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Production of Eicosapentaenoic Acid and Fucoxanthin in Cold-Tolerant Diatom Strains

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***Abstract*—**Diatoms hold great potential for nutraceutical production as they are source of high value products such as eicosapentaenoic acid (EPA) and pigment fucoxanthin. EPA has proven human health benefits, and fucoxanthin can be used as both medicinal and nutritional ingredient to prevent and treat chronic diseases. The economic perspective of commercial production of a single product from microalgae is not attractive due to the high production cost. To improve the economic viability, we explore the concept of combining the production of both EPA and fucoxanthin in a single process. In our current study, we isolated twelve new microalgae isolates from Ofotfjord. Eight of them are diatoms and 4 of them are cyanobacteria and microalgae. Screening the new diatom isolates revealed that two strains are cold-tolerant diatom which can grow at 10 °C. They accumulated significant amount of lipid, which was up to 40-60% of the dried mass. The EPA contents from the two strains ranged from 15-19% of the total fatty acid, while fucoxanthin concentrations were between 1-1.4% of the dried biomass. Comparing with other studied diatom, this is promising result. The two strains hold promise as source for EPA and fucoxanthin production.

***Keywords*—**Microalgae, fucoxanthin, eicosapentaenoic acid, diatom, fatty acid.

I. Introduction

EPA is one of the central nutrients for human consumption to counteract cardiovascular disease, diabetes, different type of carcinoma, and brain disorders [1]-[3]. EPA is also a key nutritional requirement during childhood, which helps improving cognitive and visual development [4]. Human cannot synthesize EPA and docosapentaenoic acid (DHA) and they must be taken through diet or nutritional supplements [5]. These polyunsaturated fatty acids (PUFAs) are mainly obtained from fatty fishes, but the global fish stocks cannot be considered as a sustainable source of PUFAs to fulfil the rising global demand. Market demand for alternative sources rich in PUFAs is therefore increasing. EPA from some diatom species is good candidate since its content has been shown to reach up to 2.5% of the dried biomass [6].

Beside PUFAs, diatom also synthesizes fucoxanthin, a pigment which plays a role in light-harvesting as part of the photosynthesis process in diatoms [7]. Fucoxanthin is high value product with many good biological properties including anti-diabetes, anti-obesity, anti-cancer, anti-oxidation, and anti-inflammatory [8]-[10]. Because of those biological properties, fucoxanthin can potentially be used for preventing and treatment of some chronic diseases [11]. Fucoxanthin is produced mainly from the brown macroalgae which however contain very low concentrations of fucoxanthin [12]. Fucoxanthin content ranged from 0.22 to 2.6% of dry weight in diatoms, which is 100 times more than that found in seaweeds [6].

Commercial production of a single product from microalgae has been hampered because of the high production cost. However, by combining the production of both EPA and fucoxanthin, the economic viability of commercial production of the high value products from diatom will be significantly improved. In this study, we would like to investigate the potential of combining production of both EPA and fucoxanthin in two promising diatom strains. In order to obtain high biomass yield, high EPA and fucoxanthin accumulation, we need to establish suitable lighting regimes as well as nitrogen conditions for the two strains. High light intensity has been demonstrated to promote the growth and lipid accumulation in diatoms [13], while low light intensity benefited EPA and fucoxanthin accumulation [6], [10]. High level of available nitrogen promoted the biomass production [13], [14] as well as the EPA and fucoxanthin accumulation in diatoms [13], [15]. Therefore, a two phases-lighting cultivation will be tested which includes a high-light intensity phase for biomass and lipid accumulation, followed by a low-light intensity phase for fucoxanthin accumulation. The biomass, EPA and fucoxanthin yield will be determined to evaluate the technical feasibility of the process.

II. Materials

Diatom Strains

Two diatom strains AL006 and AL009 isolated from surface seawater in Ofotfjord, Norway, were used for studying the production of fatty acids and fucoxanthin.

Culture Medium

Medium f/2 was prepared according to [16] using 40 g of sea salt (Merck Millipore) per litre of medium. The medium was adjusted to pH 8.2 using 0.1 N solution of sodium hydroxide or hydrochloric acid.

