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Extracellular terpenoid hydrocarbon extraction and quantitation from the green microalgae *Botryococcus braunii* var. Showa

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ABSTRACT

Mechanical fractionation and aqueous or aqueous/organic two-phase partition approaches were applied for extraction and separation of extracellular terpenoid hydrocarbons from *Botryococcus braunii* var. Showa. A direct spectrophotometric method was devised for the quantitation of botryococcene and associated carotenoid hydrocarbons extracted by this method. Separation of extracellular botryococcene hydrocarbons from the *Botryococcus* was achieved upon vortexing of the micro-colonies with glass beads, either in water followed by buoyant density equilibrium to separate hydrocarbons from biomass, or in the presence of heptane as a solvent, followed by aqueous/organic two-phase separation of the heptane-solubilized hydrocarbons (upper phase) from the biomass (lower aqueous phase). Spectral analysis of the upper heptane phase revealed the presence of two distinct compounds, one absorbing in the UV-C, attributed to botryococcene(s), the other in the blue region of the spectrum, attributed to a carotenoid. Specific extinction coefficients were developed for the absorbance of triterpenes at 190 nm ($\varepsilon = 90 \pm 5$ mM^{-1} cm^{-1}) and carotenoids at 450 nm ($\varepsilon = 165 \pm 5$ mM^{-1} cm^{-1}) in heptane. This enabled application of a direct spectrophotometric method for the quantitation of water- or heptane-extractable botryococcenes and carotenoids. *B. braunii* var. Showa constitutively accumulates ~30% of the dry biomass as extractable (extracellular) botryococcenes, and ~0.2% of the dry biomass in the form of a carotenoid. It was further demonstrated that heat-treatment of the *Botryococcus* biomass substantially accelerates the rate and yield of the extraction process. Advances in this work serve as foundation for a cyclic *Botryococcus* growth, non-toxic extraction of extracellular hydrocarbons, and return of the hydrocarbon-depleted biomass to growth conditions for further product generation.

1. Introduction

The genus *Botryococcus* encompasses a great variety of hydrocarbon-accumulating green microalgae. These are classified in three major races on the basis of the chemical structure of the hydrocarbons produced. Race A produces odd-numbered (C_{23}–C_{33}) *n*-alkadienes (mainly diene and triene hydrocarbons), race B produces triterpenoid hydrocarbons such as C_{30}–C_{37} botryococcenes and C_{31}–C_{34} methylated squalenes, whereas race L produce lycopadienes, which are single tetraterpenoids (Metzger and Largeau, 2005). The B-race comprises a group of micro-colony-forming green microalgae with individual cell sizes of about 10 μm in length. These microalgae synthesize long-chain terpenoid hydrocarbons via the plastidic DXP-MEP pathway (Lichtenthaler, 1999; Koppisch et al., 2000) and deposit them in the extracellular space, thus forming a hydrophobic matrix to which multiple individual cells adhere (Banerjee et al., 2002; Sato et al., 2003; Metzger and Largeau, 2005). Botryococcene hydrocarbons are modified triterpenes, having the chemical formula C_nH_{2n-10} (Banerjee et al., 2002). They could account for up to 30–40% of the dry cell biomass w/w (Metzger and Largeau, 2005). The high level of botryococcene hydrocarbons and the ability of these colonial microalgae to form blooms have raised the prospect of their commercial exploitation for the generation of synthetic chemistry and biofuel feedstocks (Casadevall et al., 1985). It was suggested that C_{30}–C_{37} botryococcenes and C_{31}–C_{34} methylated squalenes, which are also produced by *Botryococcus* B-race strains, could be converted via catalytic cracking into shorter-length fuel-type hydrocarbons, such as C_7H_n through $C_{11}H_m$ for gasoline, C_{12}–C_{15} for kerosene (jet fuel), or C_{16}–C_{18} for diesel, (Hillen et al., 1982). Interestingly, geochemical analysis of petroleum has shown that botryococcene- and methylated squalene-type hydrocarbons, presumably generated

Abbreviations: Btc, botryococcene; Car, carotenoid; ε, extinction coefficient, or specific absorbance; dcw, dry cell weight; wcw, wet cell weight; MW, molecular weight; Showa, *Botryococcus braunii* var. Showa (the Berkeley strain).

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by microalgae ancestral to *B. braunii*, may be the source of today's petroleum deposits (Moldowan and Seifert, 1980). Accordingly, botryococcene and methylated squalene hydrocarbons production by photosynthetic CO_2 fixation in microalgae could help provide a source of renewable fuel, mitigate emission of greenhouse gases in the atmosphere, and prevent climate change (Metzger and Largeau, 2005).

Colonies of *B. braunii* typically have amorphous three-dimensional structures, with a morphology resembling a "botryoid" organization of individual grape-seed-like, or pyriform-shaped cells, held together by a thick hydrocarbon matrix. The matrix surrounding individual cells forms an outer cell wall and the bulk of *B. braunii* hydrocarbons are stored in these extracellular containment structures (Largeau et al., 1980). Botryococcene hydrocarbons are initially sequestered in vesicles within the cells, where the biosynthesis and initial segregation of these molecules take place. Intracellular hydrocarbons are only a small fraction of the total micro-colony hydrocarbon content and they are more difficult to isolate compared to the extracellular matrix (Largeau et al., 1980; Wolf et al., 1985).

