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Enhancement of Growth and Lipid Accumulation by *Chlorella sorokiniana* 1019 under Mixotrophic Culture Conditions

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ABSTRACT

The enhancement of the growth and lipid accumulation by a microalga, *Chlorella sorokiniana* 1019, was investigated under mixotrophic conditions. The results showed that the growth and lipid production in this alga were notably increased under mixotrophic conditions using a limited-nitrogen medium compared to phototrophic and heterotrophic conditions. The biomass yield and lipid production were approximately 3.9 and 2.6 times more than under phototrophic growth. Among the three carbon sources of glucose, potassium acetate and glycerol, 1% glucose promoted the highest growth rate at 7.58 g of cell dry weight.L^{-1} after 7 d of cultivation, while 3% acetate yielded the highest cellular lipid reaching 56% of the dried biomass. The fatty acid composition of the alga was slightly changed from the predominant linoleic acid in the glucose medium to palmitic acid in the acetate medium.

Keywords: lipid accumulation, mixotrophic culture conditions, *Chlorella sorokiniana*, biodiesel

INTRODUCTION

Microalgae have been suggested as potential candidates for biodiesel production because of their several advantages over other energy crops; microalgal lipid properties were similar to those obtained from oil seeds, with several species of *Chlorella* (*C. vulgaris, C. protothecoides, C. emersonii, C. sorokiniana and C. minutissima*) being shown to be good options for biodiesel production (Illman *et al*., 2000; Miao and Wu, 2006; Qiao and Wang, 2009; Mallick *et al*., 2011; Muthukumar *et al*., 2012). Some microalgae can grow under various conditions such as phototrophic, heterotrophic and mixotrophic growth modes. The phototrophic mode is well known and occurs in most microalgae, although under phototrophic conditions with nitrogen deficiency, microalgae substantially increased their cellular lipid levels but their growth rate and biomass were greatly reduced (Illman *et al*., 2000). The heterotrophic and mixotrophic growth modes in which organic carbon serves as the carbon source are alternatives to improve lipid production from microalgae. These modes of growth offer the possibility of greatly improving the productivity of microalgal cultures. *Chlorella* is a member of the Trebouxiophyceae that are able to utilize organic compounds as a carbon source. Xu *et al*. (2006) reported that *C. protothecoides* produced extremely high biomass and lipid yields under completely heterotrophic conditions. However, the end product of the respiration reaction is carbon dioxide that is unavoidably released from the process. In the mixotrophic mode, the algae exhibit photosynthesis and respiration concurrently. It is assumed that the carbon dioxide released from respiration is re-fixed via

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a photosynthetic mechanism and enhances the growth rate accordingly (Lee *et al*., 1996).

An inexpensive source of simple organic compounds is needed to produce biodiesel economically. Therefore for the present study, glucose was chosen because it is a common sugar found in wastewater from the food industry, while acetic acid is used widely as a fixer in the textile industry. On the other hand, glycerol is a waste material from the transmethylation of fatty acids into biodiesel. However, there is little published information on the glycerol uptake of the *Chlorella* strain (Liang *et al*., 2009).

Chlorella sorokiniana is a freshwater microalga that can grow quickly and has been used for research and industrial applications. The current investigated mixotrophic culture conditions with three carbon sources at various concentrations to increase the biomass and lipid production of *C. sorokiniana* 1019. The fatty acid composition that was produced from the different growth modes was also observed.

METHODS

Microalgal culture, culture medium and culture conditions

Chlorella sorokiniana 1019 was obtained from the culture collection of the Institute of Food Research and Product Development of Kasetsart University, Thailand.

NSIII (Payer and Trützsch, 1972), a complete inorganic synthetic medium, was used as the basal medium under the phototrophic and mixotrophic conditions. However, the nitrate concentration of the medium was reduced to 0.25 $g.L^{-1}$ (one fourth of the original value) and used as an N-limited medium throughout the study unless otherwise stated. The composition of the heterotrophic medium was 1% glucose, 0.25% yeast extract and 0.25% peptone. These nutrients were also added to the mixotrophic culture medium.

The inoculum was prepared under mixotrophic conditions. It was grown axenically at 25°C for 3 d in 125 mL Erlenmeyer flasks containing 50 mL of medium and the flasks were mounted on an orbital shaker set at 120 rpm. Light was provided with a 12:12 h light/dark cycle.

