Potential Fucoxanthin Production from a Marine Diatom

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ABSTRACT

Potential production of fucoxanthin from a marine diatom (*Chaetoceros* sp.) isolated from the Pranburi River mouth, Gulf of Thailand was investigated. Results showed that *Chaetoceros* sp. had a high growth rate. Specific growth rate (μ) was approximately 0.59 day^{-1}, while doubling time was approximately 1.18 days under irradiance of 70 μmol photons·m^{-2}·s^{-1}. Specific growth rate increased with increasing irradiance (μ=1.04 at 140 μmol photons·m^{-2}·s^{-1}). The doubling time of this *Chaetoceros* sp. was only 0.67 days under irradiance of 140 μmol photons·m^{-2}·s^{-1}. Results from HPLC analysis demonstrated that *Chaetoceros* sp. gave maximum fucoxanthin production at 7.68 mg·g^{-1} dry weight or 5.87 pg·$cell^{-1}$ under irradiance of 140 μmol photons·m^{-2}·s^{-1}. Findings implied that the selected *Chaetoceros* sp. showed higher potential for commercial-scale fucoxanthin production than other seaweeds and diatoms.

Keywords: *Chaetoceros*, Fucoxanthin, Growth rate, Marine diatom

INTRODUCTION

Diatoms are the most abundant phytoplankton in aquatic habitats. They have a strong effect on global climate and play a major role in the biochemical cycles of carbon, nitrogen, phosphorus and silicon of both oceanic and freshwater environments (Medlin and Kaszmarska, 2004). In the oceans, diatoms are the dominant phytoplankton to bloom under nutrient-rich conditions, whereas in fresh water, high turbulence and low temperature combine with high nutrients to promote diatom blooming. De La Rocha (2004) suggested that diatoms are successful in high nutrient conditions of coastal oceanic regions, and account for 75% of their primary productivity and more than 40% of the total annual marine primary productivity. Moreover, half of the total global primary production is from marine sources, and marine diatoms carry out 20% of the primary productivity each year. Kooistra *et al*. (2003) noted from fossil evidence that centric diatoms appeared at least 180 million years ago, whereas pennate diatoms first appeared 90 million years ago and evolved from centric diatoms. Under different light conditions (intensity and spectrum), diatoms adapt by varying their total amount of cellular pigments and/or ratios of different pigments. Phytoplankton utilize and absorb excess energy during stress conditions in high level light by pigments contained in the cells (Vincent *et al*., 1984; Cogdell and Frank, 1987; Demmig-Adams, 1990). This excess energy

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Received 14 May 2019 / Accepted 8 August 2019

can cause damage to intracellular materials or metabolic processes, e.g., destruction of chloroplast membranes or inactivation of enzymes, although some carotenoids quench the excess energy as photoprotection against photoinhibition. Hager (1975) demonstrated that the xanthophyll cycle dissipates excess light energy by mutual transformations between epoxy-containing xanthophylls (oxy-derivatives of carotenes) and epoxy-free xanthophylls.

Normally, diatoms and other eukaryotic algae show a characteristic brown color resulting from high amounts of the fucoxanthin (Stauber and Jeffrey, 1988). Bertrand (2010) also suggested that fucoxanthin-Chl *a*-Chl *c2* protein-complexes (FCP) are mostly located in fucoxanthin and diadinoxanthin molecules and have functions related to the light-harvesting complexes (LHC) of green algae and higher plants. Carotenoids function as accessory molecules for light harvesting, in prevention from photo-damage, and as antioxidants in reaction centers under stress conditions (Lichtenthaler, 2007). Fucoxanthin is an important antioxidant as a colorant that is useful in stimulating the immune system (Murakami *et al*., 2002). It also stimulates apoptosis and inhibits growth of colorectal cancer cells in humans (Hosokawa *et al*., 2004). Fucoxanthin is used as a dietary supplement made from seaweed (*Undaria pinnatifida*). Several researchers have reported amounts of fucoxanthin pigment in plankton (Chromophyta) higher than in seaweed. Normally, marine phytoplankton in the Division Chromophyta are important sources of carotenoids in the form of xanthophylls, such as brown phytoplankton or diatoms, producing diatoxanthin, diadinoxanthin and fucoxanthin (Lorenz and Cysewski, 2000; Lohr and Wilhelm, 2001). Suitable varieties of phytoplankton in the Division Chromophyta insolated in Thai waters could be mass cultured for fucoxanthin production. However, to produce large volumes of phytoplankton with high productivity, it is first necessary to find a species that has rapid growth and high tolerance to changes of both physical and chemical factors. Here, a rapid-growth species with high cellular fucoxanthin composition and potential for mass culture was investigated to produce a nutritional supplement for medical benefits.

