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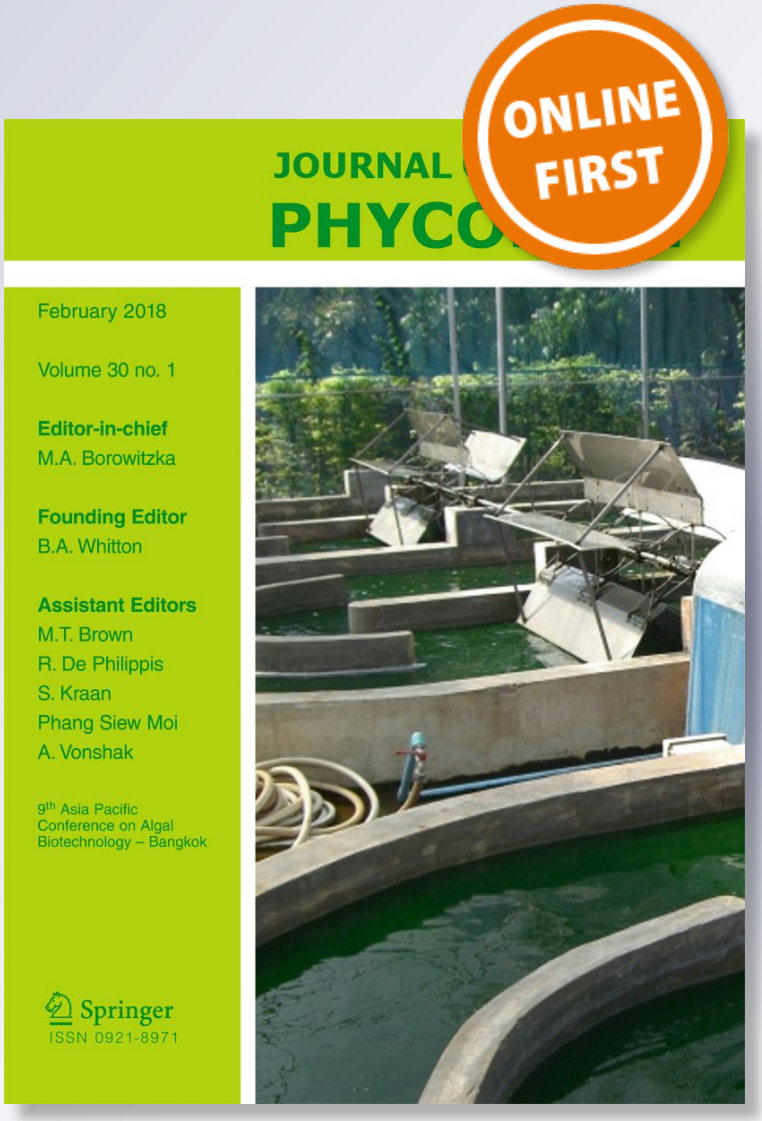

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Differential carbon partitioning and fatty acid composition in mixotrophic and autotrophic cultures of a new marine isolate *Tetraselmis* sp. KY114885

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Abstract
Biochemical composition of microalgae, a promising feedstock for biodiesel production, can be modified under different nutritional modes. The objective of this study was to investigate the influence of autotrophy and mixotrophy on the growth and biochemical composition of *Tetraselmis* sp. KY114885. Time-course variations of lipids and starch were determined using gravimetric and colorimetric methods. After direct transesterification, lipid profile on days 12 and 20 of cultivation period was determined. The results revealed higher growth of microalgae in mixotrophic cultures containing 5 g L^{-1} glucose, whereas 7.5 g L^{-1} glucose suppressed growth. Mixotrophy enhanced the lipid and starch yields in a distinctive trend. The fatty acid composition changed with the different nutritional regimes and the length of growth in batch culture. Glucose supplementation elevated the portions of monounsaturated fatty acids, particularly oleic acid, and decreased the percentage of saturated and polyunsaturated fatty acids. The present study suggests that mixotrophic cultivation of *Tetraselmis* sp. KY114885 may be a feasible strategy to improve growth parameters and the biochemical composition of the alga for biofuel production. The results also shed light on carbon allocation in the algae grown under two different trophic modes.

Keywords Chlorophyta · Microalgae · Mixotrophy · Glucose · Lipid · Starch

Introduction

Microalgae are versatile and widespread microorganisms that because of their metabolic flexibility are considered as unique candidates for commercial biosynthesis of multipurpose products (Borowitzka 2013; Bellou et al. 2014). The major biochemical constituents of microalgae determine their further applications. For example, algal biomass with high protein levels can be utilized for nutritional purposes, and carbohydrate-rich algae are interesting for bioethanol production (Benemann 2013). The algae rich in fatty acids can be considered as a source of biodiesel as well as products with wide nutritional and/or medicinal applications (Milledge 2011).

In addition to autotrophy, which is the main metabolic route of algae, some species are able to utilize organic carbon sources in the presence of light (mixotrophy) or in the dark (heterotrophy). Mixotrophy typically leads to rapid growth and overproduction of some metabolites, primarily carbohydrates and lipids. Some studies have reported the enhancement of algal growth and lipid accumulation using different organic carbon sources such as glucose (Ngangkham et al. 2012; Sabia et al. 2015). Both biomass production and lipid accumulation were enhanced by glucose in *Nannochloropsis* sp. (Cheirsilp and Torpee 2012) and *Scenedesmus quadricauda* (Zhao et al. 2012).

External carbon addition in mixotrophic conditions influences carbon partitioning and production of different metabolites (Kong et al. 2012; Yeh and Chang 2012). Glucose supplementation of *Chlorella vulgaris* increased the biosynthesis of lipids and soluble carbohydrates, whereas protein and chlorophyll productions were decreased (Kong et al. 2013). The nutritional mode can also affect different forms of lipid as well as fatty acid composition of algae (Selvakumar and Umadevi

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2014). For example, mixotrophic cultivation of *Auxenochlorella protothecoides* with 5 g L^{-1} glucose enhanced the proportions of monosaturated fatty acids (MUFAs), while the percentages of saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) declined (Krzemińska and Oleszek 2016). Glucose supplementation elevated the ratio of neutral lipids to polar lipids and also decreased the unsaturation degree of fatty acids in *Chlorella protothecoides* (= *A. protothecoides*) (Ren et al. 2016).

