Effects of Temperature and Temperature Shift on Docosahexaenoic Acid Production by the Marine Microalga *Crypthecodinium cohnii*

Yue Jiang and Feng Chen*

Department of Botany, The University of Hong Kong, Hong Kong, People's Republic of China

ABSTRACT: The effects of temperature and temperature shift on the fatty acid composition and docosahexaenoic acid (DHA, C22:6n-3) content and productivity of the marine microalga *Crypthecodinium cohnii* ATCC 30556 were investigated. The microalga grew well over the entire range of temperatures (15–30°C) studied. High temperature favored the growth of the microalga with the highest specific growth rate of 0.092 h^{-1} at 30°C. In contrast, low temperature favored the formation of polyunsaturated fatty acids. The highest DHA content was obtained at 15°C in the early stationary phase (i.e., 72 h). In order to achieve high DHA productivity, a shift from high temperature to low temperature at a later stage of cultivation (i.e., 48 h) was also attempted. A temperature shift from 25°C (for 48 h) to 15°C (for 24 h) resulted in an increase in cellular DHA content by 19.9% and productivity by 6.5% as compared to that maintained at 25°C (for 72 h).

Paper no. J9272 in *JAOCS 77,* 613–617 (June 2000).

KEY WORDS: *Crypthecodinium cohnii,* docosahexaenoic acid, growth phase, n-3 fatty acids, temperature, temperature shift.

Docosahexaenoic acid (DHA, C22:6 n-3), as a primary structural fatty acid, exists in most of the highly active neural and retina tissues of humans. Maintaining a high content of DHA in the diet is important for proper nervous and visual functions because man does not have the ability to synthesize this fatty acid *de novo* (1). Marine algae are believed to be the primary producers of DHA (2), which is the main polyunsaturated fatty acid found in heterotrophic marine dinoflagellates, especially *Crypthecodinium cohnii* (2,3). The DHA content in *C. cohnii* is up to nearly 30–50% of the total fatty acids, and no other polyunsaturated fatty acids are in excess of 1% (5).

Temperature affects algal growth and the formation of temperature-dependent components within the cells such as fatty acids. The physiological adaptations of microalgae to growth temperature are considered to be regulated by diverse biochemical characteristics, enzyme reactions, cell permeability, and cell composition. Adaptations of the membrane transport system and membrane fluidity that relate to the content of unsaturated fatty acids play key roles in microalgae in response to changes in growth temperature (6). To keep normal physiological functions of the membrane, an increased synthesis of unsaturated fatty acids at lower temperatures has been observed in many microbial species (7).

We have recently obtained a high-yielding DHA-producing strain of *C. cohnii* with industrial production potential after extensive screening and have determined an optimal growth medium for the alga (4). Biosynthesis of DHA in *C. cohnii* is a result of chain elongation and desaturation *de novo* (8). To increase DHA production, it is important to investigate the factors that affect the biosynthetic enzymes and the metabolites. Temperature is one of the important environmental factors affecting the formation of DHA. It is also known that growth phase might affect fatty acid content and composition (9) and thus productivity of polyunsaturated fatty acids including DHA. The aim of the present work was to investigate the effect of temperature and temperature shift as well as growth phase on the DHA production by *C. cohnii*.

MATERIALS AND METHODS

Microalga and culture conditions. Crypthecodinium cohnii ATCC 30556 was obtained from the American Type Culture Collection (Rockville, MD). The culture was maintained on liquid Porphyridium medium with 5 g/L glucose at 20°C (10) and subcultured every 7 d.

An inoculum was prepared by growing the microalga in a 100-mL Erlenmeyer flask containing 20 mL of the Porphyridium medium and incubating at 25°C for 48 h with orbital shaking at 150 rpm. Erlenmeyer flasks (250 mL), each containing 50 mL of the medium, were inoculated with 5% (vol/vol) of an exponentially growing inoculum and incubated at the various temperatures in an orbital shaker at 200 rpm.