MATERIALS AND METHODS

Algal collection and classification

Marine diatoms were collected from the coastline close to the Pranburi River mouth (12º 24'30.28"N, 99º59"25.66"E) in the western Gulf of Thailand. Single-cell diatoms were then isolated by micropipette under compound microscope in the laboratory. Diatoms were maintained in 10 ml culture tubes containing 25 psu filter-sterilized enriched seawater medium (ESM) according to Watanabe *et al*. (1988). Temperature was maintained at 25 °C. Illumination was provided by a daylight fluorescence lamp (Philips 40 W) with L:D (light: dark) cycles of 12:12 h. Light intensity was provided at 70 µmol photons·m^{-2}·s^{-1}. For classification, a selected diatom was sub-inoculated and cultured in a 250 ml Erlenmeyer flask containing 100 ml ESM at 25 psu. The pH of ESM was adjusted to 8.0 before autoclaving (121 ºC, 1 atm, 20 min), and then cooled before adding a mixture of penicillin G and streptomycin (Liu *et al*., 2017). The temperature was kept at 25 °C. Shaking was performed three times at 8.00, 12.00 and 16.00 for 48 h. The selected diatoms were collected by filtering with a GF/F glass fiber filter. Diatom cells on the fiber filter were then prepared before identification using a scanning electron microscope (SEM).

Sample preparation for SEM

Selected marine diatoms on the GF/F filter were washed three times with sterile filtered seawater. Then, they were dehydrated with absolute ethyl alcohol (99 %) after dehydrating by a concentration series of 25, 50, 75, 90 and 95 %, respectively. Samples were then dried by CPD (critical point drying) using a Quorum series K850, gold plated with a Sputter Coater, Balzers series SCD 040, and photographed using an SEM (JEOL series JSM-6610LV).

Algal culture and experimental design

Selected marine diatoms were cultured in a 250 ml Erlenmeyer flask containing 100 ml sterile ESM at 25 psu according to Watanabe *et al*. (1988)

under the laboratory conditions described above with varying light intensity. Selected diatoms were cultured at 25 °C. All experiments had two levels of irradiation, 70 µmol photons·m^{-2}·s^{-1} and 140 µmol photons·m^{-2}·s^{-1}, providing daylight fluorescence under L:D cycles of 12:12 h. Triplicate sampling was conducted every day for the first 11 days for chlorophyll *a* analysis and at days 3, 12 and 17 for fucoxanthin analysis.

Algal growth determination

Growth of cells in the culture medium was measured by daily cell counts using a Neubauer type hemocytometer (ISOLAB, Laborgerate GmbH). Maximum cell density was determined during the stationary phase, while specific growth rate (µ) was calculated according to the equation:

$$\mu = \text{In } (N_t\text{-}N_0)/ \Delta t$$

where N_0 and N_t are cell numbers at two different times during the experiment, and Δt is the time interval (in days) between N_t and N_0, according to Gagnon *et al*. (2005) and Guillard and Siercki (2005). Doubling time, T_2, for the culture was expressed in the same units of time as µ and the yield rate (Y), according to Andersen (2005).

$$T_2 = 0.6931/ \mu$$

$$Y = (N_t\text{-}N_0)/ \Delta t$$

Chlorophyll a analysis

Water samples obtained from each experiment were filtered through a glass fiber filter (Whatman GF/F). Diatom cells on the filter were extracted with 5 ml 90% acetone. Extracts of chlorophyll *a* were kept at -20 ºC for 24 h before analyzing with a spectrophotometer (CECIL; Model CE1010/1000). Chlorophyll *a* is assumed to have absorbance of zero at 750 nm to correct for turbidity and contaminating colored compounds according to Ritchie (2006). After standing for 24 h, the extracts were placed in a sonicator for 5 min; thereafter, they were centrifuged at 3,000 rpm for 5 min before collecting the supernatant for chlorophyll *a* analysis. Chlorophyll *a* concentration was then determined by the spectrophotometric method (Parsons *et al*., 1984).

