

Auto-flocculation through cultivation of *Chlorella vulgaris* in seafood wastewater discharge: Influence of culture conditions on microalgae growth and nutrient removal

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Nowadays, the pretreatment of wastewater prior to discharge is very important in various industries as the wastewater without any treatment contains high organic pollution loads that would pollute the receiving waterbody and potentially cause eutrophication and oxygen depletion to aquatic life. The reuse of seafood wastewater discharge in microalgae cultivation offers beneficial purposes such as reduced processing cost for wastewater treatment, replenishing ground water basin as well as financial savings for microalgae cultivation. In this paper, the cultivation of *Chlorella vulgaris* with an initial concentration of $0.01 \pm 0.001 \text{ g} \cdot \text{L}^{-1}$ using seafood sewage discharge under sunlight and fluorescent illumination was investigated in laboratory-scale without adjusting mineral nutrients and pH. The ability of nutrient removal under different lighting conditions, the metabolism of *C. vulgaris* and new medium as well as the occurrence of auto-flocculation of microalgae biomass were evaluated for 14 days. The results showed that different illumination sources did not influence the microalgae growth, chemical oxygen demand (COD) and biochemical oxygen demand (BOD) significantly. However, the total nitrogen (total-N) and total phosphorus (total-P) contents of microalgae were sensitive to the illumination mode. The amount of COD, BOD, total-N and total-P were decreased by 88%, 81%, 95%, and 83% under sunlight mode and 81%, 74%, 79%, and 72% under fluorescent illumination, respectively. Furthermore, microalgae were auto-flocculated at the final days of cultivation with maximum biomass concentration of $0.49 \pm 0.01 \text{ g} \cdot \text{L}^{-1}$, and the pH value had increased to $\text{pH } 9.8 \pm 0.1$ under sunlight illumination.

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[Key words: *Chlorella vulgaris*; Nutrient removal; Wastewater treatment; Growth condition; Green energy]

It is well known that microalgae contain various types of valuable compounds such as pigments, lipids and polysaccharides. Microalgae have been investigated for its potential in food processing and animal nutrition applications, clean energy production (biofuels), green chemistry and wastewater treatment in recent years (1–4). With the urge to develop a sustainable, safe, ecological and cost effective innovative technology, the society is focusing on biotechnology methods in treating the domestic and urban wastewater. In fact, microalgae have demonstrated a high capability to eliminate organic and inorganic nutrients from the wastewater and yielding biomass which can be used to produce biofuels, fertilizers or green chemical products (2–7). Therefore, new techniques were launched to combine the wastewater treatment process with the microalgae cultivation for two purposes: renewable energy production and reduction of overhead costs (8–10).

In addition, microalgae are able to derive nutrient in particular nitrogen and phosphorus compounds from wastewater during

cultivation (11–16). Aravantinou et al. (17) have selected 10 freshwater and marine microalgae strains, including *Chlorella* sp. to treat wastewater and produce lipid for biofuel. Their results have illustrated that the microalgae growth was associated with nutrient removal but freshwater species exhibited a better performance of total phosphorus (total-P) removal compared to the marine microalgae strain. Besides, the *Chlorella* sp. strain was also studied for the nutrient elimination and biofuel production. Li et al. (18) have demonstrated that this strain was well adapted to high concentrated urban sewage. Out of all species of microalgae, *Chlorella vulgaris* strain is known to be a promising nutrient removal and high lipid induced microalgae (19–21). *C. vulgaris* is a single-celled, non-flagella and green freshwater microalgae which is widely cultivated for alimentation or supplement purposes. It is due to the rapid growth rate of microalgae and high content of nutritious component such as proteins, pigments and lipid (22–24). Although various studies have been conducted to investigate the nutrient elimination of sewage using this strain, the cultivation of *C. vulgaris* using seafood wastewater discharge (SWD) has not given much attention in current research area. Meanwhile, wastewater from seafood industry is a potential resource for green

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energy production, especially used for microalgae cultivation due to its high nutrient content. The SWD is categorized as pollutants if it is discharged into lakes, rivers or sea without any treatment (25–28).

In this study, we aimed to reduce the loading rate on wastewater treatment plants and microalgae harvesting cost by using SWD as the nutrient-rich source for *C. vulgaris* cultivation. Up to date, this new culture medium was firstly reported in this microalgal research. Therefore, the nutrient removal in the sewage and microalgae growth rate using SWD were investigated accordingly in different cultivation conditions. The important parameters that regulate the growth rate of microalgae are pH, light intensity, nutritional ingredients and biochemical compounds (29,30). These parameters are of high concern to apply a feasible establishment and reduce the overall cost of cultivation including post production process of microalgae (31,32). This paper focuses on evaluating the adaptability of microalgae into a new medium by observing the growth rate and capability of microalgae in eliminating nutrient of the suggested medium. In addition, the illuminating condition for the photosynthesis in microalgae was altered and investigated in order to prepare a feasible set-up for outdoor cultivation instead of the traditional method, which is cultivating in photobioreactors using artificial light. Furthermore, the microalgae cultured in new condition including type of culture medium, light intensity and pH value would affect the harvesting process, where the main concern could be solved. Several studies have stated that the cost spent in harvesting process has contributed to high production cost of algae biofuel (33,34). Hence, an auto-flocculation of microalgae will minimize processing cost significantly, and decrease the chemical load in harvest stage. This phenomena will occur due to an increase of pH value during photosynthesis of microalgae.

MATERIALS AND METHODS

Microalgae strain and mediums The *C. vulgaris* 211–19 [The Culture Collection of Algae at the University of Göttingen (SAG), Göttingen, Germany] was selected due to its capacity to assimilate both ammonium (NH_4^+) and nitrate ions (NO_3^-) yet resulted in the approval of ammonium (35,36). This strain was cultured in a Sueoka medium which contained ammonium ion as a source of nitrogen, that was accommodated with the autotrophic Sueoka's medium (37) described by Harris (38). The nutrient and mineral contents of this medium (Table 1) were regulated to a total mass of microalgae concentration of $0.8 \pm 0.02 \text{ g}_{\text{DM}} \cdot \text{L}^{-1}$ (DM, dry matter) in the elaborated batch system.

Another culture medium was taken from SWD and the removal of its nutrient content through *C. vulgaris* growth was investigated. The SWD was collected from a collection tank which gathered the flows from the secondary settlers at the seafood wastewater treatment factory in Tho Quang industry zone (Danang City, Vietnam). The SWD was collected using a 20 L disposable sampling container and brought to the experimental set-up. The principal characteristics of this effluent are shown in Table 2.