Determination of cell concentrations. Cell concentration (optical density) in the culture fluids was determined at 520 nm with a Spectronic Genesys spectrophotometer (Milton Roy, Rochester, NY). Cell dry weight concentration was determined by drying the cells at 80°C in a vacuum oven until a constant weight was reached.

*To whom correspondence should be addressed at Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong.
E-mail: sfchen@hkusua.hku.hk

Determination of specific growth rate. Specific growth rate was determined by plotting the natural logarithm of culture optical density or dry weight concentration against time. Readings within the exponential phase were then used to obtain correct values of the specific growth rate by linear regression (11).

Analysis of fatty acids. Fatty acid methyl esters were prepared from the lyophilized cells by transmethylation with methanol/acetyl chloride (12). The esters were extracted with hexane and analyzed by an HP 6890 capillary gas chromatographer (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector (FID) and a Supelco (Bellefonte, PA) Omegawax™ 250 capillary column (30 × 0.25 mm). Nitrogen was used as the carrier gas. The initial column temperature was set at 170°C and was subsequently raised to 225°C at 1°C/min. The FID temperature was kept at 270°C. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO). The quantities of fatty acids were estimated from the peak areas on the chromatogram using $C_{17:0}$ (heptadecanoic acid) as the internal standard.

RESULTS AND DISCUSSION

Effect of temperature on cell growth. For the majority of microbial strains grown at temperatures below their upper limits and without nutrient limitation, the maximal growth rate can be described solely as a function of temperature by applying an Arrhenius-type equation (13,14). This suggests that a higher temperature may result in a higher growth rate. It has been reported that cell growth of *C. cohnii* is inhibited when the culture temperature is below 14°C or above 31°C (15). According to Tuttle and Loeblich (16), the optimal temperature for *C. cohnii* is 27°C. However when maintaining the culture at 30–34°C, an abnormal division and a longer generation time of the cells were observed (16). In this study, the growth of *C. cohnii* was investigated over a temperature range of 15–30°C. Cell growth curves are shown in Figure 1, and values of the corresponding growth parameters are tabulated in Table 1. The maximum specific growth rate of the alga ranged from 0.076 h^{-1} to 0.092 h^{-1}. It was obvious that the specific growth rate of *C. cohnii* increased as the temperature was increased within the range of temperatures investigated.

TABLE 1
Cell Growth Parameters of *Crypthecodinium cohnii* ATCC 30556 at Various Temperatures[a]

Parameters	15°C	20°C	25°C	30°C
Specific growth rate μ (h^{-1})	0.076	0.078	0.084	0.092
Biomass concentration (g/L)	1.75	1.79	2.24	2.42
Cell growth yield (g/g)	0.35	0.36	0.45	0.48

[a]Cell growth yield: g cell dry weight produced per g glucose used; each value represents a mean of two determinations; relative standard deviations are less than 5%; growth medium was Porphyridium medium consisting of 1 g/L yeast extract, 1 g/L tryptone, 100 mL of soil water, 500 mL of artificial seawater, and 400 mL of distilled water supplemented with 5 g/L glucose.

The biomass concentration increased in parallel with the specific growth rate. As shown in Table 1, the maximum biomass concentrations of *C. cohnii* were from 1.75 to 2.42 g/L with the highest biomass concentration achieved in the culture grown at 30°C after 56 h of cultivation. The highest biomass concentration was influenced by culture temperature. Low temperatures reduced the enzyme activity in glycolysis and the Krebs cycle and consequently the metabolism of carbon sources decreased (17). The substrate (glucose) consumption rate and growth yield on glucose were thus affected (17).

