Bioprocess and Biosystems Engineering (2019) 42:1225–1234
https://doi.org/10.1007/s00449-019-02120-x

RESEARCH PAPER

Effect of trophic conditions on microalga growth, nutrient removal, algal organic matter, and energy storage products in *Scenedesmus* (*Acutodesmus*) *obliquus* KGE-17 cultivation

Wook Jin Choi[1,2] · A. Na Chae[1] · Kyung Guen Song[1] · Joonhong Park[2] · Byung Chan Lee[3]

Received: 11 January 2019 / Accepted: 2 April 2019 / Published online: 15 April 2019

Abstract

This study compared the performance of microalga growth, nutrient removal, algal organic matter, and energy storage products in mixotrophic, photoautotrophic, and heterotrophic conditions. *Scenedesmus obliquus* was used as a model species. Mixotrophic condition showed the highest specific growth rate of 0.96 d^{-1} as well as the fastest nitrogen and phosphorus removal rate of 85.17 mg-N g-cell^{-1} day^{-1} and 11.49 mg-P g-cell^{-1} day^{-1}, respectively, compared with photoautotrophic and heterotrophic conditions. Mixotrophic microalgae had relatively higher carbohydrates and lipids contents (21.8 and 24.0%) than photoautotrophic and heterotrophic conditions. Meanwhile, algal organic matter (AOM) in the medium was produced at the highest level under photoautotrophic condition. Mixotrophic condition was more efficient in terms of microalga growth, nutrient removal, production of energy storage products, and suppression of AOM, and would be adaptable for wastewater treatment process.

Keywords Microalgae · Heterotrophic · Mixotrophic · Photoautotrophic · *Scenedesmus obliquus*

Introduction

Since Oswald proposed [1], microalgae-based wastewater treatment has been extensively studied in a wide variety of wastewaters [2–4]. Microalgae cultivation coupled with wastewater treatment is considered to be a feasible strategy for saving considerably large amounts of water and nutrients required for microalgae cultivation [5]. Furthermore, carbohydrates and lipids contained in microalgal biomass cultured for wastewater treatment could be utilized for renewable energy sources, such as bioethanol, biodiesel, and biogas [6].

As widely known, microalgae can convert both nitrogen and phosphorus into algal biomass through photosynthesis with CO_2 used as an inorganic carbon (OC) source under photoautotrophic condition. Many studies on photoautotrophic cultivation of microalgae using wastewater have been conducted to remove nutrients and to produce valuable by-products. Martinez et al.[7] cultured *Scenedesmus obliquus* in secondary treated urban wastewater under photoautotropic condition. The highest specific growth rate, and percentages of nitrogen and phosphorus elimination were 0.0438 h^{-1}, 98% and 100%, respectively and saturated fatty acids constitute a mean of 31.4% by weight of the total content of fatty acids. Sydney et al. [8] showed that photoautotrophic culture of *Botryococcus braunii* is able to remove 79.63% of N and 100% of P from treated domestic wastewater, and accumulate oil with a dry biomass of up to 36%. Schulze et al. [9] suggested that *Tetraselmis* sp. is an alternative for wastewater treatment as a replacement of the nitrification process and observed that the nutrients uptake rates were 31.4 ± 0.4 mg N L^{-1}

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00449-019-02120-x) contains supplementary material, which is available to authorized users.

✉ Kyung Guen Song
kgsong@kist.re.kr

✉ Joonhong Park
parkj@yonsei.ac.kr

[1] Center for Water Resource Cycle Research, Korea Institute of Science and Technology, Hwarangno 14-gil 5, Seongbuk-gu, Seoul 02792, South Korea

[2] Department of Civil and Environmental Engineering, Yonsei University, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, South Korea

[3] Department of Civil Engineering and Landscape Architecture, Suncheon Jeil College, Jeildaehak-gil 17, Suncheon, Cheonnam 57997, South Korea

day^{-1} and 6.66 ± 1.57 mg P-PO_4^{3-} L^{-1} day^{-1}. However, the growth of autotrophic microalgae is slower than heterotrophic or mixotrophic microalgae and they need sufficient light for cultivation. Heterotrophic and mixotrophic microalgae can grow at high rates even under limited light conditions, and can offset disadvantages of autotrophic microalgae. Furthermore, wastewater contains a lot of organic matters available for heterotrophic or mixotrophic microalgae. Thus, many investigators have been concerned with culturing microalgae in wastewater under heterotrophic or mixotrophic conditions.

Changes in carbon source and light condition due to different trophic conditions affect microalgal carbon pathways and intracellular organelle activity related to nitrogen assimilation and fatty acid synthesis [10, 11]. Many studies have shown changes in biomass productivity, lipid content, and nutrient removal with different trophic conditions. Babaei et al. reported that *Chlorella vulgaris* under mixotrophic condition fed with glucose was the appropriate condition for ammonium and nitrate removal compared with photoautotrophic and heterotrophic conditions [12]. On the other hand, heterotrophic condition with glucose was appropriate condition for *Chlorella sorokiniana* on biomass productivity and nutrient removal [13]. In case of *Scenedesmus obliquus*, mixotrophic condition with acetate was showed fastest growth rate and ammonium removal rate [11]. Ogawa and Aiba indicated that the specific growth rate of *Chlorella vulgaris* in the presence of glucose (mixotroph) was always larger than that in autotroph when the light intensity was less than 10 klux. However, *Scenedesmus acutus* behaved differently, i.e., when the light intensity was more than 6 klux, the specific growth rate in mixotroph became smaller than that in autotroph [14]. These imply that suitable trophic conditions changes according to diverse factors, such as algal species, cultural conditions, and growth environment. Moreover, some studies implied that mixotrophic growth corresponded well to a sum of microalgae growth in autotrophic and heterotrophic cultivation in terms of dry weight, specific growth rate, or lipid content [15, 16]. However, other studies revealed that mixotrophic growth was not a simple combination of heterotrophic and autotrophic growth [17, 18]. Thus, understanding growth behavior happening in various trophic conditions is helpful for process design and establishing operational strategies.

This study was conducted to investigate the effect of trophic conditions on microalgal biomass production, nutrient removal rate, and contents of energy storage products, such as carbohydrates and lipids, as well as the production of algal organic matter (AOM) related to discharging limits for organic pollutants. Microalga, *Scenedesmus* (*Acutodesmus*) *obliquus* KGE-17 was selected for this study, since it was known to be an adequate strain for wastewater treatment and the production of biofuels, and may be capable of growth under mixotrophic, photoautotrophic, and heterotrophic conditions [19, 20].

