Journal of Bioscience and Bioengineering
VOL. 120 No. 2, 205–209, 2015

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Characterization of lipid and fatty acids composition of *Chlorella zofingiensis* in response to nitrogen starvation

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Received 20 August 2014; accepted 18 December 2014
Available online 14 March 2015

Cellular biochemical composition of the microalga *Chlorella zofingiensis* was studied under favorable and nitrogen starvation conditions, with special emphasis on lipid classes and fatty acids distribution. When algal cells were grown in nitrogen-free medium (N stress), the increase in the contents of lipid and carbohydrate while a decrease in protein content was detected. Glycolipids were the major lipid fraction (50.7% of total lipids) under control condition, while neutral lipids increased to be predominant (86.7% of total lipids) under N stress condition. Triacylglycerol (TAG) content in N stressed cells was 27.3% dw, which was over three times higher than that obtained under control condition. Within neutral lipids fraction, monounsaturated fatty acids (MUFA) were the main group (40.6%) upon N stress, in which oleic acid was the most representative fatty acids (34.5%). Contrarily, glycolipids and phospholipids showed a higher percentage of polyunsaturated fatty acids (PUFA). Lipid quality assessment indicated the potential of this alga as a biodiesel feedstock when its neutral lipids were a principal lipid fraction. The results demonstrate that the neutral lipids content is key to determine the suitability of the microalga for biodiesel, and the stress cultivation is essential for lipid quality.

[**Key words:** *Chlorella zofingiensis*; Nitrogen starvation; Lipid; Fatty acids; Biodiesel]

Over-consumption of fossil fuels has led to energy crisis and environmental problems. In light of this, sustainable biofuels, especially biodiesel, have attracted much attention in recent years. Biodiesel is a mixture of fatty acid methyl esters (FAME) which is conventionally produced by transesterification of vegetable oils or animal fats (1). Currently, microalgae have been recognized as a promising feedstock for biodiesel production due to several advantages, such as high photosynthetic efficiency, rapid growth rate, and high lipid content (2,3).

Chlorella has long been commercially applied for human food, animal feed, and bioactive compounds. In recent years, there were increasing reports demonstrating the potential of *Chlorella* for biodiesel production due to their high lipid productivity and environmental adaptation (4–6). Generally, algal cell growth and metabolism are highly influenced by environmental conditions and can be physiologically manipulated. It has been reported that nutrient limitation or starvation could significantly increase lipid accumulation of many species of *Chlorella* (7–9). As far as we are concerned, not only is the lipid quantity affected by stress condition in microalgae, but variations of lipid quality are occurred as well. However, much of the information about lipid and fatty acid composition corresponds to the total lipids. Few studies focused on the lipid composition as well as the distribution of fatty acids in individual lipid class in *Chlorella* species (1). The limited information hinders the comprehensive understanding of microalgal lipid metabolism and evaluation of the microalgal lipid suitability for biodiesel (10).

Our previous studies have shown that the green microalga *Chlorella zofingiensis* could be used as a cell factory for algal oil under nitrogen starvation (11) and utilize various wastewater for growth (12,13), thus being promising in commercial applications. Other studies have also reported the high lipid content and biomass concentration of *C. zofingiensis* in the past few years (4,14), although this alga was found to produce astaxanthin initially (15,16). Liu et al. (1,4) intensively compared lipid classes and fatty acids composition of *C. zofingiensis* under photoautotrophic and heterotrophic conditions, and suggested that heterotrophic *C. zofingiensis* was more feasible for biodiesel production. However, autotrophic algal cells in their studies were cultivated under normal growth condition rather than environmental stress condition, which cannot thoroughly reflect the lipid characteristics of autotrophic cells.

Based on the aforementioned information, it is essential to study neutral lipids accumulation in microalgae under photoautotrophic conditions. Though the total lipids are an important indicator for selecting oleaginous species, neutral lipids and their fatty acids profiles provide a more specific signal of suitable substrate for biodiesel production. Up to now, a detailed characterization of lipid was not carried out for *Chlorella* grown under nitrogen starvation condition. Thus, in this study, *C. zofingiensis* was tested to further investigate the variations of cellular biochemical composition, especially lipid classes and the distribution of fatty acids in the lipid pool under nitrogen starvation (stress) condition in comparison to favorable growth (control) condition. The results from this study would be helpful for enhancing our understanding of lipid biosynthesis and evaluating the lipid suitability for biodiesel production.

