J. Phycol. **40,** 651–654 (2004)

DOI: 10.1111/j.1529-8817.2004.03112.x

NOTE

EFFECTS OF LOWERING TEMPERATURE DURING CULTURE ON THE PRODUCTION OF POLYUNSATURATED FATTY ACIDS IN THE MARINE DIATOM *PHAEODACTYLUM TRICORNUTUM* (BACILLARIOPHYCEAE)[1]

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The composition of fatty acids and contents of eicosapentaenoic acid (EPA) and polyunsaturated fatty acids (PUFAs) of the economically important marine diatom, *Phaeodactylum tricornutum* (Bohlin), were investigated to see whether reducing the culture temperature enhances the production of EPA and PUFAs. The contents of EPA and PUFAs of *P. tricornutum* were found to be higher at lower temperature when cultured at 10, 15, 20, or 25° C. When the cells grown at 25° C were shifted to 20, 15, or 10° C, the contents per dry mass of PUFAs and EPA increased to the maximal values in 48, 24, and 12 h, respectively. The highest yields of PUFAs and EPA per unit dry mass (per unit volume of culture) were 4.9% and 2.6% (12.4 and 6.6 mg · L^{-1}), respectively, when temperature was shifted from 25 to 10° C for 12 h, both being raised by 120% compared with the control. The representative fatty acids in the total fatty acids, when temperature was lowered from 25 to 10° C, decreased proportionally by about 30% in $C_{16:0}$ and 20% in $C_{16:1(n-7)}$ but increased about 85% in EPA. It was concluded that lowering culture temperature of *P. tricornutum* could significantly raise the yields of EPA and PUFAs.

Key index words: **eicosapentaenoic acid; EPA; fatty acids; *Phaeodactylum tricornutum*; polyunsaturated fatty acids; PUFA; temperature**

Abbreviations: **EPA, eicosapentaenoic acid ($C_{20:5(n-3)}$); PUFAs, polyunsaturated fatty acids; TFAs, total fatty acids**

Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid, are gaining increasing attention because of their important values for human health as well as for aquaculture (Borowitzka 1995, Wen and Chen 2000). The main commercial source of PUFAs is fish oil; however, its odor and high percentage of cholesterol have triggered the search for alternative sources of PUFAs. Microalgae have been considered as a promising source (Yongmanitchai and Ward 1991a), and a number of studies have focused on optimizing microalgal culture conditions to produce biomass rich in PUFAs (Taguchi et al. 1987, Thompson et al. 1992, Reitan et al. 1994, Otero et al. 1997, Lu et al. 2001, Goldberg et al. 2002, Hu and Gao 2003). It has been recognized that temperature plays an important role in regulating the production of PUFAs. An inverse relationship has been recognized between temperature and fatty acid unsaturation not only in microalgae (Wada et al. 1990, Zhu et al. 1997), but also in bacteria (Yano et al. 1994), yeasts (Brown and Rose 1969), and bryophytes (Saruwatari et al. 1999). However, little has been documented on the pattern of generation of PUFAs after a constant temperature-maintained culture was shifted to lower temperatures. This knowledge would be useful in raising yields of PUFAs from microorganisms by lowering temperature after its maximum biomass has been achieved.

Phaeodactylum tricornutum, a marine diatom, has been widely used as a food organism in aquaculture and considered as a potential source for EPA production. EPA as a percentage in total fatty acids (TFAs) produced by this diatom increased at decreased levels of irradiance (Thompson et al. 1990), and yield per unit volume of culture was increased and the maximal outcome was obtained with urea as nitrogen source (Yongmanitchai and Ward 1991b). Shifting temperature from a high to a lower level might enhance EPA production by this economically useful diatom. This study aims to investigate the change patterns of EPA and PUFAs contents and fatty acid composition of *P. tricornutum* after the cells grown at 25° C were shifted to lower temperatures.

