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Pyropia Conchocelis: Potential as an Algal Source for Carotenoid Extraction

Lin Rulong

Key Laboratory of Global Change and Marine-Atmospheric Chemistry, State Oceanic Administration, and Third Institute of Oceanography, State Oceanic Administration, Xiamen, China

Email address:
linrulong@yahoo.com

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Abstract: As shade adapted organisms the conchocelis of *Pyropia* contain high concentrations of photosynthetic pigments, making the conchocelis a potential source for the extraction of bioactive pigments such as phycoerythrin, phycocyanin and carotenoids. The pigment content of *Pyropia* conchocelis in response to environmental factors is poorly known. Investigations were performed on the production of carotenoid pigments as a function of environmental variables by the conchocelis phase of Alaskan *Pyropia* species: *Pyropia abbottiae, P. hiberna*,*P. torta*and*P.* sp. Conchocelis fragments were cultured under different irradiance, and nutrient concentrations for up to 60 days. Results indicate that carotenoid pigments were significantly affected by irradiance, nutrient concentrations and culture age, with some interactions of these factors. Carotenoid pigment content varied in a similar manner for each species. Light had the most obvious influence on carotenoid content. For all four species, the highest carotenoid content (3.4-7.0mg·gdw^{-1}) generally occurred at 0-10μmol photons·m^{-2}·s^{-1}. Higher irradiances, low nutrients and longer culture age generally caused a decline of carotenoid pigment content. There were significant differences in carotenoid pigment content for different species. *P. abbottiae* and *P.* sp. produced higher pigment content than the other two species. Maximal carotenoid content for *P. abbottiae* was 7.0mg·gdw^{-1}. *P. torta* contained the least carotenoid pigment under all culture conditions. Carotenoid pigments remained highest under continuous darkness for as long as 60 days for all tested species. The present study investigated the effects of environmental variables on the carotenoid content of *Porphyra* conchocelis and determined the optimal cultural conditions, which would helpful for obtaining algal material with higher pigment content and extraction of high value pigment.

Keywords: *Porphyra*, *Pyropia*, Conchocelis, Photosynthetic Pigment, Carotenoid Content

1. Introduction

Carotenoids are light-harvesting accessory pigments in the plastids of marine red algae (Schubert et al. 2006, Graham et al. 2008, Sangha et al. 2013， Indriatmoko et al. 2015).These accessory pigments (including phycobilins) transfer light energy to the reaction centers responsible for converting the absorbed light energy into chemical energy in the form of ATP and NADPH for carbondioxide fixation(Nurachman et al. 2015). The amounts of these pigments are crucial in determining physiological responses of marine red algae to environmental change. One potentially useful indicator of the quality of the conchocelis stage can be the photosynthetic pigment content (Amano and Noda 1978, Figueroa et al. 1995).

The importance of marine algae as sources of bioactive compounds has been well recognized due to their health and pharmacological benefits. Isolation and investigation of biochemicals with biological activities from marine algae have attracted much attention recently (Lanfer-Marquez et al. 2005, Maeda et al. 2008, Cornish and Garbary 2010, Yabuta et al. 2010, Holdt and Kraan 2011, Pangestutia and Kim 2011, Borowitzka 2013， Herrero et al. 2015, Kellogg et al. 2015, Yen et al. 2015). Recent studies have demonstrated that the carotenoids are natural bioactives that have antioxidant, anti-inflammatory and anti-cancer properties. It has also been found that these pigments are strong superoxide radical scavengers, inhibit growth of tumor cells and can prevent negative effects of UV radiation exposure (Okuzumi et al. 1990, Kotake et al. 2001, Maeda et al. 2005, Sachindra et al. 2007, Sangha et al. 2013),

The physiology and biochemistry of the conchocelis stage of Porphyra and Pyropia species have received little attention (Korbee et al. 2005a, 2005b, Sampath-Wiley et al. 2008).

From an applied phycological standpoint, determination of culture conditions for the optimal production of carotenoids of Pyropia conchocelis is helpful for the large-scale preparation and production of these high value bioactives (Lin and Stekoll 2011).Studies are needed on the basic information concerning how environmental factors affect the pigment content of the Pyropia conchocelis stage. We report here how the carotenoid content of Alaskan Pyropia conchocelis responds to variations of environmental variables and the potential for high valued carotenoid pigment extraction from cultured conchocelis.

2. Materials and Methods

2.1. Culture of Pyropia Conchocelis

Unialgal cultures of each Pyropia species were obtained from zygotospore release. Species collected were Pyropia abbottiae Krishnamurthy- strain PaSGS01, P. hiberna S. C. Lindstrom et K. M. Cole, strain - PeJB03,P. torta Krishnamurthy-strain PtCH13a and P. sp., strain PiSC14. (Note: the species we identify as P. sp. is morphologically indistinct from Porphyr apseudolinearis Ueda, and it will be described as a distinct species as per S. Lindstrom, personal communication.). Mature blades of the gametophyte stage of each species were collected from the field. Blades were washed and scrubbed with sterile seawater to remove surface contamination. The cleaned blades were placed in sterile seawater in petri dishes for zygotospore release. After 24-36 hours the blades were removed and the dishes incubated in Provasoli's enriched seawater (McLachlan 1973) under 16L:8D photoperiod at 11°C. Conchocelis segments (around110-250 μm) of each species were placed in 24 cell well plates (one piece per 3 mL well)and incubated at 30 psu salinity and 11°C(100-120 μmol photons·m^{-2}·s^{-1}irradiance) for the culture of pure genotypes. These clones were used for the generation of bulk amounts of conchocelis to provide material for specific experiments. Bulk conchocelis were incubated at 11°C and 25 μmol photons·m^{-2}·s^{-1}irradiance with f/2 culture medium (Guillard and Ryther 1962).

