because the dry biomass of the algae acts as natural barrier for astaxathin from light exposure. Similar to those of the dry biomass, the most suitable conditions for the storage of *H.pluvialis* extract was the storage under nitrogen atmosphere at -18 ^{O}C in the dark, followed by the storage under air atmosphere at -18 ^{O}C in the dark.

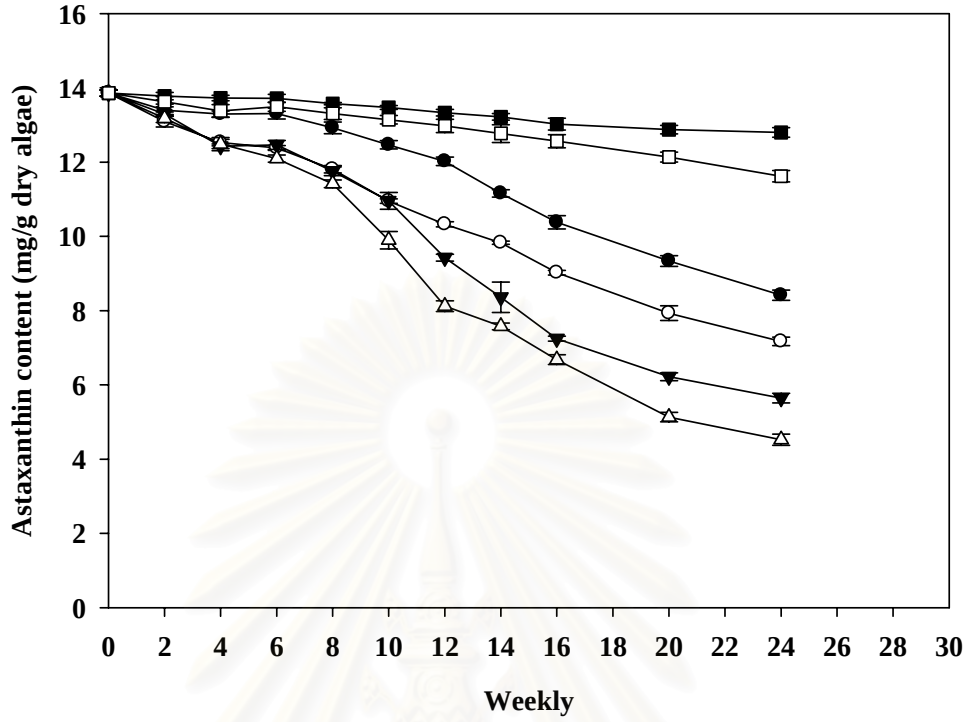


Figure 4.6 Astaxanthin content in *H. pluvialis* stored under different conditions

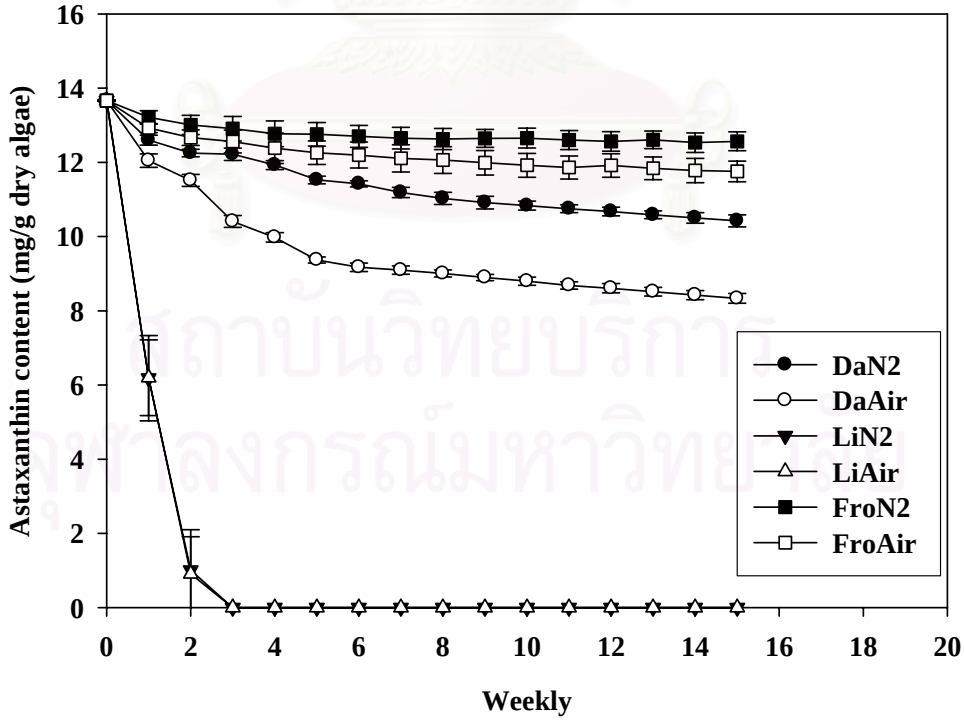


Figure 4.7 Astaxanthin content in acetone extract *H. Pluvialis* stored under different conditions.

4.4 Comparisons of stability of astaxanthin in dry biomass, beads, and capsules

4.4.1 Stability under accelerated condition (T=80 ^{O}C)

For a quick preliminary determination of the ability of chitosan encapsulation to protect the stability of astaxantin in *H.pluvialis* cells, the stability of the compounds was evaluated under an accelerated condition (T=80 ^{O}C). The chitosan coated capsules were stored in an oven at temperature of 80 ^{O}C and the amount of astaxanthin was measured and compared with that of the beads and dry biomass stored at the same condition. The results shown in Figure 4.8 indicated that astaxanthin in dry biomass, beads, and capsules is very sensitive to high temperature condition. The stability of astaxanthin in capsule was however higher than those of the beads and dry biomass. This demonstrated that the chitosan matrix protects the astaxanthin from thermal degradation and could potentially be used for enhancing the stability of the compounds. In the next section, the results for the stability of the compound under long term normal storage conditions would be presented.

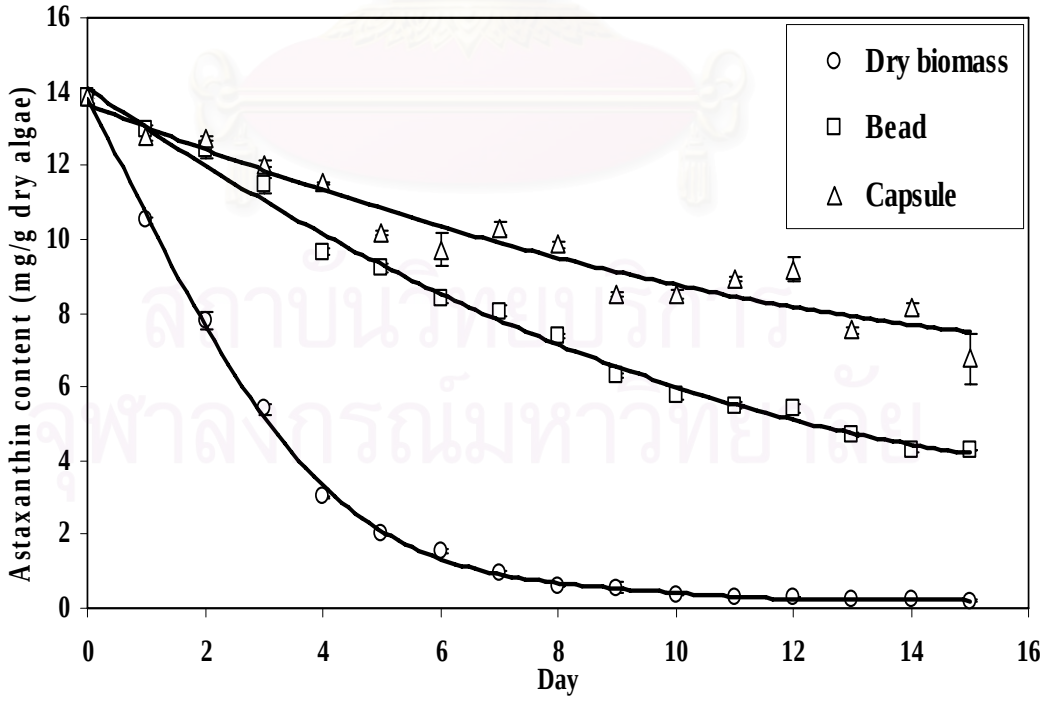


Figure 4.8 Astaxanthin content in *H. pluvialis* of dry biomass, beads, and capsules stored at 80 ^{O}C.

