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Response of growth and fatty acid compositions of *Nannochloropsis* sp. to environmental factors under elevated CO_2 concentration

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Abstract *Nannochloropsis* sp. was grown with different levels of nitrate, phosphate, salinity and temperature with CO_2 at 2,800 μl l^{-1}. Increased levels of $NaNO_3$ and KH_2PO_4 raised protein and polyunsaturated fatty acids (PUFAs) contents but decreased carbohydrate, total lipid and total fatty acids (TFA) contents. *Nannochloropsis* sp. grew well at salinities from 22 to 49 g l^{-1}, and lowering salinity enhanced TFA and PUFAs contents. TFA contents increased with the increasing temperature but PUFAs contents decreased. The highest eicosapentaenoic acid (EPA, 20:5ω3) content based on the dry mass was above 3% under low N (150 μM $NaNO_3$) or high N (3000 μM $NaNO_3$) condition. Excessive nitrate, low salinity and temperature are thus favorable factors for improving EPA yields in *Nannochloropsis* sp.

Keywords CO_2 · Eicosapentaenoic acid · *Nannochloropsis* sp. · Nutrient · Polyunsaturated fatty acids · Salinity · Temperature

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Introduction

The eustigmatophyte, *Nannochloropsis*, is a potential source of eicosapentaenoic acid (EPA, 20:5ω3) (Sukenik 1999). In recent years, there is a growing interest in improving the EPA production of this alga by optimizing environmental parameters. Elevated CO_2 or added organic carbon sources dramatically enhanced its cell growth and EPA production (Hu and Gao 2003; Hoshida et al. 2005). Changes in environmental factors may, though, alter polyunsaturated fatty acids (PUFAs) content in microalgal species. EPA content in *Ellipsoidion* sp. increased with increasing nitrate concentration (Xu et al. 2001). Phosphate-starved *Thalassiosira pseudonana* resulted in a decrease of EPA (Harrison et al. 1990). The PUFAs contents of *Porphyridium cruentum* increased when NaCl in the medium was increased from 0.8 to 1.5 M (Lee et al. 1989). High salinity (1.7 M NaCl) gave rise to a slightly higher EPA content in *Navicula* sp. (Al-Hasan et al. 1990). Compared with a salinity of 30 g l^{-1}, however, a salinity of 20 g l^{-1} increased PUFAs of *Nannochloropsis* sp. by more than 13% and EPA content by 0.5% of the dry biomass (Chini Zittelli et al. 1999). In general, there is an inverse relationship between the percentage of PUFAs in the lipid and temperature (Zhu et al. 1997). The total ω3 PUFAs content in *Chlorella* sp. tended to increase with the decreasing temperature but that

in *Nannochloropsis* sp. showed an increasing trend with increasing temperature up to 25°C (James et al. 1989). Manipulation of nitrate and phosphate levels, salinity or temperature at elevated CO_2 conditions are expected to be more efficient to improve the cell growth and EPA production. However, little is known about the environmental and nutritional effects on EPA production by *Nannochloropsis* sp. under elevated CO_2 concentrations (Hoshida et al. 2005).

In this study, we have investigated the effect of nitrate and phosphate concentrations, salinity and temperature on the growth and biochemical composition of *Nannochloropsis* sp. grown under high CO_2 (2,800 μl CO_2 l^{-1}) condition in order to optimize its EPA production under enriched CO_2 condition.

Materials and methods

Organism and growth conditions

Nannochloropsis sp. (PP983) was provided by the First Institute of Oceanography of the State Oceanic Administration, Qingdao. The strain was cultured in f/2-enriched artificial seawater (f/2AW, pH 8.5) medium (Harrison et al. 1980) in 250 ml Erlenmeyer flasks containing 200 ml medium at 22°C under continuous illumination of 50 μmol photons m^{-2} s^{-1}, aerated with 2,800 μl l^{-1} CO_2 at 200 ml min^{-1}, and grown to its late growth phase for use as inoculum.

