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Salt stress induced lipid accumulation in heterotrophic culture cells of *Chlorella protothecoides*: Mechanisms based on the multi-level analysis of oxidative response, key enzyme activity and biochemical alteration

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ABSTRACT

Salt stress as an effective stress factor that could improve the lipid content and lipid yield of glucose in the heterotrophic culture cells of *Chlorella protothecoides* was demonstrated in this study. The highest lipid content of 41.2% and lipid yield of 185.8 mg/g were obtained when *C. protothecoides* was stressed under 30 g/L NaCl condition at its late logarithmic growth phase. Moreover, the effects of salt and osmotic stress on lipid accumulation were comparatively analyzed, and it was found that the effects of NaCl and KCl stress had no significant differences at the same osmolarity level of 1150 mOsm/kg with lipid contents of 41.7 and 40.8% as well as lipid yields of 192.9 and 186.8 mg/g, respectively, whereas these results were obviously higher than those obtained under the *iso*-osmotic glycerol and sorbitol stresses. Furthermore, basing on the multi-level analysis of oxidative response, key enzyme activity and biochemical alteration, the superior performance of salt stress driving lipid over-synthesis was probably ascribed to the more ROS production as a result of additional ion effect besides the osmotic effect, subsequently mediating the alteration from carbohydrate storage to lipid accumulation in signal transduction process of *C. protothecoides*.

1. Introduction

It is believed that climate change is currently the most pressing global environmental problem and another issue is the energy crisis, in which the world suffers from lack of energy security due to depletion of the finite fossil fuel resources (Ahmad et al., 2011). Producing energy from renewable biomass can simultaneously solve these two problems by reducing CO_2 emissions and decreasing the use of fossil fuels (Ahmad et al., 2011; Chisti, 2007; Hu et al., 2008). Recently, microalgae as a potential source of biodiesel which is a potential renewable and carbon neutral alternative to petroleum fuels attracts many attentions due to its high photosynthesis efficiency, high lipid content and the additional ability of waste water treatment.

Lipids are usually accumulated in the microalgae cells under unfavorable environmental or stress conditions by altering their lipid biosynthetic pathways toward the neutral lipids formation (Courchesne et al., 2009; Hu et al., 2008). The stress conditions, however, are disadvantageous for the cell growth resulting in a low lipid productivity. Aiming at high lipid productivity, using organic carbon as carbon source in the heterotrophic culture of microalgae is promising because this strategy can not only significantly increase the algal growth rate but also facilitate the lipid productivity and efficiency for cost-effective lipid production (Bumbak et al., 2011; Kosa and Ragauskas, 2011; Perez-Garcia et al., 2011). Additionally, the massive organic carbon sources originating from the hydrolysate of crop straw, sugar-rich crops and industrial by-products can satisfy the heterotrophic culture of microalgae for the low cost biodiesel production (Gao et al., 2010; Lu et al., 2010; Yan et al., 2011).

Chlorella protothecoides is a microalgae that can grow photoautotrophically or heterotrophically under different culture conditions. The concerns on heterotrophic growth of *C. protothecoides* resulting in high production of biomass and high lipid

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content in cells have grown increasingly (Bumbak et al., 2011; Perez-Garcia et al., 2011). Xiong et al. (2008) performed high density fermentation of *C. protothecoides* with the cell density of 51.2 g/L and lipid content of 50.3% in a 5 L bioreactor using fed-batch and nitrogen starvation strategy. Besides the nitrogen starvation, a couple of strategies were investigated in order to promote the lipid accumulation in the heterotrophic culture cells of microalgae (Fan et al., 2012; Li et al., 2013b; Zheng et al., 2013). The salt stress, especially the salinity, can effectively induce the lipid accumulation in many autotrophic species of microalgae including freshwater and marine algae (Campenni et al., 2013; Ho et al., 2014; Takagi et al., 2006; Xia et al., 2014). However, the data on the effect of salt stress on the heterotrophic culture cells of *C. protothecoides* for the high lipid production has scarcely been reported. Hence, the heterotrophic culture of *C. protothecoides* coupling with the salt stress in the interest of high lipid content and yield was investigated in this study.

It has been confirmed that instant responses of plants and microalgae to salt stress involve excess production of reactive oxygen species (ROS) (Munns and Tester, 2008; Rasool et al., 2013). Recent researches indicated that ROS was not only associated with cell damage, but also played important roles in regulating the expression of many genes and signal transduction pathways (Apel and Hirt, 2004; Foreman et al., 2003; Rasool et al., 2013). On the other hand, algal cells are able to change the physiological status including antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) as a response to the oxidative stress. The biochemical compositions of the cell would also be altered to improve the adaptation and tolerance to salt stress (Kumar et al., 2010; Parida and Das, 2005).

The present study focused on the lipid synthesis, oxidative responses, alterations of cellular compositions and key enzymes activities in the metabolic pathways when *C. protothecoides* was heterotrophically cultured coupling with the salt stress for high lipid content and high lipid yield production. Furthermore, the effects of salt stress and osmotic stress were comparatively analyzed in order to further elucidate the biological mechanisms on lipid overproduction in the heterotrophic culture process of *C. protothecoides* under stress conditions.

