Research Article

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Potential fuel oils from the microalga *Choricystis minor*

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Abstract

BACKGROUND: Continuous culture of the freshwater microalga *Choricystis minor* was investigated for possible use in producing lipid feedstock for making biofuels. The effects of temperature (10–30 °C) and dilution rate (0.005–0.017 h^{-1}) on lipid productivity in a nutrient sufficient medium in a 4 L stirred tank bioreactor under continuous illumination at an incident irradiance level of 550 $\mu E \cdot m^{-2}s^{-1}$ and a controlled pH of 6 under carbon dioxide supplemented conditions are reported.

RESULTS: The maximum lipid productivity was 82 mg L^{-1} d^{-1} at 25 °C and a dilution rate of 0.014 h^{-1}. Lipid contents of the biomass were 21.3 ± 1.7 g per 100 g of dry biomass, irrespective of the culture temperature and dilution rate. After the biomass had been grown in nutrient sufficient conditions in continuous culture, it was recovered and subjected to various postharvest treatments. With the best postharvest treatment, the neutral lipid contents of the algal biomass were raised ∼6-fold relative to untreated biomass.

CONCLUSION: At 82 mg L^{-1} d^{-1}, or 21 000 L ha^{-1} $year^{-1}$, the lipid productivity of *C. minor* was nearly four times the lipid productivity of oil palm, a highly productive crop. Therefore, *C. minor* is potentially a good source of renewable lipid feedstock for biofuels.

Keywords: *Choricystis minor*; biofuels; biodiesel; microalgae; photobioreactors

NOTATION

A_b	Projected tube area (m^2)
C_b	Biomass concentration (mg L^{-1} or g L^{-1})
d	Diameter of photobioreactor tube or vessel (m)
D	Dilution rate (h^{-1})
OD_{680}	Optical density at 680 nm
P_{areal}	Areal oil productivity (L ha^{-1} $year^{-1}$)
P_b	Volumetric biomass productivity (mg L^{-1} d^{-1})
P_{oil}	Volumetric oil productivity (mg L^{-1} d^{-1})
t	time (h or days)
V_b	Volume of the cylindrical bioreactor (m^3)
x_{oil}	Mass fraction of lipids in the biomass
μ	Specific growth rate (h^{-1})

INTRODUCTION

Microalgae are a potential source of carbon neutral and renewable biofuels, particularly biodiesel.[1–3] Biodiesel is currently produced from vegetable oils.[4] Unfortunately, oil crops cannot sustainably provide sufficient biodiesel to displace conventional transport fuels;[2] hence the interest in microalgae. Estimates suggest that less than 6% of the cropping area of the USA can provide all its transport fuel needs if devoted to algal culture.[2] Algae produce many kinds of oils, or lipids, not all of which are suitable for conversion to biodiesel. Conventionally, only the neutral triglyceride lipids are used to make biodiesel; however, using existing chemistry, all algal lipids can be used to make diesel, gasoline and jet fuel. Algae produce lipids during rapid growth, but production is often greatly enhanced under nutrient deficient conditions that do not favor growth.[5] Understanding the conditions that enhance lipid production by algae and influence the biochemical profile of the lipids, is essential to making biodiesel from microalgae a feasible option.

Here we report on biomass and oil productivity of the green microalga *Choricystis minor*. *C. minor* is a freshwater picoplankton that occurs worldwide[6–8] and is specially common in nutrient-poor lakes.[9] In natural habitats, *C. minor* has been shown to tolerate well changes in pH, temperature and nutrient content of the water.[7,10,11] It has the potential to do well in nutrient-rich waters because of its high surface area to volume ratio that facilitates nutrient uptake. No data exist on photobioreactor culture of this alga, its oil productivity, biochemical profiles of its lipids, and how its lipid production might respond to culture conditions. Two different methods of lipid extraction from the algal biomass are assessed. The effects of different postharvest treatments on the biomass lipid level and biochemical profiles of the lipids, are reported.

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MATERIALS AND METHODS

Microorganism and culture conditions

The freshwater green microalga *Choricystis minor* (H. Skuja) B. Fott (phylum Chlorophyta, class Trebouxiophyceae), a picoplankton, was grown as continuous monoculture. Continuous cultures were carried out in a 4 L (3.5 L working volume) bioreactor (New Brunswick Scientific, BioFlo 110; Edison, NJ, USA) stirred by a six-bladed Rushton turbine (0.059 m in diameter) at a constant speed of 200 rpm. The fully baffled (4-equidistant baffles) bioreactor vessel had a diameter of 0.178 m. The culture medium and the vessel were sterilized by autoclaving at 120 °C for 15 min. The bioreactor was continuously illuminated using 12 fluorescent lamps (10 W each, NEC FL10EX-N-HG; NEC Corporation, Tokyo, Japan) placed around its periphery. A cylindrical reflector was placed just outside the circle of lamps. The incident irradiance (photosynthetically active) at the surface of the bioreactor vessel was 550 μE m^{-2} s^{-1}. Irradiance was measured using a QSL-100 quantum scalar irradiance sensor (Biospherical Instruments, San Diego, CA, USA).