2.2. Experimental Procedure

Pigment experiments of conchocelis were conducted at 11°C illuminated with cool-white fluorescent lamps. Irradiance gradients were obtained by wrapping the culture containers with varying layers of white paper and determined using a Li-Cor Radiation Sensor (Li-190SB Quantum Sensor). The pH of the culture medium was adjusted to 7.8-8.0(the ambient pH of the seawater in the inside waters of SE Alaska)using6 MHCl or 6 M NaOH. The salinity of experimental seawater was set at 30 psu. Culture media were changedevery7 days. Longday(16L: 8D)photoperiods were used. Nutrients were added as an f culture medium concentration, which has a nitrogen concentration of 5.87 mM. Therefore, nutrient levels of 0, f/4, f/2 and f concentrations represented 0.02, 1.47, 2.94 and 5.87 mM nitrogen concentration, respectively (conchocelis at 0 nutrient concentration represented those incubated in natural seawater with a nitrogen concentration of0.02 mM, i.e., no f culture medium was added).In order to ensure sufficient inorganic carbon source available to the conchocelis, culture media were supplemented with5 mM $NaHCO_3$. For pigment experiments different levels of three environmental factors were employed: nutrient levels of 0, f/4, f/2, f concentration; irradiances of 0, 10, 40, 160 μmol photons·m^{-2}·s^{-1}irradiance and culture age of 10, 20, 30, 60 days.

2.3. Measurement and Analysis of Pigment Content

Pyropia conchocelis were grown in 200 ml flasks under the different culture conditions. After being incubated for 10, 20, 30,60 days, about 4-6 mg fresh weight of conchocelis were used for carotenoid measurements. Four replicates of conchocelis samples from each combination of culture conditions were used for carotenoid measurement. One corresponding sample was used for the measurement of the ratio of dry weight to fresh weight. After being rinsed with sterile seawater and ground at low temperature and lowlight, conchocelis samples were extracted with 90% acetone containing one drop of saturated $MgCO_3$at 4 ºC in the dark for 12 h and then centrifuged at 14,000 x g for 30 minutes. The supernatant was used for carotenoid measurement using a Gilford spectrophotometer 250. The following formula from hEocha (1971) was used to estimate carotenoid content in conchocelis samples: carotenoid (mg·gdw^{-1})= (7.14 A_{445} -3.85A_{670})/sample amount (gdw)

2.4. Statistical Analyses

Data (including potential factor interactions) were analyzed using a three-way modelI ANOVA(pigment content as a function of light, nutrient, culture age)with S-Plus 4.5 for windows (Statistical Sciences Inc., Seattle, Washington) .The Newman-Keuls multiple comparison test (Zar 2010) was performed to identify which tested factors were important in determining pigment content of Pyropia conchocelis.

3. Results

3.1. Comparison of Absorption Spectra

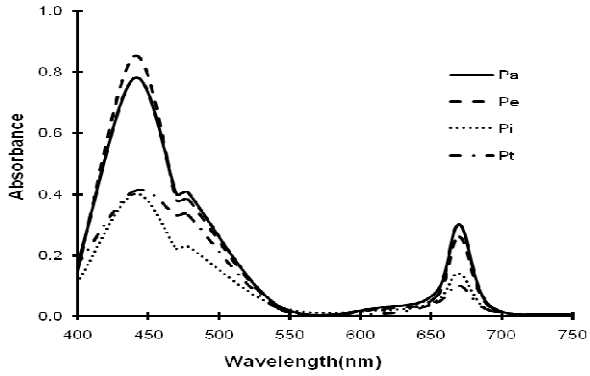

Fig. 1. *Comparison of absorption spectra of carotenoids extracted from the conchocelis of four species of Alaskan Pyropia. Pa: Pyropia abbottiae, Pe: P. hiberna, Pi: P. sp., Pt: P. torta.*

Absorption spectra of conchocelis extracts for four species of Pyropia show only slight variations (Fig. 1). The peak absorption of chlorophylla occurred at 670 nm and carotenoids had maximal absorption at 445 nm with a shoulder absorption at 475 nm. Pigments extracted from the conchocelis of all four species of Pyropia tested showed virtually identical spectra and had uniform peak absorptions at corresponding wavelengths.

3.2. Carotenoid Content of P. Abbottiae

The carotenoid content of the conchocelis of P. abbottiae was significantly influenced by all three factors (Table 1). Higher irradiance levels and longer days of culture correlated with a general decrease in carotenoid content of the conchocelis. Conchocelis cultures with no nutrients added showed a decline in carotenoids after 10 days of culture compared to cultures with added nutrients (Fig. 2). At high irradiances (40-160µmol photons·m^{-2}·s^{-1})cultures with no nutrients added usually had the lowest carotenoid content (Figs. 2 & 3). Cultures in the darkness had the highest carotenoid content. Irradiances of greater than40 µmolphotons·m^{-2}·s^{-1} resultedin a remarkable decline in carotenoid content. Carotenoid content remained highest for the first 10-20 days of cultures and then declined subsequently. The maximal carotenoid content (6.8-7.0 mg·gdw^{-1}) were achieved at 0µmolphotons·m^{-2}·s^{-1}, f/4-f/2nutrient concentration and 10-20dayscultureage.

