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Double CO_2 fixation in photosynthesis–fermentation model enhances algal lipid synthesis for biodiesel production

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ABSTRACT

In this study, a photosynthesis–fermentation model was proposed to merge the positive aspects of autotrophs and heterotrophs. Microalga *Chlorella protothecoides* was grown autotrophically for CO_2 fixation and then metabolized heterotrophically for oil accumulation. Compared to typical heterotrophic metabolism, 69% higher lipid yield on glucose was achieved at the fermentation stage in the photosynthesis–fermentation model. An elementary flux mode study suggested that the enzyme Rubisco-catalyzed CO_2 re-fixation, enhancing carbon efficiency from sugar to oil. This result may explain the higher lipid yield. In this new model, 61.5% less CO_2 was released compared with typical heterotrophic metabolism. Immunoblotting and activity assay further showed that Rubisco functioned in sugar-bleaching cells at the fermentation stage. Overall, the photosynthesis–fermentation model with double CO_2 fixation in both photosynthesis and fermentation stages, enhances carbon conversion ratio of sugar to oil and thus provides an efficient approach for the production of algal lipid.

1. Introduction

Lipids including animal fats and plant oils are the main feedstock for biofuel (biodiesel) production. Animals and most microorganisms are heterotrophs. They are able to efficiently synthesize a compact storage of energy-fat whilst releasing a certain amount of CO_2. Plants, including algae, are autotrophs and they function with bulky storage of energy-starch. Oils (lipids) are generally formed in plant seeds and constitute a very small amount of the whole plant. However, absorbance of CO_2 is one of the main advantages of autotrophy, benefiting both the environment and the economy via biomass production.

The study of algae-for-fuel has become a hot topic in recent years with energy prices fluctuating widely and green house gas emissions increasingly becoming a cause for concern (Gouveia and Oliveira, 2009; Jorquera et al., 2009; Pruvost et al., 2009; Yoo et al., 2009). Microalgae are regarded as a good source of biofuel (especially biodiesel) that has the potential to completely displace fossil fuels because of its rapid biomass production, high photosynthetic efficiency and, in some species such as *Botryococcus*, high lipid content (Haag, 2007). However, cultivation of autotrophic microalgae for biodiesel production still faces some technical challenges. For example, in a photosynthesis growth model (PM), rapidly growing cells contain lower amounts of lipids (<20% of dry weight), whereas algal cells accumulating high lipid contents (40–50% of dry weight) exhibit little growth. Heterotrophic fermentation of *Chlorella protothecoides* provides an alternative way to solve these problems (Miao and Wu, 2004, 2006; Xu et al., 2006). The cell density and lipid content achieved in a 5-L bioreactor was up to 51.2 g/L and 50.3% of dry cell weight (DCW) (Xiong et al., 2008). However, the fermentation growth model (FM) consumes organic carbon (sugar or starch) and is associated with more CO_2 release than the PM.

To develop an integrated strategy for cost-effective and environmentally-friendly production of microalgal biofuels, we adopted a photosynthesis–fermentation model (PFM) for algal cultivation. This model involves the photosynthetic growth of *C. protothecoides* to increase biomass and subsequent heterotrophic fermentation to maximize cell density and lipid accumulation. In the PFM, not only was CO_2 used for biomass production in the photosynthesis stage, but lipid biosynthesis was also enhanced in the fermentation stage compared with the FM. A theoretical analysis suggested that the CO_2 re-fixation in fermentation stage resulted in enhancing lipid synthesis. This conclusion has been supported by further experimental data, confirming that the PFM is a novel approach for more efficient biodiesel production from microalgae.

2. Methods

2.1. Cell strains and culture medium

Microalga *C. protothecoides* strain 0710 originally obtained from the Culture Collection of Alga at the University of Texas (Austin,

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Texas, USA) was screened in the Microalgal Fermentation and Bioenergy Laboratory at Tsinghua University, Beijing, China. Basic media composition was: KH_2PO_4 0.7 g/L, K_2HPO_4 0.3 g/L, $MgSO_4{\cdot}7H_2O$ 0.3 g/L, $FeSO_4{\cdot}7H_2O$ 3 mg/L, glycine 0.1 g/L, vitamin B1 0.01 mg/L, A5 trace mineral solution 1 mL/L. Glycine (5 g/L) was added to phototrophic culture broth as a nitrogen source; 45 g/L glucose was used as an organic carbon source for heterotrophic growth and glycine, limited up to 2 g/L, was used as a nitrogen source for lipid biosynthesis.

2.2. Culture conditions for the three models

2.2.1. PM

Plate grown green *C. protothecoides* was used to inoculate 500 mL media and exposed to light at 100 μmol/(m^2 s). The incubation temperature was 28 °C. Aseptic air was supplied through a pipeline located centrally at the bottom of the column. Log-phase growth was then transferred to a new tube or photo-bioreactor (Infors, Switzerland) at a 1:100 (V/V) dilution. The parameters of the photo-bioreactor were set as: pH 6.5, gas mixture comprising air and CO_2 at a flow rate of 250–300 L/h with a CO_2 concentration of 2%.

2.2.2. FM

The heterotrophic culture was incubated at 28 °C in either shaking flasks at a rate of 200 rpm or a 5-L stirring tank (Infors, Switzerland). The flow rate of aseptic air and stirring speed in the fermenter were set as 240 L/h and 250 rpm, respectively. This allowed the dissolved oxygen (DO) value to be kept over 20% air saturation. pH of the medium was automatically controlled at 6.3 ± 0.1.