Fucoxanthin analysis

Subsamples for analysis of fucoxanthin were filtered through a glass fiber filter (Whatman, GF/F) and cells that collected on the filters were extracted in 5 ml acetone (90%), stored at -20 ºC for 24 h until required for further analysis. The extracts were analyzed using high performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity, UV Array Detector, reversed-phase column of Agilent ZORBAX Eclipse Plus C_{18} Analytical 4.6x150 mm) with temperature maintained at 28 ºC. Mobile phase A was 85% methanol added with 0.5 M ammonium acetate (v/v), while mobile phase B was 90 % acetonitrile. Mobile phase C was ethyl acetate. Flow rate was set at 0.8 ml·min^{-1}. The analytical program followed the method of Van Leeuwe *et al*. (2006) with the gradient modified as follows: 0 min: A: 60 %, B: 40 %, C: 0 %; 2 min: A: 0 %, B: 100 %, C: 0 %; 7 min: A: 0 %, B: 80 %, C: 20 %; 17 min: A: 0 %, B: 50 %, C: 50 %; 21 min: A: 0 %, B: 30 %, C: 70 %; 28.5 min: A: 0 %, B: 30 %, C: 70 %; 29.5 min: A: 0 %, B: 100 %, C: 0 %; 30 min: A: 60 %, B: 40 %, C: 0 %; 35 min: A: 60 %, B: 40 %, C: 0 %. Peaks were quantified using standards for fucoxanthin acquired from Sigma-Aldrich PN:16337 and detected at 450 nM by UV array.

RESULTS

Morphology

A marine diatom was isolated from the estuary of the Pranburi River in the western Gulf of Thailand. Cell morphology is shown in Figure 1. The cell had long setae on the edges of all four corners and was approximately 4 µm in width and 10 µm in length.

Cell growth

Growth of the selected marine diatom was impacted by light intensity (Figure 2). *Chaetoceros* sp. was exposed to different irradiance treatments