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.12.018

MATERIALS AND METHODS

Algal strain and culture conditions *C. zofingiensis* was obtained from the Microalgae Culture Collection in Guangzhou Institute of Energy Conversion (Guangzhou, China) and maintained in BG11 medium. The culture conditions were performed according to our previous study (11). For the analysis of biochemical components, the cultures grown in N-free medium for 6 days (N stress condition) and in exponential phase in BG11 medium (control condition) were harvested, respectively. Each experiment was performed in triplicate.

Protein measurement Freeze-dried samples were extracted with 1 M NaOH at 80°C for 20 min and then centrifuged at 4500 rpm for 10 min. The supernatant was used for protein determination by Bradford method (17). A calibration curve was prepared using BSA dissolved in distilled water.

Carbohydrate measurement Carbohydrate concentration was determined by the phenol-sulfuric acid method. Briefly, freeze-dried biomass was reconstituted in water to prepare a known sample concentration for each sample. Aliquots of 2 mL sample were reacted with 5 mL of concentrated sulfuric acid (98 wt%) and 1 mL of phenol (6%, w/v). After cooled to room temperature, the absorbance of the final mixture was measured on a spectrophotometer at 490 nm. Samples were then quantified by comparison to a calibration curve made from glucose under the same conditions.

Total lipids extraction Total lipids were extracted according to Bigogno et al. (18). Freeze-dried algal biomass was extracted with methanol containing 10% DMSO for 50 min under stirring. Then the mixture was centrifuged, and the supernatant was removed. The residua were re-extracted with a mixture of hexane and diethyl ether (1:1, v/v). After extraction, water was added to the combined supernatants to separate the organic phase. The mixture was shaken and then centrifuged for 5 min and the upper phase was collected. The water phase was re-extracted twice with a mixture of hexane and diethyl ether (1:1, v/v). The organic phase was combined and evaporated to dryness under a stream of N_2. The lipid content was determined gravimetrically.

Lipid fractionation and fatty acids profile Total lipid extracts were fractionated into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) by solid-phase extraction according to Liu et al. (4). A 500 mg Sep-Pak cartridge of silica gel (Waters) was initially equilibrated with 5 mL of chloroform. Subsequently, 1 mL chloroform solution containing about 50 mg of lipid was applied to it. The cartridge was eluted with 10 mL of chloroform to collect NL, 10 mL of acetone to collect GL, and 10 mL of methanol to collect PL. The different fractions obtained were evaporated and weighed.

For analysis of TAG, neutral lipids were separated on a silica gel 60 TLC (Merck) using a solvent system of hexane/diethyl ether/acetic acid (70:30:1, v/v/v). After solvent evaporation, the plates were sprayed with phosphomolybdic acid solution (10% in ethanol) and heated at 90°C for 10 min. TAG was quantified using densitometry and image analysis scaled to a dilution series of TAG standard (triolein).

Fatty acid methyl esters were prepared by incubating freeze-dried biomass or lipid extracts in methanol containing 2% (v/v) H_2SO_4 at 80°C for 2.5 h. Fatty acid analysis was performed according to our previous work (11).

The analysis of algal biodiesel properties The saponification number (SN), iodine number (IN) and cetane number (CN) were estimated by empirical equations shown below (19).

$$SN = \Sigma(560 \times Pi)/MWi \quad (1)$$

$$IN = \Sigma(254 \times D \times Pi)/MWi \quad (2)$$

$$CN = 46.3 + 5458/SN - 0.225 \times IN \quad (3)$$

where P*i* is the weight percentage of each FAME, MW*i* is the molecular mass of individual FAME, D is the number of the double bonds in each FAME.