The marine microalga *Tetraselmis* (Chlorophyta, family Chlorodendraceae) has been proposed as a candidate for biofuel production due to its ability to accumulate lipid and starch (Moheimani 2013; Sing et al. 2014; Fon-Sing and Borowitzka 2016). Some strains of *Tetraselmis* are capable to grow in glucose-supplemented media heterotrophically (Lu et al. 2017b), photoheterotrophically (Selvakumar and Umadevi 2014), and mixotrophically (Mohamed et al. 2014).

The effect of mixotrophy on alga metabolism has been previously studied in some *Tetraselmis* species. Mixotrophy with glucose enhanced the biomass production in *T. suecica* and affected the biochemical composition of algae in the stationary phase. In this strain, glucose enhanced the production of carbohydrates and proteins more than lipids (Cid et al. 1992). On the other hand, Mohamed et al. (2014) showed that in the presence of glucose (30 g L^{-1}), lipid productivity of *Tetraselmis* sp. FTC 209 was 4.35 times more than its value in autotrophic cultures. In another study, increased accumulation of both polysaccharides and lipids in the stationary phase was indicated in all mixotrophic cultures of *T. chuii* with different exogenous carbon to nitrogen (C/N) ratios (Lu et al. 2017a).

Despite previous studies, no comprehensive research considering carbon allocation between starch and lipid as well as their time-course variations in mixotrophic cultures of *Tetraselmis* has been published to date. In this study, the long-term impacts of glucose on the accumulation of starch and lipid at the specific time intervals were investigated in a new isolate of *Tetraselmis* for the first time. The effects of mixotrophy on fatty acid profile of the algae as a function of time were also compared with autotrophic culture to indicate the influence of growth phase and glucose supplementation on fatty acid composition. The results can be helpful to determine the best harvesting time for the highest biomass and starch/lipid yields, regarding biofuel production.

Materials and methods

Microalga culture conditions

Tetraselmis sp. KY114885 was obtained from the Research Institute for Applied Sciences, Academic Center for Education, Culture and Research (ACECR) Tehran, Iran. It was grown axenically on Guillard's (F/2) marine enrichment medium (Guillard 1975). The pH was adjusted to 6.8 prior to autoclaving. For mixotrophic treatments, different concentrations of glucose (2.5, 5, and 7.5 g L^{-1}) were added to the F/2 medium before autoclaving. Optimum glucose concentrations for mixotrophic growth of different strains of *Tetraselmis* have been reported to be between 2.5 and 30 g L^{-1}. Utilization of higher glucose levels is not recommended because it is expensive and increases the risk of contamination. Therefore, in this study, 2.5 and 7.5 g L^{-1} were selected as the lowest and highest glucose concentrations. Autotrophic cultures without glucose were used as control groups. All cultures were incubated at 26 ± 2 °C in the 1-L cotton-stopped Erlenmeyer flasks containing 500 mL of growth medium. The cultures were aerated with e air s sterilized using 0.22-μm pore size membrane filters at a flow rate of 0.5 vvm. Light was provided by cool white fluorescent lamps with an intensity of 45 μmol photons m^{-2} s^{-1} and a 16/8-h light/dark photoperiod.

Determination of growth parameters

Alga growth was monitored regularly by measuring the optical density (OD) at 750 nm. For drawing a calibration curve showing OD versus dry cell weight (DCW), the dry weights of serial diluted samples (in the range of 6.75–100%) were determined. To measure DCW, a 10-mL aliquot of the sample was centrifuged at 7000×*g* for 5 min, rinsed twice with ammonium formate, and dried in a glass vial at 70 °C for 24 h to reach constant weight. The difference between final and initial weights of vial was considered as DCW (g L^{-1}). Biomass concentration (y) was calculated using the equation:

$$y = 1.6988x - 0.0726 \quad (r^2 = 0.9989)$$

where x represents OD_{750}.

The specific growth rate (SGR) for the cells harvested in the middle of exponential phase was calculated using Eq. (1):

$$\mathrm{SGR} = \mathrm{Ln}\frac{X2 - X1}{\mathrm{t2} - \mathrm{t1}} \tag{1}$$

The biomass productivity (BP; g L^{-1} day^{-1}) was also calculated based on Eq. (2) where X_1 and X_2 were the dry cell mass concentrations (g L^{-1}) at time t_1 and time t_2, respectively (Kong et al. 2013).

$$\mathrm{BP} = \frac{X2 - X1}{t2 - t1} \tag{2}$$

The algal samples were harvested on 8, 12, 16, and 20 days after inoculation for biochemical assays. All experiments were carried out in three replicates for each treatment (biological replicates).

Lipid extraction and quantification

A volume of 10 mL of algal suspension was harvested and centrifuged at 4000× *g* for 10 min. After rinsing the algal biomass with distilled water, lipid extraction was carried out using a mixture of hexane-ethanol. The procedure of lipid extraction was performed after the optimization of different solvent systems (unpublished data). The alga cell walls were disrupted after 5 min of ultrasonication (40 MHz) followed by vortexing for 1 min. A mixture of n-hexane, ethanol, and water (2:1:0.8 *v*/*v*) was added to the algal cells, vortexed for 30 s, and then centrifuged at 3000× *g* for 10 min. The extraction process was repeated three times, and each time, the upper layer containing hexane and lipids was transferred to a preweighed glass vial. The organic phase was evaporated in a 60 °C oven for 20 h, and the difference between initial and final weight of glass vial was measured as lipid yield (mg L^{-1}). To calculate the lipid content at each sampling time, lipid yield was divided by the dry weight of biomass (Kong et al. 2013).