Hydrocarbon recovery from *Botryococcus* can be achieved by extraction of the dry biomass with solvents (Metzger and Largeau, 2005). However, dewatering and drying of the biomass is not a cost-effective method for industrial applications. Supercritical CO_2 extraction has also been employed and the extraction was found to be optimal at a pressure of 30 MPa (Mendes et al., 2003). Contact of the wet biomass with high molecular weight and high boiling point solvents was also reported to be an approach for hydrocarbon extraction (Frenz et al., 1989).

In the present work, a new protocol was applied for the extraction and spectrophotometric quantitation of *B. braunii* extracellular hydrocarbons. Vortexing of the *B. braunii* wet cell biomass with glass beads was employed to dislodge extracellular hydrocarbons from the tightly-packed micro-colonies. Aqueous density equilibrium or aqueous/heptane two-phase partition were employed to successfully separate these extractable hydrocarbons from the biomass. A novel spectrophotometric approach was devised, with suitable extinction coefficients that permit, for the first time, quantitative determination of the amount of botryococcenes and carotenoid extracted from *B. braunii* cultures.

2. Methods

2.1. Cell growth media, culture conditions, and biomass quantitation

Batch cultures of *B. braunii* var. Showa (Nonomura, 1988) were grown in the laboratory in 2 L conical Fernbach flasks. Cells were grown in 500 mL of modified Chu-13 medium (Largeau et al., 1980). Approximately 50 mL of a two-week old *B. braunii* var. Showa culture was used to inoculate new cultures. Cells were grown at 25 °C under continuous cool-white fluorescent illumination at an intensity of 50 μmol photons m^{-2} s^{-1} (PAR) upon orbital shaking at 60 rpm (Lab-Line Orbital Shaker No. 3590). Fernbach flasks were capped with Styrofoam stoppers, allowing for sufficient aeration, i.e., gas exchange between the culture and the outside space.

Growth of *B. braunii* was measured gravimetrically and expressed in terms of both wet cell weight (wcw, based on packed cell volume measurements) and dry cell weight (dcw) per volume of liquid culture (g L^{-1}). Cell weight analysis was carried out by filtering *B. braunii* cultures through Millipore Filter (8 μm pore size), followed by washing with distilled water. Excess filter moisture was removed by ventilation. Filters were weighed before and after drying at 80 °C for 24 h in a lab oven (Precision), and dry cell matter was measured gravimetrically. This analysis suggested a dcw/wcw ratio of about (0.225 ± 0.025):1 for *B. braunii* var. Showa micro-colonies.

2.2. Hydrocarbons extraction and separation

Cells were harvested from the liquid media by filtration (Millipore Filter 8 μm pore size). Approximately 1 g wet cell weight of *B. braunii* wet cake was mixed with 1 g of glass beads (0.5 mm diameter), and suspended upon addition of 10 mL heptane (HPLC Grade – Fischer Scientific). The cells-in-heptane suspension was vortexed for different periods of time, as indicated, at maximum vortexing speed (Fisher Vortex Genie-2). Following this vortexing, 10 mL of growth medium was added to the mixture, resulting in a prompt aqueous-heptane two-phase partition. The lower aqueous phase contained the green cells, whereas the top heptane phase contained the extracted hydrocarbons. The heptane layer was removed and collected for measurement of the absorbance spectra in a UV–visible spectrophotometer (Shimadzu UV 160U). Prior to spectrophotometric analysis, samples were diluted so that absorbance values at the peak wavelength did not exceed 0.5 absorbance units. The heptane solution of extractable Showa hydrocarbons was carefully collected and evaporated to dryness under a stream of air for hydrocarbon gravimetric quantitation.

2.3. Chlorophyll measurements

A known amount of culture pellet was mixed with equal weight of glass beads (0.5 mm diameter) and with a known volume of methanol. The glass bead-methanol-biomass mixture was vortexed until the color of the biomass became white, indicating full extraction of intracellular pigments. The crude extract was filtered and the absorbance of the green methanolic phase was measured at 470, 652.4 and 665.2 nm. Total carotenoid, chlorophyll ($a + b$) content, and the Chl a/Chl b ratio were determined according to Lichtenthaler and Buschmann (2001).

2.4. Reproducibility and statistical analyses

Reproducibility of results shown was confirmed with multiple and independent cultures. Statistical analysis of the results is based on a minimum of three independent measurements. Where indicated, measured values are expressed as a mean ± standard deviation (SD), $n = 3$.

3. Results and discussion

3.1. Determination of molecular extinction coefficients

The molecular extinction coefficients of squalene, botryococcene and β-carotene were first determined in heptane, as the solvent of these hydrophobic molecules. Heptane was selected as the solvent of choice both because it can remove lipophilic molecules from the growth medium without undue adverse effect on the cells (non-toxic), and also because it does not significantly absorb in the UV and blue regions of the spectrum, where hydrocarbons of interest absorb.

The UV–visible absorbance spectrum of squalene (ACROS Organics, 99% purity) in heptane showed a single absorbance band with a peak at about 190 nm (Fig. 1a). The dependence of this absorbance at 190 nm on the concentration of squalene in heptane was determined in order to obtain an extinction coefficient for this triterpene in this solvent. Absorbance values at 190 nm were measured across a concentration range of 0–6 μM squalene. The slope of the straight line in the measurement of absorbance versus squalene concentration (Fig. 1b, solid circles) defined the molecular extinction coefficient of squalene in heptane at 190 nm to be 90 ± 5 mM^{-1} cm^{-1}. This squalene extinction coefficient in heptane