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BIOENERGY AND BIOFUELS

Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*

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Abstract We tested 10 different *Chlorella* and *Parachlorella* strains under lipid induction growth conditions in autotrophic laboratory cultures. Between tested strains, substantial differences in both biomass and lipid productivity as well as in the final content of lipids were found. The most productive strain (*Chlorella vulgaris* CCALA 256) was subsequently studied in detail. The availability of nitrates and/or phosphates strongly influenced growth and accumulation of lipids in cells by affecting cell division. Nutrient limitation substantially enhanced lipid productivity up to a maximal value of 1.5 gl^{-1} day^{-1}. We also demonstrated the production of lipids through large-scale cultivation of *C. vulgaris* in a thin layer photobioreactor, even under suboptimal conditions. After 8 days of cultivation, maximal lipid productivity was 0.33 gl^{-1} day^{-1}, biomass density was 5.7 gl^{-1} dry weight and total lipid content was more than 30% dry weight. *C. vulgaris* lipids comprise fatty acids with a relatively high degree of saturation compared with canola oil offering a possible alternative to the use of higher plant oils.

Keywords *Chlorella* · Cultivation · Fatty acids · Lipid · Oil · *Parachlorella* · Photobioreactor · Productivity

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Introduction

Increasing energy consumption worldwide, accompanied by fossil fuel depletion, has led to a search for alternate biofuel sources (bioethanol and biodiesel). For biodiesel, biofuel feedstocks include oil seeds such as soy or rapeseed in the USA or EU, respectively (Wright 2006). Unfortunately, biodiesel produced from standard sources cannot realistically satisfy even a small fraction of the existing demand for transport fuels and crops utilized for biofuels compete with the demand for food production. Therefore, efforts have been focused on identifying new organisms, principally microorganisms that are suitable for biodiesel production. Under specific growth conditions, storage lipids can accumulate in a relatively limited number of microorganisms. Among them, microalgae are highly productive and industrially important owing to their ability for photoautotrophic growth (Wood 1988; Chisti 2007, 2008). Under specific conditions (mostly nitrogen deficiency), some microalgae can accumulate substantial amounts of intracellular neutral lipids, predominantly triacylglycerols (TAGs). These are primarily composed of saturated and monounsaturated fatty acids (FAs, Piorreck et al. 1984; Henderson and Sargent 1989; Roessler 1990) that provide more energy upon oxidation than polyunsaturated fatty acids.

Microalgae appear to be one of the few renewable sources of biodiesel that can be produced without compromising production of food, fodder, and other products derived from crops (Chisti 2007, 2008). The ability of microalgae to grow photoautotrophically also makes them far more flexible, since algal cultivation can be directly coupled with carbon dioxide elimination from power plant flue gas (Kosaric and Velikonja 1995; Chisti 2007, 2008) and other sources of waste carbon dioxide in flue gases from burning waste oil (Doucha et al. 2005), incinerators of domestic solid waste (Doušková et al. 2009), etc. The use of microalgae as a potential renewable

liquid fuel source was first proposed several decades ago (Meier 1955). In spite of many partial advances (Sheehan et al. 1998 and references therein), producing microalgal biomass for biodiesel is still more expensive than growing crops. To minimize costs, biodiesel production must rely on freely available sunlight. Growth medium must be inexpensive and waste waters can be utilized advantageously (Feng et al. 2011; Park et al. 2011; Pittman et al. 2011). Carbon dioxide is often available at little or no cost from flue gases (Doucha et al. 2005; Doušková et al. 2009). Efficient harvesting and processing of algal biomass to extract oil is another obstacle. The economics of producing biodiesel from microalgae needs to improve substantially in order to make it competitive with petrodiesel (Chisti 2007, 2008).

The main photobioreactors (PBRs) used for large-scale production of microalgae are open raceway ponds and closed (tubular or flat panel) systems. Although raceways are low cost, they have low biomass potential compared with closed PBRs due to losses of carbon dioxide to atmosphere, contamination, biomass recovery problems, and the lower biomass yield. However, in closed PBR systems, the dissolved oxygen must be removed, which limits the continuous length of a PBR (Chisti 2007, 2008). In addition, both open raceway ponds and closed PBRs share the disadvantage of producing a relatively thick layer of microalgal suspension, thus limiting the light energy available for biomass yield and oil production. In this work, we demonstrate the potential of a thin layer PBR that has been used for decades for commercial algal biomass production with high efficiency of incident sunlight utilization (Doucha et al. 2005; Doucha and Lívanský 1995, 2006, 2009). Recently, several efforts were made to develop an algal culture system that fulfils demands for high growth rate, coupled with high lipid and biomass production. Development of improved PBRs (Chisti 2007) and cultivation techniques has made many advances; nevertheless, the main focus for the large-scale production of algal oils still relies on the selection of high biomass productive strains accompanied by growth conditions directed towards oil production.

Considering the return on investment needed to produce algal oils suitable as a feedstock for industry, as high as possible yields of crude algal oil are necessary. From this point of view, it is important to select algal strains that not only produce high biomass concentrations within relatively short cultivation periods, but are also capable of achieving a high lipid content (Li et al. 2007). However, the accumulation of lipids as an energy-demanding process and the accumulation of biomass at a high growth rate are mutually exclusive characteristics. Therefore, the inherent low growth rate of oleaginous species is the greatest obstacle to the use of microalgae as a biofuel feedstock. In this paper we describe rapidly growing microalgal species, non-oleaginous in principle that is able to overproduce lipids after nutrient depletion caused by a rapid production of biomass in batch cultures. *Chlorella vulgaris*, strain CCALA 256, was selected as a result of screening of 10 different *Chlorella* and *Parachlorella* strains for both high biomass and lipid yield, and high productivity in a photoautotrophic mode of cultivation. *C. vulgaris* CCALA 256 was successfully tested under lipid-inducing conditions both in the laboratory and in a large-scale PBR and demonstrated the highest lipid productivity of any microalgal species reported to date.

Materials and methods

Experimental material

All algal strains were provided by the Culture Collection of Autotrophic Organisms (CCALA) in Třeboň, Czech Republic (http://www.butbn.cas.cz/ccala/index.php). A summary of strains used in these experiments is given in Table 1. In the collection, strains have been maintained on agar slants under an irradiance of ~23 μmol m^{-2} s^{-1} and a temperature of 12–15°C.

Growth and lipid production experiments

To acclimatize the cultures, the selected algal strains were batch pre-cultivated for several days in 50 ml cultivation tubes (diameter 3 cm) filled with sterilized nutrient medium 1/2 SŠ. The concentration of nitrates (NO_3^-) and phosphates (PO_4^{3-}) in the full medium SŠ was 40 and 5 mM, respectively (Zachleder and Šetlík 1982). Continuous irradiance of 500 μmol m^{-2} s^{-1} incident on the tube surface was provided by a panel of light tubes Osram L 36W/830 Lumilux (Osram, Germany); a temperature of 28±0.5°C was maintained using a thermostatic water bath. Cultures were bubbled vigorously with air enriched with 2% CO_2 (*v*/*v*) from a pressure can. Within the exponential-log growth phase, cultures were diluted with fresh medium 1/2 SŠ to obtain a cell density of 1–2·10^6 cells ml^{-1} under the same conditions as described above. To boost the growth rate, the dilution was repeated two to three times each 24 h. The resulting cultures ("starting inocula") were diluted with medium 1/4 SŠ (1/2 SŠ with half nutrient concentrations) to obtain a cell density of ~2·10^6 cells ml^{-1} and were cultivated for 7 days; other conditions remained unchanged. Distilled water was added to the cultures to replenish that lost by evaporation. After 7 days, oil bodies were stained and both dry weight (DW) and lipid content were assessed.