In all cultivation modes, the microalga was cultivated in 200 mL conical culture tubes (4 × 30 cm) filled with 150 mL of sterilized medium. After inoculation with 1% (volume per volume; v/v) algal culture, the tubes were incubated in a water bath at 30 ± 1°C for 7 d. For phototrophic and mixotrophic cultivation, light was provided laterally by daylight fluorescent lamps with a mean photon flux density intensity of 62.5 $\mu mole.m^{-2}.s^{-1}$. For the heterotrophic conditions, light was omitted by wrapping the culture tubes with aluminum foil. Mixing and aeration were provided by bubbling air containing 5% (v/v) CO_2 for the phototrophic and mixotrophic conditions, whereas only air was used for the heterotrophic conditions. Samples were taken daily for analysis of the growth, lipid content and carbon source consumption.

Experiments

Chlorella sorokiniana 1019 was primarily cultivated in three different culture conditions—namely, phototrophic, heterotrophic and mixotrophic—using 1% glucose as the organic carbon source to determine the effects of each nutritional mode.

In the later stage, the N-limited medium was supplemented under mixotrophic conditions with each of the three carbon sources—namely, glucose, glycerol and potassium acetate at 1% (weight per volume; w/v).

The effect of carbon concentration under mixotrophic conditions was investigated by varying the glucose and potassium acetate concentration at 1, 2 and 3% (w/v).

Analyses

Cell growth was determined by measuring

OD_{600} using a spectrophotometer (UV-1700; Shimadzu; Kyoto, Japan) and converting this to cell dry weight (measured in grams per liter) by a predetermined linear relationship (Equation 1):

Cell dry weight ($g.L^{-1}$) = 0.2466 * OD_{600} + 0.0002 (R^2 = 0.998) (1)

For fatty acid analysis, each algal sample was centrifuged at 3,300 rpm for 15 min and the cell pellet was collected for fatty acid analysis. Lipid extraction was carried out based on the procedure of Bligh and Dyer (1959). Transmethylation of the lipid was accomplished by reaction with 6% sulfuric acid in methanol at 80°C for 15 h (Holub and Skeaff, 1987). Fatty acid methyl esters were then analyzed by a gas chromatographer (GC-14B; Shimadzu; Kyoto, Japan) equipped with a flame ionization detector and a split injector, using a capillary column 30 m in length with 0.25 mm internal diameter and 0.25 μm of film thickness (DB-225; Agilent J&W; Santa Clara, CA, USA). The fatty acid profiles were identified by a comparison of their retention times with authentic fatty acid standards (Sigma; St. Louis, MO, USA). An internal standard (pentadecanoic acid) was used to assist in the quantification of the fatty acids.

Glucose and glycerol were analyzed by high performance liquid chromatography (1100 series; Agilent Technologies; Foster City, CA, USA) equipped with a refractive index detector using an 8 × 300 mm Ultron PS-80C column (Shinwa Chemical Industries; Kyoto, Japan). The column temperature was kept at 80°C and filtered deionized water was used for elution at 1 $mL.min^{-1}$.

The concentration of acetate was analyzed by gas chromatography (HP6890 series; Hewlett Packard; Palo Alto, CA, USA) with a flame ionization detector equipped with a 25 m × 0.32 mm × 0.52 μm HP-FFAP capillary column (Agilent Technologies; Foster City, CA, USA). The supernatant was acidified by formic acid to pH 2 prior to analysis according to the procedure of Barbosa *et al*. (2001). Butanol at 1% (v/v) was used as the internal standard.

RESULTS AND DISCUSSION

Effect of culture condition on growth, lipid production and lipid accumulation of *C. sorokiniana* 1019

The biomass, lipid production and cellular lipid content of *Chlorella sorokiniana* 1019 under different growth conditions are compared in Figure 1. Clearly, the *C. sorokiniana* 1019 grew very well under mixotrophic conditions with 1% glucose as the additional carbon source. The biomass yield and lipid production were 7.58 $g.L^{-1}$ and 1.49 $g.L^{-1}$ which were approximately 3.9 and 2.6 times more than the phototrophic growth, respectively. Heterotrophic conditions showed moderate growth and lipid production. In fact, the cell dry weight and lipid accumulation under mixotrophic conditions were almost equal to the combined values of the other two culture conditions throughout the cultivation period.