4.4.2 Stability of astaxanthin under normal storage conditions

The ability of encapsulation of *H. pluvialis* algae with chitosan matrix was evaluated under different storage conditions. The astaxanthin contents in dry biomass, beads, and capsules were measured periodically during the 24 weeks of the study and the results are shown in Figure 4.9. It is demonstrated that after 24 weeks under all different storage conditions, the content of astaxanthin in unencapsulated beads was higher than that of dry biomass and the astaxanthin content in the chitosan encapsuled beads was higher than the unencapsulated beads. The percentages of astaxanthin loss at week 24 for all sample conditions were summarized in Table 4.1. From these results, it can be concluded that the formation of algal cells into spherical beads could protect the cells from oxidative stresses due to the decrease in the contact area. In addition, chitosan matrix could provide additional protective layers for the compound encapsulated and therefore resulted in the higher stability. Other than providing the protective film, chitosan has been shown to possess scavenging ability for hydroxy radicals inhibits lipid peroxidation of phosphatidylcholine and linoleate liposomes in vitro (Xue et al., 1998; Chiang et al., 2000; Xie et al., 2001; Jeon etal., 2003). This could therefore be another possibility attributed to the enhanced stability of the encapsulated beads

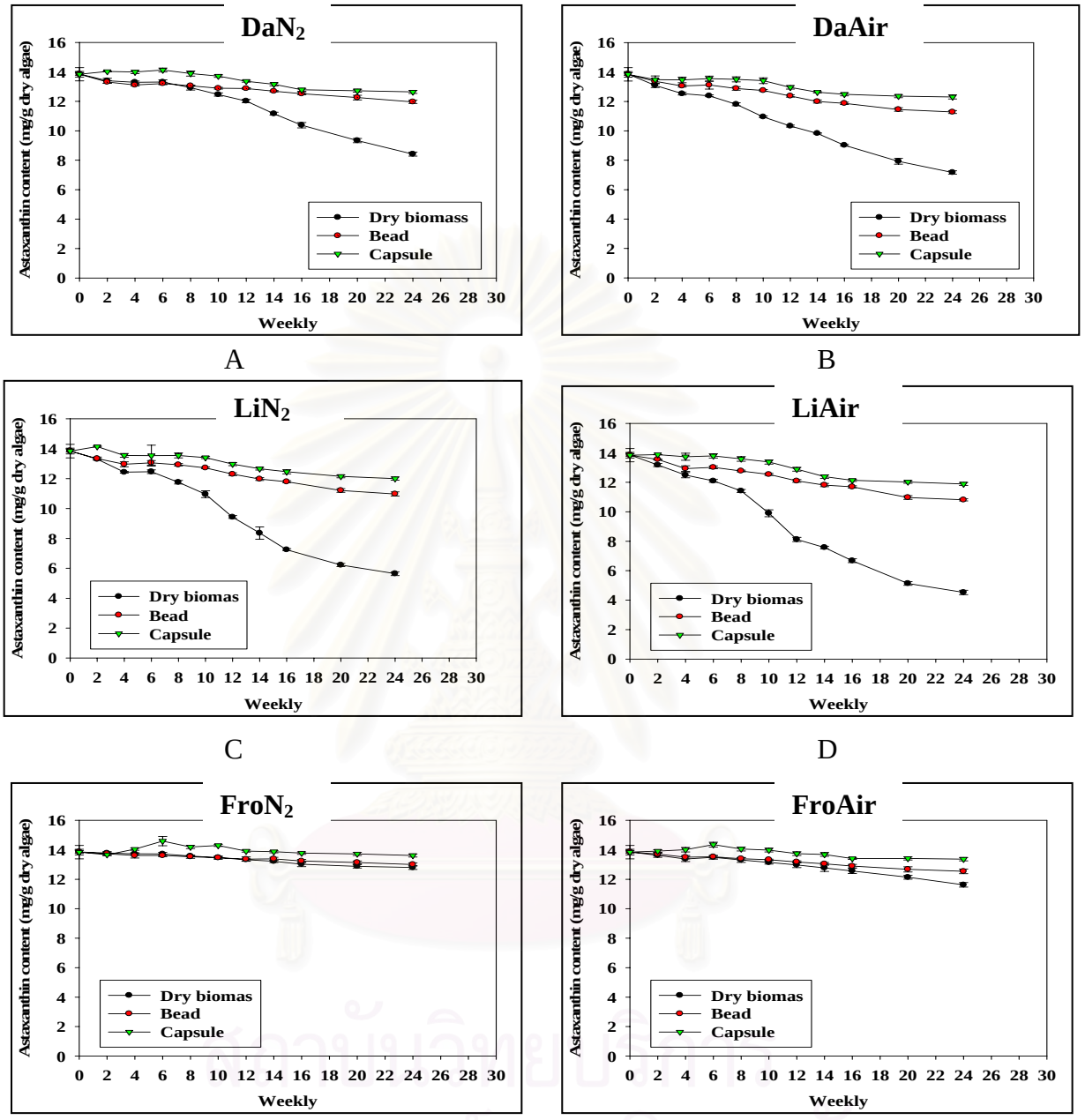


Figure 4.9 Comparison of the stability of astaxanthin in dry biomass, beads, and capsules under different conditions. (A) 30 ^{O}C under nitrogen atmosphere in the dark; (B) 30 ^{O}C under air atmosphere in the dark; (C) 30 ^{O}C under nitrogen atmosphere under light exposure; (D) 30 ^{O}C under air atmosphere under light exposure; (E) -18 ^{O}C under nitrogen atmosphere in the dark; (F) -18 ^{O}C under air atmosphere in the dark.

Table 4.1 Percentage of astaxanthin loss of dry biomass beads and capsules under different storage conditions after 24 weeks.

Algae	Storage condition (%)					
	Dark at 30^{O}C		Light exposure at 30^{O}C		Frozen at -18^{O}C in the dark	
	N2	Air	N2	Air	N2	Air
Dry biomass	39.3	48.3	59.3	67.4	7.6	16.2
Bead	13.6	18.5	20.8	21.9	6.1	9.5
Capsule	6.3	8.6	10.3	11.0	1.3	2.7

4.5 Antioxidant activity under different storage conditions

Beside the astaxanthin content, the quality of the extract of *H. pluvialis* dry biomass, beads, and capsules stored under different conditions were evaluated by measuring the antioxidant activity. The procedure for antioxidant test was explained in chapter 3 in which the radical-scavenging activity of antioxidants against free radicals, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, was measured at 734 nm after a 10 min of incubation in the dark at room temperature. The antioxidant activity of the extract was quantified by the IC_{50} value, which is the concentration of the extract producing 50 % reduction of the radical absorbance. The antioxidant activities of chitosan in dry biomass, bead, and capsule were measured at 0 week and 24 weeks and the results are shown in Figure 4.10. After the 24 weeks of experiment, the IC_{50} values of extract from dry biomass, bead and capsule increased (the antioxidant activity decrease) under all storage conditions. As expected, under light condition, the IC_{50} increased most significantly and the smallest increase in IC_{50} was found for the dark storage condition at -18 ^{O}C under nitrogen atmosphere. Although under this condition, the algae was protected against light and oxygen, the degradation of astaxanthin was still observed (Figure 4.10 E), possibly due to the fact that such condition employed was not completely free from oxidative stresses. Moisture in the algae cells might play a role in the decrease in antioxidant activity observed. Nevertheless, as shown in Figure 4.10(A-F) for all conditions, the decrease in antioxidant activity was found to be the smallest for the encapsulated beads,

indicating chitosan matrix is attributed to the protection of oxidative conditions such as temperature, light, and oxygen. From these results, it is clear that although the encapsulation process caused a 3% loss in antioxidant activity, the long term study showed that encapsulation of algae with chitosan film could enhance the stability of the algae at various storage conditions.