The best lipid producer, *C. vulgaris* strain CCALA 256, was chosen for more detailed investigations of growth and production. The "starting inoculum" was prepared as described above, and subsequently diluted either with 1/4 SŠ or 1/2 SŠ growth medium to obtain a cell density of 1–2·10^6 cells ml^{-1}; cultures were grown for 10 or 21 days (respectively) under identical conditions. Samples were taken at regular intervals; cell number, dry weight, lipid content and NO_3^- and PO_4^{3-}

Table 1 Strains used in experiments

Acronym	Species	Identical strains	Notes, EMBL accession codes
CCALA 259	*C. sorokiniana* Shihira et Krauss	ATCC 22521; UTEX 1230; SAG 211-8k; CCAP 211/8k	Type species, X62441, AY323464, FM205859, FM205834
CCALA 260	*C. sorokiniana* Shihira et Krauss		FM205860, AY323463, EF030563, EF030600, X74001
CCALA 918	*C. sorokiniana* Shihira et Krauss	CAUP H 1986	
CCALA 269	*C. vulgaris* Beijerinck	SAG 211-11b; CCAP 211/11b; UTEX 259	Type species, AY591506, AY591507, X13688, AY876300, FM205832
CCALA 256	*C. vulgaris* Beijerinck	CCAP 211/52; UTEX 2219	EF030601, EF030581, AF360745, EF030564
CCALA 262	*C. vulgaris* Beijerinck	SAG 211-1e	AY591512, AY591513
CCALA 898	*C. vulgaris* Beijerinck	SAG 211-11j; CCAP 211/11j; UTEX 265	AY591497, AY591498
CCALA 251	*P. kessleri* Krienitz et al.	UTEX 398	
CCALA 255	*P. kessleri* Krienitz et al.	SAG 13.80	
CCALA 253	*P. kessleri* Krienitz et al.		

uptakes were determined, and oil bodies were stained. Distilled water was added to the cultures before each sampling to replenish evaporated water. The experiments were carried out in duplicate, statistically significant differences in data were computed by analysis of variance (ANOVA) and *F* test, and non-linear regressions were computed by SigmaPlot 11.0 (Systat Software Inc., USA). This strain was also grown in the thin layer photobioreactor (volume 150 l) located in a temperature-uncontrolled greenhouse in Třeboň, Czech Republic (49°0′21.546″ N, 14°46′21.538″ E) in August 2010. The PBR consisted of two glass lanes (each 6 m long and 1.1 m wide) with an inclination of 1.6%, arranged in a meandering way and connected by a trough. Algal suspension was delivered from a retention tank by pump to the upper rim of the glass plate. The culture was enriched with CO_2 released directly into the cultivation medium immediately behind the pump at a flow rate of 2 lh^{-1}. A precise description of the culture system and schematic was published previously (Doucha et al. 2005). The "starting inoculum" was prepared as described above and run in the PBR containing nonsterile medium 1/4 SŠ for 8 days. Atmospheric conditions during the experiment are shown in Table 2. During these experiments, cell number, dry weight and lipid content were determined and oil bodies were stained at regular intervals.

Growth determination

Cell number was determined using a Bürker counting chamber (Assistent, Germany); at least 400 cells were counted. DW was determined by filtration with pre-weighed Synpor filters (pore diameter 0.6 μm) and dried to a constant weight at 105°C. The relative growth rate (RGR) was calculated from the dry weight by the following equation: $RGR=(DW_t-DW_0)/DW_t\times t$, where DW_t is the dry weight in the given time, DW_0 is the initial dry weight and t is the time of measurement in days. Doublings of cell number were calculated as $\log_2 (CC_t/CC_0)/(t-t_0)$, where CC_t/CC_0 is the ratio of cell number within the given time

Table 2 Atmospheric conditions of the photobioreactor environment during experiment in August 2010

Date	Max irradiance (μmol m^{-2} s^{-1})	Ambient temp. 24 h (°C)	Culture temp. 24 h (°C)	Weather daylong
19 August 2010	880	27–30	23.2–24.6	Sunny intervals
20 August 2010	883	22–35	19.5–30	Sunny intervals–sunny
21 August 2010	892	23–34	22–32.5	Sunny
22 August 2010	960	24–35	22.5–33	Sunny
23 August 2010	788	25–36	21.5–32	Sunny–cloudy
24 August 2010	645	22.5–33	23–31.5	Showers–sunny intervals
25 August 2010	812	24–28	22–26	Sunny–sunny intervals
26 August 2010	759	20–34	20–31.5	Cloudy–sunny intervals
27 August 2010	100	23–30	24–28	Cloudy

interval and $t-t_0$ is the given time interval. Statistically significant differences between treatments were computed by ANOVA and *F* test using Sigma Plot 11.0 (Systat Software Inc., USA).

Extraction and analyses of lipids

Total extractable lipids were assessed using the method of Bligh and Dyer (1959) modified as follows: a mixture of 0.5 ml of PBS (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and 1 ml of glass beads (diameter 0.5 mm) were added to a glass test tube with the sediment (approx. 10 mg ml^{-1}). Cells in the pellet were disintegrated using a high-speed disintegrator for 4 min, interrupted every 1 min by tap water cooling of the test tubes. Three millilitres of extraction solution (MeOH/chloroform, 1:2 *v*/*v*) were added, samples were shaken briefly and lipids were extracted overnight at room temperature. To produce a biphasic layer, 1 ml of distilled water was added and samples were centrifuged (5,000×*g*, 10 min., 20°C). The lower organic phase was drained using a micropipette, and the extraction procedure was repeated with another 2 ml of extraction solution (extraction time 2 h). The pooled organic phases were gathered into a pre-weighed small petri dish, chloroform was evaporated at 50°C and the lipid extract was weighed (Mettler AE 163).

Visualization of intracellular oils

Intracellular oil droplets were stained using the neutral lipid specific dye Nile Red (9-diethylamino-5*H*-benzo{a}phenoxazine-5-one; Eltgroth et al. 2005) with slight modifications. Briefly 1 ml of the cell suspension was fixed with 10 μl of glutaraldehyde and stained with 6 μl of Nile Red stock solution (500 μg ml^{-1} of MeOH). Samples were observed after 5 min using an epifluorescence OLYMPUS BX 60 LM microscope equipped with the filter combination U-MNU2 (360–370 nm excitation and >515 nm barrier filter). Photomicrographs were taken with a digital camera OLYMPUS CAMEDIA C-5050 ZOOM and processed using Adobe Photoshop 7.0.