Experimental set-up The microalgae strain, *C. vulgaris* were harvested from the original medium at a concentration of $0.8 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ and subsequently adjusted to the initial concentration of $0.01 \pm 0.001 \text{ g} \cdot \text{L}^{-1}$ in a 2 L Erlenmeyer flask which contained 1.5 L of medium. The adjustment of initial concentration was performed under continuous aeration mode for well mixing purposes. One part of the biomass was processed with original Sueoka medium and the other was processed with the effluent of SWD to evaluate the microalgae growth rate and the

TABLE 1. Ionic ingredient of the original culture medium ($\text{mg} \cdot \text{L}^{-1}$).

	Na^+	K^+	NH_4^+	Mg^{2+}	Ca^{2+}	Cl^-	NO_3^-	HCO_3^-	SO_4^{2-}	PO_4^{3-}
Sueoka based	230	28.7	243.9	13.8	6.8	493.1	–	610	54.6	69.8

TABLE 2. The initial concentration of seafood wastewater discharge characteristic ($\text{mg} \cdot \text{L}^{-1}$).

	Mg^{2+}	Ca^{2+}	HCO_3^-	PO_4^{3-}	COD	BOD	Total-N	Total-P
Concentration	14.01 ± 0.1	92.08 ± 0.7	607.15 ± 5.15	11.5 ± 0.05	306.5 ± 2.43	206.2 ± 2.36	92.7 ± 2.15	9.5 ± 0.15

performance of nutrient removal by the microalgae. During the inoculation of microalgae into SWD, the flasks were closed to avoid the loss of splashed matter due to the aeration of 0.02 vvm (volume to volume per minute) air. In order to determine the capacity of wastewater nutrient elimination by *C. vulgaris*, the experiment was set up with Erlenmeyer flask containing the SWD with no microalgae under the same condition of all assays as control sample. Meanwhile, the light source of cultivation would be supplied by fluorescent tubes or sunlight as the main energy source.

In order to further investigate the capacity of self-decantation of microalgae depending on illumination condition, the experiments were set up with two types of conditions. The first condition was conducted using the fluorescent tubes with a light intensity of $150 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (measure instantaneous light in $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) (39). As for second condition, the microalgae were illuminated by the sunlight with similar intensity in daytime and by the fluorescent light in the rest of the day. In addition, the sunlight intensity was averaged by the time interval of 0.5 h during daytime due to the change of temperature as well as solar irradiation. A total number of 18 experimental flasks were examined under 2 different illumination conditions, with 3 setups for control samples, 3 setups for SWD with microalgae and 3 setups for microalgae cultivation in Sueoka medium. The experimental temperature was provided at $27 \pm 2^\circ\text{C}$.

In the first day of experiment, the analysis for initial concentration of effluent characteristics was performed before adjusting the concentration of *C. vulgaris* to $0.01 \text{ g} \cdot \text{L}^{-1}$. As for the following days, the biomass concentration and SWD characteristics were recorded to the day that *C. vulgaris* has reached the death phase or the biomass concentration decreased rapidly to a constant value. The concentration of microalgae was analyzed by measuring the optical density at wavelength of 682 nm (OD_{682}). All experiments were performed triplicate and the experimental errors were calculated as the average absolute deviation. The analytical methods used to determine the characteristics of SWD, quality of outlet and microalgae growth were started in following section. The microalgae harvesting method was based on the methodology proposed by Nguyen et al. (40). The aeration mode was stopped when microalgae were found to be auto-flocculated. The auto-flocculation process was indicated by decreased value of the OD_{682} . In summary, the cultivation of *C. vulgaris* in SWD was implemented for nutrient removal purpose as well as biofuel production, fertilizer industry or green production. The treatment process is shown in Fig. 1.

Microalgae growth Cell concentration was interpreted on DM basis. The microalgae growth rate was monitored by checking the cell concentration in flasks through the analysis of total chlorophyll, carotenoids and cell number. Total chlorophyll in microalgae is proportional to the cell number and any instance of a difference between carotenoid-to-chlorophyll proportions represents the stress in microalgae cultivation. The measurement is available in the absorbance ranging from 652 to 665 nm according to Ritchie's study (41). The extraction of pigments was performed by using pure methyl alcohol, followed by incubation for 45 min at 45°C and centrifugation before analysis. The presence of microalgae was measured at the absorbance of 682 nm (OD_{682}). This method is the simplest analytical method to determine the microalgae cell concentration by using UV-Vis spectrophotometer (Ultrospec 7000 PC, Biochrom Spectrophotometer, Biochrom, Cambridge, UK). The absorbance of 682 nm was calibrated and the microalgae weight was measured as biomass content determination. The biomass content was obtained from the calibration curve. A calibration graph of dry cell weight against OD_{682} with the Sueoka and SWD blank mediums was constructed.

Biomass content was measured by using gravimetry method. Microalgae were filtered through glass fiber filters (Whatman GF/F, Fisher Scientific France) which were dried and weighed beforehand. These filters with microalgae were cooled in a desiccator and weighed subsequently after drying within 24 h at 105°C . This experiment was performed triplicate and the standard error was calculated.

pH In order to determine the dependence of the microalgae settling efficiency on pH change, a pH meter (HI 2211 Hanna) was used in the experiment. The pH value was recorded every two days to evaluate the capacity of flocculating microalgae through the increment of pH value.

Organic content in SWD The pollution level of SWD depends on several parameters. The most important parameter was considered in this work, which is the estimated organic content of effluent. Analysis related to biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (total-N) and total phosphorus (total-P) were carried out to estimate the contamination level of carbonaceous compounds and nitrogen-containing compounds in the effluent. Besides, these parameters also indicated the degree of microalgae metabolism, where the nutrients were consumed for growth. The samples were collected daily from the Erlenmeyer flasks with a volume of 15 ml microalgae suspension. The suspension was then centrifuged at 6000 rpm for 15 min to eliminate most of the *C. vulgaris* biomass. The parameters such as COD, BOD, total-N and total-P were performed using the supernatant obtained through APHA method (42). These parameters were measured at the initial of each experimental set-up until the end