Under mixotrophic conditions, when cells were exposed to an N-limited culture medium, the presence of an additional carbon source has been widely reported to increase the lipid content of microalgae (Cerón Garc´ıa *et al*., 2005; Qiao and Wang 2009; Qiao *et al*., 2009). This is largely due to the fact that under limited nitrogen supply, the algae convert the oversupply of carbon into storage lipids. Yeast extract and peptone which provide many essential amino acids were also reported to stimulate growth (Liang *et al*., 2009). In addition, Lee *et al*. (1996) suggested that with mixotrophic growth, light might serve as an energy source for carbon assimilation leading to the photosynthetic fixation of CO_2 for use in the formation of certain algal products. All results indicated that *C. sorokiniana* 1019 could utilize both organic and inorganic carbon as a carbon source.

Under heterotrophic growth conditions, *C. sorokiniana* 1019 exhibited rapid growth

Table 1 Comparison of biomass and lipid content in several microalgal species grown under phototrophic, heterotrophic and mixotrophic culture conditions.

Culture conditions and microalgal strain	Harvest time	Carbon source (concentration)	Biomass ($g.L^{-1}$)	Lipid content	References
Phototrophic conditions					
Chlorella sorokiniana 1019	7 d	None	1.93	30.20%; 0.58 $g.L^{-1}$	This paper
Chlorella vulgaris	23 d	None	0.32	33.00%	Liang *et al*. (2009)
Chlorella vulgaris	25 d	None	nd*	56.60%	Liu *et al*. (2008)
Chlorella vulgaris	20 d	None	0.86	29.53%	Widjaja *et al*. (2009)
Heterotrophic conditions					
Chlorella sorokiniana 1019	7 d	Glucose (1% w/v)	2.93	13.90%; 0.41 $g.L^{-1}$	This paper
Chlorella sorokiniana GXNN01	48 h	Glucose (20mmol.L^{-1})	1.15	23.70%; 0.27 $g.L^{-1}$	Qiao and Wong (2009)
	48 h	Sodium acetate (60mmol.L^{-1})	1.01	28.70%; 0.29 $g.L^{-1}$	
Chlorella protothecoids	144 h	Glucose (10 $g.L^{-1}$)	3.74	54.70%	Xu *et al*. (2006)
	144 h	Corn powder hydrolyzed	3.92	55.30%	
Chlorella protothecoids	184 h	Glucose (10 $g.L^{-1}$)	15.50	46.10%	Xu *et al*. (2006)
Chlorella protothecoids	103 h	Glucose (15 $g.L^{-1}$)	4.20	13.00%	Heredia-Arroyo *et al*. (2010)
	103 h	Acetate (20.5 $g.L^{-1}$)	3.10	23.00%	
	103 h	Glycerol (20.4 $g.L^{-1}$)	3.60	19.00%	
Chlorella protothecoids	6 d	Glucose (30 $g.L^{-1}$)	15.30	50.00%; 7.70 $g.L^{-1}$	Chen and Walker (2011)
	6 d	Pure glycerol (30 $g.L^{-1}$)	19.20	51.00%; 9.80 $g.L^{-1}$	
	6 d	Crude biodiesel glycerol (30 $g.L^{-1}$)	23.50	62.00%; 14.60 $g.L^{-1}$	
Chlorella vulgaris	6 d	Glucose (1%w/v)	1.20	23.00%	Liang *et al*. (2009)
	12 d	Acetate (1%w/v)	0.90	36.00%	
Chlorella saccharophila	15 d	Glucose (2.5 $g.L^{-1}$)	nd*	46.70%; 0.29 $g.L^{-1}$	Tan and Johns (1991)
Mixotrophic conditions					
Chlorella sorokiniana 1019	7 d	Glucose (1% w/v)	7.58	19.70%; 1.49 $g.L^{-1}$	This paper
	7 d	Potassium acetate (3% w/v)	1.48	56.50%; 0.83 $g.L^{-1}$	This paper
Chlorella vulgaris	6 d	Glucose (1%w/v)	1.70	21.00%	Liang *et al*. (2009)

*nd = No data.