Materials and methods

Microalga pre-cultivation

Scenedesmus obliquus KGE-17 was isolated from a livestock wastewater treatment facility in Gangneung, South Korea. The species was identified using the same method reported in our prior work [19]. This microalga was inoculated into 1000 mL tubular photobioreactor-containing 800 mL BG-11 medium [21]. The photobioreactors were incubated under white fluorescent light illumination at 75 µmol photon m^{-2} s^{-1} at 25 °C for 2 weeks, while continuously supplementing 5% CO_2 (v/v) into the reactors.

S. obliquus KGE-17 cultivation with modified BG-11 medium

To investigate cell growth and nitrogen removal efficiency in different trophic conditions, modified BG-11 medium was used for inducement of mixo-, photoauto-, and heterotrophic conditions. The modified BG-11 medium containing 200 mg N L^{-1} of ammonium chloride and 5 mg P L^{-1} of disodium hydrogen phosphate was replaced from the original N and P source of BG-11 medium. In mixo- and heterotrophic cultivation condition, 500 mg C L^{-1} sodium acetate was added to the modified BG-11 medium for OC source. In heterotrophic cultivation condition, the bioreactor was covered with aluminum foil to block the light supplement. In heterotrophic cultivation condition, the bioreactor was covered with aluminum foil to block the light supplement. Each experiment was performed in triplicate for photoautotrophic, mixotrophic and heterotrophic conditions.

Two-week-old 500 mg dry weight L^{-1} of *S. obliquus* KGE-17 was inoculated into tubular borosilicate reactors (34 mm inner diameter and 520 mm height, described at supplementary materials Fig. S1) that had 400 mL modified BG-11 medium, respectively. To prevent microbial contamination, the photobioreactors, reactor parts, and mediums were autoclaved at 121 °C for 20 min. Ammonium stock solution was added through 0.2 µm polypropylene syringe filter in clean bench after cooling for 3 h. Microalgae inoculation also carried out in clean bench. The bioreactors were incubated at 25 ± 2 °C for 84 h in a constant temperature room, with aeration through an inlet port at the conical bottom of the reactor. Illumination with 150 µmol m^{-2} s^{-1} of light intensity and CO_2 gas diluted with ambient air to 5% (v/v) were applied for algal growth in this study. During the incubation, a 10 mL mixed liquor was collected several times at intervals from

the bioreactor for the measurement of dry cell weight, dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), COD, dissolved nitrogen, and dissolved phosphorous after 0.2 µm cellulose acetate membranes filtration (C020A047A, Advantec, Japan).

The cell growth was determined by measuring the dry cell weight concentration using an equivalent procedure to that found in the Standard Methods for the Examination of Water and Wastewater [22]. The growth coefficient (μ, day^{-1}) and the specific nutrient consumption rate (q, mg-C or -N or -P g-cell^{-1} day^{-1}) for a specific period were calculated using the following Eqs. (1) and (2), respectively:

$$\mu = \ln\left(AB_2/AB_1\right)/\left(t_2 - t_1\right) \quad (1)$$

$$q = \left(S_1 - S_2\right)/\left[AB' \times \left(t_2 - t_1\right)\right], \quad (2)$$

where, AB_1 and AB_2 are the algal biomass concentrations as dry cell weight (g L^{-1}) at times t_1 and t_2, respectively; S_1 and S_2 are the assimilable nutrient concentrations (mg-C or -N or -P L^{-1}) at time t_1 and t_2.; and AB' is the average algal biomass concentration between moment times t_1 and t_2. Therefore, the maximum value of the growth coefficient (μ_{max}) and the maximum specific nutrient consumption rate (q_{max}) were obtained between the two data points of start and end of log growth (without lag and stationary phase) on the dry cell weight concentrations and the assimilated nutrient concentrations plotted against time, respectively.

Characterization of dissolved organic matter

Size-exclusion column (SEC) equipped liquid chromatography–organic carbon detector/ultra violet detector (LC–OCD/UVD) (DOC-Labor, Germany) was used to investigate the compositional and functional properties of organic components, and to discriminate organics originating from algal cell growth. The organic matter introduced LC system, described in previous work [23], primarily distributed the molecular weight (MW), and secondarily the electric charge of organic matter by SEC. Separated organic compounds were transferred to UVD to measure the UV absorption at 254 nm (UV254), and then IC purged by N_2 for 20 s supported by acidification solution (pH < 2). After purging, the organic matters were oxidized by UV radiation in Gräntzel thin-film reactor. The converted CO_2 gas from organic matter was monitored by non-dispersive infrared (NDIR) detector (OCD). Biopolymers (> 20 kDa), humic substances [(1–20 kDa], building blocks [(300–500) Da], low-molecular-weight (LMW) acids (< 350 Da), and LMW neutrals (< 350 Da) are separated by LC-OCD/UVD as the major five fractions of DOC [23]. The signal responses (chromatogram) presented in supplementary materials Fig. S2 and it processed by LabVIEW-based software from manufacturer.

Energy storage products' measurement

After cultivation, all algal biomass was collected by centrifugation at 3000×g for 10 min, and freeze dried (FDB-5503, Operon, South Korea), to estimate the storage products content. Phenol–sulfuric acid method [24] was applied to estimate carbohydrate. Diluted 0.5 mL samples (0.1 mg DW biomass mL^{-1}) with deionized water (DIW) and 0.5 mL of 0.5% phenol solution were introduced to test tubes, and then, 2.5 mL of commercial sulfuric acid was directly added to liquid surface. The absorbance was measured at 490 nm (DU 730, Beckman Coulter, USA), and calibrated using glucose solution.

The modified Bligh and Dyer method [25] was used for lipid analysis contained in algal biomass. Dried algal biomass of 25 mg was resuspended with 1 mL of chloroform, 2 mL of MeOH, and 0.8 mL of DIW solution, and then ultrasonicated with 60% amplification for 1 min (VCX130, Sonics & Materials, USA). After sonication, 1 mL of chloroform and DIW were added, and ultrasonication was applied again at the same condition as previously described. The mixed sample was separated by centrifugation (3000×g for 5 min). Bottom layer of 1 mL was moved to glass test tubes and evaporated for the gravimetric measurement of lipid weight.