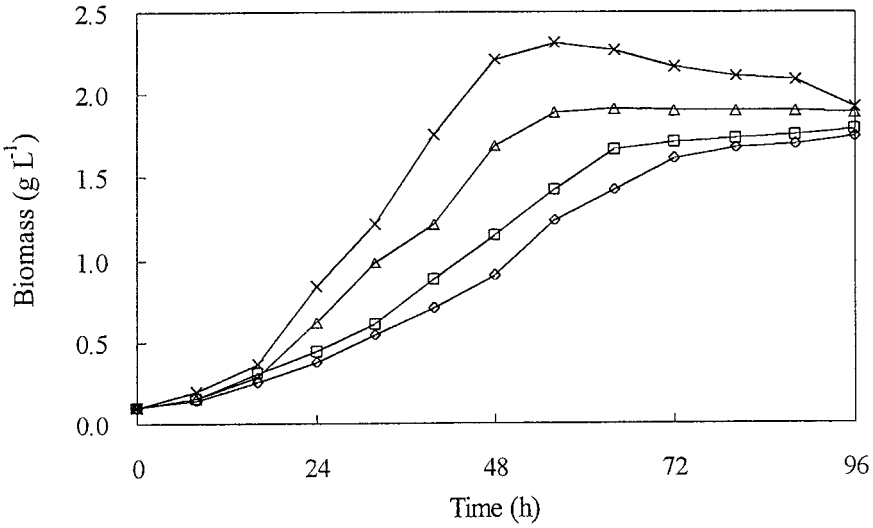

FIG. 1. Cell growth curves of *Crypthecodinium cohnii* ATCC 30556 at various temperatures. ×, 30°C; △, 25°C; □, 20°C; ◇, 15°C. Each value represents a mean of two determinations; relative standard deviations are less than 5%. Growth medium was Porphyridium medium consisting of 1 g/L yeast extract, 1 g/L tryptone, 100 mL of soil water, 500 mL of artificial seawater, and 400 mL of distilled water supplemented with 5 g/L glucose.

Effect of temperature and growth phase on DHA production. From Table 2, it can be seen that all the cells contained $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{22:5}$, and $C_{22:6}$ fatty acids regardless of the culture ages and temperatures. Lower temperatures resulted in more polyunsaturated fatty acids within the temperature range of 15–30°C. All cultures except the 30°C culture had the maximal degrees of unsaturation and DHA proportions at their early stationary phases (*ca*. 72 h). At 15°C, the culture had the highest degree of unsaturation (Δ/mole = 3.51) and the highest DHA proportion (57.64% of total fatty acids).

When the temperature was increased to 30°C, there was a drastic drop in the maximal degree of unsaturation and DHA proportion (i.e., 1.51 and 24.49%, respectively). The decrease in DHA proportions was accompanied by an increase in saturated fatty acids such as $C_{14:0}$ (*ca*. 26%) and $C_{16:0}$ (*ca*. 30%). This suggested that the cells grown at higher temperatures adapted themselves to the growth temperature by producing more saturated fatty acids (18). In contrast, the alga responded to the culture temperature by increasing the degree of fatty acid unsaturation and DHA proportions at lower temperatures. In order to maintain proper membrane lipid fluidity and functions at low temperatures, the proportions of unsaturated fatty acids, particularly polyunsaturated fatty acids, increase (19). There is a phenotypic adaptation of fatty acid composition according to the growth temperature. The effect of polyunsaturated fatty acids to cold adaptation derives from the stabilization of the lipid phase at low temperatures. Increased unsaturation of fatty acids within phosphatidylethanolamine species has been found to increase their ability to form hexagonal phases so as to stabilize the fluid bilayer phase at lower temperatures (20). The increased synthesis of unsaturated fatty acids at lower temperatures was also observed in other eukaryotic algae and Cyanobacteria (7). It was also suggested that the increase in polyunsaturated fatty acid production at low temperatures in *Chlorella* was due to the temperature sensitivity of the fatty acid biosynthetic enzymes (21).

On the other hand, according to the fatty acid biosynthesis pathways, the introduction of a double bond is an oxidative process requiring molecular oxygen (22). It is known that dissolved oxygen (DO) concentration at lower temperatures is higher than that at higher temperatures. Thus at lower temperatures a greater amount of intracellular molecular oxygen is available that allows the oxygen-dependent enzymes to catalyze the fatty acid desaturation (23). The desaturation of saturated or monounsaturated fatty acids to unsaturated fatty acids is effective if the supply of DO is adequate (22). In this study, the changes in DHA proportion might be also due to the changes in DO concentration.