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III. Methods

Cultivation Conditions

The diatom strains were pre-cultured in 200 ml of medium f/2 under 70 µmol photon m^{-2} s^{-1} with lighting cycle of 16 hours light and 8 hours dark. The pre-cultures were supplied with air at 1.5 l/min and cultivated at 20 °C for 8 days. The pre-culture was subsequently inoculated in 850 ml of fresh medium using 10% of inoculum. The cultures were then supplied with 1% (v/v) carbon dioxide in synthetic gas at 0.8 l/min and incubated at 20 °C for 10 days. The lighting regimes for different experiments are described in Table I.

TABLE I
Lighting Regimes and Nitrogen Conditions for EPA and Fucoxanthin Production

Experiment	Lighting condition	NO_3-N concentration (mg/l)	EPA	Fucoxanthin
1	70 µmol photon m^{-2} s^{-1} (16 h light, 8 h dark) for 10 days	12.4 (1N)	+	+
2	150 µmol photon m^{-2} s^{-1} (16 h light, 8 h dark) for 10 days	25 (2N)	+	+
3	70 µmol photon m^{-2} s^{-1} for 12 days, followed by 10 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark)	25 (2N)	+	+
4	150 µmol photon m^{-2} s^{-1} for 12 days, followed by 30 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark)	25 (2N)	+	+

Dried Biomass Yield

Biomass yield was estimated based on the dried biomass which was harvested from 2 l of culture at the end of the cultivation period. The culture was centrifuged at 5000 rpm, 4 °C for 45 min to collect the cells. The biomass was frozen at -18 °C for overnight and subsequently was transferred to a FreeZone 1 Liter Freeze-Dryer (Labconco), which operated at -53 °C and 0.016 mbar for 24-48 hours until the biomass reached a constant mass to estimate the dried biomass.

Lipid Extraction

Exactly 100 mg of freeze dried microalgal biomass was weighted in a falcon tube and kept in -80 ºC refrigerator for overnight. Glass beads were added to the cells and vortexed for 10 min. The cells were then suspended in 15 ml methanol/ chloroform (2:1, v/v) and incubated at 37 °C overnight. The suspension was then sonicated at 45 kHz for 45 min and centrifuged at 3000 rpm for 15 min. The supernatant was collected, and the residual biomass was extracted twice with methanol/chloroform. All the supernatants were then combined. Chloroform and 1% sodium chloride solution were then added the supernatant to achieve a final volume ratio of 1:1:0.9 (chloroform: methanol: water), and mixed. The organic phase was carefully transferred to a vial and dried to constant mass under nitrogen flow. The lipid quantity was calculated as the difference of the weight of the vial with and without lipid. The total lipid content was calculated as a percentage of the dry mass of the biomass.

Fatty Acid Profiling

Fatty acid profiling was performed by esterification of lipids to fatty acid methyl esters and analysed on GC-MS. The samples were analysed on a Waters Quattro Micro GC mass spectrometer. The standard curves for quantification were set up for all fatty acids with a branched C19 fatty acid as internal standard. The concentrations were calculated as the relative content of the identified fatty acids in the sample. The identified compounds generally constituted more that 95% of the peaks in the chromatograms, so unknown compounds did not contribute to the result in any significant way.

Fucoxanthin Analyses

Fucoxanthin was analysed by extracting the pigments from freeze dried material using 90% acetone in water and ultra-sonication for 30 minutes. Two consecutive extractions were performed for optimal extraction recovery. The analysis was performed on a Thermo IDX Tribrid Orbitrap mass spectrometer with a Thermo Vanquish UPLC. The quantification was performed by setting up an external standard curve that was run together with the microalgal samples.

IV. Results and Discussions

Nitrogen Utilization

The nitrogen contents in the cultures of strain AL006 and AL009 were presented in Fig. 1. For one phase lighting at low light intensity (70 µmol photon m^{-2} s^{-1}), the nitrogen concentration in the cultures of the two strains were depleted after 4 days (Fig. 1 A). Similar trend was observed for the two-phases lighting experiment (150/30 µmol photon m^{-2} s^{-1}). However, in two-phases lighting experiment, the same amount of $NaNO_3$ was supplemented at day 5 so that the nitrogen did not run out during the experiment (Fig. 1 B).