FA analysis

The lyophilized biomass (100 mg) was extracted using 2–5 ml of dichloromethane to methanol (1:1, *v*/*v*), purified by Supelclean ENVI-Carb (approx. 200 mg), centrifuged and evaporated in a reaction vial. The sample was then saponified in 10% KOH–MeOH at 80°C for 30 min and treated with 14% MeOH, catalyzed by BF3 at 80°C for 30 min. The fatty acid methyl esters (FAME) were then extracted by heptane. Analysis was carried out using a gas chromatograph coupled with a flame ionization detector. Samples of FAME (1 μl) were injected into the column SP-2560 (100 m×0.25 mm×0.2 μm), which was held at 140°C for 5 min, then programmed from 140°C to 240°C (at a rate of 4°C/min). Helium was the carrier gas at a flow rate of 1.2 ml/min. Injector and detector temperatures were 250°C and 260°C, respectively. The identification of FAs was accomplished by comparing peak retention times with those of the corresponding standards (PUFA No. 2—animal source; Supelco, USA).

Analysis of nitrates and phosphates

At regular intervals during batch cultivation, we followed the cellular scavenging of both NO_3^- and PO_4^{3-} from the growth media. The concentrations of NO_3^- and PO_4^{3-} in the full medium SŠ were 40 and 5 mM, respectively (Zachleder and Šetlík 1982). The samples were centrifuged at 1,400×*g* (centrifuge Janetzki T23) at room temperature for 10 min. The supernatant was then carefully drained, and both cell supernatant and pellet were stored separately at −12°C for analyses. The content of both nitrate and phosphate ions in supernatants were determined by flow injection analysis coupled with spectrophotometric detection using a UV-1650PC spectrophotometer (Shimadzu, Japan). Nitrates were reduced to nitrites on a Cd column and then detected (wavelength 540 nm). Phosphates were converted to molybdenphosphoric acid using ammonium molybdenane at low pH. Afterwards it was reduced using H_2SO_4 with $SnCl_2$ and detected at 690 nm.

Results

Comparison of lipid production

We cultured 10 *Chlorella* and *Parachlorella* strains for 7 days in nutrient-depleted growth medium as described in the "Material and methods" section. This procedure promoted rapid growth of cultures followed by intracellular lipid accumulation, and permitted a fast and reproducible comparative assessment of productivities between various algal strains. Biomass, total lipid productivity and the final content of lipids are shown in Table 3. We found large differences in all production characteristics. Generally, *Chlorella sorokiniana* showed the lowest biomass productivity (0.409±0.021 gl^{-1} day^{-1}), lipid productivity (0.161±0.014 gl^{-1} day^{-1}; strain CCALA 259) and also the lowest lipid content (22.97±1.22% of DW; strain CCALA 918). The best growing alga was *Parachlorella kessleri* (strain CCALA 253) with biomass productivity of 1,291±0.032 gl^{-1} day^{-1}, and the best lipid producer was *C. vulgaris* (strain CCALA 256) having the highest lipid productivity of 0.604±0.001 gl^{-1} day^{-1}, gaining 57.25±0.17% of DW (Table 3). Since the latter strain also showed relatively high biomass

Table 3 Comparison of biomass and total extractable lipid production in several *Chlorella* and *Parachlorella* species batch cultivated in the laboratory under nutrient depletion conditions for 7 days

Strain	Overall biomass productivity (g l^{-1} day^{-1})	Overall lipid productivity (g l^{-1} day^{-1})	Lipid content (% of DW)
CCALA 259 *C. sorokiniana*	**0.409±0.021**	**0.161±0.014**	39.54±4.04
CCALA 918 *C. sorokiniana*	0.905±0.012	0.208±0.011	**22.97±1.22**
CCALA 260 *C. sorokiniana*	1.026±0.016	0.424±0.007	41.37±0.94
CCALA 255 *P. kessleri*	0.936±0.022	0.478±0.004	51.06±1.27
CCALA 251 *P. kessleri*	1.134±0.015	0.576±0.003	50.80±0.72
CCALA 253 *P. kessleri*	**1.291±0.032**	0.526±0.027	40.76±2.34
CCALA 262 *C. vulgaris*	0.865±0.028	0.434±0.001	50.18±1.62
CCALA 269 *C. vulgaris*	0.981±0.012	0.469±0.015	47.86±1.64
CCALA 898 *C. vulgaris*	0.721±0.000	0.346±0.009	47.92±1.19
CCALA 256 *C. vulgaris*	1.054±0.003	**0.604±0.001**	**57.25±0.17**

Mean values and SD are given; limit values are in bold

productivity (1.054±0.003 gl^{-1} day^{-1}), we chose it as the model strain for subsequent experiments.

Laboratory growth experiments

The experimental strain, *C. vulgaris* CCALA 256, was batch cultivated at constant light, temperature and CO_2 supply; two different concentrations of growth medium (1/2 SŠ and 1/4 SŠ) were tested. Growth was determined as both DW of biomass and cell number; the content of lipids was also assessed. Detailed growth characteristics of CCALA 256 are shown in Fig. 1. The "starting inoculum" of the alga possessed an extraordinarily high RGR within the first day of batch cultivation (always >0.9 day^{-1}), which subsequently decreased exponentially to a value of about 0.1 day^{-1}. The culture dynamics for both media were almost identical (Fig. 1a, c). When 1/2 SŠ medium was used, vigorous cell multiplication (exponential growth phase) was conspicuous within the first 5 days of growth; maximal and average doublings were 3.410±0.140 and 1.741±0.033 day^{-1}, respectively. After 5 days of cultivation, the cell number reached its maximum and then remained unchanged until the end of the experiment (Fig. 1b). A similar time course was measured in 1/4 SŠ medium with maximum growth within 2–3 days and approx. half the cell number (Fig. 1d). The rapid increase in cell number during the exponential growth phase was accompanied by a steep decrease in both main nutrient ions (NO_3^- and PO_4^{3-}) in the culture medium for both media concentrations (Fig. 1b, d). The cell number and NO_3^- and/or PO_4^{3-} contents showed strong negative correlation coefficients of −0.933 and −0.945 for NO_3^- and PO_4^{3-}, respectively. Moreover, when 1/4 SŠ medium was used, the cell number reached maximal values when the concentrations of both nitrates and phosphates in the medium were close to zero (Fig. 1d). Depletion of nutrients resulted in cessation of cell division, while both growth of biomass and lipid accumulation proceeded. When 1/2 SŠ medium was used, both biomass DW and lipid content reached maximal values (12 and 6 gl^{-1}, respectively) after approx. 10 days and then remained unchanged till the end of the cultivation (Fig. 1a). In 1/4 SŠ medium, the culture dynamics were similar with lower maximal values for DW and lipid content (7.5 and 4.2 gl^{-1}, respectively) attained after approx. 6 days (Fig. 1c). However, the relative content of lipid was slightly, although not significantly ($P<0.12$), higher in 1/4 SŠ than in 1/2 SŠ medium (Fig. 1a, c). This cannot be explained only by a lower biomass attained in diluted medium, because the cellular content of lipids was significantly ($P<0.01$) higher in 1/4 SŠ medium: 15 pg $cell^{-1}$, compared to 11 pg $cell^{-1}$ in 1/2 SŠ medium (Fig. 1b, d); the cell size remained unchanged. Moreover, all growth and lipid maxima were attained sooner in 1/4 SŠ than in 1/2 SŠ medium (Fig. 1a–d). The time shift between cell number, and growth and/or lipid content for entering stationary phase demonstrates the typical biphasic growth of asynchronous cultures. This biphasic growth is defined by vigorous multiplication of cell number within the first phase of cultivation, when biomass increments were much lower. After reaching a steady state for cell number, cells increased their biomass. Strongly positive correlations between DW and lipid content were found in both 1/2 SŠ and 1/4 SŠ media; the correlation coefficients (r) were 0.921 and 0.931, respectively. However, the relationship between DW and lipid content was not linear, but showed hyperbolic regression (Fig. 2) confirming the biphasic growth data. This indicates the substantial de novo synthesis and accumulation of lipids during biomass production. Even during rapid cell division (exponential growth phase), some lipids were synthesized and accumulated (Figs. 1a–d and 4b, c). As cell division ceased and the biomass maximum was attained (after 2–4 days of cultivation), lipid productivity started to accelerate, reached its maximum and then gradually decreased. When the 1/2 SŠ medium was used, the attainment of both DW and lipid productivity maxima was delayed compared to the 1/4 SŠ medium. Maximal lipid productivities were 0.905±0.044 and 1.425±0.135 gl^{-1} day^{-1} in 1/2 SŠ and 1/4 SŠ medium, respectively. Maximal biomass

