Enhanced biomass and CO$_2$ sequestration of *Chlorella vulgaris* using a new mixotrophic cultivation method

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**Abstract**

CO$_2$ sequestration using microalgae has been deemed as a promising way, which can fix CO$_2$ and simultaneously produce valuable bio-products. However, large scale open-pond production of *Chlorella vulgaris* is normally cultured using acetic acid as a carbon source, which is costly and shows a low efficiency in CO$_2$ fixation. Here, a new mixotrophic culture strategy using both CO$_2$ and acetic acid was developed and evaluated for CO$_2$ sequestration by a *C. vulgaris* culture in an open pond. Our results show that the growth rate of *C. vulgaris* under this new mixotrophic condition reaches 0.24 g/L/d, which is higher than the 0.15 and 0.21 g/L/d of photoheterotrophic culture with acetic acid and photoautotrophic culture with CO$_2$, respectively. The averaged CO$_2$ fixation rate was determined as 0.29 g/L/d, which is much higher than heterotrophic method but slightly lower than photoautotrophic method. This result was further confirmed in a 125 m$^2$ open raceway pond. Physiological and biochemical characterization showed that the cell quality of *C. vulgaris* under mixotrophic conditions is better than those of photoautotrophic method and photoheterotrophic cultures. The enzyme activity assay and transcriptome sequencing analysis revealed that the metabolism of carbohydrates and amino acids was significantly enhanced under mixotrophic condition compared with other groups, which may attribute to the increased biomass and CO$_2$ sequestration of *C. vulgaris*. Our results suggest that this mixotrophic strategy can be applied in large-scale cultivation of *C. vulgaris* for biomass production and CO$_2$ sequestration.

1. Introduction

The wide use of fossil fuels has significantly contributed to the increase in atmosphere CO$_2$ concentration [1], which causes many environmental problems, such as global warming, climate changes and deterioration of ecological environment [2]. Therefore, sequestration of CO$_2$ from atmosphere has attained great attention [3]. Recently, various carbon sequestration methods have been developed, including physical, chemical and biological methods [4]. However, CO$_2$ sequestration with physical and chemical methods are costly and incompatible with sustainable development requirements [5]. Biological CO$_2$ sequestration using microalgae or plants has been considered to be a promising method [6], which can fix CO$_2$ through photosynthesis and meanwhile produce valuable bioactive products [7].

*Chlorella vulgaris* is a kind of single-cell eukaryotic green algae [8], which has been reported to possess a high CO$_2$ sequestration rate [9] and biomass productivity, favourable adaptability under different temperature, nutrient and climatic conditions [10,11]. It can grow well in oceans, lakes, ponds and other water environments. *C. vulgaris* is also rich in proteins, lipids, polysaccharides, and other biologically active metabolites [12], which shows its excellent applicability in the fields of food, health care, feed, and medicines. Besides, it is widely studied as a potential bio-energy feedstock. Therefore, culture of *C. vulgaris* has been deemed as an ideal way for CO$_2$ fixation and production of valuable bioactive substances. Although CO$_2$ fixation using *C. vulgaris* has been widely studied, the large scale commercial application is still not realized, which is mainly due to the high cost in closed photo-bioreactor and low efficiency in open raceway pond [13].

Recently, *C. vulgaris* has become the second largest cultured algal in China [14], and it is normally cultured in open raceway ponds using acetic acid [15,16], where acetic acid act as a carbon source and also keeps the pH within neutral range [17]. Compared with acetic acid, CO$_2$ can also be used by *C. vulgaris* as carbon source with the advantages of higher CO$_2$ fixation efficiency, and lower-risk of contamination [18]. However, *C. vulgaris* cells cultured only with CO$_2$ are inferior to those with acetic acids or glucose in terms of cell size and density, and are difficult to harvest [19]. Therefore, improvement of biomass production and CO$_2$ sequestration rate is an area of great interest.
concern for application of \textit{C. vulgaris}. Recently, mixotrophic cultivation of microalgae has been proved to be a preferable cultivation mode for biomass, bioenergy production and bioremediation [20]. Tamarys et al. [16] showed that the mixotrophic culture of \textit{Chlorella protothecoides} using glucose and acetate grew better than under autotrophic conditions. Monika et al. showed that biomass and lipid productivity of \textit{Chlorella pyrenoidosa} increased significantly under mixotrophic culture conditions of sodium acetate and glycerol.