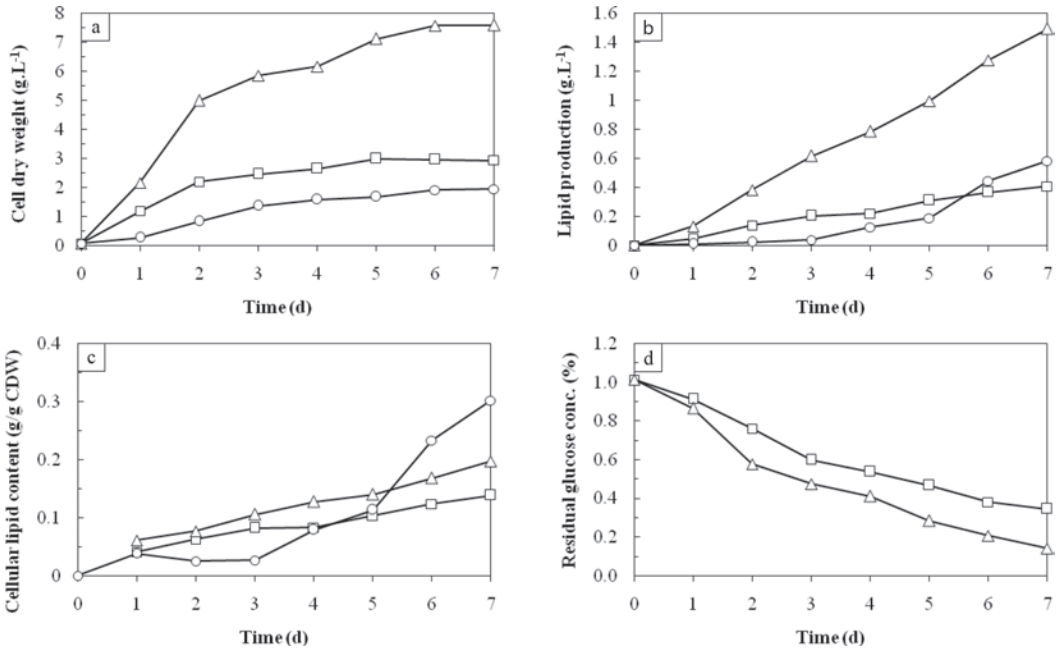


Figure 1 Time course of *C. sorokiniana* 1019 grown under three culture conditions—namely, phototrophic (-○-), heterototrophic (-□-) and mixotrophic (-△-): (a) Biomass; (b) Lipid production; (c) Cellular lipid content (CDW = Cell dry weight); and (d) Residual glucose concentration.

and high lipid accumulation at the beginning of the growth phase in response to the addition of glucose. However, it seemed that 1% glucose could support growth for only 2 d and beyond that, the algal biomass appeared to be unchanged although lipid accumulation increased slightly toward day 7. Residual glucose concentrations were slightly diminished after day 3 (Figure 1d) which explained the decline of further growth.

Under phototrophic conditions, *C. sorokiniana* 1019 and several microalgae showed moderate to high cellular lipid contents (Table 1) but their low biomass contributed to relatively low lipid yields and rendered them unattractive for commercial production. A high biomass and lipid content are necessary and can be achieved with the additional carbon sources as shown by the strain 1019 in the mixotrophic mode.

Effect of carbon sources and carbon concentration on growth and lipid accumulation of *C. sorokiniana* 1019

There are several organic carbon types that can be used for cultivation of *Chlorella* species (Table 1) but they should be readily available and not in competing demand for human consumption. In this study, the three carbon sources (glucose, glycerol and potassium acetate) had different levels of influence on growth and lipid accumulation by *C. sorokiniana* 1019. As shown in Figure 2, among the three compounds, the maximum biomass was obtained from the medium supplemented with glucose, whereas the highest cellular lipid content was attained from the acetate medium. The high biomass achieved from glucose resulted in the highest overall lipid production. This was probably due to its simple organic carbon source that could be easily assimilated into the cells and metabolized to energy and cell mass. On the other hand, while acetate might not have supported

growth very well, it could be utilized directly in the fatty acid synthesis pathway (Buchanan *et al*., 2000; Qiao and Wang 2009). Hu and Goa (2003) showed that in *Nannochloropsis* sp., acetate might play a role in increasing the buffering capacity of the system rather than participating in metabolic improvement.

Several microalgae have been reported to utilize glycerol for growth and lipid and pigment production (Liang *et al*., 2009). Chen and Walker (2011) reported that the biomass and lipid concentration of *C. protothecoides* were improved