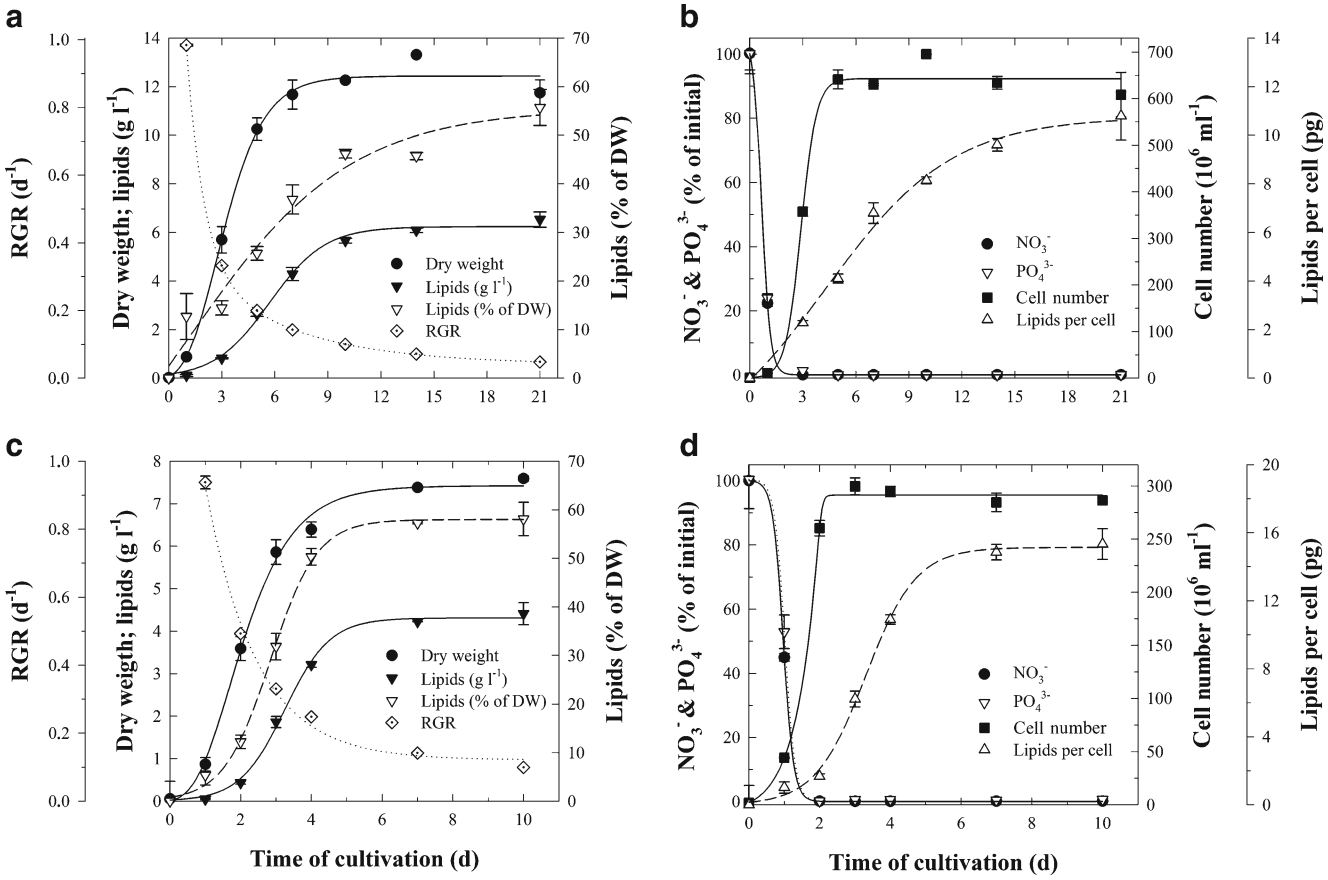

Fig. 1 Growth characteristics of CCALA 256 *C. vulgaris* cultivated either in 1/2 SŠ (**a**, **b**) or in 1/4 SŠ medium (**c**, **d**). Dry weight, content of lipids and relative growth rate (*RGR*) are shown in **a** and **c**; nitrate and phosphate uptake, cell number and intracellular content of lipids are shown in **b** and **d**

productivities were 2.412±0.274 or 2.722±0.322 gl^{-1} day^{-1} in 1/2 SŠ and 1/4 SŠ medium, respectively, with no significant difference between them (Fig. 3). The time course of lipid accumulation in algal cells cultivated in 1/4 SŠ medium is shown in Fig. 4. Within the first days of cultivation, newly synthesized oils were seen as numerous small droplets (Fig. 4b–d), which fused gradually into two to four larger globules (Fig. 4e), one large and some additional globules (Fig. 4f, g) and after 10 days only one huge oil globule taking up most of the individual cell volume (Fig. 4h). At the end of this experiment, cultures were left to settle either at low light (5 μmol m^{-2} s^{-1}) or in darkness, both at room temperature (20–23°C), and after 23 days, lipids were assessed again. No changes in either culture were observable using fluorescence visualization of oil bodies (Fig. 4i). There was only a slight decrease in lipid content: 4.11±0.02 or 4.10±0.10 gl^{-1} at low light or in darkness, respectively. The relative lipid content was nevertheless higher: 59.26±1.56% of DW or 63.79±4.07% of DW at low light or in darkness, respectively. The relative lipid content increased due to the decrease in total biomass DW caused probably by respiration processes involving other storage compounds.