To maintain the growth of the diatom, the nitrogen supplement should be at 25 mg/l or supplemented twice with half of the concentration.

Effect on Biomass Yield

Effect of the lighting intensity and the nitrogen supplement on the diatom growth was evaluated through the diatom biomass yield. For the strain AL006, the biomass yield was higher when the culture was supplemented with nitrogen at the concentration of 25 mg/l (2N) (Table II). This is because the nitrogen was depleted in the medium using only 12,5 mg/l of nitrogen (1N) after 4 days as described in previous section, so double amount of nitrogen supplement did support the diatom growth better. The strain AL006 grew best at two-phases lighting at low light intensity (70/10 µmol photon m^{-2} s^{-1}). The biomass yield was highest, 0.63 g/l.

Double nitrogen supplement did not enhance the biomass yield of the strain AL009 (Table II). However, the two-phases lighting enhanced the growth of strain AL009. The biomass yields of 0.55 and 0.41 g/l were achieved under two-phases lighting compared to 0.35 and 0.4 g/l for one phase lighting.

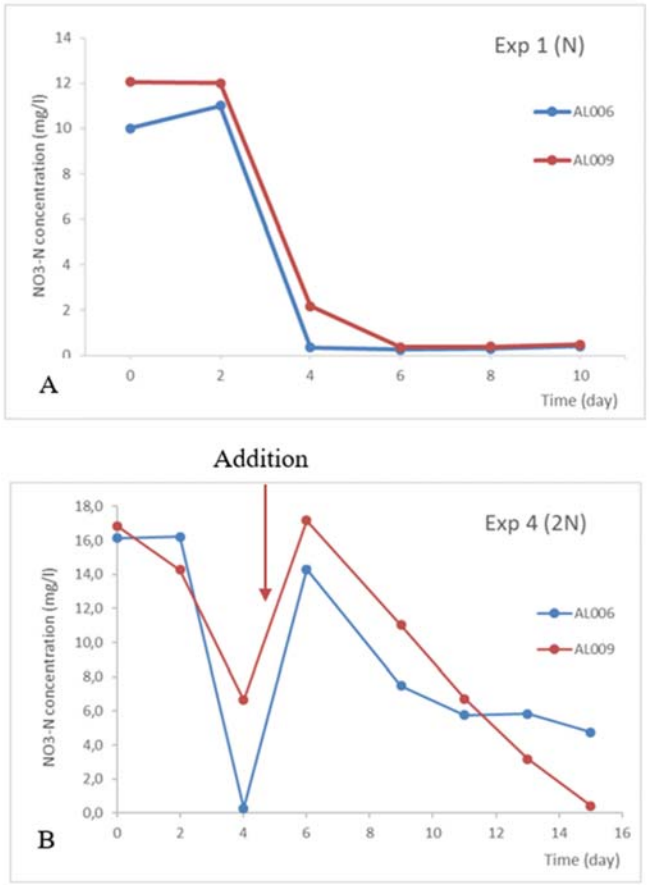

Fig. 1 Nitrogen content in the cultures of the two diatom strains AL006 and AL009 during the cultivation period: A. 70 µmol photon m^{-2} s^{-1} (16h light, 8 h dark) and 1fold nitrogen (12.5 mg/l); B. 150 µmol photon m^{-2} s^{-1} for 8 days, followed by 30 µmol photon m^{-2} s^{-1} for 3 days (16h light, 8 h dark), nitrogen was supplemented twice (25 mg/l)

TABLE II
BIOMASS YIELDS UNDER DIFFERENT LIGHTING INTENSITY AND NITROGEN SUPPLEMENT

Strain	Biomass yield (g/l)			
	150 µmol -2N	150/30 µmol -2N	70/10 µmol -2N	70 µmol -1N
AL006	0.53 ± 0.03	0.45 ± 0.02	0.63 ± 0.03	0.4 ± 0.02
AL009	0.35 ± 0.01	0.55 ± 0.03	0.41 ± 0.02	0.4 ± 0.02

Effect on Fucoxanthin Production

The fucoxanthin contents in the diatom cells under studied conditions were shown in Fig. 2. The pigment content was not affected by the lighting intensity or the supplemented nitrogen level alone. It was, however, enhanced under the two-phases lighting. The fucoxanthin content of strain AL006 increased from 4 to 8-14 mg/g, while for the strain AL009, the fucoxanthin increased from 5 to 10 mg/g under two-phases lighting compared to one phase lighting. Compared to the know fucoxanthin producers, the amount of fucoxanthin produced by strain AL006 and AL009 were rather high. Most of the diatom strains only produced less than 10 mg/g [9].