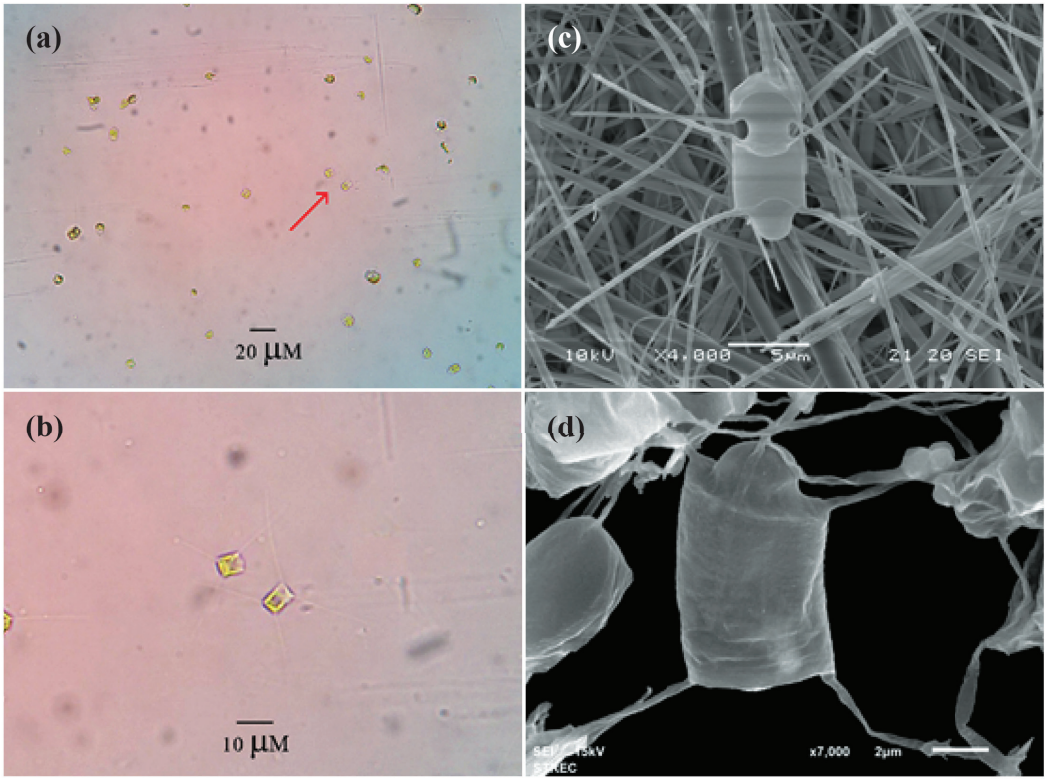

Figure 1. Morphology of the selected marine diatom (*Chaetoceros* sp.) cells under light microscope 400X (a), 1,000X (b), and SEM (scanning electron microscope) 4,000X (c) and 7,000X (d).

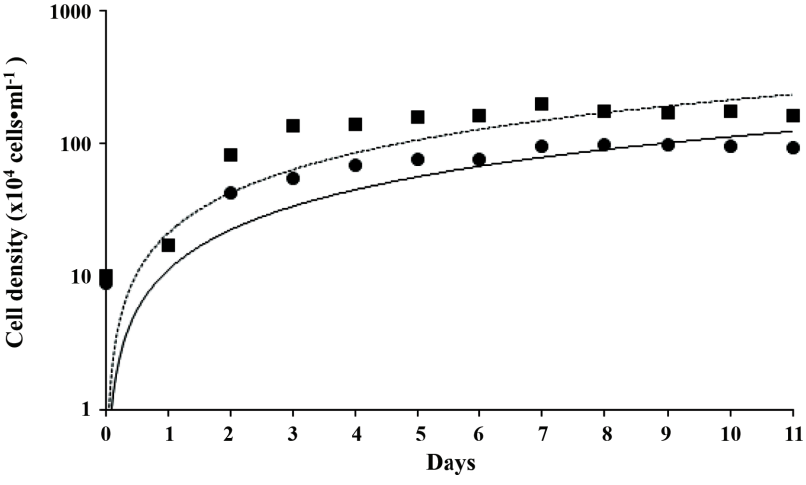

Figure 2. Growth curve of the selected marine diatom cultured under irradiance of 70 (——●) and 140 (----■) µmol photons·m^{-2}·s^{-1}.

and showed disparate final cell density. Cell density during the stationary phase increased at higher light intensity. Under high irradiance (140 µmol photons·m^{-2}·s^{-1}), cell entrance to the stationary phase was at day 8 after sub-inoculation, whereas it occurred at day 10 after sub-inoculation under low irradiance (70 µmol photons·m^{-2}·s^{-1}). No lag phase was shown for this selected diatom; initial density was 100,000 cells·ml^{-1}.

Cell density of the two experimental levels of irradiance showed no significant difference during the first two days of the experiment (43x10^4 cells·ml^{-1} under irradiance of 70 µmol photons·m^{-2} ·s^{-1} and 83x10^4 cells·ml^{-1} under irradiance of 140 µmol photons·m^{-2}·s^{-1}). After two days, the diatom cells divided rapidly and cell density increased logarithmically, passing through the exponential phase at day 8 under low irradiance and at day 7 of high irradiance (Table 1). Maximum cell density was observed on day 8 at approximately 97x10^4 cells·ml^{-1} for low irradiance and on day 7 at 198 x10^4 cells·ml^{-1} for high irradiance.

Ratio of cell density under high:low irradiance during the first day was almost equal to one. The cell density ratio increased to higher than 1.5 after day 2 of the experiment and reached a maximum value of 2.47 on day 3 (Table 1).