Phaeodactylum tricornutum 2038 (obtained from the Institute of Oceanography, the Chinese Academy of Sciences, Qingdao) was grown in a modified F/2 medium, in which urea was increased 4-fold and phosphate 2-fold to avoid possible nutrient limitation, and sterilized seawater was used. The cells were cultured in 5-L flasks with 3.5 L medium, and all cultures were aerated with ambient air and maintained in plant growth chambers (EF7 Conviron, Winnepeg, Canada) at 130 μmol photons · m^{-2} · s^{-1} on a 16:8-h light:dark

[1]Received 14 June 2003. Accepted 17 February 2004.
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cycle. The temperature was controlled at 25, 20, 15, or 10° C constantly for 9 days or at 25° C for 6 days and then transferred to 20, 15, or 10° C in different chambers for 3 days. The cell suspensions were collected on the ninth day at the early stationary phase to avoid the effects of advanced age of the culture (Alonso et al. 2000) and centrifuged at 4000 *g* for 10 min, and the pellets were then frozen and stored at −20° C before the analysis of fatty acids.

The fatty acids were extracted by mixing 200 mg cell pellets with 4 mL KOH-CH_3OH (1 M) under N_2 at 75° C for 10 min, reextracting the residue twice, methylating the combined extracts in 12 mL HCl-CH_3OH (2 M) at 75° C for 10 min, adding 4 mL hexane into the cooled suspension, drying the separated fatty acid esters under N_2, and redissolving them in 1 mL hexane. $C_{17:0}$ (Sigma, ST. Louis, MO, USA) was added as an internal standard. Fatty acid esters were analyzed by a gas chromatograph (GC-17A Shimadzu, Kyoto, Japan) with a flame-ionization detector and Carbowax capillary column (15 m × 0.53 mm). The initial column temperature was set at 165° C for 1 min and then increased to 235° C at 10° C · min^{-1}. Detector and injector were maintained at 235° C. Sample of 2 μL were injected under split mode (split ratio, 1:10). Fatty acid methyl esters were identified on a basis of retention times referring to the standard samples. The quantities of fatty acids were calculated on the basis of the known amount of the internal standard. Optimum temperature for the growth of *P. tricornutum* was 20° C, and it grew slower at higher or lower levels of temperature, hardly showing any growth at 30° C (Fig. 1).

Proportions of $C_{16:0}$ and $C_{16:1(n-7)}$ in TFAs decreased, respectively, by 30% and by 20%, but EPA and PUFAs as percentages of TFAs increased by about 80% and by about 50% when temperature was decreased from 25° C to 10° C (Table 1). The EPA contents based on dry mass also increased as temperature decreased (Fig. 2), increasing by almost 90% at 10° C compared with 25° C.

When the cells grown at 25° C for 6 days were shifted to 20° C, the proportion of $C_{14:0}$ in the TFAs increased by about 15%, whereas that of $C_{16:1(n-7)}$ decreased in 36 h and then increased markedly (Table 2). The EPA and PUFAs as proportions of TFAs increased 24 h later after the temperature shift by about 20% and 25%, respectively (Table 2). The EPA and PUFAs contents reached their maximal values (18.0 and 26.1 mg · g^{-1}dry weight) in 48 h (Fig. 3), being raised, respectively, by 50% and 20% after the temperature shift. The EPA content increased by about 40%, but the amount of PUFAs decreased by about 13% compared with the values at 20° C without shifting. When the cells grown at 25° C were switched to 15° C, the percentage of $C_{16:0}$ in TFAs decreased from 29.9% to 21.7% in 36 h and then increased to 25.9%; however, that of EPA increased from 12.0% to 20.2% in 36 h and then decreased to 15.7% (Table 2). The contents of EPA and PUFAs per dry mass (per unit volume of culture) showed the highest values of 22.0 and 33.0 mg · g^{-1}dry weight (5.4 and 8.1 mg · L^{-1}) in 24 h, being, respectively, 2.2% and 3.3% (Fig. 3), increased by about 80% and 50% compared with the control and by about 20% and 5% higher when compared with a constant 15° C. When the cells grown at 25° C were shifted to 10° C, the proportion of $C_{16:0}$ in TFAs decreased from 30.6% to 25.7% in 72 h and that of $C_{16:1(n-7)}$ hardly changed; the proportions of EPA and PUFAs increased correspondingly by 40% and 30% in 48 h (Table 2). Their contents per dry mass (per unit volume of culture) reached the highest values of 2.6% and 4.9% (6.6 and 12.4 mg · L^{-1}) in 12 h (Fig. 3), both being raised by 120% compared with the control.