Here we report a new mixotrophic cultivation of \textit{C. vulgaris}, which contains two stages, one photoautotrophic cultivation stage with CO\textsubscript{2} as carbon source during daytime, and another heterotrophic cultivation stage using acetic acid during nighttime. The biomass production, CO\textsubscript{2} bio-fixation rate, and transcriptome analysis were used to evaluate the effectiveness of the new strategy and its potential application for the large-scale carbon sequestration using \textit{C. vulgaris}.

2. Materials and methods

2.1. Strains and culture conditions

The \textit{C. vulgaris} strain was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (http://algae.ihb.ac.cn/), and cultured in conical flasks containing BG11 medium [21]. The cultures were divided into four groups: 1) photoautotrophic culture where 5% CO\textsubscript{2} (95% air) was bubbled continuously into the BG11 medium with gas flow rate of 10 L/h; 2) photoheterotrophic culture where 0.05 M of acetic acid was added to act as carbon source and regulate the pH of BG11 medium within 6.5–7.5; 3) mixotrophic culture, where 5% CO\textsubscript{2} was continuously bubbled during daytime, and acetic acid was added at night to regulate the pH value within neutral range; 4) control group, where \textit{C. vulgaris} was cultured in BG11 medium without supplement of CO\textsubscript{2} or acetic acid. All the algal cultures were incubated under LED fluorescent lamp with illumination intensity of 120 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}, and day: night cycle at 12 h:12 h. The culture temperature was maintained at 25 ± 1 °C by the temperature-controlled incubator.

The pilot scale cultivation was conducted in a 125 m\textsuperscript{2} open raceway pond by mixing with a six-blade paddlewheel. The \textit{C. vulgaris} cells were cultured under natural light and temperature conditions with the depth of 20 cm. The 5% CO\textsubscript{2} was continuously bubbled during daytime, and acetic acid was added at night to regulate the pH value within 6.5-7.5. The pond cultured with acetic acid was chosen as control.

2.2. Determination of biomass

The biomass of \textit{C. vulgaris} was determined by measuring the absorbance at 750 nm (OD\textsubscript{750}) using a Shimadzu 2450 UV–Vis spectrophotometer. To obtain the dry biomass during cultivation, 20 mL samples were dewatered by centrifugation at 8000 g for 10 min and dried at 80°C for 24 h. Biomass yield (g/L) was calculated from the microalgae dry weight produced per liter.

The algal cell size and density of \textit{C. vulgaris} was evaluated by measuring the forward scatter (FSC) and side scatter (SSC) on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) [22]. Briefly, after 10 days of culturing, algal cells were taken and diluted to 5 × 10\textsuperscript{6}–1 × 10\textsuperscript{7} cells/mL, and then filtered through a 40 μm mesh screen. The microalgae cells were flowed through the light beam of a 488 nm argon laser. A total of 30,000 algal cells were taken, and two-parameter scatter plots of \textit{C. vulgaris} under different culture conditions were recorded using flow cytometry. The averaged statistics of FSC and SSC were determined by measuring their intrinsic fluorescence signal intensity [23]. The acquisition and analysis of experimental data were performed using CellQuest software according to manufacturer’s instruction.

2.3. Determination of chlorophyll and protein content

The chlorophyll content of \textit{C. vulgaris} was determined as previously reported [24]. Briefly, 2 mL algae culture samples were centrifuged at 6000 rpm for 10 min to harvest the cells. Then 96% methanol was added, and the sample was ultrasonicated for 40 min on ice. The sample was centrifuged again, the supernatant was collected and absorbance was measured at 653 (OD\textsubscript{653}) and 666 nm (OD\textsubscript{666}), respectively, with 96% methanol as blank. The chlorophyll content of chlorophyll a (Ca) and b (Cb) was calculated according to the following formula [24]:

\[
Ca (mg/L) = (15.65 \times OD_{666} - 7.34 \times OD_{653}) \times dilution \text{ factor} \\
Cb (mg/L) = (27.05 \times OD_{653} - 11.21 \times OD_{666}) \times dilution \text{ factor}
\]

The protein content of \textit{C. vulgaris} cells was determined using the Bradford method [25].

