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Photosynthetic light reactions increase total lipid accumulation in carbon-supplemented batch cultures of *Chlorella vulgaris*

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HIGHLIGHTS

- *C. vulgaris* generates equivalent biomasses in hetero/photomixotrophic batch cultures.
- Light enhances lipid production in *C. vulgaris* batch cultures.
- Differences in lipid to biomass ratio are a result of photosynthetic light reactions.

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ABSTRACT

Microalgae are an attractive biofuel feedstock because of their high lipid to biomass ratios, lipid compositions that are suitable for biodiesel production, and the ability to grow on varied carbon sources. While algae can grow autotrophically, supplying an exogenous carbon source can increase growth rates and allow heterotrophic growth in the absence of light. Time course analyses of dextrose-supplemented *Chlorella vulgaris* batch cultures demonstrate that light availability directly influences growth rate, chlorophyll production, and total lipid accumulation. Parallel photomixotrophic and heterotrophic cultures grown to stationary phase reached the same amount of biomass, but total lipid content was higher for algae grown in the presence of light (an average of 1.90 mg/mL vs. 0.77 mg/mL over 5 days of stationary phase growth).

1. Introduction

The nonrenewable nature of fossil fuels necessitates the development of renewable and sustainable energy sources. Biodiesel is a promising petroleum alternative because it is compatible with current infrastructure and can be produced from a variety of feedstocks including vegetable oils, animal fats, seed oils, or other lipid-rich biomasses (Mata et al., 2010; Ho et al., 2014). Unfortunately, many of these sources of biodiesel have significant limitations. Much like the corn grain feedstocks used for ethanol production, oil seed crops compete for agricultural land, require fertilizers (often petrochemical-based) and have low oil yields relative to total biomass (Searchinger et al., 2008). Similarly, the use of animal fats or recycled vegetable oils is volume limited as they are produced as industrial byproducts; however, they could provide a dependable source for a smaller portion of the energy profile (Rittman, 2008). A biomass source that has a high lipid-to-biomass ratio, is scalable, and does not compete with food crops is microalgae (Dismukes et al., 2008; Chisti, 2008). Many of these unicellular organisms can produce large quantities of lipids that are easily extracted and suitable for biodiesel production. In order to optimize algal lipid yield and composition, more information is needed about the factors that influence total lipid accumulation (Chisti, 2007). In addition to lipid production, algae may be grown in carbon- and nitrogen-rich wastewater, providing a green water treatment strategy (Feng et al., 2011; Cho et al., 2011), or used as a protein supplement for animal feeds (Ursu et al., 2014).

Chlorella vulgaris, the microalga of interest in this study, is capable of growing autotrophically, heterotrophically, or photomixotrophically and can synthesize high levels of lipids per biomass (Heredia-Arroyo et al., 2011). During autotrophic growth, cellular energy is generated via the light reactions of photosynthesis and carbon demands are met by the fixation of carbon dioxide. The associated proton gradient drives adenosine triphosphate (ATP) production, while the resulting glyceraldehyde 3-phosphate

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(G3P) is incorporated into central metabolic pathways. Autotrophically grown batch cultures, however, take a significant amount of time for large biomasses to accumulate versus cultures that are continuously aerated or grown heterotrophically. This slower growth is likely caused by a lower amount of available carbon in the autotrophically grown batch cultures; a limitation caused by the rate of gas exchange at the media–air interface (Chen and Chen, 2002).

In heterotrophic batch cultures, growth is not limited by carbon availability because the algal cells fulfill energy and carbon demands through a supplied carbon source rather than the light reactions of photosynthesis coupled to the Calvin cycle. These exogenous carbon sources can range from simple saccharides to complex mixtures like orange peel extracts, waste water, or brewer fermentation waste (Park et al., 2014; Feng et al., 2014). In this study, heterotrophic cultures are defined as carbon-supplemented *C. vulgaris* cultures grown in the absence of light.

A third (photomixotrophic) growth mode is also possible for *C. vulgaris*, in which cellular energy is generated through the light reactions of photosynthesis and/or oxidation of an exogenous carbon source; carbon requirements are fulfilled via the incorporation of this exogenous carbon source and, potentially, any available carbon dioxide. In this study, dextrose was used as a carbon source for heterotrophic and photomixotrophic growth modes. Cells grown in dextrose-supplemented media accumulate biomass at higher rates than autotrophic cultures (Heridia-Arroyo et al., 2011), and this work investigates the influence of light on algal biomass composition under these carbon-supplemented conditions.

Industrial scale algal growth strategies frequently take advantage of heterotrophism to compensate for light limitation within large bioreactors or growth ponds. Provision of an extracellular carbon source facilitates continued cell growth and eliminates the need for carbon assimilation through the Calvin cycle, but it is unclear to what degree photosynthesis and lipid metabolism are coupled during heterotrophic or photomixotrophic growth modes. To investigate how the availability of light may influence lipid synthesis for downstream biodiesel production, batch cultures of *C. vulgaris* were grown heterotrophically and photomixotrophically. Analysis of total lipid levels in these heterotrophic and photomixotrophic cultures revealed that the absence of light during growth results in decreased total lipid levels but highly similar biomass accumulation and dextrose utilization, demonstrating that the light reactions of photosynthesis directly contribute to biomass composition. To the best of the authors' knowledge, this is the first study directly demonstrating that lipid production enhancement in photomixotrophically vs. hetrotrophically grown *C. vulgaris* is a result of these reactions.