RESULTS

Cellular biochemical composition Table 1 shows the biochemical composition of *C. zofingiensis* under control and N stress conditions. The algal strain was grown well in N-rich medium and showed the biomass concentration on day 4 was 2.27 g L^{-1}. However, nitrogen depletion severely inhibited cell growth with a low biomass on day 6 (0.64 g L^{-1}). The protein content showed a significant decrease under N stress (16.56% dw) compared with control (33.15% dw). In contrast, the levels of carbohydrate and total lipids under control were 31.73% dw and 26.69% dw, respectively, and increased significantly to 47.73% dw and 34.99% dw, respectively, under N stress condition. The difference of other composition of biomass except the sum of protein, carbohydrate and total lipids between two conditions may be derived from the restriction of analytical methods. For example, Bradford method was only appropriate for the measurement of soluble proteins rather than insoluble proteins.

Lipid fractions GL were the main fraction of total lipids under control condition, accounting for 50.7% of total lipids (Fig. 1) with a value of 12.95% dw (Table 1). Another kind of membrane lipid (PL) were 7.5% of total lipids (Fig. 1) and 1.92% dw (Table 1), much lower than GL. NL were the second most abundant lipid class, occupying 36.4% of total lipids (Fig. 1) and 9.29% dw (Table 1) under control condition.

Under N stress condition, NL content increased dramatically to 30.75% dw, while GL and PL were both reduced to less than 1% dw (Table 1). As shown in Fig. 1, NL became the major fraction that accounted for 86.7% of total lipids under N stress condition, in which TAG was the predominant component, accounting for 75% of the total lipids. Although TAG was also the most abundant component of NL under control, its cellular content was merely 7.67% dw, which was much lower than that obtained from N stressed cells (27.28% dw).

Regarding lipid productivity which is an important indicator for practical application, the productivities of total lipids and NL are listed in Table 1. Total lipid productivity under control (138.60 mg L^{-1} d^{-1}) was much higher than that under N stress (25.37 mg L^{-1} d^{-1}), mainly attributed to the substantial biomass production. However, NL productivity under control (48.31 mg L^{-1} d^{-1}) was less than twice that under N stress (28.88 mg L^{-1} d^{-1}).

Fatty acids profiles in total lipids and individual lipid fraction Fatty acids profiles in total lipids and major lipid fractions under control and N stress conditions are listed in Table 2. Most fatty acid species were composed of 16 and 18 carbon atoms in all lipid fractions regardless of culture conditions, but specific fatty acid species have considerable changes upon N stress. C18:0 and C18:1 species in NL increased while C16:0, C16:1, C18:2 and C18:3 species decreased under N stress condition. Especially oleic acid (C18:1) became the predominant species in NL after N stress condition, which was beneficial for producing high quality biodiesel, since high content of oleic acid can balance the oxidative and low-temperature stability (20). Fatty acids profile in total lipids has a similar pattern with that in NL in response to N stress except for C16:0 and C18:0 species. However, there were quite different patterns in GL and PL.

In order to achieve clearer understanding of variations of fatty acid profiles in each lipid fraction and total lipids upon N stress, fatty acids were categorized into three groups and summarized in Fig. 2. Interestingly, the fatty acids groups of total lipids and NL followed similar trends when exposure to N stress. N starvation led

TABLE 1. Protein, carbohydrate, total lipids and lipid fractions (in percentage of dry weight biomass = % dw), biomass and lipid productivity of *C. zofingiensis* growing under different culture conditions.

Condition	Biomass (g L^{-1})	Protein (% dw)	Carbohydrate (% dw)	Total lipids (% dw)	Neutral lipids (% dw)	Glycolipids (% dw)	Phospholipids (% dw)	Total lipid productivity (mg L^{-1} d^{-1})	NL producitivity (mg L^{-1} d^{-1})
Control	2.27 ± 0.03	33.15 ± 1.77	31.73 ± 1.40	26.69 ± 0.90	9.29 ± 0.67	12.95 ± 0.28	1.92 ± 0.39	138.60 ± 5.69	48.31 ± 2.59
N stress	0.64 ± 0.01	16.56 ± 1.23	47.73 ± 3.63	34.99 ± 0.95	30.75 ± 0.50	0.68 ± 0.17	0.73 ± 0.04	25.37 ± 2.01	28.88 ± 0.26

Values are means ± standard deviations of triplicates. NL, neutral lipids.