Starch determination

Starch production was measured according to the procedure previously described by (Mizuno et al. 2013). The starch yield (mg L^{-1}) was determined according to Eq. (3) that was obtained from a glucose standard curve ranging in concentration from 20 to 200 mg L^{-1}.

$$y = 0.0071x - 0.1264, r^2 = 0.9687 \quad (3)$$

Δstarch/Δt and Δlipid/Δt were also calculated by dividing the difference in lipid or starch yield to a defined time period.

Direct transesterification and gas chromatography analysis of fatty acid methyl esters

Algal cells from autotrophic (control) and mixotrophic (5 g L^{-1} glucose) cultures on days 12 and 20 post inoculation were harvested and lyophilized. The dried biomass was subjected to in situ transesterification based on a previously described protocol with minor modification (Talebi et al. 2013). Briefly, 450 μL of extraction solvent containing 2% (*v*/*v*) solution of concentrated H_2SO_4 in methanol was added to 4 mg of the alga sample. The reaction mixture was incubated for 4 h in 70 °C in a shaker incubator at 250 rpm. After the addition of hexane (150 μL), the reaction mixture was vortexed and then mixed with 600 μL of 1% (*w*/*v*) NaCl solution. The mixture was centrifuged at 7000× *g* for 5 min and the organic phase was collected. The fatty acid profile was analyzed using a gas chromatograph (6890, Agilent, USA) equipped with a flame ionization detector. One microliter of sample was injected with split ratio 20:1 into a H88 capillary column (100 m, 0.25 mm I.D., film thickness 0.2 μm). Both the injector and detector temperatures were maintained at 260 °C. The oven temperature was maintained at 140 °C for 5 min, then programmed to increase to 240 °C at a rate of 4 °C min^{-1}. Helium (1.5 mL min^{-1}) was utilized as carrier gas. Identification of individual fatty acids was done by retention time comparison with a mixture of 37 fatty acid methy ester (FAME) standards (Supelco, USA, Catalog No: 18919-1AMP).

Statistical analysis

All experiments were conducted with three independent biological replicates for each treatment. Biochemical assays were performed on each replicate (three technical replicates), and the data are presented as mean ± standard deviation (s.d.) values. Statistical analysis was performed using SPSS version 22 software (IBM, USA). One-way analysis of variance (ANOVA) and Tukey's test at the confidence level of 95% were used to compare differences among treatments.

Results

Three concentrations of glucose (Fig. 1 and Table 1) were used to supplement the growth media of *Tetraselmis* sp. KY114885. Algal cells supplied with 5 g L^{-1} glucose grew more rapidly compared with the cells grown under the other levels of glucose and control group. In the presence of 5 g L^{-1} glucose, the specific growth rate was the highest (0.519 ± 0.003), whereas 7.5 g L^{-1} glucose gave the lowest specific growth rate (0.300 ± 0.380). The supplementation of cultures with glucose shortened the lag phase and exponential phase and accelerated the speed to reach the stationary phase.

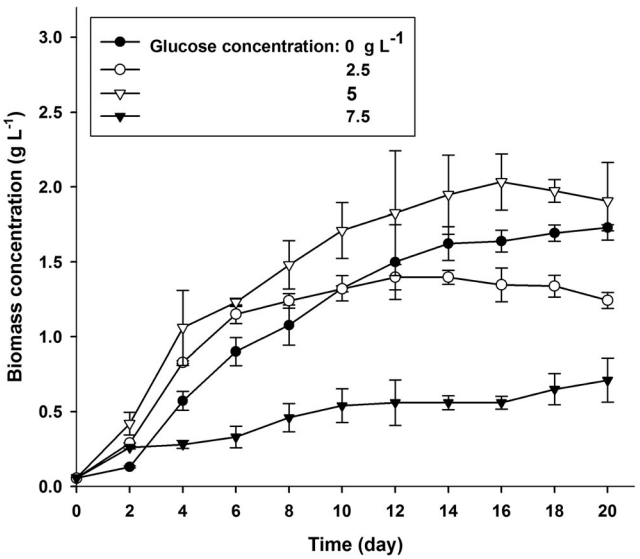

Fig. 1 Effects of glucose concentrations (g L^{-1}) on biomass production of *Tetraselmis* sp. KY114885. Values are mean ± s.d. of three replicates

Table 1 Effects of glucose concentrations on biomass concentration, specific growth rate, and biomass productivity of *Tetraselmis* sp. KY114885. Values are mean ± s.d. of three replicates. The significance of differences is denoted by different letters ($P \leq 0.05$)

Glucose concentration (g L^{-1})	Maximum biomass concentration (g L^{-1})	Maximum biomass productivity (mg L^{-1} day^{-1})	Specific growth rate (day^{-1})
0	1.73 ± 0.02^{b}	86.49 ± 1.02^{b}	0.466 ± 0.016^{c}
2.5	1.40 ± 0.84^{c}	116.49 ± 7.03^{a}	0.507 ± 0.080^{b}
5	2.03 ± 0.18^{a}	127.08 ± 11.75^{a}	0.519 ± 0.003^{a}
7.5	0.71 ± 0.24^{d}	35.69 ± 12.34^{c}	0.300 ± 0.380^{d}

Mixotrophically grown cells entered stationary phase at the end of days 8, 14, and 10 in the presence of 2.5, 5, and 7.5 g L^{-1} glucose, respectively. The growth phase of autotrophic algae changed from the exponential to stationary on day 16 of cultivation (Fig. 1).

The biomass production of this strain was significantly ($P \leq 0.05$) enhanced to as high as 2.03 ± 0.18 g L^{-1} at the concentration of 5 g L^{-1} glucose and decreased to 0.71 ± 0.24 g L^{-1} with 7.5 g L^{-1} glucose (Table 1). The maximum biomass productivities of 116.49 ± 7.03 and 127.08 ± 11.75 mg L^{-1} day^{-1} with 2.5 and 5 g L^{-1} glucose were 1.34 and 1.46 times higher than those obtained for control culture (Table 1). The lowest biomass productivity (35.69 ± 12.34 mg L^{-1} day^{-1}) was obtained in the presence of 7.5 g L^{-1} glucose. A gradual increase in biomass production was observed for this treatment after 16 days of cultivation.

