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Encapsulation of *Haematococcus pluvialis* using chitosan for astaxanthin stability enhancement

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Abstract

Astaxanthin is receiving commercial interest due to its use as a preferred pigment in aquaculture feeds. Its antioxidant 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. *Haematococcus pluvialis* were formed into beads, which were then coated with 5 layers of chitosan film, resulting in chitosan-algae capsules that have a 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. The results of stability under different storage conditions showed that although encapsulation caused 3% loss of antioxidant activity, the longer term stability of the dried algae biomass, beads, and capsules indicated that encapsulation of *H. pluvailis* in chitosan film was capable of protecting the algae cells from oxidative stresses.
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Keywords: Encapsulation; *Haematococcus pluvialis*; Chitosan; Astaxanthin; Stability; Antioxidant activity

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-β′-carotene-4-4′-dione) is a ketocarotenoid, used as a preferred pigment in aquaculture feeds. Due to high antioxidant activity, it 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, Gong, & Chen, 1999). The compound can be produced by a number of microorganisms, such as the green algae *Haematococcus pluvialis* and *Chlorella zofingiensis*, the red yeast *Phaffia rhodozyma* and the marine bacterium *Agrobacterium aurantiacum* (Yuan & Chen, 2000). The microalgae *H. puvialis*, however, is believed to be the world's richest source of astaxanthin.

As most carotenoids, astaxanthin is a highly unsaturated molecule and thus it is highly sensitive to high temperature, light, and oxidative conditions which may promote the isomerization of astaxanthin into *cis* form which possesses less activity than their corresponding *trans* configuration (Higuera-Ciapara, Valenzuela, Goycoolea, & Monal, 2004).

The production and accumulation of intracellular astaxanthin in *H. pluvialis* 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

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conditions such as high light, temperature, and oxygen exposure, as was reported by Gouveia and Empis (2003). On the other hand, the thick impermeable cell walls make the carotenoids unavailable when the whole cells are taken due to the low product release (Pinto, Raposi, Bowen, Young, & Morais, 2001). Therefore in manufacturing the agal cells for aquaculture or for human consumption, algal cells are generally homogenized to enhance the product bioavailability. This in turns lower the product stability.

Generally, when handling substances 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 (Higuera-Ciapara et al., 2004). 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. First the characteristics of these capsules were determined, such as efficiency of encapsulation, size and size distribution, membrane thickness, and the amount of astaxanthin and antioxidant activity lost during process. Then, the stability of the encapsulated cells was evaluated under different storage conditions both in terms of astaxanthin content and antioxidant activity of the extract.

2. Experiments

2.1. Material and chemicals

Astaxanthin standard, chitosan (80 mol% deacethylation), 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 peroxidisulfate) were obtained from Sigma-Aldrich (Poole, Dorset, UK). The *H. 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.

2.2. 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 *H. pluvialis* powder (1 ml of water per g dry algae powder). The mixture was then rolled into a long cylindrical rod of approximately 0.4 cm in diameter and 20 cm long. The rod was then placed horizontally on a traditional Chinese pill making apparatus which was used for slicing the algal rod into several small pellets and molding them into spherical beads. The Chinese pill making apparatus is a rectangular cutting board whose surface was carved out to obtain several small connected troughs. The edges of the adjacent troughs form long ridges which functioned as cutters when the horizontal wood piece on the top of the board was sliced over. The algal rod placed on the apparatus was then cut into small pellets and each of the pellets was molded along each through as the wood piece was sliced over to form spherical beads. 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 h to improve the gel strength. The capsules were then washed 3 times with distilled water and air dried.

2.3. Characterization of chitosan 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. For the size and weight of the capsules, the diameters of 150 chitosan coated beads were measured with a vernier ruler and the weights were measured using an electric balance. 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. For 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.

2.4. Evaluation of astanxanthin stability 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 1. For the acetone extracts, only Runs #2–7 were tested, and the measurement of stability was continued for 9 weeks. In the study of astaxanthin stability in 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

Table 1
Storage conditions tested for the stability of the algae powder, beads, and capsules

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

extract were conducted every week. All experiments were performed in triplicates.

