Pretreatment of brewery effluent to cultivate *Spirulina* sp. for nutrients removal and biomass production

Qian Lu, Hui Liu, Wen Liu, Yuming Zhong, Caibing Ming, Wei Qian, Qin Wang and Jianliang Liu

ABSTRACT

Due to the low concentration of nitrate and high contents of organics, brewery effluent was not suitable for the cultivation of *Spirulina* sp. This work changed the nutrient profile of brewery effluent effectively by dilution, addition of nitrate, and anaerobic digestion. The result showed that the optimum dilution rate and $NaNO_3$ addition for brewery effluent were 20% and 0.5 g/L, respectively. *Spirulina* sp. grown in pretreated brewery effluent produced 1.562 mg/L biomass and reduced concentrations of nutrients to reach the permissible dischargeable limits. In addition, *Spirulina* sp. grown in pretreated brewery effluent had much higher protein content and oil content. So the appropriate treatment converted brewery effluent into a nutrient balanced medium for algae cultivation and alleviated the potential environmental problems. Pretreatment procedure developed in this work is an effective way to realize the sustainable utilization of brewery effluent and produce algal biomass with valuable nutrients.

Key words | biomass, brewery effluent, nutrients removal, pretreatment, *Spirulina* sp.

Qian Lu†
Hui Liu†
Wen Liu
Yuming Zhong
Caibing Ming
Wei Qian
Jianliang Liu
Department of Environment Science and Engineering,
Zhongkai University of Agriculture and Engineering,
Guangzhou 510225,
China

Hui Liu
Guangdong Provincial Engineering and Technology Research Center for Agricultural Land Pollution Prevention and Control,
Guangzhou 510225,
China

Qin Wang (corresponding author)
Guangdong Provincial Engineering and Technology Research Center for Fruit Tree,
Guangzhou 510225,
China
E-mail: *liuhui@zhku.edu.cn*

†First two authors contributed equally to this work.

INTRODUCTION

Removal of excessive nutrients and remediation of waste effluent to alleviate potential environment pollution have become a hot research topic in recent years. Various treatment technologies, such as sorbents for decontamination, ion exchange, and denitrification, have been developed for waste stream remediation (Elwakeel *et al.* 2012; Liu *et al.* 2016). Removal of nutrients in food processing wastewater by using the algae technology has been widely explored in previous studies since algae cultivation could not only remove nutrients but also produce valuable biomass (Bezerra *et al.* 2011; Shin *et al.* 2015; Lutzu *et al.* 2016). Many studies have cultivated algae in various food processing effluents, including cheese production wastewater, juice processing effluent, corn milling effluent and so on, for both nutrients removal and biomass production (Comino *et al.* 2011; Lin *et al.* 2011; Lu *et al.* 2016). Compared with industrial wastewater and municipal wastewater, food processing wastewater contains no heavy metal or other toxic compositions (Darpito *et al.* 2015). Therefore, algae cultivated in food processing wastewater could be specially utilized for food or feed use.

In practice, however, not all types of raw food processing wastewater could be utilized for algae cultivation (Ji *et al.* 2015). The research of Lu *et al.* (2015) showed that due to the lack of some essential nutrients, meat processing wastewater could not support the growth of algae. So it has become a hot topic to improve the nutrient bioavailability of food processing wastewater in the cultivation of algae. Previous studies have applied various pretreatment technologies to make food processing wastewater to be suitable for algae cultivation (Zhou *et al.* 2013; Prajapati *et al.* 2014). Lu *et al.* (2016) improved the biomass of algae grown on dairy wastewater by appropriate dilution. Hu *et al.* (2013) effectively converted solids in animal breeding wastewater into

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nutrients absorbed algae by applying anaerobic digestion (Hu *et al.* 2013). Other methods used in pretreatment of food processing wastewater include acid hydrolysis, aeration, and fermentation (Mata *et al.* 2012; Nam *et al.* 2014; Castro *et al.* 2015).

Brewery effluent is a type of food processing wastewater obtained from a beer production factory. Thousands of beer factories in China produced about 0.3 billion m^3 brewery effluent annually, which is around 2.0% of the total wastewater production of this country (Feng *et al.* 2008). A few studies have tried to cultivate algae by using brewery effluent. For example, Mata *et al.* (2014) grew *Chlamydomonas* sp. on brewery effluent but only produced 0.229 g/L algal biomass at the end of cultivation. Biomass yield of *Scenedesmus dimorphus* grown on raw brewery effluent was only 0.63 g/L (Lutzu *et al.* 2016). The low biomass yield prevented the wide application of algae technology in the treatment of brewery effluent. To solve this problem, Darpito *et al.* (2015) treated brewery effluent anaerobically before algae inoculation and improved the biomass yield of *Chlorella protothecoides* to 1.88 g/L. Lutzu *et al.* (2016) mixed brewery effluent with artificial medium to optimize the nutrient profile and improved biomass yield of *Scenedesmus dimorphus* to 3.49 g/L. Literature review suggested that raw brewery effluent is not suitable to algae cultivation, but appropriate pretreatment techniques could make brewery effluent available in algae production.

Spirulina sp. is an algal strain, which contains valuable protein (Lu *et al.* 2015). It was reported that in artificial medium (Paoletti medium), protein content of *Spirulina* sp. was 56.17% and percentage of essential amino acids was 52.10% of algal biomass (Volkmann *et al.* 2008). *Spirulina* sp. contains high content of lipid, particularly unsaturated fatty acids, as well. The research of Gupta *et al.* (2008) revealed that content of unsaturated fatty acids in the lipid of *Spirulina* sp. could reach 53.3% (Gupta *et al.* 2008). Due to the high contents of essential amino acids and unsaturated fatty acids, in food and agricultural industry, *Spirulina* sp. is regarded as a potential alternative for protein and lipid production (Mata *et al.* 2014). However, studies on the utilization of brewery effluent in the cultivation of *Spirulina* sp. were rare.

The main aims of this work included finding out the factors limiting algae growth in brewery effluent and developing efficient pretreatment methods to promote the algae growth and nutrients removal. The specific objectives were as follows: (1) cultivating *Spirulina* sp. on raw brewery effluent and finding out factors limiting the growth of algae; (2) conducting appropriate methods to pretreat brewery effluent; (3) evaluating effects of pretreatment on the biomass yield of algae and nutrients removal; (4) assessing effects of pretreatment methods on the nutrients profile of *Spirulina* sp. At the end of this work, an effective and efficient pretreatment method will be established to promote the growth of *Spirulina* sp. in brewery effluent.