Table 1. *ANOVA table for carotenoid content of the conchocelis of four Pyropia species grown under various combinations of nutrient concentration(NC),irradiance (Light) and culture age(Day).[a]0, f/4,f/2, f; [b]0,10, 40,160µmolphotons $m^{-2}.s^{-1}$; [c] 10, 20, 30, 60 days. (*P<0.05; **P<0.01).*

Source of variation	df	Sum of squares	Mean square	F
P. abbottiae				
Nutrient Conc.[a]	3	26.8694	8.9565	5.424**
Light[b]	3	389.5719	129.8573	78.639**
Day[c]	3	57.8462	19.2821	11.677**
NC x Light	9	12.4006	1.3778	0.834
NC x Day	9	14.6073	1.6230	0.983
Light x Day	9	8.8640	0.9849	0.596
NC x Light x Day	27	7.3458	0.2721	0.165
Residuals	192	317.0494	1.6513	
P. hiberna				
Nutrient Conc.[a]	3	20.6513	6.8838	14.544**
Light[b]	3	3.4866	1.1622	2.455
Day[c]	3	23.9163	7.9721	16.843**
NC x Light	9	4.8898	0.5433	1.148
NC x Day	9	2.2420	0.2491	0.526
Light x Day	9	11.6384	1.2932	2.732**
NC x Light x Day	27	7.8932	0.2923	0.618
Residuals	192	90.8746	0.4733	
P. sp.				
Nutrient Conc.[a]	3	74.6694	24.8898	46.391**
Light[b]	3	72.4945	24.1648	45.040**
Day[c]	3	5.4269	1.8090	3.372*
NC x Light	9	23.4517	2.6057	4.857**
NC x Day	9	16.6328	1.8481	3.445**
Light x Day	9	16.1365	1.7929	3.342**
NC x Light x Day	27	20.0840	0.7439	1.386
Residuals	192	103.0122	0.5365	
P. torta				
Nutrient Conc.[a]	3	9.9316	3.3105	6.758**
Light[b]	3	58.2839	19.4280	39.661**
Day[c]	3	10.1840	3.3947	6.930**
NC x Light	9	5.9471	0.6608	1.349
NC x Day	9	0.5849	0.0650	0.133
Light x Day	9	4.4003	0.4889	0.998
NC x Light x Day	27	6.5450	0.2424	0.495
Residuals	192	94.0521	0.4899	

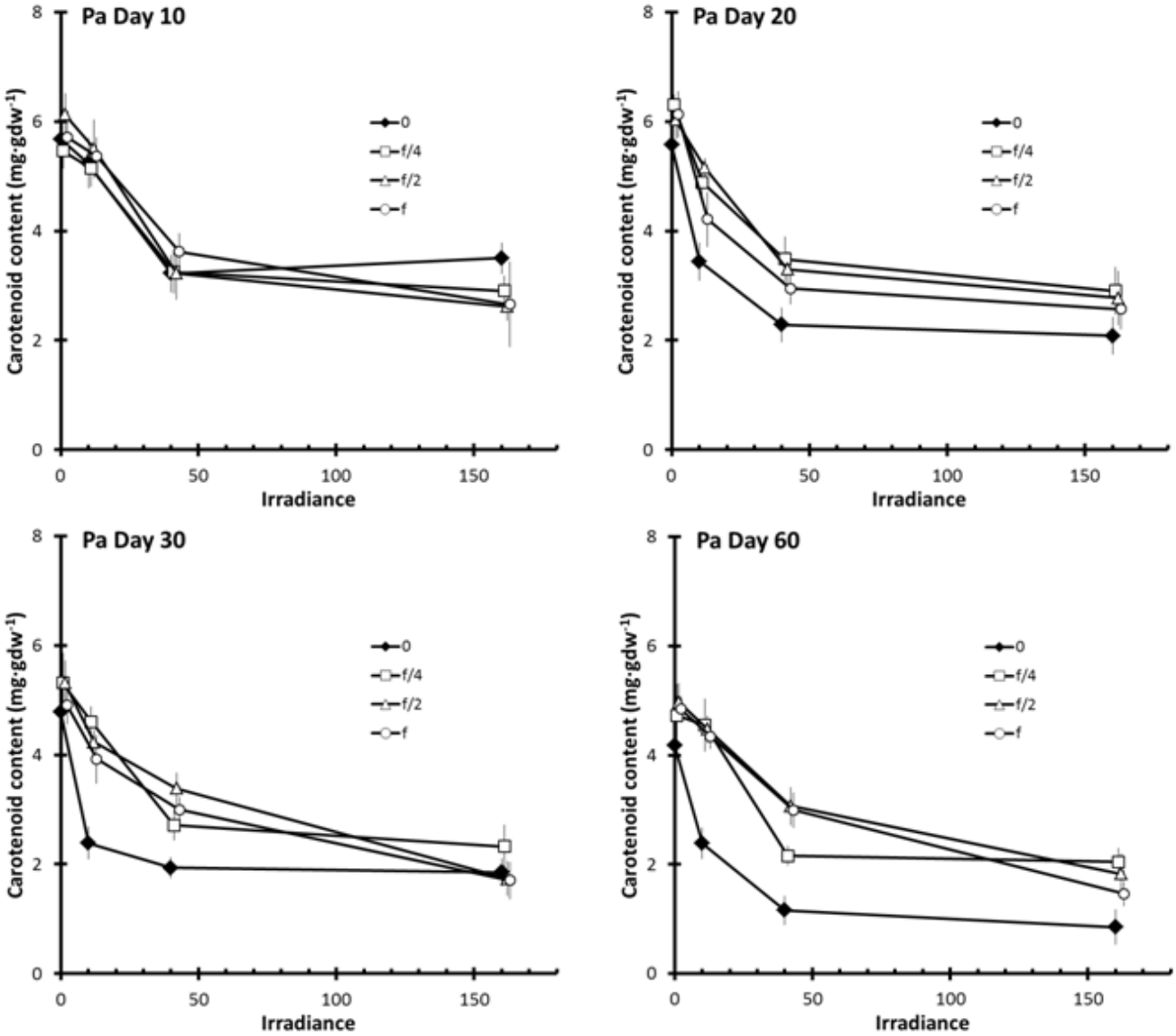

Fig. 2. *P. abbottiae (Pa). Carotenoid content of the conchocelis as a function of irradiance, nutrient concentration (◆, 0; □, f/4; △, f/2; ○, f) and culture duration. Error bars are ± S.E. Data points are slightly offset (dithered) in order to see the error bars.*