Specific growth rate

Growth rate increased rapidly during the first three days. The selected diatom cultured under low (70 µmol photons·m^{-2}·s^{-1}) and high (140 µmol photons·m^{-2}·s^{-1}) irradiance provided maximum specific growth rates (μ_{max}) of approximately 0.59 and 1.18, respectively, while doubling times (T_2) were 1.04 and 0.67 days, respectively. Yield rates (Y) were approximately 19.0x10^4 cells·day^{-1} and 59.50x10^4 cells·day^{-1}, respectively.

Chlorophyll a content

Chlorophyll *a* content of the selected marine diatom on the first day was 28.93 µg·l^{-1} and 28.18 µg·l^{-1} for irradiation at 70 and 140 µmol photons ·m^{-2}·s^{-1}, respectively. The chlorophyll *a* content gradually increased during the first three days and reached maximum values of 274.42 µg·l^{-1} after inoculation at day 6 under low irradiance and 198.77 µg·l^{-1} at day 4 under high irradiance (Table 2).

Table 1. Changes in cell density and cell density ratio of the selected marine diatom (*Chaetoceros* sp.) cultured under irradiance of 140 and 70 µmol photons·m^{-2}·s^{-1}.

Time (Day)	Cell Density (x10^4 cells•ml^{-1})		Cell Density Ratio (140:70 µmol photons•m^{-2}•s^{-1})
	Irradiance 70 µmol photons•m^{-2}•s^{-1}	Irradiance 140 µmol photons•m^{-2}•s^{-1}	
0	9±1.00	10±1.15	1.11
1	17±2.08	17±3.00	1.00
2	43±4.04	83±2.08	1.93
3	55±11.68	136±3.06	2.47
4	69±5.00	141±9.71	2.04
5	76±6.93	157±14.84	2.07
6	76±15.50	162±10.12	2.13
7	95±1.53	198±3.79	2.08
8	97±6.56	175±11.02	1.80
9	97±0.58	171±5.69	1.76
10	96±2.65	174±7.02	1.81
11	94±8.08	163±15.72	1.73

Chlorophyll *a* content of the diatoms under high irradiance declined to 176.52 $\mu g \cdot l^{-1}$ at five days after sub-inoculation, while cells under low irradiance continued to increase over the six days following sub-inoculation (Figure 2).

Chlorophyll *a* content in cells of *Chaetoceros* sp. cultured under low irradiance (70 µmol photons $\cdot m^{-2} \cdot s^{-1}$) was higher than under high irradiance (140 µmol photons $\cdot m^{-2} \cdot s^{-1}$) throughout the experiment. Chlorophyll *a* content in diatom cells cultured under low irradiance ranged between 0.26 and 0.36 $ng \cdot cell^{-1}$. Chlorophyll *a* content reached the highest value at day 6 after sub-inoculation. Chlorophyll *a* content decreased gradually from 0.28 to 0.11 ng $\cdot cell^{-1}$ over the six days following sub-innoculation (Table 2).

Fucoxanthin content

Figure 3 shows retention time of fucoxanthin analyzed by HPLC. Fucoxanthin content of the selected marine diatom during cultivation under irradiance at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ on days 3, 12 and 17 was 1.13, 2.46 and 3.20 $\mu g \cdot l^{-1}$, respectively. Based on these values, the amount of fucoxanthin that the selected diatom could produce was calculated to be 2.70, 5.91 and 7.68 $mg \cdot l^{-1}$ dry weight or 2.81, 2.60 and 5.87 $pg \cdot cell^{-1}$ after cultivation for 3, 12 and 17 days, respectively.

Table 2. Changes in chlorophyll *a* content of the selected marine diatom (*Chaetoceros* sp.) under irradiance of 70 and 140 µmol photons $\cdot m^{-2} \cdot s^{-1}$.