The culture medium was always BG11.[12] The values of the dilution rate varied from 0.005 to 0.017 h^{-1}. Each dilution rate was tested at multiple temperatures that ranged from 10 to 30 °C. The pH was controlled at 6 by automatic addition of 1 mol L^{-1} sulfuric acid. The bioreactor was aerated at 1.58 L min^{-1} with a mixture of air containing carbon dioxide to the level of 4.7% by volume. The aeration gas was sterilized by passing through a 0.2 μm Teflon membrane filter cartridge (Midisart® 2000; Sartorius AG, Goettingen, Germany).

For each set of operating conditions, after a steady state had been attained, two 2 L samples of the broth were harvested. Samples (1 L each) of this broth were subjected separately to the following treatments:

1. The biomass was centrifuged (7000g, 10 min), washed with BG11 medium that excluded nitrate and phosphate, recovered by centrifugation as above, resuspended to the original biomass concentration in BG11 medium that was free of nitrate and phosphate and grown in this medium for 2, 5 or 10 days.
2. The biomass was recovered as above, resuspended in the standard BG11 medium and the initial pH was adjusted to 9 using NaOH.
3. The biomass was recovered and resuspended in the standard BG11 medium and NaCl was added to a concentration of 10 or 30 g L^{-1}.
4. The biomass was recovered and resuspended in the standard BG11 medium and air without CO_2 supplementation was used for sparging.

In all of the above treatments, unless stated otherwise, the other conditions were as follows: 1 L aerated vessels (no mechanical agitation); 1 vvm flow of air enriched with 5% of CO_2; and 330 μE m^{-2} s^{-1} incident irradiance at the surface of the vessels.

Analytical methods

Biomass concentration

Biomass concentration was monitored by measuring the optical density at a wavelength of 680 nm. Samples were appropriately diluted if the optical density value exceeded 0.4. The following relationship between optical density (OD_{680}) and washed dry cell weight concentration (C_b, g L^{-1}) was established: $C_b = 13.19 \times 10^{-2} OD_{680}$.

Total lipid content

Sampling. For each steady state of the bioreactor and postharvest treatment, triplicate samples of the algal broth were collected directly from the culture vessel. The biomass was recovered by centrifugation (7000g, 10 min), washed twice with distilled water (each time with a volume that was the same as the volume of the original sample), and freeze dried. Total lipids in the dry biomass (at least 50 mg per triplicate sample) were measured.

For selected steady states, the biomass slurry from the outlet of the continuous flow bioreactor was collected for several hours each day in a container that was held at 0 °C in an ice bath. The biomass was recovered and washed as above and saved in a freezer at −15 °C to accumulate 6 g of freeze-dried biomass. Samples of this dry biomass (∼2 g each) were extracted and further processed in triplicate for separating the lipids into various fractions and profiling of fatty acids.

A 2 g washed sample of the biomass was freeze-dried and an identical sample was oven dried (105 °C, to constant weight) for comparison so that lipid concentrations could be expressed on a biomass dry weight (oven dried) basis.

Lipid extraction. For comparison, the total lipids in the biomass were extracted by the following two methods:

1. three consecutive extractions with hot isopropanol (50–60 °C) and then with a 1 : 1 v/v mixture of isopropanol and chloroform, as recommended by Kates;[13]
2. two consecutive extractions with the Bligh and Dyer[14] solvent system (chloroform, methanol and water in a proportion of 1 : 2:0.8 by volume) as recommended by Kates[13] for microorganisms followed by a further extraction with only chloroform as suggested by Bligh and Dyer[14] to quantitatively extract the neutral lipids.

The solvent in the extracts was evaporated in a rotary evaporator and the lipids were resuspended in 30 mL of 1 : 1 by volume mixture of methanol and chloroform. This solution was transferred to a 50 mL Teflon centrifuge tube and distilled water (13.5 mL) was added to obtain a biphasic system. The tube was mixed three times by inverting and then centrifuged (20000g, 3 min). The upper aqueous-methanolic phase containing water-soluble components was discarded. The lower chloroform phase was washed with 28.5 mL of a mixture of methanol and distilled water (1 : 0.9 by volume), as above, and recovered. The volume of the recovered chloroform phase was measured in a graduated tube and an aliquot of this phase containing between 20 and 40 mg lipids was evaporated in a preweighed aluminum container to determine the exact total lipid concentration by gravimetry.

