Enzyme-assisted extraction of astaxanthin from *Haematococcus pluvialis* and its stability and antioxidant activity

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Abstract The release of bioactive pigments could be potentially improved by enzyme degradation of plant cell wall polysaccharides. In this study, the objective was to evaluate enzyme type (cellulase and pectinase), pH values, hydrolysis temperature and time on the release of astaxanthin from *Haematococcus pluvialis* (*H. pluvialis*). The results showed that pre-treated *H. pluvialis* with enzymes could improve the separation yield of astaxanthin. Pectinase release rate of astaxanthin from *H. pluvialis* was significantly higher than cellulase ($p < 0.05$), and enzyme hydrolysis time was also shorter. The stability study of astaxanthin oleoresin and microcapsule during storage at different temperature, oxygen and illumination was found that the degradation rate of astaxanthin rose with increasing temperature and illumination time, and the retention in oxygen environment decreased. The stability of astaxanthin microcapsules was better than astaxanthin oleoresin.

Keywords *H. pluvialis* · Astaxanthin · Enzyme assisted extraction · Stability and antioxidant activity

Introduction

Astaxanthin from *Haematococcus pluvialis* (*H. pluvialis*) is a red natural pigment and carotenoid. The chemical name is called 3,3'-dihydroxy-4,4'-diketo- β - β carotene (Herrera-Andrade et al., 2011), the molecular formula is $C_{40}H_{52}O_4$ with a molar mass of 594.84 g, the two asymmetric carbons located at the 3' and 3' β -ionone ring are presented (Rao et al., 2014). Astaxanthin constitutes an interesting alternative due to biological activity as a potent antioxidant and protecting against fatty acid oxidation (Herrera-Andrade et al., 2011; Rao et al., 2014). Recently, astaxanthin has been an important functional ingredient in food products. Five of the commonly used methods of extracting astaxanthin include ultrasound-assisted and microwave-assisted extraction, maceration, enzyme-assisted solvent extraction, supercritical fluid extraction (Kobayashi et al., 1997; Sarada et al., 2006; Ruen-ngam et al., 2011; Wang et al., 2012). The ultrasound-assisted and microwave-assisted extraction would degrade the bioactivity of astaxanthin (Zhao et al., 2016). While the cost of supercritical CO_2 extraction is higher, and the solubility of astaxanthin also is lower in supercritical carbon dioxide (SC- CO_2) or organic solvent because of a tough cell wall of *H. pluvialis* (Thana et al., 2008). However, enzyme-assisted extraction has wide range of application and cheap investment, has been regarded as the most potentially feasible means for extracting *H. pluvialis*.

The cell wall of *H. pluvialis* is a complex network composed of fibrin and pectic substances, the thickness is 14–21 μ m. If the cell wall is degraded, the physical barrier

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will be eliminated and opens up the cell, which can improve the release of astaxanthin from *H. pluvialis*. The hydrolytic enzymes are an efficiency method to degrade the cell wall constituents, which will be helpful to release the intracellular nutritional contents. Some references have widely reported this method for the extraction of various kinds of substances (Zhou et al., 2013; Zuorro et al., 2011). The extraction of lycopene from tomato using petroleum ether and acetone as solvent has employed the enzymes (Zuorro et al., 2011). Now that the cellulose and pectins are the main components of the plant cell wall, the cellulases and pectinases have been applied because of this purpose. In the same studies, the enzymatic pretreatment has also been offered to extract carbohydrate compound from Huangqi and pectin from passion fruit peel (Vasco-Correa and Zapata, 2017; Zhou et al., 2013).

Few researchers reported the application of cell wall hydrolyzing enzymes, such as cellulase and lytic enzyme, to release astaxanthin from *H. pluvialis* (Kobayashi et al., 1997; Zhou et al., 2008), but the influences of the process parameters did not be explored in those studies. Therefore, it is important to evaluate the effect of enzyme hydrolysis of *H. pluvialis* on the astaxanthin release rate after hydrolysis and stability.