The dried tubes that contained lipid were used for the analysis of fatty acid methyl ester (FAME). Chloroform of 1 mL and 1 mL of 15% sulfuric acid containing MeOH was added to test tubes, respectively. DIW of 1 mL was added to test tube after 20 min heating to 120 ºC, and cooling to near RT. To separate FAME solution from mixed liquor, the mixture was vortexed for 1 min, and then transferred to gas chromatograph (GC) sample vials. The GC–Mass Spectroscopy (GCMS-QP2010, Shimadzu, Japan) equipped with capillary column (Omegawax 250, Supelco, USA) was conducted to FAME calibrated with FAME standard (FAME mixture C4-C24, Supelco, USA). The sample was introduced to split mode 100:1, 53.5 kPa Helium gas (99.9999%), and 250 ºC condition injection port. The temperature of oven was set to 50 ºC, held for 2 min, then increased to 220 ºC at 4 ºC min^{-1} rate, and then held at 220 ºC for 15 min.

Analytical methods

The Acid Persulfate Digestion and Dichromate Methods were employed to measure dissolved phosphorous and chemical oxygen demand (COD), respectively, in the water samples. These methods are equivalent to Standard Methods 4500 P. B. 5, and 5220 C, respectively for water and wastewater [22]. DOC (TOC-V CPH) and dissolved nitrogen (TNM-1) were also determined (Shimadzu, Japan) using whole samples. To measure UV254, DU 730 spectrophotometry (Beckman Coulter, USA) was used, after filtration with 0.2 µm cellulose acetate membranes. The specific UV

absorbance (SUVA) value is calculated from the UV254 divided by the DOC of the water sample, where the UV254 is mainly generated by electron-rich sites, such as aromatic functional groups and double-bonded carbon groups, in an organic molecule [26, 27]. Light intensity was determined using LI-250A (LI-COR, USA). The pH was measured by pH meter (Orion 3 STAR, Thermo Scientific, USA). Each measurement was carried out in triplicate and average values were reported.

Statistical analysis

One-way analysis of variance (ANOVA) was used to examine the differences among average values. EXCEL (Microsoft Office, 2013) was used for all statistical analyses, and differences in the variables were considered significant at the $P < 0.05$ level of confidence.

Results and discussion

Cell growth and carbon behavior in different trophic conditions

Figure 1a shows the growth of *S. obliquus* KGE-17 in mixotrophic, photoautotrophic, and heterotrophic conditions. The maximum biomass production was accomplished in mixotrophic condition, followed by photoautotrophic condition, and finally, it showed the lowest biomass production in heterotrophic condition. Unlike this study, heterotrophic microalgae presented higher biomass production than

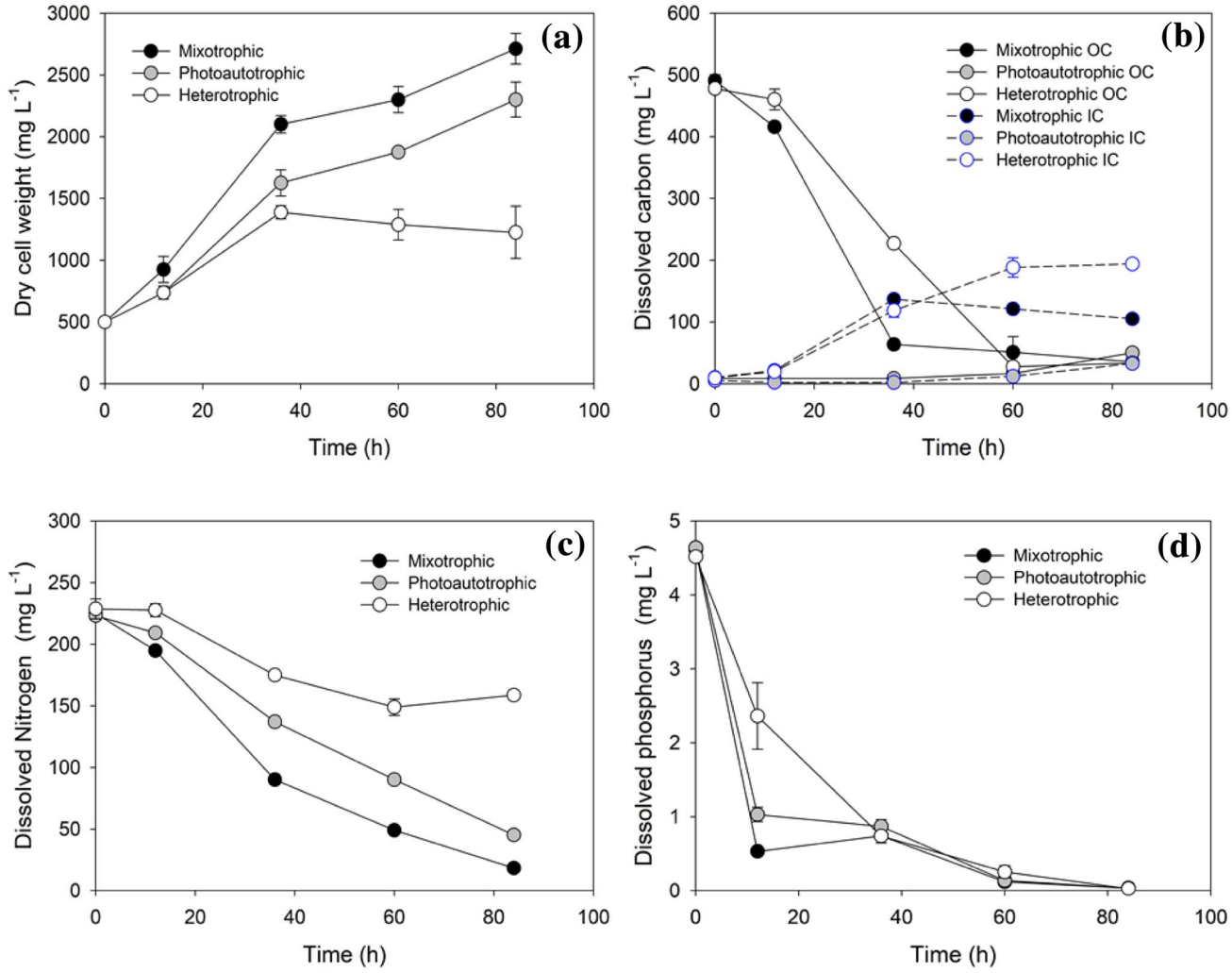

Fig. 1 *S. obliquus* KGE-17 cultivation under different trophic conditions using modified BG-11 medium. **a** Growth curves of mixotrophic (black), photoautotrophic (grey), and heterotrophic (white). **b** DOC (black edge and solid line) and DIC (blue edge and dash line) concentration of mixotrophic (black), photoautotrophic (grey), and heterotrophic (white). **c** TN mixotrophic (black), photoautotrophic (grey), and heterotrophic (white). **d** TP mixotrophic (black), photoautotrophic (grey), and heterotrophic (white) (color figure online)