2.4. Lipid extraction and FAME (Fatty acid methyl ester) analysis

Total lipid extraction from microalgal cells was determined according to the following procedure. The lyophilized cells (W\textsubscript{1}) were suspended in 5 mL of chloroform/methanol (2/1, v/v) and sonicated for 30 min in an ultrasonic cleaner (QK-100KDE, Kunshan, China) at 25 °C. After agitation, the mixture was centrifuged at 8000 g for 20 min at 4 °C and the supernatant was transferred to another pre-weighted tube (W\textsubscript{2}). The residues were further extracted twice and the chloroform phase was collected together. After evaporating and drying to constant weight under nitrogen atmosphere, the lipids were gravimetrically quantified (W\textsubscript{3}). The lipid content was expressed as % dw (dry cell weight) as according to the following formula:

\[
\text{Lipid content(% dw)} = \frac{(W_3 - W_2)}{W_1} \times 100
\]

The fatty acid content of \textit{C. vulgaris} was characterized according to the previously reported [26,27]. Briefly, 20–35 mg algae pellets were thoroughly mixed with 5 mL of sulfuric acid-methanol solution in a 10 mL round bottom flask. After the solution was methylated using condensation reflux at 70 °C in a water bath for 1 h. The sample was cooled to room temperature, 0.75 mL of deionized water and 2 mL of n-hexane was added into the flask for extraction. Then the upper liquid was extracted and analyzed on an Agilent 1100 gas chromatography.

2.5. Carbonic anhydrase and Rubisco activity assay

The activity of carbonic anhydrase (CA) was determined using the following procedure: moderate amounts of algal cells were harvested and suspended in 10 mM Tris-HCl buffer (pH 8.3) [28]. Then cells were disrupted by ultrasonic treatment and centrifuged to collect the supernatant. The CA activity was measured using the esterase method [29]. Briefly, 0.2 mL of enzyme solution and 1 mL of 3 mM p-nitrophenyl acetate (p-NPA) solution were added into a vial containing 1.8 mL of 50 mM Tris-SO\textsubscript{4} buffer (pH 7.4) and mixed thoroughly. The absorbance was monitored at 400 nm within 3 min of reaction using a Shimazu 2450 UV–Vis spectrophotometer at 25 °C.

The activity of Rubisco enzyme was assayed according to the following procedure [30]. Briefly, algal cells were harvested and suspended in chilled extraction buffer (40 mM Tris–HCl, 0.25 mM EDTA, 10 mM MgCl\textsubscript{2}, 5 mM glutathione, pH 7.6) [30]. After cell disruption, the supernatant was collected for enzyme activity assay. The reaction mixture was prepared containing 0.25 mM NADH, 2 mM ATP, 10 mM phosphocreatine, 20 units/mL of creatine phosphokinase, 40 units/mL of phosphoglycerate kinase (PGK), 20 units/mL of glyceraldehyde phosphate dehydrogenase (GAPDH) and 2.5 mM ribulose-1,5-diophosphate (RuBP). Reactions without RuBP were employed as negative controls. The absorbance changes within 5 min of reaction mixtures were measured at 340 nm [31].

2.6. Transcriptome analysis

About 300 mg algal pellets (150 mL when OD\textsubscript{750} as 1.0) were
harvested and washed twice with sterile PBS. Total RNA was extracted by TRIzol reagent (Invitrogen) for cDNA library construction and the Illumina sequencing was carried out in Personalbio Company (Shanghai, China). Quality reads were assembled into contigs, transcripts and unigenes using Velvet and Oases software. RPKM (reads per kilobase of exon model per million mapped reads) was used to normalize the abundances of transcripts. A 2-fold differential was used to identify the genes differentially expressed between two growth phases. All unigenes were used as queries in searching Non-redundant, SwissProt and Cluster of Orthologous Groups of proteins databases and functionally annotated by Gene Ontology analysis with Blast2GO software. Metabolic pathways were predicted by Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping [32].

2.7. Statistical analysis

All the experiments were performed in three biological replicates. The results presented were the average values and expressed as mean values (±SD). The eff ; sects of diff ; rent experimental conditions were analyzed using t-test and one-way analysis of variance (ANOVA) at the confidence level of 95% (p < 0.05).