Lipid fractionation. The residual extract from the previous section was concentrated to 7–8 mL by evaporation and an aliquot containing 250–280 mg of total lipids in ≤5 mL of chloroform was fractionated using a prepacked silica gel column (Extract Clean™ SPE silica 20 g, 75 mL; W.R. Grace & Co., Deerfield, IL, USA). Separation was performed as described by Kates,[13] by sequentially eluting the column with chloroform, acetone and methanol to obtain neutral lipids, glycolipids and polar lipids, respectively.

Fatty acid profiles. To quantify different fatty acids in a lipid fraction, an appropriate weight of the lipid fraction was derivatized to free fatty acid methyl esters and analyzed by gas chromatography in accordance with ISO 15304[15].

RESULTS AND DISCUSSION

C. minor has not been previously investigated for production of lipids. In earlier unpublished studies in batch cultures, this alga was observed to have a high specific growth rate of about 0.046 h^{-1} (a doubling time of ~15 h) under nutrient sufficient conditions and a lipid content of around 47.5% (w/w) in the dry biomass under nutrient limited conditions. This suggested *C. minor* as potentially attractive alga for producing lipids for conversion to renewable liquid transport fuels. Therefore this detailed study was undertaken to quantify the lipid productivity under various conditions of growth and postharvest treatments of the biomass.

Choice of the oil extraction method

In order to evaluate lipid production in detail, methods for effectively extracting the lipids from the biomass must be established. Therefore the hot isopropanol method[13] and the Bligh and Dyer[14] extraction protocol were compared using triplicate samples of a single batch of the freeze-dried biomass. The results are shown in Table 1. Both methods extracted similar levels of total lipids. Furthermore, the compositions of the total lipid extracts from the two methods were similar in terms of the contents of neutral lipids, glycolipids and polar lipids (Table 1), suggesting that both methods were equally effective in extracting the various classes of lipids. In addition, both the methods were equally effective in extracting lipids from freshly harvested moist biomass paste and from an identical sample of the freeze-dried biomass (results not shown). In view of this, the hot isopropanol extraction from freeze-dried biomass was used for all subsequent work: compared with the Bligh and Dyer[14] method, the hot isopropanol method required less chloroform and produced a monophasic solvent system that was easier to separate from the residual biomass.

Effect of dilution rate and temperature on biomass and oil productivity

In continuous culture at steady state, the specific growth rate μ of the alga, the biomass concentration C_b in the bioreactor, and the dilution rate D are related as follows:

$$\frac{dC_b}{dt} + \mu C_b - DC_b = 0 \qquad (1)$$

At steady state, therefore, $D = \mu$.

At any given dilution rate and the other specified constant operating conditions, the bioreactor always attained a steady state. Attainment of a steady state could be demonstrated by measuring the concentration of the biomass in the bioreactor at various intervals of time.[16] The steady state concentrations of biomass and the total lipid contents in the biomass samples at steady state in the nutrient sufficient BG11 medium, are shown in Table 2 for the various dilution rates and culture temperatures used. At any given dilution rate, the highest concentration of the biomass was attained generally at 25 ± 2.5 °C (Table 2), suggesting this to be the optimal growth temperature. This concurred with an optimal growth temperature value of 23.5 °C previously reported by Zidarova and Pouneva.[17]

The total lipid contents of the biomass were not significantly affected by the dilution rate, i.e. the specific growth rate, or by the temperature in the ranges tested (Table 2). On average the biomass had a total lipid contents of 21.3 ± 1.7 g per 100 g of dry biomass, irrespective of growth temperature and dilution rate (Table 2), even though the dilution rate varied more than 3-fold. A high temperature of growth has been linked to a significantly increased lipid level in some algal species, but in others, cultivation at medium and low temperatures has led to an elevated lipid level.[18–20] How the lipid level is affected by temperature depends on the algal species. An analysis of variance of the lipids contents of *C. minor* biomass at the steady states attained at various dilution rates and temperatures, indicated that the variability in lipid levels was not explained by changes in culture temperature (ANOVA P-value $\gg 0.05$ and F-ratio < 1). Furthermore, at a constant culture temperature of 25 °C, the variability in the oil contents was not explained by changes in dilution rate (ANOVA P-value $\gg 0.05$ and a low F-ratio).

