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The Effects of Nutrients and Temperature on Biomass, Growth, Lipid Production, and Fatty Acid Composition of *Cyclotella cryptica* Reimann, Lewin, and Guillard

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ABSTRACT

Batch cultures of *Cyclotella cryptica* Reimann, Lewin, and Guillard were grown at two different nutrient (nitrogen/silica) levels and at two different temperatures (20 and 30°C). Biomass and cell yields decreased with decreased levels of both the nutrients (nitrogen and silica) at 20 and 30°C; whereas lipids (total, neutral, and polar) increased with decreased levels of nutrients at these two temperatures. Changes in fatty acid composition were also noted; the diatoms produced increased amounts of fatty acids C14:0 and C16:0 when grown in nutrient-deficient medium. The influence of nutrient stress and temperature is discussed.

Index Entries: *Cyclotella cryptica* Reimann, Lewin, and Guillard; nutrients; temperature; biomass; lipids; fatty acids.

INTRODUCTION

The worldwide energy shortage following the energy crisis of the 1970s forced many nations to explore the possibilities of new sources of alternate energy. Lipid-yielding microalgae were considered one promis-

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ing source for alternate energy. This optimism was based on the fact that microalgae exhibit exceptional tolerance to environmental fluctuation, high growth rates, and high levels of lipid production. Additionally, the algal oils are readily converted into gasoline and fuels (*1,2*). Recently, it has been predicted that the prices of the liquid fuels produced from mass-cultured microalgae will be competitive with those of conventional fuels by 2010 (*3*).

The unique capability of microalgae to produce large amounts of lipids has led to increased research on the distribution and availability of lipid-producing algae and the feasibility of their use for the production of liquid fuels (*4,5*). Although in individual studies lipid production had been demonstrated to be influenced by nutritional stress, a search for a generalization that can be extended leads to difficulties, since this process has been shown to be quite species-specific (*6*). Therefore the present investigation is aimed at elucidating the lipid composition of the diatom *Cyclotella cryptica* Reimann and its response to nutritional stress under different temperatures in an attempt to identify potential high-energy, oil-producing microalgae.

MATERIAL AND METHODS

Culture Maintenance

The diatom *Cyclotella cryptica* was received from Mahasin Tadros, A & M University, Normal, AL. The organisms were cultivated in synthetic medium containing the following nutrients (g/L): Rila Mix, 20.0 (Rila Marine Products, Teaneck, NJ); $NaNO_3$, 0.15; $Na_2SiO_3 \cdot 9H_2O$, 0.04; $NaH_2PO_4 \cdot H_2O$, 0.004. This medium was supplemented with 1 mL of vitamin solution containing (g/L) thiamine-HCl, 0.1; biotin, 0.5; B12, 0.5; and 1 mL of trace-element solution containing (g/L) H_3BO_3, 0.55; $ZnCl_2$, 0.61; $CuC1 \cdot {}_2H_2O$, 0.23; $Na_2MoO_4 \cdot 2H_2O$, 0.21; and $FeSO_4$, 1.35. The pH of the medium was adjusted to 7.5. The diatoms were grown under continuous illumination (180 $\mu E/M^2s$). Stock cultures were maintained in 50 mL of medium in 125-mL Erlenmeyer flasks in a growth chamber held at a constant temperature of 30°C. To measure lipid production under stressed and nonstressed conditions, the algae were grown in 2800-mL Fernbeck flasks (for nitrogen treatments) or in 2-L polycarbonate bottles (for silica treatments), each containing 1500 mL of medium. Cultures were kept at 30 or 20°C. The cultures were aerated with a 3% mixture of carbon dioxide in air (150 mL/min).

The effect of nitrogen on growth and lipid production in *Cyclotella cryptica* was studied with two different concentrations of $NaNO_3$. Addition of 0.0606 g/L (600 μM) of this chemical was considered nitrogen-sufficient (NS) and 0.0303 g/L (300 μM) was considered nitrogen-deficient (ND).

The influence of silica was studied by the addition of sodium metasilicate. In silica-sufficient (SS) and silica-deficient (SD) media, the amounts of sodium metasilicate added were 0.176 g/L (1 m*M*) and 0.044 g/L (250 μM), respectively. These concentrations of $NaNO_3$ and sodium metasilicate were added to batch cultures and grown at 20 and 30°C. Each treatment was replicated three times.

