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Environmental Control of Lipids and Fatty Acid Production in the Diatom *Navicula saprophila*

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ABSTRACT

The unique ability of microalgae to accumulate large amounts of lipids has led to an increase in the research on the cultivation of these organisms for the production of fuels. This paper describes the accumulation of cellular lipids by the diatom, *Navicula saprophila*, as a result of culture management strategies. The cells were grown in two different nutrient concentrations (nitrogen and silica) maintained at 20° and 30°C. The biomass, lipids, and fatty acids were measured. Biomass yield was higher in a nutrient (nitrogen/silica) nonstress treatment; however, the production of lipids was enhanced in a nutrient stress medium at 30°C. The influence of the environmental conditions on the distribution of fatty acids was also observed.

Index Entries: Nutrients; temperature; biomass; lipids and fatty acids.

INTRODUCTION

In recent years, research has been directed toward exploring the potential for the large-scale production of gasoline and diesel oils from microalgal lipids (*1–3*). Microalgae that thrive in high salinity water and accumulate storage lipids in great quantities (*4–7*) are among the best biomass feedstocks available for producing renewable, high-energy liquid fuels such as gasoline and diesel fuel.

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The unique ability of diatoms to accumulate large amounts of lipids has led to an increase in research on the cultivation of algae for the production of fuels (*5–9*). It has been noticed that these organisms exhibit an exceptional tolerance to environmental fluctuations (*1–5,8*). Environmental conditions such as carbon dioxide supply, light intensity, temperature, nutrient concentration, and salinity have been observed to influence metabolism in diatoms (*4–6,8*). It has been shown in the diatoms *Chaetoceros moelleri* var *subsalsum* and *Cyclotella cryptica* (*5,8,10*), that nutritional deficiencies can lead to an increase in energy rich products such as lipids. In particular, nitrogen starvation can lead to remarkable changes in algal cell composition (*11,12*).

The Aquatic Species Program at the Solar Energy Research Institute, Department of Energy is directing research efforts aimed at developing microalgal strains that have an increased production of biomass and lipids for liquid fuels (*1,3*). This has led to a surge in research on the distribution and availability of other oil producing algae. Microalgae such as like *Chlorella* sp., *Monoraphidium minutum* and *Cyclotella cryptica*, *Dunaliella* sp., *Isochrysis* sp., *Nannachloris* sp. Thomas, *Nitzschia* sp. and *Ankistrodesmus* sp. have been tested for their biomass and lipid yields by the induction of environmental strategies (*4,8,10–13*). Enhanced lipid accumulation in algal cells was observed in some microalgae when they were grown in nitrogen limited (*1–4,7,8,10–16*) and silica limited media (*1–3,5,8,10,14–19*). The freshwater green alga *Botryococcus brauni*, accumulated lipid concentrations of up to 75% of its total dry weight when subjected to nutritional deficiencies (*15–17*). It has been suggested that with aging, nutrients become exhausted; this is then reflected in increase in a lipid content and changes in fatty acid composition (*14,15*). An 18:1 increase in the fatty acid content of *Botryococcus* sp. and *Dunaliella salina* (*15*) was noted when these microalgae were grown in nitrogen-starved medium.

The objective of this study was to evaluate the production of lipids and fatty acids by the diatom, *Navicula saprophila* in response to changes in nutritional factors (nitrogen and silica) and temperature (20 and 30°C).

MATERIALS AND METHODS

Culture Maintenance

The cultures of *Navicula saprophila* were obtained from the Solar Energy Research Institute (SERI), Golden, CO. The diatoms were grown under continuous illumination (180 $\mu E/m^2$ sec^1) in SERI Type II medium. The medium was composed of the following nutrients (mg/L): $CaCl_2$ (28), $MgCl_2.6H_2O$ (3,026), Na_2SO_4 (5,870), KCL (965), $NaHCO_3$ (2,315), Na_2CO_3 (876), NaCl (8,078), $Na_2SiO_3.9H_2O$ (500 mg), Biotin (2), Thiamine-HCl (1), vitamin B12 (1), KNO_3 (128), and 1 mL of 0.6 *M* KH_2PO_4, and was adjusted to pH 7.5. Stock cultures were maintained at 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 under optimal conditions of growth 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° and 20°C.