At otherwise fixed conditions, changes in dilution rate of course had a significant impact on the steady state biomass concentration (Table 2). Typically, at a fixed temperature, increasing dilution rate reduced the steady state biomass concentration. This behavior is characteristic of photosynthetic cultures that must become dilute to improve light penetration to sustain an increased average specific growth rate of the biomass as a consequence of the increased dilution rate. Although, the total incident irradiance at the surface of the photobioreactor did not change at all, in a dilute culture light was accessible to more cells than in a concentrated culture. In a dense culture, light penetrates only to a shallow depth and the few cells that receive a relatively high light level do not use it effectively so that much of the incident light is lost as biochemically unproductive heat and fluorescence.[21]

The local irradiance values at a depth of 1 cm from the wall of the bioreactor and at its deepest point (i.e. the center; depth = 0.089 m) are shown in Fig. 1 for various concentrations of the suspended cells. At any steady state biomass concentration in Table 2, Fig. 1 reveals that the central zone of the bioreactor was dark, but an illuminated zone always existed up to a depth of at least 1 cm from the vessel wall. A dark zone cannot sustain growth as it lacks light. Therefore, for any alga in an optically dense culture

Table 1. Comparison of oil extraction methods for total lipids and lipid classes

Extraction system[a]	Total lipid content (g per 100 g dry biomass)	Neutral lipid content (g per 100 g total oil)	Glycolipid content (g per 100 g total oil)	Polar lipid content (g per 100 g total oil)
Bligh and Dyer	26.0	34.4	29.5	30.4
Bligh and Dyer	22.8	30.1	30.4	27.8
Bligh and Dyer	22.9	38.5	28.6	30.2
Hot isopropanol	27.2	32.5	30.5	34.2
Hot isopropanol	29.7	33.8	29.9	30.2
Hot isopropanol	25.0	30.4	29.7	35.3

[a] Each extraction protocol was used on triplicate samples of the same batch of biomass.

Table 2. Steady state biomass concentration and lipid content in the biomass at various dilution rates and temperatures

	Dilution rate (h^{-1})												
	0.005				0.010				0.014				0.017
Temperature (°C)	10	15	25	30	10	25	15	20	23.5	25	30	25	30
Biomass (mg L^{-1})	1290	1409	2020	1915	703	1356	595	904	936	1046	942	815	702
Total lipids[a] (g per 100 g)	–	20.6 ± 0.5	18.2 ± 2.3	19.5 ± 0.8	21.0 ± 0.5	23.3 ± 0.7	22.2 ± 1.1	20.5 ± 0.8	23.5 ± 1.4	23.3 ± 2.3	21.1 ± 0.9	21.4 ± 2.1	–

[a] Standard deviation (g per 100 g) values are shown.

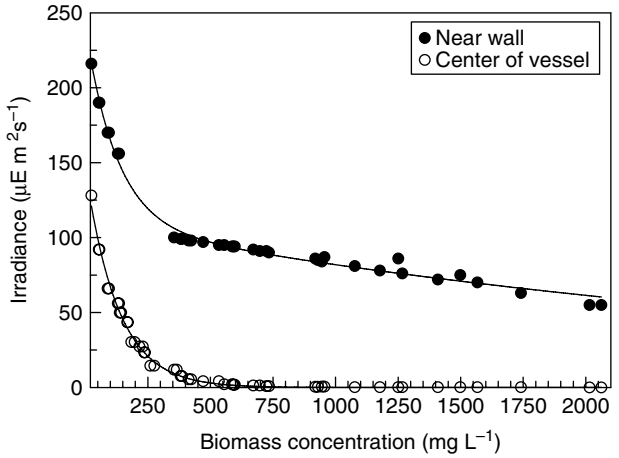

Figure 1. Average measured irradiance at various biomass concentrations: (a) at a depth of 1 cm from the wall of the bioreactor (filled symbols); (b) at the center of the bioreactor vessel (hollow symbols). The incident irradiance at the outer wall was a constant 550 μE m^{-2} s^{-1}.

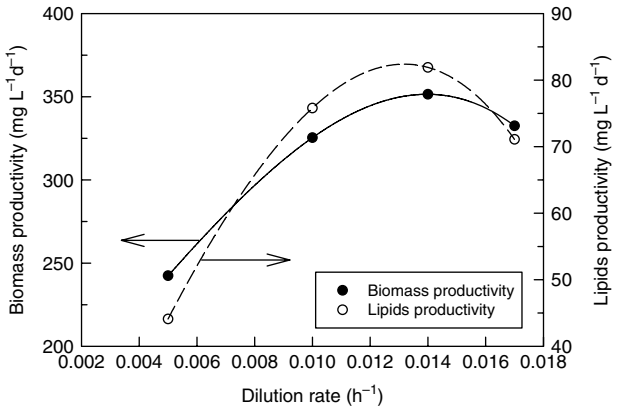

Figure 2. Steady state biomass and lipid productivities at various dilution rates at 25 °C.

in a bioreactor, the observed specific growth rate can never approach the biochemically possible maximum specific growth rate. In contrast, in a batch culture, the biomass concentration at inoculation is generally so low that light penetrates to the entire depth of the bioreactor. For example, the maximum biomass concentration in the batch reactor at the above mentioned maximum specific growth rate value of 0.046 h^{-1}, was 135 mg L^{-1}. As shown in Fig. 1, for this low concentration of biomass, a totally dark zone could not exist. Although the incident irradiance for the case in Fig. 1 was 550 μE m^{-2} s^{-1}, or nearly 65% higher than for the above mentioned batch culture, light penetrated to the entire depth of the batch culture vessel at the above noted concentration of the biomass.