In this study, the work aimed to develop a process for enzymatic extraction of astaxanthin from *H. pluvialis*, and was designed to systematically investigate the influences of extraction parameters on the astaxanthin yields. The study comprehensively carried out the enzymatic release of astaxanthin from *H. pluvialis* by evaluating enzyme types (cellulase and pectinase) in order to find the optimum enzyme assisted extraction conditions for the preparation of astaxanthin. And the stability and antioxidant activity of astaxanthin was compared with encapsulated product for commercial application.

Materials and methods

Materials

Red dried samples of feed grade *Haematococcus pluvialis* (*H. pluvialis*) were purchased from Jingzhou Natural Astaxanthin Inc. (Hubei, China). The content of astaxanthin in *H. pluvialis* dry powder was 3% by HPLC analysis. The sample was stored in a dry, tight aluminum pack at -18°C to prevent degradation. Acetic ether used for extraction was reagent grade, purchased from Shanghai Guoyao Chemicals Co. (Shanghai, China). Calibration curves of astaxanthin in various types of solvent were provided from a standard compound of $>98\%$ purity purchased from Sigma Chemical Co. (St. Louis, MO, USA). Two different enzymes were selected on the basis of

the structural composition of *H. pluvialis*. Cellulase ($\geq 700\text{U/g}$) and pectinase (3500 U/g) was purchased from Sigma–Aldrich (St. Louis, MO).

Enzyme-assisted extraction astaxanthin

In order to examine the effects of enzyme on the release of astaxanthin from *H. pluvialis*, 1.00 g of *H. pluvialis* dry powders was incubated with a mixture of cellulase (100 U/g dry matter, 100 enzyme-activity units/g dry weight sample) or pectinase (7 U/g dry matter) in 0.2 mol/L Na acetate buffer at certain temperature and time. After 1 h of shaking extraction in triangular flask, the samples were centrifuged at $4000\times g$ for 10 min, and the residue after enzyme hydrolysis was mixed with acetic ether, shaking at 40°C for 60 min. Then, the supernatant was collected and filtered ($0.45\text{ }\mu\text{m}$) (Zhao et al., 2016). The extract solution of astaxanthin was concentrated by the rotary evaporator at 50°C for removing the solvent (Zhao et al., 2016). The oleoresin containing astaxanthin was obtained and used as the analysis of astaxanthin stability and antioxidant activity.

Astaxanthin assay

The astaxanthin content was measured by the method of Wang et al. (2012). The astaxanthin sample was dissolved in mixture of dichloromethane and methanol, which the ratio of dichloromethane to methanol was 1:9 (v/v). The solution was filtered through a $0.45\text{ }\mu\text{m}$ pore size polyethylene terephthalate (PET) syringe filter (Whatman, USA). The solution containing astaxanthin was injected into the High Performance Liquid Chromatography (HPLC) (Agilent HP 1100 Series, C18 $4.6\times 150\text{ mm}$, $5\text{ }\mu\text{m}$ HPLC column, Palo Alto, CA, USA). The conditions were as following: a mobile phase 90:10 (v/v) methanol/water, flow rate 1 mL/min, the injection volume $20\text{ }\mu\text{L}$ and the detection wavelength was kept at 475 nm. The extraction efficiency of astaxanthin was calculated by the ratio of astaxanthin content in the extract to the total astaxanthin content in the algae. The astaxanthin content in oleoresin was 9.18%.

Experimental design of extraction

Astaxanthin from *H. pluvialis* was extracted using the enzyme-assisted extraction (EAE) method adapted from Xu et al. (2014) and Zhou et al. (2008), and their values were assessed using single factor and orthogonal analyses. Single factor analysis involved the determination of each factor: the amount of cellulase and pectinase enzyme was evaluated at 0.5, 1, 1.5, 2, 2.5% and 0.03, 0.05, 0.08, 0.1, 0.3%, respectively, and agitation speeds were set at