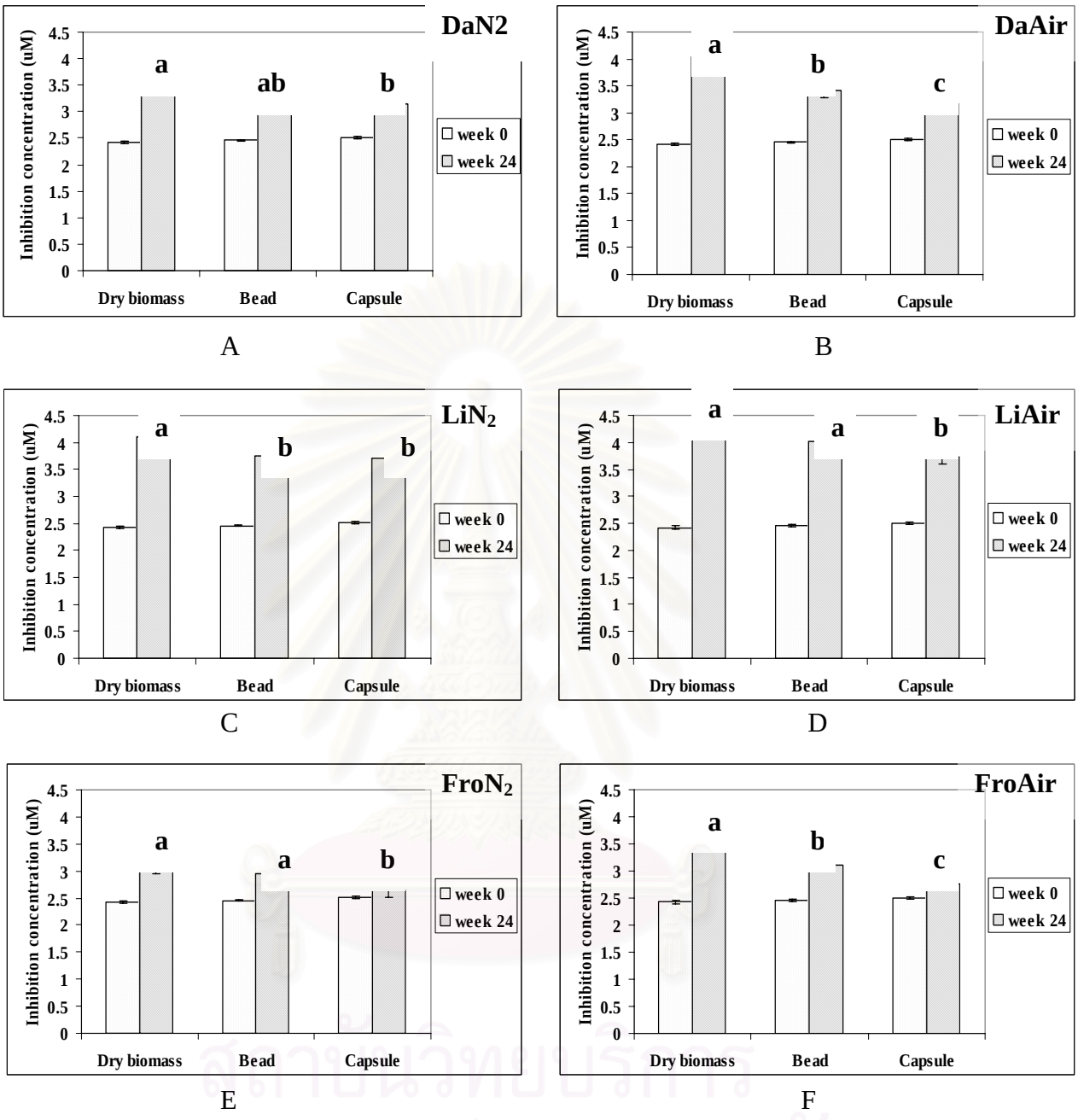


Figure 4.10 antioxidant activities of astaxanthin in dry biomass, bead and capsule under different condition at 0 week and 24 weeks. (A) 30 ^{O}C under nitrogen atmosphere in the dark; (B) 30 ^{O}C under air atmosphere in the dark; (C) 30 ^{O}C under nitrogen atmosphere and light exposure; (D) 30 ^{O}C under air atmosphere and light exposure; (E) -18 ^{O}C under nitrogen atmosphere in the dark; (F) -18 ^{O}C under air atmosphere in the dark. * Different alphabets a,b,c indicates statistically significant difference ($P \leq 0.05$).

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. Encapsulation of *H.pluvialis* beads by immersion of algal beads repeatedly into chitosan solution resulted in algal capsules of reasonably uniform size and weight.
2. The encapsulation technique employed in this study did not cause significant loss in biomass and astaxanthin content. However, 3% decrease in antioxidant activity was found as a result of the encapsulation process.
3. The best storage condition for *H.pluvialis* was found to be under nitrogen atmosphere at -18 ^{O}C in the dark. At this condition, only 8% loss of astaxanthin content was found after 24 weeks of storage.
4. Under accelerated condition at 80 ^{O}C, chitosan matrix protects astaxanthin from thermal degradation. This result suggests the potential use of encapsulation of *H.pluvialis* cells in chitosan for enhancing the stability of the compound.
5. Under various storage conditions, chitosan matrix provided protective layers for *H.pluvials* astaxanthin from oxidative stresses, and thus enhanced the stability of the compound.

5.2 Recommendations

1. The results in this study demonstrated the feasibility of encapsulation of *H.pluvialis* in chitosan film to enhance the stability of astxanthin and the antioxidant activity of the extract. The mechanism that controls the stability of the compound is important information for the development of the encapsulated beads. From the results of this study, the enhancement in the stability could possibly be the result of the formation into spherical beads which provide the protection of the algae cells from oxidative stress, and/or the result of the resistance provided by the chitosan film. The relative importance of these effects on the astaxanthin stability enhancement should be determined by investigating the stability of algae beads of varying size and chitosan film thickness.
2. Based upon the mechanism study, the optimal conditions for preparation of algae capsules should be determined. The variables suggested for the future study are such as the bead size, the concentration of chitosan solution, the degree of deacetylation of chitosan, the concentration of crosslinking agent, the number of film layers, methods of encapsulation, and types of polymers used for encapsulation.

REFERENCES

Gouveia, L., and Empis, J., Relative stabilities of microalgal carotenoids in microalgal extracts, biomass and fish feed: effect of storage condition. Innovative Food Science and Emerging Technologies (4) 2003: 227-233.

Ciapara, I. H., Valenzuela, L. F., Goycoolea, F. M., and Monal, W. A., Microencapsulation of astaxanthin in a chitosan matrix. Carbohydrate Polymers 56 (2004): 41-45.

