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Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp. under nitrogen depletion

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HIGHLIGHTS

- Above 3% biomass phosphorus content was attained under high P condition.
- Excess P reduced starch synthesis via inhibition of AGPase under N-depletion.
- Lipid synthesis was stimulated by high P uptake under N-depletion.
- P can regulate carbon allocation between starch and lipid synthesis pathway.

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ABSTRACT

The aim of this research was to study the effect of phosphorus supply on starch and lipid production under nitrogen starvation using *Chlorella* sp. as a model. High phosphate level had marginal effect on cell density but increased biomass growth. Massive phosphorus was assimilated quickly and mainly stored in the form of polyphosphate. The algal cells ceased phosphorus uptake when intracellular phosphorus reached a certain level. 5 mM phosphate in the culture rendered a 16.7% decrease of starch synthesis and a 22.4% increase of lipid synthesis relative to low phosphate (0.17 mM). It is plausible that phosphate can regulate carbon partitioning between starch and lipid synthesis pathway by influencing ADP-glucose pyrophosphorylase activity. Moreover, high phosphate concentration enhanced the abundance of oleic acid, improving oil quality for biodiesel production. It is a promising cultivation strategy by integration of phosphorus removal from wastewater with biodiesel production for this alga.

1. Introduction

Sustainable biofuels, especially biodiesel, have attracted much attention in the past several years. Microalgae have emerged as a promising feedstock for biodiesel production, due to several advantages, such as high photosynthetic efficiency, rapid growth rate, and high lipid content (Chisti, 2007; Hu et al., 2008). Basically, algal cell growth and lipid accumulation are susceptible to environmental conditions, such as nutrient availability, temperature and irradiance. Among these factors, nitrogen (N) deficiency is one of the most commonly adopted strategies to enhance lipid accumulation (Damiani et al., 2014; Mujtaba et al., 2012). N-starvation can significantly increase lipid content in microalgae, but decrease algal growth rate, resulting in low lipid productivity (Zhu et al., 2014a). In spite of this, N-deficiency conditions are very vital to induce neutral lipid, mainly in the form of triacylglycerides (TAGs), which is a more suitable substrate for biodiesel production (Zhu et al., 2015). Therefore, lipid accumulation mechanisms and strategies required to be further explored in order to improve algal lipid productivity under N-deficient conditions.

Phosphorus (P) is another important nutrient for both algal growth and metabolism. It is an essential element for cellular constituents such as phospholipids, nucleotides and nucleic acids. Moreover, it exerts a significant role in many cellular processes, especially those involved in energy transfer and signal transduction (Raghothama, 2000). Though P-deficiency could also induce lipid accumulation in many microalgae, it was less efficient than N-deficiency due to relatively low biomass and lipid content (Feng et al., 2012; Li et al., 2014). Furthermore, co-deficiency of N and P led to much lower lipid productivity because the algal growth was severely inhibited (Chu et al., 2013; Li et al., 2014). That is to say P-deficiency is not a highly efficient strategy to enhance lipid production. On the other hand, many microalgae can uptake much more phosphorus than required for survival under unfavorable growth conditions and usually stored these

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phosphorus in the form of polyphosphate (Poly-P) in cells (Brown and Shilton, 2014). Recently, some researchers demonstrated that luxury uptake of phosphorus motivated lipid accumulation in several microalgae under N-deficiency (Chu et al., 2014; Li et al., 2014). However, how sufficient phosphorus supply regulates lipid synthesis in microalgae is still less understood.

Most researchers exploring the effect of phosphorus on lipid production usually ignore another role that orthophosphate (Pi) is an inhibitor of ADP-glucose pyrophosphorylase (AGPase), which catalyzes the first committed step of starch synthesis in the plastid, by converting glucose 1-phosphate and ATP to ADP-glucose and pyrophosphate (PPi) (Ball and Morell, 2003). That is to say Pi supply can affect starch synthesis in photosynthetic organisms. For example, Li et al. (2011) reported a 39.2% and 45% decrease of AGPase activity and starch content, respectively, in *Pseudochlorococcus* sp. with the addition of 1 mM Pi under high light and N-limitation. As major carbon and energy storage compounds, starch and lipid synthesis share common carbon precursors from Calvin cycle (Li et al., 2010). Thus there may be interaction between these two pathways, regulating photosynthetic carbon partitioning into starch and lipid synthesis pathway. Our previous study demonstrated that N-deficiency triggered both starch and lipid biosynthesis in *Chlorella* sp., but starch synthesis occurred prior to lipid synthesis (Zhu et al., 2014a). Understanding biosynthetic pathways and carbon allocation in algal cells could be significant for cultivation strategies and biofuels development. So far, less attention has been paid to the regulation of excess phosphorus supply on both starch and lipid accumulation under N-deficiency.