Glucose enrichment of the cultures enhanced the lipid yield and content of *Tetraselmis* sp. KY114885 (Fig. 2). The maximum lipid yield of 380.00 ± 20.81 mg L^{-1} (18.77% of dry biomass) was obtained in the presence of 5 g L^{-1} glucose. Lipid content at all glucose levels was maximized on day 16 of cultivation, whereas autotrophic cultures reached their peak

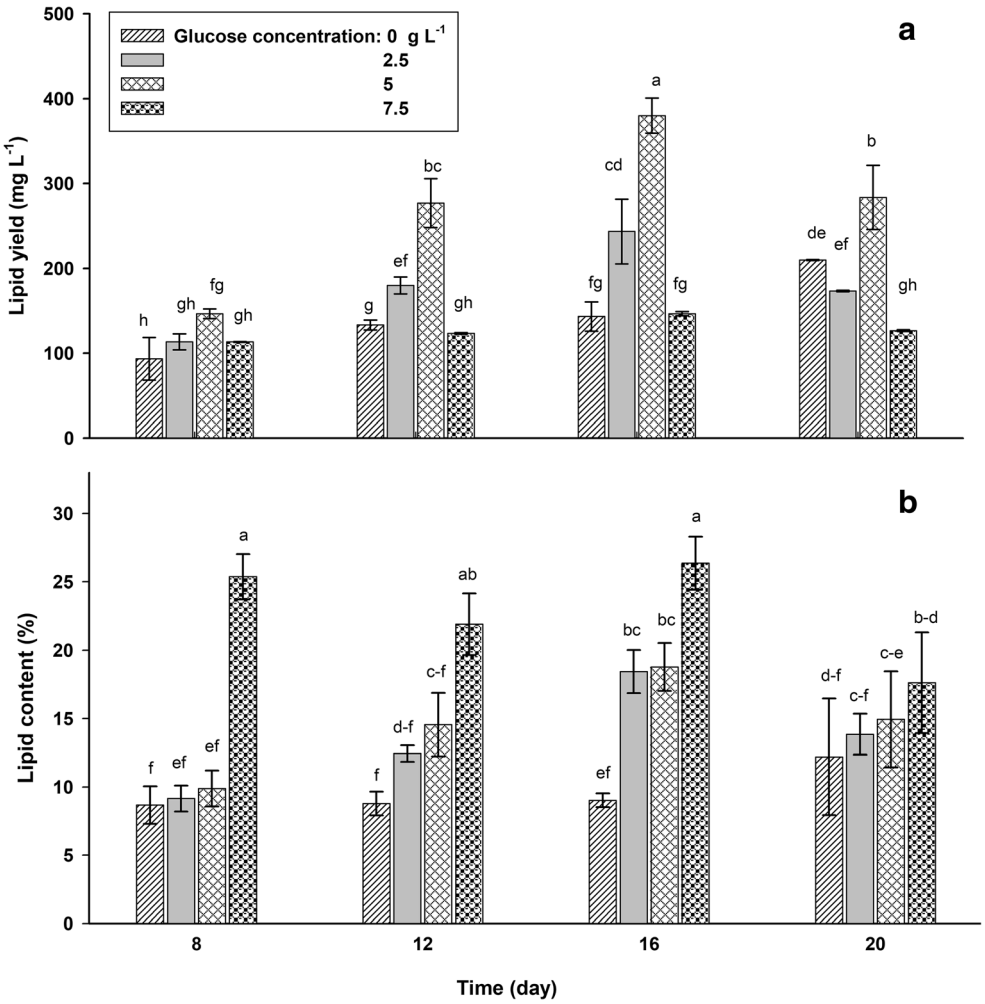

Fig. 2 Time-course variations of lipid yield (**a**) and content (**b**) of *Tetraselmis* sp. KY114885 grown on different glucose concentrations. Values are mean ± s.d. of three replicates. Similar lower case letters are not significantly different at the confidence level of 95%

of lipid content after 20 days. The highest lipid content (26.37 ± 3.50%) was obtained in the 16-day-old cultures supplemented with 7.5 g L^{-1} glucose. The lowest lipid content (8.67 ± 1.37%) was determined in 8-day-old autotrophic cultures (Fig. 2b).

The effects of glucose concentration on starch yield and content in *Tetraselmis* sp. KY114885 are shown in Fig. 3. The starch yield reached its maximum (485.03 ± 10.40 mg L^{-1}) on day 12 for the cultures grown with 5 g L^{-1} glucose. It was 1.35 times more than the value (357.38 ± 17.95 mg L^{-1}) observed in the autotrophic cells at the same incubation time. The lowest starch yield (156.58 ± 9.34 mg L^{-1}) was obtained in the 20-day-old cultures supplemented with 7.5 g L^{-1} glucose (Fig. 3a).

The maximum starch content (40.70 ± 0.95%) was measured in 12-day-old cultures supplemented with 7.5 g L^{-1} glucose. This value was 1.74, 1.54, and 1.59 times higher than those measured in 0.0 (23.37 ± 1.71%), 2.5 (26.37 ± 0.65%), and 5 g L^{-1} (25.50 ± 0.41%) glucose-treated cultures at the same incubation time, respectively (Fig. 3b). In all treatments, the highest starch content was attained in 12-day-old cultures.

Even though mixotrophy with glucose enhanced both starch and lipid contents in *Tetraselmis* sp. KY114885, the accumulation patterns of these two storage metabolites were distinctive. Under mixotrophic conditions, the time needed to accumulate the maximum lipid content (16 days after cultivation) was longer than the required time to obtain the greatest starch content (12 days after cultivation). In autotrophic cultures, the highest lipid content was attained on day 20 and the starch content was maximized on the day 12 (Figs. 2 and 3).