3. Results

3.1. Biomass production

The biomass production of *C. vulgaris* under different culture conditions is shown in Fig. 1. From the results, the three cultures with supplement of CO2, acetic acid, and both CO2 and acetic acid, show a significant increase in the biomass production of *C. vulgaris* compared with control group. The highest growth rate of *C. vulgaris* was found in mixotrophic culture supplemented with CO2 and acetic acid as 0.24 g/L/d, which was higher than the 0.15 and 0.21 g/L/d of photoautotrophic culture with CO2 and photoheterotrophic culture with acetic acids, respectively. After 10 days of culturing, the total biomass production was 2.37 g/L under mixotrophic conditions, which was 7.2-fold higher than that of the control group (P < 0.05). Followed with the photoautotrophic culture supplemented with CO2 and photoheterotrophic culture supplemented with acetic acid [27], with the biomass production as 2.1 and 1.54 g/L, which was 6.4 and 4.7-fold higher than that of the control group (P < 0.05) respectively.

From Table 1 and Fig. 2, it can be seen that the averaged FSC was highest under mixotrophic conditions, which suggest that the averaged cell size under mixotrophic condition was higher than the other three groups. Similarly, the highest SSC value of *C. vulgaris* was also found under mixotrophic conditions, which is slightly higher than that of photoheterotrophic culture, suggesting a better cell density under mixotrophic conditions. Cells cultured under the photoautotrophic condition with only CO2 show the smallest SSC value, which suggests that photoautotrophic culture of *C. vulgaris* can grow well but the cell density was lower, which would increase the difficulty of downstream cell harvesting. Taken together, our results show that this new mixotrophic strategy can give the best cell growth rate, highest cell size and density compared to the other three groups, which is could be applicable for large-scale cultivation of *C. vulgaris* with CO2 sequestration.

3.2. Physiological assay

*C. vulgaris* belongs to the Chlorophyta, rich in chlorophyll, proteins, and fatty acids [33], which are also the important indexes for quality control of algae. As showed in Fig. 3A, the chlorophyll contents of *C. vulgaris* increased with prolonged culture time under each culture condition. After a 10 day’s cultivation, the chlorophyll content of *C. vulgaris* was highest under mixotrophic condition at 6.11 mg/L/d, which was 6.4-fold higher than that of the control group (P < 0.01). And it was followed by photoautotrophic and photoheterotrophic cultures at 5.13 and 3.52 mg/L/d, which was 5.3 and 3.6-fold higher than the control group (P < 0.05), respectively. Similarly, the highest protein content of *C. vulgaris* was also found under mixotrophic conditions as 36.15 mg/L/d, which was 6.4-fold higher than the 5.65 mg/L/d of the control group (P < 0.01) (Fig. 3B). So was the photoautotrophic, photoheterotrophic culture as 25.67 and 17.56 mg/L/d respectively, which was 4.5 and 3.1-fold higher than the control group (P < 0.05), respectively.

The addition of different carbon sources also has a significant effect on the lipid contents of *C. vulgaris* (Fig. 3C). After 8 days cultivation, the lipid content and components were analyzed by gas chromatography. The highest lipid content was found as 33.52% when cultured under photobutautrophic condition. The mixotrophic and photoheterotrophic cultures showed a lipid content of 26.3% and 20.45%, respectively. GC analysis showed that the C16 and C18 fatty acids were the main components of *C. vulgaris* (Fig. 3D). According to fatty acid components analysis, the content of unsaturated fatty acids in each group is much higher than those of saturated fatty acids. Interestingly, the content of C18:1 fatty acid was significantly higher than other fatty acids in each group, which is the main antioxidant component in vivo and can also be used as main feedstock for preparation of biodiesel.