photoautotrophic microalgae in several studies [13, 28, 29]. Microalgal biomass productivity in photoautotrophic condition could be improved by increasing light intensity [30]. Furthermore, a continuous IC supplement helps to produce more microalgae biomass, because of enhanced photosynthesis. However, in heterotrophic condition where photosynthesis is not possible, OC is the sole carbon source; hence, the lower biomass productivity of heterotrophic microalgae was observed due to OC limitation in this study.

Figure 1a shows that unlike mixotrophic and photoautotrophic conditions, which showed a continuously increasing pattern, microalgal growth in heterotrophic condition decreased after 36 h, even though half of the acetate still remained in the medium. In heterotrophic condition, where photosynthetic OC could not be obtained, most of the remained acetate was utilized for survival after 36 h. This seemed to be the main reason for the decrease of dry cell weight after 36 h. On the other hand, the fastest growth in mixotrophic condition was achieved by assimilation of acetate from medium, and carbohydrate generated through photosynthesis with high light intensity (150 μmol m^{-2} s^{-1}) and continuous IC supplement, simultaneously. Both these organic matters were utilized for cell materials, as well as energy source, for their growth and survival.

The calculated specific growth rate in mixotrophic condition was 0.96 day^{-1} on 36 h cultivation, and 1.22 and 1.41 times higher than the photoautotrophic and heterotrophic conditions, respectively (Table 1). Similar results were reported in several studies. The previous study [11] reported that the growth rate of *S. obliquus* in acetate-containing medium was higher in mixotrophic condition than in heterotrophic condition. In addition, when *Chlamydomonas humicola* was cultured with acetate-containing medium in mixotrophic, photoautotrophic, and heterotrophic conditions, the growth rate in mixotrophic condition was higher compared to the photoautotrophic or heterotrophic conditions [28]. Since microalgae cultured under mixotrophic condition could metabolize both heterotrophically and photoautotrophically, this resulted in a stimulated growth rate.

Figure 1b shows that the IC concentration in photoautotrophic condition was maintained at a level close to zero until 36 h, when a depressed growth rate began due to the shading effect, and then slightly increased to lower than 40 mg L^{-1}. This is probably because the supplied CO_2 gas was mostly used for photosynthesis. However, in heterotrophic condition, due to the generated CO_2 gas by respiration and the supplied CO_2 gas, the IC concentration continuously increased. In mixotrophic condition, IC concentration increased as in heterotrophic condition; however, a decrease of IC concentration was observed after 36 h, at which OC was almost consumed. The added external IC as 5% (v/v) CO_2 gas and CO_2 from respiration of microalgae led to the increment of IC concentration until OC depletion. After the OC depletion, the IC concentration decreased as the photosynthesis progressed using a portion of the IC. Therefore, after 36 h, the difference in IC concentration between heterotrophic and mixotrophic conditions could be determined as the amount of IC consumed by photosynthesis. Similar results of IC accumulation and consumption were shown in the *Micractinium inermum* cultivation experiment with acetate and aeration. The substantial increases in dissolved gases during log growth were synchronous with significantly enhanced rates of both photosynthesis and respiration. The mixotrophic endogenic production of CO_2 and O_2 was significantly stimulated metabolic activity [29].

The pH of medium under photoautotrophic condition declined from 8 to near 3 in the early cultivation period (see supplementary materials Fig. S3), because ammonium was used as nitrogen source [31]. However, no significant decline showed under mixo- and heterotrophic conditions due to pH buffering effect of produced CO_2 by respiration. Therefore, to efficiently use IC and buffer pH decline, IC supplement should be after OC is consumed in front of the reaction and/or process.

Nitrogen and phosphorus removal in different trophic conditions

Figure 1c shows the nitrogen removal in various trophic conditions. In mixotrophic condition, the nitrogen removal rate of 104.0 mg N L^{-1} day^{-1} for the (12–36) h cultivation period (OC remained condition) decreased to 41.1 mg N L^{-1} day^{-1} for the (36–60) h cultivation period (IC utilized condition). In photoautotrophic condition, the nitrogen removal rate, which was initially 72.2 mg N L^{-1} day^{-1}, remained at a certain level, although it decreased somewhat. However,

Table 1 Comparisons of microalga *S. obliquus* KGE-17 cell growth and nutrient removal under different trophic conditions

Trophic conditions	μ (day^{-1})	q_{max} (mg-C g-$cell^{-1}$ d^{-1})	q_{max} (mg-N g-$cell^{-1}$ d^{-1})	q_{max} (mg-P g-$cell^{-1}$ d^{-1})
Mixotrophic	0.96 ± 0.02	219.01 ± 1.27	85.17 ± 6.24	11.49 ± 0.60
Photoautotrophic	0.79 ± 0.03	N.A.	54.14 ± 4.08	10.67 ± 0.58
Heterotrophic	0.68 ± 0.02	183.32 ± 6.93	38.04 ± 6.35	6.95 ± 0.96
p value	0.007	0.071	0.043	0.049

N.A. not applicable

nitrogen removal rate in the heterotrophic condition was interrupted by OC depletion, and after 60 h, the concentration of nitrogen increased rather slightly. Considering that the nitrogen removal in Fig. 1c was almost consistent with the microalgae growth (Fig. 1a), the nitrogen removal was presumed to be dependent on the microalga productivity.