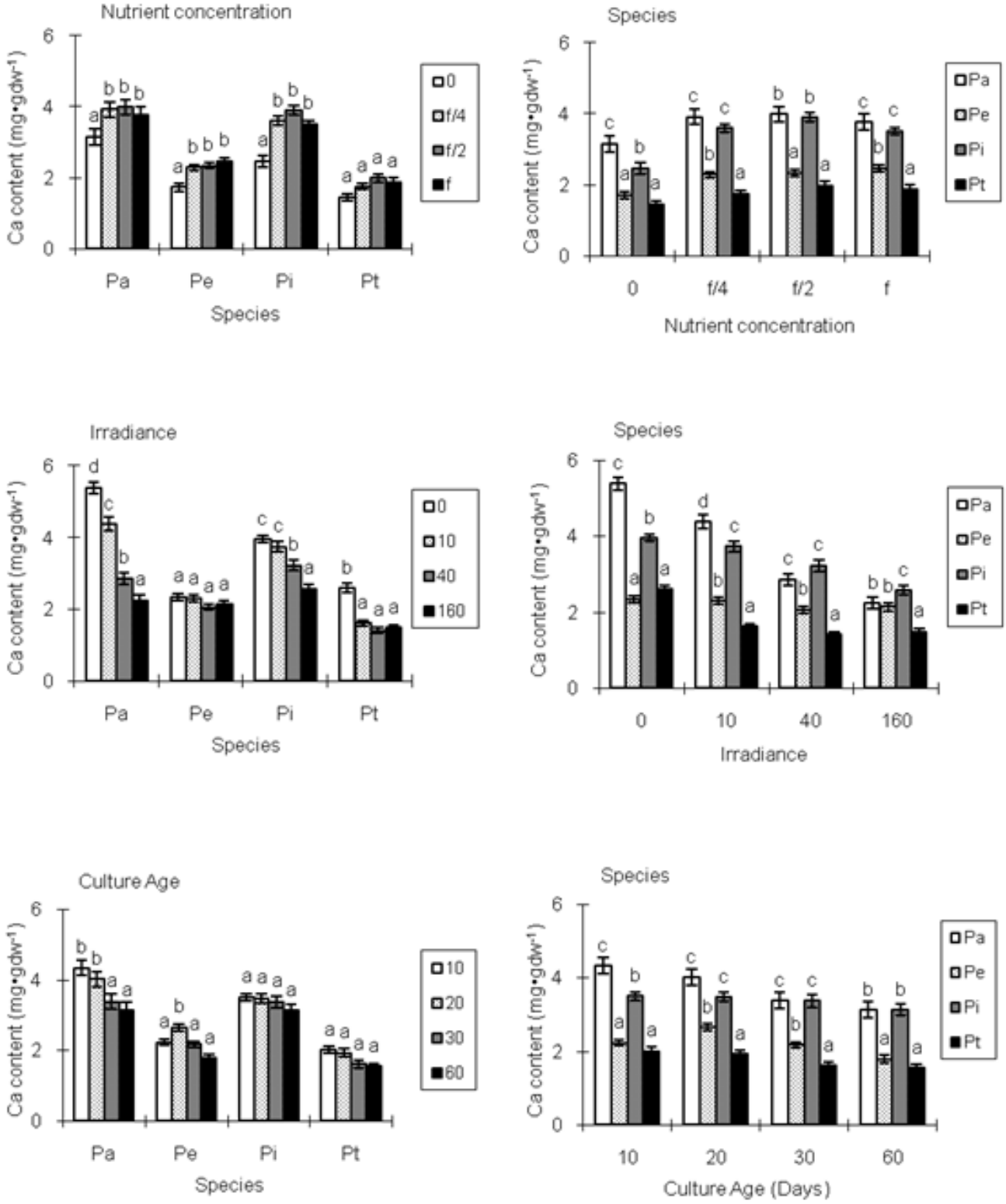

Fig. 3. *Comparison of pooled carotenoid (Ca) content of Pyropia conchocelis for each parameter tested. Error bars are ± S.E. Different letters above the bars indicate significant difference (P<0.01) based on multiple comparisons using the Newman-Keuls test. Letter comparisons are relevant within a species (for left figures) and relevant between species (for right figures). Units of parameters tested are: irradiance (µmol photons·m-2·s-1), nutrient concentration (expressed as the f fraction) and culture duration (day).*

3.3. Carotenoid Content of P. Hiberna

The carotenoid content of conchocelis ofP. Hiberna was affected by nutrients and culture age but not by light. However, there was an interaction between light and culture age (Table1). Generally speaking, this species contained low carotenoid

content (about1-4 mg·gdw^{-1}) (Fig. 4). Nutrients betweenf/4 and f concentrations did not significantly affect carotenoid content of P. hiberna, with the pooled mean of carotenoid contentbeing2.3-2.5 mg·gdw^{-1}. However, cultures with no nutrients added had significantly lower carotenoid content than those with nutrients added (Figs. 3 & 4). The carotenoid content of P. Hiberna peaked at 20 days(Fig. 3) having the highest carotenoid content (4.0 mg g·dw^{-1}) at the 20 day culture age under 0 μmolphotons·m^{-2}·s^{-1} with f nutrient concentration (Fig. 4).

