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The synergistic energy and carbon metabolism under mixotrophic cultivation reveals the coordination between photosynthesis and aerobic respiration in *Chlorella zofingiensis*

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ABSTRACT

Microalgal biofuels have attracted much attention in recent years. Nevertheless, the relatively low growth rate and low cell density limits its industrial application. Mixotrophic cultivation has been considered as a good strategy to increase the specific growth rate and biomass yield in some microalgal species. However, the metabolic mechanism has not been well elucidated. To address it, *Chlorella zofingiensis* was employed in the present study. The biomass productivity and maximum dry weight achieved under mixotrophic condition were greater than the sum of those under photoautotrophic and heterotrophic conditions. Interestingly, compared with photoautotrophic cultures, the RuBisCO activity was reduced for mixotrophic cultures, indicative of the down-regulation of the Calvin cycle. When compared with heterotrophic cultures, the citrate synthase activity was attenuated for mixotrophic cultures, indicative of the down-regulation of Tricarboxylic Acid cycle. Biochemical analysis showed that mixotrophic cells contained more starch but less total fatty acid content than heterotrophic cells. Besides, the biomass yield on glucose was 0.789 g g^{-1} for mixotrophic cultures, 63.7% higher than that for heterotrophic cultures, and the enhancement effect of light under mixotrophic cultivation was 7.35-fold. Based on these results, a novel mechanism for the synergistic effects in mixotrophic cultivation was proposed, in which glucose was more efficiently utilized for biomass production by the possible coordination of energy and carbon metabolism between photosynthesis and aerobic respiration.

1. Introduction

Microalgal biofuels have attracted much attention in recent years and are regarded as the third generation of biofuels, since microalgae can accumulate a high amount of lipids and can be cultured on non-arable land [1]. Nevertheless, no application in practice on the industrial production of microalgal biofuels has been reported. The relatively low growth rate and low cell density are considered as the two main limitations for the industrial application [2–6].

It is thought that mixotrophic cultivation is a good strategy to increase the specific growth rate and cell density [7]. In *Chlorella sorokiniana*, the specific growth rate and maximum dry weight (DW) in mixotrophic cultivation were 1.8- and 2.4-fold of those in heterotrophic cultivation, and 5.4- and 5.2-fold of those in photoautotrophic cultivation [7]. Similarly, in marine *Chlorella* sp. and *Nannochloropsis* sp., the biomass and lipid production were notably increased in the mixotrophic culture compared with photoautotrophic and heterotrophic cultures [8]. Meanwhile, mixotrophic cultivation can elevate the tolerance to high light intensity. Photo-inhibition was observed in *Spirulina* sp. at 50 W m^{-2} under photoautotrophic cultivation, whereas it was not observed within the experimental light intensity (0–65 W m^{-2}) under mixotrophic cultivation [9]. With these advantages, mixotrophic cultivation has been applied on the treatment of organic carbon-rich waste water. By this method, the water body could be purified, and a significant amount of microalgal biomass would be acquired [10–12].

Mixotrophy was firstly recognized as a simple combination of photoautotrophy and heterotrophy. However, in some microalgal species, the maximal DW under mixotrophic cultivation was higher than the sum of those under photoautotrophic and heterotrophic cultivation under certain culture conditions, which indicated the existence of synergistic effects of photosynthesis and aerobic respiration in mixotrophy [7,13,14]. Since CO_2 aeration can highly promote cell growth in some microalgae species [15], some researchers believe that

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the increase of biomass in mixotrophic cultivation may due to the reutilization of the CO_2 released from aerobic respiration [7,13]. However, there is a contradiction in this hypothesis, the process of aerobic respiration is employed for decomposing organic carbon with CO_2 releasing, and CO_2 reutilization means reusing the CO_2 from respiration to synthesize organic carbon with the utilization of energy generated from photoreaction. These two processes are reverse repeated and some intermediates are both involved in these two pathways. Thus, other metabolic mechanism might also exist under mixotrophic cultivation in certain microalgal species. In order to uncover the synergistic effect of mixotrophy in certain microalgal species, more works need to be done in the future.

C. zofingiensis has been considered as a potential candidate for biofuels production in recent years, which was due to its ability to accumulate large amount of lipids and certain amount of high valued astaxanthin at the same time [16,17]. Besides, *C. zofingiensis* can be cultured under three trophic modes, which makes this strain a prefect model for the interpretation of the mechanism involved in mixotrophic cultivation [17,18]. Ip et al. showed that, compared with heterotrophic cultivation, higher biomass production and astaxanthin yield were achieved under mixotrophic cultivation [20]. Moreover, Chen et al. illustrated that light can attenuate lipids accumulation while enhance cell proliferation and starch accumulation in *C. zofingiensis* [19]. However, the cellular mechanism of increased biomass production under mixotrophic cultivation in *C. zofingiensis* is still unclear.

In the present research, as not all mixotrophy is synergistic, only several microalgal strains have been found to possess the synergistic effect [7,13], the growth curve, biomass productivity and maximum DW were firstly characterized under three trophic modes to confirm the existence of synergistic effect in mixotrophy in *C. zofingiensis*. More importantly, enzyme activities of Calvin cycle and Tricarboxylic Acid (TCA) cycle were measured to give a fresh perspective on the coordination between photosynthesis and aerobic respiration under mixotrophic cultivation. Then, starch content, total fatty acid content, energy fixation, enhancement effect of light and biomass yield on glucose were measured and compared between three cultivation modes. Taken these together, our results showed that mixotrophic cultivation could highly increase the biomass productivity and maximum DW, which are the main limiting factors for biofuels production during photoautotrophic cultivation. In addition, a novel mixotrophic metabolic mechanism was proposed, which may provide new strategies for photoautotrophy to overcome its limitations.