Yuan, J. P., and Chen, F., Purification of *trans*-astaxanthin from a high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis*. Food Chemistry 68 (2000): 443-448.

Kobayashi, M., Kurimura, Y., Kakizono, T., Nishio, N., and Tsuji, Y., Morphological Changes in the Life Cycle of the Green Alga *Haematococcus pluvialis*. Journal of Fermentation and Bioengineering 84, 1 (1997), 94-97.

Richard, C., Astaxanthin - Antioxidant from the Sea [online]. 1999. Available from: http://www.naturalhealthweb.com[2005, January 20].

Muzzarelli, M., University of Southern Mississippi Department Polymer of Science [online]. 2000. Available from: http://www.psrc.usm.edu[2005, January 20].

Amerchol., Amerchol Expands Methyl Glucoside Capacity to Meet Worldwide Market Demand[online].1998.
Available from: http://www.chitosan.com.cn/chitosan/news[2004, November 8].

Lang, G., Wendel, H. & Konrad, E. Cosmetic Composition Based upon Chitosan Derivatives as well as Processes for the Production Thereof. US Patent 4,528,283 (1985).

Anon., Use of Chitosan as Preservative Coating and Biofungicide for the Preservation of Fresh Fruits and Vegetables[online]. 1999.
Available from: http://aceis.agr.ca/icar/docs/96000269.html[2004, November 8].

Hirano, S., Chitin biotechnology applications. *Biotechnol. Annu. Rev*. 2 (1996) , 237-258

Kanauchi, O., Deuchi, K., Imasato, Y., Shizukuishi, M. & Kobayashi, E., Mechanism for the inhibition of fat digestion by chitosan and for the synergistic effect of ascorbate. Biosci. Biotechnol. Biochem.59, 5 (1995), 786-790.

CECON Consulting Group Science and Engineering Consulting Network., Controlled Release Technology and Microencapsulation Consulting[online]. 1996.
Available from: http://cecon.com/res/microencap_2.html[2004, November 8].

Lertsutthiwong, P., Chitosan as a dry strength agent for paper. Appita Journal. 55, 3 (2002), 208-212.

Tripathi, U., Rao, S. R., and Ravishankar, G.A., Biotransformation of phenylpropanoid compounds to vanilla flavor metabolites in cultures of *Haematococcus pluvialis*. Process Biochemistry 38 (2002), 419-426.

Poncelet, D., Babak. VG., Neufeld. RJ., Goosen. MF., Burgraski. B., Advanced Colloid Science (1998).

Kaya, V. M., and Picard, G., Stability of chitosan gel as entrapment matrix of viable *Scenedesmus bicellularis* cells immobilized on screens for tertiary treatment of wastewater. Bioresource Technology 56 (1996), 147-155.

Hata, N., Ogbonna, J. C., Hasegawa, Y., Taroda, H., and Tanaka, H., Production of astaxanthin by *Haematococcus pluvialis* in a sequential heterotrophic-photoautotrophic culture. Journal of Applied Phycology 13 (2001), 395-402.

Zhang, XW., Gong, X-D., and Chen, F., Kinetic models for astaxanthin production by high cell density mixotrophic culture of the microalga *Haematococcus pluvialis*. Journal of Industrial Microbiology and Biotechnology 23 (1999), 691-696.

Sinha, V. R., Singla, A. K., Wadhawan, S., Kaushik, R., and Kumria, R., Chitosan microspheres as a potential carrier for drugs. International Journal of Pharmaceutich 274 (2004), 1-33.

Jameela, S. R., Kumary, T. V., Lal, A. V., and Jayakrishnan, A., Progesterone-loaded chitosan microspheres: a long acting biodegradable controlled delivery system. Journal of Controlled Release 52 (1998), 17-24.

Lamosa, M. L. L., Lopez, C. R., Jato, J. L. V., Alonso, M. J., Design of microencapsulated chitosan microspheres for colonic drug delivery. Journal of Controlled Release 52 (1998), 109-118.

Ko, J. A., Park, H. J., Hwang, S. J., Park, J. B., and Lee, J. S., Preparation and characterization of chitosan microparticles intended for controlled drug delivery. International Journal of Pharmaceutics 249 (2002), 165-174.

Sezer, A.D., and Akbuga, J., Controlled release of piroxicam from chitosan beads. International Journal of Pharmaceutics 121 (1995), 113-116.

Park, J. H., Ye, M., and Park, K., Biodegradable polymers for microencapsulation of drugs. Molecules 10 (2005), 146-161.

Genta, I., Costantini, M., Asti, A., Conti, B., and Montanari, L., Influence of glutaraldehyde on drug release and mucoadhesive properties of chitosan microspheres. Carbohydrate 36 (1998), 81-88.

Sriamornsak, P.,and Nunthanid, J., Calcium pectinate gel beads for controlled release drug delivery: I. Preparation and in vitro release study. International Journal of Pharmaceutics 160 (1998), 207-212.

Pinto, M. M. M., Raposi, M. F. J., Bowen, J., Young, A. J., and Morais, R., Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-avilability. Journal of Applied Phycology 13 (2001), 19-24.

Joo, D. S., Cho, M. G., Lee, J. S., Park, J. H., Kwak, J. K., Han, Y. H., and Bucholz, R., New strategy for the cultivation of microalgae using microencapsulation. J. Microencapsulation 18, 5 (2001), 567-576.

Poberta, R., Nicolegrini, P., Nana, P., Ananth P., Min, Y., and Catherine, R., Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine 26 (1999), 1231-1237.

Cinar. I., Storage stability of enzyme extracted carotenoid pigments from carrots**.** Electronic Journal of Environmental, Agricultural and Food Chemistry 3, 1(2004), 609-616.

Xue. C. H., Yu. G. L., Hirata. T., Terao. J., Lin. H., Biosci. Biotechnol. Biochem. 2(1998), 206.

Chiang. M. T., Yao. H. T., Chen. H. C., Biosci. Biotechnol. Biochem. 5(2000), 965.

Xie. W., Xu. P., Liu. Q., Antioxidant activity of water-soluble chitosan derivatives. Bioorganic & MedicinalChemistry Letters 11(2001), 1699-1701.

Jeon. T., Hwang. S.G., Park. N.G., Jung. Y.R., Shin. S.I., Choi. S.D., Park. D.K., Antioxidative effect of chitosan on chronic carbon tetrachloride induced hepatic injury in rats. Toxicology 187 (2003), 67-73.

APPENDICES

Appendix A

EXPERIMENTAL DATA ANALYSIS

A-1 Standard calibration curve for spectroscopic determination of astaxanthin

Table A-1.1 Standard calibration curve data of Astaxanthin

Concentration of astaxanthin (mM)	Absorbance at 435 nm.			
	Exp.1	Exp.2	Exp.3	Average
0.0184	2.468	2.483	2.496	2.482
0.00368	0.527	0.563	0.550	0.547
0.002208	0.360	0.358	0.365	0.361
0.001472	0.179	0.206	0.198	0.194
0.0005888	0.071	0.075	0.079	0.075
0.00023552	0.033	0.031	0.034	0.033