Determination of Growth Rate

The growth was determined by measuring the optical density of an aliquot of the culture at a wavelength of 750 nm using a Beckman Spectrophotometer (Beckman Instruments, CA). The cells were allowed to grow for 5–7 d before growth was measured. In each treatment, three cultures (replicates) were monitored for growth rate. The growth is reported as doublings/d according to the equations described by A. Vonshak and H. Maske (*7*), as shown below.

$$\text{doublings/d} = \log OD_2 - \log OD_1/T_2 - T_1(h) \times 34.632$$

(OD = optical density, T = time, h = hour)

Harvesting of Cells

The cells were examined for lipid globules under a microscope at regular intervals. When the cells appeared to be full of lipid globules, they were harvested. Harvesting was accomplished by centrifugation (10,000 rpm) for 30 min followed by a wash with sterile growth medium. The wet and dry cell masses were recorded. The wet cells were stored in a freezer at −20°C until they were used. Dry weight of the cells was obtained by drying to a constant weight of 60°C.

Analytical Methods

Determination of Ash-Free Dry Weight

Ash-free dry weight (AFDW) was determined by placing the dry cells in a furnace at 500°C for 5 h and then cooling them in a desiccator. The weight of the samples was measured before and after the cells were placed in the furnace. The difference between the two weights was considered to be the AFDW.

Determination of Lipids

The total lipids were extracted according to the method of Roessler (*8*) which was a modification of Bligh and Dyer's method (*9*). The Bligh-Dyer method was modified for reextraction of the algal pellet. The phase separation was carried with methanol–chloroform–water (10:10:9, v/v/v). The chloroform phase was evaporated to dryness under a gentle stream of nitrogen, dried under vacuum, and then weighed. This was the dry weight of total lipids. The total lipids were than fractionated into neutral

and polar lipids by silicic acid chromatography (*10*). The neutral lipids were eluted with chloroform and the polar lipids with methanol. The elutes were reduced in volume on a rotary evaporator and later evaporated to dryness under a gentle stream of nitrogen. The weight of neutral and polar lipids was then evaluated.

Fatty Acids Analysis

Fatty acids were analyzed by gas–liquid chromatography after transesterification (*11*). Two milliliters of chloroform–methanol (2:1, v/v) was added to 100 mg of lyophilized cells of diatoms, and the mixture was mechanically shaken for 10 min (*12*). After centrifugation, the lower phase was collected, and then 2 mL of chloroform–methanol (2:1, v/v) was added to the precipitate and the same procedure was repeated. The lower phases were pooled, and 0.084 g/L NaCl was added in order to separate the methanol and chloroform phases (*13*). Following centrifugation, the lower phase containing the lipids was evaporated to dryness at room temperature (25°C) under a gentle stream of nitrogen. The residue was solubilized in 1 mL of methanol–benzene (3:2, v/v), and later 1 mL of acetyl chloride–methanol (5:100, v/v) was added. The mixtures were then subjected to methanolysis at 100°C for 1 h (*14*). The specimens were shaken, centrifuged, and stored at 4°C until injection into the chromatograph.

Fatty acid analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector and coupled to a HP 3990A integrator. A nonpolar HP 5, 12-m-long, 0.21-mm-id column (from Hewlett-Packard, Evandale, CA) was utilized. Nitrogen was used as the carrier gas at a flow rate of 28 mL/min. The injection port temperature was 200°C, and that of the detector was 300°C. The column temperature was held at 80°C for 2 min and then increased in a stepwise fashion to a maximum of 215°C. The lipid standards Fatty Acid Methyl Esters Mixtures (FAME) (available as Bacterial Acid Methyl Esters Mix CP from Supelco, Inc., Bellfonte, PA) containing a selection of methyl esters of the fatty acids was used for comparison and identification of peaks in the algal methyl esters.