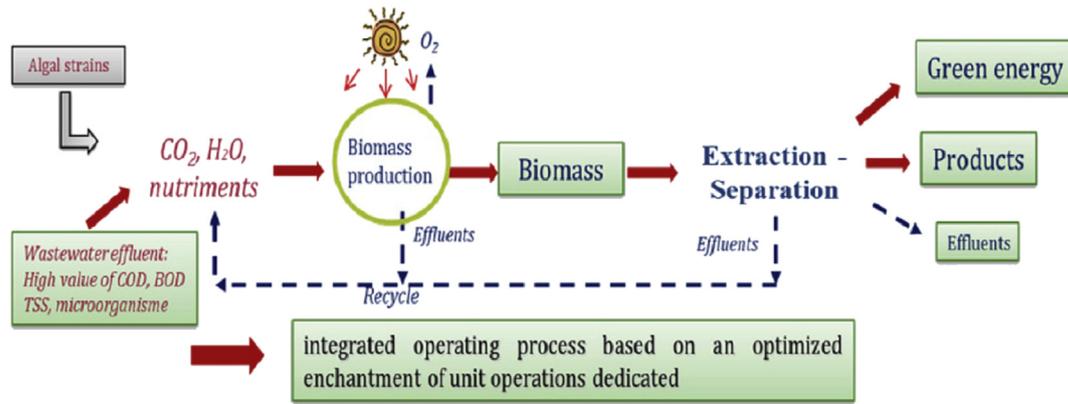


FIG. 1. Schematic of microalgae cultivation using seafood wastewater.

of experiment. Each experimental time was 14 days until the start of *C. vulgaris* biomass flocs decanting in the bottom of the flasks. The microalgae settling efficiency (E_s) was computed as the following Eq. 1:

$$E_s = \frac{OD_{682m} - OD_{682f}}{OD_{682m}} \quad (1)$$

where OD_{682m} and OD_{682f} are the optical density of processed suspension measured at the maximum cell number and supernatant at the final day of microalgae culture at 682 nm, respectively. Meanwhile, the efficiency of nutrient removal (E_r) was calculated as follows:

$$E_r = \frac{C_i - C_f}{C_i} \times 100\% \quad (2)$$

where C_i represents the initial concentration of organic content ($mg \cdot L^{-1}$) and C_f represents the final concentration of organic content ($mg \cdot L^{-1}$).

RESULTS AND DISCUSSION

Microalgae growth To evaluate the growth rate of *C. vulgaris* in SWD medium, this strain was cultivated in two types of media: Sueoka medium and SWD. In Fig. 3, the growth rate of microalgae was determined by OD_{682} , chlorophyll *a* or cell concentration (biomass concentration). The relationship of these three parameters released a linear correlation facilitated the determination of cell mass for a given value of optical density at 682 nm as observed from the calibration curve shown in Fig. 2. The results shown in Fig. 3 demonstrate the normal growth curve

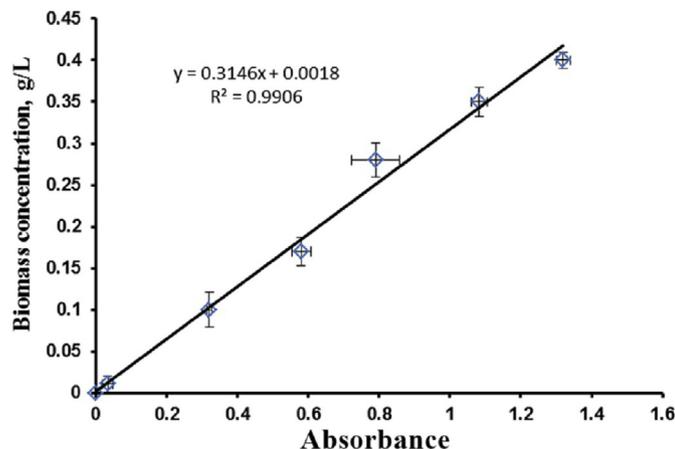


FIG. 2. Calibration curve of *C. vulgaris* at 682 nm. Mean \pm SD of three biological replicates.

of *C. vulgaris* in both Sueoka mediums under different lighting conditions. It was observed that, at the initial concentration ($0.01 \pm 0.001 \text{ g} \cdot \text{L}^{-1}$), the growth rate of microalgae increased rapidly to the stabilize phase. The analyzed results showed that the concentration of microalgae was approximately $0.46 \pm 0.015 \text{ g} \cdot \text{L}^{-1}$ under the sunlight while at fluorescent mode, the result has figured a slightly lower content. However, the growth rate of *C. vulgaris* in SWD medium was less stable than the original medium. The microalgae were decanted rapidly in the bottom of flask at the 6th day of cultivation. Based on these observations, the aeration mode was not conducted to 8-day of the cultivation. After the 8th day, the auto-flocculation of *C. vulgaris* were occurred in non-aeration mode because the microalgae have stopped consuming nutrients.

On the other hand, the tested samples of SWD containing microalgae were found that the growth of *C. vulgaris* did not reach stationary phase after reaching maximum OD_{682} on day 5 and greatly declined in the death phase. It is suggested that the presence of bacteria in the effluent could affect the occurrence of microalgae cell division. The presence of large numbers of bacterial species in the wastewater could result into the difficulty of microalgae assimilation nutrients (43,44). Previous studies have demonstrated that the bacterial contaminants were found on the surface of microalgae cells (45) and the concentration of bacterial cells would not decrease with the increment of microalgae density.

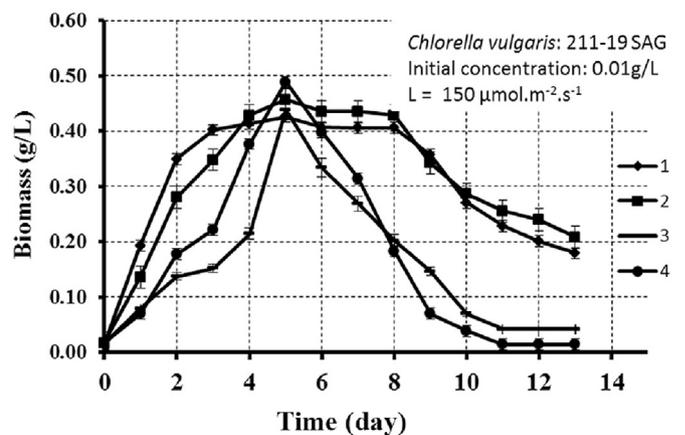


FIG. 3. The growth rate of *C. vulgaris* in two mediums with respect to the function of time in batch mode. Line 1, *C. vulgaris* in Sueoka medium with fluorescent illumination; line 2, *C. vulgaris* in Sueoka medium with sunlight illumination; line 3, *C. vulgaris* in SWD with fluorescent illumination; line 4, *C. vulgaris* in SWD with sunlight illumination. Mean \pm SD of three biological replicates.