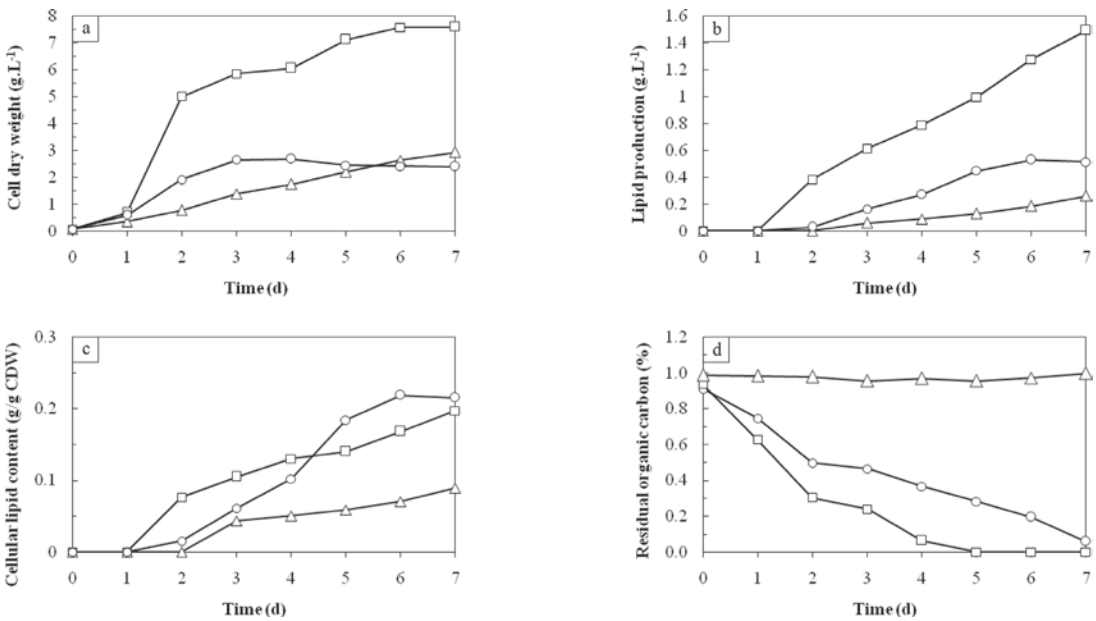


Figure 2 Time course of *C. sorokiniana* 1019 grown under mixotrophic culture conditions with 1% each of glucose (-□-), glycerol (-△-) and potassium acetate (-○-): (a) Biomass; (b) Lipid production; (c) Cellular lipid content (CDW = Cell dry weight); and (d) Residual organic carbon concentration.

Table 2 Fatty acid composition (% of total fatty acid) of *Chlorella sorokiniana* 1019 grown under various culture conditions.

Fatty acid composition	Culture conditions and carbon source			
	Phototrophic (n=7)	Heterotrophic with glucose (n=7)	Mixotrophic with glucose (n=18)	Mixotrophic with acetate (n=18)
Palmitic acid	37.80 ± 7.42	38.50 ± 4.02	22.70 ± 2.78	30.40 ± 3.44
Stearic acid	4.30 ± 1.81	5.60 ± 1.93	2.10 ± 0.72	14.70 ± 5.22
Oleic acid	16.00 ± 2.49	5.90 ± 0.71	9.20 ± 1.86	19.00 ± 3.08
Linoleic acid	28.40 ± 9.55	21.90 ± 2.60	32.60 ± 3.60	22.00 ± 4.34
α-Linolenic acid	6.30 ± 2.61	14.70 ± 2.30	14.40 ± 1.71	6.60 ± 3.42
Others	7.10 ± 3.05	13.40 ± 6.53	18.90 ± 1.23	7.40 ± 3.42

Data are reported as mean ± SD.

Others = Myristic acid, palmitoleic acids and two unknown fatty acids.

when using crude biodiesel glycerol as the carbon substrate under heterotrophic culture condition. However, in the present study, *C. sorokiniana* 1019 appeared to be unable to use glycerol as a carbon source (Figure 2d); glycerol in the culture medium was not assimilated at all. Microalgal growth might have resulted mainly from photosynthetic activity.

The effects of the levels of glucose and potassium acetate concentration on growth and lipid production of *C. sorokiniana* 1019 are shown in Figure 3. There were no apparent differences in the levels of both the growth and lipid content among the three glucose concentrations applied. In contrast, the effect of high glucose concentration on growth was observed with *C. vulgaris* and *C. protothecoides* (Xiong *et al*., 2008; Liang *et al*., 2009). However, the lipid yields of all glucose samples tested were highest at 1.38 to 1.49 $g.L^{-1}$. High acetate concentrations at 2 and 3% seemed to suppress growth, as it is well known that the acetate ion is normally toxic to most microorganisms. However, the cellular lipid content of *C. sorokiniana* 1019 in the medium containing 3% acetate abruptly increased to 56.5% after 6 d of cultivation, although the overall lipid yield under this condition was at a moderate level of 0.834 $g.L^{-1}$. However, the acetate residue at about 1.3% remaining in the culture medium was not acceptable (Figure 3d).