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 of up to 24 weeks 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 °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 (6000 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 exposure. 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 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.

2.5. Analytical determination of astaxanthin content

For the determination of astaxanthin content in the algal samples, 20 mg of the dry biomass sample was extracted repeatedly with 5 ml of acetone. The process was 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 beads were first crushed using a mortar and a pestle, 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 spectrophotometric method adapted from Choi, Yun, and Park (2002), in which the absorbance of the extract was measured at 475 nm.

2.6. Antioxidant analysis of H. pluvialis extracts

The antioxidant activity of the *H. 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 be found from the plot of percent

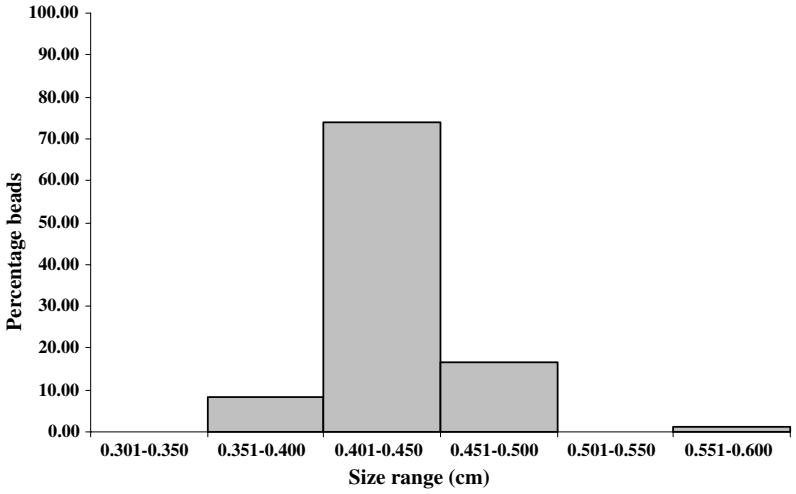

Fig. 1. Size distribution of encapusulated beads.

inhibition (PI) versus the corresponding concentration of astaxanthin, in which the values of PI can be calculated using the following equation:

$$\mathrm{PI}(\%) = [1 - (A_t/A_r)] \times 100$$

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

3. Results and discussion

3.1. 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 Fig. 1. All capsules are below 0.600 cm in diameter. Out of these, 8.23% are between 0.351 and 0.400 cm, 74.05% were between 0.401 and 0.450 cm, 16.46% were between 0.451 and 0.500 cm, and 1.27% were between 0.551 and 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 Fig. 2. All capsules are below 0.08 g. Out of these, 0.65% are between 0.0301 and 0.0400 g, 20.65% are between 0.0401 and 0.0500 g, 55.48% are between 0.0501 and 0.0600 g, 18.06% are between 0.0601 and 0.0700 g and 5.16% are between 0.0701 and 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.

The other characteristics of chitosan beads that is important for the purpose of maintaining astaxanthin stability would be the ability of the chitosan film to 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. Fig. 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.

3.2. Evaluation of encapsulation method

3.2.1. Encapsulation efficiency

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 found to be higher than 99%.

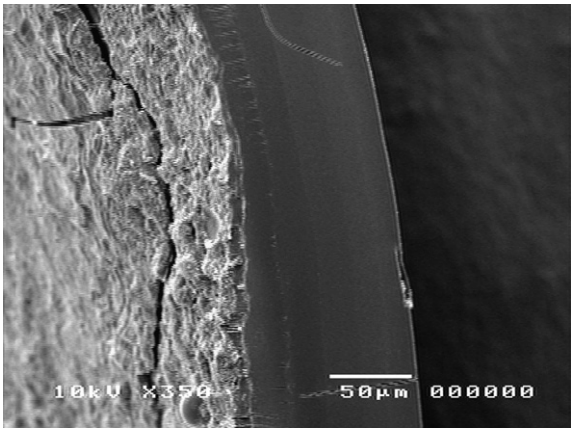

Fig. 3. Scanning electron micrograph (SEM) of encapsulated beads.