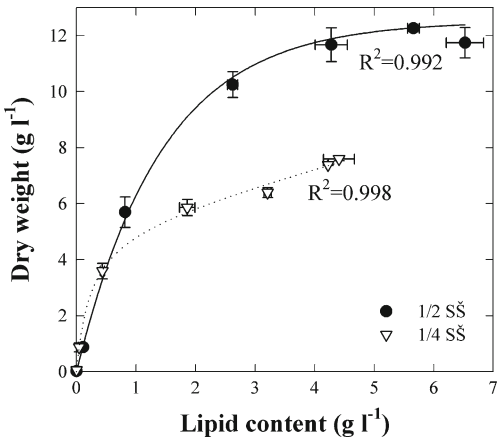

Fig. 2 Relationship between dry weight and content of lipids in CCALA 256 *C. vulgaris* cultivated either in 1/2 SŠ or in 1/4 SŠ medium

Large-scale cultivation trial

A culture of *C. vulgaris* CCALA 256 was batch cultivated for 8 days in 1/4 SŠ medium in a thin layer PBR (Fig. 5). Most growth characteristics were lower compared to laboratory

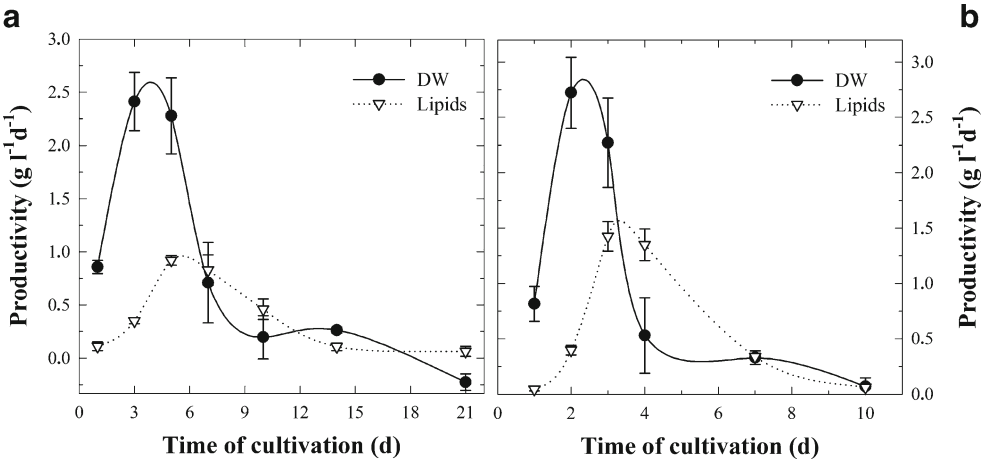

Fig. 3 Both dry weight and lipid productivities of CCALA 256 *C. vulgaris* cultivated either in 1/2 SŠ (**a**) or in 1/4 SŠ medium (**b**). Higher medium concentration delayed achievement of maximum of both dry weight and lipid productivity. Maximal lipid productivity was about 50% higher in the lower medium concentration, whereas maximal dry weight productivity was only slightly influenced

experiments in bubble tubes, as seen from Fig. 6 and compared to Fig. 1c, d. The RGR within the first day of cultivation was significantly ($P<0.01$) lower (only 0.7 day^{-1}), however the time course was similar to laboratory experiments (RGR over 0.9 day^{-1}). The maximal cell number attained was, interestingly, the same as in laboratory cultivation (~300× 10^6 cells ml^{-1}); however, it reached its maximum much later, not before the seventh day of cultivation. Compared to laboratory experiments, there was only a slightly slower decrease in both main nutrients ions (NO_3^- and PO_4^{3-}) in the culture medium. Neither biomass DW nor lipid content reached a stationary phase after 8 days of cultivation; values attained were 5.7 and 1.7 gl^{-1}, respectively. Similarly, the relative content of lipids exceeded 30% of DW and its time course was almost linear. These results indicate a potentially continuing accumulation of lipids during cultivation in a thin layer photobioreactor. Both biomass and lipid productivities were substantially lower compared to laboratory values. Maximal biomass

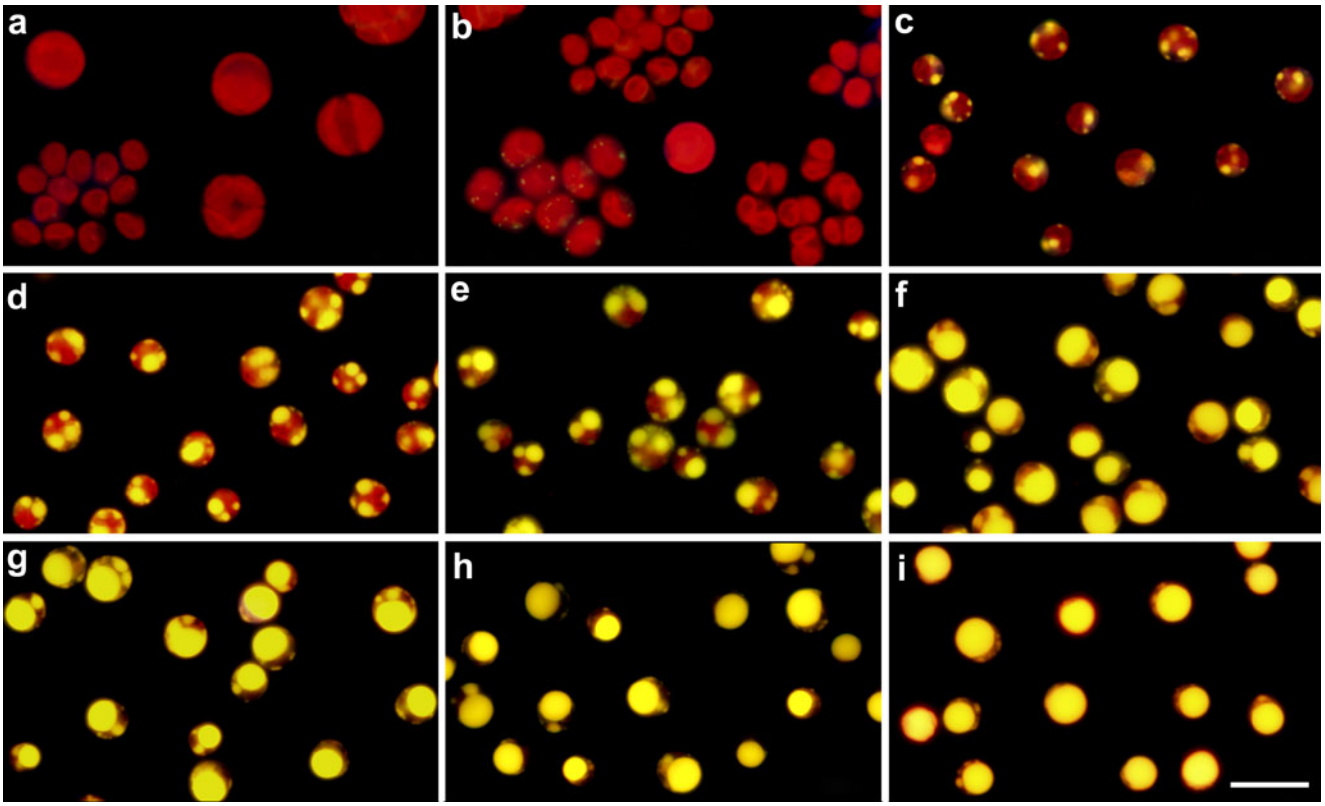

Fig. 4 Time course of neutral lipids accumulation during batch cultivation of the strain CCALA 256 *C. vulgaris*. Fluorescence images; neutral lipids were stained using Nile Red (*yellow-crimson*); autofluorescence of chloroplasts is seen as *red*. **a** Start of cultivation (time 0); **b**–**h** cultures after 1, 2, 3, 4, 7, 8 and 10 days, respectively; **i** culture as in **h** allowed to settle for following 23 days. *Scale bar* 10 μm

