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Production of biomass and lipid by the microalgae *Chlorella protothecoides* with heterotrophic-Cu(II) stressed (HCuS) coupling cultivation

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HIGHLIGHTS

- Heterotrophic-Cu(II) stressed coupling cultivation was developed for microalgae.
- Heterotrophic-Cu(II) stress achieved considerable lipid accumulation in cells.
- Glycolysis pathway was important contributor for lipid accumulation by proteomics.

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ABSTRACT

This work for the first time investigated lipid accumulation by a two-stage regime namely heterotrophic-Cu(II) stressed (HCuS) and underlying molecular basis of lipid biosynthesis in *Chlorella protothecoides* cells. The results showed that the optimized biomass and lipid yield were achieved by 6.47 g/L and 5.78 g/L with this strategy. The fatty acids compositions (almost 100% of them are C15 to C20) are ideal for preparing high quality biodiesel. Further, 30 differentially expressed proteins response to HCuS were involved in carbohydrate metabolism, carbon fixation, TCA cycle, lipid metabolism, protein biosynthesis, transportation and regulation, ATP and RNA biosynthesis, nucleotide metabolism, ROS scavenging. Especially, glycolysis pathway might be the important contributor for lipid accumulation. In future, further functional analysis of these altered proteins would help to reveal more concerning lipid biosynthesis pathway.

1. Introduction

As one of renewable, biodegradable, nontoxic bio-energy, biodiesel is becoming the focus in energy field (Chisti, 2007). The conventional biodiesel was derived from plants oil (i.e. soybean oil, rapeseed oil, palm oil, and corn oil), animal fat, and waste cooking oil (Mata et al., 2010). However, these biomasses could not meet the requirements of promising feedstock for biodiesel production. In this regard, microalgae, as one of the most promising feedstock, have received considerable attentions (Hill et al., 2006).

However, biodiesel production from microalgae is not economically feasible because rapid-growing cells contain low lipid, whereas the cells with high lipid content have slow growth rate (Lu et al., 2010). Recently, a novel regime-heterotrophic cultivation has been developed for high quality biodiesel production from microalgae. Compared to classical photoautotrophic culture, heterotrophic algal cells can reach higher biomass concentrations and accumulate moderate lipid contents (Fan et al., 2012). For example, the high lipid content (54.7%) in heterotrophic cultivation of *Chlorella protothecoides* was reported, but the glucose concentration used was relatively low (⩽10 g/L), thereby resulting in low biomass (3.74 g/L) and the low lipid yield of 2.05 g/L (Xu et al., 2006). While a high biomass of 15.8 g/L was published with 30 g/L glucose, the corresponding lipid content was only 26.5% and the final lipid yield was 4.19 g/L (Wei et al., 2009). Thus, although a high biomass or lipid content could be achieved in heterotrophic cultures by the foregoing achievements, the low lipid yield cannot meet the feedstock demands of large-scale biodiesel production.

To address this contradiction, the notion that a two-stage culture regime was initiated by many researchers (Xiong et al., 2010; Mujtaba et al., 2012; Li et al., 2013). This two-stage regime was to merge the positive aspects of high biomass condition (heterotrophic) and high lipid content conditions (environmental stresses) to enhance lipid productivity. Fan et al. (2012) have adopted a two-step culture model comprising sequential heterotrophy-dilution-photoinduction for high lipid accumulation by

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Chlorella vulgaris, *Chlorella pyrenoidosa* and *Chlorella ellipsoidea*, respectively. Li et al. (2013) have investigated a two-stage process for lipid accumulation by *C. protothecoides*: growth under heterotrophic condition and cultivation under N deprivation. Through the two-step culture systems, the cell biomass and lipid content can achieve considerable high levels.

It is worth noting that Cu(II) is an essential micronutrient required by all microalgae because it is the component of some key enzymes involved in metabolic pathway. In recent years, the function of copper in microalgae growth and intracellular valuable components accumulation has been noted by many investigators. In fact, the concentration of copper in the medium for an efficient algal growth varies depending on particular species of microalgae. For example, *Stichococcus minor* accumulated lower amounts of copper (0.38 mM) than *Gymnopilus terricola* (4.20 mM). The low intracellular Cu-accumulation and maintenance of high GSH level concomitant with PCs production seem to be responsible for a higher Cu-resistance of *S. minor* than *G. terricola* (Kalinowska and Pawlik-Skowronska, 2010). *Rhizopus arrhizus* showed high sensitivity to low Cu(II) ion induction (Uslu et al., 2003). *C. onubensis*, living in highly stressful conditions of copper, appears to be able to withstand higher concentrations of copper than other similar species (Vaquero et al., 2012). And an average biomass productivity of 0.42 g/L/d and the lutein content of 50% were obtained under 0.2 mM Cu(II) stress. However, many studies concerned the microalgae absorption to copper, few of them investigate the effects of Cu(II) concentrations on microalgal lipids production, which are regarded as one of the most promising feedstock for biodiesel production.