Fatty acid composition of algal cells may be influenced by growth phase as well. As shown in Table 2, DHA content increased as the culture aged up to the early stationary phase. The changes in fatty acid composition, such as an increase in DHA content and a decrease in saturated fatty acid content in the late exponential phase or early stationary phase, might be the results of complete consumption or starvation of some specific nutrients in the medium that induced qualitative and quantitative changes in fatty acids. Sufficient nutrient supply might lead to the synthesis of more saturated fatty acids (22). On the contrary, nitrogen limitation might result in the formation of more unsaturated fatty acids (22).

Effect of temperature shift on DHA production. Although low temperature is the principal regulating factor for the increased synthesis of polyunsaturated fatty acids, low temperature is disadvantageous for practical reasons because of the resulting low specific growth rate and the high energy costs for cooling. In order to shorten cultivation period, temperature shift from high temperature to low temperature after optimal growth has been achieved may be effective for the industrial production of DHA.

The effect of temperature shift on the fatty acid composition was investigated using two temperature shift experiments. In the first experiment, the alga that had been grown at 30°C for 48 h was transferred to a lower temperature (15°C) environment and maintained at that temperature for

TABLE 2
Fatty Acid Composition (% of total fatty acids) of *Crypthecodinium cohnii* ATCC 30556 at Various Temperatures and Culture Ages[a]

	15°C				20°C				25°C				30°C			
Fatty acid	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
12:0	2.10	1.86	0.75	1.08	2.12	1.73	1.78	1.09	2.57	2.23	2.12	2.95	5.81	5.97	5.23	5.66
14:0	15.15	14.12	13.92	15.72	15.43	14.73	13.98	15.98	16.65	14.94	14.17	15.54	25.67	26.03	28.75	29.98
16:0	17.14	15.24	14.83	16.43	17.70	11.23	14.73	16.61	19.44	18.65	18.35	19.46	29.89	30.16	31.45	32.05
18:0	12.13	11.90	10.80	13.12	12.06	1.25	10.29	12.49	9.56	9.10	8.26	11.10	12.71	13.31	14.03	14.72
18:1	0.97	1.07	1.24	0.77	1.01	1.25	1.52	0.89	0.43	0.52	0.43	0.37	0.85	0.58	0.47	0.33
18:2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18:3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20:5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22:5	0.20	0.18	0.82	0.16	0.08	0.10	0.38	0.19	0.32	0.25	0.26	0.23	0.58	0.34	0.26	0.48
22:6 (DHA, n-3)	52.31	55.63	57.64	52.97	51.60	55.55	57.32	52.75	51.03	54.31	56.41	50.95	24.49	23.61	19.81	16.79
TSF	46.52	43.12	40.30	46.35	47.31	43.10	40.78	46.17	48.22	44.92	42.90	49.05	74.08	75.47	79.46	82.41
Δ/mole	3.19	3.36	3.51	3.19	3.11	3.35	3.47	3.18	3.08	3.28	3.40	3.07	1.51	1.44	1.21	1.03

[a]—, not detected; DHA, docosahexaenoic acid; TSF, total saturated fatty acids; Δ/mole, the degree of unsaturation. The value was calculated according to Reference 22: Δ/mole = [1.0 (% monoene) + 2.0 (% diene) + 3.0 (% triene)]/100.

