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A chemical approach to manipulate the algal growth, lipid content and high-value alpha-linolenic acid for biodiesel production

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ABSTRACT

Microalgal cultivation systems for energy production are not yet economically viable due to the low yield of lipids and biomass. In this study, we propose a chemical approach, using exogenous bioactive molecules for improving algal growth, lipid content and the production of high-value alpha-linolenic acid (ALA), using *Chlorella protothecoides* as a model. Based on our findings, among the cytokinin and auxin classes, Kinetin increased the biosynthesis of ALA by 26.5% at 1 ppm concentration, and indole-3-butyric acid at 2 ppm improved the biomass and lipid productivity up to 29.15 ± 2.02 g m^{-2} day^{-1}, and 6.69 ± 0.56 g m^{-2} day^{-1}, respectively. Methyl jasmonate as a stress-related phytohormone also showed a positive stimulatory effect on growth of *C. protothecoides*, and the biomass productivity was increased up to 25.26 ± 1.48 g m^{-2} day^{-1} at the concentration of 5 ppm (lipid content: 219.66 ± 13.20 mg g^{-1} dw; lipid productivity: 5.61 ± 0.25 g m^{-2} day^{-1}; ALA: 0.81 ± 0.16 g m^{-2} day^{-1}). A difference in the composition of fatty acids, due to the type and concentrations of stress-related phytohormones was observed, and under the influence of stress-related phytohormones, the biosynthesis of saturated fatty acids increased, and polyunsaturated fatty acids decreased in *C. protothecoides*. Butylated hydroxyanisole, at a concentration of 0.05 ppm, increased the ALA content by 60.5%. Our study shows that the proposed bioactive molecules could increase the biomass productivity of *C. protothecoides* at a lower cost compared to the use of acetate as a carbon source.

1. Introduction

Microalgae have been identified as an attractive source for production of feedstock, biofuels and different types of high-value compounds. During the past decades, the prospective applications of microalgae in the sectors of pharmaceuticals, cosmetics, food, and bioenergy have been widely studied [1,2]. Nevertheless, the potential of microalgae as a source of bioenergy is not as yet economically feasible, due to low yields of biomass and lipids. Accordingly, researchers recommend more investment in research and development, with precise policies and plans, including metabolic and genetic engineering methods to tap into this resource [3,4].

Microalgae have simpler structures than plants, and the simplicity of their genetic manipulation is very attractive. However, genetic manipulation should be closely monitored, in the case of a potential threat to the ecosystem. As such, it may be banned for outdoor cultivation [5]. Alternatively, researchers have proposed chemical approaches, which are flexible, easy to use, and eco-friendly strategies to improve the low biomass and lipid yield of microalgae. These approaches directly influence the microalgae and modify the function of the protein in real time, without disrupting the gene function, as occurs with genetic manipulation. The chemical approach can be effectively applied to increase the biosynthesis of microalgal lipids as well as high-value products, such as alpha-linolenic acid (ALA), which may offer a gateway for biodiesel production [6–8]. Furthermore, this approach can be applied to decrease the amount of undesirable polyunsaturated fatty acids (PUFAs) from feedstock without affecting the lipid productivity of the microalgal system for biodiesel production. Franz et al. [8] reported that these bioactive molecules were effective when tested in large volumes of cultures, and in the first proof of the concept from this approach an increase up to 84% in lipid content of 4 nM forskolin was reported. Similarly, the biomass, total lipid and ALA content of three *Chlorella* species increased by up to 126, 80 and 60%, respectively by the use of exogenous acetylcholine (ACh), its precursor and analogs [9]. Park et al. [10] reported that the growth of *Chlamydomonas reinhardtii* cultured in a nitrogen-limited medium was successfully increased by up to 54–69% using indole-3-acetic acid (IAA) gibberellic acid (GA), kinetin (Kin), 1-triacontanol, and abscisic acid (ABA). Also, Mekhalfi

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et al. [11] reported that bioactive molecules, such as IAA and diethyl amino ethyl hexanoate are effective to enhance (3.5–5%) the biosynthesis of the triacylglycerols in the freshwater diatom *Asterionella formosa*. The exogenous GA and methyl jasmonate (MeJA) bioactive molecules were also used by Lu et al. [12] to induce the biosynthesis of astaxanthin (ASX) in *Haematococcus pluvialis*. The mode of action of GA and MeJA was their regulatory mechanism on the expression of the bkts gene, which catalyzes β-carotene to canthaxanthin in the ASX biosynthetic pathway. Jiang et al. [13] proposed the use of diethyl aminoethyl hexanoate (DAH) for stimulation of growth, fatty acids profile and amino acids composition of *Chlorella ellipsoidea* SDEC-11 and *Scenedesmus quadricauda* SDEC-13. At a very low concentration (10^{-7} M), DAH improved the lipid productivity of the algae strains up to 39 and 33 mg L^{-1} day^{-1}, respectively. It also altered the fatty acids profile, so that the quality of the microalgal biodiesel was guaranteed to meet EN 14214 standard.

Although the reports on the increases in microalgal biomass, lipid productivity, and high-value compounds via bioactive molecules demonstrate a sustainable, scalable and economical production pathway, the calculation of input costs of using bioactive molecules is undoubtedly vital for building a cost-effective microalgal system at a commercial-scale [14]. An example is a proposed optimized microalga system using ACh and its analogs (dosage from 1 to 200 μg L^{-1}) at the cost of 0.2 to 17.2 USD per 10 mg of compound, which in return generates a net increase in ALA yield by 140 mg. The profit of the system per liter reaches 34 USD (ALA) at the cost of 0.02 USD for the bioactive molecules [9].