Although the temperature and growth rate did not affect the lipid contents of the biomass (Table 2), the lipid and biomass productivities were quite sensitive to these variables, as shown in Fig. 2. Productivities increased with increasing dilution rate until the biomass concentration declined so much that it negated the productivity enhancing effect of the increasing dilution rate. At 25 °C, the maximum values of the biomass and lipid productivities were 351 mg L^{-1} d^{-1} and 82 mg L^{-1} d^{-1}, respectively. Therefore, the optimal dilution rate under the specified conditions of agitation and incident irradiance was 0.014 h^{-1} (Fig. 2).

Based on ANOVA, and quite obvious in Fig. 2, there was a strong association between the productivity of lipids and the biomass productivity (F-ratio = 154.8, $P < 0.0001$, degrees of freedom, df = 13). Thus, for *C. minor*, the lipid productivity can be maximized by focusing on improving the productivity of the biomass.

In unpublished prior work mentioned earlier, *C. minor* was found to have a maximum specific growth rate of 0.046 h^{-1} in batch culture in a 2 L aerated bioreactor that was illuminated at 330 μE m^{-2} s^{-1} at the surface. In principle, therefore, the dilution rate of a continuous culture can be increased to nearly 0.046 h^{-1} without washout, but such a culture would be exceedingly dilute and have a low productivity.

The biomass and oil productivities in Fig. 2 were calculated using the following well-known equations:[22]

$$P_b = C_b D \tag{2}$$

$$P_{\text{oil}} = P_b x_{oil} \tag{3}$$

where P_b is the volumetric productivity of the biomass, C_b is the concentration of the biomass, P_{oil} is the volumetric productivity of the lipids in the bioreactor and x_{oil} is the mass fraction of lipids in the biomass.

The above mentioned volumetric oil productivity of 82 mg L^{-1} d^{-1} can be converted to an approximate areal oil productivity of 42 000 L ha^{-1} year^{-1} in a tubular photobioreactor. This conversion assumes: (1) a tube diameter of 0.178 m, or the same as for the stirred vessel used in this work; (2) a tube layout in which the tube's length is parallel to flat ground; (3) an annual operational period of 330 days; and (4) a density of 900 kg m^{-3} for the crude algal oil. The volumetric oil productivity P_{oil} was converted to areal productivity P_{areal}, as follows:

$$P_{\text{oil}} V_b = P_{\text{areal}} A_b \tag{4}$$

where V_b is the volume of the cylindrical bioreactor that can be viewed as a section of a tube with the projected area A_b. For such a system, Equation (4) can be simplified to:

$$P_{\text{areal}} = \frac{P_{\text{oil}} \pi d}{4} \tag{5}$$

In Equation (5), d is the diameter of the tube or the bioreactor vessel. The estimated areal productivity for the continuous illumination mode was about 42 000 L ha^{-1} year^{-1}. This was multiplied by 0.5 as a correction for the inevitable diurnal cycle that would occur outdoors. Therefore, the final estimated outdoor productivity was 21 000 L ha^{-1} year^{-1}.

The estimated productivity of 21 000 L ha^{-1} year^{-1} is high in comparison with the maximum oil productivity of 5950 L ha^{-1} year^{-1} for oil palm, one of the most productive oil crops.[2] In practice, because the average outdoor irradiance would be significantly greater than the light level used indoors, the oil productivity outdoors should be substantially greater than the estimated value. Indeed, outdoor cultures of algae commonly have higher biomass productivities when compared to the typically used indoor culture conditions.

Effect of dilution rate and temperature on the fatty acid profiles

As noted in the previous section, the dilution rate and temperature did not affect the total lipid level in the biomass; however, as shown

Table 3. Fatty acid profiles of total lipids at different temperatures and dilution rates at steady states