The enzyme released by the bacteria from the sewage could destroy the microalgae cell wall, causing *Chlorella* cell lysis (43). The assimilation of nutrient for bacterial metabolism may inhibit microalgae growth as well. Most of the bacterial species in SWD were found high in density, and their metabolism is able to affect the microalgae growth rate due to the competition for essential nutrients such as nitrogen or phosphorus (46).

Effect of illumination mode on algae cultivation After the mass culture of *C. vulgaris* in SWD under sunlight and fluorescent illumination, microalgae biomass have reached the maximum concentration of $0.49 \pm 0.01 \text{ g L}^{-1}$ and $0.44 \pm 0.004 \text{ g L}^{-1}$, respectively, and subsequently biomass concentration began to decline to a constant value. The reduction indicated that the nutrient of SWD was eliminated from the effluent. Furthermore, Fig. 3 shows that the microalgae density under the sunlight mode was slightly higher than the sunless mode. The difference of these two modes in each medium was insignificant. The microalgae cultivated in Sueoka medium had small deviation in the value of cell density under two different illumination modes, the latter illustrated slightly lower cell numbers of microalgae. Microalgae density in SWD under the sunlight was higher than microalgae that cultivated under another lighting condition, but the microalgae density was greatly reduced after reaching a peak with the pH of 8.22 ± 0.14 . The higher microalgae density has led to quicker settling process of microalgae in the bottom of flasks with the pH value of 9.71 ± 0.17 on day 9. The obtained results showed that the microalgae biomass in SWD under two types of lighting modes was easily estimated as described by Nguyen et al. (40) at $47.00 \pm 5.5 \text{ g L}^{-1}$ which corresponded to a complete settling at pH 9.80 ± 0.10 , and the settling efficiencies were close to 99% for sunlight and 92% for fluorescent light.

Meanwhile, the microalgae in the original medium was difficult to be auto-decanted. The settling efficiencies of the microalgae in Sueoka medium obtained were 55%, and 60% with fluorescent lighting and sunlight, respectively. The pH value measured was around 7.5 until *C. vulgaris* has entered the death phase. The auto-flocculation process of microalgae would definitely not accurate at this pH value. This result was accorded with the findings of previous researches (47,48), stated that the lighting condition did not affect the algal growth rate significantly, at which the light intensity up to $50\text{--}60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ did not lead to the increment of algal cell density, particularly for *C. vulgaris*. On the other hand, the experiments were launched under the similar intensity of $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for the sunlight as well as fluorescent light. Therefore, the difference of results between two types of lighting conditions would solely depend on the solar irradiance of daytime (26). Sunlight is inexpensive but the instability in irradiance as well as temperature changes following solar irradiance could limit the lighting source of microalgae cultivation whereas the fluorescent light is a continuous lighting source.

Effect of temperature and pH on algae cultivation The changes in temperature and pH can have significant effects on biomass production and microalgae harvesting (40,49,50). The early researches have started the temperature for the efficient microalgae cultivation ranged from 25°C to 28°C . The higher the solar radiation, the higher the temperature during daytime is, which is a limiting factor of photosynthesis (51). This relationship, which exists naturally, might reduce the microalgae density. Apparently, the summer temperature of a tropical country, which often changes drastically throughout the day, may rise to 35°C in the morning and gradually reduces in the afternoon, causing an instability in the irradiance as well as inconstant lighting mode around the algal inoculation flask. Likewise, temperature associated with pH changes during the algal cultivation process. The pH of wastewater could reach up to

pH 11 due to the presence of alkaline composition. Effluents from fish processing plants, which are not frequently acidic, exhibit neutral or alkaline condition due to the presence of organic proteinaceous decomposition as well as ammonia release (52–54). Compared to the Sueoka medium, the concentration of calcium and magnesium contained in studied medium is high due to collection of effluents from seafood processing plants. Based on the ingredients of SWD, the concentration of calcium in this effluent is high which resulted in auto-flocculation process. In addition, the pH values were ranged from pH 9 to 11 during the production of seafood meal. This high pH range may cause the mineral precipitation, leading to the flocculation of microalgae cells (40,53,55). Furthermore, the process of photosynthesis by microalgae has resulted the increasing pH levels in wastewater, creating a favorable condition for microalgae to flocculate in SWD without any base addition (56–58). When pH values of the wastewater have increased over pH 9, microalgae cells were tend to aggregate, which allowed the auto-flocculation to be occurred. Therefore, the density of *C. vulgaris* in SWD has increased gradually on day 5 at pH 8.22 ± 0.14 under sunlight and 8.0 ± 0.01 under fluorescent lighting, then decreased significantly from day 9 at the pH values of 9.71 ± 0.17 and 9.25 ± 0.04 (Fig. 4). This result has suggested that the auto-flocculation of microalgae could happen for algal inoculum in the outdoor cultivation using SWD as cultivation medium. The obtained results have shown that this naturally flocculate microalgae is a potential solution to the high cost of wastewater treatment process.

Effluent parameter The nutrient elimination of a wastewater medium was identified by the parameters such as COD, BOD, total-N and total-P. With the initial concentration of $0.01 \pm 0.001 \text{ g L}^{-1}$ (C_0) in the effluent, the *C. vulgaris* growth rate was observed through the reductions of these parameters and presented in Figs. 5A and B, and 6A and B. The error bars in these results were calculated from triplicate experiments. These parameters were also measured at the same time during the determination of microalgae growth that is presented in Fig. 3. After the 7th day of the microalgae cultivation, the auto-flocculation began to occur after stopping aeration at day 8. Besides, the lighting conditions did not influence much to the effluent parameters. The results obtained for sunlight mode and fluorescent light were not differ much. Hence, the following discussions include the outputs of

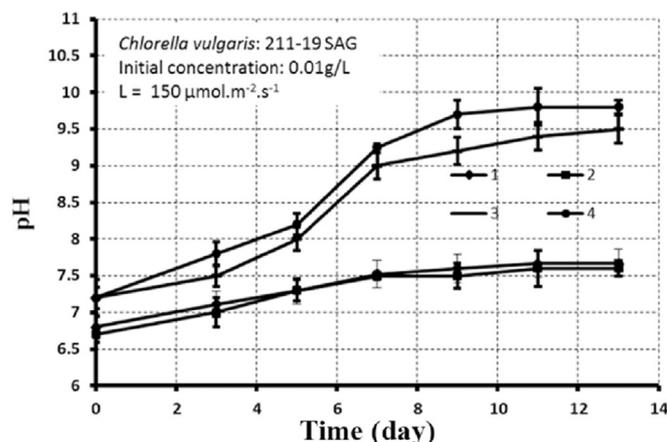


FIG. 4. Natural pH increased in two mediums during algal culture process. Line 1, *C. vulgaris* in Sueoka medium with fluorescent illumination; line 2, *C. vulgaris* in Sueoka medium with sunlight illumination; line 3, *C. vulgaris* in SWD with fluorescent illumination; line 4, *C. vulgaris* in SWD with sunlight illumination. Mean \pm SD of three biological replicates.