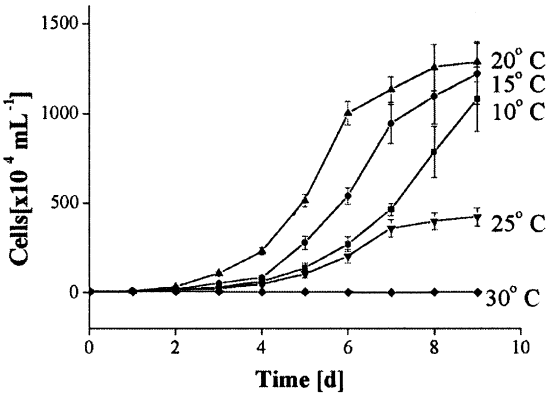

FIG. 1. Growth of *Phaeodactylum tricornutum* at different levels of temperature.

The content of PUFAs based on dry mass showed insignificant changes 72 h after the temperature shift ($P > 0.1$, one-way analysis of variance) among the levels of temperature, though it was significantly higher ($P < 0.05$, one-way analysis of variance) than before the temperature change. The highest EPA and PUFAs contents were found at the lowest temperature shifted to in the shortest time period (Fig. 3).

In the present study, EPA and PUFAs were accumulated at lower temperatures in *P. tricornutum* 2038,

TABLE 1. The fatty acids composition as the percentage of total fatty acids in *Phaeodactylum tricornutum* 2038 cultured at different levels of temperature.

	10° C	15° C	20° C	25° C
$C_{14:0}$	5.9 ± 0.4	6.5 ± 0.0	5.4 ± 0.1	4.5 ± 0.1
$C_{16:0}$	18.8 ± 0.6	25.2 ± 0.3	26.5 ± 0.2	25.8 ± 0.1
$C_{16:1(n-7)}$	30.1 ± 1.0	30.5 ± 0.5	37.1 ± 0.3	37.5 ± 0.1
$C_{18:0}$	4.5 ± 0.3	2.0 ± 0.0	1.0 ± 0.1	1.3 ± 0.1
$C_{18:2(n-6)}$	4.9 ± 0.2	4.5 ± 0.1	5.4 ± 0.1	5.1 ± 0.3
$C_{18:3(n-6)}$	2.7 ± 0.1	1.5 ± 0.0	1.8 ± 0.2	2.0 ± 0.3
$C_{20:4(n-6)}$	nd	nd	0.9 ± 0.2	1.6 ± 0.0
$C_{20:5(n-3)}$[a]	24.2 ± 0.8	18.5 ± 0.2	14.8 ± 0.3	13.1 ± 0.2
$C_{22:6(n-3)}$	nd	0.5 ± 0.1	nd	nd
Others	8.9 ± 0.3	10.6 ± 0.4	7.1 ± 0.1	9.0 ± 0.7
PUFAs	31.9 ± 1.0	24.9 ± 0.3	23.8 ± 0.2	21.8 ± 0.4

Values are the means ± SD for triplicate cultures. nd, not detected.

[a]EPA.