TABLE 3
DHA Content and DHA Productivity of *Crypthecodinium cohnii* ATCC 30556 Under Different Temperatures and Temperature Shifts at 72 h[a]

	15°C			20°C	25°C	30°C
	Shift from 25 (48 h) to 15°C (24 h)	Shift from 30 (48 h) to 15°C (24 h)	(72 h)	(72 h)	(72 h)	(72 h)
DHA content (%TFA)	57.81	49.49	57.64	57.32	56.41	19.81
DHA content (% d.wt.)	6.21	2.56	5.79	5.31	5.18	1.44
DHA productivity (mg /L h)	1.47	0.79	1.37	1.27	1.38	0.39

[a]TFA, total fatty acids; d.wt., dry weight. See Table 2 for other abbreviations.

another 24 h. In the second experiment, the culture was grown at 25°C for 48 h before changing the temperature to 15°C for incubation for another 24 h. For comparison, DHA contents and productivity of these two temperature-shift cultures and the constant temperature cultures are presented in Table 3. The DHA content and productivity of cultures grown at 15, 20, and 25°C as well as in the second temperature shift experiment (25°C for 48 h followed by 15°C for 24 h) were all higher than those achieved at 30°C or in the first temperature shift experiment (30°C for 48 h followed by 15°C for 24 h). The highest DHA content of 6.21% of dry weight and the highest productivity of 1.47 mg/Lh were achieved using the second temperature shift strategy (25°C for 48 h followed by 15°C for 24 h). These values represented a 19.9% increase in cellular DHA content and a 6.5% increase in DHA productivity as compared to those achieved at the culture maintained at 25°C for 72 h. Although the DHA productivity (0.79 mg/Lh) was low using the first temperature shift strategy (30°C for 48 h followed by 15°C for 24 h), it was twice that cultured at 30°C for 72 h (0.39 mg/Lh). These results demonstrate that *C. cohnii* has the capacity to rapidly modify its fatty acid composition in response to the temperature decrease. These results further supported the conclusion that low temperature was necessary for the synthesis of DHA and DHA might be the temperature-dependent fatty acid (9).

At 15°C, *C. cohnii* had a higher DHA proportion of 57.64% (of total fatty acids), but a lower biomass concentration resulted. So the DHA productivities were similar for all cultures grown at 15–25°C. Results clearly indicate that a temperature shift from 25 to 15°C is advantageous for DHA production.

In conclusion, the results obtained from this investigation indicate that temperature greatly affects the cell growth and DHA content and productivity of *C. cohnii* ATCC 30556. Low temperature favors the formation of polyunsaturated fatty acids including DHA. Temperature shift to a lower temperature promotes the accumulation of cellular DHA. The highest DHA content of 6.21% (of dry weight) and the highest DHA productivity of 1.47 mg/Lh were obtained from the culture whose temperature was shifted from 25°C to 15°C. Research is continuing in this laboratory to further enhance DHA productivity by investigating the effect of temperature shock and a more broad temperature shift on the DHA formation of *C. cohnii* ATCC 30556.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the Research Grants Council of Hong Kong and the Industrial Department of the Government of Hong Kong Special Administrative Region.