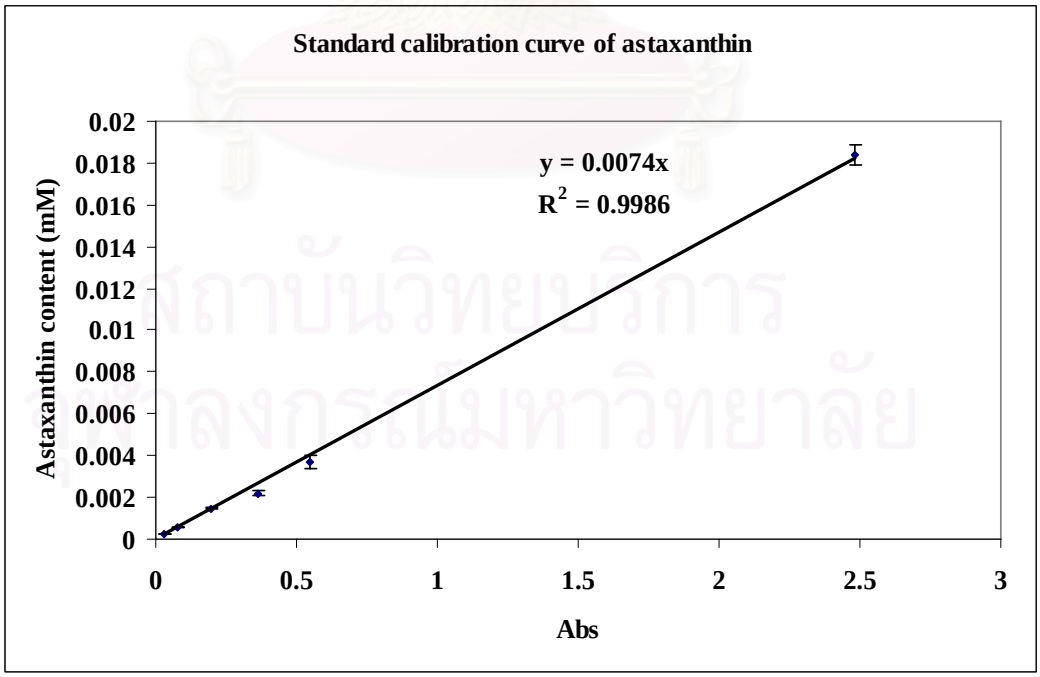


Figure A-1 Standard calibration curve data of astaxanthin

Appendix B

B-1 Antioxidant activities

Antioxidant activity is an important property for determination of the stability of astaxanthin. In this experiment, the antioxidant activities of dry biomass, beads, and capsules against 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS method) free radicals were measured. The reduction in the absorbance of ABTS solution in ethanol with starting absorbance of 0.70 (±0.02) at the wavelength of 734 nm was measured, when series of samples of different concentrations were added to the free radical solution at the ratio of sample to ABTS solution of 1:2 by volume, after a 10 min. incubation period at 30 °C. The percentages inhibition (PI%) were calculated from reduction in absorbances which were then plotted versus the concentration of the sample to determine the value of IC_{50}, the concentration of the sample that results in 50% reduction in the ABTS absorbance.

B-2 Sample calculation for astaxanthin antioxidant activity of dry biomass (week 0)

Table B-2.1 Astaxanthin antioxidant activity of dry biomass in 0 week

Conc. (μM)	Abs	PI%
4.29	0.065	86.11
3.43	0.131	72.01
2.57	0.233	50.21
1.71	0.279	40.38
0.85	0.362	22.65
IC_{50} = 2.43	Ref = 0.468	

The plot of PI (%) versus the concentration of the astaxanthin extract allows the determination of the IC50 as shown in Figure B-1. From the above example, the equation for the plot of PI (%) and concentration was y = 20.578x, which gives the IC_{50} value of 2.43 μM.

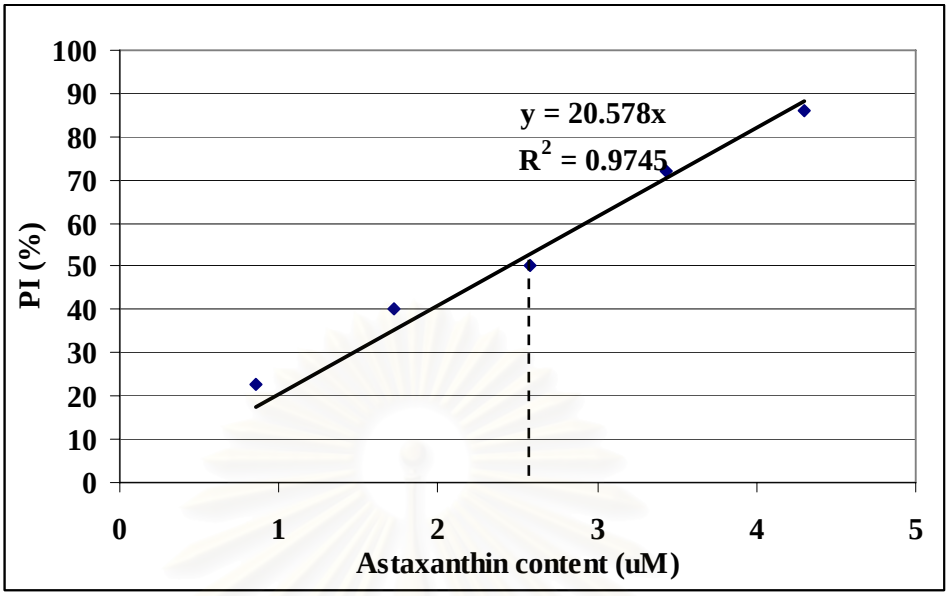


Figure B-1 Sample of IC_{50} determination

APPENDIX C

STATISTICAL ANALYSIS

C-1 The analysis of variance (ANOVA)

Suppose we have a different levels of a single factor that we wish to compare. The observed response at each of the factor levels is a random variable. The data would appear in general form as in Table B-2.1. Each entry of the table, denoted by X_{ij}, represents the j_{th} observation taken under treatment *i*. We consider the case where there is an observation, *n*, for each factor level.

Table C-1.1 Typical data for One-Way classification analysis of variance

Observation	**Treatment**					
	1	2	…	j	…	k
1	X_{11}	X_{12}		X_{1j}		X_{1k}
2	X_{51}	X_{22}		X_{2j}		X_{2k}
.	.	.		.		.
.	.	.		.		.
n	X_{n11}	X_{n22}		X_{njj}		X_{nkk}
Total	X_1	X_2		X_3		X_4

Where : X_1, X_2, X_3 and X_4 is summation of each treatment

We now present test for comparing variances with suppose that the F statistic

$$F = \frac{MS_B}{MS_W} \qquad \text{(C-1.1)}$$

Where: MS_B is mean square between- group

MS_W is mean square within- group

Table C-1.2 The analysis of variance for the one-way classification fixed effects model

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F**
Between factor levels	k-1	$SS_B = \frac{\sum_{j=1}^{k} T_j^2}{n_j} - \frac{T^2}{N}$	$MS_B = \frac{SS_B}{k-1}$	$F = \frac{MS_B}{MS_W}$
Error (within Factor levels)	k(n-1)	$SS_W = SS_T - SS_B$	$MS_W = \frac{SS_w}{k(n-1)}$	
Total	nk – 1	$SS_T = \sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 - \frac{T^2}{N}$		

Where: SS_T = Sum square total

SS_B = Sum square between group

SS_W = Sum square within group

T_j = The total of observations under the j_{th} treatment

n_j = Number of observation

k = Number of treatment

N = The total number of observations

T = The grand total of all observations

Step calculation

Step 1: the following null (H_0) and alternative hypotheses (H_1) are

H_0: $\mu_1=\mu_2=...=\mu_k$ (where k is number of group)

H_1: $\mu_1\neq\mu_2\neq\ldots\neq\mu_k$

Step 2: set significant studentized range (α) = 0.05

Step 3: calculate the value of the F-test statistic from $F = \frac{MS_B}{MS_W}$

Step 4: evaluation F value from the distribution of F table, we must know this values

1) significant studentized range (α) = 0.05
2) df of MS_B , $df_1 = k-1$: where df is degree of freedom
3) df of MS_W , $df_2 = N-k$: where df is degree of freedom

Step 5: compare F value between step3 and step 4

Step 6: If $F_{calculate} < F_{table}$, null hypothesis ($H_0$) is accepted, i.e. there is no evidence of a statistically significant difference between the two groups.