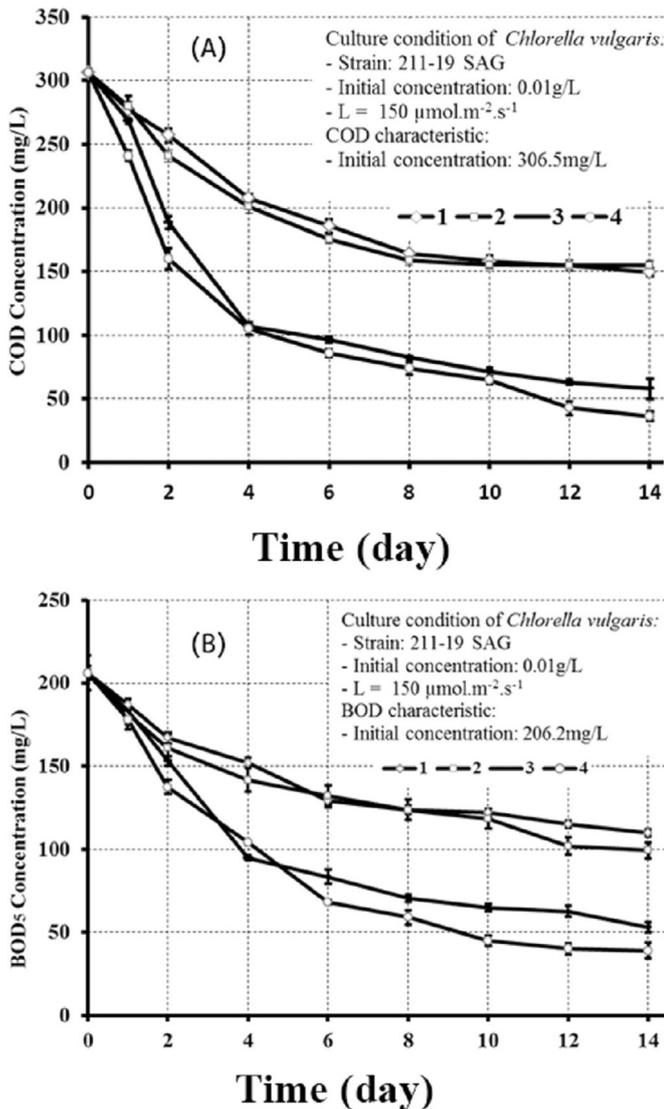


FIG. 5. (A) Evaluation of COD with *C. vulgaris* inoculum at the initial concentration of $0.01 \pm 0.001 \text{ g}\cdot\text{L}^{-1}$. Line 1, SWD without *C. vulgaris* under fluorescent illumination; line 2, SWD without *C. vulgaris* under sunlight illumination; line 3, SWD with *C. vulgaris* under fluorescent illumination; line 4, SWD with *C. vulgaris* under sunlight illumination. Mean \pm SD of three experimental replicates. (B) Evaluation of BOD₅ with *C. vulgaris* inoculum at the initial concentration of $0.01 \pm 0.001 \text{ g}\cdot\text{L}^{-1}$. Line 1, SWD without *C. vulgaris* under fluorescent illumination; line 2, SWD without *C. vulgaris* under sunlight illumination; line 3, SWD with *C. vulgaris* under fluorescent illumination; line 4, SWD with *C. vulgaris* under sunlight illumination. Mean \pm SD of three experimental replicates.

SWD medium with no cells of microalgae (control sample) and SWD with microalgae.

Evaluation COD and BOD The analysis results of COD and BOD₅ in SWD with microalgae inoculum under two types of lighting conditions showed a sharp decrease of the COD and BOD₅ (Fig. 5A,B). It has indicated that the amount of the organic matter reduced rapidly to less than $40.00 \pm 3.87 \text{ mg}\cdot\text{L}^{-1}$ of COD as well as for BOD₅, where the corresponding removal efficiencies of COD and BOD₅ were found to be 88% and 81% at the final day of algal inoculums. However, this reduction was more pronounced in SWD with cells of *C. vulgaris* inoculum than the one without microalgae. These COD and BOD₅ values were relatively low on the day when *C. vulgaris* began to settle and stopped absorbing

