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Mariculture Wastewater Treatment with Bacterial-Algal Coupling System (BACS): Effect of Light Intensity on Microalgal Biomass Production and Nutrient Removal

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Abstract:

Mariculture wastewater generated from the mariculture industry has increased public concern due to its impact on the sustainability of aquatic environments and aquaculture practices. Herein, the Bacterial-Algal Coupling System was applied for mariculture wastewater treatment. Microalgae growth in heterotrophy and mixotrophy (2000-8000 lux) were first compared. The best microalgal growth and nutrient removal were obtained at 5000 lux, where biomass productivity of microalgae was 0.465 g L$^{-1}$ d$^{-1}$, and 98.1% of chemical oxygen demand, 70.7% of ammonia-nitrogen, and 90.0% of total phosphorus were removed. To further understand the nutrient removal through microalgae cultivation, the enzyme activities involved in the Calvin cycle and the Tricarboxylic Acid cycle at different light intensities were determined. Under mixotrophic cultivation, there was a coordination between photosynthesis and heterotrophic metabolism in algal cells, which resulted in a high algal biomass production and removal efficiency of nutrients. This study provided a novel insight into the bioremediation of mariculture wastewater and microalgae cultivation.

Keywords:
Mariculture wastewater; Bacterial-Algal Coupling System; Mixotrophic cultivation; Light intensity; Enzyme activity
Note

This research does not involve human subjects or animal research.

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1. Introduction

In recent years, the mariculture industry has been developed rapidly due to the increasing demand for seafood and the continuous decrease of wild fishery resources (Barnharst et al., 2018; Lang et al., 2020; Zheng et al., 2016). Meanwhile, it has led to an increase in mariculture wastewater (Song et al., 2020; Zhimiao et al., 2019). Mariculture wastewater mainly contains suspended solids, soluble organic matter, nitrogen (N), and phosphorus (P), which are derived from uneaten feed and fish fecal material (Barnharst et al., 2018; Zheng et al., 2016). This wastewater will inevitably pose threats to the marine ecology (e.g., oxygen depletion, red tide, and disease) if discharged directly into offshore and coastal zones (Guo et al., 2014; Jin et al., 2018).

For the cost concerns, conventional biological methods are more suitable for the removal of contaminants from mariculture wastewater (Lang et al., 2020; Zhimiao et al., 2019). However, the pollutant removal efficiency of these methods (e.g., activated sludge wastewater treatment process) cannot meet the requirements of discharge or recycling (You et al., 2020). Furthermore, the existence of solid wastes in mariculture wastewater will also restrict the performance of wastewater treatment (Gao et al., 2020a; Zhao et al., 2018). This means that an alternative and effective biological process for treating mariculture wastewater is urgently needed.

Acidogenic fermentation is an important step in anaerobic digestion (Huang et al., 2018), and could convert complex organic pollutants contained in wastewaters into
ammonium (NH$_4^+$-N), phosphates, and volatile fatty acids (VFAs) (Bertin et al., 2010; Eskicioglu et al., 2006; Ren et al., 2018). These fermentation products present in acidogenic fermentation effluents can be further used as the substrates for the cultivation of microorganisms (Qi et al., 2017). On the other hand, microalgae are considered as a promising energy feedstock owing to their high intracellular lipid content (30-60% of dry weight), efficient cost, rapid growth rate, and non-secondary pollution (Cao et al., 2018; Ren et al., 2015). This type of microorganisms can grow in nutrient-rich waters, such as ponds and even fermentation effluents, due to their ability to utilize simple dissolved organics (e.g., VFAs), N and P for cell growth and metabolism (Chalima et al., 2017; Yang et al., 2018; Zhang et al., 2018). In this sense, the Bacterial-Algal Coupling System (BACS), a technology combining both acidogenic fermentation and microalgae cultivation, can be a potential and alternative solution to treat mariculture wastewater.

It is noteworthy that microalgae utilize organic carbon under both heterotrophic and mixotrophic cultivations (Cheirsilp and Torpee, 2012). Under mixotrophic cultivation, light and organic carbon are simultaneously exploited within a single algal monoculture (Patel et al., 2019; Zhan et al., 2017). Thus, compared with heterotrophic cultivation, it helps to obtain a faster algal growth rate and accumulate more biomass in a given growth spell (Chen et al., 2018; Nagarajan et al., 2017), which will facilitate the nutrient removal from wastewaters and minimize the contamination risks (Patel et al., 2020). Mixotrophy involves complex processes, in which light plays a critical role in microalgae growth by regulating
nutrients absorption, cell protoplasm synthesis, and product accumulation (Alkhamis and Qin, 2013; Cai et al., 2013; Liu et al., 2010). Normally, microalgal biomass production increases with increasing of light intensity below the optimum value, while it decreases at light intensity exceeding the optimal value due to the damage of the photosystem (Patel et al., 2019; Vanlerberghe et al., 2020). Furthermore, light intensity varies with the alternation of day and night under natural conditions. Thus, it is necessary to evaluate the performance of BACS in the treatment of mariculture wastewater under different light intensities. It is noteworthy that photosynthesis and heterotrophy may have a coordination on energy and carbon metabolisms at the cellular level under mixotrophy (Alkhamis and Qin, 2013; Nair and Chakraborty, 2020; Zhang et al., 2017). However, the underlying pathway of coordination between two metabolic processes is still unclear, and few studies were conducted for the effect of light intensity on this synergism.