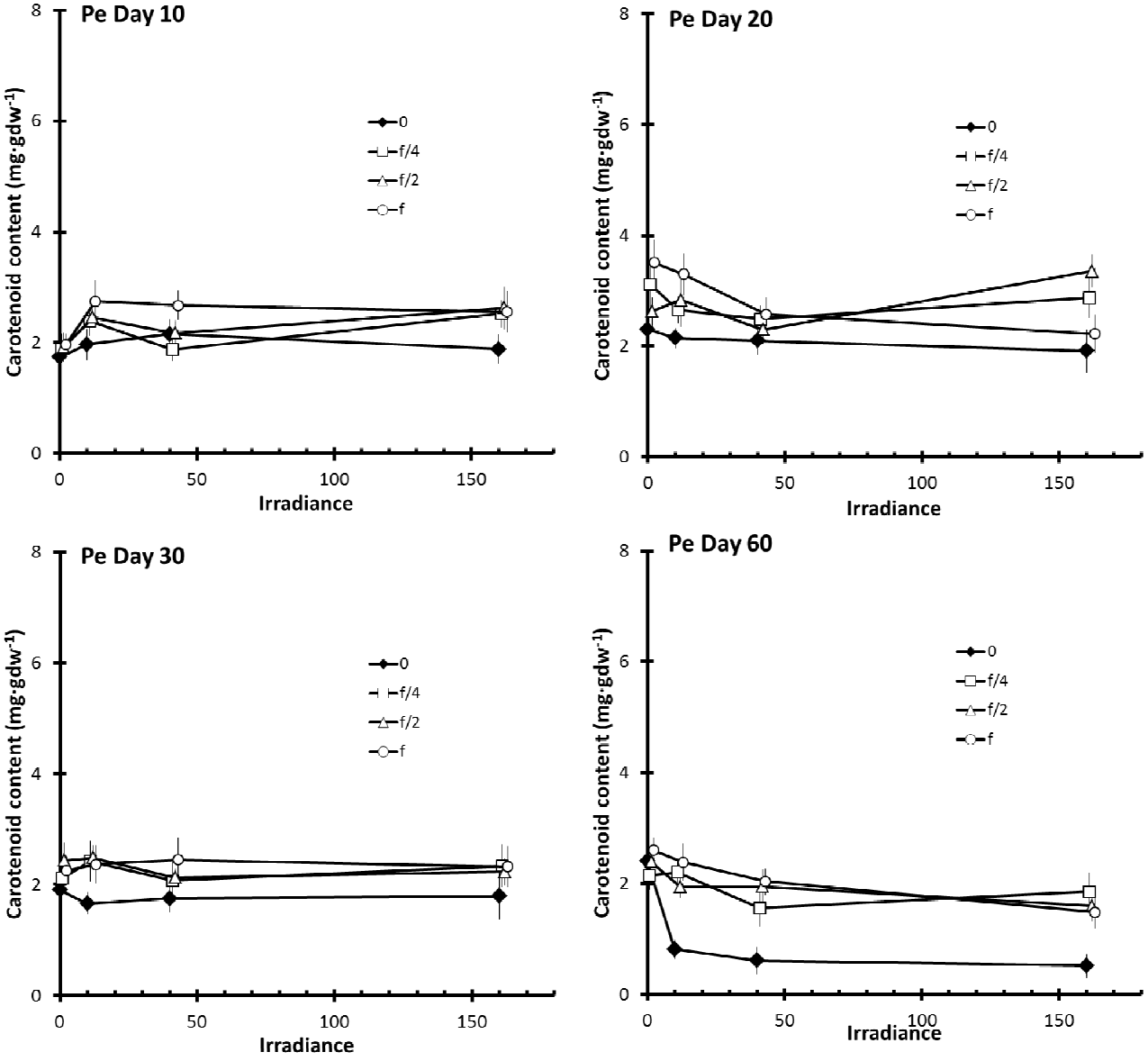

Fig. 4. *P. hiberna (Pe). Carotenoid content of the conchocelis as a function of irradiance, nutrient concentration (◆, 0; □, f/4; △, f/2; ○, f) and culture duration. Error bars are ± S.E. Data points are slightly offset (dithered) in order to see the error bars.*

3.4. Carotenoid Content of P. Sp

The carotenoid content of the conchocelis of P. sp. Was influenced by all three factors tested, including all interaction effects between these factors with the exception of no three-factor interaction occurring (Table1). Conchocelis cultures with no nutrients added generally had lower carotenoid content, particularly for older culture ages. At higher irradiances (40-160 μmolphotons·m^{-2}·s^{-1}) cultures with no nutrients added showed particularly low carotenoid content (Fig. 5). Carotenoid content of P. sp. varied with different light environments. Cultures in the darkness or at the low irradiance with nutrients generally had higher carotenoid content. High irradiances resulted in a marked decline of carotenoid content (Figs.3 & 5). Cultures in the darkness usually had the highest carotenoid content, but it was not significantly higher than that at 10μmolphotons·m^{-2}·s^{-1} (Fig. 3). Although there was a slight decrease in carotenoid content in older cultures, this decline was not statistically significant (Fig$_{-1}$3). The maximum carotenoid content (5.6 mg·gdw) was achieved at10μmolphotons·m^{-2}·s^{-1}, f/2 nutrient concentration at 20 and 30 days culture age.