The experimental design was as follows.

Nitrogen Concentration

The nitrogen source was potassium nitrate (KNO_3). Two separate concentrations of KNO_3 were added to batch cultures maintained at 20 and 30°C as shown below.

Nitrogen

Sufficient (NS)		Deficient (ND)	
600μM		300μM	
20°C	30°C	20°C	30°C

Silica Concentration

Sodium metasilicate was added as the silica source to batch cultures kept at 20 and 30°C separately as shown below.

Silica

Sufficient (SS)		Deficient (SD)	
1 m*M*		250 u*M*	
20°C	30°C	20°C	30°C

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, 3 cultures (replicates) were monitored for growth rate. The growth is reported as doublings per day according to the equations described by A. Vonshak and H. Maske as shown below.

$$\text{Doubling/Day} = \text{Log O·D·}_2 - \text{Log O·D·}_1 \,/\, T_2 - T_1\,(\text{h}) \times 34.632$$

Harvesting the Cells

Before harvesting, the cells were examined for lipid globules under a microscope. Only after the cells appared to be full of lipid globules were they harvested. Harvesting was accomplished by centrifugation (10,000 rpm) for 30 min followed by a wash with sterilized growth medium. The wet and dry cell mass of cells was 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 at 60°C.

Analytical Methods

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 these two weights was considered to be the ash free dry weight.

Determination of Lipids

The total lipids were extracted according to the modified method of Bligh and Dyer (*20*), as described for algal lipids. Enough chloroform and water were added to give the Bligh-Dyer ratio for phase separation (chloroform:methanol:water of 10:10:9).

Total lipids were separated into neutral lipids and polar lipids by silicic acid chromatography (*13*). The neutral lipids were eluted with chloroform and the polar lipids with methanol. The eluants were reduced in volume on a rotary evaporator and later evaporated to dryness under air. The weight of neutral lipids and polar lipids was then evaluated.

Fatty Acids Analysis

The cells of the diatoms were lyophilized and the fatty acids were analyzed by gas-liquid chromatography after transesterification (*21*). Two ml of chloroform:methanol [2:1 (v/v)] were added to 100 mg of lyophilized cells, and the mixture was mechanically shaken for 10 min (*22*). After centrifuging, the lower phase was collected. Two mL of chloroform:methanol [2:1 (v/v)] was added to the precipitate and the same procedure was repeated. The lower phase was pooled and 145 m*M* NaCl was added in order to separate the methanol and chloroform phases (*23*). After centrifuging, the lower phase containing the lipids was evaporated to dryness at room temperature (25°C+1°C) under a gentle stream of nitrogen. The residue was solubilized in 1 mL of methanol:benzene [3:2 (v/v)] and 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 (*24*). The specimens were shaken, centrifuged, and stored at 4°C. Eventually, they were injected 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. Nitrogen was used as the carrier gas at a flowrate of 28 mL/min. The injection port temperature was 200°C and the detector was 300°C. The column temperature was held at 80°C for 2 min and then increased in a step-wise fashion to a maximum of 215°C. The standard FAME mix (available from Supelco, Inc., Bellefonte, PA) containing a

selection of methyl esters of the fatty acids, was used for comparison. The identification of peaks in the algal methyl esters was done by comparison with the standard FAME mix.

