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Effects of Nutrients and Temperature on Lipid and Fatty Acid Production in the Diatom *Hantzshia* DI-60

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ABSTRACT

The ability of microalgae to accumulate considerable amounts of lipids has led to an increase in research on the cultivation of these organisms for the production of fuels. The accumulation of cellular lipids by the diatom, *Hantzschia* DI-60, as a result of culture management strategies is described. The cells were grown in various nitrogen and silica nutrient concentrations at 20 and 30°C. Biomass, lipids, and fatty acids were measured. Biomass yield was higher in a non-stress nutrient (nitrogen/silica) treatment; however, the production of lipids was enhanced in a nutrient stress medium at 30°C. The influence of the culture conditions on the distribution of fatty acids was also observed.

Index Entries: Nitrogen; silica; temperature; biomass; lipids; fatty acids.

INTRODUCTION

Chrysophytes are known to accumulate large quantities of lipids. Microalgae grown in high salinity water accumulate storage lipids in great quantities (*1–3*), and as such, these strains are among the best biomass feedstocks available for producing high-energy liquid fuels, such as gasoline and diesel fuel. The influence of environmental conditions, such as carbon dioxide supply, light intensity, temperature, nutrient concentration, and salinity on metabolism of algae, has been reported (*4–7*). Temper-

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ature, nitrogen concentration, and silica concentration are factors that are known to influence lipid composition and quantity (*8–11*). Owing to the influence of these culture conditions on the accumulation of storage lipids, research has been directed toward exploring the potential for the large scale production of gasoline and diesel oils from microalgal lipids (*10–15*).

In order to fully realize the potential of this technology, research efforts in recent years have been aimed at developing microalgal strains that have an increased production of biomass and lipids for liquid fuels (*11–14*). In microalgae such as *Monottantus salina* (*11*), *Nannochloris sp.* and *Isochrysis sp.* (*15*), *Nitzchia sp.* (*3,13*), *Navicula saprophila* (*16*), *Monoraphidium minutum* and *Cyclotella cryptica* (*8*), *Chaetoceros moelleri var subsalsum* (*14*), and *Ankistrodesmus sp.* (*7*), increased lipid production in response to nitrogen starvation was observed. Similarly the effects of silica stress on enhanced lipid production in some algal strains has been reported (*17–20*). An increase in synthesis of fatty acids in strains such as *Butryococcus sp.* (*21*) *Dunaliella salina* (*22*), and *Chlorella sp.* (*23*) and *Euglena sp.* (*24*) has been reported. The increase in lipid content and changes in fatty acid composition in these microalgae has been correlated with nutritional deficiencies (*21–24*).

The simple growth requirements by microalgae and their ability to produce large amounts of storage lipids under different culture conditions encouraged us to evaluate the production of lipids and fatty acids by the diatom *Hantzschia* DI-60 in response to varying nutrients concentrations and temperatures. The present study describes the growth response, lipid content, and fatty acids composition in this strain cultured in two different concentrations of nitrogen and silica and at two temperatures (20 and 30°C).

MATERIALS AND METHODS

Culture Maintenance

The cultures of *Hantzschia* DI-60 were btained from M. Tadros, Alabama A & M University, Normal, AL. Organisms were grown on synthetic medium containing the following nutrients (mg/L): KNO_3 (300), $Na_2SiO_3 \cdot 9H_2O$ (30), $NaH_2PO_4 \cdot H_2O$ (5), and Rila Mix (40,000) and vitamins (μg), thiamine-HCl (100), biotin (0.50), and B_{12} (0.5) at a final pH of 7.5. The trace elements stock solution (1 mL) was added. The trace element stock solution contained (mg/L): Na_2EDTA(4630), $FeCl_3 \cdot 6H_2O$(3150), $MnCl_2 \cdot 4H_2O$(180), $CuSo_4 \cdot 5H_2O$(10), $ZnSo_4 \cdot 7H_2O$ (22), $CoCl_2 \cdot 6H_2O$(10), and $Na_2MoO_4 \cdot 2H_2O$ (6). Algae were grown in a temperature-controlled growth room at 30°C under continuous illumination with Cool White Westinghouse fluorescent lamps (light intensity of about 180 $\mu E/M^2/s$). The cultures were aerated with 3% carbon dioxide. To measure lipid production under stressed and nonstressed conditions, the microalgae were

grown under optimal conditions of growth in 2800 mL Fernback flasks (for nitrogen treatments), or in 2 L polycarbonate bottles (for silica treatments), each containing 1500 mL of medium. Two concentrations of potassium nitrate (600 and 300 μM) and sodium metasilicate (1 mM and 250 μM) were added to batch cultures and grown at 20 and 30°C. Each treatment was replicated three times.

Determination of Growth Rate

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, Irvine, 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. Daily sampling for growth measurement was done on aliquots of the culture suspension and included cell counting, using a Peteroff Housing Chamber. The growth is reported as doublings per day according to the method of Vonshak and Maske (*25*), as shown below.