For the experiments to test the effects of nutrients at 22°C, nitrate or phosphate levels (on the basis of f/2AW) were set at 150 (low N), 600 (middle N) and 3000 μM NO_3^- (high N) with 36 μM PO_4^{3-} or 6 (low P), 25 (middle P) and 120 μM PO_4^{3-} (high P) with 882 μM NO_3^-. $NaNO_3$ and NaH_2PO_4 were used for the adjustment of N or P levels. Triplicate cultures were implemented at each nutrient level. For salinity gradient experiments, f/2AW was prepared with NaCl of 0.20, 0.36, 0.72, 1 or 1.5 M, and cells were cultured at 22°C. For temperature experiments, cultures were grown at 14°C, 22°C and 30°C with f/2AW medium. All the cultures were grown in 10 Schott glass bottles containing 9 l medium under continuously illuminated (50 μmol photons m^{-2} s^{-1}) plant growth chambers (E7 Conviron, Winnipeg, Canada) that was equipped with CO_2 controlling system. The cultures were aerated with air of 2,800 μl CO_2 l^{-1} at 200 ml min^{-1}, and harvested on 10th day after the inoculation, freeze-dried and analyzed for their biochemical compositions. The pH values of the media aerated with enriched CO_2 (2,800 μl l^{-1}) decreased by less than 2.0 pH units from the initial pH of 8.5 during the whole growth period compared with those for normal CO_2 (350 μl l^{-1}), however, pH shift did not affect the EPA content of *Nannochloropsis* sp. (Hoshida et al. 2005). Therefore, the data about the pH change were not included in this study.

Growth monitoring

Cell density was measured turbidimetrically at 665 nm and converted from an appropriate calibration curve to dry mass (DW). All cultures were initiated with an OD_{665} of about 0.1. OD_{665} was monitored every 24 h until the stationary phase was reached.

Analytical methods

Protein content was assayed by the Lowry method after hydrolysis in 1 M NaOH for 2 h at 100°C. Total carbohydrate was analyzed by phenol/sulphuric acid method (Kochert 1978). Total lipid was extracted by the method of Bligh and Dyer (1959). The extracts were transesterified in 1 M sodium methoxide (60°C, 20 min) and re-extracted with hexane. After drying under N_2 and redissolving in chloroform, the fatty acid esters were analyzed by gas chromatography equipped with a glass column (1.8 m × 2 mm) packed with 5% (w/v) diethylene glycol succinate. The flow rate was at 30 ml min^{-1} with N_2 as the carrier gas. The injector and detector were set at 240°C, with an injection volume of 1 μl. The column was initially held at 180°C for 15 min and then increased to 200°C at 2°C min^{-1}. Fatty acids were identified by comparison with retention times of known standards. Quantitative analysis was based on known amount of internal standard (17:0 fatty acid) added to the sample before injection. Data were analyzed by *t*-test or one-way analysis of variance (ANOVA).

Table 1 Biomass yield and biochemical composition of *Nannochloropsis* sp. grown for 10 days with different levels of $NaNO_3$, KH_2PO_4, NaCl and temperature with 50 μmol photons m^{-2} s^{-1} and aerated with 2,800 μl CO_2 l^{-1}. Data are the means ± SD of three replicates

[a]Dry weight

[b]Lipid refers to total extractable lipid material which will be therefore higher than the corresponding values given in Table 2 for total fatty acids

Treatment	Biomass yield (mg l^{-1})[a]	Lipid[b] (% w/w)	Carbohydrate (% w/w)	Protein (% w/w)
$NaNO_3$ (μM)				
150	220 ± 10.4	62 ± 2.8	15 ± 1.1	23 ± 0.6
600	305 ± 20.5	23 ± 0.9	13 ± 0.7	30 ± 0.9
3,000	296 ± 15.6	13 ± 0.6	7 ± 0.3	50 ± 1.2
NaH_2PO_4 (μM)				
6	238 ± 15.3	25 ± 3.1	17 ± 0.8	39 ± 1.3
25	318 ± 17.2	19 ± 0.8	10 ± 0.9	50 ± 0.7
120	308 ± 19.7	11 ± 0.3	9 ± 0.3	48 ± 2.1
NaCl (M)/Salinity (g l^{-1})				
0.20/22	275 ± 13.9	12 ± 0.4	7 ± 0.8	54 ± 1.8
0.36/31	308 ± 15.4	11 ± 0.3	5 ± 0.7	57 ± 2.5
0.72/49	237 ± 10.3	11 ± 0.4	6 ± 0.3	52 ± 4.0
1.00/64	36 ± 5.1	18 ± 0.6	8 ± 0.5	59 ± 3.3
1.50/88	10 ± 2.3	–	–	–
Temperature (°C)				
14	388 ± 17.8	19 ± 0.8	12 ± 0.9	55 ± 4.1
22	403 ± 15.2	9 ± 0.5	11 ± 1.0	41 ± 2.9
30	318 ± 10.8	15 ± 0.7	11 ± 0.5	44 ± 2.7

Results

Growth

Elevating nitrate concentration from 150 μM (low N) to 600 μM (middle N) or phosphate concentration from 6 μM (low P) to 25 μM (middle P) raised the biomass yield by 39% or 34%, respectively ($P < 0.05$). However, further elevation to 3,000 μM N (high N) or 120 μM P (high P) slightly decreased the biomass yield. *Nannochloropsis* sp. grew best at 31 g l^{-1} salinity (0.36 M NaCl), and sustained good growth within a range of 22–49 g l^{-1} salinities (0.2–0.72 M NaCl). *Nannochloropsis* sp. grew well within a range of 14–30°C (Table 1).