Similar to the growth rate, nitrogen removal rate was the fastest as 85.17 ± 6.24 mg-N g-cell^{-1} day^{-1} in mixotrophic condition, followed by photoautotrophic and heterotrophic conditions (54.14 ± 4.08 and 38.04 ± 6.35 mg-N g-cell^{-1} day^{-1}, respectively) (see Table 1). This indicates that the microalgae cultivation under mixotrophic condition can remove nitrogen 1.57 and 2.24 times faster, compared to cultivation under photoautotrophic and heterotrophic conditions, respectively. In particular, the change in trophic condition from photoautotrophic or heterotrophic to mixotrophic resulted in a relatively low increase of 1.21 and 1.41 times in the microalgae growth rate, respectively, while a larger increase in nitrogen removal rate occurred. The previous work [11] also reported similar results in which the ammonium uptake rate of *S. obliquus* cultivated with acetate was 3.7 times higher in mixotrophic condition than in other conditions, while the microalgae growth rate was only 1.8 times higher. This suggests that the mixotrophic condition is effective for increasing the microalgae growth rate, and at the same time, it is more effective in increasing the nitrogen removal rate. Carbon and nitrogen metabolism is linked in microalgae, because they share carbon supplied directly from respiration of fixed CO_2 (autotrophic growth) or assimilated OC (heterotrophic growth) and the energy generated in the TCA cycle and in the mitochondrial electron transport chain. The primary assimilation of inorganic nitrogen (ammonium) to form amino acids requires carbon skeletons in the form of keto-acids (2-oxaloglutarate and oxaloacetate) and energy in the form of ATP and NADPH to synthesize the amino acids [10]. Dark respiration of nitrogen-starved microalgae cells correlated with inorganic nitrogen assimilation. Ammonium-enhanced respiration continued until either ammonia concentration in the suspending medium dropped to an undetectable concentration or intracellular carbohydrate energy reserves were almost completely exhausted. Addition of organic carbon allow ammonium assimilation to continue, as well as amino acid and protein synthesis [32]. The addition of acetate in the light or in the dark increase the activity of isocitrate lyase in *S. obliquus* cells, which is a key enzyme for the regulation of the glyoxylate cycle responsible for acetate catabolism, and could relieve limitation of carbon skeleton as well as increased ammonium uptake rate [11]. Thus, light with acetate allowed higher ammonium uptake rate than dark condition with acetate.

Figure 1d shows that regardless of trophic conditions, nearly complete phosphorus removal was achieved in the final culture stage. However, the initial rate of phosphorus removal showed a completely different pattern, depending on the trophic conditions. The phosphorus removal rates in mixotrophic and photoautotrophic conditions were higher at 11.49 and 10.67 mg-P g-cell^{-1} day^{-1}, respectively, while, in heterotrophic condition, it was the lowest at 6.95 mg-P g-cell^{-1} day^{-1} (see Table 1). The slowest phosphorus removal was observed in heterotrophic condition, because photophosphorylation (ADP to ATP) was absent. The conversion of ADP to ATP is carried out in mitochondria and chloroplasts; thus, in the absence of light, the removal of phosphorus appears to be slow, because the synthesis of ATP occurs only in the mitochondria, due to the absence of the photosystem [33].

The phosphorous removal using *S. obliquus* KGE-17 being affected by the kind of OC suggests that high concentration of phosphorous-containing wastewater (carbon/phosphorous ratio unbalanced) should be treated in mixotrophic, or in combination of heterotrophic and photoautotrophic, condition cultivation.

Algal organic matter in different trophic conditions

Effluents from biological wastewater treatment systems contain a variety of soluble organic compounds, and the majority of the soluble organic matter is actually soluble microbial products (SMPs). In particular, a significant portion of the SMPs are hardly degradable by natural environmental conditions [34]. Therefore, their presence is of particular interest in terms of achieving discharge consent levels for BOD and COD, as well as the performance of the treatment system [35]. For this reason, this study characterized algal organic matter (AOM) produced through microalgae cultivation in different trophic conditions. Bulk organics can be classified as shown in Fig. 2, based on the fact that the retention time of organic components in the SEC of LC-OCD depends on their MW. Comparing before and after cultivation, the acetate (represented as LMW acids before cultivation) in the medium under mixotrophic and heterotrophic conditions was completely consumed by *S. obliquus* KGE-17. After cultivation, various fractions of AOM were produced, as shown in Fig. 2. In particular, among the fractions of AOM, humic substances and building blocks were observed at significantly higher level in photoautotrophic condition, compared to the other trophic conditions, while biopolymers and LMW neutrals presented similar concentration in all trophic conditions. Since the highest concentration of humic substances and building blocks was observed in photoautotrophic condition, in which photosynthetic metabolism was exclusively performed, and the lowest in heterotrophic condition, in which no photosynthesis occurred at all, production of humic substances and building blocks appears to be directly related to photosynthesis, even though the exact mechanism could not be explained, and further study is required.

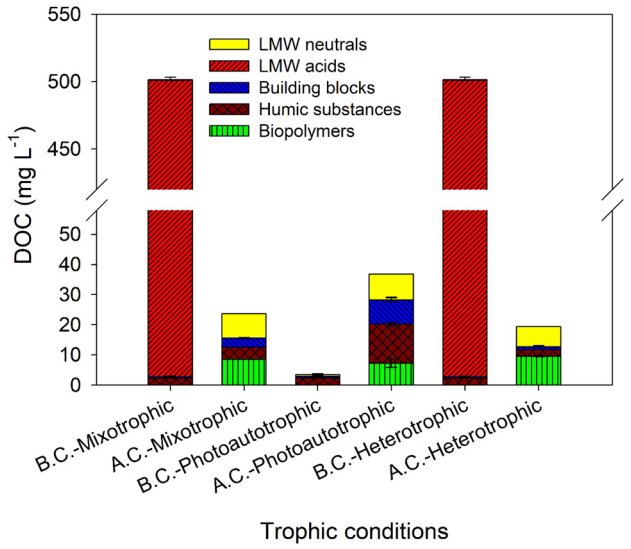

Fig. 2 Organic matter elimination and release (*A.C.* after cultivation, *B.C.* before cultivation) from *S. obliquus* KGE-17 cultivation under different trophic conditions by LC-OCD analysis. Biopolymer (green and vertical line), humic substances (brown and mesh line), building block (blue and left-to-right diagonal line), LMW acids (red and right-to-left diagonal line), and LMW neutrals (yellow) represent fractionated DOC components (color figure online)

