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The boosted biomass and lipid accumulation in *Chlorella vulgaris* by supplementation of synthetic phytohormone analogs

Tingting Liu, Fei Liu, Chao Wang, Zhenyao Wang, Yuqin Li *

Biological Engineering Department, School of Chemical Engineering, Xiangtan University, Xiangtan, China

HIGHLIGHTS

- Synthetic hormone analog NAA was tested to boost algal biomass and lipid accumulation.
- NAA performed remarkable promoting effect on cell growth and lipid biosynthesis.
- NAA modified proportions of fatty acids which were prone to high-quality biofuels.
- NAA-treatment manipulated endogenous phytohormones metabolism.
- Economic-estimation of NAA indicated possibility in developing lipid for biofuels.

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ABSTRACT

This study attempted at maximizing biomass and lipid accumulation in *Chlorella vulgaris* by supplementation of natural abscisic acid (ABA) or synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) hormone analogs. Amongst three tested additives, NAA-treatment performed remarkable promoting effect on cell growth and lipid biosynthesis. The favorable lipid productivity (418.6 mg/L/d) of NAA-treated cells showed 1.48 and 2.24 times more than that of 2,4-D and ABA. NAA-treatment also positively modified the proportions of saturated (C16:0 and C18:0) and monounsaturated fatty acids (C18:1) which were prone to high-quality biofuels-making. Further, NAA-treatment manipulated endogenous phytohormones metabolism leading to the elevated levels of indole-3-acetic acid, jasmonic acid, and salicylic acid and such hormone accumulation might be indispensable for signal transduction in regulating cell growth and lipid biosynthesis in microalgae. In addition, the economic-feasibility and eco-friendly estimation of NAA additive indicated the higher possibilities in developing affordable and scalable microalgal lipids for biofuels.

1. Introduction

As part of endeavors to tackle the energy crisis and global warming, oleaginous microalgae were increasingly being recognized as renewable and environment-friendly alternative to fossil energy. This interest toward microalgae is principally due to its fast growth, non-arable cultivation, and potential to modify metabolism pathway for a higher lipid production (Chisti, 2007). However, a series of challenges have to be conquered before commercial application of microalgae-based biodiesel, one of which is how to effectively improve the lipid productivity.

The versatile chemical and physical stressors such as nitrogen-deficiency, high-salinity, and high irradiance have been verified to effectively stimulate lipid biosynthesis in microalgae (Cheng et al., 2016). However, even large promotions in lipid content will not pay off lipid productivity because cell growth is severely impeded under adverse environment conditions. Subsequently, the ample evidence indicated that manipulation of algal growth media could also achieve improvements in lipid biosynthesis (Jusoh et al., 2015a). In this regard, some organic carbon sources (e.g., sweet sorghum juice, corn powder and cassava starch hydrolysates), nitrogen (e.g. urea and amino acid), and other nutrients ingredients (e.g. phosphorus and iron) have been well-documented to exert positive effects on lipid productivity (Salama et al., 2014). However, these achieved lipid productivities was still being a big gap with the theoretical value proposed by Hu et al. (2008). Therefore, the cost-effective and biologically-acceptable cultivation additives for microalgae lipids production were needed to be further explored.

* Corresponding author at: School of Chemical Engineering, Xiangtan University, Xiangtan 411105, China.
E-mail address: yuqinli2004@126.com (Y. Li).

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Hormones in microalgae presented new opportunities in developing microalgal lipids for biodiesel production (Lu and Xu, 2015). However, the manipulation of hormones metabolism in microalgae is still in its infancy as well as the concentration of hormones present in microalgae is considerably lower than in plants (Sulochana and Arumugam, 2016). Therefore, considering the evolution relationship between microalgae and plants, the majority of scientific literatures focused on exogenous application of hormones for microalgal lipids. For instance, Cho et al. (2015) reported an increment in lipid productivity of *D. salina* by supplement of myo-inositol; the exogenous applications of diethyl aminoethyl hexanoate boosted the cell growth and fatty acids content of *Scenedesmus obliquus* (Salama et al., 2014). Indole-3-acetic acid, phenylacetic acid, indole-3-butyric acid, and abscisic acid have also been highlighted as potentially valuable culture supplements for microalgae to promote cell growth and lipid metabolite biosynthesis (Piotrowska and Bajguz, 2014; Bajguz and Piotrowska, 2014). However, the precise mechanisms of lipid accumulation by exogenous application of phytohormones remained elusive. The existing evidences suggested that the exogenous phytohormones could effectively regulated fatty acid biosynthesis genes and manipulated endogenous phytohormones metabolism, which might be directly or indirectly related with lipid biosynthesis (Stirk et al., 2014; Jusoh et al., 2015a,b).