Time (Day)	Chlorophyll *a* ($\mu g \bullet l^{-1}$)		Chlorophyll *a* content ($ng \bullet cell^{-1}$)	
	Irradiance 70 µmol photons $\bullet m^{-2} \bullet s^{-1}$	Irradiance 140 µmol photons $\bullet m^{-2} \bullet s^{-1}$	Irradiance 70 µmol photons $\bullet m^{-2} \bullet s^{-1}$	Irradiance 140 µmol photons $\bullet m^{-2} \bullet s^{-1}$
0	28.93±4.45	28.18±2.57	0.32	0.28
1	48.95±1.54	39.16±6.72	0.29	0.23
2	120.15±3.34	144.63±13.89	0.28	0.17
3	140.18±24.06	194.32±13.59	0.26	0.14
4	212.12±15.63	198.77±5.14	0.31	0.14
5	247.72±6.80	176.52±11.2	0.33	0.11
6	274.42±16.85	182.45±29.18	0.36	0.11

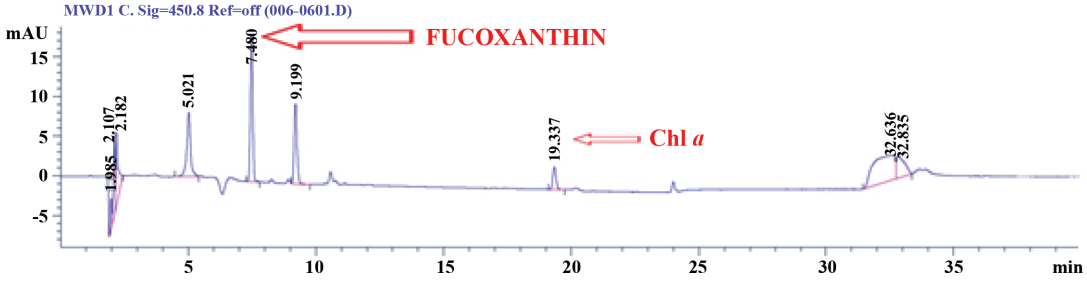

Figure 3. Retention time of fucoxanthin and chlorophyll *a* from the selected marine diatom (*Chaetoceros* sp.) from HPLC analysis.

DISCUSSION

After attempting for several years to discover a new marine diatom with high fucoxanthin production potential, we finally selected a species from the Pranburi River mouth estuary. The specimen had small cell size of only 4 µm width and 10 µm length. The girdle view of the cell was generally square or rectangular with radial symmetry; therefore, we classified the specimen as a centric diatom. It was identified as *Chaetoceros* sp. because of the long setae on the edges of all four corners. Most girdle views were rectangular, corresponding to Sunesen *et al*. (2008) who determined phytoplankton in the Division Chromophyta, Class Bacillariophyceae, Order Biddulphiales, Suborder Biddulphineae and Family Chaetocerotaceae.

Growth

The two light intensity levels resulted in different cell densities of *Chaetoceros* sp. in the same time period, although initial densities of these two treatments were similar. Creswell (2010) and Anderson (2005) suggested that phytoplankton normally show growth curves which include a lag phase, exponential phase, stationary phase and declining phase. Here, there was no evidence of the lag phase because initial cell density was quite high (ca.10x10^4 cells·ml^{-1}). In addition, the selected diatom grew very fast and entered the exponential phase only two days after inoculation. Under high light intensity (140 µmol photons·m^{-2}·s^{-1}), cell density of this diatom was markedly higher than under low light intensity (70 µmol photons·m^{-2}·s^{-1}). Richmond *et al*. (1980) reported that light was the energy source which converted nutrients in the photosynthesis process for algal biomass, while cell density was the primary variable regulating the efficient use of light. Cell density ratio for high to low light intensity was generally higher than 2.0 during the exponential phase, indicating that light intensity stimulated the cell density of this diatom.

In this study, all parameters of culture conditions, including nutrient levels, temperature, and salinity were fixed, and only light intensity was varied. Results showed that increasing light intensity enhanced the specific growth rate (µ) of our selected diatom. This phenomenon concurred with Strzepek and Price (2000) who reported that high irradiance increased the specific growth rate of *Thalassiosira weissflogii*, while specific growth rates were reduced at low light intensity. Isolated *Chaetoceros* sp. in our study grew very fast (µ=0.59 day^{-1} under irradiance of 70 µmol photons·m^{-2}·s^{-1} and 1.04 day^{-1} under 140 µmol photons·m^{-2}·s^{-1}) compared with several previous studies. Anderson (2005) suggested that the specific growth rate of *Cyclotella cryptica* was as high as 0.94 day^{-1} and doubling time (T_2) was 0.74 days. Here, doubling time of the selected *Chaetoceros* sp. was only 0.67 days when cultured under high light intensity. Moreover, our selected *Chaetoceros* sp. achieved a specific growth rate higher than bacteria in biofilm that had specific growth rate of 0.77 day^{-1} (Anderl *et al*., 2003).