MATERIALS AND METHODS

Algal strain

Algal strain was preserved in Zarrouk medium (with 15% agar), which is a commonly used artificial medium for the cultivation of *Spirulina* sp. The major compositions of Zarrouk medium are listed as follows: $NaHCO_3$ (16.80 g/L), K_2HPO_4 (0.50 g/L), $NaNO_3$ (2.50 g/L), K_2SO_4 (1.00 g/L), $MgSO_47H_2O$ (0.20 g/L), $CaCl_2$ (0.04 g/L), $FeSO_47H_2O$ (0.01 g/L), H_3BO_3 (2.86 mg/L), $MnCl_2H_2O$ (1.81 mg/L), $ZnSO_4$ (0.00 mg/L), $CuSO_4$ (0.08 mg/L), and MoO_3 (0.01 mg/L).

Spirulina sp., in this experiment, was cultivated in 250 mL Erlenmeyer flasks with 100 mL culture medium at 28 °C. Erlenmeyer flasks were placed on a shaker with rotating speed of 200 rpm. Continuous fluorescent light (300 μmol photon m^{-2} s^{-1}) was provided for algae growth.

Algae growth and nutrients analysis

Brewery effluent used in this study was obtained from a beer factory in Guangzhou (China). Before algae inoculation, brewery effluent was centrifuged at 3,000 rpm for 3 min to remove large solid particles and sterilized for 20 min at 121 °C to kill bacteria. Analysis of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), NH_3-N, and NO_3-N was conducted according to the Chinese National Standards, GB11914-89, GB11894-89, GB11893-89, GB7974-87, and GB7480-87, respectively (Wang *et al.* 2007). Concentrations of nutrients were expressed as mg/L. Removal efficiencies of nutrients were calculated according to Equation (1).

$$R = \frac{(C_i - C_t)}{C_i} \times 100\% \quad (1)$$

where R refers to the removal efficiency of a certain nutrient in brewery effluent; C_i is the initial concentration of nutrient before algae inoculation; and C_t is the concentration of nutrient in brewery effluent on Day t.

Biomass yield of algae was measured according to published method (Lu *et al.* 2016) and expressed as g/L. Average growth rate was calculated according to Equation (2).

$$S = \frac{(W_t - W_i)}{t} \quad (2)$$

where S refers to the average growth rate (g/L/d); W_t is the dry weight of algal biomass on Day t; W_i is the dry weight of algae inoculated in the culture medium; and t is the cultivation period (days) of algae.

Algal compositions analysis

Two compositions, protein and oil, in algal cells were measured in this experiment. Content of protein was measured by Folin-Lowry method using spectrophotometer (López *et al.* 2010). The measurement was carried out at 750 nm. Content of oil was measured by the method described in a previous publication (Lu *et al.* 2015). In this experiment, contents of oil and protein were expressed as a percentage (%).

Experimental design

Experiments were carried out in five steps. The first step was to measure the nutrients profile of brewery effluent and compare the differences between brewery effluent and artificial medium. The second step was to evaluate the effects of dilution on the biomass yield of *Spirulina* sp. and nutrients removal. The third step was to find out the most appropriate amount of $NaNO_3$ added into diluted brewery effluent. The fourth step was to assess the effects of anaerobic digestion on the characteristics of brewery effluent. The fifth step was to cultivate algae in digested brewery effluent and measure the biomass yield and nutrients removal. The final step was to analyze the compositions of harvested algal cells. In this work, pretreatment of brewery effluent consisted of three parts, including dilution rate, addition of $NaNO_3$, and anaerobic digestion. Biomass yield of algae and nutrient removal efficiency were two major factors that were considered in the evaluation of this pretreatment strategy.

Anaerobic digestion process

A glass bioreactor with the volume of 2 L was used as anaerobic digestion in this study. Since the pH of brewery effluent is favorable to the growth of most microorganisms, the effluent and 150 mL activated sludge were put into the digester without pH adjustment. The bioreactor outlet was sealed with glue to create an anaerobic environment for the digestion (Liu *et al.* 2016). In the digestion process, temperature was controlled at 35 ± 1°C. Effluent in the bioreactor was mixed continuously by agitation at 200 rpm.

Statistical analysis

In this study, all experiments were conducted in triplicate. The results were expressed as means ± standard values. Analysis of variation was utilized to evaluate the data.

RESULTS AND DISCUSSION

Characteristics of brewery effluent

Comparison of nutrient profiles of artificial medium (Zarrouk medium) and brewery effluent (Table 1) showed that two nutrient profiles were very different. Firstly, brewery effluent contained high concentrations of COD (10,120 ± 233 mg/L) while the COD concentration of artificial medium was zero. The main reason is that brewery effluent from the brewery factory contained organic carbon while no organic carbon was added into the artificial medium. The research of Lu *et al.* (2015) revealed that high concentration of organics in food processing wastewater might prohibit the growth of microalgae. Secondly, nitrogen sources in brewery effluent and artificial medium were ammonia and nitrate, respectively. Research of Rodrigues *et al.* (2011) showed that metabolisms of *Spirulina* sp. would alkalize the culture medium and cause the ammonia evaporation. So previous studies mainly used nitrate, rather than ammonia, in artificial medium for the cultivation of *Spirulina* sp. (Rodrigues *et al.* 2011). Thirdly, TN of brewery effluent was much lower than that of artificial medium.

Table 1 | Nutrient profiles of artificial medium and brewery effluent

mg/L	COD	NH_3-N	NO_3-N	TN	TP
Artificial medium	0	0	407.9 ± 3.7	411.3 ± 5.4	89.7 ± 2.8
Brewery effluent	10,120 ± 233	129.4 ± 3.9	0	207.6 ± 4.3	128.7 ± 4.5

Spirulina sp. with high protein content requires higher concentration of nitrogen, which is necessary for the protein synthesis in algal cells, in culture medium.

Based on the analysis of the nutrient profile, we assumed that due to the exceedingly high concentration of COD, existence of ammonia, and lower concentration of TN, brewery effluent is not a good culture medium for the cultivation of *Spirulina* sp. This is one of the reasons why rare publications tried to cultivate *Spirulina* sp. in brewery effluent for biomass production. This assumption was verified by the following experiments. To apply brewery effluent in the cultivation of *Spirulina* sp., two objectives, reducing the concentration of COD to a lower level and improving the concentration of absorbable nitrogen, should be achieved.