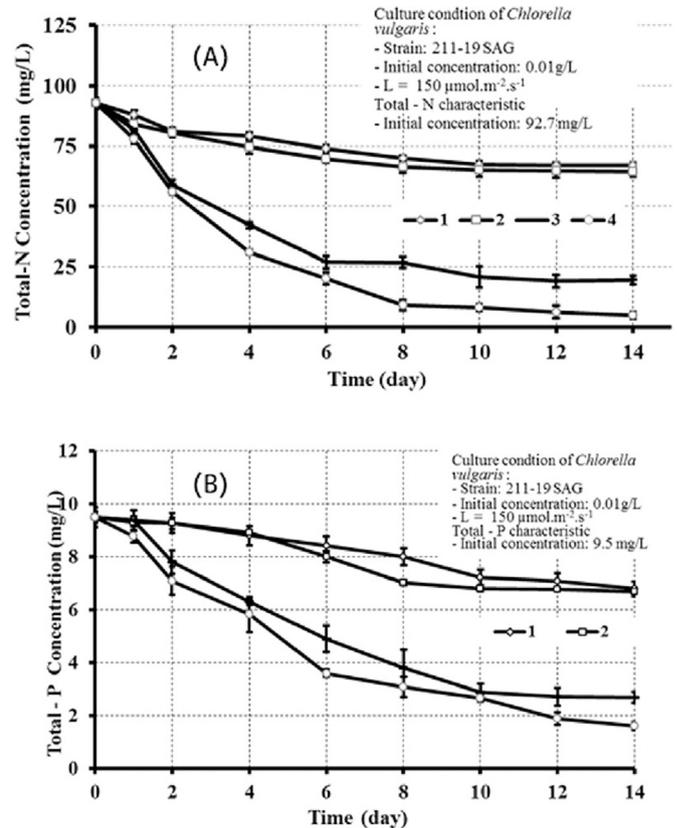


FIG. 6. (A) Evaluation of total nitrogen of effluent with *C. vulgaris* inoculum at the initial concentration of $0.01 \pm 0.001 \text{ g}\cdot\text{L}^{-1}$. Line 1, SWD with no cell of *C. vulgaris* under fluorescent illumination; line 2, SWD with no cell of *C. vulgaris* under sunlight illumination; line 3, SWD with *C. vulgaris* under fluorescent illumination; line 4, SWD with *C. vulgaris* under sunlight illumination. Mean \pm SD of three experimental replicates. (B) Evaluation of total phosphorus of effluent with *C. vulgaris* inoculum at the initial concentration of $0.01 \pm 0.001 \text{ g}\cdot\text{L}^{-1}$. Line 1, SWD with no cell of *C. vulgaris* under fluorescent illumination; line 2, SWD with no cell of *C. vulgaris* under sunlight illumination; line 3, SWD with *C. vulgaris* under fluorescent illumination; line 4, SWD with *C. vulgaris* under sunlight illumination. Mean \pm SD of three experimental replicates.

the nutrients from wastewater. Obviously, the organic content of wastewater gradually sloped down from day 2 since microalgae was inoculated in wastewater medium, suggesting that *C. vulgaris* has consumed the excess nutrient of effluent for cell division.

In comparison to the nutrient elimination by microalgae inoculum in SWD, the control samples with no cells of microalgae were also analyzed for the COD and BOD₅ removal efficiencies. The results showed that these values were around 47%–50%, illustrating a weakness in organic matter removal without algal inoculum. The removal of nutrient occurred due to the decomposition of organic compounds by microorganisms in the sewage, causing a decrement of COD and BOD₅ (59). It is obvious that the presence of microalgae in cultivation medium has increased the consumption of organic as well as inorganic matters. These results were concurring with previous studies where 90% of nutrient in wastewater, represented by the COD as well as BOD₅, was consumed by microalgae (18,43,60). In addition, previous studies have shown that the microorganisms in wastewater could cause the formation of small molecules from the organic and non-organic matters, which act as nutrients for microalgae (43,61). This has suggested that the wastewater has provided sufficient nourishment to the algal cell for metabolism process.

Evaluation of total-N and total-P Similarly, the total-N and total-P removal data are illustrated in Fig. 6A and B. The

concentration of total-N was significantly decreased during the growth of *C. vulgaris*. After the 14th day of cultivation, concentration of total-N was less than $5.00 \pm 0.15 \text{ mg}\cdot\text{L}^{-1}$ for the sunlight mode and $20.00 \pm 1.5 \text{ mg}\cdot\text{L}^{-1}$ for the fluorescent illumination, which indicates 95% and 79% of total-N has been removed. Meanwhile, the total-P removal was only 83% for the sunlight mode and 72% for the fluorescent lighting after stopping aeration and *C. vulgaris* began to settle down. The COD and BOD results showed role in biofuel production as the lipid content increases when microalgae were facing the deprivation of nitrogen during cultivation stage (17). Phosphorus is an essential nutrient for microalgae cell construction as it acts as a component of phospholipids for cell membrane and adenosine triphosphate (ATP) to provide energy for cell activities. Compared to the COD and BOD values obtained, total-N and total-P results have shown that the difference between two lighting conditions was statically significant and the energy for photosynthesis supplied by the natural lighting was more efficient than the one with artificial light. The nitrogen and phosphorus removal efficiency for the sunlight mode was 15% and 10%, respectively, which is higher than the results obtained in the fluorescent mode. The removal efficiencies were found to be higher in the experiments using sunlight than with fluorescent lighting, suggesting that algal growth process required nitrogen and phosphorus to be the essential nutrients for protein and lipid production but also as colloidal compounds for aggregation. As discussed in section above, the greater pH value was associated with sunlight mode. In the beginning of day 9 for algal inoculum, pH value of wastewater gradually rose over 9.0 that attributes to the appearance of lime precipitation containing wastewater (40). The previous studies showed that calcium and/or magnesium started precipitating at a high pH value, inducing flocculation of algal cell to be occurred at the pH values up to pH 10.0. In addition, precipitation of phosphates which was caused by phosphate anion contained in wastewater, would occur at the pH ranged from pH 10.5 to 11 (54). With this reason, total-P in the wastewater has decreased significantly to provide sufficient nutrient to microalgae cell production and induced lime precipitation. From day 9 of algal inoculums under sunlight mode, the pH values were exceeded $\text{pH } 9.00 \pm 0.05$ as illustrated in Fig. 4. It was observed that the lime precipitation occurred easily at high pH value, inducing algal flocculation, and resulting 99% of algal cells to be eliminated from SWD. After the decantation of *C. vulgaris* in 2 days, all characteristics of effluent as well as the number of cells stopped decreasing or remained constant. The microalgae were collected to determine the cell concentration using the method stated by Nguyen et al. (40). The biomass concentration was $47.00 \pm 5.5 \text{ g}_{\text{DM}}\cdot\text{L}^{-1}$.

In this work, the cultivation of *C. vulgaris* in SWD and the effects of various cultivation conditions have been investigated. The results indicated that microalgae are able to consume organic compounds, nitrogen and phosphorus efficiently for their cell construction. It has been shown that the changes in lighting condition have not noticeably affected the microalgae biomass productivities in Sueoka and SWD mediums. Lastly, the sunlight illumination and the new medium are favorable for microalgae harvesting. This is an important finding for reducing the final cost of the wastewater treatment process as well as the harvesting cost of microalgae production, where auto-flocculated *C. vulgaris* at harvesting stage is one of the key processes.