In this study, *Chlorella* sp. was used as a model to elucidate the influence of luxury uptake of phosphorus on starch and lipid production in microalgae under N-deficiency. Specifically, three different phosphate levels were adopted in the culture. Algal growth, nutrient assimilation, AGPase activity, and starch/lipid production were monitored. Poly-P granules were observed by confocal laser scanning microscopy. Kinetics of starch and lipid synthesis and their productivities were also analyzed. The results may provide further insight into the relations among phosphorus assimilation, starch and lipid accumulation as well as cultivation strategies for biodiesel production.

2. Methods

2.1. Strain and culture conditions

Chlorella sp. was obtained from the Microalgae Culture Collection in Guangzhou Institute of Energy Conversion (Guangzhou, China) and maintained in BG11 medium. The algal cultivation device was designed as described in a previous study (Zhu et al., 2014a). In order to investigate the effect of phosphorus on starch and lipid production in *Chlorella* sp. under N-depletion, cells were initially grown to the logarithmic phase, and then collected by centrifugation (4000 rpm × 5 min). Subsequently, these pre-cultured cells were inoculated into N-depleted BG11 medium containing different Pi concentrations. The Pi concentrations in the culture were 0.17, 5 and 10 mM, respectively. It should be noted that 0.17 mM is the regular phosphorus concentration in BG11 medium. The initial biomass concentration was approximately 210 mg L^{-1}. Each experiment was performed for 10 days with three duplicates.

2.2. Growth measurement

Cell numbers were counted using a hemocytometer after appropriate dilution under a light microscope (CX31, Olympus). The cell dry weight (dw) was measured by filtering culture through pre-weighed Whatman GF/C filters. Then the filter paper was dried in oven at 80 °C until constant weight. The specific growth rate (μ) and biomass productivity were calculated as described in a previous study (Zhu et al., 2014b).

2.3. Phosphorus analysis

The intracellular total phosphorus was measured according to Chu et al. (2014). Briefly, aliquots of lyophilized algal biomass were resuspended in deionized water and then digested using 5% potassium persulfate solution. After all types of phosphorus converted into orthophosphate, the measurement of PO_4^{3-}-P was conducted using a water quality autoanalyzer (DR 2700, Hach) according to the manufacturer's instructions.

2.4. Observation of Poly-P

Poly-P granules were observed via 4,6-diamidino-2-phenylindole (DAPI) staining. The cells were fixed with 4% paraformaldehyde in PBS for 15 min, and then washed three times with PBS. Afterwards, cells were stained with DAPI (50 μg mL^{-1} final concentration; Sigma) and incubated for 30 min in the dark. The stained cells were mounted on a slide and observed with a confocal laser scanning microscope (LSM 700, Carl Zeiss) with a plan apo 63×/1.4 oil objective lens. Fluorescence was excited using a 405 nm laser and emission was collected using a 530 nm long pass emission filter for Poly-P detection.

2.5. Starch analyses

Starch was determined by the method described by Branyikova et al. (2011), based on the total hydrolysis of starch by 30% perchloric acid and quantification of liberated glucose by colorimetry. The details are given in a previous study (Zhu et al., 2014a).

2.6. AGPase activity measurement

AGPase activity was determined by a modified method described by Nakamura et al. (1989). Cells were disrupted in liquid nitrogen and 2 mL of the extraction buffer (100 mM HEPES-NaOH (pH 7.4), 8 mM $MgCl_2$, 2 mM EDTA, 50 mM β-mercaptoethanol, 12.5% (v/v) glycerol and 5% (w/v) insoluble polyvinylpolypyrrolidone-40) was added. The extract was centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was retained for AGPase assay.

AGPase assay was conducted in 100 mM HEPES-NaOH (pH 7.4), 1.2 mM ADP-glucose, 3 mM sodium pyrophosphate, 5 mM $MgCl_2$, 4 mM DTT, and enzyme extract in a reaction mixture of 650 μL. After 20 min, the reaction was terminated by heating the mixture in boiling water for 2 min. The resulting solution was centrifuged at 10,000*g* for 10 min. A portion (500 μL) of the supernatant was mixed with 15 μL of 10 mM $NADP^+$. The activity was recorded as the increase in absorbance at 340 nm after addition of 1 μL each of phosphoglucomutase (0.4 U) and G-6-P dehydrogenase (0.35 U). A unit of AGPase activity was defined as the amount of the enzyme required to generate 1 μmol of NADPH per min (Nakamura et al., 1989).

Protein content was determined spectrophotometrically at 595 nm using the Bradford Protein Assay (Sangon, China), with bovine serum as a standard.

2.7. Lipid analyses

For lipid analysis, fatty acid methyl esters were prepared by incubating lyophilized biomass in methanol containing 2% (v/v) H_2SO_4 at 80 °C for 2.5 h. Heptadecanoic acid (C17:0, Sigma) was added as an internal standard. Fatty acid methyl esters were