Optimization of dilution rate of brewery effluent

Biomass yields and changes of pH values

In the 5-day cultivation, *Spirulina* sp. grown in 20% diluted brewery effluent had the highest biomass yield (0.925 g/L) (Figure 1(a)). The research of Prajapati *et al.* (2014) indicated that some wastewater with a large quantity of solids could prohibit the growth of algae by preventing the light transmission and limiting the photosynthesis. In this experiment, the solid particles in brewery effluent with no dilution or low dilution rate may be the major factor that limited the growth of *Spirulina* sp. The possible reason for the low biomass yield of algae in 10% diluted brewery effluent is that the dilution reduced the concentrations of nutrients and the deficiencies of some nutrients limited the growth of algae.

Figure 1(b) indicates that with the growth of *Spirulina* sp., the pH value of brewery effluent increased significantly. At the end of the cultivation period, pH values of brewery effluent with different dilution rates ranged from 8.17 to 10.78. This result is in accordance with the research conclusion that metabolisms of *Spirulina* sp. would alkalize the culture medium (Rodrigues *et al.* 2010). Compared with pH values of original and 50% diluted brewery effluents, pH values of 20% diluted and 10% diluted brewery effluents were improved. Two possible reasons could explain this phenomenon. Firstly, some chemicals and components improved the pH buffering capacity of brewery effluent. The dilution reduced the concentrations of these chemicals and components and seriously damaged the pH buffering capacity of brewery effluent. Accordingly, in 20% diluted and 10% diluted brewery effluents, pH values were improved by the metabolisms of *Spirulina* sp. Secondly, Figure 1(a) indicates that biomass yields of algae in 20% diluted and 10% diluted brewery effluents were much higher than biomass yields of algae in the original and 50% diluted brewery effluents. Therefore, we assumed that the active metabolisms of *Spirulina* sp. contributed to the higher pH values in 20% diluted and 10% diluted brewery effluent.

Removal efficiencies of nutrients

Results in Figure 1(c) suggest that dilution not only improved the removal efficiencies of COD, but also reduced the concentration of COD left in brewery effluent after algae cultivation. As shown in Figure 1(d), the removal of NH_3-N was closely related with the pH value of brewery effluent. Therefore, we assumed that the alkalization of brewery effluent was the major factor that contributed to the removal of ammonia since under the base environment, ammonia will be converted into ammonia gas and evaporate into the atmosphere. Due to the fact that 62.33% of TN in brewery effluent was NH_3-N (Table 1), it was observed that the removal of TN (Figure 1(e)) was similar with the removal of NH_3-N. Compared with the removal efficiencies of NH_3-N, removal efficiencies of TN were much lower. The most possible reason is that a portion of nitrogen in brewery effluent was organic nitrogen that existed in the solid particles which could not be absorbed by algal cells (Liu *et al.* 2011). The existence of these indigestible nitrogen sources in brewery effluent led to the low removal efficiencies of TN. *Spirulina* sp. grown in brewery effluent showed great ability to remove TP (Figure 1(f)). At the end of cultivation, concentrations of TP left in the original, 50% diluted, 20% diluted, and 10% diluted brewery effluents were 63.3 mg/L, 36.1 mg/L, 3.9 mg/L, and 5.3 mg/L, respectively.

Evaluation of brewery effluent

According to the regulation of wastewater discharge, the concentrations of COD, TP, and NH_3-N in wastewater should be lower than 300 mg/L, 1 mg/L, and 25 mg/L, respectively. However, the concentrations of COD, TP and NH_3-N in brewery effluent without any pretreatment were 5,025 mg/L, 63.3 mg/L, and 104.2 mg/L, respectively, which were much higher than the permissible dischargeable limits. In addition, biomass yield of algae grown in brewery effluent without any pretreatment was only 0.489 g/L. The low biomass yield would reduce the economic benefits and prevent the commercial application of brewery effluent in algae cultivation. This result supports the assumption that