Effect of culture condition on fatty acid composition of *C. sorokiniana* 1019

The fatty acid profiles of *C. sorokiniana* 1019 grown under the three culture modes are shown in Table 2. Palmitic acid made up the largest proportion in this algal growth under phototrophic and heterotrophic conditions, while linoleic acid was the second principal acid. It should be noted that the proportion of oleic acid in phototrophic cells (16%) was reduced substantially compared to heterotrophic conditions (5.9%). Consequently,

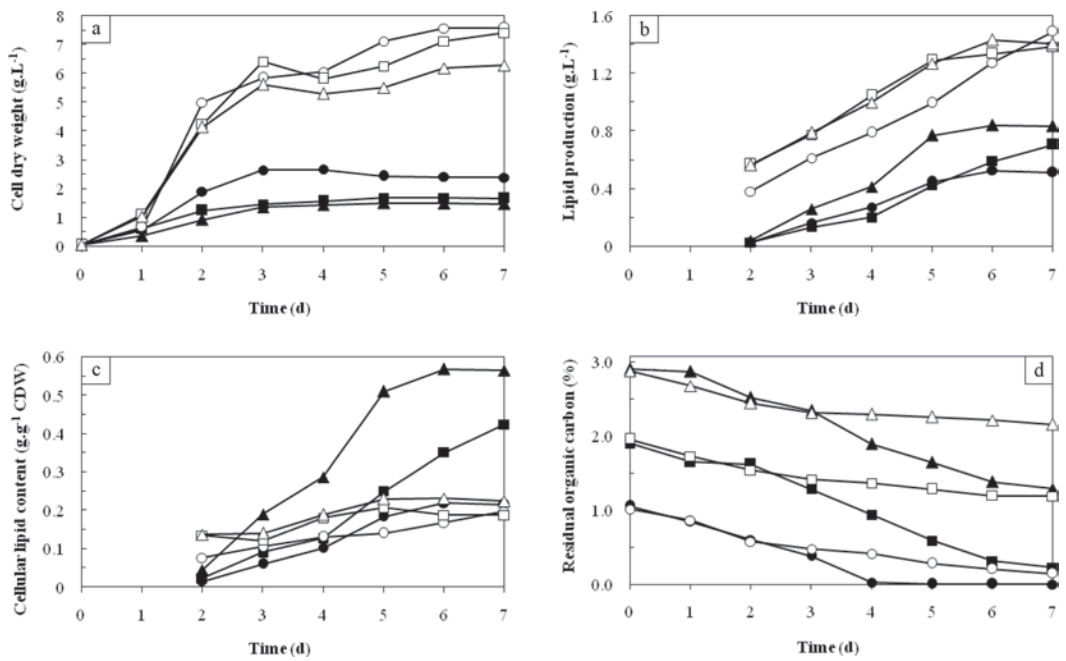


Figure 3 Time course of *C. sorokiniana* 1019 grown under mixotrophic culture conditions with glucose at 1% (-○-), 2% (-□-), 3% (-△-) and potassium acetate at 1% (-●-), 2% (-■-), 3% (-▲-): (a) Biomass; (b) Lipid production; (c) Cellular lipid content (CDW = Cell dry weight); and (d) Residual organic carbon concentration.

there was a rise in the content of α-linolenic acid which was a result of further chain elongation of the two former fatty acids. The fatty acid composition of strain 1019 was slightly changed under mixotrophic conditions. It appeared that the amounts of palmitic, linoleic and α-linolenic acid were compromised compared to the two former conditions. The percentage of palmitic acid was reduced by approximately 10–15% while the contents of linoleic and α-linolenic acid increased by the same proportion (Table 2).

CONCLUSIONS

Among the three cultivation modes, the growth and lipid content of *C. sorokiniana* 1019 were significantly enhanced under mixotrophic conditions in a nitrogen-limited medium. Glucose was more effective in promoting the growth, achieving a biomass amount of 7.58 $g.L^{-1}$, while the lipid content decreased substantially (19.7%). However, the overall lipid production remained highest among the conditions tested. Acetate produced normal growth but enhanced the lipid content substantially (56.5%). Glycerol produced negative effects on both the growth and lipid production. Under phototrophic and heterotrophic conditions, palmitic acid constituted the largest proportion in the profile while linoleic was prominent under mixotrophic conditions.

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