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Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency

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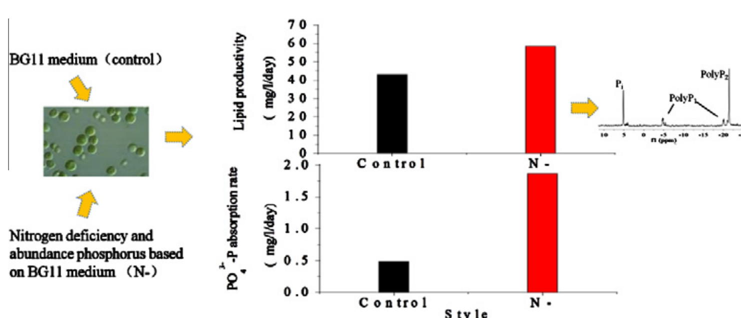
HIGHLIGHTS

- P is important to enhance algal lipid productivity under nitrogen deficiency (N−).
- Lipid productivity under N− with sufficient P supply is highest as 58.39 mg/L/day.
- P uptake rate under N− is 3.8 times faster than that of sufficient N&P supply.
- ^{31}P NMR shows that uptake P is accumulated as polyphosphate in algal cells.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate the role of phosphorus in lipid production under nitrogen starvation conditions, five types of media possessing different nitrogen and phosphorus concentrations or their combination were prepared to culture *Chlorella vulgaris*. It was found that biomass production under nitrogen deficient condition with sufficient phosphorus supply was similar to that of the control (with sufficient nutrition), resulting in a maximum lipid productivity of 58.39 mg/L/day. Meanwhile, ^{31}P NMR showed that phosphorus in the medium was transformed and accumulated as polyphosphate in cells. The uptake rate of phosphorus in cells was 3.8 times higher than the uptake rate of the control. This study demonstrates that phosphorus plays an important role in lipid production of *C. vulgaris* under nitrogen deficient conditions and implies a potential to combine phosphorus removal from wastewater with biodiesel production via microalgae.

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1. Introduction

As one of the outstanding alternative sources of renewable energy, biodiesel has attracted growing attention from researchers worldwide (Hongyang et al., 2011; Hu et al., 2008; Wijffels and Barbosa, 2010). In this context, biodiesel production from microalgae is considered an important future direction. Microalgae, especially some green algal species, grow faster than many traditional oil crops and can be cultivated in non-arable areas without competing with crop plants for land. For these reasons, some researchers even regard microalgal biodiesel as potentially the only form of renewable energy for the future that can replace fossil oils while fully meeting the demand for global transport energy (Chisti, 2007; Demirbas and Demirbas, 2011). Unfortunately, no commercial-scale production of biodiesel production from microalgae has been achieved yet (Demirbas and Demirbas, 2011; Tang et al., 2011). One main obstacle is the high investment required for maintaining optimized growth conditions for microalgae in open ponds or photo-bioreactors (Rodolfi et al., 2009).

In order to reduce the cost of biodiesel production from microalgae, it is important to increase the biomass yield and/or the lipid content of algal cells. Many strategies have previously been

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employed, such as nitrogen starvation, high iron strength, intensive illumination, mixotrophic cultivation, etc. (Liu et al., 2008; Wang et al., 2012; Yeesang and Cheirsilp, 2011). Among these, nitrogen starvation is one of the most commonly adopted strategies. Under nitrogen-deficient conditions, many microalgae change their lipid metabolic pathways to accumulate neutral lipids, mainly in the form of triacylglycerides (TAGs) which are the carbon and energy storage in the cytoplasm of microalgal cell (Hu et al., 2008). Spoehr and Milner (1949) discovered that *Chlorella pyrenoidosa* accumulated a lipid content of up to 85.6% in nitrogen starved culture, as compared with 4.5% in a nitrogen sufficient culture. However, while a limitation of nitrogen can induce high lipid content, nitrogen limitation has also been found to lower biomass productivity. As a consequence, the overall lipid productivity of microalgae under nitrogen deficient conditions was actually lower than that under normal growth conditions (Griffiths and Harrison, 2009; Widjaja et al., 2009; Wijffels and Barbosa, 2010).

Phosphorus is another essential ingredient in the process of photosynthesis, and an important nutrient for algal growth. Use of microalgae to remove nutrient in water and produce biomass was studied as well (Hu et al., 2012; Wang et al., 2012). However, it was found that some microalgae could assimilate more phosphorus than required for growth under nutritional conditions unfavorable for growth. Further investigations using ^{31}P NMR analysis demonstrated that assimilated phosphorus was in the form of polyphosphate (Poly-P) (Harold, 1966). The phenomena of luxury phosphorus uptake have also been found in algae of natural ecosystems such as eutrophic lakes and rivers (Schelske and Sicko-Goad, 1990). Therefore, in order to enhance phosphorus removal, more studies have been undertaken focusing on Poly-P metabolism in algae and these studies found that environmental factors such as light, osmotic shocks, nutrient availability all affected phosphorus accumulation (Eixler et al., 2006). For example, Kuesel et al. (1989) reported that *Chlorella fusca* accumulated Poly-P as energy storage under nitrogen starvation. However, to our knowledge, no attention has so far been paid to the relationship between luxury phosphorus uptake and lipid production under nitrogen starvation condition in microalgae.