If $F_{calculate} > F_{table}$, null hypothesis ($H_0$) is rejected, i.e. there is evidence of a statistically significant difference between the groups. We must obtain confidence intervals for all pairs of group mean differences, described in the next section.

C-2 Comparing pairs of treatment means

When statistic test F-value in variance analysis table has significant different, Duncan's new multiple range test is used for Comparing pairs of treatment means. The step calculation can following

1. Determine the number of intervals to be constructed

$$ {}^{n}C_2 = n(n-1)/2 \qquad \text{(C-2.1)} $$

where n = number of group

2. Calculate statistic test following

$$ LSR_\alpha = r_\alpha(p, f)\sqrt{\frac{MS_W}{2}\left(\frac{1}{r_i}+\frac{1}{r_j}\right)} \quad ; \ r_i \neq r_j \qquad \text{(C-2.2)} $$

$$ LRS_\alpha = r_\alpha(p, f)\sqrt{\frac{MS_W}{r}} \quad ; \ r_i = r_j \qquad \text{(C-2.3)} $$

Where: LSR = least significant ranges

$r_\alpha(p, f)$ = critical range of Dancan at significant studentized range for α and

p = number of means for range being tested (different level +1) and

f = degree of freedom of error

MS_W = error mean square in table analysis

ri, rj = repeat of treatment i and j

3. Comparison different highest means and lowest means with LSR. If different between highest means and lowest means more than LSR, the means are significantly different.

C-3 Example statistic analysis calculation for Comparisons of astaxanthin content in *Haematococcus pluvialis* before and after encapsulation.

Table C-3.1 Astaxanthin content from dry biomass, bead and capsule

Observations	Treatment (mg/g dry algae)		
	Dry biomass	Bead	Capsule
1	13.84828	14.05497	13.33685
2	13.77673	13.68133	14.21131
3	13.94367	13.78468	13.97282
Total	**41.56868**	**41.52098**	**41.52098**
Average	**13.85623**	**13.84033**	**13.84033**

Calculation step can be following:

1. Degree of freedom between group = k-1 = 3-1 = 2 (k = number of treatment = 3)

2. Degree of freedom in group = k(n-1) = 3*(3-1) = 6 (n = number of observation = 3)

3. Calculate square of the grand total of all observations (T^2)

$= (41.56868+41.52098+41.52098)^2$

$= 15527.81265$

And then, calculate $\frac{T^2}{N} = \frac{15527.81268}{9} = 1725.31252$

4. Calculate $\sum_{j=1}^{k} T_j^2$ which T_j in each treatment are 41.56868, 41.52098 and 41.52098

$$\sum_{j=1}^{k} T_j^2 = (41.56868)^2 + (41.52098)^2 + (41.52098)^2$$

$$= 1727.95517 + 1723.99197 + 1723.99193$$

$$= 5175.93907$$

After that calculate $\frac{\sum_{j=1}^{k} T_j^2}{n} = \frac{5175.93907}{3} = 1725.31302$

5. Calculate SS_B (sum square between group) from equation $SS_B = \frac{\sum_{j=1}^{k} T_j^2}{n_j} - \frac{T^2}{N}$

So $SS_B = 1725.31302 - 1725.31252 = 0.0005056$

6. Calculate $\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = (13.84828)^2 + (13.77673)^2 + (13.94367)^2 +$

$(14.05497)^2 + (13.68133)^2 + (13.78468)^2 +$

$(13.33685)^2 + (14.21131)^2 + (13.97282)^2$

$\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = 1725.81017$

7. Calculate SS_T (sum square total) from equation $SS_T = \sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 - \frac{T^2}{N}$

So $SS_T = 1725.81017 - 1725.31252 = 0.4976539$

8. Calculate SS_W (sum square within group) from equation $SS_W = SS_T - SS_B$

So $SS_W = 0.4976539 - 0.0005056 = 0.4971483$

9. Calculate MS_B (Mean square between- group) from equation $MS_B = \frac{SS_B}{k-1}$

So $MS_B = \frac{0.0005056}{2} = 0.000252789$

10. Calculate MS_W (Mean square within- group) from equation $MS_W = \frac{SS_w}{k(n-1)}$

So $MS_W = \frac{0.4971483}{6} = 0.082858045$

11. Calculate $F_{calculate}$ from equation $F = \frac{MS_B}{MS_W}$

So $F_{calculate} = \frac{0.000252789}{0.082858045} = 0.003050874$

12. Evaluation F value from the distribution of F table (สุรพล อุปดิสสกุล, 2523) with significant studentized range (α) = 0.05, which degree of freedom of MS_B (df1) = k–1 = 2 and degree of freedom of MS_W (df2) = k(n-1) = 3*(3-1) = 6

So $F_{0.05\ (2,6)} = 5.14$

13. $F_{calculate}$ is less than 5.14, thus astaxanthin content in *Haematococcus pluvialis* before and after encapsulation does not significantly affect to astaxanthin content of *Haematococcus pluvialis.*

C-4 Example statistic analysis calculation for Comparisons of astaxanthin content in dry biomass, storage under different storage conditions (at week 24).

Table C-4.1 Astaxanthin content in dry biomass, storage under different storage conditions (at week 24).

Week 24	**Treatment (mg/g dry algae)**					
Observation	**DaN_2**	**DaAir**	**LiN_2**	**LiAir**	**$FroN_2$**	**FroAir**
1	8.54587	7.21033	5.76349	4.38025	12.80687	11.64623
2	8.41867	7.04338	5.50910	4.68234	12.92612	11.45543
3	8.27558	7.25802	5.66015	4.50745	12.66378	11.75752
Total	**25.24011**	**21.51173**	**16.93274**	**13.57004**	**38.39677**	**34.85918**
Average	**8.41337**	**7.17058**	**5.64425**	**4.52335**	**12.79892**	**11.61973**

Calculation step can be following:

1. Degree of freedom between group = k-1 = 6-1 = 5 (k = number of treatment = 6)

2. Degree of freedom in group = k(n-1) = 6*(3-1) = 12 (n = number of observation = 3)

3. Calculate square of the grand total of all observations (T^2)

= $(25.24011+21.51173+16.93274+13.57004+38.39677+34.85918)^2$

= 22653.43456

And then, calculate $\frac{T^2}{N} = \frac{22653.43456}{18} = 1258.52414$

4. Calculate $\sum_{j=1}^{k} T_j^2$ which T_j in each treatment are 25.24011, 21.51173, 16.93274, 13.57004, 38.39677 and 34.85918

$$\sum_{j=1}^{k} T_j^2 = (25.24011)^2 + (21.51173)^2 + (16.93274)^2 +$$

$$(13.57004)^2 + (38.39677)^2 + (34.85918)^2$$

$$= 637.06339 + 462.75463 + 286.71763 +$$

$$184.14597 + 1474.31216 + 1215.16256$$

$$= 4260.15634$$

After that calculate $\frac{\sum_{j=1}^{k} T_j^2}{n} = \frac{4260.15634}{3} = 1420.05211$

5. Calculate SS_B (sum square between group) from equation $SS_B = \frac{\sum_{j=1}^{k} T_j^2}{n_j} - \frac{T^2}{N}$

So $SS_B = 1420.05211 - 1258.52414 = 161.52797$

6. Calculate $\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = (8.54587)^2 + (8.41867)^2 + (8.27558)^2 + (7.21033)^2 +$

