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Kelp waste extracts combined with acetate enhances the biofuel characteristics of *Chlorella sorokiniana*

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HIGHLIGHTS

- Higher biomass and lipid yields in cells were acquired by co-substrates culture.
- The synthesis of C18:3n3 in cells was markedly reduced by co-substrates culture.
- The synthesis of C18:1n9c in cells was stimulated by co-substrates culture.
- Higher carbohydrate content in cells was induced by co-substrates culture.
- The biofuel property of *C. sorokiniana* was enhanced by co-substrates culture.

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ABSTRACT

To probe the effect of kelp waste extracts (KWE) combined with acetate on biochemical composition of *Chlorella sorokiniana*, the cultures were performed under independent/combined treatment of KWE and acetate. The results showed that high cell density and biomass were obtained by KWE combined with acetate treatments, whose biomass productivity increased by 79.69–102.57% and 20.04–35.32% compared with 3.0 g L^{-1} acetate and KWE treatments respectively. The maximal neutral lipid per cell and lipid productivity were gained in KWE combined with 3.0 g L^{-1} acetate treatment, which increased by 16.32% and 129.03% compared with 3.0 g L^{-1} acetate, and 253.35% and 70.74% compared with KWE treatment. Meanwhile, C18:3n3 and C18:2n6c contents were reduced to 4.90% and 11.88%, whereas C16:0 and C18:1n9c were improved to 28.71% and 37.76%. Hence, supplementing appropriate acetate in KWE cultures is supposed to be a great potential method for large-scale cultivation of *C. sorokiniana* to generate biofuel.

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1. Introduction

Microalgae have a great potential to produce substantial amounts of biomass, lipids and carbohydrate suitable for biodiesel or bioethanol production (Ho et al., 2012). Green unicellular microalgae have been proposed as a potential renewable biofuel feedstock for a long time and attract considerable interests in bio-energy (Pittman et al., 2011). However, the economic viability of microalgal biofuel production greatly rests with microalgae species and strains for their own properties and culture conditions (Pulz and Gross, 2004). *Chlorella* is one of the promising microlagal species for biofuel production (Spolaore et al., 2006; Zhang et al., 2014). To date, many researches focused on optimizing conditions to induce high lipids accumulation in microalgae, such as nutrient deficiency (Mujtaba et al., 2012; Sun et al., 2014), high salinity (Yang et al., 2014) and high light intensity (He et al., 2015a; Ho et al., 2012), or to stimulate microalgae growth to gain high biomass, such as supply of single carbon sources (Najafabadi et al., 2015; Perez-Garcia et al., 2011a). However, a main shortcoming of these approaches is that the simultaneous improvement on high biomass and lipid productivities cannot be achieved (Ho et al., 2014). Screening the method to obtain high lipid and biomass productivity in microalgae is still needed.

Kelp waste is the residue after extracting alginate from kelp. Considering the low extracting efficiency and the worldwide intensive requirements for alginate production, a great quantity of kelp residues are being produced each year in China (Yue et al., 2014).

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These residues still contain massive crude fiber, protein, as well as residual alginic acid. The degradation of these substances could generate a mass of organic nutrients and nutrient salts, which could be used to stimulate the growth of plant or microbe (Möller and Smith, 1998; Zhang et al., 2010). Moreover, most of these solid wastes are directly released with the landfill in recent years, which gives rise to serious waste of resources and occupation of much land (Zhang et al., 2010). It has been illustrated, in previous study (Zheng et al., 2016), that 8.0% of kelp waste extracts (KWE) substantially improved biomass yield, lipid content and fatty acids composition of *C. sorokiniana*. However, the content of C18:3n3 in treated cultures is higher and cannot meet the requirement of European standard EN 14214 for biodiesel production (The permissible level of C18:3n3 is $\leqslant$12%). Thus, the reuse of kelp waste has great promise in alleviating waste pollution and developing large-scale culture of microalgae for biofuel production.

The pre-experiments found that the marked reduction of linolenic acid (C18:3n3) in *C. sorokiniana* was observed under the presence of acetate, while the biomass productivity was not dramatically stimulated in this research. It was also found that the decrement of C18:3n3 through supply of acetate cannot be achieved when the equivalent glucose as carbon source was added. Acetate is one of the most commonly used carbon sources for microalgae cultivation, which is a readily available and inexpensive substrate originated from various industrial applications (Perez-Garcia et al., 2011b). De-Bashan et al. (2005) pointed out that acetate was the most economical supplement for wastewater treatment using microalgae. It has been confirmed that, to some extent, the growth and lipid content could be improved in the presence of acetate (Liang et al., 2009; Najafabadi et al., 2015; Perez-Garcia et al., 2011b). Moreover, acetate has been used for industrial-scale cultivation of microalgae (Perez-Garcia et al., 2011a). The flux of acetate from chloroplast to cytoplasm is closely related to the acetyl-CoA transport, which has a great importance in fatty acids synthesis of *Chlorella* (Fan et al., 2015; Zuniga et al., 2016). Hence, the combined effect of KWE and acetate in *C. sorokiniana* was approached to improve the biomass yield and fatty acids compositions simultaneously in this study. This research aims to explore an alternative measure to cultivate *C. sorokiniana* with high biomass and lipid productivity coupled with low level of C18:3n3.

2. Material and methods

2.1. Algal strains and cultivation conditions

A strain of *Chlorella sorokiniana* (FACHB-275) provided by Institute of Hydrobiology, Chinese Academy of Sciences was used in this research. Cells were cultivated in Bold's Basal medium (BBM) (Najafabadi et al., 2015). The working volume was 200 mL in 250 mL flask. The cells were grown at 25 ± 3 °C with cool-white fluorescent lamps (45 μmol m^{-2} s^{-1}) and a 14/10 h light/dark cycle. Each flask was shaken three times every day.

2.2. Experimental design

Kelp waste extracts (KWE) was prepared with enzymolysis as described by Zheng et al. (2016). The composition of BBM and KWE (Zheng et al., 2016) was shown in Table 1. To explore the combined effect of KWE and acetate (Ac) on the growth and biochemical compositions of *C. sorokiniana*, cultures were incubated with 8.0% (v/v) KWE and different concentrations of acetate (0.5, 1.0, 2.0 and 3.0 g L^{-1}). The inoculum was ~10% (v/v) microalgae cultures with an initial cell density of $1.42 \pm 0.13 \times 10^6$ cells mL^{-1}. The following two experiments were carried out in this study. Treatment without KWE and acetate was set as the control. Each set of treatments was conducted in triplicate.

Table 1
The composition of Bold's Basal medium (BBM) and kelp waste extracts (KWE).