RESULTS AND DISCUSSION

Growth of *Cyclotella cryptica* was measured in terms of cell yields under the influence of nutrients and temperature (Fig. 1). When the cells of this diatom were grown in sufficient nutrients (nitrogen and silica), an increase in cellular yields was observed as compared to nutrient-stress conditions. The increase in growth rate was influenced by higher temperatures, such as 30°C. Nutrient (nitrogen and silica) deficient culture conditions and lower temperature resulted in decreased growth response. Similar observations have been noted in marine microalgae (*6,20–22*). Darley and

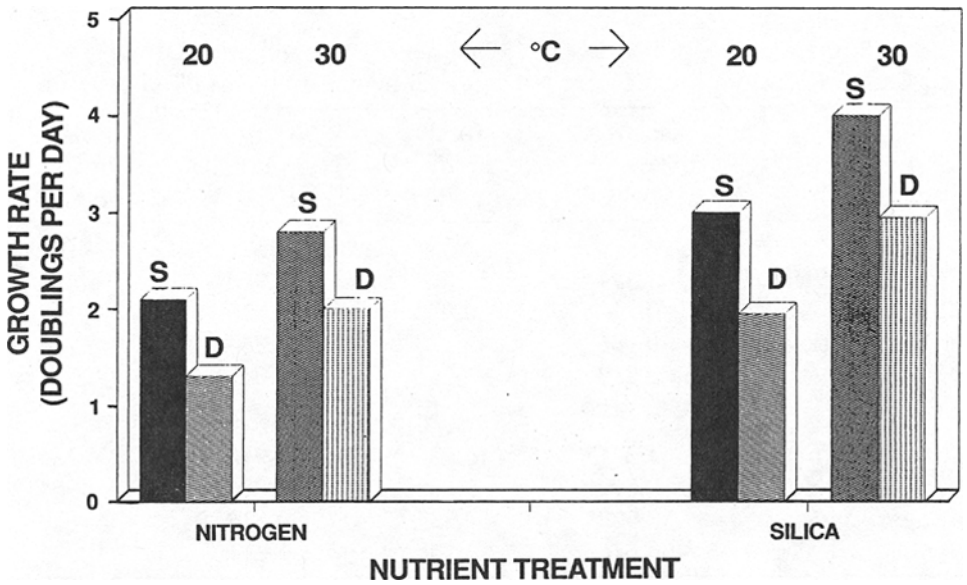

Fig. 1. Growth response (doublings/d) of *Cyclotella cryptica* Reimann, Lewin, and Guillard grown in nutrient-sufficient and nutrient-deficient conditions at 20 and 30°C.

Volcani (*23*) reported that the first state of DNA synthesis in *Chaetoceros calcitrans* (Paulsen) Tak. was silica-dependent. Similarly, decreased growth response was reported in various species of green and blue-green algae grown under nitrogen (*16*) or silica (*18,25,26*) stress conditions. Earlier studies attributed the decreased growth response mainly to the restriction in the size of cells and inhibitory effect on the cell-division rate (*27*). It has been demonstrated that nutrients play an important role in growth and metabolism of such diatoms as *Chaetoceros muelleri* var *subsalsum* (Lemm.) Johan. et Rush, *Navicula saprophila* Lange-Bertalot and Bonik, *Monoraphidium minutum* (Naeg) Kom.-Leg, *Nitzschia dissipata* (Kutz) Grunow, and some microalgae (viz., *Chlorella* sp., *Dunaliella* sp.) (*29–33*). The results of our experiments with *Cyclotella cryptica* also suggest that when such nutrients as nitrogen and silica are addded in sufficient quantities, an appreciable increase in cell number will be noticed compared to cells grown in nutrient-deficient conditions.

The role of nutrients in production of biomass and lipids in algae was recognized in recent years (*6,33,34*), and therefore several items were made to establish the correlation between nutrient stress conditions and increased yields by several conditions (*6,10,15,16,28–36*). In the experiments described here, when the diatom *Cyclotella cryptica* was cultured in sufficient and deficient concentrations of nutrients (nitrogen and silica), it was observed that biomass accumulation is higher when the cells were grown in nutrient-sufficient conditions (Tables 1 and 2). The biomass production (expressed as g/L of AFDW) is three- to fourfold higher than the

Table 1
Biomass and Lipid Yields of *Cyclotella cryptica* Reimann, Lewin, and Guillard Grown in Different Nitrogen Concentrations and Temperatures