Biochemical composition

Contents of lipids, carbohydrates and proteins ranged 9–62%, 5–17% and 23–59%, respectively, on a basis of dry weight (Table 1). Lipid and carbohydrate contents increased ($P < 0.05$) with decreasing nitrate or phosphate concentration. Both high N and P treatments induced higher protein contents. Lipid contents of *Nannochloropsis* sp. grown at 22–49 g l^{-1} salinities were around 11%. Elevation of salt concentration to 64 g l^{-1} raised the lipid content by over 50%. Protein and carbohydrate contents for different salt concentrations showed insignificant difference ($P > 0.05$). Both low and high temperatures resulted in the increase of total lipids and protein contents ($P < 0.05$).

Fatty acid profiles

The predominant fatty acids of *Nannochloropsis* sp. were palmitic acid (16:0), palmitioleic acid (16:1) and EPA (20:5ω3), irrespective of the culture conditions (Tables 2, 3). Percentages of oleic acid (18:1) tended to decrease with increasing nitrate or phosphate levels and decreasing salt concentration, however, EPA showed the contrary. Increased temperature enhanced the percentage of palmitic acid, however, that of EPA decreased ($P < 0.05$).

Elevation of nitrate, phosphate or salt concentration in media decreased ($P < 0.05$) the amount of total fatty acids (TFA), and temperature stress (high and low temperature) resulted in the increase of TFA content ($P < 0.05$). The polyunsaturated fatty acids (PUFAs: 18:2, 20:4, 20:5, 22:6) increased ($P < 0.05$) as percentages of

Table 2 Fatty acid composition (% w/w total fatty acid) and EPA productivity (mg l^{-1} d^{-1}) of *Nannochloropsis* sp. grown for 10 days with different levels of $NaNO_3$ and KH_2PO_4 with 2,800 μl CO_2 l^{-1} at 22°C and 50 μmol photons m^{-2} s^{-1}. Data are the means ± SD of three replicates

DW, dry weight; EPA, eicosapentaenoic acid; TFA, total fatty acids; tr, value below 1%

Treatment	$NaNO_3$ (μM)			NaH_2PO_4 (μM)		
	150	600	3,000	6	25	120
TFA (mg g^{-1} DW)	403 ± 8.5	136 ± 5.7	105 ± 5.3	148 ± 6.0	88 ± 3.5	29 ± 2.1
Fatty acid						
14:0	3.5 ± 0.2	3.7 ± 0.2	3.6 ± 0.3	3.8 ± 0.1	4.3 ± 0.4	3.3 ± 0.1
16:0	38.2 ± 1.2	33.9 ± 0.8	22.7 ± 1.4	29.8 ± 1.5	25.3 ± 0.5	23.1 ± 0.7
$16:1\Delta^{9c}$	28.3 ± 0.9	23.7 ± 0.6	22.7 ± 1.1	23.2 ± 0.2	24.0 ± 0.8	23.0 ± 0.9
18:0	tr	tr	tr	1.2 ± 0.1	tr	tr
$18:1\Delta^{9c}$	16.4 ± 0.8	13.4 ± 0.6	4.1 ± 0.1	21.6 ± 1.3	4.4 ± 0.5	6.3 ± 0.7
$18:2\Delta^{9c,12c}$	2.7 ± 0.5	4.2 ± 0.1	7.0 ± 0.8	3.2 ± 0.4	5.1 ± 0.2	7.6 ± 0.6
$20:1\Delta^{11c}$	tr	tr	3.3 ± 0.5	tr	3.3 ± 0.2	1.9 ± 0.1
$20:4\Delta^{5,8,11,14c}$	1.1 ± 0.1	2.5 ± 0.2	3.6 ± 0.1	2.5 ± 0.4	3.5 ± 0.2	4.4 ± 0.2
$20:5\Delta^{5,8,11,14,17c}$	7.9 ± 0.3	15.7 ± 0.8	29.9 ± 0.9	12.8 ± 1.0	27.9 ± 1.3	27.4 ± 0.5
$22:6\Delta^{4,7,10,13,16,19c}$	tr	tr	tr	tr	tr	tr
Others	tr	1.1 ± 0.2	2.3 ± 0.3	1.1 ± 0.1	1.5 ± 0.2	1.7 ± 0.1
EPA productivity	0.6 ± 0.2	0.6 ± 0.4	0.9 ± 0.4	0.4 ± 0.2	0.7 ± 0.3	0.2 ± 0.1