REFERENCES

1. Gill, I., and R. Valivety, Polyunsaturated Fatty Acids, Part I: Occurrence, Biological Activities and Applications, *Trends Biotechnol. 15*:401–409 (1997).
2. Radwan, S.S., Sources of C_{20}-Polyunsaturated Fatty Acids for Biotechnological Use, *Appl. Microbiol. Biotechnol. 35*:421–430 (1991).
3. Harrington, G.W., D.H. Beach, J.E. Dunham, and G.G. Holz, Jr., The Polyunsaturated Fatty Acids of Marine Dinoflagellates, *J. Protozool. 17*:213–219 (1970).
4. Jiang, Y., F. Chen, and S.Z. Liang, Production Potential of Docosahexaenoic Acid by the Heterotrophic Marine Dinoflagellate *Crypthecodinium cohnii*, *Process Biochem. 34*:633–637 (1999).
5. Henderson, R.J., J.W. Leftley, and J.R. Sargent, Lipid Composition and Biosynthesis in the Marine Dinoflagellate *Crypthecodinium cohnii*, *Phytochemistry 27*:1679–1683 (1988).
6. Richmond, A.E., Microalgaculture, *Crit. Rev. Biotechnol. 4*: 368–438 (1986).
7. Yongmanitchai, W., and O.P. Ward, Omega-3 Fatty Acids: Alternative Sources of Production, *Process Biochem. 24*:117–125 (1989).
8. Beach, D.H., G.W. Harrington, J.L. Gellerman, H. Schlenk, and G.G. Holz, Jr., Biosynthesis of Oleic Acid and Docosahexaenoic Acid by a Heterotrophic Marine Dinoflagellate *Crypthecodinium cohnii*, *Biochim. Biophys. Acta 369*:16–24 (1974).
9. Hamamoto, T., N. Takata, T. Kudo, and K. Horikoshi, Effect of Temperature and Growth Phase on Fatty Acid Composition of the Psychrophilic *Vibrio* sp. Strain no. 5710, *FEMS Microbiol. Lett. 119*:77–82 (1994).
10. Starr, R.C., and J.A. Zeikus, UTEX—The Culture Collection of Algae at the University of Texas Austin, *J. Phycol. (suppl.). 29*: 90–95 (1993).
11. Chen, F., and M.R. Johns, Relationship Between Substrate Inhibition and Maintenance Energy of *Chlamydomonas reinhardtii* in Heterotrophic Culture, *J. Appl. Phycol. 8*:15–19 (1996).
12. Cohen, Z., D. Shiran, I. Khozin, and Y.M. Heimer, Fatty Acid Unsaturation in the Red Alga *Porphyridium cruentum*. Is the Methylene Interrupted Nature of Polyunsaturated Fatty Acids an Intrinsic Property of the Desaturases? *Biochim. Biophys. Acta 1344*:59–64 (1997).
13. Pirt, S.J., *Principles of Microbe and Cell Cultivation*, Blackwell Scientific, Oxford, 1975.
14. Goldman, J.C., and E.J. Carpenter, A Kinetic Approach to the Effect of Temperature on Algal Growth, *Limnol. Oceanogr. 19*: 756–766 (1974).
15. Beach, D.H., and G.G. Holz, Jr., Environmental Influences on

the Docosahexaenoate Content of the Triacylglycerols and Phosphatidylcholine of a Heterotrophic, Marine Dinoflagellate, *Crypthecodinium cohnii*, *Biochim. Biophys. Acta 316*: 56–65 (1973).
16. Tuttle, R.C., and A.R. Loeblich, III, An Optimal Growth Medium for the Dinoflagellate *Crypthecodinium cohnii*, *Phycologia 14*:1–8 (1975).
17. Kruger, G.H.J., and J.N. Eloff, The Effect of Temperature on Specific Growth Rate and Activation Energy of *Microcystis* and *Synechoccocus* Isolates Relevant to the Onset of Natural Blooms, *J. Limnol. Soc. S. Afr. 4*:119–124 (1978).
18. Vonshak, A., and A. Richmond, Problems in Developing the Biotechnology of Algal Mass Production, *Plant Soil 89*: 129–135 (1985).
19. Gounot, A.M., Bacterial Life at Low Temperature: Physiological Aspects and Biotechnological Implications, *J. Appl. Bacteriol. 71*:386–397 (1991).
20. Epand, R.M., R.F. Epand, N. Ahmed, and R. Chen, Promotion of Hexagonal Phase Formation and Lipid Mixing by Fatty Acids with Varying Degrees of Unsaturation, *Chem. Phys. Lipids 57*: 75–80 (1995).
21. Seto, A., H.L. Wang, and C.W. Hesseltine, Culture Conditions Affect Eicosapentaenoic Acid Content of *Chlorella minutissima*, *J. Am. Chem. Soc. 61*:892–894 (1984).
22. Chen, F., and M.R. Johns, Effect of C/N Ratio and Aeration on the Fatty Acid Composition of Heterotrophic *Chlorella sorokiniana*, *J. Appl. Phycol. 3*:203–209 (1991).
23. Singh, A., and O.P. Ward, Microbial Production of Docosahexaenoic Acid, *Adv. Appl. Microbiol. 45*:271–312 (1997).

[Received June 1, 1999; accepted March 28, 2000]