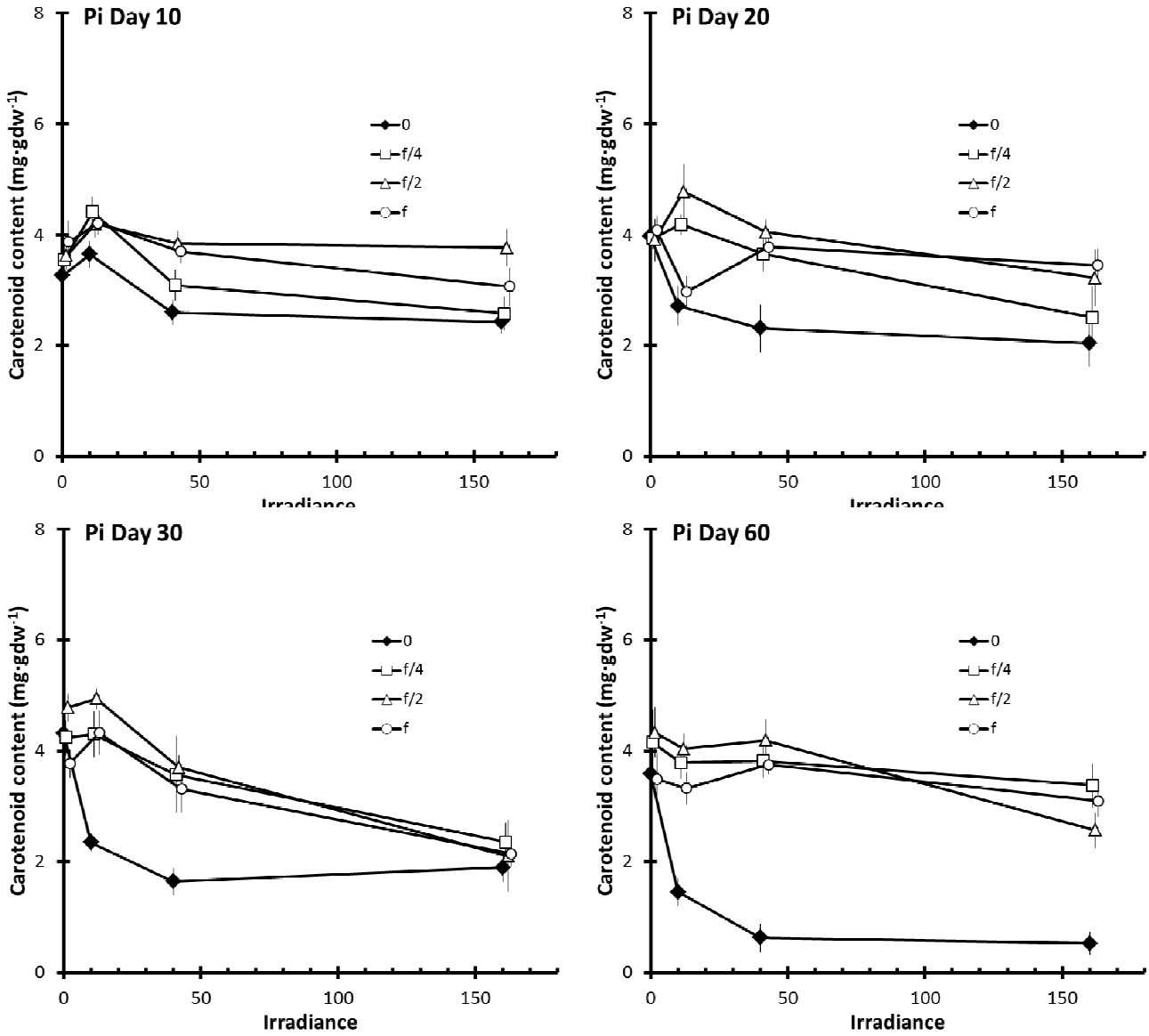

Fig. 5. *P. sp. (Pi). Carotenoid content of the conchocelis as a function of irradiance, nutrient concentration (◆, 0; □, f/4; △, f/2; ○, f) and culture duration. Error bars are ± S.E. Data points are slightly offset (dithered) in order to see the error bars.*

3.5. Carotenoid Content of P. Torta

All three factors affected the carotenoid content of the conchocelis of P.torta. However, there was no interaction occurring among factors (Table 1). Carotenoid content of this species varied little with nutrient conditions. Conchocelis cultures with no nutrients added generally had the same pigment contents as those cultures with nutrients added (Fig. 6). Similar to P. abbottiae, P. torta produced more carotenoids in the dark environment. Cultures grown in the various light environments showed little difference in the carotenoid content (Fig. 6).Similar to P. sp.,a decrease in the carotenoid content of P. torta in older cultures was not statistically significant, although a slight decline with culture age was observed (Figs.3 &6).The maximal carotenoid content (4.3 $mg{\cdot}gdw^{-1}$)was obtained at 0µmolphotons$\cdot m^{-2}\cdot s^{-1}$, f nutrient concentration at 10dayscultureage.