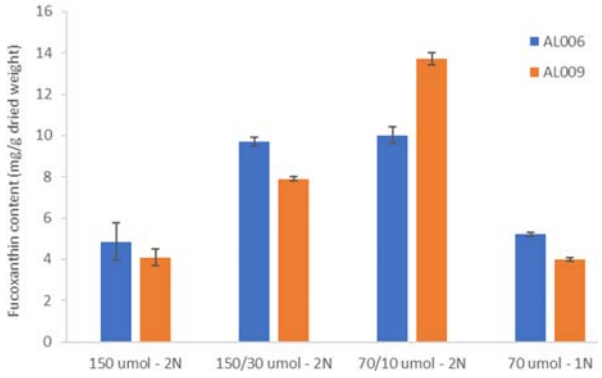

Fig. 2 Fucoxanthin contents of the two diatom strains AL006 and AL009 under 4 lighting conditions: i) 150 µmol photon m^{-2} s^{-1} (16 h light, 8 h dark); ii) 150 µmol photon m^{-2} s^{-1} for 8 days, followed by 30 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark); iii) 70 µmol photon m^{-2} s^{-1} for 8 days, followed by 10 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark); iv) 70 µmol photon m^{-2} s^{-1} (16 h light, 8 h dark) and nitrogen concentration of 2 folds (2N-25 mg/l) or 1 fold (1N-12.5 mg/l)

Effect on Lipid Yield and Fatty Acid Profiles

The lipid contents of two diatom strains under different lighting conditions and double nitrogen supplement are presented in Fig. 3. Under the tested conditions, the lipid content ranged from 26-42% for strain AL006 and 20-60% for strain AL009, which is rather high mong diatom. The higher lighting intensity (150 µmol photon m^{-2} s^{-1}) enhanced the lipid content of strain AL006 (42%) compared to the lower light intensity (26%) (70/10 µmol photon m^{-2} s^{-1}). For the strain AL009, the two-phases lighting enhanced the lipid accumulation in the strain better (39 & 69%) than the one phase lighting (20%).

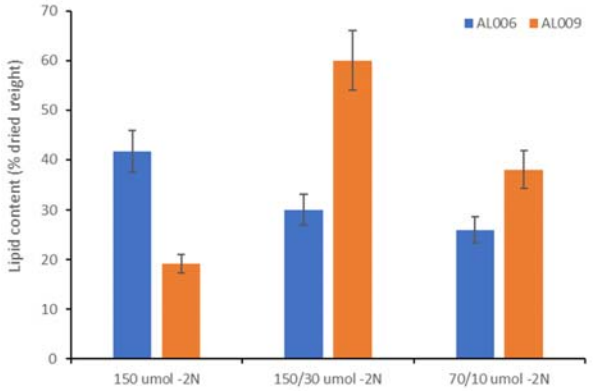

Fig. 3 Lipid contents of the two diatom strains AL006 and AL009 under 3 lighting conditions: i) 150 µmol photon m^{-2} s^{-1} (16 h light, 8 h dark); ii) 150 µmol photon m^{-2} s^{-1} for 8 days, followed by 30 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark); and iii) 70 µmol photon m^{-2} s^{-1} for 8 days, followed by 10 µmol photon m^{-2} s^{-1} for 3 days (16 h light, 8 h dark) and nitrogen concentration of 2 folds (25 mg/l)

The fatty acid composition of the two strains under different growth condition were also analysed to study the effect of lighting and nitrogen concentration (Table III). The main fatty

acids which were found in high percentages in both strains were 16:0, 16:1, 18:1a, 20:5 (EPA) and 21:0. Among them, palmitoleic acid (16:1) and EPA (20:5) are valuable fatty acids. The palmitoleic acid content was higher under low nitrogen concentration (or harvested before 8 days) for both strains, but the EPA content was higher when the cultures were supplemented with high nitrogen concentration (2N) and two-phases lighting.