RESULTS AND DISCUSSION

The growth response as determined on the basis of cell counting is shown in Fig. 1. It was noted that there was an appreciable increase in cell number when cells of *Navicula saprophila* were grown in sufficient nutrients (nitrogen and silica) as compared to those grown in a nutrient deficient medium. The higher temperature (30°C) caused a greater increase in cellular yields than the lower temperature (20°C). The influence of environmental factors such as temperature, pH, light intensity, and humidity on the growth of algae, e.g., *Chlorella* sp., *Isochrysis* sp., *Nitzschia* sp., *Cyclotella cryptica*, *Dunaliella* sp., and *Chaetoceros moelleri* var *subsalsum* have already been reported (*7,10–13*). Likewise, nitrogen and silica are also known to have a strong correlation relative to growth and metabolism in a number of microalgae (*5,9,10*). An increased biomass accumulation (expressed as g/L of AFDW) was noticed in the diatoms cultured at 30°C under optimal nitrogen and silica conditions as compared to the yields of biomass in diatoms cultured at lower temperature (20°C), and to those supplied with suboptimal doses of nitrogen and silica (Tables 1 and 2). However, the accumulation of total, neutral, and polar lipids (expressed as percent AFDW) increased sufficiently when this diatom was cultured under suboptimal doses of nitrogen and silica. Generally, it was observed that the lipid yields were higher at increased temperature (30°C). A recent study reported that the biomass accumulation in some of the most commonly used algae, viz: *Chlorella* sp. *Scenedesmus obliques* and *Spirulina platensis* cultured with 0.1% supply of KNO_3 was 287, 403, and 235 mg/L. (*1–4,24*).

The present study indicates a remarkable change in the amount of biomass accumulation under nonstressed conditions of nitrogen and silica. The total lipid yields of *Navicula saprophila* were generally higher than that of *Monoraphidium minutum*, *Chaetoceros moelleri* var *subsalsam*, and *Cyclotella cryptica* (*1–3,8,10*) (Table 3). The results show that under nitrogen and silica stressed conditions, this diatom also produced more neutral and polar lipids. This was true at both 30 and 20°C; however, the lipid production in general was higher at 30 than at 20°C, as shown in Tables 1 and 2. Nitrogen and silica are known to have a strong influence on the accumulation of biomass and lipids (*5,7,10*). As shown in Table 4, the fatty acids present in *Navicula saprophila* were C12:0, C14:0, C16:1, C16:0, C18:2, C18:1, C18:0, C19:0, C20:0, and C22:0. Both qualitative and quantitative variations in the fatty acids were observed when the cells were grown according to nutrient (nitrogen and silica) stress and non-