Based on the above considerations, BACS was applied in this study to strengthen the pollutants elimination from mariculture wastewater. Firstly, acidogenic fermentation effluents obtained from mariculture wastewater were prepared and used for cultivating microalgae. Subsequently, the effects of light intensity on algal growth and nutrient removal during the microalgae cultivation were analyzed. Finally, the pathway of coordination between photosynthesis and heterotrophy under mixotrophic cultivation and the effect of light intensity on this coordination were investigated to further understand the nutrient removal through microalgae cultivation. The results of this study were expected to
provide a new perspective on algal mixotrophic cultivation and mariculture wastewater treatment.

2. Materials and methods

2.1. Wastewater and algal strain

Original mariculture wastewater was collected from the mariculture center of the Marine Biology Institute of Shandong Province (Qingdao, China). The wastewater was sieved (2.0 mm) and subsequently stored in a refrigerator (4 °C) for future use. The main characteristics of mariculture wastewater are as follows. Salinity: 2.2 ± 0.1%, volatile suspended solids (VSS): 35.2 ± 1.8 g/L, pH: 7.32 ± 0.4, chemical oxygen demand (COD): 714.0 ± 35.7 mg/L, total phosphorus (TP): 3.7 ± 0.2 mg/L, NH$_4^+$-N: 47.6 ± 2.4 mg/L.

Marine Chlorella vulgaris (C. vulgaris), a unicellular green microalga, was used as the model algal strain in this study. It was obtained from the Institute of Oceanology, Chinese Academy of Sciences (Qingdao, China). C. vulgaris strain was maintained in autoclaved f/2 medium and stored at 4 °C for later use.

2.2. Mariculture wastewater treatment with BACS

2.2.1. Acidogenic fermentation of mariculture wastewater

Thermophilic bacteria (Bacillus sp. AT07-1) pretreatment method was applied to enhance the hydrolysis of mariculture wastewater as previous study (Gao et al., 2020b). Mariculture wastewater was equivalently put into four vessels (1.0 L) and the bacterial
suspensions were inoculated at a ratio of 1:50 (v/v). Then mariculture wastewater was hydrolyzed at 65 °C, 120 rpm (revolutions per minute) for 12 h in a water bath shaker.

Initial pH can change the path of acidogenic fermentation, which affects the final compositions of VFAs and the release of N and P (You et al., 2020). Hence, the initial pH of hydrolyzed mariculture wastewater in four vessels was adjusted to 4.0, 6.0, 8.0, and 10.0, respectively, and anaerobic sludge obtained from the secondary settling tank of Haibohe wastewater treatment plant (Qingdao, China) was inoculated at a ratio of 1:10 (v/v). After that, the anaerobic environment in the vessels was established by sparging with nitrogen gas. The fermentation was conducted at 36 °C, 120 rpm for 72 h in a water bath shaker. Finally, the acidogenic fermentation effluents were centrifuged (5000 rpm, 10 min) to discard the pellet and the supernatant was used as the culture medium. The main components of the ultimate acidogenic fermentation effluents are listed in Table 1.

2.2.2. Pre-culture of microalgae

*C. vulgaris* was firstly cultivated in Erlenmeyer flasks containing 0.8 L autoclaved f/2 medium. Cultivation was executed in a 25 °C light incubator (GHQ-160, Putian, China), and lighting parameters were as follows: cool white LED light; the light intensity of 3000 lux; and the light/dark cycle of 12 h /12 h. The growth was monitored by daily measuring optical density ($OD_{680}$) of algal cells. At the exponential phase, microalgae were collected by centrifuging (5000 rpm, 15 min) and used as the inoculum.
2.2.3. *Microalgae cultivation with the acidogenic fermentation effluents*

In our previous study (You et al., 2020), different dilution ratios (5%, 10%, 15%, and 20%) of the acidogenic fermentation effluents for microalgae cultivation were analyzed. Based on the nutrient removal performance and microalgal growth, the optimum dilution ratio of the acidogenic fermentation effluents was 10%, which was used in the followed experiment.

Microalgae cultivation was conducted in four groups, and the diluted acidogenic fermentation effluents obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 were used as the culture medium in group I, II, III, and IV, respectively. 1.0 L conical flasks (containing 0.8 L culture medium) were applied to cultivate *C. vulgaris*. Before cultivation, the pH of each flask was adjusted to 7.0 by 1.0 M HCl or 1.0 M NaOH, and then the flasks were autoclaved at 120 °C for 20 min. Afterward, algae seeds collected at the pre-culture stage were inoculated in culture mediums to make an initial OD$_{680}$ of 0.1 in all flasks. Finally, the four group flasks were put in a 25 °C light incubator (GHQ-160, Putian, China), in which four light intensities of 0, 2000, 5000, and 8000 lux were applied under an L/D cycle of 12 h:12 h. During the cultivation period, the flasks were shaken three times a day. Samples were taken every day to monitor algal growth and nutrient removal. At the end of the exponential phase, algal cells were extracted to determine enzyme activity.