2. Material and methods

2.1. Algal species, medium and culture conditions

C. protothecoides was kindly provided by Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences. *C. protothecoides* was maintained in a 250 mL Erlenmeyer shaking flask containing 100 mL BG-11 medium (Ge et al., 2011) under continuous light radiation (approximately 50 μmol photons/m^2/s) in a rotary shaker (125 rpm, 25 °C). For heterotrophic seeds culture, single colony of *C. protothecoides* obtained by the serial dilution plating technique was inoculated in a 500 mL Erlenmeyer shaking flask containing 200 mL heterotrophic medium without illumination in a rotary shaker (200 rpm, 30 °C). The optimized medium of the heterotrophic culture contained glucose 45.0 g/L, $NaNO_3$ 5.0 g/L, $K_2HPO_4{\cdot}3H_2O$ 1.0 g/L and $MgSO_4{\cdot}7H_2O$ 0.3 g/L, the remaining compositions were the same with the BG-11 medium. Both the autotrophic and heterotrophic media were autoclaved for 30 min at 115 °C.

In the stress experiments, 20 mL heterotrophic inoculum was inoculated in a 500 mL Erlenmeyer shaking flask containing 200 mL heterotrophic medium and cultured in a rotary shaker (200 rpm, 30 °C). When cell growth entered into late logarithmic phase (97 h), culture was treated with salt or osmotic stresses. In NaCl stress groups, concentrations of 10, 20, 30, 40 and 50 g/L in the culture were carried out. In the comparative study on salt and osmotic stresses, 30 g/L NaCl was the reference concentration. Different dosages of sterile KCl, glycerol or sorbitol saturated solution were added to the culture making the osmolaritiy equal to that of treatment with 30 g/L NaCl stress (1150 mOsm/kg). All experiments were conducted in three independent biological replicates.

2.2. Analytical methods

Cells of *C. protothecoides* were harvested by centrifugation, washed twice with distilled water, then the precipitate was dried at 105 °C till the cell weight was constant, thus dry cell weight (DCW) was obtained. Glucose concentration was analyzed using an enzymatic bio-analyzer (SBA-40C, Shan dong Academy of Sciences, China). The osmolarity of the culture was determined by the auto freezing point osmometer (FM-8P, Shanghai Medical University Instrument Factory, China). The total protein content of cells was determined using the Kjeldahl nitrogen determination method, as described by the national standard of the People's Republic of China (GB/T5009.5-2003). The carbohydrate content was analyzed based on anthrone-sulfuric acid colorimetric method (Roe, 1955). The chlorophyll content was determined using a modified method described by Lichtenthaler and Wellburn (1983), and 80% (w/w) acetone was used as extraction solution, then chlorophyll content was calculated by Eq. (1):

$$\text{Total chlorophyll (aandb)(mg/L)} = 20.2 \times A_{645} + 8.02 \times A_{663} \quad (1)$$

where A_{645} and A_{663} are the optical absorbance at 645 and 663 nm determined with a spectrophotometer (Unico 7200, Shanghai, China).

Cellular lipid content and fatty acids profile in *C. protothecoides* were determined by GC–MS after direct transesterification. Briefly, the microalgae powder was suspended in 2 mL 0.4 M KOH-methanol solution, the mixture was then heated at 70 °C for 30 min in a water bath. After cooling, 2 mL 0.6 M H_2SO_4-methanol solution and 1 mL 14% BF_3-methanol solution (Sigma-Aldrich, USA) were added, and then the mixture was heated in water bath at 70 °C for 30 min again. After cooling, the fatty acid methyl esters (FAMEs) were extracted with 2 mL *n*-hexane, vortexing, then centrifuged at 4000 rpm for 5 min. The *n*-hexane layer was transferred to a vial. The prepared sample was analyzed by GC–MS (Agilent Technologies 7890C GC System and 5975C inert MSD with Triple-Axis Detecter, Santa Clara, CA, USA) with a fused silica capillary column HP-Innowax (Thickness 0.25 μm, I.D. 0.25 mm, Length 30 m, Agilent Technologies, USA). The injection volume was 1 μL at a split ratio of 10:1. The initial oven temperature was 160 °C held for 2 min and then increased by 5 °C/min to 230 °C and held for 2 min. The temperatures of the injector port and the FID-detector were set at 250 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 1.0 mL/min. Quantitation of individual FAME was accomplished by incorporating a known amount of internal standard (400 mg/L), methyl nonadecanoate (C19:0) (Sigma-Aldrich, USA). Peaks were identified by comparing retention times and ion fragments information to those of reference standards (Sigma-Aldrich, USA). Cellular lipid content was expressed as total FAMEs percentage of dry cell weight.

2.3. Measurements of intracellular ROS, MDA and enzyme activity

2′,7′-Dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, USA) was used to detect intracellular ROS level (Cash et al., 2007). DCFH-DA was added to culture samples with the final concentration of 10 mM and incubated at 30 °C for 30 min in the dark, then the samples were washed with potassium phosphate buffer (pH 7.0) by centrifugation at 10000 rpm for 5 min and re-suspended with