Apart from a few exceptions, no significant differences in the lipid and starch yields were found between the control and 2.5 g L^{-1} glucose-treated cultures. In addition, the algal cell growth was inhibited by 7.5 g L^{-1} glucose. Therefore, the fatty acid profile was just only analyzed for the cultures treated with 5 g L^{-1} glucose as well as autotrophic cultures on days 12 and 20 of cultivation.

The dominant FAs in autotrophic cultures on day 12 included arachidic (C20:0), oleic (C18:1), and linoleic (C18:2n6) acids. The main fatty acids on day 20 were oleic, arachidic, and alpha linolenic (C18:3n3) acids. In the mixotrophic cultures, oleic and arachidic acids were the two

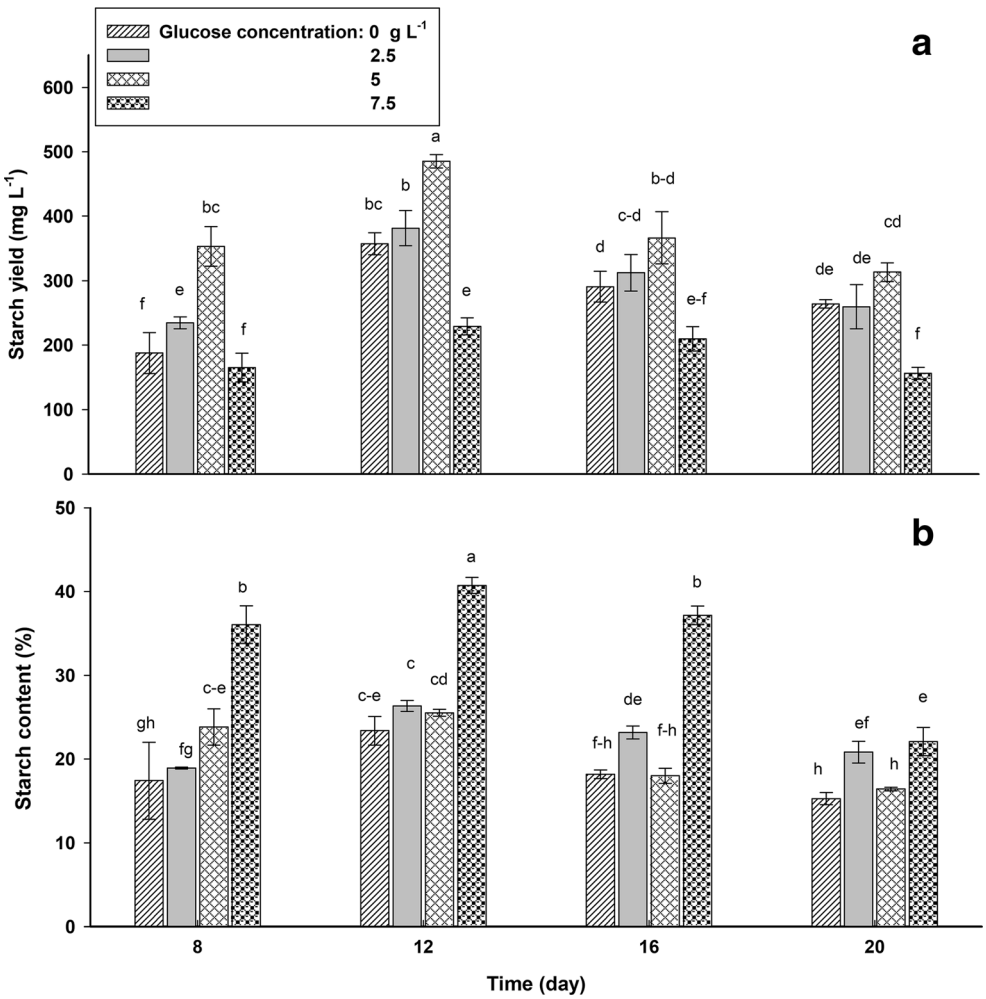

Fig. 3 Time-course variations of starch yield (**a**) and content (**b**) of *Tetraselmis* sp. KY114885 grown on different glucose concentrations. Values are mean ± s.d. of three replicates. Similar lower case letters are not significantly different at the confidence level of 95%

main fatty acids. The third rank in the FA profile on days 12 and 20 of mixotrophic cultures were lignoceric (C24:0) and palmitic (C16:0) acids, respectively (Table 3).

The percentage of individual FAs changed significantly based on the cultivation period and trophic mode (Table 3). The ratio of oleic acid to total FAs in both autotrophic and mixotrophic samples was higher on day 20 compared with those on day 12. Mixotrophy enhanced the percentage of oleic acid significantly, so that the contents of oleic acid on day 12 (45.26 ± 1.86%) and day 20 (50.48 ± 1.59%) of growth period was 2.23 and 1.26 times higher than those obtained from autotrophic cultures on day 12 (20.22 ± 3.41%) and day 20 (39.90 ± 0.60%).

Compared to autotrophic cultures, treatment with 5 g L^{-1} glucose led to a 81.1% increase in the proportion of MUFAs on day 12, while a 147 and 17.5% decrease in the ratios of PUFAs and SFAs was observed, respectively. The ratio of Σ(SFA+MUFA)/Σ(PUFA) was 2.69 ± 0.38% in 12-day-old and 4.84 ± 0.95% in 20-day-old autotrophic cultures. This value reached 8.64 ± 0.96% on day 12 and 8.96 ± 0.99% on day 20 of cultivation under mixotrophic condition.