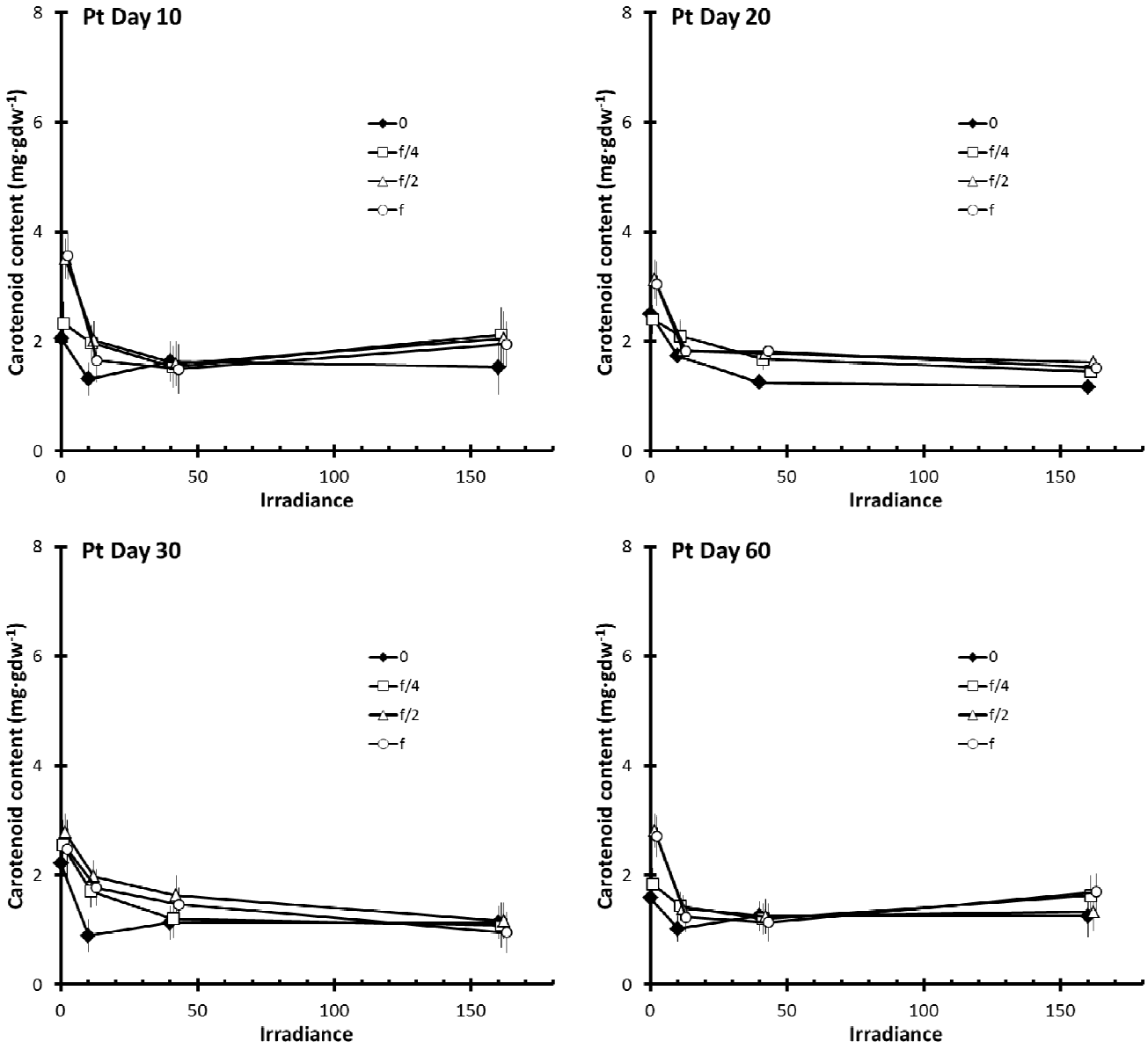

Fig. 6. *P. torta (Pt). Carotenoid content of the conchocelis as a function of irradiance, nutrient concentration (◆, 0; □, f/4; △, f/2; ○, f) and culture duration. Error bars are ± S.E. Data points are slightly offset (dithered) in order to see the error bars.*

3.6. Comparisons Among Species

Results from pooled data analyses showed that the conchocelis of P. abbottiae and P.sp. contained significantly higher carotenoid content than the other two species under all of the experimental conditions (Fig. 3).P. hiberna had the lowest carotenoid content for all levels of the three factors (Fig. 3).

Light had the most obvious influence on carotenoid content. For all four species, the higher carotenoid content (3.4-7.0 $mg \cdot gdw^{-1}$) generally occurred at 0-10μmol$photons \cdot m^{-2} \cdot s^{-1}$, f/2-f nutrient concentration at10 days culture age. Higher irradiances (≥40 μmol$photons \cdot m^{-2} \cdot s^{-1}$), low nutrients and longer culture age generally resulted in a decrease of carotenoid content.

4. Discussions

4.1. Environmental Conditions Determine Pigment Content

Because photosynthetic pigments are crucial for sustaining all life activities in the algal cell and are essential components for algal photosynthesis, variations of pigment content are important aspects of the growth, development, physiological responses and survival of the algae (Waaland et al. 1974, Fortes and Lüning 1980, Korbee et al. 2005b, Sefyabadi et al. 2011, Wahidin et al. 2013，Wondraczek et al. 2013, Ota et al. 2015). Our experimental findings show that photosynthetic pigments of the conchocelis of four Pyropia species are significantly influenced by environmental factors such as irradiance, nutrient concentration and culture age, including some interactions among these factors. Pigment content of the

conchocelis appears to be sensitive to environmental change and could be used to indicate the physiological state of the sporophytic stage of Pyropia.

Sampath-Wiley et al..(2008) reported that the highest carotenoid content in Porphyra umbilicalis thalli was about 0.4mg·gfw^{-1}(~4mg·gdw^{-1}). In this paper we report levels of carotenoid pigments (6.8-7.0 mg·gdw^{-1}) as much as 75% greater. However, the main finding here is that levels of these pigments can vary widely depending on the culture conditions.