180 rpm for 3 and 2.5 h at 45 °C and pH 5.0, respectively; the processing time of cellulase and pectinase enzyme was 1, 2, 3, 6, 10 h and 1.5, 2, 2.5, 3 and 5 h, respectively, and agitation speeds were set at 180 rpm at 45 °C and pH 5.0, cellulase and pectinase amount of 3% and 0.08%, respectively; the pH values of cellulase and pectinase enzyme were 3.5, 4, 4.5, 5, 5.5 and 6, and agitation speeds were set at 180 rpm for 3 and 2.5 h at 45 °C, cellulase and pectinase amount of 3% and 0.08%, respectively; the temperatures of cellulase and pectinase enzyme were 30, 40, 45, 50 and 60 °C, and agitation speeds were set at 180 rpm for 3 and 2.5 h at pH 5.0, cellulase and pectinase amount of 3% and 0.08%, respectively. Optimal results considered both the extraction rate and cost. For evaluating of the overall values, a four factor, three level (3^4) was selected for each factor to the best design extraction and an orthogonal analytical method was applied in the extreme difference to verify the data (Tables 1, 2).

Astaxanthin microencapsulation by spray drying

Microcapsules of astaxanthin were prepared by spray drying method. Wall materials (maltodextrin: gelatin = 2:1 (w/w)) were dissolved in distilled water to form an aqueous solution, and then astaxanthin oleoresin was dripped into the aqueous solution. The emulsion (30% total solid content) was stirred by a magnetic bar. A BILON-6000Y, spray dryer (Bilon experimental Co. Ltd., Shanghai, China) was used to convert the solution into encapsulated powder. The feed flow was 300 mL/h. The inlet and outlet temperature was set at 180 and 80 °C, respectively. Thus, the microencapsulation astaxanthin was obtained and collected in plastic containers.

Stability of astaxanthin

Effect of temperature on astaxanthin stability

40 g of astaxanthin microcapsule and oleoresin were vacuum sealed using aluminum foil and kept in dark conditions at 4, 25, 40 and 60 °C for different durations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days), respectively. Take 1.00 g microcapsule and oleoresin of astaxanthin as sample every 24 h. Astaxanthin retention was determined by spectrophotometric (UV-2401PC, Shimadzu Corporation,

Kyoto, Japan) method at wavelength 478 nm. The concentration of astaxanthin was calculated using a standard curve. The astaxanthin retention was calculated according to the following equation (Otálora et al., 2015):

$$\text{Astaxanthin retention (\%)} = \frac{m_1}{m_0} \times 100 \quad (1)$$

where m_1 and m_0 were the absolute amounts of astaxanthin at storage time and before starting the experiment (zero storage time), respectively.

Effect of oxygen on astaxanthin stability

The samples were placed in brown glass vials covered with aluminum foil, and stored at 25 °C. The stability study was performed after a storage time of 10 days. Quantification was conducted every 24 h, employing the same methodology used to calculate astaxanthin retention (Otálora et al., 2015).

Effect of illumination on astaxanthin stability

40 g of dried microcapsule and oleoresin of astaxanthin was exposed to room light. Then the sample was stored for 10 days at room temperature. 1.00 g of microcapsule and oleoresin were taken as samples every 24 h and astaxanthin retention was determined according to the above methodology (Otálora et al., 2015).

Evaluation of the antioxidant capacity of astaxanthin oleoresin from different methods

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical DPPH was used to evaluate the antioxidant activity of astaxanthin with different methods. The DPPH scavenging activity of samples was determined according to the described method of Rockenbach et al. (2011).

ABTS radicals scavenging assay

ABTS assay was used to measure the antioxidant ability of radical cation scavenging capacity of samples, which was measured according to the methods of Zhang et al. (2014).

Table 1 Three level orthogonal analysis of cellulase-assisted extraction of astaxanthin from *H. pluvialis*

Level	Factor			
	A: Temperature (°C)	B: Time (min)	C: Enzyme content (%)	D: pH
1	40	2	1	4.5
2	45	3	1.5	5.0
3	50	6	2	5.5

Table 2 Three level orthogonal analysis of pectinase-assisted extraction of astaxanthin from *H. pluvialis*

Level	Factor			
	A: Temperature (°C)	B: Time (h)	C: Enzyme content (%)	D: pH
1	45	2	0.05	4.5
2	50	2.5	0.08	5.0
3	55	3	0.10	5.5

Statistical analysis

Data for the extracts were reported as mean $\pm$ standard deviation (S.D.) of three replicates. The comparison between different treatments was performed by Least—Significant Difference (LSD) method using DPS ver. 13.0 software (Zhejiang University, Hangzhou, China). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Effect of pH, temperature, cellulase and pectinase amount and time on astaxanthin extraction