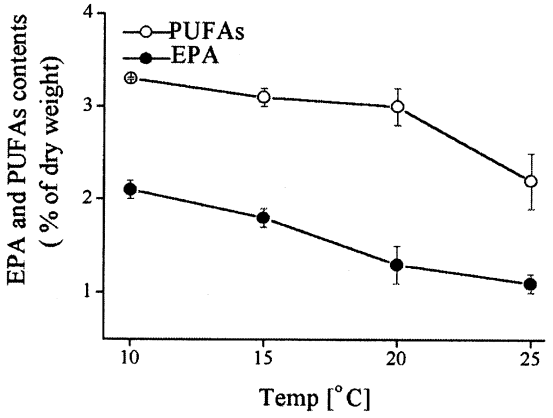

FIG. 2. EPA and PUFAs contents of *Phaeodactylum tricornutum* 2038 grown at different levels of temperature. Data are the means ± SD for triplicate cultures.

and their yields were further raised by lowering the culture temperature. However, the yields were lower than those reported by Yongmanitchai et al. (1991b), which could be due to differences in the strains or culture conditions. Within the temperature range tested, a shift to the lowest temperature brought about the highest EPA and PUFA contents in the shortest time. Increased unsaturation of fatty acids at low temperature in *P. tricornutum* 2038 might be a response to maintain membrane fluidity and function, allowing the diatom to acclimate to low temperature stress, as reported in some cyanobacteria (Sato and Murata 1981, Sakamoto et al. 1994). The elevated level of unsaturated fatty acids in *P. tricornutum* 2038 at low temperatures might be associated with the activity of desaturase, which is a temperature-sensitive enzyme. The level of *des*B desaturase gene transcription increased about 10-fold when the temperature was lowered from 34 to 22° C in the cyanobacterium *Synechococcus* sp. PCC 7002 (Sakamoto et al. 1994), which was suggested to be an acclimation that compensates for the decrease in membrane fluidity. The increased level of PUFAs at low temperature might be attributable to desaturase genes that received signals from a specific sensor in the cytoplasmic membrane (Vigh et al. 1993). On the other hand, lowering temperature reduces both the metabolic rates and photosynthetic activity of the cells and the unsaturation level of thylakoid membrane lipids, partly compensating for the lower metabolic rates (Kis et al. 1998).

Lower temperatures bring about higher contents of EPA and PUFAs per unit mass; however, microalgal growth rate and biomass accumulation are decreased. Therefore, the highest overall production yields of PUFAs and EPA could not be achieved at low temperature levels. Culturing an alga at its optimal tempera-

TABLE 2. Changes with time in fatty acid composition after the *Phaeodactylum tricornutum* 2038 cells grown at 25° C were shifted to 20, 15, or 10° C.