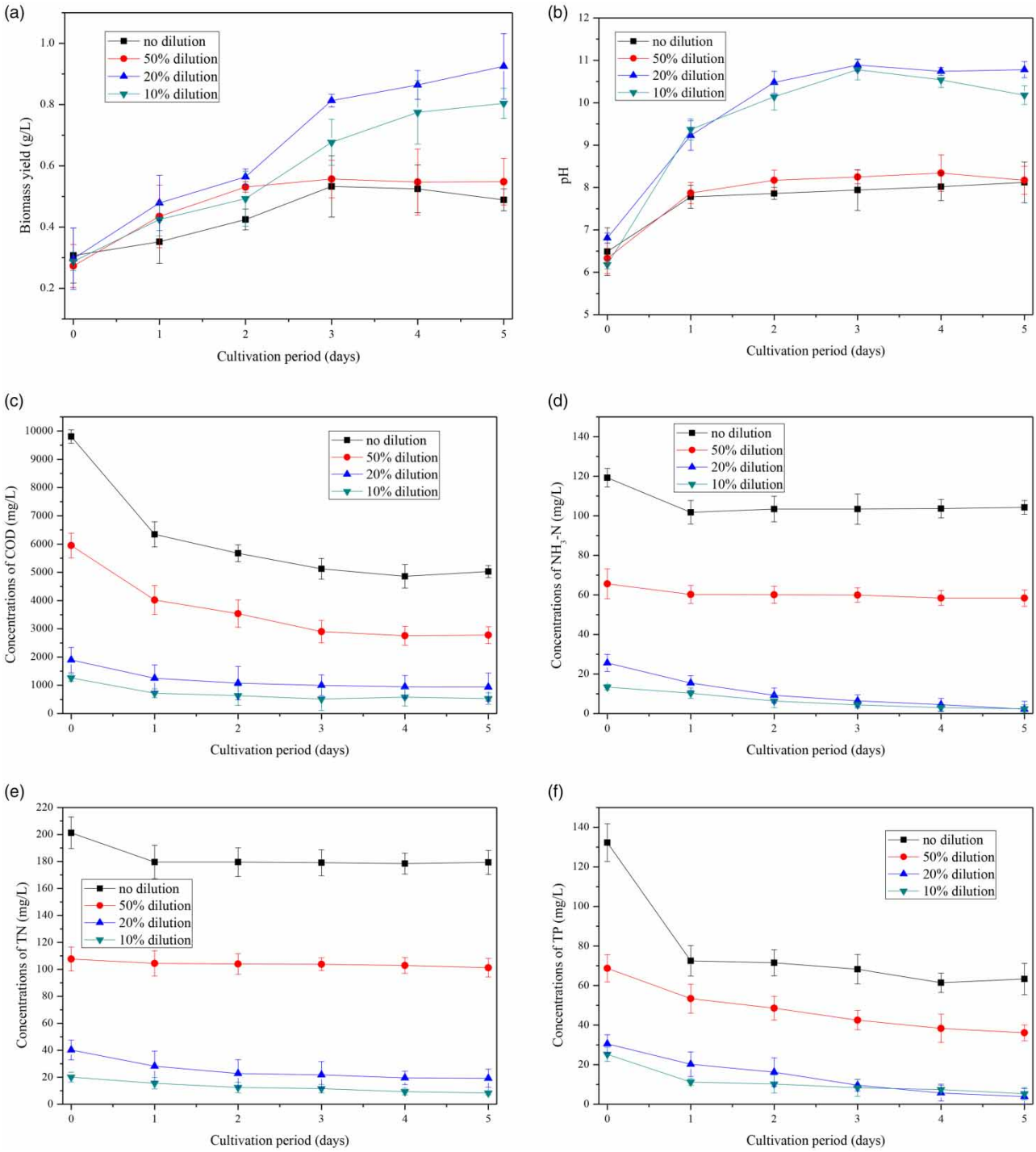

Figure 1 | Growth of *Spirulina* sp. and nutrients removal efficiencies in diluted brewery effluent.

brewery effluent without appropriate pretreatment is not a good culture medium for the growth of *Spirulina* sp.

Discussion of biomass yields of *Spirulina* sp. and removal efficiencies of nutrients indicated that in 20% diluted brewery effluent, biomass yield of *Spirulina* sp. and removal efficiencies of nutrients reached peak values. Therefore, in this experiment, the optimum dilution rate of brewery effluent is 20%.

Addition of nitrate in brewery effluent

In the 20% diluted brewery effluent, concentrations of COD, NO_3-N, NH_3-N, TN, and TP were 1,897 mg/L, 0 mg/L, 25.6 mg/L, 40.3 mg/L, and 30.5 mg/L, respectively. Compared with the artificial medium, 20% diluted brewery effluent had lower concentrations of TN and TP and did not contain NO_3-N. It was reported that NO_3-N is a critical nutritional factor in culture medium that determines the protein synthesis of *Spirulina* sp. (Rodrigues *et al.* 2010). We assumed that the lack of nutrients, particularly NO_3-N, became a bottleneck for algae growth in diluted brewery effluent. To reduce the negative effects of deficiency of nitrate on the growth of *Spirulina* sp., different amounts of sodium nitrate ($NaNO_3$) were added into 20% diluted brewery effluent.

Biomass yields and changes of pH values

Figure 2(a) indicates that addition of $NaNO_3$ in brewery effluents improved the biomass yield of algae. This result verified the assumption that adding nitrate into diluted brewery effluent is a good way to eliminate the limitation of nutrients deficiency on algae growth and improve the biomass yield of *Spirulina* sp. Statistical analysis indicated that biomass yield was not improved significantly when the concentration of $NaNO_3$ increased from 0.5 g/L to 2.0 g/L.

Data in Figure 2(b) suggest that the addition of $NaNO_3$ improved the pH values of diluted brewery effluents at the end of cultivation. The main reason is that under the environment with more nitrate, cells of *Spirulina* sp. had more active metabolisms which contributed to the increase of pH values. Since the alkalization is favorable to the removal of ammonia in culture medium, higher pH values of diluted brewery effluents were preferred in this experiment.

Removal efficiencies of nutrients

As shown in Figure 2(c), with the increase of $NaNO_3$ addition, removal efficiencies of COD were improved. Accordingly, the concentration of COD left in brewery effluent after algae growth was reduced to a lower level with the increase of $NaNO_3$ addition. For example, at the end of cultivation, the concentration of COD in diluted brewery effluent with no $NaNO_3$ addition was 853 mg/L while the concentration of COD in diluted brewery effluent with 0.5 g/L $NaNO_3$ addition was only 679 mg/L. Therefore, adding the appropriate amount of $NaNO_3$ in diluted brewery effluent is an effective way to reduce the concentration of COD.

Figure 2(d) shows that removal efficiencies of NH_3-N in diluted brewery effluents without $NaNO_3$ and with $NaNO_3$ were high, ranging from 89.70% to 95.75%. In the alkalized brewery effluent, most NH_3-N was removed. As shown in Figure 2(e), removal efficiency of TN in diluted brewery effluent with 0.5 g/L $NaNO_3$ addition was the highest (64.06%). When the concentration of added $NaNO_3$ increased from 0.5 g/L to 2.0 g/L, removal efficiencies of TN decreased from 64.06% to 37.34%. The main reason for this phenomenon is that if the concentration of $NaNO_3$ exceeds certain range, the nitrogen source could not be absorbed by algae. Therefore, at the end of cultivation, the concentration of TN in diluted brewery effluent with 2.0 g/L $NaNO_3$ addition was 216.8 mg/L, which is 341.69% higher than that (49.1 mg/L) in diluted brewery effluent with 0.5 g/L $NaNO_3$ addition. To improve the biomass yield of algae and control the environmental contamination caused by the underutilized nitrogen source at the same time, $NaNO_3$ should be added into brewery effluent but the concentration of $NaNO_3$ should be controlled.

At the end of cultivation, concentrations of TP ranged from 1.3 mg/L to 2.2 mg/L. Since the concentrations of TP left in brewery effluent after algae growth were slightly higher than the regulation of wastewater discharge regulation (1.0 mg/L), the removal of TP was not regarded as a serious technical problem in this experiment.

Optimum addition amount of $NaNO_3$

Data in Figure 2 show that addition of $NaNO_3$ could improve the biomass yield of *Spirulina* sp., but the biomass yield was not improved significantly when the concentration of $NaNO_3$ increased from 0.5 g/L to 2.0 g/L. For example, biomass yield (1.256 g/L) of *Spirulina* sp. grown in diluted brewery effluent added with 2.0 g/L $NaNO_3$ was only 1.54% higher than that (1.237 g/L) of *Spirulina* sp. grown in diluted brewery effluent added with 0.5 g/L $NaNO_3$. In addition, addition of $NaNO_3$ improved the concentration of TN in brewery effluent after algae cultivation. In this work, after algae cultivation, concentrations of TN in diluted brewery effluents added with 0.5 g/L and 2.0 g/L $NaNO_3$ were 49.1 mg/L and 216.8 mg/l, respectively. High concentration of TN in culture medium after algae cultivation could not only reduce utilization efficiency of nutrients, but also cause environmental pollutions. To prevent these problems, in this work, the optimum addition amount of $NaNO_3$ in 20% diluted brewery effluent was 0.5 g/L.