2.4. *Analytical methods*

The pH was measured with a digital pH-meter (PHB-5, Aolilong, Hangzhou). COD,
VSS, NH$_4^+$-N, and TP were analyzed according to the methods described previously (Guo et al., 2014; Ren et al., 2015). VFAs were detected by GC2010 gas chromatography (Shimadzu, Japan) following the method described in the previous study (Gao et al., 2020b). The samples for analysis of COD, NH$_4^+$-N, TP, and VFAs were filtered with a 0.45 µm acetate cellulose membrane. OD$_{680}$ of algal cells was measured at 680 nm. Microalgal biomass concentration was indicated by dry cell weight (DCW) and determined by filtering samples through a 0.45-micron filter, drying at 80 °C for 24 h.

2.5. Enzyme activity determination

2.5.1. Rubisco activity

Rubisco activity was determined using the chemical assay kits (Solarbio, Beijing, China). 10 mL algal cultures were collected and centrifuged (5000 g, 4 °C, 5 min), and the supernatants were removed. Cell pellets were grinded and homogenized in extraction buffer (pH 7.8, 10 mM Tris, 1 mM EDTA, 20 mM KCl). The homogenates were centrifuged (10000 g, 4 °C, 10 min) and kept in an ice bath for enzyme activity measurement. The reaction mixture was prepared, which contained 1 M Tris buffer (pH 7.2), 6.0 mM NADH, 0.5 M KHCO$_3$, 0.1 M GSH, 0.5% glyceraldehyde-3-P dehydrogenase, 0.025% 3-P-glycerate kinase, 0.05% α-glycerophosphate dehydrogenase-triose-P isomerase, 0.025 M ribulose diphosphate, 0.5 M MgCl$_2$, 0.2 M ATP, and 0.1 mL homogenates supernatant (You et al., 2020; Zhang et al., 2017). Finally, the absorbance (340 nm) of the mixture was measured.
2.5.2. *Citrate synthase activity*

Citrate synthase activity was determined using the chemical assay kits (Solarbio, Beijing, China). The extraction procedure was same as in “Rubisco activity” section, except that the buffer (pH 7.4) was replaced with 10 mM Tris, 5.0 mg/mL BSA, 250 mM sucrose, 1 mM PMSF, and 3 mM EDTA. The reaction mixture was prepared, which contained 100 mM Tris, pH 8.0, 100 mM 5,5′-di-thiobis-(2-nitrobenzoic acid), 0.1% triton X-100, 100 mM Acetyl-CoA, and 35 μL sample (You et al., 2020; Zhang et al., 2017). Finally, the absorbance (412 nm) of the mixture was monitored.

2.6. *Statistical analysis*

Each test was run in triplicate and the average data was reported. Experimental results were also analyzed by one-way ANOVA, and p < 0.05 was considered to be statistically significant.

3. *Results and discussion*

3.1. *Microalgal growth at various light intensities*

Light intensity is a crucial factor to regulate microalgae growth and biomass production (Cai et al., 2013; Liu et al., 2010). The growth patterns of *C. vulgaris* under different light intensities are depicted in Fig. 1. The results showed that the microalgae grew fast during the cultivation process (Fig. 1), suggesting that acidogenic fermentation effluents obtained from mariculture wastewater were suitable for *C. vulgaris* growth. In
heterotrophy (0 lux), the final OD$_{680}$ of *C. vulgar* was 0.299-0.308 (Fig. 1). When light intensity was 2000 lux, signaling that *C. vulgaris* entered mixotrophic growth mode, the final OD$_{680}$ of *C. vulgaris* increased to 0.368-0.459 ($p < 0.05$) (Fig. 1). This confirmed that mixotrophic cultivation is a more effective approach to harvest microalgal biomass, since organic and inorganic sources can be utilized simultaneously by mixotrophic microalgae, providing more intermediates and energy for building up cells (Ren et al., 2019; Salati et al., 2017). By increasing light intensity from 2000 to 5000 lux, the final OD$_{680}$ of *C. vulgaris* reached 0.548-0.601, much higher than that under 2000 lux condition ($p < 0.05$) (Fig. 1). It revealed that a proper light intensity increment could facilitate the mixotrophic microalgal growth (Patel et al., 2020). However, when light intensity was 8000 lux, the final OD$_{680}$ of the microalgae decreased to 0.328-0.538 (Fig. 1), which was mainly because the absorption rate of light energy exceeded its consumption rate, causing the inhibition for microalgal growth (Alkhamis and Qin, 2013; Li et al., 2014).