BBM		KWE	
$NaNO_3$ (mg L^{-1})	250.00	N (mg L^{-1})	5723.93 ± 75.21
$MgSO_4 \cdot 7H_2O$ (mg L^{-1})	75.00	P (mg L^{-1})	5529.45 ± 33.94
NaCl (mg L^{-1})	25.00	K (mg L^{-1})	60.54 ± 0.43
K_2HPO_4 (mg L^{-1})	75.00	Ca (mg L^{-1})	54.91 ± 4.51
KH_2PO_4 (mg L^{-1})	175.00	Mg (mg L^{-1})	75.64 ± 5.94
$CaCl_2 \cdot 2H_2O$ (mg L^{-1})	25.00	Fe (mg L^{-1})	ND
$ZnSO_4 \cdot 7H_2O$ (mg L^{-1})	8.82	Mn (mg L^{-1})	0.65 ± 0.06
$MnCl_2 \cdot 4H_2O$ (mg L^{-1})	1.44	Cu (mg L^{-1})	0.04 ± 0.09
MoO_3 (mg L^{-1})	0.71	Zn (mg L^{-1})	8.30 ± 1.75
$CuSO_4 \cdot 5H_2O$ (mg L^{-1})	1.57	B (mg L^{-1})	6.04 ± 0.85
$Co(NO_3)_2 \cdot 6H_2O$ (mg L^{-1})	0.49	Amino acids (mg L^{-1})	194.03 ± 0.75
H_3BO_3 (mg L^{-1})	11.42	Reducing sugars (g L^{-1})	19.55 ± 0.13
EDTA (mg L^{-1})	50.00	Total sugars (g L^{-1})	23.19 ± 0.65
KOH (mg L^{-1})	31.00	Alginic acid (g L^{-1})	6.09 ± 0.44
$FeSO_4 \cdot 7H_2O$ (mg L^{-1})	4.98		
H_2SO_4 (conc., mL)	1.00		

Experiment 1: This experiment aimed to compare the independent impacts of 8.0% KWE and various acetate concentrations on the growth performance, lipid accumulation and fatty acids composition of *C. sorokiniana*. The treatments were individually conducted as 8.0% KWE, 0.5 g L^{-1} Ac (Ac1), 1.0 g L^{-1} Ac (Ac2), 2.0 g L^{-1} Ac (Ac3) and 3.0 g L^{-1} Ac (Ac4), respectively.

Experiment 2: To probe the effects of KWE combined with different concentrations of acetate on the growth and lipid accumulation of *C. sorokiniana*, the treatments were performed as follows: 8.0% KWE; Group 1 (G1), 8.0% KWE + 0.5 g L^{-1} Ac; Group 2 (G2), 8.0% KWE + 1.0 g L^{-1} Ac; Group 3 (G3), 8.0% KWE + 2.0 g L^{-1} Ac and Group 4 (G4), 8.0% KWE + 3.0 g L^{-1} Ac.

2.3. Measurement of cell density and pigments

To evaluate the growth kinetics of *C. sorokiniana* under different treatments, cell density and chlorophylls of cultures were determined in this study. The number of cells was counted using a hemacytometer under an optical microscope (Nikon YS 100, Japan). The specific growth rate (μ) was calculated according to the following equation:

$$\mu(/d) = (\ln X_2 - \ln X_1)/(t_2 - t_1),$$

where X_1 and X_2 are the cell density at time t_1 and t_2 respectively.

The content of chlorophyll (a + b) was determined using a modified methanol method (Lichtenthaler and Wellburn, 1983). The process was as described by Zheng et al. (2016). The absorbance of extracts at 653 and 666 nm was analyzed with SpectraMax M5 Microplate Reader (Molecular Device, USA).

2.4. Measurement of neutral lipid content

The content of neutral lipids was measured by Nile red staining method. Cells were stained using a modified method described by Kimura et al. (2004). Each sample of 1 mL cultures was mixed with 330 μL dimethyl sulfoxide (25%, v/v) and sonicated for 2 min using an ultrasonic cleaner (KQ5200B, China). Then 15 μL Nile red (0.1 mg mL^{-1} acetone) was added into the mixture, which was stained at 40 °C for 10 min in a water bath. The fluorescence intensity of mixture was analyzed by a SpectraMax M5 Microplate Reader with the wavelengths of excitation and emission at 480 nm and 575 nm respectively.

2.5. Determination of biomass, total lipids content and lipid classes

The algal cells at a stationary phase were collected by centrifugation at 4 °C, 6800 *g* for 10 min and dried by a lyophilizer for 48 h. The weight of pellets was recorded to calculate biomass productivity. The content of total lipids was measured with chloroform-methanol based on a modified method (Bligh and Dyer, 1959). The process was as described by Zheng et al. (2016). The lipid content was assessed gravimetrically and expressed as dry cell weight percentages (% DCW). The lipid extracts were also used to analyze the lipid classification.

Lipid classification was conducted with a solid phase extraction column (Silica cartridges, Waters). The neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLs) were successively eluted with three different eluents. The preparation of eluents and the elution process were as described by Zhang et al. (2016). The content of each lipid fraction was calculated according to the differential gravimetric analysis and expressed as total lipids percentage (% TL).

2.6. Analysis of fatty acids composition

To analyze the composition content of fatty acids in microalgal cells with a gas chromatography (GC; Thermo Trace GC Ultra, USA) method, fatty acids were primarily transformed into fatty acid methyl esters (FAME) with H_2SO_4-CH_3OH (2.0%, v/v). An aliquot of 25 mg dried microalgal powder was added into a 4 mL gas bottle with 2 mL H_2SO_4-CH_3OH and incubated in a water-bath at 80 °C for 60 min (Kumari et al., 2011). Then 1 mL distilled water and 1 mL n-hexane were added into the gas bottle, and the mixture was separated into two phase. The n-hexane layer was collected by centrifugation at 1700 *g* for 5 min to analyze the fatty acid profile. The process of GC was performed as described by Zheng et al. (2016). Based on the retention time of a Supelco 37 component FAME mix (Sigma-Aldrich, USA), chromatograph peaks of each sample were identified. The content of each fatty acid was calculated according to the peak area and expressed as total FAME percentages (% of total FAME). All samplings were performed in triplicate.

2.7. Analyzing the biochemical composition using Fourier transform infrared spectroscopy (FT-IR)

To further explore the changes of lipids, protein and carbohydrate contents in *C. sorokiniana*, the FT-IR analysis was conducted as reported by Feng et al. (2013). A sample of 2 mg dried microalgal cells was mixed with 200 mg potassium bromide (KBr) and ground for 5 min in agate mortar. Then, approximately 100 mg of the mixture was pressed into a tablet with a sheet embosser. The absorbance spectrum between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans was recorded and analyzed using Thermo Scientific OMNIC software (Thermo Nicolet 380, USA). The contents of lipid, protein and carbohydrate in algal cells were quantitatively determined according to the bands around 3000–2800, 1650 and 1200–950 cm^{-1} (Meng et al., 2014). To minimize the difference between the absorption peaks caused by the inhomogeneous thickness of tablet, the peak around 1724–1585 cm^{-1} represented amide I was considered as an internal reference to evaluate the lipid and carbohydrate contents relative to protein. Based on the peak area ratio, relative lipid content and relative carbohydrate content were expressed as lipid/amide I and carbohydrate/amide I respectively.

2.8. Measurement of carbohydrate content

To further validate the result of FT-IR analysis, the carbohydrate content was measured using a modified Phenol-sulfuric acid method (Dubois et al., 1956). Before measuring carbohydrate content, each sample of 10 mL cultures was repeatedly freeze-thawed for three times and sonicated for 10 min in an ice bath. Then the supernatants were collected by centrifugation at 4 °C, 6800 *g* for 10 min. One mL of supernatants was mixed with 0.5 mL phenol solution (6.0%, v/v) and 2.5 mL concentrated sulfuric acid. Then the mixture was incubated at 45 °C for 30 min in a water bath. The absorbance of mixture at 490 nm was measured by SpectraMax M5 Microplate Reader.

2.9. Statistical analysis

All experiments were conducted in triplicates. The data were expressed as means ± standard deviation (SD). Statistical significance was evaluated by the analysis of variance (ANOVA) with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) with a threshold values (α) of 0.05. All figures were created by origin 8.5 (OriginLab Corporation, Northampton, MA).

3. Results and discussion

3.1. Effect of KWE and acetate on the growth of C. sorokiniana

The cell density and specific growth rate of *C. sorokiniana* exhibited different responses to various treatments (Fig. 1). As shown in Fig. 1a, under the conditions of acetate treatment, the highest cell density (7.10×10^7 cells mL^{-1}) was obtained by the treatment of Ac4, which was slightly higher than that of Ac3 treatment (6.63×10^7 cells mL^{-1}). However, it was markedly lower than that of KWE treatment (9.45×10^7 cells mL^{-1}). Similarly, the specific growth rate of *C. sorokiniana* treated with KWE was dramatically higher than that of cells treated by acetate (Fig. 1b). The difference of the specific growth rate among acetate treatments was non-significant within 4 d. However, the specific growth rate of Ac1 treatment was notably lower than that of other acetate treatments after cultivation for 5 d.

Interestingly, further enhancement on cell density of *C. sorokiniana* was observed under KWE combined with acetate treatments (Fig. 1c). The maximal cell density (11.42×10^7 cells mL^{-1}) of *C. sorokiniana* was achieved in treatment of G2. The cell density in treatment of KWE was markedly lower than that of G1-G4 treatments after incubation for 7 d. The optimal specific growth rate of *C. sorokiniana* was also gained at G2 treatment (Fig. 1d). However, except for the first day, there was no significant discrepancy between KWE and combination treatments on the specific growth rate of *C. sorokiniana*. These improvements on the cell density and specific growth rate of *C. sorokiniana* by cooperating of KWE with appropriate acetate, compared with the corresponding independent treatment, indicated the response of *C. sorokiniana* followed Monod-type kinetics with increasing substrate concentration (Kumar et al., 2014).

It was found that biomass and biomass productivity of *C. sorokiniana* under different treatments were inclined to increase with the augmenting concentration of acetate (Fig. 2). As shown in Fig. 2a, the increments of biomass (0.76 g L^{-1}) and biomass productivity (84.62 mg L^{-1} d^{-1}) in treatment of Ac4 were significantly higher than that of other acetate treatments compared with the control. However, they were markedly lower than those of KWE treatment. Higher biomass and biomass productivity of *C. sorokiniana* were obtained under treatment of KWE compared with acetate treatments. Interestingly, further improvement on biomass and biomass productivity in *C. sorokiniana* was observed when KWE was combined with different concentrations of acetate (Fig. 2b). The optimal biomass (1.54 g L^{-1}) and biomass productivity (171.41 mg L^{-1} d^{-1}) of *C. sorokiniana* were achieved in G4 treat-

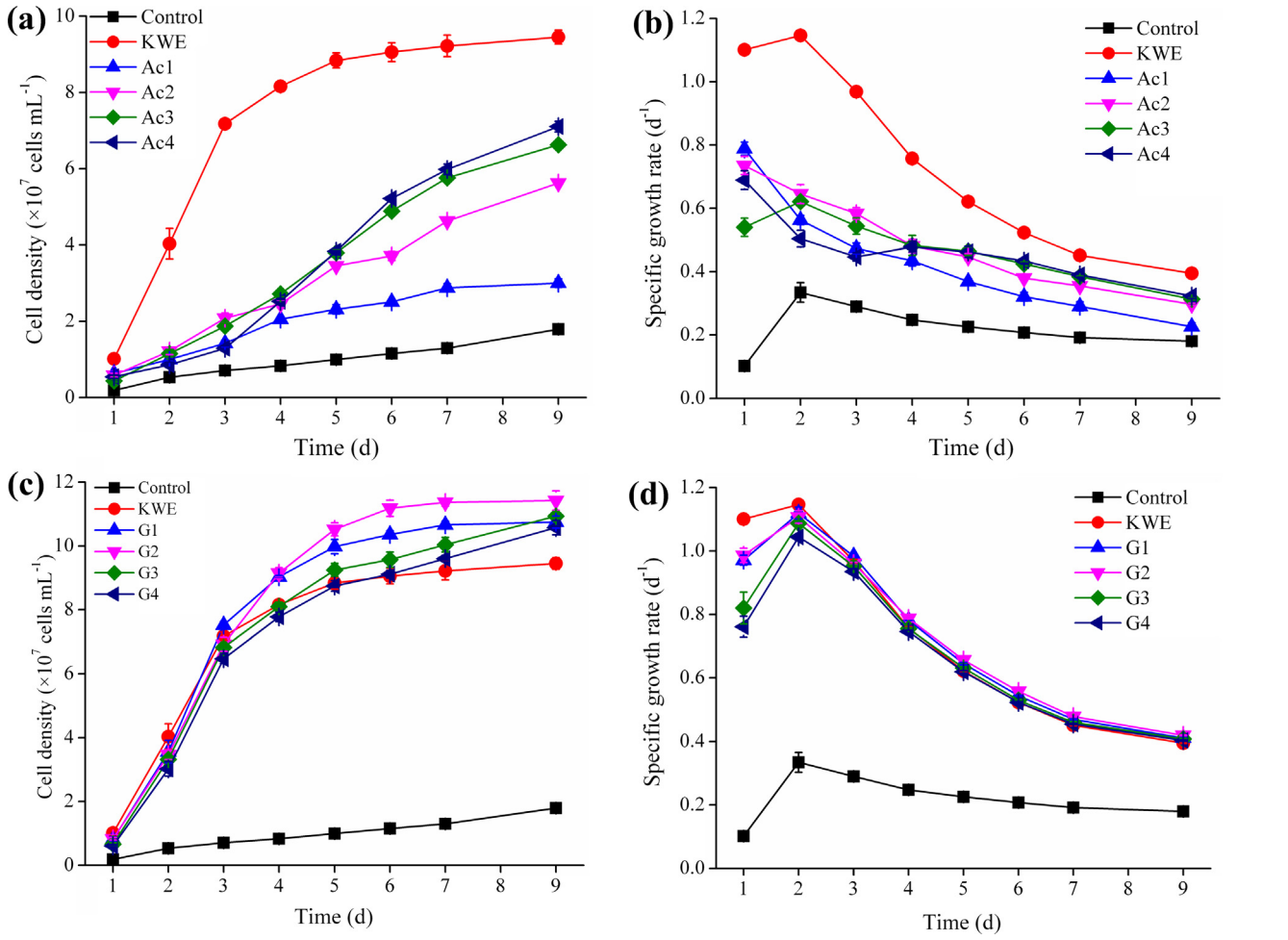

Fig. 1. Growth kinetics of *C. sorokiniana* under various treatments. (a) and (b) are the time-course changes of cell density and specific growth rate of *C. sorokiniana* under acetate treatment, where Ac1-Ac4 consecutively represents the different concentrations of acetate (0.5, 1.0, 2.0 and 3.0 g L^{-1}, w/v). (c) and (d) are the time-course variations of cell density and specific rate of *C. sorokiniana* under treatment of KWE combined with acetate, where G1-G4 orderly represents the different combinations of KWE and acetate. The means of Ac1-Ac4 and G1-G4 in the following figures are the same.

ment, which increased by 0.78 g L^{-1} and 86.79 mg L^{-1} d^{-1} compared with Ac4 treatment, and 0.40 g L^{-1} and 44.74 mg L^{-1} d^{-1} compared with KWE treatment. These were 124.39% and 149.32% higher than that of the treatment with supplementing 2 g L^{-1} glucose as carbon source in a local isolate *C. sorokiniana* (Juntila et al., 2015). These results elucidate that the desired biomass productivity could be achieved under treatment of KWE combined with moderate concentration of acetate.

Compared with the control, the content of chlorophyll (a + b) in treated cultures has increased notably (Fig. 3). Higher chlorophyll (a + b) content in cells was obtained under Ac2 treatment compared with other acetate treatments in 5–9 d (Fig. 3a). However, chlorophyll (a + b) content was further enhanced by the treatment of KWE, which was significantly higher than that of all acetate treatments during the cultivation time. Surprisingly, the contents of chlorophyll (a + b) in treated cells with G1 and G2 were far higher than that of KWE treatment in 5–9 d (Fig. 3b). There was no significant difference between independent KWE and G3-G4 treatments in the culture time. This may be due to substrates inhibition in G3-G4 treatments impeding the synthesis of chlorophylls (Abreu et al., 2012; Liu et al., 2008). Yan et al. (2012) pointed out that the reduction of chlorophylls in mixotrophic cells cultured with 2 g L^{-1} acetate or 4 g L^{-1} glucose resulted from the inhibition by organic carbon. These results imply that the optimal chlorophyll (a + b) content in cultures was achieved by G2 treatment. This trend was in accordance with the changes of cell density in treated cultures.

Above results indicate that KWE combined with appropriate acetate could further improve the growth and chlorophylls content of *C. sorokiniana*, compared with the independent treatment of KWE and acetate. It may be largely attributed that more available carbon, nitrogen (N) and phosphorus (P) sources in combination treatments were supplied for microalgae growth. It has been reported that KWE contained massive organics such as soluble sugars, amino acids and diverse mineral elements (Zheng et al., 2016). Reducing sugars were the main composition of soluble sugars in KWE. And nitrogen (N) was the most abundant macroelement in KWE, followed by phosphorus (P). KWE could provide abundant carbon (C), N and P sources for the growth and lipid accumulation of microalgae. However, there is no carbon source in BBM, which consists of N, P, potassium (K), calcium (Ca), magnesium (Mg) and other essential trace elements. BBM cannot supply carbon source for stimulating the growth of microalgae. The contents of N and P in BBM are significantly lower than those of KWE. Through comparing the effects of carbon, nitrogen and phosphorus sources in KWE on microlagae growth, it was found that carbon source in KWE was the main factor to stimulate the growth of *C. sorokiniana*. The positive effect induced by nitrogen source was markedly lower than that of carbon and phosphorus sources. The C/N ratio of culture solution was increased by supplementing KWE cultures with

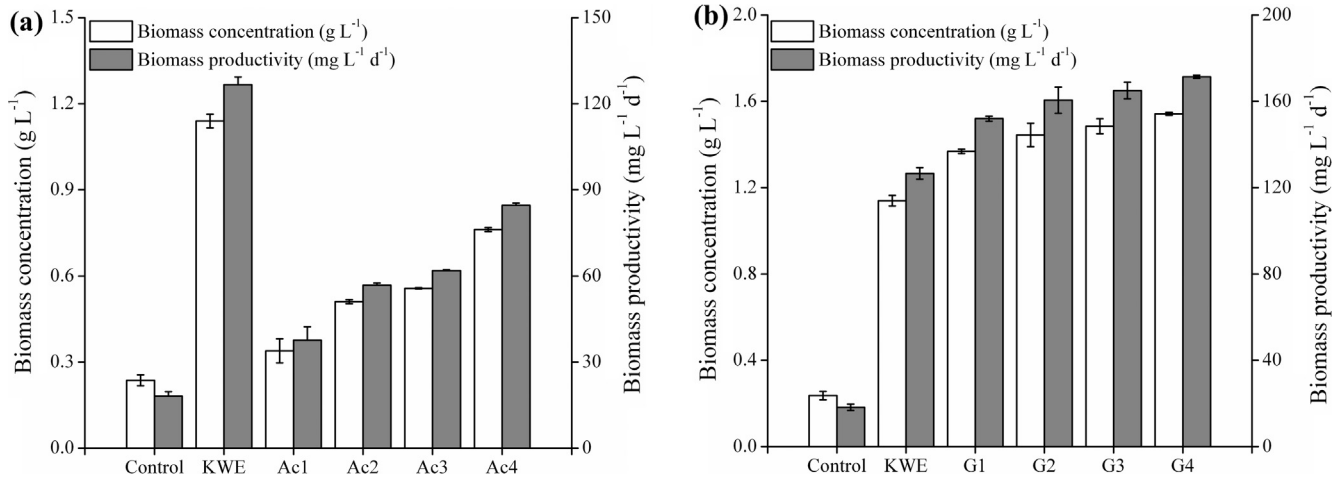

Fig. 2. Biomass changes of *C. sorokiniana* under treatments of acetate (a) and KWE combined with acetate (b).

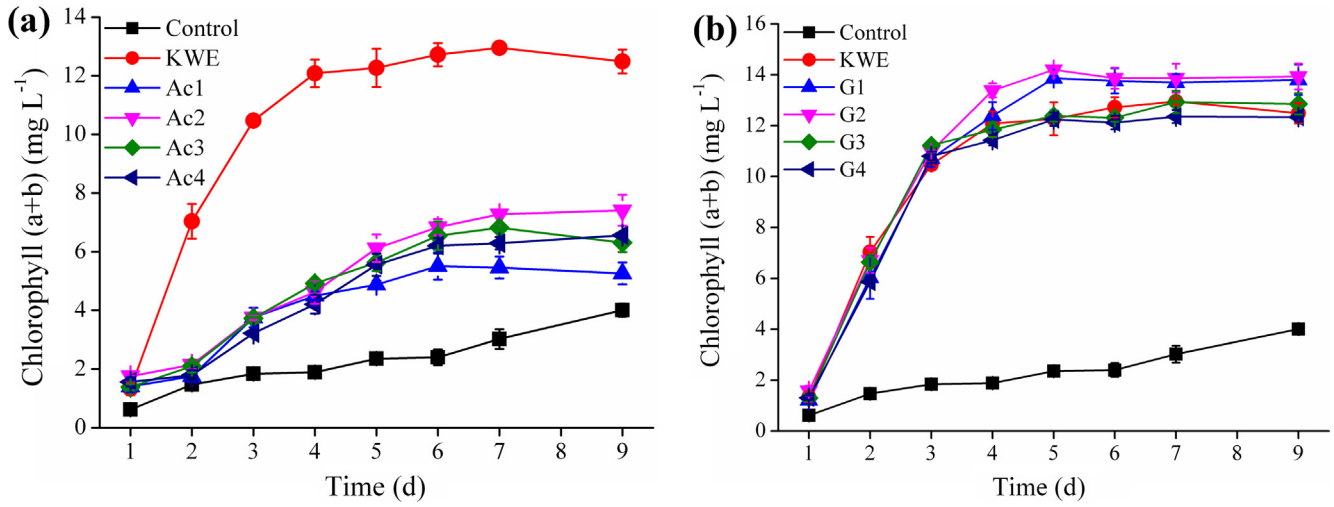

Fig. 3. The chlorophyll (a + b) content of *C. sorokiniana* under treatments of acetate (a) and KWE combined with acetate (b).

acetate, which could improve the biomass production and lipid accumulation (Bhatnagar et al., 2011; Najafabadi et al., 2015).

3.2. Effect of KWE and acetate on lipid accumulation of C. sorokiniana

Microalgal lipids are comprised of neutral lipids (NLs) and polar lipids including glycolipids (GLs) and phospholipids (PLs). NLs are the main feedstock of biodiesel production (Damiani et al., 2010). The enhancements on NLs and total lipid contents in *C. sorokiniana* were observed in all treatments compared with the control (Fig. 4). The content of NLs was indicated by the relatively fluorescence intensity of Nile red staining cultures. As shown in Fig. 4a and b, the synthesis of NLs in cells was dramatically stimulated by all treatments compared with the control. Higher NLs per volume or per cell of microalgae was obtained in treatment with high concentration of acetate. The NLs per volume of cultures in treatment of KWE was higher than that of Ac1 and Ac2 treatments (Fig. 4a). The NLs per cell of cultures in treatment of KWE was slightly lower than that of Ac1 treatment. Further improvement on NLs per volume or per cell was attained under KWE combined with acetate treatments (Fig. 4b). The maximal NLs per volume and per cell of cultures were achieved in treatment of G4, which were 2.93 and 2.53 times higher than that of KWE treatment respectively. They were improved by 82.98% and 16.32% compared with Ac4 treatment individually.

Interestingly, the total lipid content of cultures treated by KWE was notably higher than that of Ac1-Ac3 treatments (Fig. 4c). The lipid productivity in treatment of KWE was dramatically higher than that of all acetate treatments. Further enhancement on total lipid content and lipid productivity were found under combination treatments (Fig. 4d). The lipid productivity in cells under treatment of G1-G4 was promoted by 15.14–29.18 mg L^{-1} d^{-1} compared with KWE treatment, and 41.76–55.80 mg L^{-1} d^{-1} in comparison with supplementing 2 g L^{-1} glucose as carbon source in a local isolate *C. sorokiniana* (Juntila et al., 2015). The maximal lipid productivity was gained in G4 treatment, which increased by 129.03% and 70.74% in comparison with Ac4 and KWE treatments respectively. The total lipid content of G1-G4 treatments was markedly higher than that of KWE treatment, which increased by 13.88–30.34%. However, the optimal total lipid content was obtained under G3 treatment, which was 47.90% and 79.11% higher than that of the treatments of supplementing 60 mM acetate and 20 mM glucose as carbon source in *C. sorokiniana* GXNN01 (Qiao and Wang, 2009).

Above results indicate that the contents of NLs and total lipid in *C. sorokiniana* were further improved under KWE combined with appropriate acetate treatments (Fig. 4). This may be attributed to

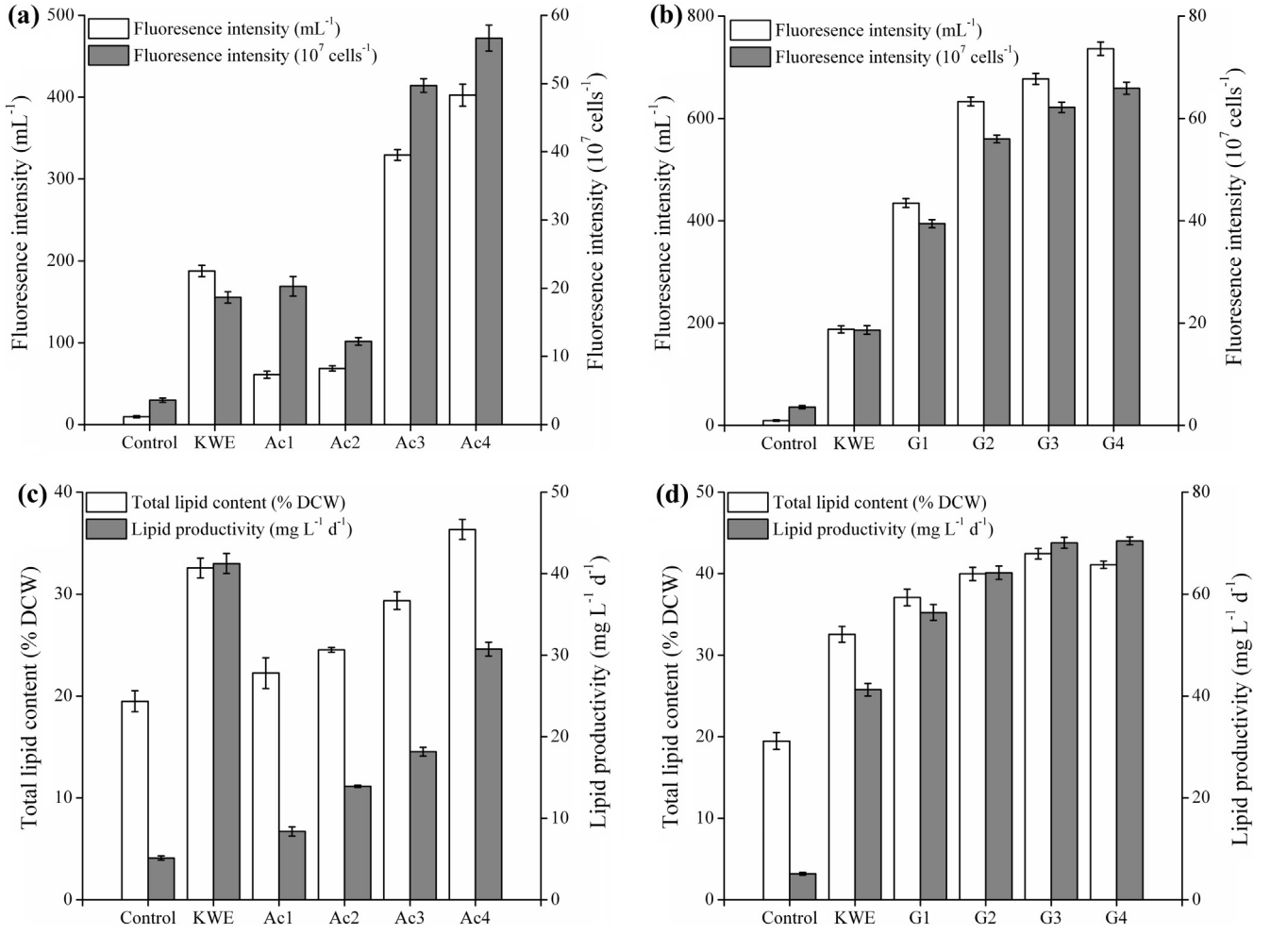

Fig. 4. The changes of neutral lipid and total lipid contents of *C. sorokiniana* under various treatments. (a) and (c) are the contents of neutral lipid and total lipid in *C. sorokiniana* in treatment of different acetate concentrations. (b) and (d) are the contents of neutral lipid and total lipid in *C. sorokiniana* under different combination treatments.

the fact that appropriate concentration of acetate and glucose contributes high lipids production in microalgae (Najafabadi et al., 2015; Xu et al., 2006). Supplementation with appropriate acetate may stimulate the synthesis of lipid in *Chlorella* (Fan et al., 2015). Under KWE combined with moderate concentration of acetate, more carbon skeletons, NADPH and ATP were supplied to generate lipids in *C. sorokiniana* (Perez-Garcia et al., 2011a). The assimilation of acetate in microalgae begins with the acetylation of coenzyme A, catalyzed by acetyl coenzyme A (acetyl-CoA) synthetase, to form acetyl-CoA (Boyle and Morgan, 2009). Then acetyl-CoA is generally oxidized metabolically through the glyoxylate cycle to form malate in glyoxysome, and through the tricarboxylic acid cycle (TCA) to citrate in the mitochondria with providing reducing power (NADPH), energy (ATP) as well as carbon skeletons (Perez-Garcia et al., 2011b). To further explain the effect of combination treatments on lipids in cells, lipid fractions were analyzed in this research. As shown in Fig. 5, the main lipid component of *C. sorokiniana* was NLs, which accounted for 46.94–64.38% of total lipids. The optimal NLs content in cells was obtained in treatment of G4, which increased by 37.17% and 20.99% compared with the control and KWE treatment respectively. Increasing acetate concentration resulted in the reduction of GLs contents. The lowest content of GLs in cultures was gained at the treatment of G4, which reduced by 37.73% and 18.64% in comparison with the control and KWE treatment. The content of PLs in all treated cells was the lowest compared with the other lipid constituents, and was non-significant discrepancy between the combination treatment and the control. These results indicate that the reduction of GLs content in *C. sorokiniana* might result from the increase in NLs synthesis at

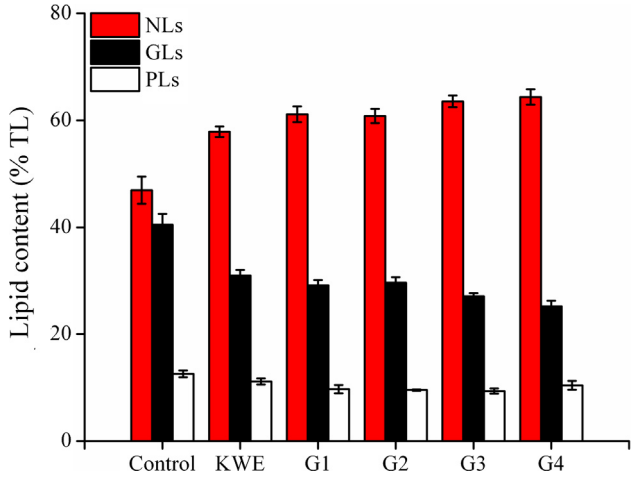

Fig. 5. The changes of different lipid classes in *C. sorokiniana* under treatment of KWE combined with acetate. NLs: Neutral lipids; GLs: Glycolipids; PLs: Phospholipids.

Table 2
The main fatty acids composition of *C. sorokiniana* under treatment of acetate (% of total FAME, n = 3).

Fatty acid (%)	Control	KWE	Ac1	Ac2	Ac3	Ac4
C16:0	21.65 ± 0.85d	29.03 ± 1.16ab	28.40 ± 0.81b	28.24 ± 0.59bc	29.81 ± 0.27a	27.18 ± 0.50c
C16:1	5.34 ± 0.02b	5.21 ± 0.15b	2.05 ± 0.69d	4.25 ± 0.45c	4.40 ± 0.63c	7.67 ± 0.42a
C18:0	0.61 ± 0.15e	2.42 ± 0.15c	1.51 ± 0.13d	1.63 ± 0.03d	5.60 ± 0.52b	19.26 ± 0.24a
C18:1n9c	23.30 ± 0.37b	24.36 ± 0.24a	21.65 ± 0.60d	22.10 ± 0.11d	22.65 ± 0.08c	20.90 ± 0.31e
C18:2n6c	16.74 ± 0.31f	22.92 ± 0.65d	24.56 ± 0.44c	27.20 ± 0.69b	31.73 ± 0.10a	19.51 ± 0.32e
C18:3n3	31.12 ± 0.83a	15.60 ± 0.47c	21.83 ± 0.60b	15.96 ± 0.80c	4.53 ± 0.16d	3.17 ± 0.32e
∑SFA	22.27 ± 0.70e	31.46 ± 1.19c	29.91 ± 0.67d	29.87 ± 0.39d	35.41 ± 0.47b	46.43 ± 0.26a
∑MUFA	28.64 ± 0.27b	29.58 ± 0.20a	23.70 ± 0.06e	26.36 ± 0.40d	27.06 ± 0.58c	28.58 ± 0.11b
∑PUFA	47.85 ± 0.37a	38.53 ± 1.08d	46.39 ± 0.73b	43.16 ± 0.07c	36.26 ± 0.09e	22.68 ± 0.05f

∑SFA: the total of saturated fatty acid; ∑MUFA: the total of monounsaturated fatty acid; ∑PUFA: the total of polyunsaturated fatty acid. Ac1-Ac4 represents 0.5, 1.0, 2.0 and 3.0 g L^{-1} (w/v) acetate treatments in sequence. The different letters within columns represent the difference is significant at a 5% level, the same as below.

the treatments of G3-G4, which was consistent with the result reported by He et al. (2015b). It has been reported that GLs contain abundant 16- and 18-carbon fatty acids, which have been considered as an important source of fatty acids and could be converted into NLs (da Costa et al., 2016; He et al., 2015b). The treatments of G3-G4 markedly facilitated the synthesis of NLs in *C. sorokiniana*.

3.3. Effect of KWE and acetate on fatty acid composition in C. sorokiniana

The biodiesel property of microalgal bioenergy greatly depends on fatty acid profile and the proportion of neutral lipid accounting for total lipids. Significant differences on fatty acid composition of *C. sorokiniana* were observed under various treatments (Tables 2 and 3). C16:0, C18:1n9c, C18:2n6c and C18:3n3 were the overriding fatty acids in *C. sorokiniana*.

The main fatty acids changes of *C. sorokiniana* under treatment of acetate were as shown in Table 2. Compared with the control, the observed marked improvements on the contents of C16:0, C18:0 and C18:2n6c were stimulated by Ac1-Ac4 treatments. The maximal contents of C16:0 and C18:2n6c were obtained in treatment of Ac3, which increased by 37.67% and 89.58%. The optimal C18:0 content was gained at Ac4 treatment, which was 30.45-fold higher than that of the control. Meanwhile, the synthesis of C18:3n3 was dramatically decreased under Ac1-Ac4 treatments. The minimal proportion of C18:3n3 (3.17%) was achieved under Ac4 treatment, which reduced by 89.81% compared with the control and was dramatically lower than the requirement of European standard EN 14214 for biodiesel production (⩽12%). This result implies that the reduction of C18:3n3 proportion might be caused by the enhancement on the syntheses of C16:0 and C18:2n6c (or C18:0 in Ac4 treatment). A similar change on fatty acids composition of *C. sorokiniana* was observed in treatment of KWE. The increments of C16:0, C18:0 and C18:2n6c proportions in total fatty acids were 34.09%, 295.86% and 36.97% compared with the control, respectively. And the decrease of C18:3n3 content was 49.86%. However, it was obvious that the effect of KWE on 18-carbon fatty acids for biodiesel production was not as efficient as Ac3-Ac4 treatments.

Interestingly, further improvement on fatty acids composition of *C. sorokiniana* was observed when KWE was combined with diverse concentrations of acetate (Table 3). The highest proportion of C16:0 in cells was obtained under G1 treatment, which increased by 50.17%, 14.51% and 11.99% compared with the control, Ac1 and KWE treatment respectively. Surprisingly, the synthesis of C18:1n9c was dramatically stimulated by G3-G4 treatments. The optimal proportion of C18:1n9c was achieved at the treatment of G4 (37.76%), which improved by 66.68–80.64% and 68.84% compared with the independent acetate and KWE treatments respectively. The minimal ratios of C18:2n6c (11.88%) and C18:3n3 (4.90%) in cultures were also obtained under G4 treatment. The proportion of C18:2n6c was markedly reduced by 39.13–62.57% and 48.20% compared with the single acetate and KWE treatments respectively. Although the proportion of C18:3n3 under treatment of G4 was higher than that of Ac3-Ac4 treatments, it was still significantly lower than the requirement of EN 14214. It was markedly decreased by 68.57% in comparison with KWE treatment, and 37.97% compared with supply of 10 g L^{-1} glucose for *C. sorokiniana* UTEX 1602 (Li et al., 2014). This result indicates that the notable improvements on C16:0 and C18:1n9c syntheses in cells could affect the syntheses of C18:2n6c and C18:3n3 under treatments of G4, particularly affecting C18:3n3 synthesis. This change of fatty acid profile was similar to the observation in *C. viscosa* and *Dunaliella salina* under continuous light condition and nutrient starvation (Lamers et al., 2012; Takeshita et al., 2014).

Fatty acid composition of biodiesel directly affects the biodiesel quality, since many biodiesel specifications (such as cetane number, oxidative stability, kinematic viscosity, heat of combustion, exhaust emissions, etc.) depend on it (Knothe, 2008). It has been reported that monounsaturated fatty acids (MUFA) are more desir-

Table 3
The main fatty acids composition of *C. sorokiniana* in treatment of KWE combined with acetate (% of total FAME, n = 3).

Fatty acid (%)	Control	KWE	G1	G2	G3	G4
C16:0	21.65 ± 0.85e	29.03 ± 1.16bc	32.52 ± 0.46a	30.31 ± 0.38b	27.62 ± 1.03d	28.71 ± 0.34 cd
C16:1	5.34 ± 0.02c	5.21 ± 0.15c	3.23 ± 0.49d	4.64 ± 0.55c	7.78 ± 1.02b	8.78 ± 0.22a
C18:0	0.61 ± 0.15e	2.42 ± 0.15d	3.58 ± 0.27c	4.14 ± 0.12b	3.43 ± 0.11c	4.66 ± 0.16a
C18:1n9c	23.30 ± 0.37e	24.36 ± 0.24d	24.31 ± 0.37d	25.34 ± 0.26c	32.02 ± 0.19b	37.76 ± 0.45a
C18:2n6c	16.74 ± 0.31c	22.92 ± 0.65a	23.64 ± 0.33a	23.23 ± 0.12a	18.57 ± 0.74b	11.88 ± 0.66d
C18:3n3	31.12 ± 0.83a	15.60 ± 0.47b	10.71 ± 0.13c	9.73 ± 0.18d	6.10 ± 0.11e	4.90 ± 0.23f
∑SFA	22.27 ± 0.70d	31.46 ± 1.19c	36.10 ± 0.60a	34.45 ± 0.26b	31.05 ± 0.99c	33.38 ± 0.37b
∑MUFA	28.64 ± 0.27d	29.58 ± 0.20 cd	27.54 ± 0.54e	29.99 ± 0.38c	39.80 ± 1.00b	46.54 ± 0.49a
∑PUFA	47.85 ± 0.37a	38.53 ± 1.08b	34.35 ± 0.46c	32.96 ± 0.08d	24.66 ± 0.81e	16.78 ± 0.84f

G1-G4 successively represents the different combination treatments between KWE and different acetate concentrations (0.5, 1.0, 2.0 and 3.0 g L^{-1}).

able than saturated fatty acids (SFA) for improving cold-flow property (Knothe, 2008). MUFA are more suitable than polyunsaturated fatty acids (PUFA) for enhancing oxidative stability, cetane number and combustion properties of biodiesel (Knothe, 2008). The above experimental results indicate that, under the single treatment of KWE and acetate, the reduction of C18:3n3 was inclined to stimulate the accumulations of C16:0, C18:2n6c or C18:0. However, the observed marked reduction of C18:3n3 and C18:2n6c syntheses in cells was achieved under G4 treatment, whereas the synthesis of C18:1n9c was dramatically stimulated. Compared with the single treatment of KWE and acetate, the decrease of PUFA in *C. sorokiniana* was further enhanced, and the content of MUFA was markedly increased under treatments of G3-G4 (Tables 2 and 3). Moreover, methyl oleate (C18:1) has been suggested to be a suitable main component of biodiesel (Knothe, 2008). In this research, it reached to 37.76% in *C. sorkiniana* under G4 treatment. Hence, the biodiesel property of *C. sorokiniana* was further improved by KWE combined with moderate concentration of acetate.

3.4. Effect of KWE and acetate on biochemical composition in microalgae

Screening microalgal strains with high lipid or carbohydrate content is vital to acquire biofuel candidates (Meng et al., 2014). FT-IR has been used to simultaneously monitor the contents of lipids, protein and carbohydrate in treated microalgal cells to depict the carbon partitioning of microalgae (Meng et al., 2014). The results of FT-IR can describe the physiological status in cultures (Meng et al., 2014). To further explore the impact of KWE combined with acetate on biofuel property of *C. sorokiniana*, the biochemical compositions in treated cells were primarily analyzed using FT-IR method in this study. As shown in Fig. 6a, there were significant differences between the treated groups and the control on ratios of lipid/amide I and carbohydrate/amide I. The ratio of lipid/amide I in treatments of G1-G4 was elevated by 0.52–0.96 and 0.26–0.44 compared with the control and KWE treatment, respectively. The maximal proportion of lipid/amide I (1.35) was obtained in treatment of G3, which was markedly higher than that of other treatments except G4. The proportion of carbohydrate/amide I in treatments of G1-G4 was improved by 0.90–1.90 and 0.48–1.01 compared with the control and KWE treatment respectively. The optimal carbohydrate/amide I ratio was also achieved under G3 treatment, which was dramatically higher than that of all other treatments.

Traditional method (Phenol-sulfuric acid) for determining the carbohydrate content was used to further validate the result of FT-IR on carbohydrate content in *C. sorokiniana*. Except for G4, the changes of carbohydrate per volume under independent KWE and G1-G3 treatments were similar to the result determined by FT-IR method (Fig. 6b). The maximal carbohydrate per volume was still gained in treatment of G3, which was 8.17-fold that of the control and 1.88-fold that of KWE treatment. Besides, the great improvement on carbohydrate per DCW was also obtained under G3 treatment, which increased by 84.25% and 122.47% compared with the control and KWE treatment respectively. The treatments of KWE and G1 couldn't increase the carbohydrate per DCW of *C. sorokiniana*. These results indicate that further improvements on lipid and carbohydrate contents in co-substrates culture resulted from addition of moderate acetate.

Biodiesel and bioethanol are two kinds of well-known biofuel products from microlagae. The bioethanol production originated from microalgae is mainly acquired by the fermentation of carbohydrates contained in microlagal biomass (Kim et al., 2011). The production of bioethanol can be processed after lipid extraction, since most carbohydrate still remain in the residual of microlagae, which is a win-win strategy for re-utilizing the waste to produce another bioenergy (Sun et al., 2014). Based on the above results, it was clear that the optimal lipid productivity and fatty acids composition in *C. sorokiniana* were gained under treatment of G4. The maximal contents of total lipid and carbohydrate were obtained at G3 treatment. Hence, desired changes on biomass, lipids, fatty acids profile and carbohydrate in *C. sorokiniana* would be achieved by replenishing 2.0–3.0 g L^{-1} acetate in KWE. Combined effect of KWE and acetate on the growth and lipid production of *C. sorokiniana* was higher than that of the independent action. Moreover, kelp waste is a massive industrial residue from alginate production in China, which indicates that the feedstock of KWE was easily available and free. The cost to produce one liter of KWE was 0.28 USD based on the used enzymes' price (Zheng et al., 2016). The price of analytical pure acetate is 0.59 USD per 500 g in China. It is much cheaper than that of glucose (1.33 USD/500 g) and bi-carbonate (0.81 USD/500 g), which are the commonly used carbon sources for microalgae growth. If the industrial-grade acetate is used, the cost will be lower. Clearly, KWE combined with appropriate acetate is an promising method for large-scale culture of microalgae to produce biofuel.

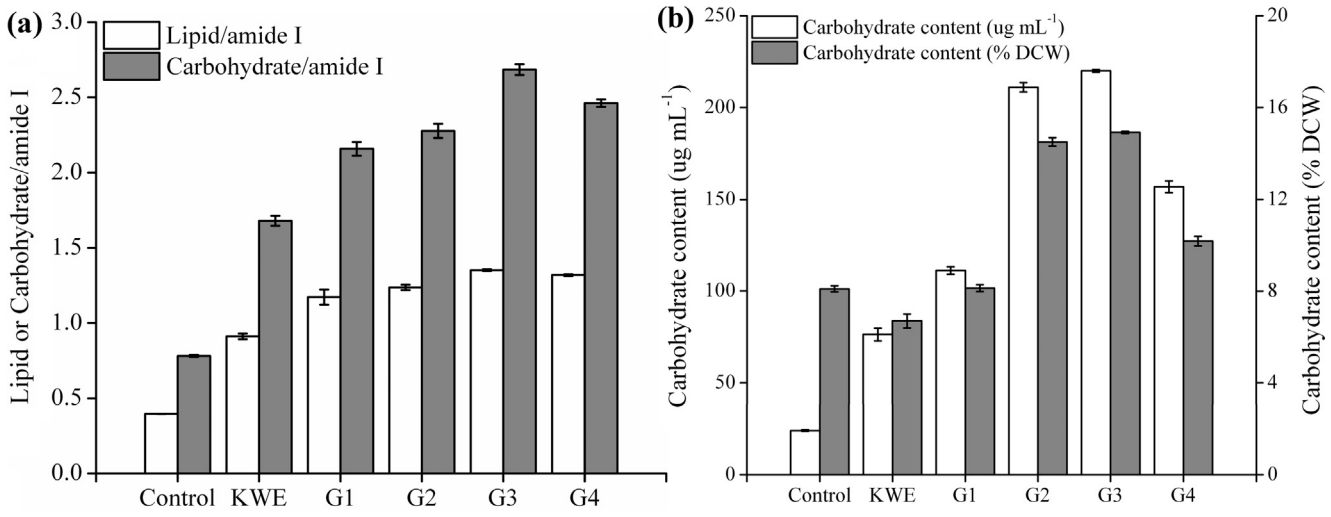

Fig. 6. The chemical compositions contents of *C. sorokiniana* under treatment of KWE combined with acetate. (a) is the result of FT-IR analysis, and (b) is carbohydrate content in *C. sorokiniana* determined with a modified phenol-sulfuric acid method.

4. Conclusions

This research illustrated that supplement of appropriate acetate in KWE cultures could further improve the biomass, lipid synthesis and fatty acid profile of *C. sorokiniana*. The increase of C16:0 and C18:1n9c in treated cells might result in the reduction of C18:2n6c and C18:3n3. Under the treatment of KWE combined with 2.0–3.0 g L^{-1} acetate, the fatty acid composition of cells was more suitable for biodiesel production than the independent treatments of KWE and acetate. This cultivation method also could dramatically improve carbohydrate production in cells. Clearly, complementing moderate acetate in KWE cultures significantly enhanced the biofuel property of *C. sorokiniana*.

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