In this study, a two-stage culture regime namely heterotrophic-Cu(II) stressed (HCuS) coupling cultivation was developed for algal biomass and lipid production by *C. protothecoides*. Further, the underlying molecular basis of lipid accumulation in response to HCus was revealed by cell proteomic analysis. Using this strategy, 31.4 mg/L Cu(II) stress (approximate 0.49 mM) is a good choice for lipid accumulation by two-stage culture regime, with the lipid yield of 5.78 g/L. The fatty acids compositions from algal cells are ideal for preparing high quality biodiesel because almost 100% of the fatty acids are C15 to C20, similar to the carbon chain length of ordinary diesel fuel. And the glycolysis pathway might be the important contributor for intracellular lipid accumulation. This study is the first to report on lipid accumulation capacity and mechanism in algal cells with HCuS, which might provide new clues for revealing and improving the lipid metabolic pathways.

2. Methods

2.1. Algae culture

C. protothecoides used in this study was obtained from the CSIRO Marine Laboratory (Hobart, Australia). The basal culture medium (BCM) was consisted of glucose 10 g, urea 3 g, KH_2PO_4 1.25 g, $MgSO_4{\cdot}7H_2O$ 1 g, EDTA 0.5 g, boron 114.2 mg, $CaCl_2$ 83.75 mg, $FeSO_4{\cdot}7H_2O$ 49.8 mg, $ZnSO_4{\cdot}7H_2O$ 88.2 mg, $MnCl_2{\cdot}4H_2O$ 14.4 mg, MoO_3 7.1 mg, $CoNO_3{\cdot}6H_2O$ 4.9 mg, $CuSO_4{\cdot}5H_2O$ 15.7 mg in per liter distilled water.

The HCuS cultivation process was carried out by two-steps: (1) firstly, the algal cells were grown in sterilized BCM in flasks for 168 h to reach the late logarithmic phase; (2) secondly, the cultures were transferred into sterilized centrifugal bottles in biological safety cabinet. The cultures were taken from biological safety cabinet and then centrifuged at 3000 rpm for 10 min. The stratified cultures were carried out with alcohol disinfection and transferred again into the biological safety cabinet to remove the supernatant. Subsequently, the fresh sterilized Cu(II)-BCM (C-BCM) containing the same components as BCM, except for 0, 15.7, 31.4, 47.1 and 62.8 mg/L $CuSO_4{\cdot}5H_2O$, were added to the collected algal pellets for induction in intelligent incubator. The cultures were taken at 12, 24, 48, 72, and 96 h in the second stage, respectively, to monitor the variations of the biomass and total lipid content caused by Cu(II) induction. All cultures were incubated in 250 mL Erlenmeyer flask containing 100 mL broth in an incubator shaker with rotation speed of 160 rpm at 28 °C.

2.2. Biochemical analysis of HCuS coupling cultivation cells

2.2.1. Measurement of algal biomass

The cultured cells were collected by centrifugation at 3000 rpm for 10 min and the algal pellets were then washed three times with distilled water. Finally, the algal cells were dried at 105 °C to constant weight and the biomass was determined by the cell dry weight method.

2.2.2. Determination of total lipid content

The dried cell biomass was blended with 0.5 mL distilled water and 3 mL chloroform/methanol (2:1, v/v). The mixtures were shaken for 20 min using a mixer and then centrifuged at 10,000 rpm for 10 min to collect the chloroform phases. The above process was repeated five times. All the chloroform phases were evaporated and dried to constant weight under vacuum conditions. Finally, the extracted total lipid yield weight was determined by the weighing method with an electronic scale.

2.2.3. Fatty acid compositions analysis

The 20 mg microalgal power was blended with 1 mL of NaOH–CH_3OH solution and the mixture was saponified in a thermostat water bath for 10 min at 75 °C. Afterwards, 2 mL of boron trifluoride-methanol (1:2, v/v) was introduced to the cooled saponified solution, the mixture was then shaken again for 10 min at 75 °C in a thermostat water bath. The phase containing the fatty acids was obtained by using 0.3 mL of saturated salt solution and 2 mL of hexane. Finally, the upper-layer fatty acid was subjected to GC–MS analysis.

2.2.4. Measurement of starch content

The starch content was measured according to anthrone–sulfuric acid colorimetric assay (Roe, 1954).

2.3. Proteomics analysis of HCuS coupling cultivation algal cells

2.3.1. Protein extraction and purification

The cultured cells were collected and washed with ice-cold phosphate-buffered saline buffer, and then directly disrupted in 1 mL lysis buffer (7 M urea, 2% CHAPS, 2 M thiourea, and 20 mM Tris; pH 8.8) containing 1 mM PMSF protease inhibitors, 50 μg/ml RNase, and 200 μg/ml DNase. The lysis mixture was subjected to ultrasonic treatment using a sonicator (Scientz-ΠD, Ningbo Biotechnology Co., Ltd., China). The supernatant was collected by centrifugation at 18,000*g* for 20 min at 4 °C. According to the manufacture' instructions, the protein samples were cleaned up and quantified with Clean-up kit and 2D Quant Kit (Bio-Rad, America).

2.3.2. Two-dimensional gel electrophoresis and 2-DE image analysis

For the first dimensional isoelectric focusing (IEF), 120 μg protein was directly applied onto IPG strips (24 cm, pH 4–7, linear), and rehydrated in a solution (2 M Thiourea, 40% w/v CHAPS, 20 mM w/v DTT, 0.5% v/v IPG buffer pH 4–7, 0.002% w/v bromophenol blue and 8 M urea) at 17 °C and 50 V for 12 h. After rehydration, isoelectric focusing was conducted by stepwise increase of the voltage as follows: voltage was held at 300 V for 30 min;