$(7.04338)^2 + (7.25802)^2 + (5.76349)^2 + (5.50910)^2 +$
$(5.66015)^2 + (4.38025)^2 + (4.68234)^2 + (4.50745)^2 +$
$(12.80687)^2 + (12.92612)^2 + (12.66378)^2 + (11.64623)^2 +$
$(11.45543)^2 + (11.75752)^2$

$\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = 1420.27402$

7. Calculate SS_T (sum square total) from equation $SS_T = \sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 - \frac{T^2}{N}$

So $SS_T = 1420.27402 - 1258.52414 = 161.74988$

8. Calculate SS_W (sum square within group) from equation $SS_W = SS_T - SS_B$

So $SS_W = 161.74988 - 161.52797 = 0.22191$

9. Calculate MS_B (Mean square between- group) from equation $MS_B = \frac{SS_B}{k-1}$

So $MS_B = \frac{161.52797}{5} = 32.30559$

10. Calculate MS_W (Mean square within- group) from equation $MS_W = \frac{SS_w}{k(n-1)}$

So $MS_W = \frac{0.22191}{12} = 0.01849$

11. Calculate $F_{calculate}$ from equation $F = \frac{MS_B}{MS_W}$

So $F_{calculate} = \frac{32.30559}{0.01849} = 1746.9953$

12. Evaluation F value from the distribution of F table (สุรพล อุปดิสสกุล, 2523) with significant studentized range (α) = 0.05, which degree of freedom of MS_B (df1) = k–1 = 5 and degree of freedom of MS_W (df2) = k(n-1) = 6*(3-1) = 12

So $F_{0.05\,(5,12)} = 3.11$

13. $F_{calculate}$ is more than 3.11, thus astaxanthin content in dry biomass does statistically significant difference between the conditions. We must obtain confidence intervals for all pairs of group mean differences. Which Duncan's new multiple range test is used for Comparing pairs of treatment means.

14. Rearrange average value from high to low

(1)	(2)	(3)	(4)	(5)	(6)
LiAir	**LiN_2**	**DaAir**	**DaN_2**	**FroAir**	**$FroN_2$**
4.5233465	5.6442461	7.1705774	8.4133715	11.619727	12.798924

15. Calculate the number of intervals to be constructed from equation

$${}^{n}C_2 = n(n-1)/2 \quad ; (n = \text{number of group})$$

$$= 6*(6-1)/2$$

$$= 15$$

16. Calculate statistic test from equation

$$LSR_\alpha = r_\alpha(p, f)\sqrt{\frac{MS_W}{r}} \quad ; (LSR = \text{least significant ranges})$$

p = number of means for range being tested (different level +1)

f = degree of freedom of error = k(n-1) = 6*(3-1) = 12

r = repeat of treatment = 3

α = significant studentized range = 0.05

$$\sqrt{\frac{MS_W}{r}} = \sqrt{\frac{0.01849}{3}} = 0.07851$$

p	2	3	4	5	6
$r_{0.05(p,12)}$	3.08	3.23	3.33	3.36	3.4
$LSR_{0.05}$	0.2418149	0.2535916	0.2614428	0.2637981	0.2669385

17. Comparison different highest means and lowest means with LSR. If different between highest means and lowest means more than LSR, the means are significantly different (shown in table C-4.2).

Table C-4.2 Comparison of means between treatment of astaxanthin content in dry biomass, storage under different storage conditions (at week 24).

Different of range	Different mean		$LSR_{0.05}$
(6)-(1)	8.28	>	0.26694*
(6)-(2)	7.15	>	0.26380*
(6)-(3)	5.63	>	0.26144*
(6)-(4)	4.39	>	0.25359*
(6)-(5)	1.18	>	0.24181*
(5)-(1)	7.10	>	0.26380*
(5)-(2)	5.98	>	0.26144*
(5)-(3)	4.45	>	0.25359*
(5)-(4)	3.21	>	0.24181*
(4)-(1)	3.89	>	0.26144*
(4)-(2)	2.77	>	0.25359*
(4)-(3)	1.24	>	0.24181*
(3)-(1)	2.65	>	0.25359*
(3)-(2)	1.53	>	0.24181*
(2)-(1)	1.12	>	0.24181*

* Treatment means have statistically significant difference between the conditions for significant studentized range (α) = 0.05

18. We take alphabet for comparison between treatment

(1)	(2)	(3)	(4)	(5)	(6)
f	e	d	c	b	a

19. In conclusion, astaxanthin content in dry biomass have statistically significant difference under storage different storage conditions (at week 24).

C-5 Example statistic analysis calculation for Comparisons of astaxanthin content in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Table C-5.1 Astaxanthin content in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Observation	**Treatment (mg/g dry algae)**		
Week24	**Cell**	**Bead**	**Capsule**
1	8.54587	11.85292	12.65318
2	8.41867	11.96421	12.52599
3	8.27558	12.07551	12.73268
Total	**25.24011**	**35.89264**	**37.91184**
Average	**8.41337**	**11.96421**	**12.63728**

Calculation step can be following:

1. Degree of freedom between group = k-1 = 3-1 = 2 (k = number of treatment = 3)

2. Degree of freedom in group = k(n-1) = 3*(3-1) = 6 (n = number of observation = 3)

3. Calculate square of the grand total of all observations (T^2)

= $(25.24011+35.89264+37.91184)^2$

= 9809.83165

And then, calculate $\frac{T^2}{N} = \frac{9809.83165}{9}$ = 1089.98129

4. Calculate $\sum_{j=1}^{k} T_j^2$ which T_j in each treatment are 25.24011, 35.89264 and 37.91184

$$\sum_{j=1}^{k} T_j^2 = (25.24011)^2 + (35.89264)^2 + (37.91184)^2$$

$$= 637.06339 + 1288.28128 + 1437.30793$$

$$= 3362.65259$$

After that calculate $\frac{\sum_{j=1}^{k} T_j^2}{n} = \frac{3362.65259}{3} = 1120.88420$

5. Calculate SS_B (sum square between group) from equation $SS_B = \frac{\sum_{j=1}^{k} T_j^2}{n_j} - \frac{T^2}{N}$

So $SS_B = 1120.88420 - 1089.98129 = 30.90290$

6. Calculate $\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = (8.54587)^2 + (8.41867)^2 + (8.27558)^2 +$

$(11.85292)^2 + (11.96421)^2 + (12.07551)^2 +$

$(12.65318)^2 + (12.52599)^2 + (12.73268)^2$

$$\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = 1120.96728$$

7. Calculate SS_T (sum square total) from equation $SS_T = \sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 - \frac{T^2}{N}$

So $SS_T = 1120.96728 - 1089.98129 = 30.98599$

8. Calculate SS_W (sum square within group) from equation $SS_W = SS_T - SS_B$

So $SS_W = 30.98599 - 30.90290 = 0.08308$

9. Calculate MS_B (Mean square between- group) from equation $MS_B = \frac{SS_B}{k-1}$

So $MS_B = \frac{30.90290}{2} = 15.45145$

10. Calculate MS_W (Mean square within- group) from equation $MS_W = \frac{SS_w}{k(n-1)}$

So $MS_W = \frac{0.08308}{6} = 0.01385$

11. Calculate $F_{calculate}$ from equation $F = \frac{MS_B}{MS_W}$

So $F_{calculate} = \frac{15.45145}{0.08308} = 1115.85999$

12. Evaluation F value from the distribution of F table (สุรพล อุปดิสสกุล, 2523) with significant studentized range (α) = 0.05, which degree of freedom of MS_B (df1) = k–1 = 2 and degree of freedom of MS_W (df2) = k(n-1) = 3*(3-1) = 6

So $F_{0.05\,(2,6)} = 5.14$

13. $F_{calculate}$ is more than 5.14, thus astaxanthin content in dry biomass, bead and capsule does statistically significant difference between the treatment. We must obtain confidence intervals for all pairs of group mean differences. Which Duncan's new multiple range test is used for Comparing pairs of treatment means.