	Concentration of fatty acid (g per 100 g total lipid)						
	Temperature (°C)[a]			Dilution rate (h^{-1})[b]			
Fatty acid	15	25	30	0.005	0.010	0.014	0.017
C 16 : 0 Palmitic	25.8	20.8	27.8	34.3	22.8	20.8	23.5
C17 : 0 Margaric	ND[c]	6.3	2.6	3.2	3.4	6.3	7.9
C 18 : 0 Stearic	ND	ND	2.2	3.5	2.3	ND	ND
C 18 : 1 cis9 Oleic	14.0	14.5	15.1	22.8	14.6	14.5	14.1
C18 : 1 cis11 Vaccenic	5.9	6.4	ND	ND	4.7	6.4	7.5
C18 : 2 cis9,12 Linoleic	28.1	30.3	30.6	15.5	26.6	30.3	24.8
C 20 : 0 Arachidic	ND	ND	ND	1.9	ND	ND	ND
C18 : 3n6 cis6,9,12 γ-Linolenic	8.0	5.8	6.6	4.0	5.9	5.8	5.3
C 18 : 3 cis9,12,15 Linolenic	15.0	15.9	9.5	8.6	13.9	15.9	16.8
C20 : 2 cis11,14 Eicosadienoic	ND	ND	ND	ND	3.8	ND	ND
C 22 : 0 Behenic	3.1	ND	5.5	6.1	1.9	ND	ND

[a] Dilution rate 0.014 h^{-1}.
[b] Temperature 25 °C.
[c] Not detected.

in Table 3, these variables did affect the fatty acid profiles of the total lipid fraction of the biomass. These effects are discussed here in terms of the average age of the algal cells in the bioreactor.

In continuous culture of freely suspended cells as in this work, the dilution rate controls the average age of the cells in the bioreactor. The average age of cells at a given dilution rate is identical to the mean residence time of the broth in the bioreactor, or to the inverse of dilution rate. For the range of dilution rates used in this study, the average cell age varied from 59 h (dilution rate = 0.017 h^{-1}) to 200 h (dilution rate = 0.005 h^{-1}). For cells with an average age of ≤100 h, irrespective of the culture temperature, the polyunsaturated fatty acids (i.e. the fatty acids with the number of C=C double bonds $n \geq 2$, or C18 : 2, C18 : 3n6, C18 : 3 and C20 : 2 in Table 3) constituted from 47% to 52% (w/w) of the total lipids (Table 3) and were by far the predominant form of the fatty acids. At the identified optimal growth temperature of 25 °C, the cells with an average age of 200 h (dilution rate = 0.005 h^{-1}), had 49% of the fatty acids as saturated ($n = 0$, or C16 : 0, C17 : 0, C18 : 0, C20 : 0 and C22 : 0 in Table 3) and only 28.1% as polyunsaturated (Table 3). Irrespective of the culture temperature, in cells with an average age of ≤100 h, saturated fatty acids were the second most abundant class of fatty acids in the total lipid fraction, constituting between 27% and 31% of the total lipids (Table 3). For all cell ages and culture temperatures, the monounsaturated ($n = 1$) fatty acids constituted from 15% to 23% of the total lipid fraction and were the least dominant class of fatty acids. Age clearly affected the distribution of the various lipid types in the cells.

At a constant average cell age of ~71 h (dilution rate = 0.014 h^{-1}) an increase of culture temperature had a tendency to elevate the level of saturated fatty acids at the expense of the polyunsaturated fatty acids. This behavior has been commonly observed in many microalgae[23,24] and has been ascribed to the cell's need to maintain the fluidity of its various lipid-based membranes. At a low temperature, a membrane would solidify unless it was made with a high proportion of polyunsaturated fatty acids that remain liquid at fairly low temperatures.

Effects of postharvest treatments

For many microalgae, a deprivation of nitrate and phosphate and exposure to other environmental stresses have been shown to markedly increase the lipid contents of the biomass relative to nonstressed controls.[5] For example, algae commonly respond to above-normal increase in salinity by enhancing the lipid contents, but the magnitude of the response is highly dependent on the species involved.[20,25,26] Our unpublished studies with batch cultured *C. minor* suggested a stress-associated enhancement in lipid levels for this alga. This led to a detailed evaluation of the effects of the various stresses imposed on live cells after they had been grown rapidly to a high concentration under nonstressed optimal conditions in continuous culture.

For all studies in this section, the biomass had been grown at the optimal growth conditions identified in the previous section: the normal BG11 medium, a dilution rate of 0.014 h^{-1}, a temperature of 25 °C, and the earlier specified level of aeration with a 4.7% supplementation with carbon dioxide. Data on the biomass concentrations and lipid contents after the various postharvest treatments are shown in Table 4 in comparison with the relevant control cultures.