TFA with increasing nitrate or phosphate concentration, which was 12%, 23% and 41% in low, middle and high N levels, respectively, and was 19%, 37% and 40% in low, middle and high P levels. However, the percentage of PUFAs decreased with the increasing salt concentration and temperature, which was 39%, 36%, 35% and 17% for 22, 31, 49 and 64 g l^{-1} salinity, respectively, and was 40%, 35% and 27% for 14, 22 and 30°C.

EPA as a percentage of dry mass was 3.2% and 3.1% in low and high N level, which increased by 50% and 46% compared with 2.1% in middle N level. Middle P or 22 g l^{-1} salinity culture resulted in the highest EPA dry mass percentage of 2.5% and 2.4%, respectively, among three phosphate levels or four salinities. EPA dry mass percentages at both high (2.4%) and low (2.8%) temperature were comparable, which were around 1.5-fold of that at 22°C (1.6%). Accordingly, the maximum EPA productivity of 1.2 mg l^{-1} d^{-1} was achieved at 14°C, and it was the lowest at 64 g l^{-1} salinity (Tables 2, 3).

Table 3 Fatty acid composition (% w/w total fatty acid) and EPA productivity (mg l^{-1} d^{-1}) of *Nannochloropsis* sp. grown for 10 days with different NaCl concentrations and temperatures at 50 μmol photons m^{-2} s^{-1} and aerated with 2,800 μl CO_2 l^{-1}. Data are the means ± SD of three replicates

	NaCl (M)/Salinity (g l^{-1})				Temperature (°C)		
	0.20/22	0.36/31	0.72/49	1.00/64	14	22	30
TFA (mg g^{-1} DW)	90 ± 3.3	78 ± 2.5	46 ± 0.9	11 ± 0.7	88 ± 1.3	64 ± 1.7	143 ± 3.7
Fatty acid							
14:0	3.1 ± 0.3	3.3 ± 0.1	4.1 ± 0.2	9.0 ± 0.3	4.7 ± 0.7	4.1 ± 0.2	5.5 ± 0.5
16:0	25.3 ± 1.1	24.9 ± 1.5	22.1 ± 1.0	29.8 ± 1.9	23.3 ± 0.9	25.4 ± 0.6	40.1 ± 1.4
$16:1\Delta^{9c}$	24.0 ± 0.7	26.0 ± 1.2	27.8 ± 0.9	23.6 ± 0.8	21.3 ± 1.1	25.6 ± 0.6	20.5 ± 1.3
18:0	tr	tr	tr	tr	tr	tr	1.0 ± 0.2
$18:1\Delta^{9c}$	4.5 ± 0.2	4.6 ± 0.3	6.2 ± 0.5	17.8 ± 1.1	8.4 ± 0.4	7.1 ± 0.3	4.2 ± 0.2
$18:2\Delta^{9c,12c}$	6.7 ± 0.3	7.8 ± 0.8	6.3 ± 0.1	4.7 ± 0.2	4.3 ± 0.1	4.8 ± 0.1	6.8 ± 0.4
$20:1\Delta^{11c}$	2.6 ± 0.3	3.5 ± 0.2	3.2 ± 0.1	tr	1.3 ± 0.1	1.2 ± 0.1	tr
$20:4\Delta^{5,8,11,14c}$	4.1 ± 0.1	4.0 ± 0.1	4.9 ± 0.2	3.9 ± 0.4	3.2 ± 0.6	3.4 ± 0.7	2.7 ± 0.4
$20:5\Delta^{5,8,11,14,17c}$	27.0 ± 0.5	23.6 ± 0.9	23.7 ± 1.1	8.4 ± 0.4	31.7 ± 1.8	25.3 ± 1.0	16.4 ± 0.4
$22:6\Delta^{4,7,10,13,16,19c}$	tr	tr	tr	tr	tr	1.4 ± 0.2	tr
Others	1.7 ± 0.3	1.5 ± 0.5	1.5 ± 0.6	1.7 ± 0.1	tr	1.4 ± 0.2	1.7 ± 0.3
EPA productivity	0.7 ± 0.4	0.6 ± 0.3	0.3 ± 0.1	0.0 ± 0.0	1.2 ± 0.7	0.9 ± 0.4	0.8 ± 0.3