Discussion

The metabolic flexibility of microalgae for changing their nutritional mode according to the substrate availability gives them an evolutionary advantage to survive and sustain their growth in different conditions (Mohan et al. 2015). The ability of some *Tetraselmis* strains such as *T. suecica* (Cid et al. 1992) and *Tetraselmis* sp. FTC 209 (Mohamed et al. 2014) to grow mixotrophically has been reported previously. Higher biomass concentration and productivity of *Tetraselmis* sp. KY114885 in the presence of 5 g L^{-1} glucose can be related to the uptake of this external organic source in the medium. An inducible hexose transport system has been characterized for *C. vulgaris* (Haass and Tanner 1974). The hexose/H^+ symporter is responsible for glucose uptake from the medium and can be induced 15–18 min after the addition of hexose sugars to the culture medium (Morales-Sánchez et al. 2013). This may provide an explanation for the quick acclimation of *Tetraselmis* sp. KY114885 to grow in glucose-fed conditions.

Maximum biomass productivity in mixotrophic cultures of the studied strain was found to be 1.46 times higher than that of autotrophic cultures. In some strains of the algae such as *Tetraselmis* sp. FTC 209, maximum biomass productivity in mixotrophic treatments was enhanced 4.29 times more than in the control group (Mohamed et al. 2014). Difference between the results of the two studies can be attributed to different culture medium compositions (F/2 versus enriched Walne) and glucose levels (5 versus 30 g L^{-1}). The impacts of external carbon source on the algal growth in mixotrophic cultures are related to the ionic concentrations and their interactions in culture media. As it was shown in *C. protothecoides*, the cell ionic environment is an important factor influencing biomass production (Ren et al. 2016).

Based on the growth indices measured in the present study, 5 g L^{-1} was the optimum concentration of glucose for improving the growth of *Tetraselmis* sp. KY11488. Deleterious effects of 7.5 g L^{-1} glucose on the growth of this strain may be due to substrate inhibition. Although there are no comprehensive biochemical studies on the mechanism of substrate inhibition, a decrease decreasing in the enzyme activities involved in the cell growth and division might be a possible explanation (Reed et al. 2010). Mohamed et al. (2014) reported 30 g L^{-1} glucose as the optimum concentration for mixotrophic growth of *Tetraselmis* sp. FTC 209, whereas the concentrations higher than 40 g L^{-1} decreased the alga growth. The different

Table 2 The rate of starch and lipid production as a function of time in *Tetraselmis* sp. KY 114885 under different glucose concentrations. Values are mean ± s.d. of three replicates. The significance of differences is denoted by different letters ($P \leq 0.05$)

		Glucose concentration (g L^{-1})			
Variable	Day of cultivation	0	2.5	5	7.5
ΔStarch/Δt	8	$23.47 \pm 8.96^{b-d}$	$29.32 \pm 1.11^{a-d}$	44.13 ± 4.30^{a}	20.66 ± 1.15^{cd}
	12	42.40 ± 6.52^{a}	36.72 ± 3.00^{ab}	$32.98 \pm 6.55^{a-c}$	15.92 ± 4.44^{d}
	16	-16.68 ± 5.69^{de}	-17.26 ± 3.05^{de}	-29.67 ± -4.80^{e}	-4.85 ± 5.31^{d}
	20	-6.66 ± 4.37^{d}	-13.21 ± 7.37^{d}	13.25 ± 5.12^{d}	-13.26 ± 3.45^{d}
ΔLipid/Δt	8	$11.66 \pm 3.14^{b-d}$	$14.16 \pm 3.22^{a-d}$	$18.33 \pm 2.75^{a-c}$	$14.16 \pm 1.18^{a-d}$
	12	$10.00 \pm 5.00^{b-d}$	$16.66 \pm 8.08^{a-c}$	32.50 ± 3.01^{a}	$4.05 \pm 2.81^{c-e}$
	16	$2.50 \pm 00/00^{c-e}$	$15.83 \pm 9.46^{a-c}$	25.83 ± 5.20^{ab}	$5.83 \pm 3.80^{b-d}$
	20	$16.66 \pm 3.81^{a-c}$	-17.50 ± 15.00^{f}	-24.16 ± 5.20^{ef}	$-5 \pm 13.22^{d-f}$
Starch/lipid	8	$2.16 \pm 1.01^{a-c}$	$2.08 \pm 0.81^{a-d}$	2.41 ± 0.24^{ab}	$1.49 \pm 0.20^{b-d}$
	12	2.69 ± 0.42^{a}	$2.12 \pm 0.32^{a-c}$	$1.76 \pm 0.40^{a-d}$	$1.86 \pm 0.13^{a-d}$
	16	$2.03 \pm 0.23^{a-d}$	1.13 ± 0.19^{cd}	0.96 ± 0.53^{d}	$1.45 \pm 0.36^{b-d}$
	20	1.26 ± 0.13^{cd}	$1.52 \pm 0.26^{b-d}$	1.11 ± 0.26^{cd}	1.29 ± 0.18^{cd}

Table 3 Fatty acid composition of *Tetraselmis* sp. KY114885 in two different trophic modes on the 12th and 20th day of growth. Values are mean ± s.d. of three replicates. The significance of differences is denoted by different letters ($P \leq 0.05$)