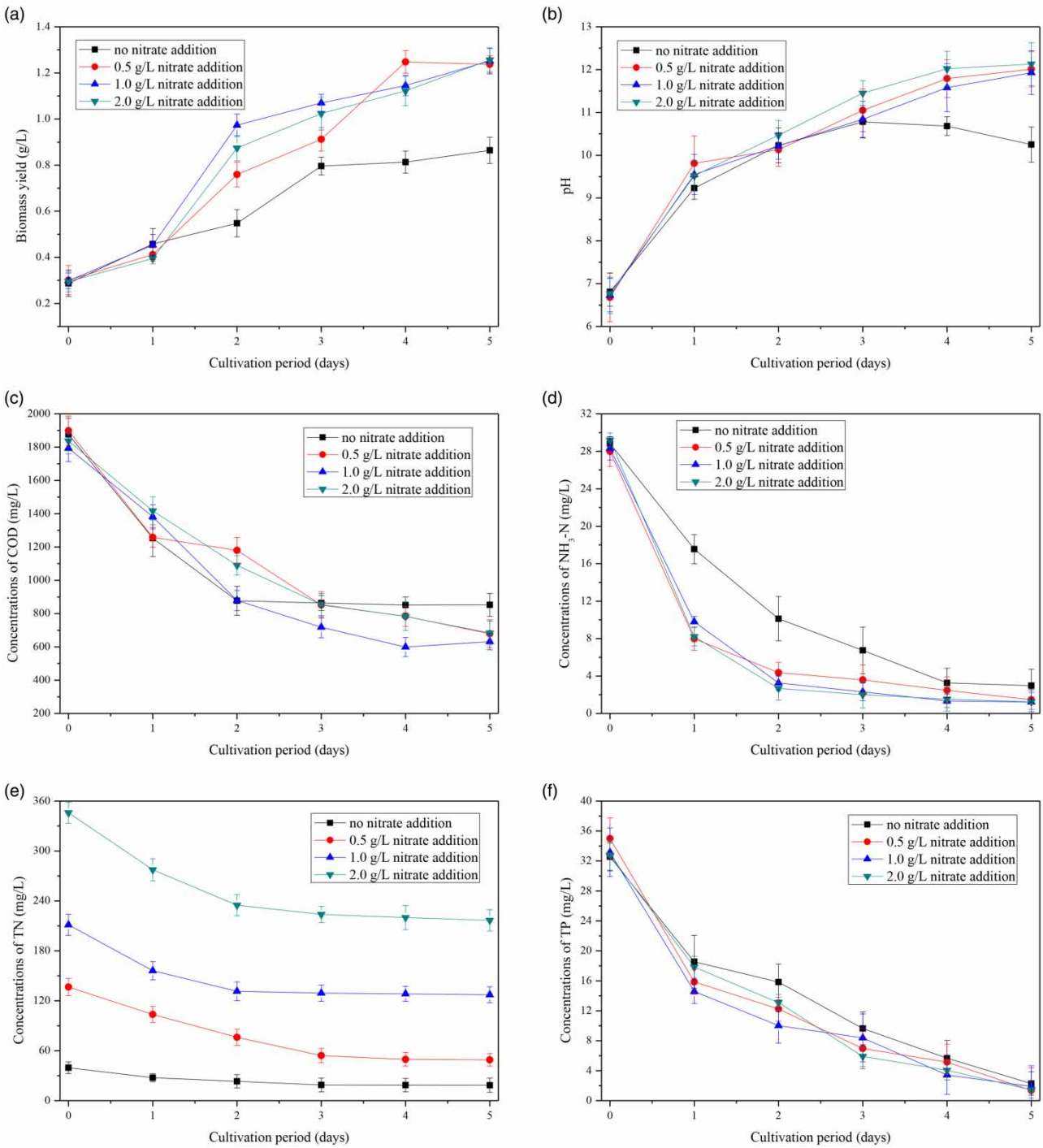

Figure 2 | Growth of *Spirulina* sp. and nutrients removal efficiencies in 20% diluted brewery effluent added with nitrate.

Effects of $NaNO_3$ addition on algal composition

Spirulina sp. is an algal strain mainly used for the production of highly valuable protein. So the protein content was an important concern in this research. Table 2 shows that, compared with the algae grown in artificial medium, algae grown in diluted brewery effluent had much lower protein content. Protein content of algae grown in diluted brewery effluent was only 37.14%, which is much lower than the protein content (49.85%) of algae grown in artificial

Table 2 | | Compositions of algal biomass

	Protein (%)	Oil (%)	Other compositions (%)
Artificial medium	49.85	9.62	40.53
20% diluted brewery effluent	37.14	11.99	50.87
20% diluted brewery effluent with 0.5 g/L $NaNO_3$	47.63	13.05	39.32
20% diluted brewery effluent with 1.0 g/L $NaNO_3$	48.44	13.44	38.12
20% diluted brewery effluent with 2.0 g/L $NaNO_3$	50.53	13.61	35.86
No anaerobic digestion	47.63	13.05	39.32
1 day anaerobic digestion	54.00	13.72	32.28
2 days anaerobic digestion	53.82	14.12	32.06
3 days anaerobic digestion	53.79	13.88	32.33

medium. With the addition of $NaNO_3$ in diluted brewery effluent, content of protein in cells of *Spirulina* sp. was improved from 37.14% to 50.53%. Addition of nitrogen source improved the protein content since nitrogen is a necessary element in the biosynthesis of protein in algal cells. Therefore, adding the appropriate amount of $NaNO_3$ could not only improve the biomass yield of *Spirulina* sp., but also increase the protein content in algal cells.

Anaerobic digestion for the conversion of ammonia

Although brewery effluent had high concentration of COD, concentration of biodegradable organics was not high since some organics in brewery effluent existed in solid particles that could not be absorbed by algal cells. This is the main reason why the concentration of COD in 20% diluted brewery effluent after algae cultivation was high. In this work, anaerobic digestion was applied to convert indigestible organics into bio-digestible nutrients.

Changes of anaerobic environment

Figure 3(a) indicates that ORP of diluted brewery effluent decreased gradually from 191 mV on Day 0 to −376 mV on Day 4. This change suggested that the environment of diluted brewery effluent changed from aerobic conditions to anaerobic conditions. The research of Ryan *et al.* (2010) revealed that the environment of which ORP value was lower than −150 mV was regarded as strict anaerobic circumstances. The anaerobic digestion process mainly consists of three steps: acidogenesis, acetogenesis, and methanogenesis (Kim *et al.* 2010; Ryan *et al.* 2010). Volatile fatty acids accumulated in acidogenic phase, which started when the ORP value was lower than 100 mV (Wang *et al.* 2014). In this experiment, the acidogenic phase, in which the volatile fatty acids accumulated, started from Day 1. In the anaerobic digestion, pH values of diluted brewery effluent decreased from 6.88 to 4.33 (Figure 3(b)). The main factor leading to the acidic environment is the accumulation of volatile fatty acids in the anaerobic process. In the anaerobic digestion, solid organics in brewery effluent were converted into volatile fatty acids.

Changes of nutrient profile

It was reported that microorganism activities in anaerobic digestion consumed energy (Mizuta & Shimada 2010; Cao & Pawłowski 2012). Changes of COD concentrations (Figure 3(c)) showed that organics were the energy source of microorganism activities in anaerobic digestion. Therefore, in this study, one portion of organics in diluted brewery effluent was converted into volatile fatty acids while another portion was utilized by microorganisms as energy source in the process of anaerobic digestion. This is in accordance with the results in the research of Cao & Pawłowski (2012), which revealed that bacterial activities in anaerobic digestion contributed to the removal of COD.

Figure 3(d) and 3(e) indicate that concentrations of NH_3-N and TN were improved while the concentration of NO_3-N did not change significantly. Some organics contained nitrogen source, which was released into brewery effluent by the microorganism activities in anaerobic digestion. Similar phenomenon was observed in the changes of TP concentration, which was improved from 32.92 mg/L to 39.01 mg/L (Figure 3(f)). Therefore, anaerobic digestion not only contributed to the accumulation of volatile fatty acids, but also improved the concentrations of TN and TP.

Given the low concentrations of TP and COD after Day 4 in anaerobic digestion, in this study, the maximum period of anaerobic digestion was set as 3 days.

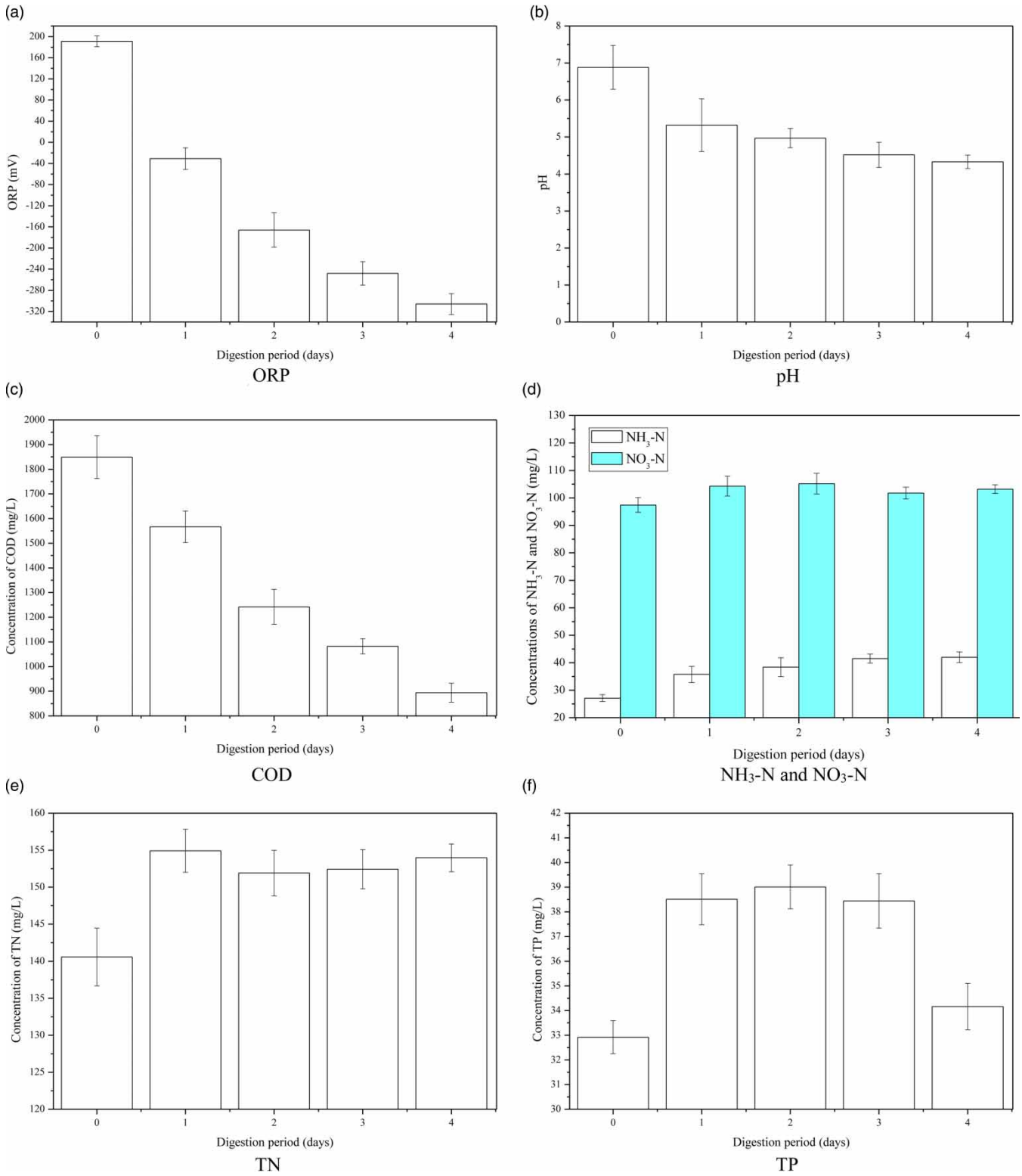

Figure 3 | Properties of brewery effluent in anaerobic digestion.

Growth of *Spirulina* sp. in digested brewery effluent

Biomass yields and changes of pH values

Results in Figure 4(a) suggest that 2-day anaerobic digestion improved the biomass yield of algae by 24.96%. The main reasons for the improvement of biomass yield include the conversion of indigestible organics into biodegradable organics and the release of nitrogen and phosphorus into culture medium. Therefore, appropriate anaerobic digestion is an efficient and effective way to improve the biomass yield of *Spirulina* sp.

Although anaerobic digestion reduced pH values of brewery effluent, metabolisms of *Spirulina* sp. still contributed to the alkalization, which led to the removal of NH_3-N, of digested brewery effluents. As shown in Figure 4(b), although initial pH values of brewery effluents with different digestion periods were different, at the end of algae cultivation period, pH values were higher than 11, which was enough to cause ammonia evaporation. Therefore, the difference of initial pH values caused by anaerobic digestion would not impact the removal efficiency of NH_3-N.

Removal efficiencies of nutrients

Figure 4(c) shows that although the concentration of COD decreased in the anaerobic digestion, utilization efficiency of organics was improved due to the accumulation of volatile fatty acids. At the end of algae cultivation period, COD in brewery effluent with 2-day digestion had the lowest concentration, 354 mg/L, which meets the requirement of wastewater discharge standard. According to the data in Figure 4(c), 2-day digestion improved the removal efficiency of COD to the highest level and reduced the concentration of COD to the lowest level. Therefore, given the removal of COD, subjecting the diluted brewery effluent to 2-day digestion is the best choice.

The high removal efficiency of NH_3-N in Figure 4(d) supports the assumption that different initial pH values would not cause significant difference in the removal of NH_3-N. Figure 4(g) shows that removal efficiency of NO_3-N in brewery effluent with longer period of anaerobic digestion was higher. It is the metabolism of algae that contributed to the improvement of removal efficiency of NO_3-N. In this experiment, the longer period of anaerobic digestion promoted the growth of algae improved the biomass yield (Figure 4(a)). Algae with more active metabolisms absorbed more nutrients in culture medium.

As shown in Figure 4(e) and 4(f), digestion improved the removal efficiencies of TN and TP. In the brewery effluent with 2-day digestion, concentration of TP after algae growth was only 0.97 mg/L.

Optimum digestion period

According to the removal efficiencies of nutrients and the biomass yield of *Spirulina* sp., 2-day anaerobic digestion is the optimum choice for the pretreatment of diluted brewery effluent prior to algae cultivation. In the diluted brewery effluent with 2-day digestion, after algae growth, concentrations of COD, TN, TP, NH_3-N, and NO_3-N were 298 mg/L, 32.92 mg/L, 0.97 mg/L, 0.65 mg/L, and 19.22 mg/L, respectively. According to the regulation of wastewater discharge, after pretreatment and algae cultivation, brewery effluent could be discharged.

Composition of algal biomass

Table 2 shows that appropriate anaerobic digestion improved the protein content in algal biomass. Two reasons could be used to explain this phenomenon. Firstly, digestion improved the concentration of TN, which determined the content of protein in algal cells. Secondly, anaerobic digestion converted the high-weight-molecular organics into low-weight-molecular organics which could be absorbed more efficiently by algal cells. Accordingly, the organic resource in brewery effluent was more likely to be utilized by algal cells. So it was observed that not only the protein content, but also the oil content, in algal cells was improved by anaerobic digestion. For example, the conversion of solid organics into volatile fatty acids improved the absorption efficiency of organic carbon in brewery effluent. Absorbed organic carbon could directly participate in the synthesis of intracellular protein and lipid (Hartong *et al.* 2008). Compared with carbon dioxide, absorbed organic carbon could be utilized by algal cells in a more efficient way. High absorption efficiency and utilization efficiency of organic carbon improved the yield of oil in algal cells slightly (Table 2). Therefore, appropriate anaerobic digestion could improve the contents of oil and protein by changing the properties of nutrients in brewery effluent.

Discussion

Biomass production of *Spirulina* sp

To reduce the production cost and improve the biomass yield of *Spirulina* sp., previous studies mainly focused on the addition of cheap nutrient resources in artificial

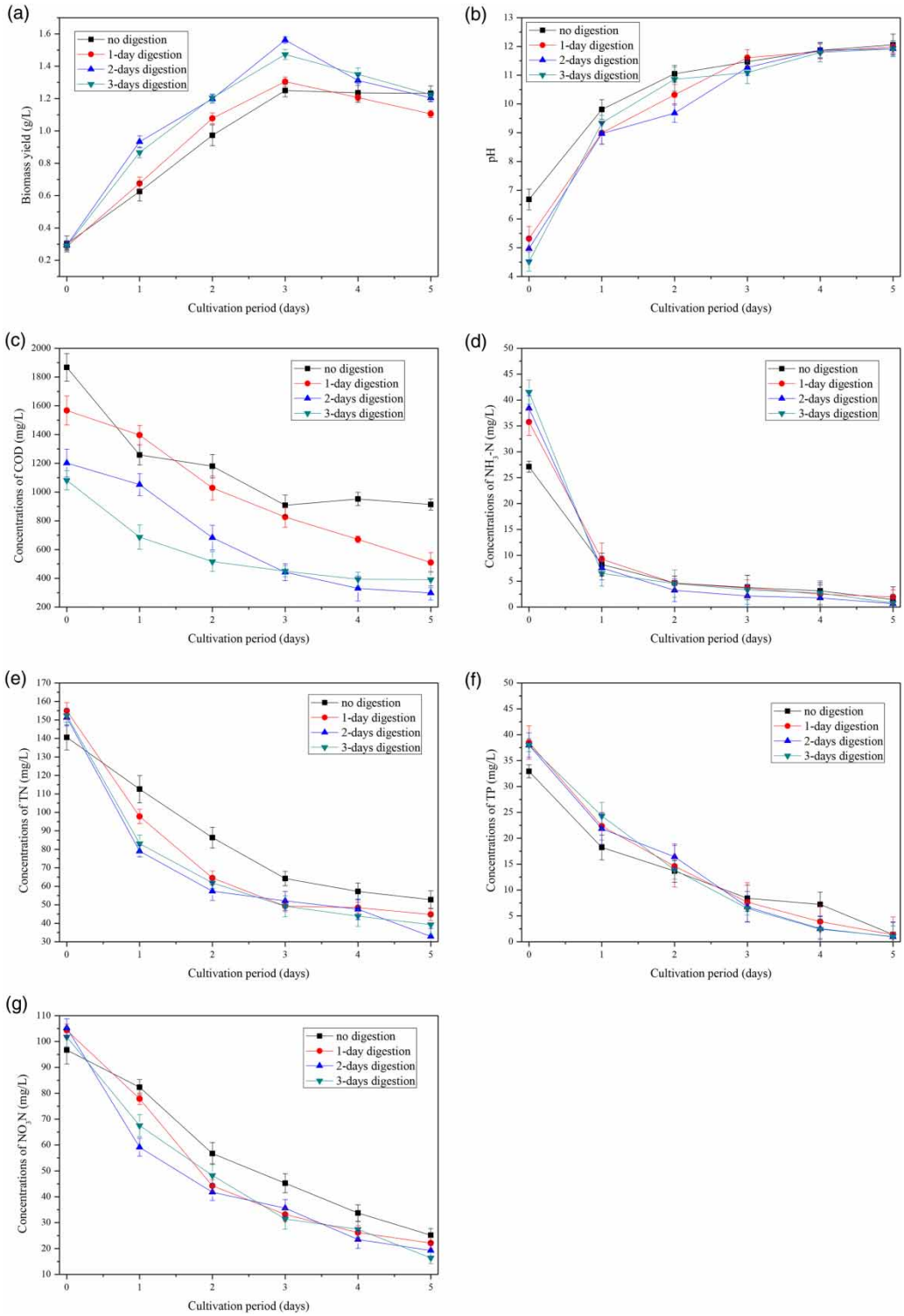

Figure 4 | Growth of *Spirulina* sp. and nutrients removal efficiencies in anaerobically digested brewery effluent.