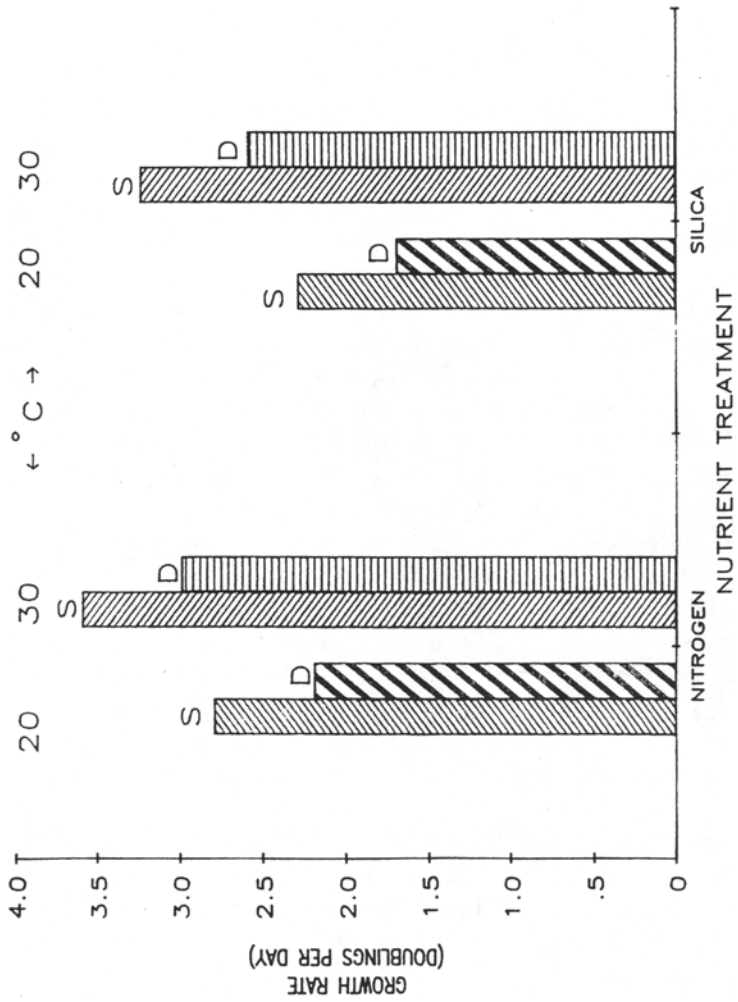

Fig. 1. Growth response (doublings/day) of *Navicula saprophila* at 20 and 30°C in nutrient sufficient (S) and nutrient deficient (D) treatments.

Table 1
Biomass and Lipid Yields of *Navicula saprophila* grown in Different Nitrogen Concentrations and Temperatures, in Percent of AFDW

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)	1.108 ±0.082	23.30 ±1.50	13.14 ±1.64	7.35 ±0.55
Nitrogen deficient (ND)	0.977 ±0.082	41.24 ±1.53	24.98 ±1.40	11.42 ±0.50
		30°C temperature		
Nitrogen sufficient (NS)	1.014 ±0.120	26.48 ±1.34	13.90 ±1.70	8.24 ±0.25
Nitrogen deficient (ND)	0.980 ±0.089	44.36 ±1.44	25.15 ±1.50	11.08 ±0.20

Table 2
Biomass and Lipid Yields of *Navicula saprophila* grown in Different Silica Concentrations and Temperatures, in Percent of AFDW

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)	1.040 ±0.079	19.98 ±1.10	12.55 ±1.72	4.20 ±0.19
Silica deficient (SD)	0.952 ±0.081	33.05 ±1.25	18.20 ±1.40	6.84 ±0.21
		30°C temperature		
Silica sufficient (SS)	1.242 ±0.984	20.02 ±1.08	13.42 ±1.85	5.29 ±0.25
Silica deficient (SD)	0.984 ±0.742	34.14 ±0.15	19.25 ±1.14	8.05 ±0.10

stress treatments. The characterization of fatty acids is important because it helps in rating these hydrocarbons as substitute liquid fuels.

On the basis of our data on *Navicula saprophila* we suggest that it would be useful to conduct further studies on biomass and lipid yields for hydrocarbon production with this strain because it has exhibited a good degree of tolerance to environmental variations. There is a considerable

Table 3
Biomass and Lipid Yields of *Monoraphidium minutum*, *Cyclotella cryptica*, and *Chaetoceros moelleri* var *subsalsum*, Grown in Different Nitrogen and Silica Concentrations and Temperatures in Percent of AFDW