Culture conditions	Ash-free dry weight AFDW g/L	Total lipids % of AFDW	Neutral lipids % of AFDW	Polar lipids % of AFDW
		20°C Temperature		
Nitrogen-sufficient (NS)	1.114 ±0.714	16.214 ±0.928	9.960 ±1.210	4.428 ±0.145
Nitrogen-deficient (ND)	0.936 ±0.812	42.428 ±1.241	27.964 ±1.042	8.240 ±0.764
		30°C Temperature		
Nitrogen-sufficient (NS)	1.321 ±0.897	16.920 ±1.085	11.242 ±1.024	4.264 ±0.964
Nitrogen-deficient (ND)	0.982 ±0.816	45.214 ±1.248	30.510 ±1.214	9.028 ±0.924

biomass content reported for some of the commonly grown algae, viz, *Chlorella vulgaris* Beig., *Scenedesums obliques* (Turp.) Kuetz., and *Spirulina platenis* (Nordst.) Geitler (*15,16*). When the cells of *Cyclotella cryptica* were grown under lower concentrations of nitrogen and silica, the biomass production decreased significantly at 20 and 30°C. Temperature has an influence on the biomass production (*18,29*). In the present studies, it was noted that, under optimal conditions of the nutrients, the biomass content was higher at 30 than 20°C.

In the present study, a remarkable change in the accumulation of total, neutral, and polar lipids (expressed as percent of AFDW) was also observed. When this diatom was cultured under suboptimal doses of nitrogen and silica, the production of these classes of lipids was significantly higher than in the cultures grown with sufficient amounts of these nutrients (Tables 1 and 2). By growing cells under nitrogen-deficient conditions, a significant increase in total lipid production was noticed. When the cells were grown in silica-stressed conditions, the total lipid content was increased appreciably. A marginal increase in total lipid production was observed when the temperature was increased from 20 to 30°C. Studies carried out with other diatoms (*Navicula saprophila, Chaetoceros moelleri* var *subsalsum, Nitzschia dissipata*) under similar culture conditions (*29–31*) also show enhanced production of the lipids when grown in nutrient-stressed conditions. The results show that, of total lipids, both neutral and polar lipid production is increased under the nutrient-stressed conditions. Nutrient deficiency also induced increased accumulation of polar lipids. It was seen that the increase was more significant in nitrogen-deficient conditions than in silica-deficient conditions. Furthermore, nitrogen-stress conditions at 30°C and silica-stress conditions at 20°C

Table 2
Biomass and Lipid Yields of *Cyclotella cryptica* Reimann, Lewin, and Guillard Grown in Different Silica Concentrations and Temperatures

Culture conditions	Ash-free dry weight AFDW g/L	Total lipids % of AFDW	Neutral lipids % of AFDW	Polar lipids % of AFDW
		20°C Temperature		
Silica-sufficient (SS)	1.161 ±0.021	19.140 ±1.082	12.640 ±1.214	3.140 ±1.048
Silica-deficient (SD)	0.978 ±0.092	31.415 ±0.985	19.060 ±0.984	7.140 ±1.046
		30°C Temperature		
Silica-sufficient (SS)	1.219 ±0.025	21.046 ±1.014	13.460 ±0.948	4.106 ±0.684
Silica-deficient (SD)	0.894 ±0.046	41.948 ±1.214	29.080 ±1.410	9.140 ±0.745

elicited higher accumulations of polar lipids. Our results on enhanced production of total, neutral, and polar lipids are in agreement with previous reports (*29–32,35–37*) for other microalgae and diatoms that were grown under similar environmental conditions (nutrients and temperature). The influence of nutrients on lipid production is species-specific, and it is interesting to note that the lipid production in *Cyclotella cryptica* is similar to that in the other diatoms that we studied (*Chaetoceros muelleri* var *subsalsum, Nitzschia dissipata,* and *Navicula saprophila*) (*28–33*).