2. Materials and methods

2.1. Strains and culture conditions

C. zofingiensis (ATCC30412) was purchased from American Type Culture Collection (ATCC, Rockville, USA). For culture conditions, 10 mL stock culture was inoculated into 500 mL Erlenmeyer flasks (with filter cap) containing 100 mL Kuhl growth medium, and cultured in dark with orbital shaking at 150 rpm [20,21]. Glucose was added and used as the carbon source at a concentration of 5 g L^{-1}. The culture temperature was maintained at 25 °C by the temperature-controlled incubator. The algal cells were allowed to grow 3 days to serve as the seed cultures for different cultivation modes. The cells were collected, washed, and re-suspended at a cell density of 0.5 g L^{-1} in (1) photoautotrophic cultivation under 40 μmol m^{-2} s^{-1} light (P + L40), (2) photoautotrophic cultivation under 100 μmol m^{-2} s^{-1} light (P + L100), (3) photoautotrophic cultivation under 100 μmol m^{-2} s^{-1} light with CO_2 aeration (1.5% CO_2 mixed in air) (P + L100 + CO_2), (4) heterotrophic cultivation with initial 5 g L^{-1} glucose in culture medium (H + G5), (5) mixotrophic cultivation under 40 μmol m^{-2} s^{-1} light with 5 g L^{-1} glucose (M + G5 + L40) and (6) mixotrophic cultivation under 100 μmol m^{-2} s^{-1} light with 5 g L^{-1} glucose (M + G5 + L100). The Illumination was provided by cool-white fluorescent lamps, which were embedded in the lids of the incubators. For the CO_2 aeration of P + L100 + CO_2, a sterile thin rubber tube was combined with the filter cap of the Erlenmeyer flask. The top of the tube was coupled with a sterile filter, and the mixed air (1.5% CO_2 mixed in air) was pumped through the tube into the culture medium at 0.2 vvm. After the glucose in heterotrophic and mixotrophic culture medium were exhausted, cells under all different modes were harvested.

2.2. Dry weight and glucose concentration measurement

After being cultivated for 12, 24, 36 and 40 h, 2 mL culture was collected from each of the cultivation modes, and centrifuged for 3 min at 5000 *g*. The supernatants were collected and glucose concentration was measured by 3,5-dinitrosalicylic acid (DNS) method [22]. The residual cell pellets were washed 3 times by distilled water, then dried at 80 °C for 12 h and weighed for DW.

2.3. Total fatty acid quantification

Cells were collected by centrifuging at 5000 *g* for 3 min at 4 °C and lyophilized in a DW3 freeze-drier (Heto Dry Winner, Denmark). The lyophilized cell samples were directly methylated to fatty acid methyl esters (FAMEs) by incubating with 1% sulphuric acid in methanol according to the procedures described by Liu et al. [23]. The FAMEs were analyzed by using a GC–MS-QP 2010 SE (Electron Ionization type) gas chromatograph-mass spectrometer (SHIMADZU, Japan) and a Rtx-2330 capillary column (30 m × 0.25 mm) (Restek, Guangzhou, China). Helium was used as the carrier gas. The injection temperature, ion temperature and interface temperature were set at 250 °C, 200 °C and 260 °C, respectively. The initial column temperature was set at 150 °C. The column temperature subsequently rose to 200 °C at 10 °C/min and then to 220 °C at 6 °C/min, followed by a hold at 220 °C for 10 min. The quantification of FAMEs was performed according to Liu et al. [17]. All measurements were done in triplicate.

2.4. Starch quantification

The starch content was measured in triplets by a modified method Davis et al. [24]. Aliquots of 50–100 mg lyophilized biomass were disrupted with mortar and pestle under liquid nitrogen condition, the pellet including starch and cell debris were collected and washed twice using 20 mM Tris/HCl before re-suspended in 4 mL of the same buffer. The sample was centrifuged and the supernatant was discharged. The glucose and maltodextrins in the collected pellet were removed by incubating in 85 °C for 5 min with 80% ethanol solution. The mixed content was centrifuged for 10 min at 12,000 *g* to collect the pellet, after which, 2 mL DMSO was added to the pellet and boiled for 5 min to digest the resistant starch. Thermostable a-amylase (Sigma) was added to the mixture and boiled again to catalyze the hydrolysis of starch at pH 6.9. After cooling down, amyloglucosidase preparation (Sigma) was added to the mixture and incubated in a 60 °C shaking water bath for 15 min at pH 4.8. The mixture was finally centrifuged at 4000 *g* for 15 min, and the starch content of each sample was measured equivalents to the glucose that was released to the supernatant.

2.5. Biomass productivity, energy fixation of DW, enhancement effect of light and biomass yield on glucose

The biomass productivity was defined as the DW increase in 1 L and 1 day under different cultivation modes. The energy fixation of DW was defined as the total energy that fixed from both light and glucose under different cultivation modes. The enhancement effect of light was defined as the ratio between light triggered the excess energy fixation under mixotrophic cultivation (energy fixation of mixotrophy–energy fixation of heterotrophy) and energy fixation under photoautotrophic