Chlorophyll a content

Results demonstrated that chlorophyll *a* content of the selected diatom cultured under low light intensity was higher than under high light intensity throughout the experiment. This phenomenon also occurred during culture of *Thalassiosira weissflogii*, as reported by Strzepek and Price (2000). They suggested that *Thalassiosira weissflogii* reduced the impact of irradiance by increasing chlorophyll *a* content in the cell. The impact of light intensity on photosynthesis was studied for potential commercial biomass culture of *Chaetoceros* sp. and chlorophyll *a* was shown to be a highly useful index for monitoring culture growth (Anderson, 2005). Moreover, the pigment ratio of diadinoxanthin plus diatoxanthin to chlorophyll *a* of *Thalassiosira weissflogii*, *Chaetoceros gracilis* and *Phaeodactylum tricornutum* increased substantially at higher irradiance (Fujiki and Taguchi, 2001).

Fucoxanthin content

Although maximum fucoxanthin production of the selected *Chaetoceros* sp. in our study was lower than the production of fucoxanthin from *Odontella aurita* reported by Xia *et al.* (2013), the selected *Chaetoceros* sp. can grow approximately

three times faster than *O. aurita*. Xia *et al*. (2013) cultured *O. aurita* in 320 l photobioreactors under light intensity of 100 and 300 µmol photons·m^{-2}·s^{-1}. Maximum fucoxanthin production was 18.47 mg ·$g^{-1}$of dry weight under light intensity at 100 µmol photons·m^{-2}·s^{-1}, while productivity was 7.96 mg ·l^{-1}·day^{-1}. Recently, fucoxanthin production was achieved from seaweed, but was still low compared to the productivity of the marine diatom in this study. Jaswir *et al*. (2012) reported that amounts of fucoxanthin in *Sargassum binderi* and *S. duplicatum* were only 0.73 and 1.01 mg·g^{-1} dry weight, while Kim *et al*. (2012) reported that *Isochrysis* aff. *galbana*, *Phaeodactylum tricornutum*, *Chaetoceros gracilis*, *Isochrysis galbana* and *Nitzschia* sp. cultured in f/2-Si medium at light intensity of 2,500 lux at 20 ºC produced maximum fucoxanthin of 18.23, 8.55, 2.24, 6.04 and 4.92 mg·g^{-1} dry weight, respectively. Compared to these results, our selected *Chaetoceros* sp. showed higher potential for fucoxanthin production. Overall results from our study indicated that the production potential of fucoxanthin of our selected diatom was significantly higher than brown algae and the other marine diatoms. Our selected *Chaetoceros* sp. showed potential for producing high quantities of fucoxanthin by rapid growth and is a candidate for commercial mass culture in the future.

CONCLUSION

Our selected marine diatom isolated from the Pranburi River estuary in the western Gulf of Thailand was identified as *Chaetoceros* sp. Results from potential production experiments of *Chaetoceros* sp. concluded that productivity of fucoxanthin can be increased by higher light intensity levels, resulting in increased biomass and high specific growth rates. The selected *Chaetoceros* sp. entered the exponential phase at 2-5 days and reached maximum cell density at 8-10 days. Overall results indicated that the selected *Chaetoceros* sp. showed greater potential to produce fucoxanthin than seaweed with the possibility of expansion to a commercial scale.

ACKNOWLEDGEMENTS

We gratefully acknowledge Assoc. Prof. Thaithaworn Lirdwitayaprasit for his helpful comments and thank all members of the Kasetsart University Marine Environment Laboratory (Department of Marine Sciences) for their help and cooperation with the laboratory analyses. Part of this research was funded by the Center for Advanced Studies in Tropical Natural Resources, Center for Advanced Studies for Agriculture and Food, and the KU Institute for Advanced Studies, Kasetsart University, Thailand.

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