3.3. Carbonic anhydrase and Rubisco activity assay

Carbonic anhydrase (CA) and Rubisco are two key enzymes in the microalgal CO2 concentrated mechanism (CCM) and play important roles in carbon transport and metabolism [34]. Therefore, the enzyme activity of CA and Rubisco was determined to characterize the effect of culture conditions on their CO2 fixation process. As shown in Fig. 4A, the CA activity was significantly higher for the control group than the other three groups (P < 0.05), which suggested that under photoautotrophic conditions without supplement of CO2, *C. vulgaris* needs to
initialize the CCM at the expense of ATP to capture enough CO2 for photosynthesis [35,36]. While the addition of 5% CO2 or acetic acid as carbon sources, the C. vulgaris is not necessary to use CCM to concentrate more CO2, and then the activity of CA was inhibited [37]. Accordingly, the highest activity of Rubisco was found under the phototrophic conditions supplemented with 5% CO2 (Fig. 4B), which suggests that the addition of 5% CO2 can significantly increase the intracellular CO2 concentration by permeation, leading to up-regulation of the activity or quantity of Rubisco, and then enhanced catalytic carbon sequestration of Rubisco [38].

3.4. CO2 biofixation

It has been reported that for each 1 g of dry algal biomass, 1.65–1.88 g of CO2 will be bio-fixed [39]. In order to characterize the CO2 biofixation by C. vulgaris under different culture conditions, the CO2 fixation was calculated and summarized in Table 2. The highest averaged CO2 bio-fixation attained was 0.37 g/L/d under phototrophic conditions and then 0.29 g/L/d under mixotrophic conditions within 10 days cultivation, which were 6.28 and 4.98 times higher than the control group (0.059 g/L/d), respectively. For the phototrophic culture, the algae mainly uses acetic acid as carbon source and their activity of CA and Rubisco were the lowest compared to the other three groups, which mean its CO2 fixation should be lower than the control group. These results show that this new mixotrophic strategy can simultaneously increase the biomass production and CO2 bio-fixation of C. vulgaris.

3.5. Pilot scale cultivation of C. vulgaris in open raceway pond

The pilot scale cultivation of C. vulgaris using mixotrophic strategy was carried out in a 125 m² open raceway pond. The pond cultured as commercial heterotrophic method with acetic acid was chosen as control. As showed in Fig. 5A, after 5 day’s cultivation, the OD750 value of C. vulgaris reached 3.20 under the mixotrophic condition, which was 1.3 times that of the control group (2.44). This indicates that the mixotrophic strategy works well in large-scale cultivation of C. vulgaris for biomass production and CO2 sequestration.

3.6. Transcriptome analysis

The differences in the expression level of genes associated with

![Fig. 2. Characterization of cell size (FSC) and cell density (SSC) of C. vulgaris cultured under different culture conditions. (a) control group, cultured in BG11 medium. (b) phototrophic group, 0.05 M of acetic acid was added to regulate the pH of BG11 medium within 6.5-7.5. (c) phototrophic group, 5% CO2 was bubbled continuously into the BG11 medium. (d) mixotrophic group, 5% CO2 was continuously bubbled during daytime, and acetic acid was added at night to regulate the pH value within neutral range.](image-url)
carbon metabolism were analyzed and compared by transcriptome analysis under the three culture conditions. Compared with the control group, the transcript abundance of the triose phosphate isomerase (EC5.3.1.1) in Calvin cycle, which reversibly converts GAP into dihydroxyacetone phosphate (DHAP), increased by 2.7-fold under photoautotrophy conditions (Fig. 6A). In addition, the transcript abundance of the succinate dehydrogenase (EC1.3.5.1), a key enzyme in tricarboxylic acid cycle, increased by 6.6-fold. These suggested that addition of 5% CO₂ can partly enhance the carbon metabolism of *C. vulgaris*.

Under photoheterotrophic conditions with supplement of acetic acid, the acetate was first converted into acetate phosphate and then acetyl-CoA, which can further participate in the tricarboxylic acid cycle (TCA cycle). Therefore, under photoheterotrophic conditions, the transcript abundance of the citrate synthase (EC2.3.3.1) and ATP citrate lyase (EC2.3.3.8), which involved catalyzing the binding of acetyl-CoA with oxaloacetate to produce citrate, increased by 5.8-fold and 4.2-fold respectively. Moreover, the transcript abundance of fructose-1,6-bisphosphatase I (EC3.1.3.11), aconitate hydratase (EC4.2.1.3), succinyl-CoA synthetase (EC6.2.1.4, EC6.2.1.5), and fumarate hydratase (EC4.2.1.2) in TCA cycle, and the glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.12), triose phosphate isomerase (EC5.3.1.1), and sedoheptulose-1,7-bisphosphatase (EC3.1.3.37) in Calvin cycle, were also significantly increased (Fig. 6B). These results indicated that the supplement of acetic acid can enhance the metabolic pathway of TCA cycle, and then increase the biomass of *C. vulgaris*.