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References

1. **Olaizola, M.:** Commercial development of microalgal biotechnology: from the test tube to the marketplace, *Biomol. Eng.*, **20**, 459–466 (2003).
2. **Mata, T. M., Martins, A. A., and Caetano, N. S.:** Microalgae for biodiesel production and other applications: a review, *Renew. Sustain. Energy Rev.*, **14**, 217–232 (2010).
3. **Htet, A. N., Noguchi, M., Ninomiya, K., Tsuge, Y., Kuroda, K., Kajita, S., Masai, E., Katayama, Y., Shikinaka, K., Otsuka, Y., and other 3 authors:** Application of microalgae hydrolysate as a fermentation medium for microbial production of 2-pyrone 4, 6-dicarboxylic acid, *J. Biosci. Bioeng.*, **125**, 717–722 (2018).
4. **Gautam, K., Pareek, A., and Sharma, D. K.:** Biochemical composition of green alga *Chlorella minutissima* in mixotrophic cultures under the effect of different carbon sources, *J. Biosci. Bioeng.*, **116**, 624–627 (2013).
5. **Cai, T., Park, S. Y., and Li, Y.:** Nutrient recovery from wastewater streams by microalgae: status and prospects, *Renew. Sustain. Energy Rev.*, **19**, 360–369 (2013).
6. **Garcia-gonzalez, J. and Sommerfeld, M.:** Biofertilizer and biostimulant properties of the microalga *Acutodesmus dimorphus*, *J. Appl. Phycol.*, **28**, 1051–1061 (2015).
7. **Whitton, R., Le Mével, A., Pidou, M., Ometto, F., Villa, R., and Jefferson, B.:** Influence of microalgal N and P composition on wastewater nutrient remediation, *Water Res.*, **91**, 371–378 (2016).
8. **Park, J. B. K., Craggs, R. J., and Shilton, A. N.:** Wastewater treatment high rate algal ponds for biofuel production, *Bioresour. Technol.*, **102**, 35–42 (2011).
9. **Cabanelas, I. T. D., Arbib, Z., Chinalia, F. A., Oliveira, C., Perales, J. A., Fernando, P., Druzian, J. I., and Andrade, L. F.:** From waste to energy: microalgae production in wastewater and glycerol, *Appl. Energy*, **109**, 283–290 (2013).
10. **Chen, C. and Chang, Y.:** Engineering strategies for enhancing *C. vulgaris* ESP-31 lipid production using effluents of coke-making wastewater, *J. Biosci. Bioeng.*, **125**, 710–716 (2018).
11. **Martinez, M. E., Yous, F. E., and Mu, L.:** Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*, *Bioresour. Technol.*, **73**, 263–272 (2000).
12. **Ruiz-marin, A., Mendoza-espinoza, L. G., and Stephenson, T.:** Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater, *Bioresour. Technol.*, **101**, 58–64 (2010).
13. **Aslan, S. and Kapdan, I. K.:** Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae, *Ecol. Eng.*, **28**, 64–70 (2006).
14. **Ji, M. K., Abou-shanab, R. A., Kim, S. H., Salama, E. S., Lee, S. H., Kabra, A. N., Lee, Y., Hong, S., and Jeon, B.:** Cultivation of microalgae species in tertiary municipal wastewater supplemented with CO₂ for nutrient removal and biomass production, *Ecol. Eng.*, **58**, 142–148 (2013).
15. **Delgadillo-mirquez, L., Lopes, F., Taidi, B., and Pareau, D.:** Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture, *Biotechnol. Rep.*, **11**, 18–26 (2016).
16. **Samorì, G., Samorì, C., Guerrini, F., and Pistocchi, R.:** Growth and nitrogen removal capacity of *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I, *Water Res.*, **47**, 791–801 (2013).
17. **Aravantinou, A. F., Theodorakopoulos, M. A., and Manariotis, I. D.:** Selection of microalgae for wastewater treatment and potential lipids production, *Bioresour. Technol.*, **147**, 130–134 (2013).
18. **Li, Y., Chen, Y. F., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., and Ruan, R.:** Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production, *Bioresour. Technol.*, **102**, 5138–5144 (2011).
19. **Lau, P. S., Tam, N. F. Y., and Wong, Y. S.:** Operational optimization of batchwise nutrient removal from wastewater by carrageenan immobilized *Chlorella vulgaris*, *Water Sci. Technol.*, **38**, 185–192 (1998).
20. **Megharaj, M., Pearson, H. W., and Venkateswarlur, K.:** Removal of nitrogen and phosphorus by immobilized cells of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil, *Enzyme Microb. Technol.*, **14**, 656–658 (1992).
21. **Zhou, W., Wang, Z., Xu, J., and Ma, L.:** Cultivation of microalgae *Chlorella zofingiensis* on municipal wastewater and biogas slurry towards bioenergy, *J. Biosci. Bioeng.*, **126**, 644–648 (2018).
22. **Shi, X., Chen, F., Yuan, J., and Chen, H.:** Heterotrophic production of lutein by selected *Chlorella* strains, *J. Appl. Phycol.*, **9**, 445–450 (1997).
23. **Hadj-romdhane, F., Jaouen, P., Pruvost, J., Grizeau, D., Van Vooren, G., and Bourseau, P.:** Development and validation of a minimal growth medium for recycling *Chlorella vulgaris* culture, *Bioresour. Technol.*, **123**, 366–374 (2012).
24. **Spolaore, P., Joannis-cassan, C., Duran, E., Isambert, A., De Génie, L., and Paris, E. C.:** Commercial applications of microalgae, *J. Biosci. Bioeng.*, **101**, 87–96 (2006).
25. **Ackefors, H. and Enell, M.:** The release of nutrients and organic-matter from aquaculture systems in Nordic countries, *J. Appl. Ichthyol.*, **10**, 225–241 (1994).
26. **Blanken, W., Cuaresma, M., Wijffels, R. H., and Janssen, M.:** Cultivation of microalgae on artificial light comes at a cost, *Algal Res.*, **2**, 333–340 (2013).