Chlorella protothecoides (Chlorellaceae) is identified as a facultative heterotrophic green alga inhabiting different environments. *C. protothecoides* is recognized as one of the potent algal species for biodiesel production, since it can grow on different carbon sources (i.e., glucose, glycerol, and acetate) and can synthesize high levels of biomass (up to 14.47 g L^{-1}), and lipid (up to 55.2% of dry weight). Indeed the considerable amount of high-value ALA (up to 23–30% of fatty acids profile) synthesized by *C. protothecoides* could be further separate as a driving force for scale up biodiesel production [9,15,16].

In this study, we investigated the functions of two classes of bioactive molecules at different concentrations of biomass, lipid productivity and fatty acids composition of *C. protothecoides*. Also, we determined the ideal concentrations of each molecule essential for improving the biomass productivity and lipid content of *C. protothecoides*. Finally, we explored the cost-effectiveness of an optimized algal production system for each of the selected bioactive molecules.

2. Materials and methods

2.1. Microalga cultivation and maintenance

C. protothecoides was purchased from UTEX (The Culture Collection of Algae at the University of Texas at Austin), and was selected as a standard microalga for the study. The microalga was grown in 100 mL of tris-acetate-phosphate (TAP) medium in 250-mL Erlenmeyer flasks with an arranged inoculation density on OD_{680} = 0.045. Cultures were illuminated by white fluorescent light (4800 lx) under a light/dark cycle of 14/10 h. The temperature was adjusted to 27 °C, and the growth of *C. protothecoides* was screened by optical density (OD_{680}) using a spectrophotometer device (UV-2600 Shimadzu). The volumetric production (g L^{-1}), total lipid content (mg g^{-1} dw), lipid productivity (g m^{-2} day^{-1}) and ALA content (g m^{-2} day^{-1}) were analyzed three days after the stationary phase (day 10, OD_{680} = 2.74 ± 0.2).

The biomass productivity was reported based on a real production (g m^{-2} day^{-1}) and calculated using the following formula, introduced by Yadala and Cremaschi [17]

$$PrA = (PrV \times V)/SA$$

where PrA is the areal production (g m^{-2} day^{-1}), PrV is the biomass volumetric productivity (g m^{-3} day^{-1}), SA is the surface area of the culture (m^2), and V is the volume.

2.2. Bioactive compounds used

Two types of synthetic and natural bioactive molecules, belonging to the phytohormones and antioxidants classes, were used in the study as follows:

(1) Phytohormones, including 6-Benzylaminopurine (BAP), kinetin (Kin), Indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), gibberellic acid (GA), methyl jasmonate (MeJA), salicylic acid (SA), and ethephon (Eth).
(2) Antioxidants, including catechin (Cath), vitamin C (Vit C), propyl gallate (PG), and butylated hydroxyanisole (BHA). The concentrations of the bioactive molecules were used based on our unpublished previous experiments as follows:

(1). 0, 0.5, 1.5, and 2 (ppm) for BAP, Kin, 2,4-D, NAA, and IBA. (2). 0, 2.5, 5, 10, and 15 (ppm) for GA, MeJA, SA, and Eth. (3). 0, 0.5, 1, 2, 4 and 8 (ppm) for vitamin C. (4). 0.05, 0.1, 0.5, 1.5, 5 (ppm) for BHA. (5). 0, 0.5, 0.1, 0.5, and 3 for Cath. (6). 0, 4, 10, 20, 60, and 100 (ppm) for PG. All the bioactive molecules were supplied by Sigma-Aldrich and were used at the initial phase of algal growth. Cath and Vit C were dissolved in water, BAP, Kin, IBA, and NAA were dissolved in NaOH, and 2,4-D, GA, MeJA, SA, Eth, PG, and BHA were dissolved in ethanol.

2.3. Lipid extraction and transesterification

The collected biomass from the stationary phase was placed in 50 mL centrifuge tubes following the addition of 4 mL distilled water and 5 mL hydrochloric acid (HCl). Afterward, the samples were heated for 20 min at 70 °C in a water bath, and 5 mL of ethanol was added. The samples were then allowed to cool down to room temperature. Ten mL diethyl ether was added to each sample, and then they were shaken and centrifuged (4500 rpm) for 2 min. The ether layer was collected and gathered into a round flask; this process was repeated three times. Finally, the total lipid content (mg g^{-1} dw) was obtained by evaporation of the ether via a rotary evaporator.

At the transesterification stage, the obtained lipid was dissolved in chloroform and transferred into a 1.5 mL glass vial. Subsequently, 1 mL of 1 M sulfuric acid-methanol was added to the samples, which were maintained for 1 h at a temperature of 100 °C. After that, the samples were cooled naturally. Finally, 500 μL distilled water was added and mixed by shaking for 2 min. Subsequently, the samples were extracted for gas chromatography (GC) analysis.

2.4. Fatty acid profiling analysis

A GC device consisting of a FID detector (Agilent 7890) and a DB-WAX column (30 m × 0.32 mm × 0.50 μm) was used to screen the fatty acid content and composition of the samples. Methyl undecanoate (C11:0, Purity = 99%) was used as an internal standard. The analysis program was set as follows: the temperature program included three phases (1) the temperature was raised from 50 °C to 150 °C at a rate of 10 °C per min and kept for 2 min. (2) The temperature was increased to 200 °C from 150 °C at a rate of 10 °C per min and held for 6 min. (3) The temperature was increased to 230 °C from 200 °C at a rate of 10 °C per min and held for 5 min. Carrier gas (N2) velocity: 3 mL per min. Detector: hydrogen flame detector, the velocity of H2 was 30 mL per min, and the velocity of air was 300 mL per min. The detector temperature and injector temperature were adjusted to 300 and 280 °C, respectively.