2. Methods

2.1. Strains and culture growth conditions

In order to promote heterotrophic and photomixotrophic growth of *C. vulgaris*, batch cultures were grown with and without light. Flasks were covered with aluminum foil to establish the heterotrophic light conditions. The medium used was a dextrose-supplemented *Chlorella* medium (DSCM). The recipe was modified from Richmond (1986): KNO_3 (1.25 g/L), KH_2PO_4 (1.25 g/L), $MgSO_4 \cdot 7H_2O$ (1.00 g/L), $CaCl_2$ (0.084 g/L), H_3BO_3 (0.114 g/L), $FeSO_4 \cdot 7H_2O$ (0.040 g/L), $ZnSO_4 \cdot 7H_2O$ (0.088 g/L), $MnCl_2 \cdot 4H_2O$ (0.014 g/L), MoO_3 (0.007 g/L), $CuSO_4 \cdot 5H_2O$ (0.016 g/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.005 g/L), EDTA (0.50 g/L), dextrose (20.0 g/L). For cell growth, 150 mL flasks containing 75 mL of medium were inoculated with 115 μL of stationary phase culture (0.15% v/v). Triplicate heterotrophic and photomixotrophic cultures were grown in parallel at 25 °C on an orbital shaker with a rotational speed of 100 rpm. Photomixotrophic cultures were grown with continuous cool-white fluorescent light (3000 K) illumination at 8000 lx. Following inoculation, culture turbidity, chlorophyll *a* levels, dry weights, total lipids, and dextrose levels in cell-free media were measured every 24 h. Cultures were started from the same liquid starter culture and upon completion of growth curves, Luria broth (LB) plates were streaked from all cultures to test for contamination.

2.2. Spectroscopic cell density measurement

Cell density (turbidity) was obtained using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, JP). Dilutions with DSCM were used for samples with a turbidity of more than 1.0 (AU). Individual cultures were measured in triplicate by absorbance at 750 nm, which was selected to prevent interference by pigment absorptivity. Statistical correlation between turbidity and dry weight was shown to be linear for photomixotrophically grown cultures ($R^2 = 0.98$) and for heterotrophically grown cultures ($R^2 = 0.99$).

2.3. Chlorophyll a extraction

Chlorophyll *a* was extracted with dimethylsulfoxide (DMSO) and measured by UV–visible spectroscopy, similar to the method of Wellburn (1994). Cells were pelleted at 13,400 rpm for 5 min using an Eppendorf Minispin Centrifuge (Hamburg, GE) and resuspended in 1 mL DMSO. Following resuspension, cellular debris was pelleted (13,400 rpm, 5 min) and the absorbance spectrum of the supernate was measured from 600 to 700 nm. Chlorophyll *a* levels were measured in triplicate and concentrations (in μg/mL) were calculated using the following equation as described by Wellburn (1994):

$$[\mathrm{Chl}] = (12.47 \times \mathrm{Abs}_{665.1}) - (3.62 \times \mathrm{Abs}_{649.1})$$

2.4. Cell dry weight measurement

The cell density approximations given by the light scattering data provided growth curves for the cell cycles, but a second growth curve was established using cell dry weights using the method of Li et al. (2011) with slight modification. Prior to use, 1 μm, 25 mm GF/B Whatman glass microfibre filters (Whatman International Ltd., Maidstone, UK) were rinsed with 1 mL of deionized water and dried at 100 °C for 24 h. Pre-weighed filters were placed on a Buchner funnel, and 0.5 mL samples of cells were filtered in duplicate. Filtered cells were rinsed with 1 mL deionized water and allowed to air dry on the filter for 90 s. Filters and cells were transferred to an incubator and dried at 75 °C for 24 h. Following the drying period, filters were weighed on an analytical balance.

2.5. Lipid extraction and lipid dry weight measurement

Lipid extractions were performed using a modified methyl tert-butyl ether (MTBE) extraction similar to methods in Matyash et al. (2008). Cells (1 mL) were pelleted in a 15 mL glass tube by centrifugation in an International Clinical Centrifuge (International Equipment/Chicago Apparatus, Chicago, US) at 1000 rpm for 10 min. Pellets were rinsed by resuspension in 1 mL of deionized water, then were collected by centrifugation (1000 rpm, 10 min). Cells were vortexed in 1.5 mL of methanol for 1 min, followed by the addition of 5 mL of MTBE. Extraction mixtures were incubated at room temperature for 1 h, then 1.25 mL deionized water was added to induce phase separation. A 10 min incubation at 20 °C was followed by phase separation by centrifugation (1000 rpm,