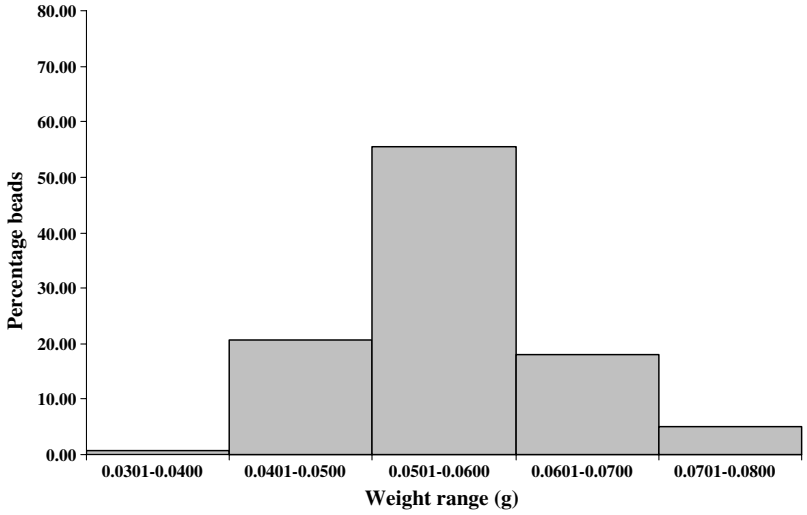

Fig. 2. Weight distribution of encapsulated beads.

3.2.2. 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 Fig. 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 Fig. 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.

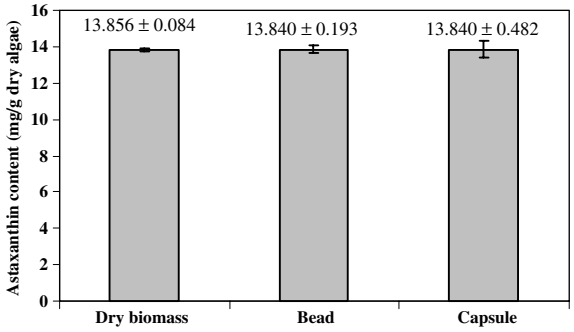

Fig. 4. Astaxanthin content in *H. pluvialis* before and after encapsulation.

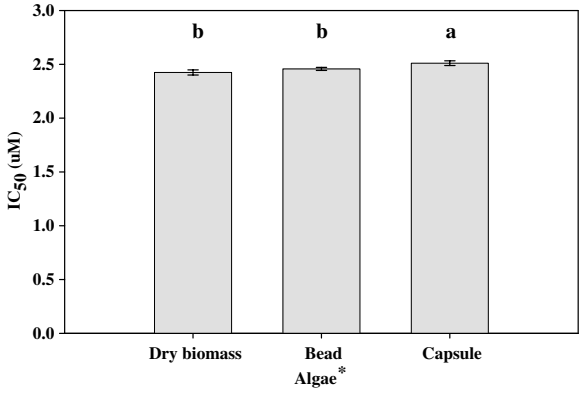

Fig. 5. Antioxidant activity of astaxanthin in *H. pluvialis* biomass, beads, and capsules. *Different alphabets a,b indicates statistically significant difference ($P \leqslant 0.05$).

3.3. Evaluation of astaxanthin stability under different storage conditions

The enhanced stability of astaxanthin during storage is a very important criteria for the encapsulated algae beads to be attractive and acceptable (Cinar, 2004). In this study, the comparison was 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.

The result of astaxanthin content in *H. pluvialis* biomass measured under different storage conditions, during the 24 weeks of the study are shown in Fig. 6. 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 most suitable storage condition was under nitrogen atmosphere at −18 °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

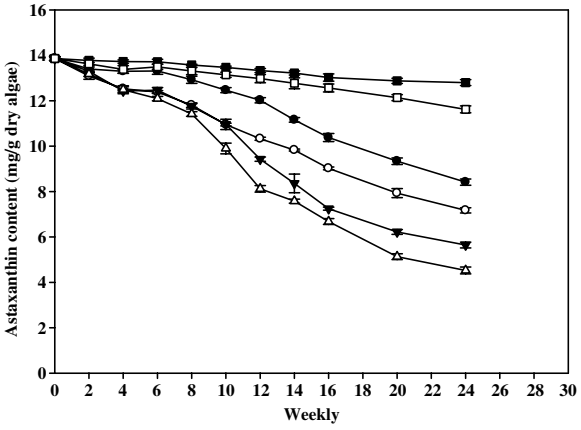

Fig. 6. Astaxanthin content in *H. pluvialis* stored under different conditions.