	Fatty acid		Trophic mode and harvesting time			
			Autotrophy-12	Autotrophy-20	Mixotrophy-12	Mixotrophy-20
PUFA	Behenic acid	C22:2	3.15 ± 1.15^{a}	3.96 ± 1.87^{a}	0.00^{b}	0.00^{b}
	Eicosatrienoic acid	C20:3n3	0.87 ± 0.38^{a}	0.45 ± 0.21^{ab}	0.87 ± 0.08^{a}	0.31 ± 0.07^{b}
	Arachidonic acid	C20:4n6	0.23 ± 0.19^{a}	0.30 ± 0.01^{a}	0.00^{b}	0.00^{b}
	Eicosadienoic acid	C20:2	0.71 ± 0.04^{a}	0.76 ± 0.09^{a}	0.42 ± 0.03^{c}	0.58 ± 0.08^{b}
	Gamma-linolenic acid	C18:3n6	0.10 ± 0.09^{ab}	0.27 ± 0.01^{a}	0.03 ± 0.02^{b}	0.00^{b}
	Alpha-linolenic acid	C18:3n3	9.53 ± 0.79^{a}	8.57 ± 0.24^{a}	5.70 ± 0.40^{b}	5.30 ± 0.64^{b}
	Linoleic acid	C18:2n6	13.99 ± 9.09^{a}	4.51 ± 0.10^{b}	3.95 ± 0.37^{b}	3.87 ± 0.21^{b}
MUFA	Paullinic acid	C20:1	0.37 ± 0.19^{a}	0.00^{b}	0.00^{b}	0.00^{b}
	Oleic acid	C18:1n9c	20.22 ± 3.41^{d}	39.90 ± 0.60^{c}	45.26 ± 1.86^{b}	50.48 ± 1.59^{a}
	Elaidic acid	C18:1n9t	0.41 ± 0.04^{b}	0.40 ± 0.02^{b}	0.48 ± 0.03^{b}	0.58 ± 0.02^{a}
	Palmitoleic acid	C16:1	6.82 ± 0.66^{a}	6.16 ± 0.14^{ab}	4.77 ± 0.24^{c}	5.56 ± 0.44^{bc}
	Myristoleic acid	C14:1	0.27 ± 0.02^{a}	0.28 ± 0.02^{a}	0.25 ± 0.03^{a}	0.28 ± 0.02^{a}
SFA	Lignoceric acid	C24:0	3.83 ± 0.19^{d}	5.08 ± 0.04^{b}	7.11 ± 0.60^{a}	4.93 ± 0.66^{c}
	Heneicosanoic acid	C21:0	1.04 ± 0.07^{a}	1.00 ± 0.02^{a}	0.55 ± 0.05^{b}	1.01 ± 0.14^{a}
	Arachidic acid	C20:0	22.78 ± 3.65^{a}	17.11 ± 0.09^{b}	19.22 ± 0.61^{b}	16.52 ± 0.71^{b}
	Stearic acid	C18:0	3.25 ± 0.33^{a}	2.65 ± 0.08^{ab}	1.91 ± 0.15^{b}	0.70 ± 0.09^{c}
	Palmitic acid	C16:0	9.58 ± 2.29^{a}	6.78 ± 0.15^{ab}	4.75 ± 0.79^{b}	6.40 ± 1.33^{ab}
	Myristic acid	C14:0	0.41 ± 0.12^{a}	0.24 ± 0.06^{b}	0.00^{c}	0.00^{c}
	Tridecylic acid	C13:0	1.25 ± 0.14^{bc}	1.08 ± 0.02^{c}	1.46 ± 0.16^{b}	1.61 ± 0.01^{a}
	Caprylic acid	C8:0	2.01 ± 1.85^{a}	0.06 ± 0.01^{c}	0.00^{d}	0.12 ± 0.04^{b}
	Caproic acid	C6:0	0.00^{c}	1.34 ± 0.13^{ab}	2.64 ± 0.32^{a}	1.28 ± 0.02^{ab}
ΣSFA			44.53 ± 5.69^{a}	35.73 ± 3.50^{b}	37.91 ± 0.46^{b}	32.98 ± 0.16^{c}
Σ MUFA			28.22 ± 8.52^{d}	46.86 ± 0.75^{c}	51.09 ± 0.69^{b}	56.93 ± 1.16^{a}
Σ PUFA			27.25 ± 2.82^{a}	17.40 ± 2.80^{b}	11.00 ± 0.90^{c}	10.09 ± 0.99^{c}
Σ(SFA+ MUFA)/Σ(PUFA)			2.69 ± 0.38^{c}	4.84 ± 0.95^{b}	8.64 ± 0.96^{a}	8.97 ± 0.99^{a}

behaviors of these two strains of *Tetraselmis* may be related to the different culture conditions such as light intensity and different abilities in glucose utilization.

With the consumption of glucose, its inhibitory effect was removed in *Tetraselmis* sp. KY114885, and after 16 days, it had decreased to a level that was no longer inhibitory and could induce growth of algae with a slow rate. The resuming growth may be the result of the release of some metabolites in the presence of glucose which have been converted to CO_2 and assimilated after glucose depletion (Ren et al. 2016).

In this study, supplementation of culture medium with 5 g L^{-1} glucose increased up the lipid yield to the maximum level. The required amount of glucose for the enhancement of lipid biosynthesis is different among algal strains. For example, the highest lipid yield was achieved with 30 g L^{-1} glucose for *Tetraselmis* sp. FTC209 (Mohamed et al. 2014). Lipid synthesis is an energy-demanding process and is favored under high ratio of ATP/ADP (Li et al. 2014). Mixotrophic cultures of *C. protothecoides* with the higher ratio of ATP/ADP showed more lipid accumulation as compared to autotrophic cultures (Ren et al. 2016). In mixotrophy, lipid production may be enhanced due to directing carbon flux toward the glycolytic pathway or the synthesis of acetyl-coA, the main precursor for fatty acid biosynthesis (Jia et al. 2015). Higher lipid content was correlated with increased expression level of acetyl-coA carboxylase (ß-subunit), the rate limiting enzyme for fatty acid biosynthesis, in mixotrophic cultures of *Chlorella sorokiniana* (Ngangkham et al. 2012). Elevated lipid production in mixotrophic cultures may be a mechanism that protects the algae against oxidative damage through the consumption of excess NADPH in fatty acid biosynthesis (Li et al. 2015).

The glucose concentration for optimum growth of *Tetraselmis* sp. KY114885 was not the best concentration for maximum lipid content. Similarly, treatment of *A.*

protothecoides with 1 g L^{-1} resulted in the highest content, whereas the highest biomass production was achieved in with 3 g L^{-1} glucose (Krzemińska and Oleszek 2016).