DW, dry weight; EPA, eicosapentaenoic acid; TFA, total fatty acids; tr, value below 1%

Discussion

When the cultures were aerated with 2,800 μl CO_2 l^{-1}, growth of *Nannochloropsis* sp. was not limited by the supply of CO_2 (Hoshida et al. 2005). Accordingly, nitrate and phosphate would be the key limiting nutrient for algal growth. Elevation of nitrate or phosphate concentration might enhance growth of algae. In the present study growth of *Nannochloropsis* sp. increased significantly between low nutrient (N or P) and middle nutrient conditions, but there was no further enhancement for high nutrient conditions. This indicated that nitrogen or phosphorus availability of *Nannochloropsis* sp. was sufficient at the medium levels of N or P, and further elevation of N or P concentration would influence C:N or N:P ratio, and therefore affected the cell growth. *Nannochloropsis* sp. could adapt to a relatively wide range of salinity and temperature and showed optimum growth at 31 g l^{-1} salinity and at 22°C, respectively. A similar salinity and temperature tolerance had been reported previously for this species (James et al. 1989; Chini Zittelli et al. 1999).

Nitrogen deficiency resulted in an increase in carbohydrate and a decrease in protein (Shifrin and Chisholm 1981; Harrison et al. 1990). *Nannochloropsis* sp. grown in the low N condition was high in carbohydrate and low in protein in the present study. However, the response of lipids to nutrient concentration appears to differ according to species. Green algae showed an apparent enhancement in the storage of lipids during N-deficient conditions (Shifrin and Chisholm 1981), while only minor differences in lipid content of *Dunaliella primolecta* were observed at different N levels (Uriarte et al. 1993). Almost a 4-fold increase in lipid content of *Nannochloropsis* sp. grown in low N level was found compared with that of high N level. Such an increase has hardly been reported elsewhere. Contents of lipids could readily be altered under the enriched CO_2 condition in the present work. It was reported that salinity had a great effect on lipid content (Al-Hasan et al. 1990; Renaud and Parry 1994), however, our results showed that within the salinity range 22–49 g l^{-1} the lipid content remained constant except that lipid content increased dramatically at extremely high salt concentration. There was no consistent pattern for production of lipids as influenced by temperature (Renaud et al. 1995). Increasing lipid content with increasing temperature had been shown in some algae (Zhu et al. 1997), however low temperature resulted in higher lipid production for *Nitzschia paleacea* (Renaud et al. 1995). The present study showed that lipid tended to accumulate at both low and high growth temperatures.

EPA only accounted for 1.6% of the dry weight for *Nannochloropsis* sp. grown with 882 μM $NaNO_3$ at 2,800 μl CO_2 l^{-1}, which increased by 28% compared with that at 350 μl CO_2 l^{-1} (Hu and Gao 2003). However, both low and high nitrate conditions brought about rather higher amount of EPA on a dry-mass basis (above 3%). Under the enriched CO_2 condition, low N and high N enhanced the amount of EPA on a dry-mass basis by increasing the TFA content and EPA as a percentage of TFA, respectively. It was evident that at the enriched CO_2 manipulation of nitrate concentration would be more efficient for enhancing EPA production for *Nannochloropsis* sp. Harrison et al. (1990) suggested that phosphate-starved condition resulted in the EPA decrease of *Thalassiosira pseudonana*, while our results showed that excessive phosphate also posed a disadvantage to the increase of EPA on a dry-mass basis. Algae responded to salinity by modifying their cellular fatty acid compositions, and the response was species-specific and different for each fatty acid (Lee et al. 1989; Renaud and Parry 1994). The present study found a significant increase in proportions of EPA at low salinity in *Nannochloropsis* sp., which has been previously reported for other species in this genus (Renaud and Parry 1994; Chini Zittelli et al. 1999). The effect of temperature on the cellular fatty acid composition seems to be quite complex and the available reports are not consistent. Zhu et al. (1997) showed that cultures grown at a low temperature were characterized by a relatively high level of PUFAs. By contrast, James et al. (1989) found maximal EPA content of *Nannochloropsis* strain MFD-2 at 25°C within the temperature range 15–35°C. In the present study, PUFAs and EPA were accumulated at lower temperatures. In conclusion, our work showed

that elevated CO_2, low temperature, low salinity, moderate phosphate and excessive nitrate levels are favorable factors for increasing the EPA yield in *Nannochloropsis* sp.

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