The objective of this research was to investigate the role of phosphorus in microalgal biodiesel production under nitrogen deficient conditions. *Chlorella vulgaris*, which was considered to be a promising candidate for the production of biodiesel (Griffiths and Harrison, 2009), was cultivated under nitrogen starved conditions with different phosphate concentrations. The relationship between phosphorus and lipid productivity was then investigated. The results may suggest a better combination of phosphorus cremoval with biodiesel production via microalgae.

2. Methods

2.1. Strains and culture conditions

C. vulgaris was obtained from Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB) in China. The algae were cultured in BG11 medium with the following compositions: 1500 mg/L $NaNO_3$, 40 mg/L $K_2HPO_4{\cdot}3H_2O$, 75 mg/L $MgSO_4{\cdot}7H_2O$, 20 mg/L Na_2CO_3, 27 mg/L $CaCl_2$, 6 mg/L citric acid monohydrate, 6 mg/L ammonium ferric citrate, 1 mg/L Na_2EDTA, with 1 mL trace metal solution (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2{\cdot}4H_2O$, 0.222 mg/L $ZnSO_4{\cdot}7H_2O$, 0.079 mg/L $CuSO_4{\cdot}5H_2O$, 0.050 mg/L $CoCl_2{\cdot}6H_2O$, 0.39 mg/L $Na_2MoO_4{\cdot}2H_2O$). All the culture medium used was autoclaved at 121 °C for 30 min.

C. vulgaris was cultivated in 2000 mL flasks plugged with perforated rubber stoppers. A glass tube was connected through the stopper and extended close to the flask bottom for gas supply. Three Philips straight fluorescent tubes (40 W/tube) were placed 6 cm away from the flasks. Illumination intensity was about 6000 lx with photoperiodicity 16:8 (light for 16 h and dark for 8 h). The temperature was maintained at 25 ± 2 °C by air conditioners. The gases were sterilized by 0.20 μm PTFE gas filter diaphragm Midisart-2000 (SRP65, Sartorius, Germany) and provided at a rate of 0.5 v/v/min with 4% CO_2 as carbon source. The solution pH was 6–8.

2.2. Experimental setup

To investigate the effects of nutrients especially nitrogen and phosphorus deficiency on algal biomass and oil productivity, five types of media were used in this study, which included: nitrogen deficient (N−), phosphorus deficient (P−), both nitrogen and phosphorus deficient (N−&P−), nitrogen deficient and phosphorus limited (N−&P−lim), and control with sufficient nutrients. Here, "nitrogen or phosphorus deficient" means no external N or P was provided, while "limited" means nitrogen or phosphorus was provided but at a level lower than what is required for normal growth.

After 6-d cultivation in BG11 medium, *C. vulgaris* biomass were recovered by centrifugation and resuspended in BG11 medium without N and P. The suspension was then reinoculated at 1:8 (v/v) into the five types of medium. The initial biomass concentrations were about 560 mg/L. The concentrations of nitrogen (NO_3^--N) in the medium of control and P− experiments were both about 200 mg/L. The concentrations of phosphorus (PO_4^{3-}-P) in the medium of control and N− experiments were about 35 mg/L. Moreover, a small quantity of phosphorus was added every few days to induce a phosphorus limited condition for the growth of *C. vulgaris*. The experiments for each type of medium lasted for 14 days and with three duplicates. Liquid and solid samples were taken every day for analysis.

2.3. Analysis

2.3.1. Growth parameters

The dry weight of algae biomass was measured by the method of suspended solid determination using a 0.45 μm filter membrane in accordance with Li et al. (2010). The filtrate obtained from biomass determination was then used to measure NO_3^--N, PO_4^{3-}-P. Certain volume of microalgal suspension was recovered for the measurement of total nitrogen (TN) and total phosphorus (TP) after high pressure dissolution. All the measurements were made according to the Chinese state standard monitoring methods (Monitoring Methods for Water and Wastewater, 2002).

2.3.2. Lipid extraction and measurement

The total lipid content was analyzed gravimetrically using a method adapted from Bligh and Dyer (Bligh and Dyer, 1959). An aliquot (100 mL) of the sample was collected and the algal pellets were then dried using a vacuum freeze dryer (HETO power Dry Pl3000, USA). Extraction reagent (chloroform: methanol 2:1 v/v) (2.25 mL) was added to lyophilized algal powder (20 mg). The samples were then placed on a shaker and kept in the dark for 14 h, and then centrifuged. The supernatant was collected in tubes. Additional extraction reagent of 1.5 mL was added for the second extraction, after shaking for 2 h. The samples were centrifuged and the supernatant was again collected. Methanol and 0.1% NaCl solution were then added to achieve a methanol:chloroform:water ratio of 1:1:0.9. The samples were vortexed for 30 s and centrifuged to form two layers. The bottom layer consisted of chloroform and lipid, while the upper layer comprised methanol, water and cell debris. The upper layer was removed with a pipette. The chloroform layer was transferred into a constant weight tube which was deposited into a pressurized gas blowing concentrator (HSC-24A, Tianjin Henao Corp. China) until all of the chloroform evaporated. Then,

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