Under mixotrophic conditions with supplement of 5% CO₂ and acetic acid, the transcript abundances of most enzymes involved in the Calvin cycle and TCA cycle increased significantly (Fig. 6C). For example, in the Calvin cycle, the glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.12) increased by 3.7-fold, the phosphoribulokinase (EC2.7.1.19), which phosphorylates Ru5P into RuBP, increased by 5.9-fold, and the Ribulose-phosphate 3-epimerase (EC5.1.3.1) increased by 51-fold. The fructose-1,6-bisphosphatase aldolase (EC4.1.2.13), which catalyzes the irreversible conversion of GAP to sedoheptulose-7-phosphate (S7P), increased more than 4-fold. Similarly, in the TCA cycle, the citrate synthase (EC2.3.3.1), which catalyzes the binding of acetyl-CoA to oxaloacetate, transcript abundance increased more than 10 times. Then, the transcript abundance of ATP citrate lyase (EC2.3.3.8) increased 13.2-fold. The aconitate hydratase (EC4.2.1.3), which catalyzes the conversion of citric acid to isocitrate, transcript abundance increased by 6.7-fold. And the isocitrate dehydrogenase (EC1.1.1.42), which catalyzes isocitrate to 2-oxoglutarate, increased by 20.4-fold. The two 2-oxoglutarate dehydrogenases (EC1.2.4.2, EC2.3.1.61), which convert 2-oxoglutarate to succinyl-CoA, transcript abundance increased by 9.1 and 7-fold, respectively. Moreover, the succinyl-CoA synthetase (EC6.2.1.4, EC6.2.1.5) also increased by 4.2 and 9.1-fold, respectively. The fumate hydratase (EC4.2.1.2), which converts the reversible transformation of fumaric acid and malic acid, increased by 5.5-fold. These results indicated that the whole carbon fixation process was significantly up-regulated. Therefore, the growth rate and protein contents of *C. vulgaris* increased obviously compared with other groups (Fig. 6).
Besides, some genes were also found to be down-regulated. Most significantly, the transcript abundance of Rubisco (EC4.1.1.39) was down-regulated in the Calvin cycle, which catalyzes the carboxylation of ribulose 1,5-diphosphate with carbon dioxide in the Calvin cycle, and then is a key enzyme in the carbon concentrating mechanisms. In control group, the carbon source was insufficient and *Chlorella* need to activate the carbon concentrating mechanisms (CCM) to maintain normal cell growth [34,35]. But in the other three groups, there are enough carbon sources (CO₂ or acetic acids), the algae do not need to activate CCM, and therefore resulted in the down-regulation of Rubisco. In addition, Rubisco was a key enzyme in photosynthesis that determined the rate of carbon assimilation. Down-regulation of the Rubisco also limited the expression of the phosphoribulokinase (EC2.7.1.19) and phosphoglycerate (EC2.7.2.3).

4. Discussion

CO₂ sequestration using micro-algae has attracted much interest and *Chlorella vulgaris* has been considered an ideal strain for CO₂ fixation based on its high photosynthesis efficiency [40]. Although *C. vulgaris* has been cultured outdoors in large scale, commercial large-scale cultivation of *C. vulgaris* does not use CO₂ as carbon source, but mainly use acetic acid or glucose [15]. Therefore, utilization of more CO₂ to substitute acetic acid or glucose during large-scale commercial cultivation of *Chlorella* is a great concern.