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

(OD = Optical Density, T = Time, h = hour).

Determination of Ash-Free Dry Weight

Harvesting was done as per the method described elsewhere (*16*). Algae were harvested by centrifugation at the end of the logarithmic phase. Part of the pellet was lyophilized for fatty acid analysis, and the remaining was kept frozen until lipid analysis. Nitrogen-deficient cultures were grown on 300 μM KNO_3 to the end of the logarithmic phase, left for about eight more days at the steady state phase, and finally harvested by centrifugation. The wet and dry cell mass was recorded. The wet cells were stored at −20°C. Ash-free dry weight (AFDW) was determined by drying the samples at 60°C and ashing at 540°C.

Determination of Lipids

The total lipids were extracted according to the modified method of Bligh and Dyer (*26*), as described for algal lipids. Adequate chloroform and water were added to give the Bligh-Dyer ratio for phase separation (chloroform:methanol). The elutants were condensed in volume on a rotary evaporator and later evaporated to dryness under air. The weight of neutral lipids and polar lipids was then evaluated. The total lipids were passed through a silicic acid column to separate neutral and polar lipids, and their weights were evaluated (*16*).

Fatty Acids Analysis

The cells of the diatoms were lyophilized, and the fatty acids were analyzed by gas-liquid chromatography after transesterification (*27*). 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 (*28*). 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 (*29*). After centrifuging, the lower phase containing the lipids was evaporated to dryness at room temperature (25±1°C) under a gentle stream of nitrogen. The residue was dissolved 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 (*30*). The samples were shaken, centrifuged, and injected into the chromatograph.

Fatty acid analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector coupled to a Hewlett-Packard 3990A integrator. Nitrogen was used as the carrier gas at a flow rate of 28 mL/min. The injection port temperature was 200°C and the detector was 300°C. The column temperature was held at 80cC for 2 min and then increased in a step-wise fashion (8.5°C/min) to a maximum of 215°C. The standard fatty acid methyl ester (FAME) mix (Supelco, Inc., Bellefonte, PA) 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

Growth was determined by cell counting and is shown in Table 1. It was observed that significant reduction in growth rates take place in cultures of *Hantzschia* DI-60 supplied with deficient concentrations of nitrogen and silica, whereas an appreciable increase in cell number was noted when the cells were grown in sufficient concentration of nutrients. At 30°C, the cellular yields were greater compared to 20°C. The influence of environmental factors, including nutrients on growth and metabolism in other microalgae, e.g., *Chlorella sp., Nitzschia sp., Cyclotella cryptica, Dunaliella sp., Chaetoceros moelleri var subsalsum, Navicula saprophila,* and *Monoraphidium minutum,* have already been reported (*3–8,11–14,16*). The present study indicated that nitrogen and silica have a strong influence on the growth of the diatom *Hantzschia* DI-60. The biomass production (expressed as g/L AFDW) also decreased when suboptimal doses of nitrogen and silica were supplied to this diatom (Tables 2 and 3). However, an increased biomass accumulation was noticed in the diatoms cultured at 30°C under optimal conditions of these nutrients compared to the yields of biomass in diatoms cultured at 20°C. The biomass accumulation in this diatom is higher than in other diatoms, viz, *Chaetoceros moelleri, var subsalsum, Cyclotella cryptica, Navicula saprophila,* and microalga *Monoraphidium minutum* and cultured with same amounts of nitrogen and silica (*14,16*) (Table 4).

Table 1
Growth Rate (Doublings/Day) of *Hantzschia* DI-60 at 20 and 30°C in Nutrient-Sufficient and Nutrient-Deficient Treatments

Temp, °C	Nitrogen		Silica	
	Sufficient	Deficient	Sufficient	Deficient
20	3.25	2.90	2.75	2.15
30	3.90	3.40	3.20	2.85

Table 2
Biomass and Lipid Yields of *Hantzschia* DI-60 Grown in Different Nitrogen Concentrations and Temperatures in Percent of AFDW

Culture conditions	Ash-free dry weight (AFDW), g/L	Total lipids, % AFDW	Neutral lipids, % AFDW	Polar lipids, % AFDW
		20°C		
Nitrogen Sufficient (NS)	1.314 ±0.924	24.268 ±1.645	14.368 ±1.024	6.381 ±0.967
Nitrogen Deficient (ND)	0.845 ±0.948	43.461 ±1.434	27.348 ±1.648	11.250 ±0.846
		30°C		
Nitrogen Sufficient (NS)	1.582 ±0.924	29.681 ±1.439	12.680 ±0.684	5.364 ±0.19
Nitrogen Deficient (ND)	1.120 ±0.867	53.384 ±1.742	32.684 ±1.046	14.680 ±0.670

Table 3
Biomass and Lipid Yields of *Hantzschia* DI-60 Grown in Different Silica Concentrations and Temperatures in Percent of AFDW