Treatments involving increased salinity (i.e. treatments 1–3, Table 4) did increase the lipid contents somewhat relative to control (i.e. treatments 9 and 10, Table 4), but this freshwater alga did not withstand elevated salinity even for relatively short periods. The treatments involving aeration without supplemental carbon dioxide either at the normal pH of 7.5 for BG11 or at an elevated pH of 9 (treatments 4 and 5 in Table 4) actually reduced the lipid contents especially under the alkaline conditions. The treatments involving nitrate and phosphate deprivation (i.e. treatments 6–8, Table 4), substantially enhanced the lipid levels relative to controls (treatments 9–11, Table 4) and the extent of enhancement increased with the length of the treatment (2–10 days). For example, 10 days of nutrient deprivation (treatment 8, Table 4) increased the lipid level by more than two-fold relative to control (treatment 11, Table 4). After the best postharvest treatment (treatment 8, Table 4), the biomass contained nearly 60% total lipids by weight. Moreover the required treatment was exceedingly simple and did not involve the use of any additives. Deprivation of nitrogen and phosphorous suppresses protein synthesis in algae so that the carbohydrate formed by photosynthesis is preferentially channeled to making lipids.

In addition to influencing the lipid contents, all nitrate and phosphate deprivation treatments and the corresponding control treatment in the complete BG11 medium, profoundly affected the profiles of the lipids produced (Table 5) compared with the lipid profile of the freshly harvested biomass (Table 3). Thus, in the postharvest treated biomass (Table 5), the proportion of saturated fatty acids was between 50% and 57% of the total lipid fraction, whereas it was only 27% in the freshly harvested biomass (Table 3). After postharvest treatment, the monounsaturated fatty acids constituted between 29% and 32% of the total lipid fraction (Table 5) whereas they constituted only 21% in the total lipids that were recovered from the fresh biomass. After postharvest treatment, the proportion of the polyunsaturated fatty acids was reduced to between 14% and 20% of the total lipids (Table 5) from 52% in the freshly harvested biomass (Table 3). A 2-day postharvest

Table 4. Effect of different postharvest treatments on the final biomass concentration and lipid contents of the biomass

Treatment[a]	Duration of treatment (days)	Biomass concentration (mg L^{-1})	Lipid contents (g oil per 100 g dry biomass)
1. BG11 medium with 30 g L^{-1} of NaCl added	2	– [b]	– [b]
2. BG11 medium with 10 g L^{-1} NaCl added	2	1540	29.3 ± 1.3
3. BG11 medium with 10 g L^{-1} of NaCl added	5	1702	28.8 ± 3.3
4. BG11 medium, no CO_2 supplementation of the sparging air	2	1670	21.0 ± 1.8
5. BG11 medium, no CO_2 supplementation of air and initial pH = 9	2	1020	19.2 ± 1.7
6. BG11 medium without nitrate and phosphate	2	1300	34.2 ± 0.4
7. BG11 medium without nitrate and phosphate	5	1860	45.3 ± 3.0
8. BG11 medium without nitrate and phosphate	10	1730	59.5 ± 1.6
9. BG11 control culture	2	1450	25.2 ± 2.1
10. BG11 control culture	5	1890	27.0 ± 0.8
11. BG 11 control culture	10	1850	26.0 ± 1.2

[a] Unless stated otherwise, the culture volume, incident irradiance, aeration (4.7% CO_2 by volume in humidified air) rate and initial pH values were always 1 L, 330 µE m^{-2} s^{-1}, 1 vvm (without mechanical agitation), and 6, respectively.
[b] No biomass was recovered as the alga did not withstand this salinity level.

treatment in the absence of nitrate and phosphate was sufficient to bring about the above mentioned changes in the lipid profiles.

The above mentioned changes in lipid profiles have important implications for the production of biodiesel from microalgal oils. Although almost all components of an algal oil can be used to make hydrocarbon fuels such as gasoline, diesel and jet fuel, only the neutral lipid fraction is suitable for making the conventional biodiesel. Within the neutral lipid fraction, a high proportion of polyunsaturated fatty acids is not wanted because it adversely impacts the stability of the biodiesel. Unfortunately, compared with most vegetable oils, algal oil extracted from freshly harvested biomass is exceptionally rich in polyunsaturated fatty acids, as the above results demonstrate. A preponderance of polyunsaturated fatty acids in microalgae enables them to remain metabolically active and survive the relatively cold conditions that often occur in their habitats.

Nutrient deprived postharvest treatments beneficially altered the lipid profile of the algal oil. The neutral lipid fraction of the total lipids extracted after 10-day postharvest nutrient deprivation treatment, had a high proportion of the desirable saturated fatty acids at 55.6% and monounsaturated fatty acids at 32.2% (Table 5). In this neutral lipid fraction, the undesired polyunsaturated fatty acids were present only to the extent of 11%, an acceptable level. In contrast, the neutral lipid fraction from the freshly harvested biomass had 44% of the total lipids in the form of polyunsaturated fatty acids and only 22% as unsaturated fatty acids. Unlike in many marine microalgae, *C. minor* lipids did not contain detectable levels of polyunsaturated fatty acids with four or more C=C double bonds, suggesting that this alga is either poorly adapted to surviving extreme cold or it synthesizes highly unsaturated fatty acids only at relatively low temperatures that were not assessed in this work. Highly unsaturated fatty acids have poor oxidative stability and are generally restricted to quite low levels in biodiesel by the various regulatory standards governing its properties.[2]

These results suggest a two-stage process for producing lipids of *C. minor*: a first stage continuous culture that is optimized to attain a maximum growth rate and concentration of the biomass, followed by a second stage that involves suspending the cells in a nitrate- and phosphate-free medium for a few days.