Although phytohormones were typically active to regulate various aspects of microalgae growth and lipid accumulation at very low concentrations, the cost impact of natural phytohormones application for microalgae cultivation must be considered from a commercial standpoint before implementation of mass production (Salama et al., 2014). Consequently, exploring economic and high-efficient natural phytohormones alternatives for large-scale lipid production is of increasing interest. Numerous studies suggested that the synthesized hormones analogs (e.g. 2,4-Dichlorophenoxyacetic acid) induce similar physiological responses as natural phytohormones in bioassays (Szechyńska et al., 2007; Piotrowska-Niczyporuk et al., 2012). However, little is known about the roles of exogenous application of synthetic hormones analogs for biomass and lipid accumulation in microalgae. This study aimed to probe the feasibility of using synthetic hormone analogs (NAA) for lipid production by microalga *Chlorella vulgaris*. The biomass, lipid content, fatty acid compositions, endogenous phytohormones, and fatty acid biosynthesis genes would be employed to identify biochemical characteristics that lead to the optimal lipid productivity of *C. vulgaris*. The economic-feasibility analysis and eco-friendly estimation of using synthetic NAA for mass production of microalgae was also evaluated.

2. Materials and methods

2.1. Materials and chemicals

The green *Chlorella vulgaris* was purchased from the Culture Collection of Algae at University of Texas at Austin (UTEX), USA. The natural indole-3-acetic acid, jasmonic acid, and salicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthetic 2,4-Dichlorophenoxyacetic acid and 1-Naphthaleneacetic acid were supplied by Hefei Bo Mei Biotechnology. CO., Ltd., (Anhui province). GF-1 Total RNA Extraction Kit (Vivantis, USA) was purchased from Solarbio Biotechnology. CO., Ltd., (Shanghai). All other chemicals were of the highest purity available.

2.2. Microalgae cultivation

The pre-culture of *Chlorella vulgaris* was initiated from a single colony taken from the stock agar plate and cultured in the improved liquid Basal medium as described in Mu et al. (2015). Subsequently, 5 mL of the pre-cultured liquid cells was inoculated into 250-mL flask containing 100 mL Basal media and cultured on a reciprocating shaker (160 rpm/min) at 28 °C and light intensity of 100 μmol/m^2/s. The natural abscisic acid (ABA), synthetic 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthaleneacetic acid (NAA) were incorporated into Basal medium from the beginning of culture at different concentrations: 0.5, 1.0, 1.5, and 2.5 mg/L. All treatments carried three biological duplicates. The algal cells cultured with Basal medium (without hormones) was treated as control group. The cultivation conditions (temperature, light intensity, and agitation) of the control cells were consistent with hormones-treated cells. The biomass and lipid content were measured at 12 ~ 24 h interval.

2.3. Analytical methods

2.3.1. Biomass measurement

The algal cultures were collected by centrifugation at 4000 rpm for 20 min. The supernatant was carefully removed and pellet washed twice with di-ionized water. Subsequently, the pellet was dried in an oven at 50 °C for 12 ~ 24 h until constant weights were achieved.

2.3.2. Total lipid content

Lyophilized algae powder was mixed with distilled water and chloroform/methanol (2:1, v/v). The mixtures were shaken for 15 min using a vortex mixing apparatus and then centrifuged at 5000 rpm for 15 min. All the chloroform phases were collected and evaporated to constant weight by nitrogen gas. The total lipid contents were determined by biomass dry weight.

2.3.3. Fatty acids compositions

The fatty acid methyl esters (FAMEs) profile of *C. vulgaris* was measured by transesterification. The algae powder was performed saponification at 75 °C in a thermostated water bath for 20 min by introducing NaOH-CH_3OH solution. The boron trifluoride-methanol (1:2, V/V) was then added to the saponified samples and shaken for 30 min. The esterified oil samples were mixed with hexane and the upper-layer FAMEs profile was characterized by an Agilent 6890 gas chromatograph (Agilent Technologies, USA) fitted with a HP-88 capillary column (0.25 mm inner diameter × 30 cm length) and a flame ionization detector. 2 μL of FAMEs solubilized in chloroform were injected at chromatograph by splitless mode using helium at 1.5 mL/min as carrier gas. The ion source and quadruple temperatures were 230 °C and 150 °C respectively. And the flame ionization detector (FID) temperature was 270 °C. The initial oven temperature was set at 130 °C for 1 min, increased to 200 °C at a rate of 5 °C/min, and then held at this temperature for 7 min. Detection was done using full scan mode between 35 and 500 m/s and EMV mode gain factor 5 and identification was performed using the NIST08.LIB mass spectral database.

2.3.4. Fatty acids biosynthesis gene expression analysis

Total RNA of *C. vulgaris* cells was extracted with GF-1 Total RNA Extraction Kit according to the manufacturer's protocol. 100 mg of algal cells treated with hormone analogs was grinded in liquid nitrogen to fine powder before adding 400 μL Buffer TR and centrifuged at 8000 rpm for 3 min. The clear lysate was then centrifuged to collect the flow-through. 350 μL of 80% ethanol was subsequently added to the flow-through before transferring the mixture into a RNA binding column and centrifuged at 8000 rpm for 1 min. The final RNA was treated with DNase I to remove contaminating DNA and then performed reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's instructions. The generated cDNA was directly used