The EPA contents were highest for both strains under two-phases lighting condition. The EPA reached 19 and 15-19% of the total fatty acid for strain AL006 and AL009 respectively. Comparing with the top known fucoxanthin-producing diatoms, our strains produced reasonable high quantity of fucoxanthin and EPA (Table IV).

V. Conclusion

The two-phases lighting (70 µmol photon m^{-2} s^{-1}, followed by 10 µmol photon m^{-2} s^{-1}) and high nitrogen supplementation, resulted in highest biomass yield of strain AL006. For strain AL009, the two-phases lighting with high lighting intensity (150 µmol photon m^{-2} s^{-1}, followed by 30 µmol photon m^{-2} s^{-1}) and high nitrogen supplementation resulted in better biomass yield. Our preliminary study suggests that the two-phases lighting can be used to enhance the production of both EPA and fucoxanthin in the two diatom strains. Moreover, additional nitrogen supply is necessary to avoid nitrogen depletion during the cultivation. Comparing to the known diatoms, the two studied strains hold great potential for commercialized production of EPA and fucoxanthin.

TABLE III
Effect of Nitrogen Concentration and Lighting Condition on the Fatty acid Composition and Content of the Two Diatom Strains

Fatty acid	AL006				AL009			
	150 µmol -2N	150/30 µmol -2N	70/10 µmol -2N	70 µmol -1N	150 µmol -2N	150/30 µmol -2N	70/10 µmol -2N	70 µmol -1N
14:0	5.59 ± 0.14	6.02 ± 0.13	7.05	7.88	6.00 ± 0.3	5.54 ± 0.02	6.90	8.92
16:0	19.35 ± 0.73	16.40 ± 0.2	16.27	27.72	19.68 ± 0.8	14.64 ± 0.04	16.02	25.69
16:1	23.04 ± 2.3	25.65 ± 0.9	23.13	41.58	30.62 ± 3.4	16.83 ± 0.5	29.84	33.34
18:0	2.62 ± 0.2	2.28 ± 0.1	2.90	0.44	1.88 ± 0.3	3.02 ± 0.09	2.08	1.49
18:1a	10.00 ± 0.3	9.87 ± 0.14	9.94	8.17	9.25 ± 0.7	10.88 ± 0.04	10.28	6.58
18:1b	2.96 ± 0.14	2.56 ± 0.1	2.54	0.73	4.91 ± 0.2	5.34 ± 0.06	5.06	3.61
18:2	6.03 ± 0.5	5.26 ± 0.2	5.66	0.58	4.64 ± 0.7	6.99 ± 0.1	5.39	0.85
18:4	1.54 ± 0.07	1.71 ± 0.02	1.27	1.17	1.21 ± 0.1	1.67 ± 0.02	0.94	0.85
20:5	15.29 ± 0.5	18.57 ± 0.07	19.31	6.71	12.58 ± 0.8	19.16 ± 0.07	14.60	9.77
21:0	13.57 ± 1.4	11.68 ± 0.6	11.94	nd*	9.24 ± 2.0	15.92 ± 0.34	8.89	nd*

*nd: not detected

TABLE IV
Comparing Some Characteristics of Studied Diatoms and the Two Diatom Strains AL006 and AL009

Species	Lipid content (%)	EPA content (% TFA)	Fucoxanthin (mg/g biomass)	References
Isochrysis aff. Galbana CCMP1324	Not known	Not known	18.2	[17]
Ondotella aurita SCCAP K-1251	Not known	Not known	21.7	[15]
Cylindrotheca closterium	Not known	Not known	15.2-22	[18]
Mallomonas sp. *SBV13*	Not known	Not known	26.6	[12]
P. tricornutum	40	14-17	7.5	[13]
Nitzschia laevis	12	19-30	14	[19]
Thalassiosira weissflogii	40-45	24-28	8-10	[20]
Cylindrotheca fusiformis	45	7-10	6	[13]
Chaetoceros sp.	Not known	9.2	18	[21]
AL006 & AL009	40-60	19	10-14	Our work

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