Meanwhile, AOM in the medium under photoautotrophic, mixotrophic, and heterotrophic conditions, which was represented as DOC, was 50.2, 35.8, and 34.1 mg C L^{-1}, respectively. AOM in photoautotrophic condition was the highest, even if the highest biomass production occurred in mixotrophic condition (see Fig. 1a). In the study of *Arthrospira platensis* extracellular polymeric substances (EPS) production, which is closely related to AOM, under the three different trophic modes of photoautotrophic, heterotrophic, and mixotrophic conditions [36], similar results were observed in which the highest specific EPS productivity was obtained under photoautotrophic culture, although the maximum specific growth rate was achieved under mixotrophic condition. Inhibitions of cell growth induced enhanced EPS productivity [37]. Light can be an excess of energy, and can generate oxidative stress in cells. To avoid this damage, cells involve a drain of excess ATP (to a reducing power) by EPS biosynthesis [38]. Since microalgae in mixotrophic culture could metabolize both heterotrophically and photoautotrophically, a part of the O_2 produced by cells growing photoautotrophically might be immediately used for cells growing mainly heterotrophically under sufficient light intensity, decreasing dissolved oxygen concentration; this can help reduce photooxidative damage. Then, during the mixotrophic culture, the induction effect of photostress is attenuated, which could explain the reduced level of EPS synthesis in mixotrophic culture compared to photoautotrophic culture, unless excess of carbon is mainly shifted to mixotrophic growth, instead of EPS biosynthesis [36]. This explanation for the highest specific EPS productivity under photoautotrophic culture might apply to the highest production of AOM in photoautotrophic condition in this study. Consequently, mixotrophic or heterotrophic cultivations that utilize OC (acetate) as a carbon source could be helpful to reduce DOC and non-biodegradable organic matter in effluent.

Energy storage products in different trophic conditions

Microalgae can effectively accumulate large quantities of energy storage products, such as lipids (for biodiesel) and carbohydrates (for bioethanol), in the cell. The carbohydrates accumulation was affected by nutrients starvation (nitrogen, phosphorus, sulfur, and iron), organic carbon supplement, light intensity, temperature, etc. [39]. The lipids accumulation was also affected by similar factors [10, 40]. Table 2 summarizes the carbohydrate and lipid content and productivity of *S. obliquus* KGE-17 under different trophic conditions. Carbohydrates contents were 21.8 ± 0.3, 18.7 ± 0.1, and 18.1 ± 1.6%, and lipids contents were 24.0 ± 0.0, 24.4 ± 0.4, and 20.0 ± 0.8% in mixotrophic, photoautotrophic, and heterotrophic conditions, respectively. Even though carbohydrate and lipid contents were not significantly different between different tropic conditions, the contents of both carbohydrate and lipid were slightly higher in mixotrophic condition than in heterotrophic condition. Cultivation under nitrogen-deficient conditions leads to a sharp increase in carbohydrate or lipid content more than 40%, because the condition of nitrogen-depletion probably transforms protein or peptides to lipids or carbohydrates [30, 41]. In this study, the lowest nitrogen concentration

Table 2 Energy storage product contents and productivity of *S. obliquus* KGE-17 under different trophic conditions

	Mixotrophic	Photoautotrophic	Heterotrophic
Carbohydrates			
Content (%)	21.8 ± 0.3	18.7 ± 0.1	18.1 ± 1.6
Productivity (mg L^{-1} d^{-1})	137.7 ± 8.2	96.1 ± 7.3	36.8 ± 5.7
Lipids			
Content (%)	24.0 ± 0.0	24.4 ± 0.4	20.0 ± 0.8
Productivity (mg L^{-1} d^{-1})	151.7 ± 12	125.6 ± 13.4	41.1 ± 9.1

(18.5 mg L^{-1}) at the end of cultivation was observed in mixotrophic condition (Fig. 1c), and this extremely low nitrogen level seemed to partially induce nitrogen starvation stress to increase carbohydrate and lipid content under mixotrophic condition. Consequently, this partial nitrogen-deficient condition in mixotrophic condition made a slight increase in carbohydrate and lipid contents, but the increment was not as much as shown in the previous studies [30, 41].

However, the productivity of carbohydrates and lipids, which considered mass productivity, showed significant difference between the trophic conditions. The highest productivity for both carbohydrate and lipid was achieved in mixotrophic condition (Table 2). In mixotrophic condition, carbohydrate productivity was 1.39 and 3.63 times higher than photoautotrophic and heterotrophic conditions, and lipid productivity was 1.21 and 3.69 times higher than photoautotrophic and heterotrophic conditions, respectively. This suggests that mixotrophic culture was beneficial to the production of energy storage products.

Figure 3 shows FAME compositions under the different trophic conditions. The compositions of palmitic acid (C16:0), palmitoleic acid (C16:1), and stearic acid (C18:0) indicated no significant differences ($p > 0.05$), whereas elaidic acid (C18:1n9t), oleic acid (C18:1n9c), and linoleic acid (C18:2n6) showed significant differences, depending on the trophic conditions (p value was 0.021, 0.007, and 0.047, respectively). Many studies mentioned that oleic acid was related to stress conditions, such as nitrogen starvation, photo-adaptation, and low temperature [30, 42–44]. The increase of oleic acid under mixotrophic condition in this study was presumed to be induced by the partial nitrogen starvation effect mentioned above. The change of FAME compositions with different trophic conditions was observed in this study, and also reported in other studies [42, 45]. However, it is unclear which factors under different trophic conditions lead to changes in FAME compositions, and further studies are needed to clarify factors changing FAME composition under different trophic conditions.