Fig. 5 Thin layer PBR in a greenhouse of the Institute of Botany, Třeboň, August 2010

productivity was less than half (1.259±0.029 gl^{-1} day^{-1}) and was attained in more than twice the time (5 days) than for laboratory experiments. Maximal lipid productivity was about 20% (0.326±0.010 gl^{-1} day^{-1}) of laboratory cultures, and the broad peak was attained in twice the time (after ~7 days, Fig. 7). Thus, the optimal time for harvesting thin layer PBR-grown oil-rich *Chlorella* cultures under the given conditions was after 1 week of growth.

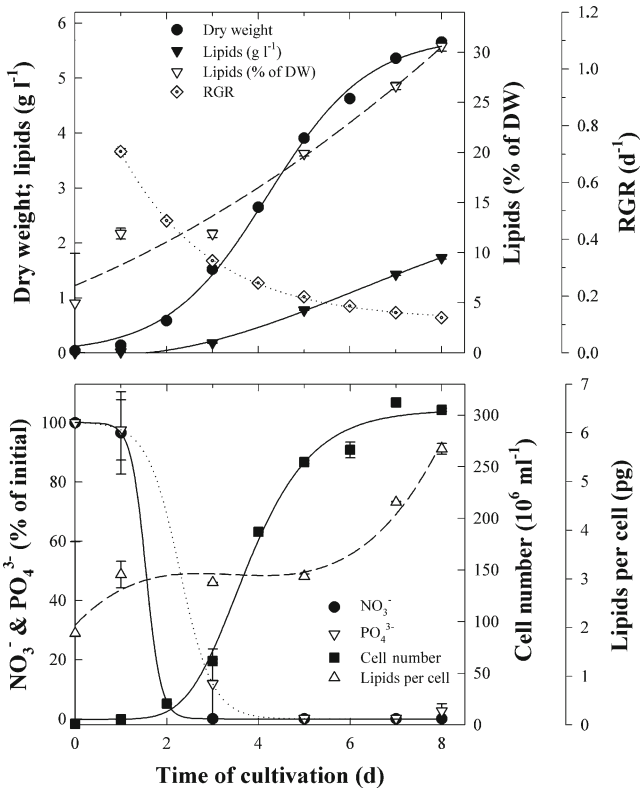

Fig. 6 Growth characteristics of CCALA 256 *C. vulgaris* cultivated in 1/4 SŠ medium in the thin layer PBR. *Upper graph* dry weight, content of lipids and relative growth rate (*RGR*); *lower graph* nitrate and phosphate uptake, cell number and intracellular lipid content

FA composition

The fatty acid composition of a *C. vulgaris* CCALA 256 culture after 8 days of cultivation in the thin layer PBR was analysed. As seen from Table 4, the predominant fraction were polyunsaturated FAs (linoleic and α-linolenic acid)—43.93% of the total FA content, followed by saturated FAs (palmitic, stearic and myristic acid)—29.10% of the total FA content and monounsaturated FAs (oleic and palmitoleic acid)—19.78% of the total FA content. The ratio of total unsaturated to saturated FAs was 2.11.

Discussion

Microalgae-based oil production is currently not economic due to high costs associated with cultivation, harvesting and processing the microalgal biomass. To overcome this limitation, a selection of algal strains possessing high lipid productivity is the first step and the prerequisite for any large-scale oil production processes. The lipid yield per unit area is a more important production characteristic than high lipid content in cells, although the latter is also desirable as it improves oil extraction efficiency (Rodolfi et al. 2009). To select high lipid-producing strains, the best growing microalgae should be tested primarily for lipid accumulation. The genus *Chlorella* belongs to those promising microalgae. *Chlorella*, one of the most investigated green microalgae, is known to be a rapidly growing species and in some strains, substantial lipid production has been documented many times (see below). Sixty years ago, *Chlorella pyrenoidosa* was laboratory cultivated, yielding 1.3–5.5 gl^{-1} of biomass DW and the basic elements content and *R* value (degree of reduction) were determined. Using these data, extraordinary high lipid contents (77–80% of DW) were calculated (Milner 1948; Spoehr and Milner 1949). However, these data have not been confirmed; maximal lipid content, determined gravimetrically following organic solvent extraction, was 40–60% of biomass DW (Richardson et al. 1969; Shifrin and Chisholm 1981; Illman et al. 2000; Ramazanov and Ramazanov 2006; Liu et al. 2008; Chen et al. 2011; Tang et al. 2011). Similarly, we found the maximal lipid content to be between 23% and 60% of DW depending on the strain used.

A large number of papers reporting recent achievements in the improvement of microalgal oil production have been published (Table 5). These works include various approaches to enhance lipid productivity, select best producer strains, nutrient limitations, autotrophic/mixotrophic/heterotrophic growth, improvement of cultivation techniques, etc. mostly under controlled laboratory conditions. Although an astonishing biomass yield of 51.2 gl^{-1} and a lipid content 57.8% of DW in *Chlorella protothecoides* (Xiong et al. 2008) or very high lipid productivity of 1.38 gl^{-1} day^{-1} in *Chlorella*

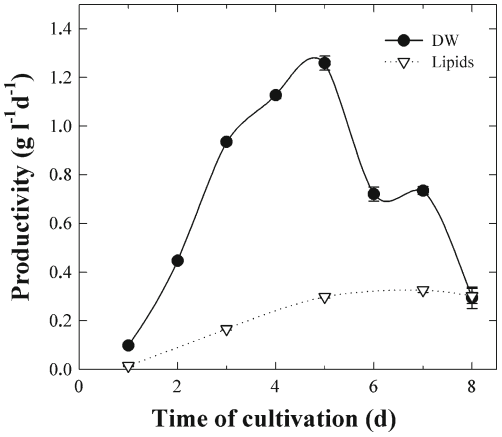

Fig. 7 Both dry weight and lipid productivities of CCALA 256 *C. vulgaris* cultivated in 1/4 SŠ medium in the thin layer PBR

zofingiensis (Liu et al. 2010) were achieved recently by glucose feeding of a heterotrophic cultures, the elevated costs due to the use of organic compounds restrict this approach to large-scale industrial use (Chisti 2007). It is largely believed that for large-scale production of oils, the major source of carbon for lipid synthesis has to be fixed photoautotrophically.