Notably, at 5000 lux the ultimate OD$_{680}$ in group I (0.601) was higher than that in group II (0.548), III (0.583), and IV (0.587) (Fig. 1), and a larger difference in final OD$_{680}$ between group I (0.538) and other groups (0.328-0.427) was found at 8000 lux (Fig. 1). It was reported that excess NH$_4^+$-N in culture systems would directly damage photosynthetic apparatus with subsequent effect on microalgal growth (Chen and Wang, 2020), which could be amplified at elevated light intensities (Rossi et al., 2020). Hence, at 5000 and 8000 lux, the relatively low growth performance of *C. vulgaris* in group II, III, and IV could be
explained by the growth-limitary effect of high concentrations of NH$_4^+$-N in the culture medium (Table 1).

Meanwhile, the biomass productivities of *C. vulgaris* at different light intensities were analyzed (Table 2). The biomass productivity was defined as the DCW of microalgae increase in one day under different cultivation modes (Zhang et al., 2017). As shown in Table 2, the biomass productivities in mixotrophic cultivation were higher than those in heterotrophic cultivation (p < 0.05). In group I, for instance, the biomass productivity of *C. vulgaris* at 0, 2000, and 5000 lux was 0.120±0.006, 0.159±0.008, and 0.465±0.023 g L$^{-1}$ d$^{-1}$, respectively (Table 2); it further revealed a higher algal growth potential under mixotrophic condition (Huo et al., 2018). Moreover, it could be found that the maximum biomass productivity of *C. vulgaris* was obtained at 5000 lux (Table 2), which was in agreement with the final OD$_{680}$ of *C. vulgaris*. These results demonstrated that among the tested light intensities, 5000 lux was best for *C. vulgaris* cultivation with the acidogenic fermentation effluents of mariculture wastewater as culture medium.

3.2. Removal of organic matters at various light intensities

3.2.1. COD removal

The changes in COD concentration and removal efficiencies at various light intensities are shown in Fig. 2. A rapid decline of COD concentration was observed at the first 6-8 days owing to the metabolism of *C. vulgaris* during the early stage of cultivation; afterward, the concentration tended to be stable (Fig. 2). It reflected that the organic matters in culture
medium could be readily utilized by *C. vulgaris*.

The initial COD concentration in four groups was 239, 345, 386, and 402 mg/L, respectively (Fig. 2A-D). In heterotrophic cultivation (0 lux), the final COD concentrations decreased to 80-140 mg/L (Fig. 2 A-D), and the removal efficiencies were approximately 65% only (Fig. 2E). In comparison, when entering mixotrophic growth mode, the final COD amounts further decreased to around 45 at 2000 lux and 30 mg/L at 5000 lux with the average removal efficiencies of 86.2% at 2000 lux and 92.2% at 5000 lux (Fig. 2E). The better COD removal performance under mixotrophy resulted from a higher microalgal biomass production in this growth mode (Fig. 1). However, when light intensity was adjusted to 8000 lux, the COD removal performance decreased with an average removal efficiency of 86.8% (Fig. 2E), which could be attributed to the photoinhibition effect on microalgal growth (Alkhamis and Qin, 2013; Li et al., 2014). Overall, *C. vulgaris* exhibited a higher organics removal performance at 5000 lux (p < 0.05), which was tightly consistent with the highest OD$_{680}$ attained under the same condition (Fig. 1).

In a previous study, non-poisonous iron intensified constructed wetlands were used for the mariculture wastewater treatment, and the obtained COD removal efficiency was 62 ± 2% (Zhimiao et al., 2019). In another study, Zhang et al. (2020) applied biofilm membrane bioreactors coupled with pre-anoxic tanks for treating mariculture wastewater, and the COD in the wastewater was efficiently utilized with removal efficiency of over 94.1%. In this study, BACS was applied to treat mariculture wastewater. The diluted acidogenic
fermentation effluents obtained from mariculture wastewater were used as culture medium for microalgae cultivation, and the COD concentration in culture medium reduced from 239-402 mg/L to about 30 mg/L (5000 lux) with the average removal efficiencies of more than 90% (Fig. 2E). It demonstrated the feasibility of treating mariculture wastewater with BACS.

3.2.2. VFAs removal

The utilizations of VFAs for C. vulgaris are presented in Fig. 3. In heterotrophy (0 lux), the VFAs utilization efficiency was 56-74%, while the efficiency was enhanced to 76.7-84.0% under the mixotrophic condition with 2000 lux (Fig. 3A-D). This enhancement in VFAs utilization efficiency was owing to the comparatively high biomass production of C. vulgaris under mixotrophic conditions (Fig. 1). However, when higher light intensities of 5000 and 8000 lux were supplied, only 25-50% of VFAs were utilized (Fig. 3A-D). Huo et al. (2018) pointed out that there is a competition between organic and inorganic carbon uptake under mixotrophic conditions. In this study, increasing light intensity to 5000 lux enhanced the inorganic carbon utilization capacity of C. vulgaris, since the highest Rubisco activity (1668.0 U/g) of C. vulgaris was obtained at 5000 lux (Fig. 6A). The Rubisco makes the functions in carbon dioxide (CO₂) fixation in photosynthesis (You et al., 2020). Considering the highest algal biomass production obtained at 5000 lux (Fig.1), it was concluded that at 5000 lux, inorganic carbon was a preferred substrate compared to VFAs during microalgae cultivation with the acidogenic fermentation effluents as culture medium,
which led to the decrease in VFAs removal efficiency at 5000 lux (Fig. 3A-D). The decrease in VFAs removal efficiency at 8000 lux was mainly related to the photoinhibition effect on microalgal growth (Li et al., 2014).