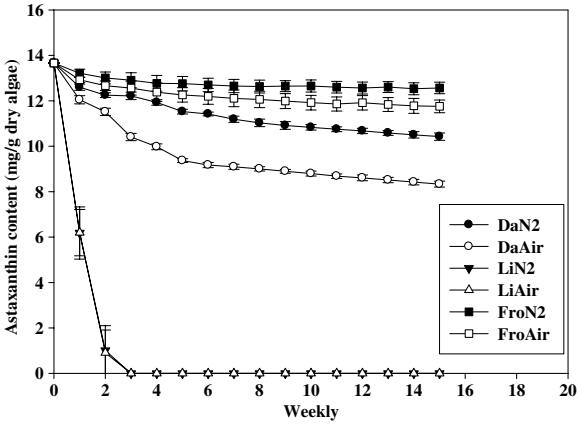

Fig. 7. Astaxanthin content in acetone extract *H. pluvialis* stored under different conditions.

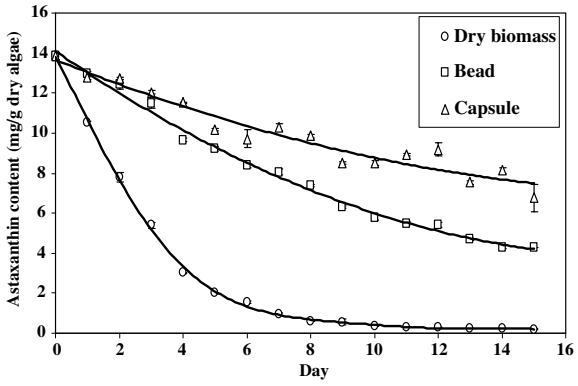

Fig. 8. Astaxanthin content in *H. pluvialis* of dry biomass, beads, and capsules stored at 80 °C.

atmosphere at −18 °C in the dark, where an approximate loss of 16% of astaxanthin was observed after 24 weeks.

The astaxanthin content in the acetone extract of *H. pluvialis* stored under different conditions are shown in Fig. 7. The results show that astaxanthin disappeared by the third weeks when stored in the presence of light at 30 °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 °C in the dark, followed by the storage under air atmosphere at −18 °C in the dark.