In this study, the maximum and minimum values of starch yield were produced in the presence of 5 and 7.5 g L^{-1} glucose, respectively. The effects of glucose on the starch yield of the algae can be attributed to its influence on the growth and biomass production. In the same way, based on the measurements of DCW, the increase in starch content in the cell cultures containing 7.5 g L^{-1} glucose can be related to the decline in algal biomass. Despite the negative effects of 7.5 g L^{-1} on the growth of this alga, it could enhance starch and lipid biosynthesis. Suppressing algal energy and carbon-demanding processes such as cell division ensures the hyperaccumulation of lipid and starch (Vitova et al. 2015). Carbon allocation either between cell growths or the accumulation of storage compounds determines the biomass production rate in mixotrophic cultures (Subramanian et al. 2013).

The accumulation trend of two main storage molecules, lipid and starch, in *Tetraselmis* sp. KY114885 was similar to that of *C. sorokiniana* in which starch obtained its maximum level faster than lipid (Li et al. 2015). It seems that in both trophic modes, starch accumulation pathways are activated earlier than the lipid biosynthesis. Furthermore, it is likely that starch is degraded to supply carbon skeletons as precursors for lipid biosynthesis. The biosynthesis of lipid and starch depends on the shared pool of 3-C molecules (de Jaeger et al. 2014), and they are interconvertible (Li et al. 2011). The required time to reach the highest lipid content in control (autotrophic) cultures was longer than that observed for mixotrophic cultures. This may also indicate that the rate of starch catabolism in mixotrophic cultures of *Tetraselmis* sp. KY114885 is greater than in autotrophic cultures.

The tendency of algae to accumulate lipid or starch is different among species (Vitova et al. 2015). The measured for starch/lipid yield in *Tetraselmis* sp. KY114885 under two nutritional regimes during the cultivation period indicated the preference of this strain to accumulate starch rather than lipid (Table 2). The negative values of Δstarch/Δt on the 16th day of cultivation versus the positive values of Δlipid/Δt at the same time suggest that some part of lipid might have originated from starch degradation. Upregulation of starch-degrading enzymes such as isoamylase and beta-amylase in *C. sorokiniana* led to increased amounts of lipids (Li et al. 2015). Similarly, simultaneous upregulation of the genes related to starch degradation (isoamylase, beta-amylase, and starch phosphatase) and fatty acid biosynthesis (malic enzyme, PEP carboxylase, and acetyl-coA carboxylase) has been shown in *Chlorella* sp. AE10 (Cheng et al. 2017).

Fatty acid composition of the alga studied here was affected by the incubation time and nutritional regime. In mixotrophic conditions, oleic acid increased more substantially as compared with other fatty acids. There are some other reports on the production of oleic acid in mixotrophic cultures (Li et al. 2014; Baldisserotto et al. 2016). Among 14 mixotrophically cultivated microalgae studied by Park et al. (2012), oleic acid was the major constituent of 10 strains. Moreover, oleic acid content of *A. protothecoides* in mixotrophic condition was approximately five times higher than that of control groups (Krzemińska and Oleszek 2016). Our results showed the higher production of oleic acid in stationary phase as compared with exponential phase in both autotrophic and mixotrophic cultures. Similarly, oleic acid content in the stationary phase of glucose-supplemented *Neochloris oleoabundans* was higher than that of the cells in exponential phase (Baldisserotto et al. 2016). Oleic acid is a very suitable fatty acid for biodiesel application, and its increasing level in algal oils improves low-temperature properties and oxidative stability of biodiesel (Knothe 2006).

The contents of arachidonic (C20:4n) and alpha-linolenic acid were decreased in the presence of glucose (Table 3). According to the ASTM D6751 and EN 14214 standard specifications, in order to utilize biodiesel as an industrialized product, the maximum content of fatty acid methyl esters with four and more double bonds should be 1%. Furthermore, linolenic acid methyl esters content should not surpass 12% (Baldisserotto et al. 2016). Glucose-treated cultures of *Tetraselmis* sp. KY114885 with increased level of oleic acid (50.98 ± 1.59%) and reduced levels of α-linolenic acid (5.30 ± 0.64%) are more suitable for biodiesel production than those cultivated autotrophically.

In stationary phase of both autotrophic and mixotrophic cultures of *Tetraselmis* sp. KY114885, the percentage of MUFA increased concomitantly with the decrease of PUFA and SFA percentages. The elevated ratio of MUFA and decreased ratios of PUFA and SFA were also reported in the stationary phase cultures of *Synedra acus* as compared to the exponential phase cultures (Shishlyannikov et al. 2014). In accordance with our results, Baldisserotto et al. (2016) reported higher production of MUFA in glucose-treated cells of *N. oleoabundans* compared to autotrophic cells. Mixotrophy also increased MUFA content (400%) and decreased PUFA content (33%) in *C. sorokiniana* (Ngangkham et al. 2012). These changes in the dominance of PUFA and MUFA levels reveal that in mixotrophic cultures, the rate of MUFA conversion to PUFA is declined. Lower PUFA percentage in mixotrophic cultures has been explained by lower level of intracellular oxygen acting as a substrate for desaturase enzymes (Smith et al. 2015). In mixotrophic cultures of *Tetraselmis* sp. KY114885, the $\Sigma(SFA+MUFA)/\Sigma(PUFA)$ was significantly increased in comparison with autotrophic cultures. This finding was in agreement with a previous report on *N. oleoabundans* where 41% increase in the ratio of $\Sigma(SFA+MUFA)/\Sigma(PUFA)$ obtained through changing the mode of nutrition (Baldisserotto et al. 2016).

In summary, *Tetraselmis* sp. KY114885 showed a good capacity to grow mixotrophically using 5 g L^{-1} glucose. Glucose supplementation increased biomass production as well as starch and lipid yields. The lipid quality of algae for biodiesel application was also improved in mixotrophic condition. The alga was able to produce the highest biomass and lipid content besides a preferential increase in MUFA and significant decrease in PUFA and SFA contents. This study suggests that mixotrophic cultivation of *Tetraselmis* sp. KY114885 can be regarded as a useful approach to produce biofuel.

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