In the acidogenic fermentation effluents, acetic acid (61.3-70.2% of VFAs) and propionic acid (17.2-22.9% of VFAs) were major fermented VFAs, plus small contents of butyric acid (7.3-11.5% of VFAs) and other fatty acids (Table 1). During mixotrophic growth in a VFAs mixture, microalgae preferentially utilize acetic acid or propionic acid to sustain a higher growth rate (Chen et al., 2018; Huo et al., 2018), and butyric acid uptake was accelerated only after acetic acid or propionic acid was depleted (You et al., 2020). A similar result was also attained in this work. For example, at 2000 lux, acetic acid and propionic acid were mainly utilized in the first 7-8 days with an average uptake rate of 13.7 and 2.2 mg L\(^{-1}\) d\(^{-1}\), respectively (Fig.3E and F). In comparison, the main utilization of butyric acid occurred in the last 3 days when acetic acid and propionic acid were depleted, and the average uptake rate was 1.5 mg L\(^{-1}\) d\(^{-1}\) (Fig.3G). It reflected that acetic acid and propionic acid were preferentially utilized by \textit{C. vulgaris} among fermented VFAs during the cultivation with the acidogenic fermentation effluents of mariculture wastewater as culture medium.

This selective preference in VFAs utilization is called the diauxic effect, which can be ascribed to the different metabolic pathways of VFAs in algal cells (Chen et al., 2018). Acetic acid has a simple structure and can be directly converted to acetyl-CoA (a central
precursor for cell substance synthesis), while butyric acid needs to be converted to butyryl-CoA and then gradually degraded to acetyl-CoA (Chen et al., 2018; Huo et al., 2018). Hence, acetic acid is more prone to increase the biomass of microalgae when compared to butyric acid. Propionic acid is utilized by microalgae by converting it into either acetyl-CoA or succinyl-CoA (Chen et al., 2018). Indeed, the more detailed metabolism of propionic acid is yet fully understood, but its utilization potential by microalgae is highly strain-dependent (Chen et al., 2018; Tan et al., 2020).

3.3. Removal of eutrophication nutrients at various light intensities

3.3.1. NH$_4^+$-N removal

NH$_4^+$-N can be easily assimilated by *C. vulgaris*, which is because NH$_4^+$-N can be directly transported into algal cells for compound synthesis (e.g., proteins, nucleic acids, and phospholipids) (Rossi et al., 2020). Fig. 4 shows the NH$_4^+$-N removal during the cultivation at different light intensities. In heterotrophic cultivation (0 lux), the utilization efficiencies of NH$_4^+$-N were 9.0%-37.9%, while in mixotrophic cultivation with 2000-5000 lux, the utilization efficiencies were enhanced to 38.7%-70.7% (Fig. 4E). It resulted from that the biomass production of *C. vulgaris* in mixotrophy was much higher than that in heterotrophy (Fig. 1), resulting in more NH$_4^+$-N assimilation. As further increment in light intensity to 8000 lux, the average utilization efficiencies of NH$_4^+$-N in four groups decreased to 46.7% (Fig. 4E), which was related to the low microalgal biomass production caused by the photoinhibition (Alkhamis and Qin, 2013; Li et al., 2014).
Indeed, the pH in culture systems would gradually increase with the substrate uptake, resulting in an accelerated ammonia-stripping (Cai et al., 2013; Mujtaba and Lee, 2017). In this study, for all tests, the final pH values in culture systems were 8.5-9.0. Consequently, it should be noted that algal assimilation and ammonia-stripping together contributed to the NH$_4^+$-N removal, even though the stripping effect in shaking flasks was weak (Mujtaba and Lee, 2017). However, NH$_4^+$-N removal performance achieved in this study was unsatisfactory due to low removal efficiency and large NH$_4^+$-N residual (Fig. 4). It is known that Chlorella shows a high potential for NH$_4^+$-N removal from wastewaters (Rossi et al., 2020). But the NH$_4^+$-N uptake capacity of Chlorella is strain-specific, which is likely determined by glutamine synthetase-glutamate synthase activity in chloroplasts (Cai et al., 2013; Chen and Wang, 2020); it would affect the final NH$_4^+$-N removal (Cai et al., 2013). Therefore, screening for Chlorella strains that can absorb and assimilate high levels of NH$_4^+$-N is an important consideration in the application of BACS on mariculture wastewater treatment.