	$C_{14:0}$	$C_{16:0}$	$C_{16:1(n-7)}$	$C_{18:0}$	$C_{18:2(n-6)}$	$C_{18:3(n-6)}$	$C_{20:4(n-6)}$	$C_{20:5(n-3)}$ (EPA)	$C_{22:6(n-3)}$	Others	PUFAs
Control (h)											
0	4.7 ± 0.2	31.6 ± 0.5	39.3 ± 1.1	1.2 ± 0.0	4.1 ± 0.3	1.7 ± 0.3	nd	11.4 ± 0.9	nd	6.0 ± 1.2	17.2 ± 0.3
12	4.8 ± 0.1	29.0 ± 0.1	38.2 ± 0.1	1.4 ± 0.2	4.4 ± 0.1	1.5 ± 0.2	nd	12.2 ± 0.7	nd	8.5 ± 0.4	18.1 ± 0.8
24	5.1 ± 0.3	26.5 ± 0.3	37.8 ± 1.6	1.4 ± 0.2	4.0 ± 0.5	1.7 ± 0.2	nd	12.8 ± 0.2	nd	11.4 ± 0.5	18.5 ± 0.9
36	5.2 ± 0.2	25.7 ± 0.4	37.9 ± 0.7	1.3 ± 0.0	4.5 ± 0.1	1.4 ± 0.0	nd	13.3 ± 0.5	nd	10.7 ± 0.8	19.2 ± 0.2
48	5.0 ± 0.1	25.1 ± 0.4	38.1 ± 0.4	1.2 ± 0.1	4.4 ± 0.1	1.6 ± 0.1	0.5[a]	13.5 ± 0.1	nd	10.6 ± 0.8	20.0 ± 0.8
72	5.0 ± 0.2	25.1 ± 0.4	38.2 ± 0.9	1.3 ± 0.2	4.4 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	13.7 ± 0.2	nd	10.3 ± 0.5	20.1 ± 0.4
To 20° C (h)											
0	4.6 ± 0.2	32.2 ± 1.2	40.8 ± 1.4	1.5 ± 0.9	3.5 ± 0.1	1.4 ± 0.1	nd	12.2 ± 0.1	nd	4.2 ± 0.4	17.1 ± 0.2
12	4.9 ± 0.1	26.5 ± 0.3	37.4 ± 0.5	1.1 ± 0.1	2.6 ± 0.4	0.9 ± 0.1	nd	16.9 ± 0.2	nd	9.4 ± 0.8	20.4 ± 0.4
24	5.3 ± 0.2	21.3 ± 0.2	34.3 ± 0.4	1.3 ± 0.2	3.7 ± 0.4	1.3 ± 0.1	nd	19.5 ± 0.3	2.1 ± 0.2	9.7 ± 0.6	24.5 ± 0.3
36	5.5 ± 0.1	27.2 ± 0.1	34.2 ± 0.6	1.1 ± 0.2	4.8 ± 0.5	1.7 ± 0.1	nd	17.1 ± 0.4	1.3 ± 0.3	7.0 ± 0.2	24.9 ± 0.4
48	5.6 ± 0.1	25.4 ± 0.1	36.9 ± 0.2	1.2 ± 0.1	3.1 ± 0.2	1.6 ± 0.1	nd	16.2 ± 0.5	0.5 ± 0.0	9.6 ± 0.1	21.4 ± 0.2
72	5.3 ± 0.1	23.5 ± 0.4	39.6 ± 0.7	1.0 ± 0.0	3.8 ± 0.1	1.7 ± 0.0	nd	14.8 ± 0.2	0.4[a]	10.0 ± 1.3	20.3 ± 0.1
To 15° C (h)											
0	5.1 ± 0.1	29.9 ± 0.6	38.4 ± 0.1	1.6 ± 0.1	3.3 ± 0.1	1.7 ± 0.2	nd	12.0 ± 0.7	nd	7.3 ± 0.5	17.0 ± 1.1
12	4.5 ± 0.0	29.7 ± 0.4	41.4 ± 0.5	1.8 ± 0.1	3.9 ± 0.2	0.9 ± 0.1	nd	12.8 ± 0.2	0.9[a]	5.2 ± 1.0	17.6 ± 0.1
24	5.2 ± 0.1	26.7 ± 0.4	35.9 ± 0.2	3.1 ± 0.1	5.1 ± 0.2	1.4 ± 0.2	nd	14.7 ± 1.4	nd	8.0 ± 0.8	21.2 ± 0.7
36	5.0 ± 0.1	21.7 ± 0.4	32.0 ± 0.6	2.8 ± 0.1	5.5 ± 0.1	0.8 ± 0.1	nd	20.2 ± 0.3	1.4 ± 0.3	9.2 ± 1.2	26.5 ± 0.3
48	6.2 ± 0.1	22.5 ± 0.5	30.1 ± 0.5	2.0 ± 0.0	5.7 ± 0.1	1.7 ± 0.0	nd	17.5 ± 0.4	2.2 ± 0.3	12.4 ± 1.0	27.1 ± 0.5
72	6.3 ± 0.2	25.9 ± 0.4	34.6 ± 0.1	1.4 ± 0.1	3.6 ± 0.1	1.4 ± 0.0	nd	15.7 ± 0.3	0.4 ± 0.0	10.9 ± 0.4	21.1 ± 0.4
To 10° C (h)											
0	4.9 ± 0.0	30.6 ± 0.8	39.0 ± 1.0	1.4 ± 0.1	3.9 ± 0.5	1.5 ± 0.2	nd	11.8 ± 0.9	nd	6.9 ± 1.2	17.2 ± 0.8
12	5.2 ± 0.3	30.0 ± 0.9	38.0 ± 0.9	2.8 ± 0.2	4.5 ± 0.1	1.9 ± 0.3	nd	13.6 ± 1.3	nd	4.2 ± 0.4	20.0 ± 1.6
24	4.8 ± 0.2	27.5 ± 0.3	37.8 ± 1.6	2.4 ± 0.1	3.7 ± 0.3	1.6 ± 0.4	nd	15.8 ± 0.2	nd	8.4 ± 0.5	21.4 ± 1.1
36	5.1 ± 0.3	26.7 ± 0.4	37.9 ± 0.7	2.4 ± 0.1	4.0 ± 0.1	1.4 ± 0.0	nd	16.4 ± 0.1	nd	6.6 ± 0.8	21.8 ± 0.0
48	5.6 ± 0.3	25.1 ± 0.6	37.7 ± 0.9	3.0 ± 0.1	4.4 ± 0.8	1.3 ± 0.1	nd	16.8 ± 0.1	nd	6.0 ± 0.8	22.6 ± 0.8
72	6.0 ± 0.4	25.7 ± 0.0	38.0 ± 1.9	2.3 ± 0.2	3.7 ± 0.3	1.2 ± 0.2	nd	14.7 ± 0.2	nd	7.2 ± 0.5	19.6 ± 0.4