The analysis of fatty acids shows that the predominant fatty acids in *Cyclotella cryptica* were C14:0, C16:1, and C16:0 (Table 3). In microalgae *Ankistrodesmus* sp., *Botryococcus braunii, Dunaliella* sp., and *Nitzschia* sp., the presence of fatty acids C14:0, C16:0, C18:1, C18:2, and C18:3 was reported (*28–34*). Very few studies have been undertaken on the influence of nutrients on fatty acid composition in algal species, particularly diatoms. In *Chaetoceros muelleri* var *subsalsum, Navicula saprophila,* and *Nitzschia dissipata,* the fatty acid composition was reported. By growing algae in nitrogen- and silica-stress conditions, the fatty acid production seems to be influenced to a certain extent (*29,30*). In the present studies, nitrogen-stress culture conditions induced an increase in the production of C14:0 and C16:0 fatty acids at 20°C as well as 30°C. However, no significant increase or decrease in levels of fatty acids C16:1, C18:1, and C22:0 was observed in nitrogen-deficient growth conditions. A similar profile of these fatty acids was observed when this diatom was cultured in silica-sufficient and -deficient media. The studies carried out earlier on diatoms like *Navicula saprophila, Chaetoceros muelleri* var *subsalsum,* and *Nitzschia dissipata* also report changes in fatty acids C14:0, C16:0, and C18:1 (*29–31*). The results of the present study and earlier observation (*29–32*) suggest

Table 3
Effects of Temperatures and Nutrients on the Fatty Acid Composition of *Cyclotella cryptica* Reimann, Lewin, and Guillard

Fatty Acid Carbon Number	Percent Fatty Acids			
	Temperature, 20°C		30°C	
	Nitrogen			
	Sufficient (NS)	Deficient (ND)	Sufficient (NS)	Deficient (ND)
C12:0	4.20	4.77	3.90	3.20
C14:0	26.16	28.09	30.59	35.20
C16:1	7.77	5.77	3.60	2.90
C16:0	43.52	46.98	46.65	47.82
C18:1	11.61	8.71	7.30	4.50
C18:0	Tr	Tr	Tr	Tr
C19:0	Tr	Tr	Tr	Tr
C20:0	3.67	1.59	3.90	2.48
C22:0	3.05	4.09	4.06	3.60
	Silica			
C12:0	2.93	3.43	3.80	3.62
C14:0	22.07	24.21	23.50	25.02
C16:1	9.30	12.82	11.63	10.95
C16:0	33.05	36.30	35.82	37.61
C18:1	11.69	8.46	10.17	8.48
C18:0	Tr	Tr	Tr	Tr
C19:0	7.68	4.34	5.82	5.02
C20:0	6.38	6.45	6.47	5.75
C22:0	6.90	3.99	2.79	3.55

that stressful conditions invariably increase the accumulation of some fatty acids. The pattern of fatty acid composition in *Cyclotella cryptica* is different from that in some other algal species, such as *Anacystis nidulans* (C16:1, C16:0, and C18:1, with traces of C17:1, C17:0, and C20:0), *Microcystis aeruginosa* (C16:1, C16:0, C18:2, C18:1, and C28:3), *Spirulina* and strains (C16:1, C16:0, C18:3, C18:2, and C18:1) (*15*). Therefore, it may be that the fatty acid pattern is species-specific.

Microalgal species are capable of producing biomass containing a high percentage of oil. However, to develop a technology base to produce liquid fuels from algal biomass, it is essential to select species with desirable characteristics. Some of the important characteristics are

1. Tolerance to a wide range of temperatures,
2. Tolerance to salinity,
3. Biomass production,
4. Ability to product lipids that constitute at least 40–50% of the biomass, and
5. High productivity at relatively low nutrient supply.

In our experiments, by varying the temperature and concentration of nutrients (nitrogen/silica) in the nutrient medium, increases in production of lipids were observed. On the basis of our data, we suggest exploitation of the diatom *Cyclotella cryptica* for lipid production, especially as this strain has exhibited a good degree of tolerance to varying temperature and salinity.

CONCLUSIONS

The results of the present study suggest that there is considerable opportunity to exploit the technology of harnessing liquid fuels from microalgae by modifying the culture conditions. By growing diatoms like *Cyclotella cryptica* in nutrient (silica and nitrogen) stressed conditions, the production of total, neutral, and polar lipids could be increased significantly. These are the cellular products that could ultimately be used for conversion into gasoline and fuels.

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