Microalgal species	Ash-free dry weight AFDW g/L		Total lipids % of AFDW		Neutral lipids % of AFDW		Polar lipids % of AFDW		Ash-free dry weight AFDW g/L		Total lipids % of AFDW		Neutral lipids % of AFDW		Polar lipids % of AFDW	
	20°C temperature								30°C temperature							
	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)	(SS)	(SD)
Monoraphidium minutum[a]	1.078 ±0.098	0.898 ±0.076	22.68 ±1.30	28.94 ±1.43	5.02 ±1.45	13.64 ±1.85	6.14 ±0.45	7.80 ±0.36	1.348 ±0.106	1.190 ±0.090	26.68 ±1.30	33.68 ±1.30	16.83 ±1.80	18.96 ±1.60	6.41 ±0.20	8.14 ±0.20
Cyclotella cryptica[b]	1.147 ±0.081	0.759 ±0.985	18.30 ±0.93	32.67 ±1.11	13.69 ±0.90	18.71 ±1.30	2.96 ±0.60	5.67 ±0.45	1.198 ±0.120	0.895 ±0.100	21.70 ±1.21	41.05 ±1.29	13.69 ±1.45	27.74 ±1.64	4.51 ±0.25	8.05 ±0.40
Chaetoceros moelleri var[b]	1.167 ±0.079	0.989 ±0.068	21.08 ±1.28	32.44 ±1.32	10.47 ±1.78	16.89 ±1.40	3.40 ±0.28	3.87 ±0.38	1.268 ±0.122	1.126 ±0.078	23.28 ±1.42	34.20 ±1.24	12.45 ±1.40	24.20 ±1.20	6.41 ±0.22	7.28 ±0.23
	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)	(NS)	(ND)
Monoraphidium minutum[a]	1.168 ±0.089	0.942 ±0.078	23.06 ±1.30	29.48 ±1.30	12.93 ±1.91	15.91 ±1.60	5.43 ±0.20	5.50 ±0.22	1.361 ±0.068	1.106 ±0.980	26.31 ±1.21	34.81 ±1.21	17.13 ±2.40	18.83 ±1.22	6.14 ±0.30	9.23 ±0.12
Cyclotella cryptica[b]	1.024 ±0.097	0.898 ±0.987	15.23 ±1.08	42.55 ±1.10	9.56 ±0.80	27.08 ±1.80	3.71 ±0.45	7.31 ±0.56	1.103 ±0.112	0.906 ±0.104	15.59 ±1.12	45.18 ±1.14	10.79 ±0.84	31.02 ±1.25	3.79 ±0.25	8.18 ±0.26
Chaetoceros moelleri var *subslasum*[b]	0.980 ±0.984	0.675 ±0.079	19.05 ±1.02	34.12 ±1.10	13.90 ±0.85	19.98 ±1.40	4.52 ±0.38	6.55 ±0.49	1.095 ±0.104	0.715 ±0.115	22.56 ±1.18	42.20 ±1.25	14.10 ±0.79	29.25 ±1.60	4.10 ±0.31	8.54 ±0.34

[a] Chlorophyta.
[b] Chrysophyta.
NS = Nitrogen sufficient, ND = nitrogen deficient, SS = Silica sufficient, and SD = silica deficient.

Table 4
Fatty Acids of *Navicula saprophila*
Grown in Nitrogen and Silica Concentrations at Different Temperatures

Fatty acid carbon number	Fatty acids temperature 20°C		30°C	
	Nitrogen			
	Sufficient (NS)	Deficient (ND)	Sufficient (NS)	Deficient (ND)
C12:0	4.10	4.38	—	2.19
C14:0	20.98	22.35	17.59	4.77
C15:0	—	—	—	—
C16:1	4.48	—	—	3.18
C16:0	34.70	40.95	—	39.92
C18:2	—	—	—	3.70
C18:1	10.20	—	—	4.69
C18:0	21.96	28.33	15.57	19.06
C19:0	—	—	—	—
C20:0	—	—	—	4.73
C22:0	3.55	3.96	66.82	17.71
	Silica			
	Sufficient (SS)	Deficient (SD)	Sufficient (SS)	Deficient (SD)
C12:0	—	3.09	—	—
C14:0	—	—	16.15	22.45
C15:0	—	—	—	—
C16:1	—	10.79	17.63	—
C16:0	39.96	57.96	42.12	46.17
C18:2	—	—	2.52	3.90
C18:1	Tr	—	1.95	1.36
C18:0	5.32	7.20	12.52	14.56
C19:0	—	—	—	—
C20:0	46.57	20.94	2.58	8.27
C22:0	5.16	—	3.05	1.97

opportunity for exploiting the correlation between diatom metabolism and environmental factors to produce higher lipid yields for the ultimate conversion of these cellular products into gasoline and fuels.

CONCLUSIONS

The growth response of *Navicula saprophila* was significantly higher in nitrogen sufficient (NS) and silica sufficient (SS) media at both 30 and 20°C. The total lipids, neutral lipids, and polar lipids were higher when

these diatoms were grown in nitrogen deficient (ND) and silica deficient (SD) media at 30 °C as well as at 20 °C. The lipid production in general was greater at 30 than at 20 °C. The nitrogen and silica concentrations affected the distribution of fatty acids (C12:0 to C22:0).

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