Values are the means ± SD for triplicate cultures. nd, not detected.
[a]Only one datum.

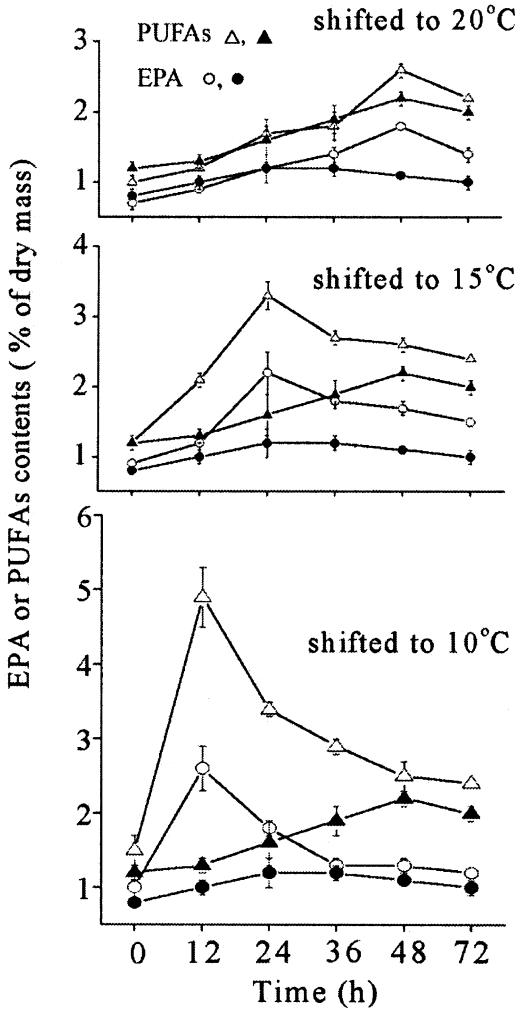

FIG. 3. Changes in EPA and PUFAs contents of *Phaeodactylum tricornutum* 2038 after the cells grown at 25° C for 6 days were shifted to 20, 15, or 10° C. Solid symbols denote controls (continuously cultured at 25° C). Data are the means ± SD for triplicate cultures.

ture for the highest biomass density and then shifting it to a lower temperature can generate higher yields of EPA and PUFAs. However, the feasibility of such a practice depends on the balance of the cost and commercial values of the product.

This study was funded by the National Natural Science Foundation of China (No. 30070582, No. 30270036) and by the Guangdong High Education Bureau. We thank the anonymous reviewers for the useful comments and Prof. J. Raven for corrections of the wordings. We are also grateful to Junhui Huang for his assistance in analysis of fatty acids.

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