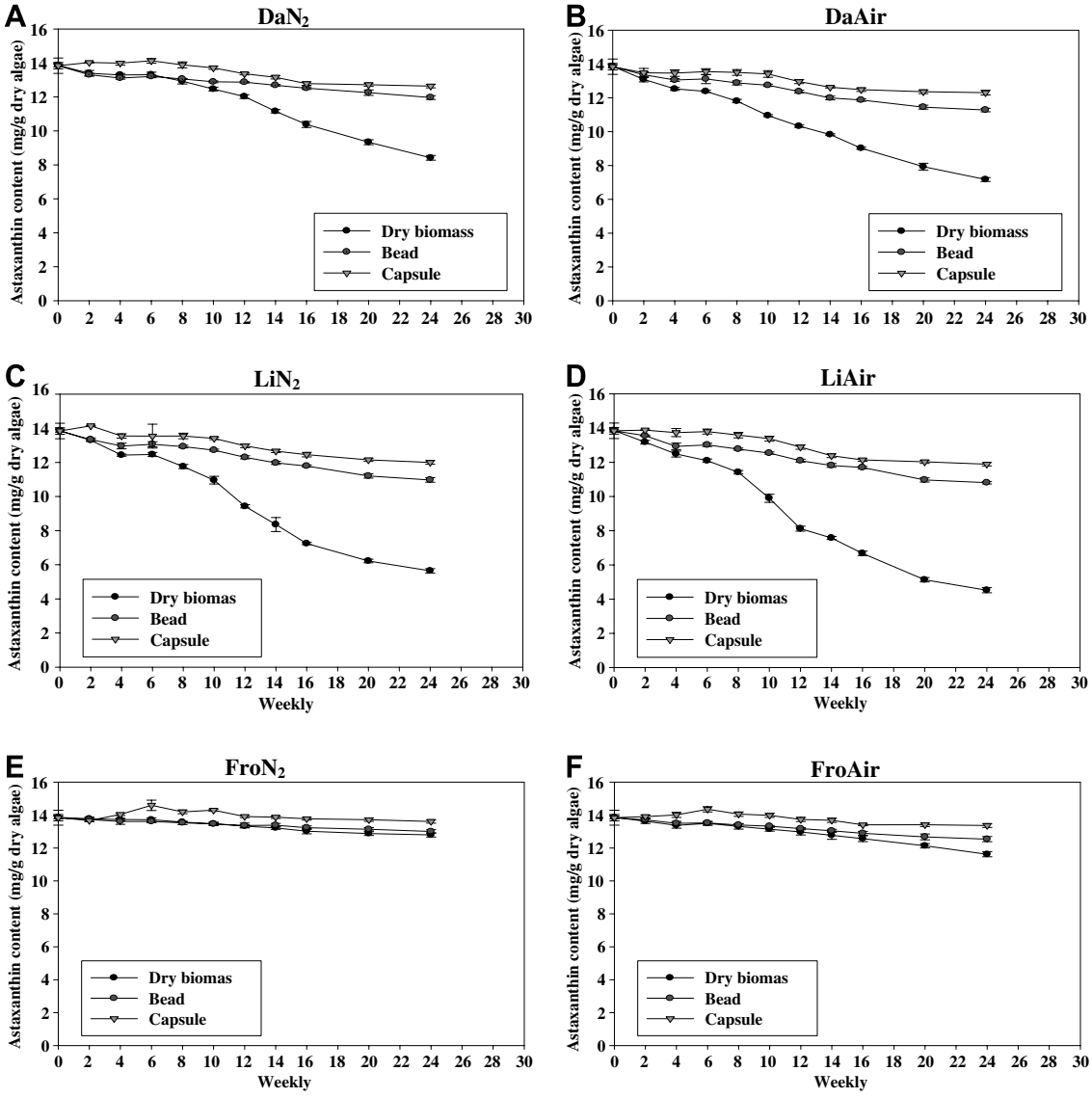

Fig. 9. Comparison of the stability of astaxanthin in dry biomass, beads, and capsules under different conditions. (A) 30 °C under nitrogen atmosphere in the dark; (B) 30 °C under air atmosphere in the dark; (C) 30 °C under nitrogen atmosphere under light exposure; (D) 30 °C under air atmosphere under light exposure; (E) −18 °C under nitrogen atmosphere in the dark; (F) −18 °C under air atmosphere in the dark.

3.4. Comparisons of astaxanthin stability in dry biomass, beads, and capsules

3.4.1. Stability under accelerated condition ($T = 80\ ^\circ C$)

The results shown in Fig. 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 non-accelerated storage conditions would be presented.

3.4.2. Astaxanthin stability under non-accelerated 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 Fig. 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

Table 2
Percentage of astaxanthin loss of dry biomass beads and capsules under different storage conditions after 24 weeks

Algae	Storage condition (%)					
	Dark at 30°C		Light exposure at 30°C		Frozen at −18°C in the dark	
	N_2	Air	N_2	Air	N_2	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