27. **Arvanitoyannis, I. S. and Kassaveti, A.:** Fish industry waste: treatments, environmental impacts, current and potential uses, *Int. J. Food Sci. Technol.*, **43**, 726–745 (2008).
28. **Carawan, R. E., Chambres, J. V., and Zall, R.:** Seafood water and wastewater management. Water and wastewater management in food processing. The North Carolina Agricultural Extension Service, Raleigh, NC (1979).
29. **Chakraborty, S., Mohanty, D., Ghosh, S., Das, D., and Ioeng, J. B. I. B.:** Improvement of lipid content of *Chlorella minutissima* MCC 5 for biodiesel production, *J. Biosci. Bioeng.*, **122**, 294–300 (2016).
30. **Kurano, N. and Miyachi, S.:** Selection of microalgal growth model for describing specific growth rate-light response using extended information criterion, *J. Biosci. Bioeng.*, **100**, 403–408 (2005).
31. **Milledge, J. J. and Heaven, S.:** A review of the harvesting of micro-algae for biofuel production, *Rev. Environ. Sci. Biotechnol.*, **12**, 165–178 (2013).
32. **Oh, S. H., Han, J. G., Kim, Y., Ha, J. H., Kim, S. S., Jeong, M. H., Jeong, H. S., Kim, N. Y., Cho, J. S., Yoon, W. B., and other 3 authors:** Lipid production in *Porphyridium cruentum* grown under different culture conditions, *J. Biosci. Bioeng.*, **108**, 429–434 (2009).
33. **González-Fernández, C. and Ballesteros, M.:** Microalgae auto-flocculation: an alternative to high-energy consuming harvesting methods, *J. Appl. Phycol.*, **25**, 991–999 (2013).
34. **Shen, Y., Zhu, W., Chen, C., and Nie, Y.:** Glycine induced culture-harvesting strategy for *Botryococcus braunii*, *J. Biosci. Bioeng.*, **121**, 424–430 (2016).
35. **Reisner, G. S., Gering, R. K., and Thompson, J.:** The metabolism of nitrate and ammonia by *Chlorella*, *Plant Physiol.*, **35**, 48–52 (1960).
36. **Schuler, J. F., Diller, V. M., and Kersten, H. J.:** Preferential assimilation of ammonium ion by *Chlorella vulgaris*, *Plant Physiol.*, **28**, 299–303 (1952).
37. **Sueoka, N.:** Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*, *Proc. Natl. Acad. Sci. USA*, **46**, 83–91 (1960).
38. **Harris, E. H.:** The *Chlamydomonas* source book: Introduction to *Chlamydomonas* and its laboratory use. Academic Press, New York (2009).
39. **Bhola, V., Desikan, R., Santosh, S. K., Subburamu, K., Sanniyasi, E., and Bux, F.:** Effects of parameters affecting biomass yield and thermal behaviour of *Chlorella vulgaris*, *J. Biosci. Bioeng.*, **111**, 377–382 (2011).
40. **Nguyen, T. D. P., Frappart, M., Jaouen, P., Pruvost, J., and Bourseau, P.:** Harvesting *Chlorella vulgaris* by natural increase in pH: effect of medium composition, *Environ. Technol.*, **35**, 1378–1388 (2014).
41. **Ritchie, R. J.:** Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents, *Photosynth. Res.*, **89**, 27–41 (2006).
42. **APHA/AWWA/WEF:** Standard methods for the examination of water and wastewater, Standard methods, 20th ed. American Public Health Association, Washington, D.C. (1998).
43. **Cole, J. J.:** Interactions between bacteria and algae in aquatic ecosystems, *Ann Rev. Ecol. Syst.*, **13**, 291–314 (1982).
44. **Zhao, P., Yu, X., Li, J., Tang, X., and Huang, Z.:** Enhancing lipid productivity by co-cultivation of *Chlorella* sp. U4341, *J. Biosci. Bioeng.*, **118**, 72–77 (2014).
45. **Blasco, R. J.:** Nature and role of bacterial contaminants in mass cultures of thermophilic *Chlorella pyrenoidosa*, *Appl. Microbiol.*, **13**, 473–477 (1965).
46. **Abdel-Raouf, N., Al-Homaidan, A. A., and Ibraheem, I. B. M.:** Microalgae and wastewater treatment, *Saudi J. Biol. Sci.*, **19**, 257–275 (2012).
47. **Sorokin, C. and Krauss, R. W.:** The effects of light intensity on the growth rates of green algae, *Plant Physiol.*, **33**, 109–113 (1958).
48. **Alam, M. A., Wan, C., Guo, S. L., Zhao, X. Q., Huang, Z. Y., Yang, Y. L., Chang, J., and Bai, F. W.:** Characterization of the flocculating agent from the spontaneously flocculating microalga *Chlorella vulgaris* JSC-7, *J. Biosci. Bioeng.*, **118**, 29–33 (2014).
49. **Sukenik, A. and Shelef, G.:** Algal auto flocculation-verification, *Biotechnol. Bioeng.*, **26**, 4–9 (1984).
50. **Hase, R., Oikawa, H., Sasa, C., Morita, M., and Watanabe, Y.:** Photosynthetic production of microalgal biomass in a raceway system under greenhouse conditions in Sendai City, *J. Biosci. Bioeng.*, **89**, 157–163 (2000).
51. **Vonshak, A., Abeliovich, A., Boussiba, S., Arad, S., and Richmond, A.:** Production of *spirulina* biomass: effects of environmental factors and population density, *Biomass*, **2**, 175–185 (1982).
52. **Gomes, H. I., Mayes, W. M., Rogerson, M., and Stewart, D. I.:** Alkaline residues and the environment: a review of impacts, management practices and opportunities, *J. Clean. Prod.*, **112**, 3571–3582 (2016).
53. **Vandamme, D., Foubert, I., and Muylaert, K.:** Flocculation as a low-cost method for harvesting microalgae for bulk biomass production, *Trends Biotechnol.*, **31**, 233–239 (2013).
54. **Vandamme, D., Foubert, I., Fraeye, I., and Muylaert, K.:** Influence of organic matter generated by *Chlorella vulgaris* on five different modes of flocculation, *Bioresour. Technol.*, **124**, 508–511 (2012).
55. **Horiuchi, J., Ohba, I., Tada, K., Kobayahi, M., Kanno, T., and Kishimoto, M.:** Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control, *J. Biosci. Bioeng.*, **95**, 412–415 (2003).
56. **Greenwell, H. C., Laurens, L. M. L., Shields, R. J., Lovitt, R. W., and Flynn, K. J.:** Placing microalgae on the biofuels priority list: a review of the technological challenges, *J. R. Soc. Interface*, **7**, 703–726 (2009).
57. **Lijklema, L.:** Factors affecting pH change in alkaline waste water treatment-III. A dynamic simulation, *Water Res.*, **3**, 913–930 (1972).
58. **González, J. F.:** Wastewater treatment in the fishery industry. Food and Agriculture Organization of the United Nations, Rome (1996).
59. **Doucette, G. J.:** Interactions between bacteria and harmful algae: a review, *Nat. Toxins*, **3**, 65–74 (1995).
60. **Woertz, I., Feffer, A., Lundquist, T., and Nelson, Y.:** Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock, *J. Environ. Eng.*, **135**, 1115–1122 (2009).
61. **Ma, X., Zhou, W., Fu, Z., Cheng, Y., Min, M., Liu, Y., Zhang, Y., Chen, P., and Ruan, R.:** Effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater-based algae cultivation system, *Bioresour. Technol.*, **167**, 8–13 (2014).