Any process for commercial production of algal oils will necessarily require outdoor culture under sunlight in open ponds[2,27] or photobioreactors.[28–31] The general behavior observed indoors in this work can be expected also in controlled continuous culture of *C. minor* in sunlight. Under natural sunlight outdoors, the biomass and oil productivities are expected to be reduced compared with the data obtained in artificial light indoors. This is mainly because in a given 24 h period, the outdoor culture will receive light for only 50% of the time and some consumption of biomass by respiration will occur during the night. This effect of diurnal cycle may be compensated to a large extent by a high average productivity during daylight because the average level of outdoor irradiance would be much higher than the level used indoors in the present study for characterizing the algal culture. The results suggest that further studies of *C. minor* are worthwhile undertaking in an outdoor production scenario.

Cells of certain algae are destroyed or otherwise damaged by shear stresses generated by eddies in the turbulent flow of a culture vessel.[16,32–34] Algae with relatively large cells are generally more susceptible to turbulence-induced damage.[34] As a picoplankton, *C. minor* has cells that are comparatively small and, therefore, less likely to be sensitive to hydrodynamic shear stresses that are typically encountered in photobioreactors.[34,35]

CONCLUDING REMARKS

The freshwater microalga *Choricystis minor* is shown to produce copious quantities of lipids in a two-step process involving a growth stage followed by a postharvest starvation stage. The continuous culture growth stage of the process maximizes the productivity of the biomass. The subsequent step subjects the live biomass to an environment that is devoid of nitrate and phosphate, to profoundly increase the lipid contents of the cells, enhance the proportion of the neutral lipids in the total lipid fraction and radically alter the fatty acid profile of the neutral lipid fraction to make it more attractive for use in biodiesel production. The postharvest starvation treatment is shown to elevate the total lipid contents in the biomass to as much as 60% by dry weight and enhance the neutral lipid fraction of the total lipids six-fold. Without the postharvest treatment, most of the fatty acids in the neutral lipid fraction were in the undesired polyunsaturated form. After

Table 5. Fatty acid profiles of the various lipid fractions after postharvest nitrate/phosphate (N/P) deprivation treatments, control treatment and nutrient sufficient continuous culture

Fatty acid	2 days N/P deprived (g per 100 g total lipids)	5 days N/P deprived (g per 100 g total lipids)	10 days N/P deprived (g per 100 g total lipids)	10 days control (BG11) (g per 100 g total lipids)	10 days N/P deprived (g per 100 g neutral lipids)	Continuous culture (25 °C, 0.014 h^{-1} dilution rate) (g per 100 g neutral lipids)	(g per 100 g glycolipids)	(g per 100 g polar lipids)
C 16 : 0 Palmitic	34.2	34.3	36.0	36.3	36.5	22.0	13.9	29.7
C17 : 0 Margaric	ND	0.7	0.4	1.1	1.3	ND	7.8	5.1
C 18 : 0 Stearic	10.2	12.2	12.3	7.3	12.2	ND	ND	2.4
C 18 : 1 cis9 Oleic	28.0	31.1	30.7	28.9	32.2	25.3	12.5	13.8
C18 : 1 cis11 Vaccenic	1.0	1.1	0.5	0.8	ND[a]	8.8	8.7	4.5
C18 : 2 cis9,12 Linoleic	12.7	9.8	9.9	10.9	8.3	20.5	36.8	28.6
C 20 : 0 Arachidic	1.7	2.1	1.9	1.6	2.1	ND	ND	ND
C18 : 3n6 cis6,9,12 γ-Linolenic	2.5	2.1	2.1	2.9	1.3	ND	ND	2.7
C 18 : 3 cis9,12,15 Linolenic	4.4	2.1	1.7	3.3	1.4	10.5	20.3	13.2
C20 : 2 cis11,14 Eicosadienoic	0.6	0.9	0.5	1.6	ND	13.0	ND	ND
C 22 : 0 Behenic	4.1	3.2	2.7	3.8	3.5	ND	ND	ND

[a] Not detected.

the treatment, the saturated form of fatty acids predominated. In summary, this study proves that both the lipid level and the lipid profile of algal biomass can be tailored for use in production of fuels, by a suitable combination of growth conditions and postharvest treatment.

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