It has been reported that *C. vulgaris* can grow well under 3 %–20 % CO₂ conditions [41,42], but the algal cells under photoautotrophic condition are not homogeneous and the averaged cell density is normally lower than that of heterotrophic cells using acetic acid or glucose, which would increase the difficulty of downstream harvesting process and lower down the quality of *C. vulgaris* as food or health products [19]. Since the *C. vulgaris* can not only be cultured based on photosynthesis (photoautotrophy), but also grows fast utilizing glucose, tryptone or acetate (heterotrophy) [43,44], to improve the biomass production and CO₂ fixation rate, a new mixotrophic cultivation method has been developed using CO₂ and acetic acid. In daytime, 5% CO₂ was bubbled into the culture medium, and *C. vulgaris* can perform photosynthesis using CO₂ as carbon source. Under this condition, the pH value of medium can be stabilized around 7 based on NaCO₃-CO₂ buffer system [45]. During nighttime, *C. vulgaris* can conduct heterotrophic growth using acetic acid as carbon source, and meanwhile the addition of acetic acid can also regulate the pH value of medium around

Table 2

| Biomass productivity and CO₂ bio-fixation rate of *C. vulgaris* under different culture conditions. |
|--------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Control                                         | Phototrophic culture            | Phototrophic culture            | Mixotrophic culture             |
| Biomass productivity (g/L/d)                    | 0.033                           | 0.154                           | 0.206                           |
| CO₂ biofixation (g/L/d)                         | 0.059a                          | –                               | 0.371 a                         |

a. The CO₂ fixation was calculated as 1.8 times of algal biomass [10,51].

b. The CO₂ fixation of mixotrophic culture was calculated as 70% of 1.8 × algal biomass.
7 based on acetic acid-sodium acetate buffer system [17]. Under this mixotrophic condition, the biomass and quality of *C. vulgaris* can be improved, and more CO₂ can be fixed during large-scale *C. vulgaris* cultivation, which would be a promising way to capture CO₂ using these valuable microalgae.

To conduct photosynthesis under aquatic environment, microalgae uses the carbonic concentration mechanism (CCM) [46] to actively transfer inorganic carbon and concentrated CO₂ for dark reaction center of photosynthesis, which then achieve a higher photosynthetic rate in the lower concentration CO₂ environment [47]. In green algae, CO₂ concentration is considered to be one of the important factors controlling CCM [48,49]. In photoautotrophic group under 5% CO₂, the CA activity was inhibited but the Rubisco activity was much higher compared with control group. The activity of Rubisco was up-regulated, which suggests that there is enough CO₂ surrounding Rubisco because of the penetration of CO₂ through cell membranes, hence no need for active uptake of CA from outside at the expense of ATP. For the mixotrophic condition, the activity of CA and Rubisco is both low compared with control group, which suggests that *C. vulgaris* can use penetrated CO₂ or acetic acid as carbon source, hence no need for higher activity of CA and Rubisco for photosynthesis.

*C. vulgaris* can grow through TCA cycle using acetic acid as carbon source both under light and matt conditions [50]. The metabolic pathway of acetate is mainly involved in the tricarboxylic acid cycle and glyoxylate cycle [50]. Based on this mechanism (Fig. 7), Chlorella can be cultured at night using acetic acid as carbon source. It should be noted that the photoheterotrophic cultivation in this study is also a kind of "mixotrophic culture", which can use both acetate and CO₂ from the air. But due to the existence of acetate, the activity of CCM in Chlorella will not be initialized to concentrate CO₂, and Rubisco activity is also kept lower, therefore, the CO₂ fixation should not be higher than the control group.

5. Conclusions

A new mixotrophic cultivation of *C. vulgaris* has been developed
using CO₂ and acetic acid as carbon source. Under this mixotrophic condition, C. vulgaris can achieve a faster growth rate, higher biomass, increased chlorophyll, and protein content compared to other photoautotrophic and phot-heterotrophic cultures. Transcriptome sequencing analysis and enzymatic activity assay demonstrated that the activity of CA and Rubisco was inhibited and the whole carbon metabolic pathway was significantly enhanced in mixotrophic condition, which attributes to the improved biomass production and CO₂ fixation of C. vulgaris. Our results suggest that this mixotrophic culture can be a promising way for large-scale production of C. vulgaris as well as CO₂ bio-fixation.

Author’s contributions

B. Ge, S. Qin and Q. Yu designed the research plan. Q. Yu, H. Wang, X. Li and B. Ge conducted the experiments. B. Ge, Q. Yu, H. Wang, X. Li and S. Qin analyzed the data and interpreted the results. B. Ge, Q. Yu and S. Qin drafted the manuscript, and all authors revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2019.11.022.

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