The quality of biodiesel is usually considered by cetane number (CN), degree of unsaturation (DU), or cold filter plugging point (CFPP) [46, 47]. Table 3 summarizes the calculated CN, DU, and CFPP values. Under all trophic conditions, CN and DU satisfied the biodiesel standard EN 14214 [48]. According to the biodiesel standard EN 14214, CN should not exceed the limit of 51, and DU, which is related to CN, iodine value, and oxidation stability [47], should be under 137. Low content of poly-unsaturated fatty acid (PUFA), which represented high oxidative stability of diesel [41] was observed in all trophic conditions (Table 3). In contrast, CFPP satisfied only the summer season limit of

Table 3 Biodiesel quality property after transesterification of extracted lipid aliquots from *S. obliquus* KGE-17 under different trophic conditions [48]

	Mixotrophic	Photoautotrophic	Heterotrophic
SFA (%)[a]	30.2 ± 0.4	28.0 ± 1.4	29.6 ± 1.2
MUFA (%)[b]	49.70 ± 0.4	48.9 ± 4.2	45.6 ± 3.1
PUFA (%)[c]	20.1 ± 0.8	23.1 ± 1.8	24.8 ± 2.4
CN[d]	58.3 ± 1.4	57.3 ± 2.8	57.3 ± 1.1
DU[e]	90.0 ± 7.3	95.2 ± 4.2	95.2 ± 5.4
CFPP (°C)[f]	− 1.2 ± 1.1	− 2.3 ± 0.4	− 2.7 ± 0.5

[a]Saturated fatty acid

[b]Mono unsaturated fatty acid

[c]Poly-unsaturated fatty acid

[d]Cetane number (minimum limit value = 51, [48])

[e]Degree of unsaturation

[f]Cold filter plugging point (0 °C in summer season, − 10 °C in winter season [48])

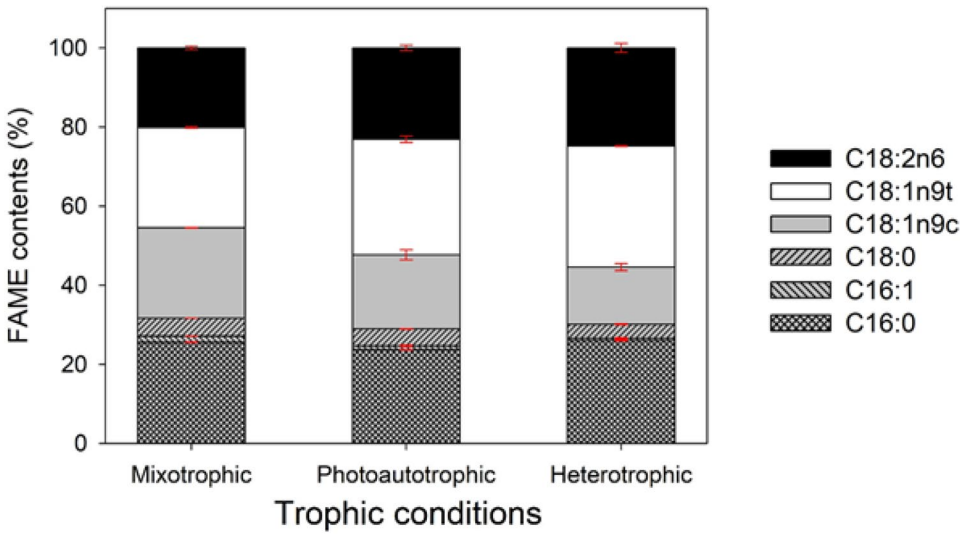

Fig. 3 FAME composition of *S. obliquus* KGE-17 under different trophic conditions. palmitic acid (C16:0, grey and mesh line), palmitoleic acid (C16:1, grey and left-to-right diagonal line), stearic acid (C18:0, grey and right-to-left diagonal line), oleic acid (C18:1n9c, grey), elaidic acid (C18:1n9t, white), and linoleic acid (C18:2n6, black) were observed on GC–MS analysis (color figure online)

EN 14214 [48] under all trophic conditions. Consequently, *S. obliquus* cultured under various trophic conditions could be considered as a potential organism for biofuel production.

Conclusions

Biomass production, nutrients removal, AOM release, and energy storage products of *S.obliquus* under different trophic conditions were compared in this study. The growth of *S.obliquus* under mixotrophic condition was 1.2 and 1.4 times higher than photoautotrophic and heterotrophic conditions, respectively. The fastest nutrient removal was also achieved for mixotrophic condition. Comparing with photoautotrophic condition, mixotrophic or heterotrophic conditions could suppress the release of AOM, which related residual organic pollutants in effluent. Considering the biomass production, the productivity of carbohydrates and lipids of *S.obliquus* for mixotrophic condition was significantly higher than photoautotrophic or heterotrophic conditions. Consequently, mixotrophic growth condition was superior in terms of the microalgae growth, nutrient removal, and energy source production as well as suppression of AOM compared to heterotrophic and photoautotrophic conditions, and could be adaptable to wastewater treatment.

Acknowledgements This work was supported by a Grant (18CTAP-C116746-03) from the Technology Advancement Research Program funded by the Ministry of Land, Infrastructure and Transport of the Korean government, and the Korea Institute of Science and Technology (KIST) Institutional Program (2E29660).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Oswald WJ, Gotaas HB (1957) Photosynthesis in sewage treatment. Trans Am Soc Civ Eng 122:73–105
2. Umamaheswari J, Shanthakumar S (2016) Efficacy of microalgae for industrial wastewater treatment: a review on operating conditions, treatment efficiency and biomass productivity. Rev Environ Sci Biotechnol 15:265–284
3. Li Y, Chen YF, Chen P, Min M, Zhou W, Martinez B, Zhu J, Ruan R (2011) Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour Technol 102:5138–5144
4. Kim HC, Choi WJ, Chae AN, Park J, Kim HJ, Song KG (2016) Evaluating integrated strategies for robust treatment of high saline piggery wastewater. Water Res 89:222–231
5. Dang NM, Lee K (2018) Recent trends of using alternative nutrient sources for microalgae cultivation as a feedstock of biodiesel production. Appl Chem Eng 29(1):1–9
6. Suali E, Sarbatly R (2012) Conversion of microalgae to biofuel. Renew Sust Energ Rev 16:4316–4342
7. Martinez ME, Sanchez S, Jimenez JM, El-Yousfi F, Munoz L (2000) Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresour Technol 73:263–272
8. Sydney EB, da Silva TE, Tokarski A, Novak AC, de Carvalho JC, Woiciecohwski AL, Larroche C, Soccol CR (2011) Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. Appl Energy 88:3291–3294
9. Schulze PSC, Carvalho CFM, Pereira H, Gangadhar KN, Schuler LM, Santos TF, Varela JCS, Barreira L (2017) Urban wastewater treatment by *Tetraselmis* sp. CTP4 (Chlorophyta). Bioresour Technol 223:175–183
10. Perez-Garcia O, Escalante FM, de Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. Water Res 45:11–36
11. Combres C, Laliberté G, Sevrin Reyssac J, de la Noüe J (1994) Effect of acetate on growth and ammonium uptake in the microalga *Scenedesmus obliquus*. Physiol Plant 91:729–734
12. Babaei A, Mehrnia MR, Shayegan J, Sarrafzadeh M-H, Amini E (2018) Evaluation of nutrient removal and biomass production through mixotrophic, heterotrophic, and photoautotrophic cultivation of chlorella in nitrate and ammonium wastewater. Int J Environ Res 12:167–178
13. Kim S, Park JE, Cho YB, Hwang SJ (2013) Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. Bioresour Technol 144:8–13
14. Ogawa T, Aiba S (1981) Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. Biotechnol Bioeng 23:1121–1132
15. Marquez FJ, Sasaki K, Kakizono T, Nishio N, Nagai S (1993) Growth characteristics of *Spirulina platensis* in mixotrophic and heterotrophic conditions. J Ferment Bioeng 76:408–410
16. Quinn J, de Winter L, Bradley T (2011) Microalgae bulk growth model with application to industrial scale systems. Bioresour Technol 102:5083–5092
17. Cheirsilp B, Torpee S (2012) Enhanced growth and lipid production of microalgae under mixotrophic culture condition: effect of light intensity, glucose concentration and fed-batch cultivation. Bioresour Technol 110:510–516
18. Mohammad-Mirzaie MA, Kalbasi M, Mousavi SM, Ghobadian B (2016) Investigation of mixotrophic, heterotrophic, and autotrophic growth of *Chlorella vulgaris* under agricultural waste medium. Prep Biochem Biotechnol 46:150–156
19. Park YT, Lee H, Yun HS, Song KG, Yeom SH, Choi J (2013) Removal of metal from acid mine drainage using a hybrid system including a pipes inserted microalgae reactor. Bioresour Technol 150:242–248
20. Hegewald EH (1997) Taxonomy and phylogeny of *Scenedesmus*. Algae 12:235–246
21. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61
22. APHA, AWWA, WEF (1998) Standard methods for the examination of water and wastewater 21st edn. American Public Health Association, Washington
23. Huber SA, Balz A, Abert M, Pronk W (2011) Characterisation of aquatic humic and non-humic matter with size-exclusion chromatography—organic carbon detection—organic nitrogen detection (LC-OCD-OND). Water Res 45:879–885

24. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Calorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
25. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem phys 37:911–917
26. Kim H-C, Lee S (2006) Pump diffusion flash mixing (PDFM) for improving coagulation process in drinking water treatment. Sep Purif Technol 52:117–125
27. Lamsal R, Walsh ME, Gagnon GA (2011) Comparison of advanced oxidation processes for the removal of natural organic matter. Water Res 45:3263–3269
28. Laliberté G, de la Noüe J (1993) Auto-, hetero-, and mixotrophic growth of *Chlamydomonas humicola* (Chlorophyceae) on acetate. J Phycol 29:612–620
29. Smith RT, Bangert K, Wilkinson SJ, Gilmour DJ (2015) Synergistic carbon metabolism in a fast growing mixotrophic freshwater microalgal species *Micractinium inermum*. Biomass Bioenerg 82:73–86
30. Ho SH, Chen CY, Chang JS (2012) Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour Technol 113:244–252
31. Huang A, Sun L, Wu S, Liu C, Zhao P, Xie X, Wang G (2016) Utilization of glucose and acetate by *Chlorella* and the effect of multiple factors on cell composition. J Appl Phycol 29(1):23–33
32. Geider RJ, Osborne BA (1989) Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth. New Phytol 112(3):327–341
33. Cardol P, Forti G, Finazzi G (2011) Regulation of electron transport in microalgae. Biochim Biophys Acta Bioenerg 1807:912–918
34. Wang ZW, Liu Y, Tay JH (2007) Biodegradability of extracellular polymeric substances produced by aerobic granules. Appl Microbiol Biotechnol 74:462–466
35. Barker DJ, Stuckey DC (1999) A review of soluble microbial products (SMP) in wastewater treatment systems. Water Res 33:3063–3082
36. Trabelsi L, Ouada HB, Zili F, Mazhoud N, Ammar J (2013) Evaluation of *Arthrospira platensis* extracellular polymeric substances production in photoautotrophic, heterotrophic and mixotrophic conditions. Folia Microbiol 58:39–45
37. Trabelsi L, Ouada HB, Bacha H, Ghoul M (2009) Combined effect of temperature and light intensity on growth and extracellular polymeric substances production by the cyanobacterium *Arthrospira platensis*. J Appl Phycol 21:405–412
38. Guillaume-Cogne JB, Gros-Dussap CG (2003) Identification of a metabolic network structure representative of *Arthrospira (spirulina) platensis* metabolism. Biotechnol Bioeng 84:667–676
39. Markou G, Angelidaki I, Georgakakis D (2012) Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Appl Microbiol Biotechnol 96:631–645
40. Dang NM, Lee K (2018) Decolorization of organic fertilizer using advanced oxidation process and its application for microalgae cultivation. J Ind Eng Chem 59:297–303
41. Mandal S, Mallick N (2009) Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Appl microbiol biotechnol 84:281–291
42. Ratha SK, Babu S, Renuka N, Prasanna R, Prasad RB, Saxena AK (2013) Exploring nutritional modes of cultivation for enhancing lipid accumulation in microalgae. J Basic Microbiol 53:440–450
43. Mendoza H, Jiménez del Río M, García-Reina G, Ramazanov Z (1996) Low temperature induced β-carotene and fatty acid synthesis, and ultrastructural reorganization of the chloroplast in *Dunaliella salina* (Chlorophyta). Eur J Phycol 31:329–331
44. Mendoza H, Martel A, Jiménez del Río M, García-Reina G (1999) Oleic acid is the main fatty acid related with carotenogenesis in *Dunaliella salina*. J Appl Phycol 11:15–19
45. Wang H, Xiong H, Hui Z, Zeng X (2012) Mixotrophic cultivation of *Chlorella pyrenoidosa* with diluted primary piggery wastewater to produce lipids. Bioresour Technol 104:215–220
46. Bamgboye AI, Hansen AC (2008) Prediction of cetane number of biodiesel fuel from the fatty acid methyl ester composition. Int Agrophys 22:21–29
47. Ramos MJ, Fernandez CM, Casas A, Rodriguez L, Perez A (2009) Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour Technol 100:261–268
48. UNE-EN 14214 (2003) Automotive fuels – fatty acid methyl esters (FAME) for diesel engines — requirement methods, European Committee for Standardization (CEN), Brussels, Belgium