2.2.3. PFM

The green cells of *C. protothecoides* were obtained by growing the alga autotrophically as described above. At the end of log-phase growth, green algal cells were left to sediment overnight. The supernatant was then discarded and cell pellet was re-suspended in the defined heterotrophic medium. Subsequent procedures for heterotrophic incubation were as detailed above.

2.3. Analytical techniques

Cell growth was monitored by optical density measurements at 540 nm using a UV/Visible spectrophotometer (Pharmacia Biotech Ultrospec 2000). Samples were diluted to an appropriate concentration to keep the OD_{540} value between 0.2 and 0.8. At the end of every batch, wet algal cells were freeze-dried and weighed. The DCW corresponded to OD_{540} value by a regression equation: $y = 0.4155x$ ($R^2 = 0.9933$, $P < 0.05$), where y (g/L) is the DCW, x is the absorbance of the suspension at 540 nm. Glucose consumption was analyzed using an enzymatic bio-analyzer (SBA-40C, Shandong Academy of Sciences). The concentration of CO_2 in the inlet-gas/off-gas was determined with an online tandem gas analyzer (Milligan Instrument, UK). Chlorophyll content was determined from the absorbance of the methanol extracts at 666 nm (MacKinney, 1941). The composition of the lipid was analyzed by GC–MS according to our previous protocols (Xiong et al., 2008). The cellular lipid content was measured by gas chromatography: cells were harvested by centrifugation, washed twice with distilled water, then lyophilized. The resulting dry algal powder was suspended in a mixture of 2 mL methanol acidified with 3% sulfuric acid and 2 mL chloroform containing 2.5 g/L capric acid (the internal standard to correct transesterification and injection volume errors). The mixture was then heated in a sealed tube at 100 °C for 4 h. After cooling, 1 mL of distilled water was added and the sample was vortexed for 20 s. After separation of phases, 1 μL was injected into the gas chromatograph (HP 6890, USA), using a 0.32-mm diameter column, 30 m in length. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. Measurements started at 80 °C for 1.5 min, then the temperature was increased to 140 °C at a rate of 30 °C/min, it then reached 300 °C at a rate of 20 °C/min, and was kept stable for 1 min before the analysis was terminated. Retention times were 4.4 min for capric acid methyl ester, 7.4 min for hexadecanoic acid and 8.5 min for octadecanoic acid methyl ester.

2.4. Electron microscopy

Sample pretreatment for autotrophic and heterotrophic cells was performed by standard protocols (Glauert and Lewis, 1998). In short, the procedures included fixation, dehydration, embedding, sectioning and staining. Cells were observed and photographed using a JEM-123O transmission electron microscope (Hitachi, Japan).

2.5. Quantitation and activity assay of Rubisco

Immunoblotting was carried out to measure Rubisco in *C. protothecoides* during the phototrophic/heterotrophic process. Cells were disrupted using an ultrasonic cell pulverizer (JY92-2D, Xinzhi, Ningbo, China) for 40 min. After centrifugation at 15,000*g* for 10 min, proteins were extracted from the precipitates using the phenol/SDS method (Nagai et al., 2008). Protein powder was dissolved in the rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT) and protein concentration was determined by the method of Bradford (Bradford, 1976) with BSA as the standard. Protein (20 μg) was separated by 9% SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The protein blots were blocked with 5% skimmed milk in TBST buffer (20 mM Tris–HCl, pH 7.6, 0.8% NaCl, 0.05% Tween 20) and incubated for 1 h at room temperature with a rabbit antibody (provided by G. Chen, dilution 1:1000) against the tobacco holoenzyme of Rubisco. The hydroxyperoxidase-labelled goat anti-rabbit IgG (Bio-Rad, dilution 1:5000) was used as a secondary antibody. After autoradiographic detection, the quantitation of band intensities was analyzed using Image software, Gelpro4.

For activity assay, cells from 250 mL cultures were suspended in 10 mL extraction buffer (pH 7.8) that contained 100 mM Tris–HCl, 20 mM KCl, 1 mM EDTA and disrupted by ultrasonic cell pulverizer (JY92-2D, Xinzhi, Ningbo, China) for 40 min. The crude preparation was clarified by centrifugation at 15,000*g* for 10 min at 4 °C. Carboxylase activity of Rubisco was assayed by measuring [^{14}C]-3-phosphoglyceric acid. Briefly, 20 μL of crude preparation that contained 2 mg/mL protein were pre-incubated for 15 min in 460 mL assay buffer (100 mM Tris–HCl [pH 8.2], 20 mM $MgCl_2$, 1 mM DTT, 10 mM $NaH^{14}CO_3$ [0.2 μCi/μmol]) to fully activate the enzyme. Reactions were initiated by addition of 20 μL RuBP [10 mM, pH 6.5] and were terminated by the addition of 200 μL HCl (2 M). Radiolabel was determined by liquid scintillation counting to calculate activity of Rubisco.

2.6. Elementary flux mode analysis

The network of biochemical reactions in *C. protothecoides* cells was assembled based on specific genomic knowledge of *Chlorella* (http://genome.jgi-psf.org/ChlNC64A_1/ChlNC64A_1.info.html and http://genome.jgi-psf.org/Chlvu1/Chlvu1.home.html) and other photosynthetic microorganisms (Shastri and Morgan, 2005; Yang et al., 2000, 2002). For network construction and elementary modes computation, Cellnetanalyzer 9.1, a MATLAB package for structural and functional analysis of biochemical networks was utilized (Klamt et al., 2007).