### 3.3.2. TP removal

P, as a key element in algal cells, is stored in lipids, nucleic acids, proteins, and carbohydrate metabolic intermediates (Cai et al., 2013). Hence, the removal of P from wastewater streams by microalgae is directly linked to metabolic activities (You et al., 2020). Fig. 5 reflects the TP variation and utilization efficiency during the *C. vulgaris* cultivation. It can be observed that P was effectively removed since only 0.1-0.3 mg/L of
TP could be detected at the ending cultivation run (Fig. 5A-D). At light intensities of 0, 2000, 5000, and 8000 lux, the average removal efficiency of TP was 54.1%, 64.4%, 66.1%, and 60.3%, respectively (Fig. 5E). This result implied that light intensity of 5000 lux was optimum for TP removal.

It was reported that soluble phosphate can be removed by the precipitation process when the pH value in the culture medium is higher than 7.5 (You et al., 2020). In this work, the final pH values in culture systems were 8.5-9.0. Therefore, the removal of TP from the acidogenic fermentation effluents was achieved by a combination of microalgae uptake and abiotic precipitation.

It can be also found that the TP removal efficiencies in group I could reach over 80%, much higher than those (28.7-66.3%) in other groups (P < 0.05) (Fig. 5E). Notably, the TP concentration (12.9±0.6 mg/L) in the acidogenic fermentation effluent with initial pH 4.0 was higher than that (5.1±0.2 - 7.6±0.4 mg/L) in the acidogenic fermentation effluents with initial pH 6.0, 8.0, and 10.0 (Table 1). It reflected that limited P concentration in substrate would affect the final TP removal. Beuckels et al. (2015) suggested that at high N supply, the P concentration in microalgal biomass was a function of the P supply. Based on the above results, the N content in the acidogenic fermentation effluents of mariculture wastewater was in excess, which could favor the TP removal difference in this study.

3.4. Rubisco and citrate synthase activities under different light intensities

Photosynthesis and heterotrophic metabolism are important cellular metabolic
processes for substance and energy cycles and coexist simultaneously under mixotrophy (Li et al., 2014). To the best knowledge, photosynthesis and respiration share many intermediates (e.g., acetyl-CoA and ATP) (Nair and Chakraborty, 2020). And mixotrophic cultivation can dramatically enhance algal biomass production, even higher than the sum of those under autotrophy and heterotrophy (Alkhamis and Qin, 2013; Nair and Chakraborty, 2020; Zhang et al., 2017). It reflected that photosynthesis and heterotrophic metabolism may act in a coordinated manner on carbon and energy metabolisms under mixotrophy. Rubisco is the key enzyme of the Calvin cycle in photosynthesis (You et al., 2020), and in heterotrophic growth, citrate synthase is the most important enzyme in the TCA cycle for organic carbon metabolism (Zhang et al., 2017). Hence, the activities of these two enzymes in algal cells (collected from group I) were measured to reveal the interaction between photosynthesis and heterotrophic metabolism in mixotrophy.

As shown in Fig. 6A, Rubisco activity in heterotrophy (0 lux) was much lower than in mixotrophy thanks to the absence of photosynthesis in heterotrophy (Fig. 6A). At 2000, 5000 and, 8000 lux, Rubisco activity was 919.8, 1668.0, and 968.5 U/g, respectively (Fig. 6A). It demonstrated that a proper light intensity increment improved the CO₂ fixation in photosynthesis, while excessive light supply (8000 lux) exhibited a converse effect, which was associated with photoinhibition (e.g., damage to photosystem II) (Nair and Chakraborty, 2020). On the other hand, citrate synthase activity showed a declined trend with increasing light intensity (Fig. 6B), since compared with 0 lux, citrate synthase activity at 2000, 5000,
and 8000 lux decreased by 19.5%, 33.8%, and 43.4%, respectively. It revealed that the TCA cycle was down-regulated under mixotrophic cultivation.

3.5. Effect of light intensity on the synergistic of energy and carbon metabolism under mixotrophic cultivation

Taken together, the aforesaid results indicated that there was a synergy of energy and carbon metabolism between photosynthesis and heterotrophy in algal cell under mixotrophic conditions (Fig. 6C).