Here, we showed very high (maximal values 1–1.5 $g l^{-1}$ day^{-1}) and stable (not lower than 0.5 $g l^{-1}$ day^{-1} for 5 days of cultivation) lipid productivity and content (up to 60% of biomass DW) and high biomass density (7–12 $g l^{-1}$) in *C. vulgaris* CCALA 256 under laboratory conditions (Figs. 1 and 3). Similarly, the extraordinarily high lipid content (56.6% of biomass DW) was achieved during autotrophic cultivation using 1.2 10^{-5} mol l^{-1} of the Fe^{3+}-treated marine species, *C. vulgaris* C7, after re-supplementation of growth medium (Liu et al. 2008). This lipid content is comparable to our results; however, Liu et al. (2008) did not report either lipid productivity or final DW of cultures; the cell density of $2 \cdot 10^7$ cells ml^{-1} does not indicate very high biomass (compared to 30–$70 \cdot 10^7$ cells ml^{-1} reported in this study). Most recently, other promising photoautotrophic production systems were reported, using *C. zofingiensis* ASU 2 with a maximal lipid productivity of 0.312 $g l^{-1}$ day^{-1}, a lipid content of 48% of biomass DW and biomass density of 6.5 $g l^{-1}$ (Chen et al. 2011), *Pseudochlorococcum* sp. LARB 1 with maximal lipid productivity of 0.35 $g l^{-1}$ day^{-1}, lipid content 52.1% of biomass DW and biomass density 5.8 $g l^{-1}$ (Li et al. 2011), or *Nannochloropsis* sp. with the maximal lipid productivity of 0.41 $g l^{-1}$ day^{-1}, lipid content 47% of biomass DW and biomass density of 8.1 $g l^{-1}$ (Pal et al. 2011). All those values were, however, markedly lower than described for *C. vulgaris* CCALA 256.

We have demonstrated the predominant FA composition of lipids in *C. vulgaris* cultivated for 8 days in a large-scale PBR. In the previous work on the same strain as CCALA 256 (=UTEX 2241), it was shown that cultivation conditions such as light intensity, photoperiod or nitrogen availability strongly influenced the fatty acid composition of lipids. When compared with values obtained from experiments with higher irradiances, nitrogen deficiency or 12/12 (light/dark) photoperiod (Tang et al. 2011), lipid composition was found to be similar to that shown in our data. The only substantial differences were C 18:0 and C 18:2, whose relative contents we found were substantially enhanced (7.56% and 22.31% of total FA, respectively) compared to 0–1.3% and 8.4–16.5% of total FA, respectively, found by Tang et al. (2011), and C 16:0, where the relative contents were opposite: 23.4–29.7% of total FA (Tang et al. 2011) and 20.51% (this work). These differences were caused by different cultivation conditions in the laboratory and PBR. Moreover, we determined a relatively low level of unsaturation of *C. vulgaris* CCALA 256 lipids; the ratio of total unsaturated to saturated FAs was only 2.11. Previously, higher values, 2.23–3.17, were found in *Chlorella minutissima* UTEX 2241 (=CCALA 256, Tang et al. 2011), 2.3 in *C. zofingiensis* ATCC 30412 (Liu et al. 2011) or 2.54 in *C. vulgaris* CCTCC M 209256 (Zheng et al. 2011), all cultivated photoautotrophically. For comparison, in canola oil, this ratio is over 13 (Stauffer 2005). Thus, the FA composition of *C. vulgaris* lipids shows a high degree of saturation, making microalgal oil a potential substitute of oils produced from some crops, f.e. for biodiesel production.

We also found an extraordinarily high RGR of *C. vulgaris* CCALA 256, 0.17–0.98 day^{-1} within the first week of cultivation, the first day being maximal and then exponentially decreasing (Fig. 1a, c). The biomass yield of *C. vulgaris* CCALA 256 was up to 12 $g l^{-1}$ after 7 days of cultivation (Fig. 1a). Higher values using autotrophic cultivation (up to 14 $g l^{-1}$ after 7 days) were reported in *C. vulgaris* supplied with flue gas containing 10–13% of CO_2 and under a light intensity of 1,150 μE m^{-2} s^{-1} (Doušková et al. 2009), which

Table 4 Fatty acid composition of the biomass of *C. vulgaris* CCALA 256 grown in the thin layer PBR for 8 days

FA	Myristic	Palmitic	Stearic	Palmitoleic	Oleic	Linoleic	α-Linolenic	Saturated FA	Unsaturated FA
Scheme	C14:0	C16:0	C18:0	C16:1	C18:1n9c	C18:2n6c	C18:3n3	Total	Total
% of total	1.03	20.51	7.56	0.52	19.26	22.31	21.62	30.26	63.88

Only FAs of amount higher than 0.5% of the total FA content are shown

Table 5 Overview of relevant recent achievements in microalgal oil production

Microalgal strain	Cultivation mode	Carbon source	Max lipid productivity ($g\ l^{-1}\ day^{-1}$)	Max lipid content (% of DW)	Max DW biomass ($g\ l^{-1}$)	References
C. vulgaris CCALA 256 (=*C. minutissima* UTEX 2219)	Laboratory, tubes 50 ml[a]	CO_2	1.425±0.135	58.1±3.5	7.59±0.06	This paper
	Laboratory, tubes 50 ml[a]		0.905±0.044	55.7±3.7	11.74±0.54	
	Thin layer PBR, 150 l		0.326±0.010	30.6±0.5	5.66±0.04	
C. minutissima UTEX 2219	Laboratory, flasks 650 ml	CO_2	<0.155	50	1.24	Tang et al. (2011)
C. vulgaris FACHB1068	Laboratory, 2 l	CO_2	0.147	42	1.7	Feng et al. (2011)
C. vulgaris C7	Laboratory, flasks 200 ml	CO_2	–	56.6	$20{\cdot}10^6$ cells ml^{-1}	Liu et al. (2008)
C. zofingiensis ASU 2	Laboratory, tubes 300 ml	CO_2	0.312	48	6.5	Chen et al. (2011)
C. protothecoides UTEX 25	Laboratory, tubes 200 ml	CO_2	0.077	16.8	6.3	Sirisansaneeyakul et al. (2011)
Chlorella sp. TISTR 8990	Laboratory bioreactor 1.6 l	CO_2	0.056	30	2.6	
Pseudochlorococcum sp. LARB 1	Laboratory, PBR 1.2 l	CO_2	0.35	52.1	5.8	Li et al. (2011)
Scenedesmus obliquus CNW-N	Laboratory, vessel 1 l	CO_2	0.079	38.9	3.5	Ho et al. (2010)
Nannochloropsis sp.	Laboratory, columns 1 l	CO_2	0.41	47	8.1	Pal et al. (2011)
Nannochloropsis sp. F&M-M24	Outdoor PBR, 110 l	CO_2	>0.250	60	<1.5	Rodolfi et al. (2009)
C. protothecoides UTEX 249	Laboratory, flasks 100 ml	Glucose/glycerol	0.25±0.03	29.45±0.84	4.76±1.50	Heredia-Arroyo et al. (2010)
C. protothecoides UTEX 255	Laboratory, flasks 150 ml	Glucose	0.85	50.5	13.1	Shen et al. (2010)
C. protothecoides UTEX	Laboratory bioreactor 5 l	Glucose	–	57.8	51.2	Xiong et al. (2008)
C. protothecoides UTEX	Laboratory bioreactors	Glucose	<0.953	48.7	15.5	Li et al. (2007)
C. sorokiniana CCTCC M209220	Laboratory	Glucose	0.056±0.006	52	1.3	Wan et al. (2011)
C. pyrenoidosa	Laboratory, flasks 250 ml	RSH	0.62±0.01	56.3±1.4	2.83±0.04	Li et al. (2011)
C. zofingiensis ATCC 30412	Laboratory, flasks 50 ml	Glucose	0.263	51.1	9.7	Liu et al. (2011)
C. zofingiensis ATCC 30412	Laboratory, fermenter 3.7 l	Glucose	1.38	52	43	Liu et al. (2010)