Culture Conditions	Ash-free dry weight (AFDW), g/L	Total lipids, % AFDW	Neutral lipids, % AFDW	Polar lipids, % AFDW
		20°C		
Silica Sufficient (SS)	1.185±0.082	22.824±1.201	12.680±0.164	7.640±0.145
Silica Deficient (SD)	0.987±0.071	40.646±1.304	24.652±1.040	10.964±0.964
		30°C		
Silica Sufficient (SS)	1.264±0.096	26.421±1.070	13.350±1.540	8.342±0.148
Silica Deficient (SD)	1.062±0.079	44.362±1.148	25.458±1.248	10.634±0.924

Table 4
Fatty Acids of *Hantzschia* DI-60 Grown in Nitrogen and Silica Concentrations at Different Temperatures

Fatty Acid Carbon Number	Percent Fatty Acids			
	Temperature, 20°C		30°C	
	Nitrogen			
	Sufficient	Deficient	Sufficient	Deficient
C12:0	3.60	3.48	4.50	6.20
C14:0	20.10	21.40	9.50	12.60
C15:0	-	-	-	-
C16:1	2.84	1.40	6.48	2.96
C16:0	28.66	35.46	18.02	16.24
C18:2	-	-	-	-
C18:1	9.25	10.50	6.14	6.53
C18:0	16.60	6.74	20.40	21.54
C19:0	-	-	-	-
C20:0	16.03	18.90	18.56	19.42
C22:0	2.90	2.12	16.40	14.51
	Silica			
C12:0	1.60	1.40	2.40	3.65
C14:0	16.26	18.45	14.56	16.43
C15:0	-	-	-	-
C16:1	7.84	11.79	17.58	16.49
C16:0	49.45	43.84	42.95	44.29
C18:2	-	-	2.19	3.20
C18:1	Tr	Tr	-	-
C18:0	8.47	6.45	10.31	11.23
C19:0	-	-	-	-
C20:0	16.38	18.03	7.60	2.14
C22:0	-	-	2.14	2.57

The accumulation of total, neutral, and polar lipids (expressed as percent of AFDW) in this diatom, when cultured in optimal and suboptimal doses of the nutrients at 20 and 30°C, was measured (Tables 2 and 3). It was noted that the yields of all these lipids were enhanced when the cells were cultured in suboptimal doses of nitrogen and silica. The increase in lipid production in *Hantzschia* DI-60, owing to nutrient deficiency, is in agreement with earlier reports (*6,8,10,13–16*). It has been reported that *Monoraphidium minutum, Cyclotella cryptica,* and *Chaetoceros moelleri var subsalsum* are high lipid-producing microalga (*16*). The present study shows that the lipid yields of *Hantzschia* DI-60 are higher than these microalgae. An increase in incubation temperature from 20 to 30°C resulted in higher lipid yields. It is interesting to note that the production of neutral and polar lipids is significantly higher in nitrogen and silica-deficient con-

centrations compared to these nutrients in sufficient concentrations. The production of total, neutral, and polar lipids was higher at 30°C than 20°C, as shown in Tables 2 and 3. The influence of nitrogen and silica on lipid accumulation microalgae has been reported earlier (*1–8,16–20*), and it appears that an increase or decrease in lipid content, exhibited by a variety of diatoms, is species dependent. The fatty acids of the diatom *Hantzschia* DI-60 were: C12:0, C14:0, C16:1, C16:0, C18:2, C18:1, C18:0, C20:0, and C22:0 (Table 4). Among them, C14:0, C16:0, and C18:0 were found to be predominant. This fatty acid composition appears to be different from that reported in other algal species, e.g., *Microcystis aeruginosa* C16:0, C16:1, C18:2, and C18:3; *Anacystis nidulans* C16:0, C16:1, and C18:1 with traces of C17:0, C17:1, and C20:0; and *Spirulina sp.* C16:0, C16:1, C18:1, and C18:2, and C18:3 (*20*). The most common fatty acids synthesized in microalgae *Ankistrodesmus sp., Botryococcus braunii, Dunaliella sp.*, and *Nitzschia sp.* were C14:0, C16:0, C18:1, C18:2, and C18:3 (*22–24*). From these observations and our study, it seems that the fatty acid composition is species specific. A comparison of the distribution of the fatty acids in *Hantzschia* DI-60, grown in optimal and suboptimal concentrations of nitrogen and silica, suggests that there is a variation both in qualitative and quantitative distribution.

CONCLUSIONS

The growth response, biomass, and lipid production in *Hantzschia* DI-60 were significantly influenced by environmental conditions, such as nutrients and temperature. Cellular and biomass yields were significantly higher in nitrogen-sufficient (NS) and silica-sufficient (SS) media at both 30 and 20°C, whereas the production of total, neutral, and polar lipids increased in nitrogen-deficient (ND) and silica-deficient (SD) media at these two temperatures. In general, the lipid production was greater at 30°C than 20°C. Considerable changes in the distribution of the fatty acids (C14:0 to C22:0) were also noted.

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