The TCA cycle was down-regulated under mixotrophic cultivation (Fig. 6B), which indicated that less absorbed organic carbons were channeled to the catabolism and thus less energy/ATP was produced through respiration under mixotrophic condition (Zhang et al., 2017). However, the biomass production under mixotrophic condition (< 8000 lux) was much higher than that under heterotrophic condition (Fig. 1), reflecting that the mixotrophic microalgae needed more energy for cell metabolism to support a faster growth rate. These results indicated that there was another pathway to generate energy for cell metabolism during mixotrophic cultivation. It was reported that under changing conditions, chloroplast linear electron transport (LET) can produce additional ATP in chloroplast with the participation of the respiration to fulfill extra-chloroplastic demand for ATP (such as needed for sucrose synthesis in cytosol) (Vanlerberghe et al., 2020). Hence, it was concluded that part of photoreaction-derived energy was exported to cytosol for cell metabolism under mixotrophic conditions (Fig. 6C). On the other hand, the removal efficiency of COD in
mixotrophic cultivation was higher than that in heterotrophic cultivation (Fig. 2), indicating more organic carbons were utilized by *C. vulgaris* under mixotrophic conditions. There were three reasons for this. Firstly, the CO₂ fixation process in photosynthesis participated in the enhancement of algal biomass production by generating autotrophically biomass under mixotrophic condition (Turon et al., 2016), which conversely promoted organic carbon removal. Secondly, the assimilation of VFAs by microalgae can be promoted by photosynthesis (Perez-Garcia et al., 2011). It is noteworthy that VFAs (carried by acetyl-CoA) can be also oxidized through the glyoxylate cycle to form malate (MA) in glyoxysomes when VFAs is available in culture medium (Perez-Garcia et al., 2011). And glyoxylate (GOX), an intermediate of photosynthesis, can condense with acetyl-CoA to MA in the glyoxylate cycle (You et al., 2020). Lastly, more absorbed organic carbons were channeled to the anabolism rather than the catabolism under mixotrophic conditions (Zhang et al., 2017), which further promoted the algal biomass accumulation and organic matter removal.

When increasing light intensity from 2000 lux to 5000 lux, the OD₆₈₀ of *C. vulgaris* and the COD removal efficiency increased (Fig.1 and Fig.2). Moreover, at 5000 lux, the Calvin cycle was up-regulated, but the TCA cycle was down-regulated and meanwhile, the VFAs utilization efficiency decreased compared with that at 2000 lux (Fig.3 and Fig.6). These results meant that CO₂ fixation in photosynthesis was strengthened while heterotrophic metabolism decreased, and photoautotrophic growth made a major
contribution to the enhancement in biomass production and nutrient removal performance at 5000 lux. When light intensity was 8000 lux, the algal biomass production and nutrient removal performance decreased (Fig.1~5), and the Calvin cycle and TCA cycle were down-regulated (Fig.6A-B), which indicated a decrease of both photosynthesis and heterotrophic metabolism at 8000 lux. It was concluded that light intensity could affect the synergy between photosynthesis and heterotrophic metabolism under mixotrophic conditions, which further affected the nutrient removal performance.

3.6. Implications in this study

With the development of aquaculture industry, a large amount of mariculture wastewater is discharged into the ocean (Guo et al., 2014; Lang et al., 2020), which seriously threaten the coastal ecology (Gao et al., 2020b). For the cost and treatment efficiency concerns, BACS, a biological technology integrating acidogenic fermentation and microalgae cultivation, was used for mariculture wastewater treatment. Mixotrophic microalgae cultivation, which uses light energy and organic carbon simultaneously, combines the advantages of photo-autotrophy and heterotrophy (Patel et al., 2019). Thus, compared with heterotrophic cultivation, mixotrophic cultivation helps to improve biomass production and nutrient removal (Chen et al., 2018; Nagarajan et al., 2017). However, light intensity is varied due to the alternation of day and night under natural conditions. Light plays a critical role in microalgae growth by regulating nutrients absorption, cell protoplasm synthesis, and product accumulation (Cai et al., 2013; Liu et al., 2010), which
will affect the removal of COD and nutrients (N and P) from waste streams (Patel et al., 2020). Based on these facts, the mariculture wastewater treatment performance with BACS at different light intensities was evaluated in this study. The aforesaid results confirmed that microalgae had a high pollutant removal performance under mixotrophy; at 5000 lux, the removal efficiency of COD, NH$_4^+$-N and TP reached 98.1% (Fig. 2), 70.7% (Fig. 3) and 90.0% (Fig. 4), respectively. Nevertheless, microalgae could also remove large amounts of organic carbons and nutrients under heterotrophy; at 0 lux (dark condition), the propionic acid removal efficiency could reach 85.2% (Fig. 3), and TP removal efficiency could reach 82.3% (Fig. 5). The results showed the feasibility of BACS to treat mariculture wastewater under both dark and lighting conditions, which provided a referential value for the application of BACS in practice. Moreover, for a thorough understanding of pollutant removal through microalgal cultivation at varied light intensities, the pathway of coordination between heterotrophy and photosynthesis in mixotrophic cultivation was proposed (Fig. 6C). Light intensity could regulate the coordination between heterotrophy and photosynthesis, and CO$_2$ fixation was strengthened while heterotrophic metabolism decreased with the increase of light intensity from 0 lux to 5000 lux, indicating that the enhancement in microalgal growth and pollutant removal was mainly contributed by photosynthesis. This result showed a novel perspective for understanding mixotrophic microalgal cultivation.
4. Conclusion

The feasibility of BACS to treat mariculture wastewater at varied light intensities was demonstrated. And 5000 lux was optimum for the mixotrophic growth of *C. vulgaris*, which conversely facilitated the removal of COD, NH$_4^+$-N, and TP. *C. vulgaris* could uptake fermented VFAs with a selective preference, in which acetic acid and propionic acid were preferentially utilized. Under mixotrophic conditions, a proper increase of light intensity could enhance CO$_2$ fixation, while the TCA cycle was down-regulated with the increment in light intensity. The synergy on carbon metabolism in cell between photosynthesis and heterotrophy resulted in a relatively high organic carbon removal performance of mixotrophic microalgae. Increasing light intensity in a certain range enhanced the contribution of photosynthesis to mixotrophic microalgal growth and final pollutant removal.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online.