Effects of different temperature, pH, enzyme (cellulase and pectinase amount and time on extraction yield of astaxanthin are shown in Fig. 1. As shown Fig. 1A, for cellulase and pectinase, the extraction yield of astaxanthin significantly increased from 30 to 40 °C (from 50.41 ± 0.56 to $60.93 \pm 1.27\%$) and from 30 to 50 °C (from 31.43 ± 0.73 to $45.38 \pm 1.12\%$) ($p < 0.05$), respectively, but was inhibited strongly by the temperatures above 40 and 50 °C due to the denaturalization of cellulase and pectinase at higher temperature. This result agreed with previous literatures (Zuorro et al., 2016). Therefore, the temperature of 40 and 50 °C was chosen as optimal and used as center point for subsequent experiments of cellulase and pectinase assisted extraction, respectively.

For the effect of pH values, the extraction yield of astaxanthin increased with an increasing pH values from 3.5 to 5.0 (Fig. 1B). The activity of enzymes with the increase of pH was improved significantly ($p < 0.05$), which was responsible for the increase of the extraction yield. When pH value exceeded 5.0, the extraction yield significantly decreased ($p < 0.05$), suggesting that a suitable pH range was needed for cellulase and pectinase to collapse cell structures and to release astaxanthin. The result was supported by the investigation of Zuorro et al. (2016), where optimal of pH was obtained in the acidic range using similar enzymes. Hence, the higher extraction of astaxanthin was obtained at pH 5.0.

The effect of using different concentrations of enzymes on the maximum extraction of astaxanthin from *H. pluvialis* is shown in Fig. 1C. The extraction yield of

astaxanthin significantly increased with increasing cellulase and pectinase amounts from 0.5 to 1.5% (from $42.98 \pm 2.27\%$ to $52.80 \pm 0.32\%$) and from 0.03 to 0.08% (from $48.13 \pm 0.73\%$ to $54.95 \pm 0.45\%$) ($p < 0.05$), respectively, then decreased with increasing the concentration of enzymes (Fig. 1C). As seen in Fig. 1C, when cellulase and pectinase amounts were 1.5% and 0.08% w/w, respectively, the highest astaxanthin extraction yield was shown, and $52.80 \pm 0.32\%$ and $54.95 \pm 0.45\%$, respectively. The enzyme concentrations required may vary depending upon the material from which the astaxanthin was extracted and the activity of the used enzymes (Zuorro et al., 2011;2016).

The effect of extraction time on the extraction yield of astaxanthin is displayed in Fig. 1D. It was found that the extraction yield for cellulase and pectinase significantly increased with the extension of time from 1 to 3 (from $47.23 \pm 1.08\%$ to $59.05 \pm 1.5\%$) and from 1.5 to 2.5 h (from $40.33 \pm 1.57\%$ to $45.83 \pm 0.12\%$) ($p < 0.05$), respectively, and after 3 and 2.5 h slight lowered. The results indicated that the enzymatic degradation of cell-wall components was very fast and occurred in the first hour of extraction time. Therefore, the vast majority of astaxanthin molecules contained in *H. pluvialis* was probably rapidly released from the protective chromoplast structures. With increasing of extraction time, the released astaxanthin was exposed to the conditions of the external environment and could undergo rapid oxidative degradation (Rao et al., 2014). So, the reduction in extraction yields observed at prolonged extraction times could be a reflection of the progressive astaxanthin loss due to oxidation (Ahmed et al., 2015). Thus, the optimal cellulase and pectinase aided extraction time of astaxanthin chosen was 3 and 2.5 h, respectively.

Orthogonal optimization of astaxanthin extraction

The single factor and orthogonal analyses of the data from the experiments using cellulase and pectinase was used to verify the influence of individual factors on astaxanthin from *H. pluvialis* extraction efficiency. The experimental data obtained from the orthogonal experiment optimization with four factors and three levels $L_9 (3^4)$ was interpreted by LSD method. The results are summarized in Tables 3 and 4 with the application of extreme difference. The extreme

Fig. 1 Effects of different pH, temperature, enzyme amount and time on extraction yield of astaxanthin ($n = 3$). Astaxanthin from *H. pluvialis* extracted by the method of enzyme-assisted extraction. **A:** under the fixed conditions including pH value of 5.0; cellulase and pectinase amount of 3% and 0.08%, respectively; time of 3 and 2.5 h, respectively. **B:** under the fixed conditions including extraction

temperature of 45 °C; cellulase and pectinase amount of 3% and 0.08%, respectively; time of 3 and 2.5 h, respectively. **C:** under the fixed conditions including pH value of 5.0; temperature of 45 °C; time of 3 and 2.5 h, respectively. **D:** under the fixed conditions including pH value of 5.0; temperature of 45 °C; cellulase and pectinase amount of 3% and 0.08%, respectively

Table 3 Results and analysis of orthogonal experiment from cellulase-assisted extraction of astaxanthin with extreme differences ($n = 3$)

No.	Temperature (°C)	Time (h)	Enzyme content (%)	pH	Extract yield (%)
1	40	2	1	4.5	53.95 ± 0.76
2	40	3	1.5	5.0	58.74 ± 1.10
3	40	6	2	5.5	63.09 ± 0.87
4	45	2	1.5	5.5	64.33 ± 1.02
5	45	3	2	4.5	56.96 ± 1.02
6	45	6	1	5.0	67.15 ± 0.53
7	50	2	2	5.0	60.69 ± 0.75
8	50	3	1	5.5	60.16 ± 0.83
9	50	6	1.5	4.5	56.08 ± 0.82
K1	58.593	59.657	60.420	55.663	
K2	62.813	58.620	59.717	62.193	
K3	58.977	62.107	60.247	62.527	
K	4.220	3.487	0.530	6.864	
R	A2	B1	C2	D3	

Ki: total extraction yield of the same factor and level in the experiments, $i = 1, 2$, or 3

K: range analysis value

Table 4 Results and analysis of orthogonal experiment from pectinase-assisted extraction of astaxanthin with extreme differences (n = 3)

No.	Temperature (°C)	Time (h)	Enzyme content (%)	pH	Extract yield (%)
1	45	2	0.05	4.5	45.25 ± 0.62
2	45	2.5	0.08	5.0	47.82 ± 0.59
3	45	3	0.10	5.5	57.32 ± 0.53
4	50	2	0.08	5.5	57.59 ± 0.71
5	50	2.5	0.10	4.5	59.00 ± 0.89
6	50	3	0.05	5.0	59.72 ± 0.93
7	55	2	0.10	5.0	62.20 ± 0.77
8	55	2.5	0.05	5.5	59.89 ± 0.72
9	55	3	0.08	4.5	75.30 ± 0.62
K1	50.130	55.013	54.953	57.850	
K2	58.770	55.570	58.237	56.580	
K3	65.797	64.113	60.237	58.850	
K	14.667	9.100	5.284	2.270	
R	A3	B3	C2	D1	

Ki: total extraction yield of the same factor and level in the experiments, i = 1, 2, or 3

K: range analysis value

difference data (K1, 2, 3) and average value (K) were calculated to compare the significance of each factor (Ge et al., 2009). As seen in Tables 3 and 4, The influences of the parameters on the cellulase assisted extraction yields in descending order were: D (pH) > A (extraction temperature) > B (extraction time) > C (enzyme content) according to the K values, while pectinase assisted extraction yields in descending order were A (extraction temperature) > B (extraction time) > C (enzyme content) > D (pH) according to the K values. Based on the results of variance analysis, extraction temperature, extraction time, extraction pH and enzyme amount significantly affected the extraction yield of astaxanthin, the maximum cellulase and pectinase assisted extraction yield was obtained under the combination of A2B1C2D3 and A3B3C2D1, respectively. Considering the astaxanthin extraction efficiency, the cost of energy and the feasibility of experiment, the optimal condition for the cellulase assisted extraction of astaxanthin was: enzyme hydrolysis temperature 45 °C, time 6 h, enzyme addition 1.0%, pH 5.0, the extraction yield of astaxanthin reached 67.15%, which was significantly high than other values ($p < 0.05$). The pectinase assisted extraction was: enzyme hydrolysis temperature 55 °C, time 3 h, enzyme addition 0.08%, pH 4.5, under these conditions, the extraction yield of 75.30% was obtained, it was significant different from other values ($p < 0.05$).

Some researchers reported that enzymes had been widely applied in enzyme-assisted extraction, which could degrade or disrupt cell walls to improve efficient extraction of bioactive compounds, such as cellulase, amylase and pectinase etc. (Puri et al., 2012; Xu et al., 2014). In general,

the main factors that influence enzyme-assisted extraction performance were extraction temperature, extraction time, enzyme amount and pH value (Ranveer et al., 2013; Zuurro et al., 2011). As shown in the Table 5, extraction temperature, pH values and temperature had the most significant effect on the cellulase assisted extraction of astaxanthin ($p < 0.05$). The result was not supported by Yin et al. (2011), who found that the temperature, pH, time and enzyme amount could markedly affect the extraction of bioactive compounds. Table 6 showed that the temperature and time had the most significant effect on the pectinase assisted extraction of astaxanthin ($p < 0.05$). Azabou et al. (2016) reported that different enzymes had different effect of extraction efficiency of lycopene. It indicated that the reason was possibly the difference in types of enzyme used.

Comparative study of pectinases and cellulases extraction of astaxanthin

The efficiency of the cellulase aided extraction was evaluated in comparison with pectinases preparation. The results showed that the pectinase assisted extraction yield (75.30%) was significantly higher than cellulase assisted extraction (67.15%) ($p < 0.05$) (Tables 3, 4). Hence, pectinase enzyme is more effective than cellulase for astaxanthin extraction from *H. pluvialis*. The result was in agreement with the investigation of Azabou et al. (2016). It suggested that the pectinase was more suitable to rapidly and totally hydrolyze polymers of the plant tissue, and then beneficial to the solvent penetration, which could increase the extraction yield of bioactive compounds. The extraction efficiency of astaxanthin obtained through two enzyme

Table 5 A Variance analysis of orthogonal test of cellulase-assisted extraction of astaxanthin from *H. pluvialis*

Variance resource	SS	df	MS	F	F(0.05)	F(0.01)	<i>p</i>
A	32.6754	2	16.3377	36.00	19.00	99.00	0.027*
B	19.2340	2	9.6170	21.20			0.045*
C	0.8056	2	0.4028	0.10			0.744
D	89.8574	2	44.9287	197.40			0.005**
Sun	142.5724						

* $p < 0.05$; ** $p < 0.01$ **Table 6** Variance analysis of orthogonal test of pectinase-assisted extraction of astaxanthin from *H. pluvialis*

Variance resource	SS	df	MS	F	F(0.05)	F(0.01)	<i>p</i>
A	369.4681	2	184.7340	406.40	19.00	99.00	0.009**
B	156.1084	2	78.0542	24.76			0.049*
C	49.1794	2	24.5897	6.10			0.234
D	16.0447	2	8.0223	1.82			0.6521
Sun	590.8006						

* $p < 0.05$; ** $p < 0.01$

assisted extraction was higher than previously used solvent leaching method (44%) ($p < 0.05$) (Ruen-ngam et al., 2011). According to Zuorro et al. (2011), improved solvent penetration and lycopene dissolution in the chromoplasts could be expected to occur when cell-wall polysaccharides are enzymatically degraded. It was inferred that the enzyme type could affect the extraction yield of astaxanthin, the result was agreed with the investigation of Xu et al. (2014) and Azabou et al. (2016). In brief, enzyme assisted extraction method was more efficient than the solvent leaching method for extraction yield of astaxanthin.

Storage stability of astaxanthin at different temperatures

Figure 2A shows the retention of astaxanthin oleoresin and microencapsulated astaxanthin obtained through enzyme treatment, astaxanthin oleoresin without enzyme treatment after 10 days at different temperatures. Three replicates were stored for each temperature. Astaxanthin retention was greatly influenced by storage temperature. The retention rate of three samples was found to decrease with increasing temperature. The results studied retention was significantly greater at 4 °C compared to storage at 25, 40 and 60 °C ($p < 0.05$). By comparison of the results shown, the largest degradation rate constant was found at 60 °C, followed by at 25 and 40 °C. It was obviously observed that the temperature was the higher, the degradation was the faster. It indicated that the astaxanthin oxidation process was greater at higher temperature (Rocha et al., 2012). Takeungwongtrakul et al. (2015) found that the lipids containing astaxanthin from shrimp (*L. vannamei*) hepatopancreas stored at 28–30 °C had a higher oxidation rate.

The retention rate of encapsulated astaxanthin at the end of 10 days storage period decreased 87.29, 79.25, 58.29 and 44.55% at 4, 25, 40 and 60 °C, respectively (Fig. 2A (a, b, c and d)), while the astaxanthin oleoresin decreased 70.92, 55.96, 39.79 and 29.47%, respectively (Fig. 2A (a, b, c and d)). These results suggested that the thermo stability of microencapsulated astaxanthin became significantly higher than that of the free astaxanthin at the higher temperature environment ($p < 0.05$). This might be due to that wall materials were could provide effective protection on astaxanthin, avoiding the damage of the thermal treatment (Rocha et al., 2012). Bustamante et al. (2016) studied the stability of the astaxanthin oleoresins and powders at 40, 50 and 70 °C, and verified that encapsulated astaxanthin was relatively stable at those temperatures. Rocha-Selmi et al. (2013) also found that the microencapsulation by complex coacervation in a gelatinegum arabic matrix could prevent the degradation of lycopene. Bustos-Garza et al. (2013) assessed the storage stability of astaxanthin microcapsule and oleoresin at 30, 40 and 50 °C, found that the micro-capsuled astaxanthin oleoresin had the higher thermal stability. It was attributed to the good thermal stability of the wall materials. However, the retention rate of astaxanthin with or without enzyme treatment was not significant ($p > 0.05$). It indicated that the astaxanthin was destroyed by enzyme.

Effect of oxygen on stability of astaxanthin

Astaxanthin is susceptible to isomerisation and oxidation during storage process due to containing a number of conjugated double bonds, so it is degraded by oxidative reactions (Christophersen et al., 1991). The effect of

Fig. 2 **A** Storage stability of astaxanthin oleoresin (I) and microencapsulated astaxanthin (II) with enzyme treatment, astaxanthin oleoresin without enzyme treatment (III) during storage at different temperature (a: 4 °C; b: 25 °C; c: 40 °C; d: 60 °C); **B** Effect of oxygen on stability of astaxanthin oleoresin (I) and microencapsulated astaxanthin (II) with enzyme treatment, astaxanthin oleoresin without enzyme treatment (III) during storage at 25 °C; **C** Effect of illumination on stability of astaxanthin oleoresin (I) and microencapsulated astaxanthin (II) with enzyme treatment, astaxanthin oleoresin without enzyme treatment (III) during storage at 25 °C. Data were expressed by mean $\pm$ standard deviation ($n = 3$)

oxygen on storage stability of astaxanthin microcapsules and oleoresin obtained through enzyme treatment, astaxanthin oleoresin without enzyme treatment shows different pattern at 25 °C in Fig. 2A (b). The experiment was carried out in triplicate. There was a positive stabilising affect on astaxanthin microcapsules and oleoresin. However, when the treatment time was increased to 10 days, the content of astaxanthin microcapsules and oleoresin obtained through enzyme treatment, astaxanthin oleoresin without enzyme treatment retained 43.98, 24.02 and 22.15%, respectively. While the astaxanthin microcapsules and oleoresin obtained through enzyme treatment, astaxanthin oleoresin without enzyme treatment that were to be stored at 25 °C without oxygen had the retention rate of 79.25, 55.96 and 53.86% at day 10, respectively (Fig. 2A (b)). By comparison with these values, the retention of astaxanthin without oxygen was higher than oxygen. The result was in agreement with the investigation of Ahmed et al. (2015). It indicated that the oxygen could lead to the degradation of astaxanthin content (Bustos-Garza et al., 2013). The oxidative stability of astaxanthin microcapsules was significantly higher than that of astaxanthin oleoresin with or without enzyme treatment ($p < 0.05$). But the retention rate of astaxanthin treated was similar with untreated. It indicated that the enzyme treatment was beneficial to improve the extraction yield of astaxanthin, and did not change the retention rate. The experimental measurements suggested that the microcapsule wall material act as a permeability barrier, which would depress oxygen transfer from the surrounding environment onto the astaxanthin surface, so the net oxidation rate was decreased (Bustos-Garza et al., 2013). Gomez-Estaca et al. (2016) found that content of non-encapsulated astaxanthin from shrimp waste in comparison with the encapsulated astaxanthin fell sharply up to day 9, the degradation rated was less at day 36, but the astaxanthin was almost fully degraded until day 43. Therefore, the wall polymer of microcapsules could be applied to improve the stability of pigments.

Effect of illumination on stability of astaxanthin

The stability of astaxanthin obtained through enzyme treatment and no enzyme treatment was evaluated at a temperature of 25 °C in the presence and absence of illumination (Fig. 2A (b) and C), which showed that samples exposed to light had lower astaxanthin content, and microencapsulation process could successfully stabilize astaxanthin against light. The experiment data were finished in triplicate. The content of astaxanthin from three samples in the presence of light was found to sharply decrease with increasing illumination time (Fig. 2C), while the astaxanthin content without light slowly reduced (Fig. 2A (b)). The astaxanthin contents of the astaxanthin microcapsules and oleoresin obtained through enzyme treatment, astaxanthin oleoresin without enzyme treatment decreased by 35.24, 70.14 and 72.39% at the end of 10 day exposure to light at 25 °C, respectively (Fig. 2C). While the retention rate of astaxanthin without light decreased by 20.75, 44.04 and 46.14%, respectively (Fig. 2A (b)). By comparison with experimental data in the absence or presence of light, the retention of astaxanthin from microcapsule was significantly higher than two oleoresins ($p < 0.05$). From day 1 at storage temperature 25 °C, the retention rate of astaxanthin oleoresin exposed to light was significantly lower than no light ($p < 0.05$). But the *H. pluvialis* treated by enzyme did not affect the retention rate of astaxanthin. Other authors also evaluated the pigment stability in relation to illumination, and showed that illumination played an important role in the degradation of pigments (Burin et al., 2011).

Antioxidant activities

DPPH and ABTS antioxidant assays were used in the present work in order to indicate the different aspects of antioxidant efficacy of astaxanthin obtained through different enzyme assisted extraction. The aim of DPPH assay was to detect antioxidant capacity of free radical-scavenging ability of astaxanthin, while ABTS assay was based on radical cation scavenging capacity. The IC₅₀ values of DPPH from astaxanthin oleoresin with different enzyme extraction on DPPH radical were shown in Table 7. The IC₅₀ values of astaxanthin oleoresin with no enzyme, pectinase and cellulase assisted extraction were 21.29, 22.01 and 21.95 µg/ml, respectively. These values were not significantly different ($p > 0.05$), implying that enzyme assisted extraction had no influence on the DPPH radical-scavenging ability of astaxanthin.

The scavenging ability of all samples on ABTS free radical is shown in Table 7. The IC₅₀ values of astaxanthin oleoresin with no enzyme, pectinase and cellulase assisted extraction were 6.16, 7.02 and 6.96 µg/ml, respectively.

Table 7 IC₅₀ values for antioxidant activities of pectinase and cellulase assisted extraction of astaxanthin from *H. pluvialis*

Astaxanthin oleoresin samples	IC ₅₀ (μg/ml)	
	DPPH radical	ABTS radical
Non enzyme extraction	21.29 ± 1.08 ^a	6.16 ± 1.08 ^a
Pectinase assisted extraction	22.01 ± 1.17 ^a	7.02 ± 1.08 ^a
Cellulase assisted extraction	21.95 ± 1.07 ^a	6.96 ± 1.08 ^a
Vitamin C	45.87 ± 1.16 ^b	14.87 ± 1.23 ^b

Values were expressed as mean ± SD (n = 3) of three replicated determinations. Means with the different letter in the same index were significantly different ($p < 0.05$)

The results were agreement with the results of DPPH radicals scavenging assay, confirming that enzyme extraction behavior did not affect the antioxidant activities of astaxanthin.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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