14. Rearrange average value from high to low

(1)	(2)	(3)
Cell	**Bead**	**Capsule**
10.37958	12.51539	12.78567

15. Calculate the number of intervals to be constructed from equation

$$^{n}C_2 = n(n-1)/2 \quad ; (n = \text{number of group})$$
$$= 3*(3-1)/2$$
$$= 3$$

16. Calculate statistic test from equation

$$LSR_{\alpha} = r_{\alpha}(p, f)\sqrt{\frac{MS_W}{r}} \quad ; (LSR = \text{least significant ranges})$$

p = number of means for range being tested (different level +1)

f = degree of freedom of error = k(n-1) = 3*(3-1) = 6

r = repeat of treatment = 3

α = significant studentized range = 0.05

$$\sqrt{\frac{MS_W}{r}} = \sqrt{\frac{0.08308}{3}} = 0.06794$$

p	2	3
$r_{0.05(p,6)}$	3.46	3.58
$LSR_{0.05}$	0.23507	0.24322

17. Comparison different highest means and lowest means with LSR. If different between highest means and lowest means more than LSR, the means are significantly different (shown in table C-5.2).

Table C-5.2 Comparison of means between treatment of astaxanthin content in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Different of range	Different mean		$LSR_{0.05}$
(3)-(1)	2.40609	>	0.24322*
(3)-(2)	0.27029	>	0.23507*
(2)-(1)	2.13580	>	0.23507*

* Treatment means have statistically significant difference between the conditions for significant studentized range (α) = 0.05

18. We take alphabet for comparison between treatment

(1)	(2)	(3)
c	b	a

19. In conclusion, astaxanthin content in dry biomass, bead and capsule have statistically significant difference storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

C-6 Example statistic analysis calculation for astaxanthin antioxidant activity in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Table C-6.1 Astaxanthin antioxidant activity in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Observation	**Treatment (mg/g dry algae)**		
Week24	**Cell**	**Bead**	**Capsule**
1	3.52435	3.24275	3.20123
2	3.27375	3.10366	3.06560
3	3.58706	3.31631	3.16216
Total	**10.38516**	**9.66272**	**9.42899**
Average	**3.46172**	**3.22091**	**3.14300**

Calculation step can be following:

1. Degree of freedom between group = k-1 = 3-1 = 2 (k = number of treatment = 3)

2. Degree of freedom in group = k(n-1) = 3*(3-1) = 6 (n = number of observation = 3)

3. Calculate square of the grand total of all observations (T^2)

$= (10.38516+9.66272+9.42899)^2$

$= 868.88617$

And then, calculate $\frac{T^2}{N} = \frac{868.88617}{9} = 96.54291$

4. Calculate $\sum_{j=1}^{k} T_j^2$ which T_j in each treatment are 10.38516, 9.66272 and 9.42899

$$\sum_{j=1}^{k} T_j^2 = (10.38516)^2 + (9.66272)^2 + (9.42899)^2$$

$$= 107.85159 + 93.36824 + 88.90582$$

$$= 290.12566$$

After that calculate $\frac{\sum_{j=1}^{k} T_j^2}{n} = \frac{290.12566}{3} = 96.70855$

5. Calculate SS_B (sum square between group) from equation $SS_B = \frac{\sum_{j=1}^{k} T_j^2}{n_j} - \frac{T^2}{N}$

So $SS_B = 96.70855 - 96.54291 = 0.16565$

6. Calculate $\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = (3.52435)^2 + (3.27375)^2 + (3.58706)^2 +$

$(3.24275)^2 + (3.10366)^2 + (3.31631)^2 +$

$(3.20123)^2 + (3.06560)^2 + (3.16216)^2$

$\sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 = 96.79659$

7. Calculate SS_T (sum square total) from equation $SS_T = \sum_{j=1}^{k}\sum_{i=1}^{n_j} X_{ij}^2 - \frac{T^2}{N}$

So $SS_T = 96.79659 - 96.54291 = 0.25368$

8. Calculate SS_W (sum square within group) from equation $SS_W = SS_T - SS_B$

So $SS_W = 0.25368 - 0.16565 = 0.08804$

9. Calculate MS_B (Mean square between- group) from equation $MS_B = \frac{SS_B}{k-1}$

So $MS_B = \frac{0.08804}{2} = 0.08282$

10. Calculate MS_W (Mean square within- group) from equation $MS_W = \frac{SS_w}{k(n-1)}$

So $MS_W = \frac{0.08804}{6} = 0.01467$

11. Calculate $F_{calculate}$ from equation $F = \frac{MS_B}{MS_W}$

So $F_{calculate} = \frac{0.08282}{0.01467} = 5.64461$

12. Evaluation F value from the distribution of F table (สุรพล อุปดิสสกุล, 2523) with significant studentized range (α) = 0.05, which degree of freedom of MS_B (df1) = k–1 = 2 and degree of freedom of MS_W (df2) = k(n-1) = 3*(3-1) = 6

So $F_{0.05\,(2,6)} = 5.14$

13. $F_{calculate}$ is more than 5.14, thus astaxanthin antioxidant activity in dry biomass, bead and capsule does statistically significant difference between the treatment. We must obtain confidence intervals for all pairs of group mean differences. Which Duncan's new multiple range test is used for Comparing pairs of treatment means.

14. Rearrange average value from high to low

(1)	(2)	(3)
Capsule	**Bead**	**Cell**
3.14300	3.22091	3.46172

15. Calculate the number of intervals to be constructed from equation

$${}^{n}C_2 = n(n-1)/2 \quad ; (n = \text{number of group})$$

$$= 3*(3-1)/2$$

$$= 3$$

16. Calculate statistic test from equation

$$LSR_{\alpha} = r_{\alpha}(p, f)\sqrt{\frac{MS_W}{r}} \quad ; (LSR = \text{least significant ranges})$$

p = number of means for range being tested (different level +1)

f = degree of freedom of error = k(n-1) = 3*(3-1) = 6

r = repeat of treatment = 3

α = significant studentized range = 0.05

$$\sqrt{\frac{MS_W}{r}} = \sqrt{\frac{0.01467}{3}} = 0.06994$$

p	2	3
$r_{0.05(p,6)}$	3.46	3.58
$LSR_{0.05}$	0.24198	0.25037

17. Comparison different highest means and lowest means with LSR. If different between highest means and lowest means more than LSR, the means are significantly different (shown in table C-6.2).

Table C-6.2 Comparison of means between treatment of astaxanthin content in dry biomass, bead and capsule storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24).

Different of range	Different mean		$LSR_{0.05}$
(3)-(1)	0.31872	>	0.25037*
(3)-(2)	0.24081	<	0.24198
(2)-(1)	0.07791	<	0.24198

* Treatment means have statistically significant difference between the conditions for significant studentized range (α) = 0.05

18. We take alphabet for comparison between treatment

(1)	(2)	(3)
b	ab	a

19. In conclusion, astaxanthin content in dry biomass and capsule have statistically significant difference storage at 30 ^{O}C under nitrogen atmosphere in the dark (at week 24), whereas bead has not statistically significant difference for dry biomass and capsule.

VITA

Mr. Prachanart Kittikaiwan was born on 14 August, 1981 in Ratchaburi, Thailand. He received a Bachelor's Degree of Industrial Chemistry from the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang in 2002. After then he subsequently completed the requirements for a Master's Degree in Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University in 2006.