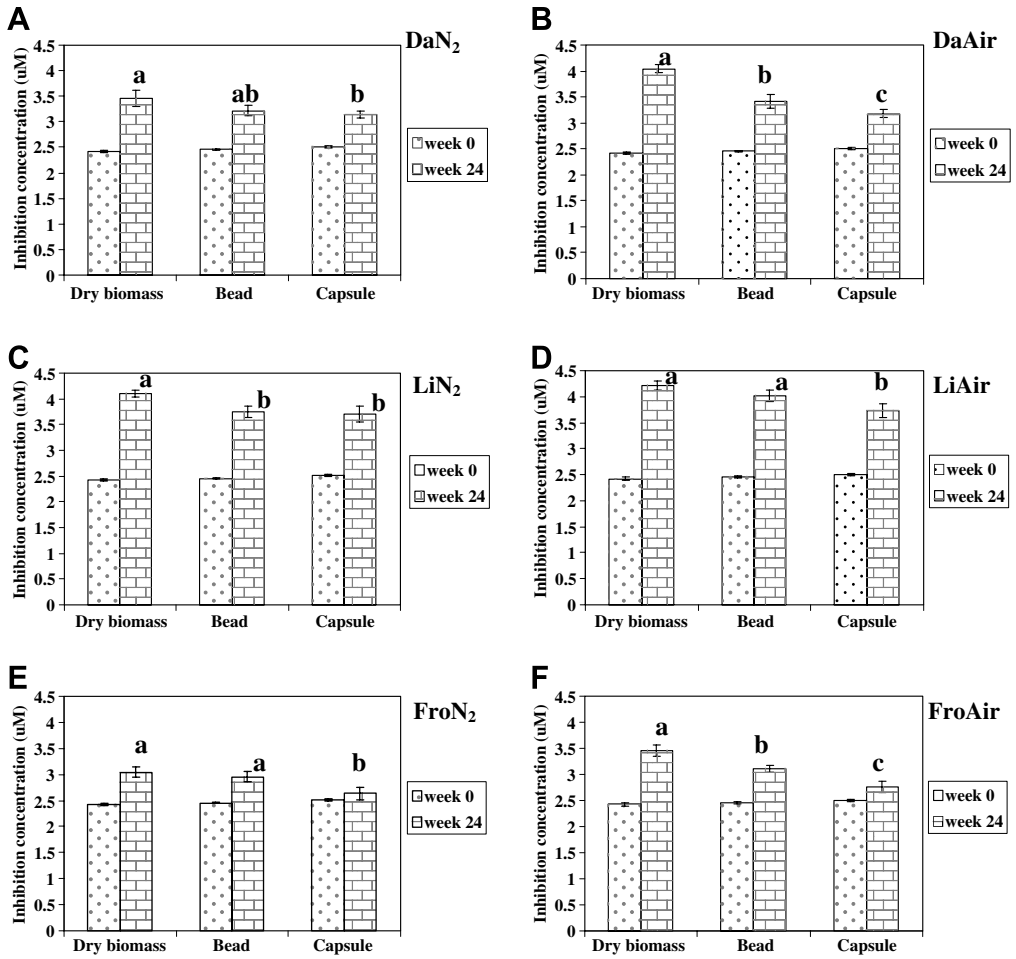

Fig. 10. Antioxidant activities of astaxanthin in dry biomass, bead, and capsule under different condition at 0 week and 24 weeks. (A) 30 °C under nitrogen atmosphere in the dark; (B) 30 °C under air atmosphere in the dark; (C) 30 °C under nitrogen atmosphere and light exposure; (D) 30 °C under air atmosphere and light exposure; (E) −18 °C under nitrogen atmosphere in the dark; (F) −18 °C under air atmosphere in the dark.

astaxanthin loss at week 24 for all sample conditions were summarized in Table 2. 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 (Chiang, Yao, & Chen, 2000; Jeon et al., 2003; Xie, Xu, & Liu, 2001; Xue, Yu, Hirata, Terao, & Lin, 1998). This could therefore be another possibility attributed to the enhanced stability of the encapsulated beads.

3.5. Antioxidant activity under different storage conditions

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 Fig. 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 °C under nitrogen atmosphere. Although under this condition, the algae was protected against light and oxygen, the degradation of astaxanthin was still observed (Fig. 10E), 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 Figs. 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.

4. Conclusions

In summary, chitosan encapsulated *H. pluvialis* beads prepared by immersion of algal beads repeatedly into chitosan solution have uniform size and shape. The encapsulation process employed was found not to cause biomass or astaxanthin loss. However, approximately 3% loss of antioxidant activity of the *H. pluvialis* was observed after encapsulation. For the study of stability under different storage conditions, it was proven that chitosan could enhance the stability of the *H. pluvialis* under different storage conditions, with the best storage condition found to be at −18 °C under N_2 atmosphere in the dark.

Acknowledgement

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