RSH rice straw hydrolysate

[a] Different amount of nutrients

was more than twice the irradiance used in our experiments. When using 2% CO_2 as a control, lower values (8–10 $g l^{-1}$ within 7 days) were reported (Doušková et al. 2009). The efficient utilization of flue gas from a municipal waste incinerator proves that the linkage between bioremediation of CO_2 and production of algal biomass is achievable and biomass yield may even be increased further. After the first 4 days of cultivation of *C. vulgaris* CCALA 256 in complete growth medium, cell number attained its maximal value $0.6–0.7{\cdot}10^9$ cells ml^{-1} (Fig. 1b). A similar cell concentration was attained in a starchless mutant of *C. pyrenoidosa* (Ramazanov and Ramazanov 2006). However, in this case, *C. pyrenoidosa* grew more slowly, because these values were not achieved before the eighth day of cultivation.

C. vulgaris CCALA 256 was batch cultivated under optimal conditions for growth as specified previously (Lukavský 1982; Kvíderová and Lukavský 2005). It has long been recognized that environmental conditions play a major role in determining the quantity and quality of lipids produced by microalgae (Roessler 1990). Triacylglycerols appear to be synthesized as an early response to growth under conditions when energy input exceeds the cellular capacity for energy utilization (cell growth and division). This situation can result from a variety of adverse environmental conditions (Roessler 1990; Mock and Kroon 2002). The dogma that maximum yields of lipids or hydrocarbons are achieved during the stationary growth phase was confirmed by our results (Fig. 1). Nitrogen availability has been shown by many investigators to influence greatly the lipid content of various microalgae. The first report that nitrogen deficiency induces an increase in lipid content of *C. pyrenoidosa* was by Spoehr and Milner (1949). To the present, many other reports of this phenomenon have been recorded, so nitrogen deficiency is believed to be the general tool to enhance lipid production in algae. Phosphorus deficiency has been less investigated, but a marked increase in cellular lipid content has also been found to be a response to phosphorus depletion (Khozin-Goldberg and Cohen 2006). Phosphorus limitation resulted in increased lipid content in *Phaeodactylum tricornutum*, *Chaetoceros* sp., *Pavlova lutheri* and *Isochrysis galbana*, whereas, in contrast, lipid levels decreased in *Nannochloris atomus* (Reitan et al. 1994). In this work, we found a steep decrease in both nitrates

and phosphates in the culture medium, accompanied by a cessation of cell division within the first 2–3 days of cultivation (Fig. 1b, d). When complete growth medium was used, cell division continued for 2–3 days after nutrient depletion, possibly due to nutrient reserves in the cells. This indicates that the availability of nitrates and/or phosphates is a prerequisite for cell division to be performed. Similar rapid removal of almost all nitrogen and phosphorus (within 2 days) was found in *C. vulgaris* cultures in artificial wastewater medium (Feng et al. 2011). Similarly, the depletion of nitrates was found to be the major signal for cessation of cytokinesis in *Trachydiscus minutus* (Přibyl et al. 2012). It seems that availability of one (or both) of these ions is required for cell division and their depletion leads to its cessation. Under high-energy input conditions, nutrient limited non-dividing cells are forced to channel excess light energy and carbon input into intracellular high-energy compounds—lipids. Thus, our results indicate an indirect effect of nitrogen or phosphorus deficiency on enhanced lipid production in *Chlorella*. The efficient nutrient removal capacity of fast-growing microalgal cultures can thus be exploited in wastewater treatment with complementary production of microalgal biofuels (Park et al. 2011; Pittman et al. 2011).

This lipid production system proved under laboratory conditions was also tested using large-scale cultivation in an inclined thin layer PBR, used successfully for more than 20 years for synthesis of algal biomass (Doucha et al. 2005; Doucha and Lívanský 2006). Although environmental conditions, especially irradiance, were suboptimal (see Table 2), we achieved maximal lipid productivity of 0.33 $g l^{-1}$ day^{-1} of lipids, up to 31% of biomass DW and biomass density of up to 5.7 $g l^{-1}$ (Figs. 6 and 7) within 8 days of cultivation. Moreover, both biomass and lipid content show potential for further increases (Fig. 6) using longer cultivation times. Although lower than in the laboratory, lipid productivity was comparable with other production systems operating under more favourable climatic conditions. Such a promising large-scale production system was reported previously: the oleaginous microalgal strain *Nannochloropsis* sp. F&M-M24 was selected and subsequently tested in an outdoor PBR of the original design. The high lipid content of this alga (over 30% of biomass DW) was increased by nitrogen deficiency to 60% within 7 days cultivation (Rodolfi et al. 2009). This feature seems to be typical for some eustigmatophycean microalgae, which employ oil as the main storage compound, in contrast to the green microalga *Chlorella*. The lipid productivity of *Nannochloropsis* sp. F&M-M24 was found to be about 0.25 $g l^{-1}$ day^{-1} after 3 days of cultivation in an outdoor PBR and then decreased markedly (Rodolfi et al. 2009). This value is only slightly lower than ours, but it was attained after a shorter time than in our case. Unfortunately, the maximal biomass density, which represents an important production characteristic, was not reported in this study (Rodolfi et al. 2009).

As we have shown in this work, the laboratory batch cultivation of *C. vulgaris* CCALA 256 under optimal growth conditions resulted in a high lipid content (over 50–60% within 5–14 days), extraordinary high and stable lipid productivity (0.5–1.5 $g l^{-1}$ day^{-1} within 3–5 days), and relatively high biomass density (7–12 $g l^{-1}$ within 7 days), depending on nutrient availability. These characteristics, together with high RGR (maximal 0.9 day^{-1}) make this alga an interesting material for energy-yielding biotechnology, as confirmed by successful testing in a large-scale PBR. Such high lipid production was achieved by an appropriate combination of algal strain and cultivation conditions. To enhance lipid production, it was not necessary to invoke nutrient deficiency, which usually reduces biomass yields and increases costs during large-scale cultivation. A suitable yield of lipid in relatively dense algal biomass was attained by "natural depletion" of nutrients during the first phase of cultivation. The present paper provides evidence that a search for new strains capable of high lipid productivity together with further research on flexible cultivation conditions seems to be the key approach to the economically sustainable industrial application of algae for biofuel production or for any other outputs requiring high levels of lipids.

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