medium. Table 3 summarizes some studies which applied this strategy in the cultivation of *Spirulina* sp. It showed that the addition of different nutrients would impact the biomass yield of *Spirulina* sp. The addition of some nutrients, such as whey protein (Salla *et al.* 2016), shell and soil extract (Jung *et al.* 2014) and monoethanolamine (da

Table 3 | Characteristics of biomass of *Spirulina* sp. grown in different culture mediums

Culture medium	Algae growth period (days)	Biomass yield (g/L)	Reference
Zarrouk medium with shell and soil extract	14	2.2	Jung *et al.* (2014)
Zarrouk medium with why protein	16	1.5	Salla *et al.* (2016)
Zarrouk medium with monoethanolamine	12	1.2	da Rosa *et al.* (2016)
Paoletti medium	23	2.5	Volkmann *et al.* (2008)
Modified Zarrouk medium	6	0.57	Raoof *et al.* (2006)
Pretreated brewery effluent	5	1.56	This study

Rosa *et al.* 2016), improved biomass yield of *Spirulina* sp. However, in some studies, one problem is that the algae growth period is still long, ranging from 12 days to 23 days. This problem would seriously reduce the economic benefits of algae cultivation and prevent the industrial application. The research of Raoof *et al.* (2006) modified the profile of Zarrouk medium and reduced the algae growth period to 6 days. This work cultivated *Spirulina* sp. by using waste resources with only a few artificial chemicals. The problem caused by high cost of culture medium was totally solved. In addition, compared with previous studies, this work had much shorter growth period. As shown in Table 3, in terms of biomass yield, pretreated brewery effluent was much better than some modified artificial mediums, such as Zarrouk medium with whey protein and Zarrouk medium with monoethanolamine. Therefore, given the high biomass yield and short cultivation period, pretreated brewery effluent is a good culture medium for the production of *Spirulina* sp.

Removal of nutrients in brewery effluent

Literature review showed that only a few publications focused on the application of algae technology in brewery effluent treatment. Table 4, which compares the nutrients removal of brewery effluent, shows that brewery effluent treatment by algae cultivation has two problems, high concentrations of nutrients after algae growth and long treatment period. Firstly, concentrations of nutrients in brewery effluent after algae cultivation were high. For example, in the studies of Mata *et al.* (2012) and Raposo *et al.* (2010), concentrations of COD in brewery effluent treated by algae were higher than 1,500 mg/L. Mata *et al.* (2012) discovered that dilution could promote algae to absorb the nutrients in brewery effluent and improve the removal efficiencies of nutrients. However, concentrations of nutrients after algae growth were still higher than the permissible dischargeable limits. Accordingly, brewery effluent treated by algae could not be discharged without further treatment. Secondly, although some studies developed methods to improve the removal efficiencies of nutrients, the treatment period was still long (more than 2 weeks) (Farooq *et al.* 2013). In the industry, to improve the capacity of wastewater treatment facilities, treatment techniques with a shorter time period are preferred. This is another reason why algae technology is applied rarely in brewery effluent treatment.

In this study, it was observed that *Spirulina* sp. performed well in the removal of COD in brewery effluent. As discussed above, after anaerobic digestion, high-weight-molecular organics in effluent were converted into low-weight-molecular organics. Digested organic carbon could be classified into two major categories, saccharide and short-chain fatty acid (Hu *et al.* 2013). After hydrolysis,

Table 4 | Removal of nutrients in brewery effluents by algae cultivation

		Removal efficiencies (%)			Concentrations of nutrients after treatment (mg/L)			
Algal strain	Algae growth period (days)	COD	TN	TP	COD	TN	TP	Reference
Scenedesmus obliquus	13	57.5	20.8	NA	1,692	47	NA	Mata *et al.* (2012)
Chlorella vulgaris	20	14.6	63	28	1,854	43	NA	Raposo *et al.* (2010)
Chlorella sp.	15	NA	87	80	NA	7	3.3	Farooq *et al.* (2013)
Spirulina sp.	5	75.2	78.3	97.4	298	32.9	0.97	This study

some saccharides were transformed into glucose, which was utilized by algal cells through glycolysis (Bogorad *et al.* 2013). Short-chain fatty acids could also be absorbed and utilized by algae. For example, acetic acid absorbed by algae was converted into acetyl-CoA, which is the substrate for fatty acids synthesis and the Krebs cycle (Hartong *et al.* 2008). Therefore, organics in brewery effluent play a critical role in algae growth and intracellular metabolisms.

Practical application

According to the experimental results, high volume of freshwater should be used for the dilution of brewery effluent. In the practice, brewery effluent could be subjected to anaerobic digestion first and then used for algae cultivation. In this way, the scale and volume of anaerobic facilities could be reduced to a lower level. In addition, to control the use of freshwater, a portion of brewery effluent after treatment will be recycled for the dilution of new batch of effluent (Liu *et al.* 2017). The recycling of treated effluent could significantly reduce the demand on freshwater and ensure the practical application of technologies developed in this study.

This study which applied dilution and anaerobic digestion in the pretreatment of brewery effluent successfully reduced the concentrations of nutrients to reach the permissible dischargeable limits and shortened the wastewater treatment period (Table 4). With the solution of problems discussed above, the application of algae technology in brewery effluent will be more applicable in practice.

CONCLUSIONS

It was concluded that: (1) raw brewery effluent is not a good culture medium for the cultivation of *Spirulina* sp.; (2) the optimum dilution rate and $NaNO_3$ addition for brewery effluent were 20% and 0.5 g/L, respectively; (3) *Spirulina* sp. grown in pretreated brewery effluent produced 1.562 mg/L biomass and reduced the concentrations of nutrients to reach the permissible dischargeable limits; (4) brewery effluent pretreatment improved contents of protein and oil in algal cells; and (5) combined pretreatment, including dilution, addition of $NaNO_3$, and anaerobic digestion, of brewery effluent is an effective way to pretreat brewery effluent for algae cultivation.

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