4.2. Light and Nutrient Are Important Factors Related to Pigment Variation

It has been well documented that changes in light intensity could resul tin a large change in the photosynthetic pigment content of algae(Beach and Smith 1996, Figueroa et al. 1997, Khoyi et al. 2009, Chaloub et al. 2015)and that lower irradiance levels require greater amounts of light harvesting molecules to perform photosynthesis (Lüning 1990). From our results, carotenoid content of the conchocelis was found to vary inversely with the amount of available light. Moreover, carotenoid content was observed to be higher in complete darkness or at low irradiances and significantly declined at higher irradiances. This result mirrors that found with phycobilins in these same species (Lin and Stekoll 2011). It appears that these species in the conchocelis stage are shade adapted plants in an environment that rarely receives high light irradiance. In fact, both P. abbottiae and P. torta exhibit photoinhibition at higher light irradiances (Lin et al.2008).

It is interesting that the conchocelis tufts maintain very high levels of carotenoid pigments even when kept in the dark for as long as 60 days. Although there is no carbon fixation happening in the dark, there is measurable respiration occurring (Lin et al. 2008), and thus, energy reserves must become depleted. In spite of the decrease in energy reserves, the photosynthetic pigments remain high, suggesting that these algae must be ready for light harvesting at anytime when light becomes available.

It is well known that the conchocelis of Porphyra/Pyropia burrow into shells or barnacle tests in nature. It is not known how long the conchocelis can persist in these environments. In Alaska, the gametophytes of P. abbottiae and P. torta first appear in late winter and early spring and are gone by mid-summer. The conchocelis of these species, in order to produce the next generation of gametophytes, must live throughout the summer when temperatures are high but nutrients are low and through the winter when there is very little sunlight. It is reasonable to conclude that the environmental constraints in the Alaskan waters have contributed to the fact that these algae can maintain their photosynthetic pigments in conditions of low nutrients and/or low light for several months.

Many studies have shown that nutrients, especially nitrogen affect both growth, development and pigment content of algae (Lapointe and Ryther 1979, Meiqin et al. 1979, Wheeler and North 1980, Hannach 1989, Grobe et al. 1998, Korbee et al. 2005a, 2010, Kim et al. 2007, Xie et al. 2013, Imaizumi et al. 2014, Chaloub et al. 2015). Our experimental results also indicate that nutrients are very important for the sporophytic stage of Pyropia. Under the culture conditions tested, especially under higher light irradiances, conchocelis grown in media with nutrients added usually had much higher content of photosynthetic pigments in contrast to cultures with no nutrients. Nitrogen source and supply in coastal waters can take place with large seasonal fluctuations. Shortage of nutrients would exert a potentially negative effect on the growth, development and survival of the natural populations of Porphyra/Pyropia sporophytes.

Sufficient nutrient supply is necessary to promote higher pigment content for Pyropia conchocelis. However, different species exhibited differences in nutrient requirements. For example, higher nutrient concentration (f concentration)was favorable for carotenoid production in P. hiberna. For the other threes pecies, intermediate nutrient concentrations (f/4-f/2) were sufficient for high pigment content. Culture age was also a factor in the production of pigments. P. abbottiae tended to synthesize significantly less photosynthetic pigments with prolonged culture age, in contrast to the other three species which had relatively constant amount of pigment production throughout the entire period of culture.

4.3. Conchocelis: Good Algal Source for Highly-Valued Pigment Extracts

There are three basic classes of natural pigments found in marine red algae, i.e. chlorophylls, carotenoids and phycobilins. Besides their roles in photosynthetic and photoprotective functions, it has been reported that these natural pigments exhibit various biological properties such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities. These properties provide health benefits and have potential applications in foods, cosmetics and pharmaceuticals(Okaiet al. 1996, Shetty et al. 2005, Yabutae t al. 2010, Pangestutia and Kim 2011, Sangha et al.2013, Wang et al. 2015). It has been shown that extracts from Porphyra/Pyropia contain important bioactive compounds. For instance, certain low molecular weight peptides containing Asp, Ala and Glu possess immunosuppressive, antioxidant and antihypertensive capacities (Qu et al. 2010，Cian et al. 2012). It is worthwhile to explore the use of these high-value bioactive properties. Of interest in this respect is that the carotenoids are not only photosynthetic accessory pigments but also possess important bioactive properties that can be beneficial to human health in many different ways(Okuzumi et al. 1990, Schubert et al. 2006, Sachindra et al. 2007).

The carotenoids are not only beneficial to human health, but also valuable as a commodity. The current price of carotenoids has been as high as $5-400 per microgram depending on different types, purities and sources (Sigma Chemical, St Louis, MO). For commercial production, there are several advantages in using Porphyra/Pyropia conchocelis material for the preparation and production of high-value phycological extracts: (i)the conchocelis stage can be grown relatively quickly using standard culture apparatus, (ii) the cultures of

the conchocelis stage can be maintained indefinitely in a nonreproductive state under the proper culture conditions,(iii) conchocelis grown under the proper conditions have relatively high concentrations of phycobiliproteins, carotenoids (Lin and Stekoll 2011),(iv) high-quality and high purity extracts can be obtained from cultures of the conchocelis stage using simple extraction procedures and (v) target products can be acquired at any time, year-round, without relying on the availability of wild algal material(Stekoll et al. 1999, Lin and Stekoll 2011).Furthermore, it is possible to produce multiple kinds of high-value components such as carotenoids , phycoerythrin and phycocyanin simultaneously from conchocelis material. Based on complete combination experiment of 4 levels with three factors (nutrient concentration, irradiance and culture age), the present study investigated the effects of environmental variables on the carotenoid content of Porphyra conchocelis and determined the optimal cultural conditions, which would helpful for obtaining algal material with higher pigment content and extraction of high value pigment. The results presented here can contribute to creating the optimal culture conditions for producing the maximal yield of carotenoids from Pyropia conchocelis with implications for the commercial production of these pigments.

Acknowledgements

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