References


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Fig. 1. Growth curves of *C. vulgaris* at different light intensities. Group I, II, III, and IV represent the microalga cultivation with diluted acidogenic fermentation effluent obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 as culture medium, respectively.
Fig. 2. Changes of COD during the cultivation of C. vulgaris (A-D) and COD removal efficiencies (E) at different light intensities. Group I, II, III, and IV represent the microalgae cultivation with diluted acidogenic fermentation effluent obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 as culture medium, respectively.
Fig. 3. Utilization of VFAs (A-D) and variations of acetic acid (E), propionic acid (F), and butyric acid (G) during the cultivation of *C. vulgaris*. Group I, II, III, and IV represent the microalgae cultivation with diluted acidogenic fermentation effluent
obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 as culture medium, respectively.
Fig. 4. Changes of NH$_4^+$-N during the cultivation of C. vulgaris (A-D) and NH$_4^+$-N removal efficiencies (E) at different light intensities. Group I, II, III, and IV represent the microalgae cultivation with diluted acidogenic fermentation effluent obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 as culture medium, respectively.
Fig. 5. Changes of TP during the cultivation of *C. vulgaris* (A-D) and TP removal efficiencies (E) at different light intensities. Group I, II, III, and IV represent the microalgal cultivation with diluted acidogenic fermentation effluent obtained at initial fermentation pH 4.0, 6.0, 8.0, and 10.0 as culture medium, respectively.
Fig. 6. The activities of Rubisco (A) and citrate synthase (B) at different light intensities. The synergy between photosynthesis and heterotrophic metabolism of C. vulgaris under mixotrophic cultivation (C): black arrows indicate metabolism processes (solid arrow: carbon metabolism; dashed arrow: energy supply), and red arrows indicate the regulation of enzyme activity or metabolism processes (up arrow: up-regulation; down arrow: down-regulation).
Table 1 Major components in the acidogenic fermentation effluents obtained from mariculture wastewater. Data are expressed as a mean ± standard deviation.

<table>
<thead>
<tr>
<th>Components</th>
<th>Units</th>
<th>Acidogenic fermentation effluents</th>
<th>Initial fermentation pH 4.0</th>
<th>Initial fermentation pH 6.0</th>
<th>Initial fermentation pH 8.0</th>
<th>Initial fermentation pH 10.0</th>
</tr>
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<tbody>
<tr>
<td>COD</td>
<td>g/L</td>
<td></td>
<td>3.5±0.2</td>
<td>4.2±0.2</td>
<td>4.7±0.2</td>
<td>5.0±0.3</td>
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<tr>
<td>NH₄⁺-N</td>
<td>mg/L</td>
<td></td>
<td>253.8±12.7</td>
<td>629.1±31.4</td>
<td>718.6±35.9</td>
<td>666.2±33.3</td>
</tr>
<tr>
<td>TP</td>
<td>mg/L</td>
<td></td>
<td>12.9±0.6</td>
<td>7.0±0.3</td>
<td>5.1±0.2</td>
<td>7.6±0.4</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>mg/L</td>
<td></td>
<td>1033.3±51.7</td>
<td>943.0±47.1</td>
<td>1126.3±56.3</td>
<td>1542.4±77.1</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>mg/L</td>
<td></td>
<td>354.6±17.7</td>
<td>231.4±11.6</td>
<td>390.0±19.5</td>
<td>516.1±25.8</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>mg/L</td>
<td></td>
<td>194.6±9.7</td>
<td>109.0±5.5</td>
<td>126.1±6.3</td>
<td>168.4±8.4</td>
</tr>
<tr>
<td>VFAs</td>
<td>mg/L</td>
<td></td>
<td>1685.7±84.3</td>
<td>1343.8±67.2</td>
<td>1700.1±85.0</td>
<td>2308.3±115.4</td>
</tr>
</tbody>
</table>
Table 2 The biomass productivities of *C. vulgaris* during cultivation at different light intensities. Data are expressed as a mean ± standard deviation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Biomass productivity (g L(^{-1}) d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 lux</td>
</tr>
<tr>
<td>I</td>
<td>0.120±0.006a</td>
</tr>
<tr>
<td>II</td>
<td>0.118±0.006c</td>
</tr>
<tr>
<td>III</td>
<td>0.140±0.007d</td>
</tr>
<tr>
<td>IV</td>
<td>0.150±0.008b</td>
</tr>
</tbody>
</table>

Note: means followed in the same row by the different lower-case letter are statistically different (p < 0.05).
Declaration of interests

✔ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: