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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

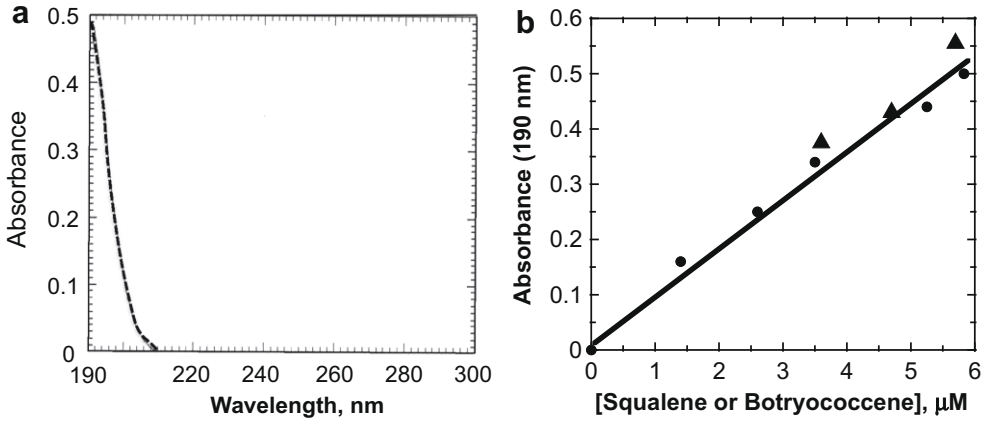

Fig. 1. (a) Absorbance spectrum of squalene in heptane. Note the single absorbance band in the 200–300 nm region, peaking at 190 nm. (b) (Solid circles) Absorbance values at 190 nm of squalene in heptane, plotted as a function of squalene concentration. The slope of the straight line defined the specific absorbance coefficient (extinction coefficient) of squalene in heptane at 190 nm, equal to 90 ± 5 mM^{-1} cm^{-1}. (Solid triangles) Absorbance at 190 nm of purified botryococcene in heptane, measured in three different samples and plotted as a function of botryococcene concentration. The latter was determined gravimetrically upon a subsequent evaporation of the heptane solvent and weighing of the residue.

was somewhat greater than that in acetonitrile, determined by Grieveson et al. (1997) to be 59 ± 2 mM^{-1} cm^{-1} at 195 nm.

Purified botryococcene extracts in heptane were also used in quantitative absorbance spectrophotometry. Absorbance of botryococcene solutions in heptane at 190 nm was measured in three different samples and plotted as a function of the botryococcene concentration (Fig. 1b, solid triangles). The latter was determined gravimetrically upon a subsequent evaporation of the heptane solvent and weighing of the residue in a suitable mg scale. The results suggested that squalene and botryococcene have the same A_{190} as a function of their concentration in heptane, hence the same extinction coefficient.

The UV–visible absorbance spectrum of β-carotene (MP Biomedicals) in heptane showed typical features of multiple carotenoid absorbance bands in the blue region (Fig. 2a). The major absorbance band occurred at 450 nm, with secondary peaks at 425 and 480 nm. The dependence of the absorbance at 450 nm on the concentration of β-carotene in heptane was determined in order to obtain the extinction coefficient for this carotenoid in such solvent. Absorbance values at 450 nm were measured across a concentration range of 0–6 μM β-carotene. The slope of the straight line in the measurement of the absorbance versus β-carotene concentration (Fig. 2b) defined the molecular extinction coefficient of β-carotene in heptane at 450 nm to be 165 ± 5 mM^{-1} cm^{-1}. This β-carotene extinction coefficient in heptane is consistent with results obtained in other solvents. For example, Zhang et al. (1999) reported ε (β-carotene at 450 nm) in hexane to be 134 mM^{-1} cm^{-1}, whereas Eijckelhoff and Dekker (1997) found an ε (β-carotene at 450 nm) in methanol to be 140 mM^{-1} cm^{-1}. Earlier, Land et al. (1970) reported on the extinction coefficient of β-carotene in hexane, in the wavelength region of 515 nm, to be 170 ± 40 mM^{-1} cm^{-1}.

Absorbance spectra of β-carotene in heptane were extended from the blue through the low UV region, down to 190 nm. The A_{190}/A_{450} ratio for β-carotene in heptane was determined to be about 4:1 (not shown). Determination of this ratio was important in order to properly partition A_{190} measurements between botryococcenes and carotenoids in heptane, following hydrocarbon extraction from Showa cultures (see below).

3.2. Micro-colony properties of *B. braunii* var. Showa

A group of Fernbach flasks on orbital shaker with Showa cultures in different phases of growth are shown in Fig. 3a. Typical in these cultures, and distinct among cultures of other unicellular microalgae, is the tendency of the Showa micro-colonies to

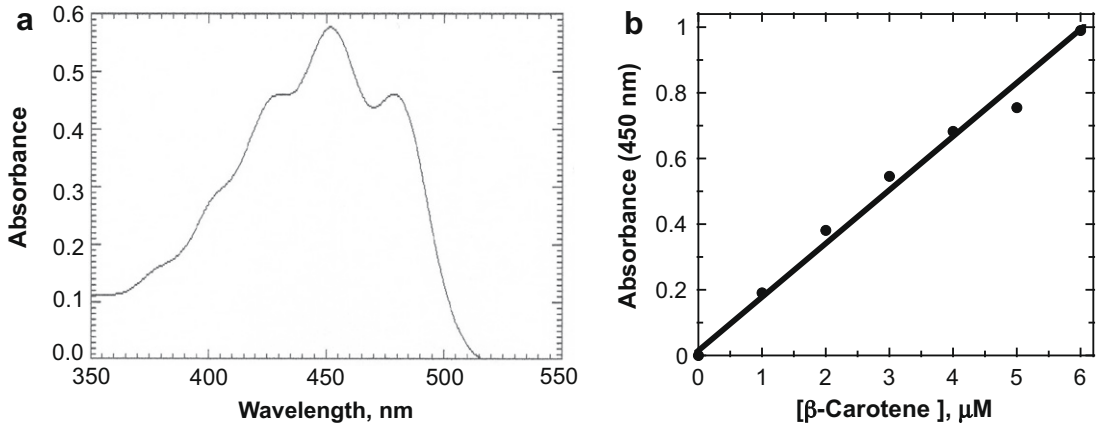

Fig. 2. (a) Absorbance spectrum of β-carotene in heptane. Note the typical carotenoid absorbance bands in the 400–500 nm region, with the prominent absorbance at 450 nm. (b) Absorbance values at 450 nm of β-carotene in heptane, plotted as a function of β-carotene concentration. The slope of the straight line defined the specific absorbance coefficient (extinction coefficient) of β-carotene in heptane at 450 nm, equal to 165 ± 5 mM^{-1} cm^{-1}.

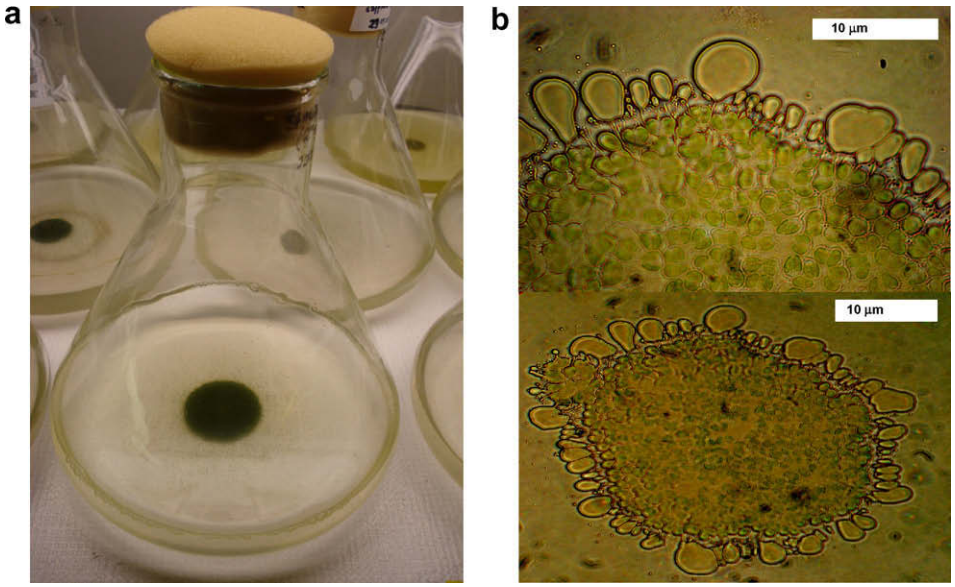

Fig. 3. (a) *B. braunii* var. Showa cultures grown in 500 mL modified Chu-13 medium in conical Fernbach flasks upon orbital shaking. Note that oil-rich micro-colonies centrifuge to the center of the H_2O-based growth medium. (b) Microscopic observation of mechanically compressed micro-colonies of *B. braunii* var. Showa, revealing droplets of triterpenoid hydrocarbons exuding from the "flattened" micro-colonies into the growth medium.

aggregate, or "centrifuge", toward the center of the growth medium, apparently a result of the orbital shaking and a consequence of the high hydrocarbon content of these micro-colonies (Eroglu and Melis, 2009). Showa micro-colonies are tightly-packed three-dimensional amorphous structures having a high density of grape-seed-like or pyriform cells. The substantial extracellular botryococcene matrix holds the cells together. A two-dimensional view of such a Showa micro-colony can be gleaned from microscopic images of "lightly compressed" individual micro-colonies, in which the three-dimensional structure is flattened and droplets of botryococcene hydrocarbons are then seen effusing from the periphery of the micro-colony (Fig. 3b).

3.3. Rates of B. braunii var. Showa growth and productivity

After approximately 10 days of growth in batch culture, Showa micro-colonies reached a biomass density of about 200 mg dry cell weight per liter culture. To measure the rate of growth under continuous culturing conditions, 40% volume (200 mL) of the initial culture was removed from the Fernbach flasks and replaced with an identical volume of fresh growth media. This removal-and-replacement was repeated every 48 h, followed by harvesting and measurement of the biomass. The dry cell weight of the harvested biomass, measured in grams per liter, was plotted as a function of time in Fig. 4a. The results suggested a rate of biomass accumulation equivalent to about 250 mg dry cell weight per liter culture per 48 h, or about 125 mg dcw L^{-1} d^{-1}. The cumulative dry cell weight from such an experiment is plotted in Fig. 4b. In this continuous growth system, and under the specific growth conditions employed, the slope of the straight line showed an algal biomass increase occurring with a rate of 125 mg dcw L^{-1} d^{-1}, in agreement with the previous measurement (Fig. 4a). By way of comparison, An et al. (2003) reported a rate of biomass accumulation of ~190 mg dcw L^{-1} d^{-1} (including about 30 mg botryococcene L^{-1} d^{-1}) from the culture of *B. braunii* UTEX-572, grown in secondarily treated piggery wastewater in a batch reactor. On the other hand, also working with the UTEX-572 strain, grown in secondarily treated sewage in a continuous bioreactor system with a daily dilution rate of 0.57, Sawayama et al. (1994) measured a biomass production rate of only about 28 mg dcw L^{-1} d^{-1}. It is evident from these results that *B. braunii* growth conditions, including bioreactor design and growth media composition, substantially impact productivity of the cultures.

3.4. Mechanical dispersion of B. braunii var. Showa micro-colonies

Mechanical dispersion studies of Showa micro-colonies were conducted to test for the behavior of the cells under such external shearing forces. This was implemented either by sonication, or glass bead vortexing of the cultures in growth media. Microscopic observations of mechanically dispersed Showa micro-colonies (Fig. 5a) revealed extensive loosening-up of the normally tightly-packed three-dimensional micro-colonies. A substantial extracellular yellowish matrix (Fig. 5a, Btc) was largely separated from the grape-seed-like green cells. Nile red staining confirmed the lipophilic nature of the colony-surrounding Btc hydrocarbons matrix, and further revealed intracellular globules of highly lipophilic matter, presumably sites of botryococcene sequestration (not shown). Showa cells retained their intactness, original grape-seed-like shape and green coloration, in spite of the mechanical dispersion of the compact micro-colony. Viability of the dispersed cells was successfully tested upon re-incubation of isolated cells in the Showa growth medium (results not shown).

It is clear from the results of Fig. 5a that the majority of the botryococcenes are extracellularly localized. These results are consistent with findings by Wolf et al. (1985), who estimated that only about 7% of the botryococcenes are intracellular, with the majority of these hydrocarbons forming the extracellular colonial matrix. Likewise, Largeau et al. (1980) reported that 95% of the botryococcenes are located in the extracellular pool of hydrocarbons. These observations raise the question of the mechanism by which such substantial amounts of long-chain hydrocarbons, synthesized and segregated in the form of globules intracellularly, are eventually excreted into the extracellular space.

Results from this work also suggest that mechanical dispersion of Showa micro-colonies might suffice to dislodge the hydrophobic botryococcene-carotene hydrocarbons from the extracellular matrix of the micro-colonies. This was further tested by a simple centrifugation in sucrose gradient of the mechanically dispersed micro-colonies. A centrifugation method in sucrose gradient was

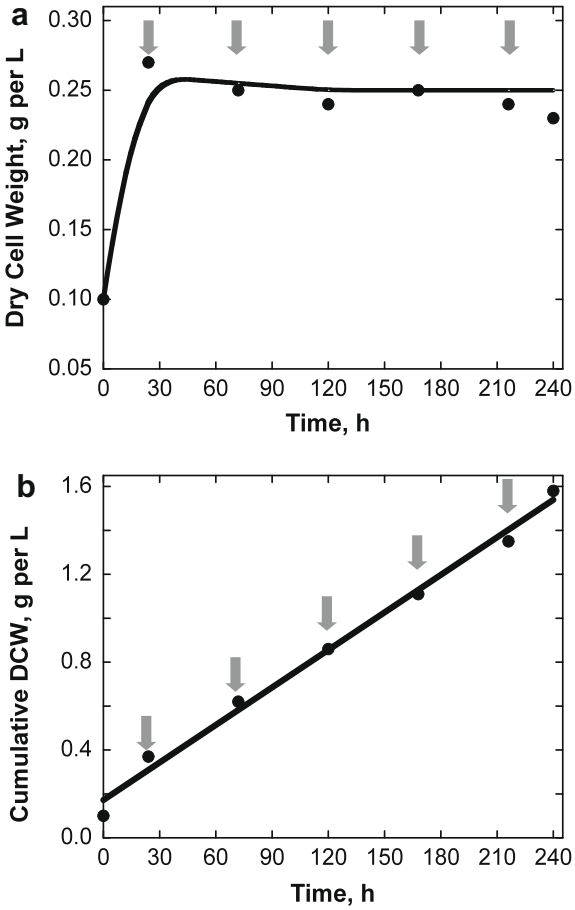

Fig. 4. (a) *B. braunii* var. Showa dry cell weight biomass harvested from a continuous fed culture. Arrows indicate the points in time, i.e., every 48 h, when a fixed fraction (40% of the culture volume) was harvested and replaced by an equal amount of fresh growth medium. Plotted is the dry cell weight in grams of the harvested biomass per liter culture as a function of growth time in the continuous culture. (b) Cumulative productivity of *B. braunii* var. Showa cultures from a continuous fed process, as shown in Fig. 3a, and according to the experimental details of (a). The slope of the straight line defined the rate of biomass accumulation, equal to 125 mg dcw L^{-1} d^{-1}.

recently designed in this lab to provide a measure of the buoyant density of biomass upon measurement of the "*density equilibrium*" of the sample (Eroglu and Melis, 2009). The outcome of such a sucrose density centrifugation, conducted with mechanically dispersed Showa micro-colonies, is seen in Fig. 5b. The results showed a clear-cut separation of the yellowish hydrocarbons fraction, which floated on top of the 10% sucrose density step, from the *B. braunii* green biomass that equilibrated in the vicinity of the 40–50% sucrose density step. This provides evidence that aqueous density equilibrium can be successfully employed to separate extractable hydrocarbons from the *Botryococcus* biomass.

3.5. Determination of the hydrocarbons productivity in B. braunii var. Showa cultures

The preceding mechanical dispersion experiment suggested that one should be able to selectively extract botryococcene and related hydrocarbons from the extracellular matrix of the micro-colonies. Vortexing Showa wet biomass with glass beads in the presence of heptane resulted in a release of extracellular hydrocarbons from the micro-colony and their subsequent solubilization in the heptane phase. The outcome of such an aqueous/organic two-phase extraction experiment was a top heptane phase, which contained a clear yellowish solution (Fig. 6a), whereas the lower water phase contained the green cell biomass (Fig. 6b). The glass beads are also seen in the bottom of the Falcon tube in Fig. 6c. Measurement of the absorbance spectrum of this heptane extract is shown in Fig. 7. Two distinct and separate absorbance bands were discerned, one peaking in the UV-C (λ_{max} = ~190 nm) attributed to triterpenoid (botryococcene) hydrocarbons, the second in the blue region of the spectrum (380–520 nm), attributed to a carotenoid, apparently associated with the extracellular hydrocarbons in *B. braunii*. Of interest was the lack of green pigmentation and absence of chlorophyll absorbance bands in this spectrum, consistent with the notion that the heptane-extracted hydrocarbons originated from the extracellular space and not from components of the photosynthetic apparatus from within the cell. The amplitude ratio A_{190}/A_{450} of the Showa extracts in heptane was measured to be in the range of 110:1; i.e., substantially greater than the 4:1 attributed to the absorbance of a carotenoid. On the basis of these spectrophotometric measurements, and the extinction coefficients

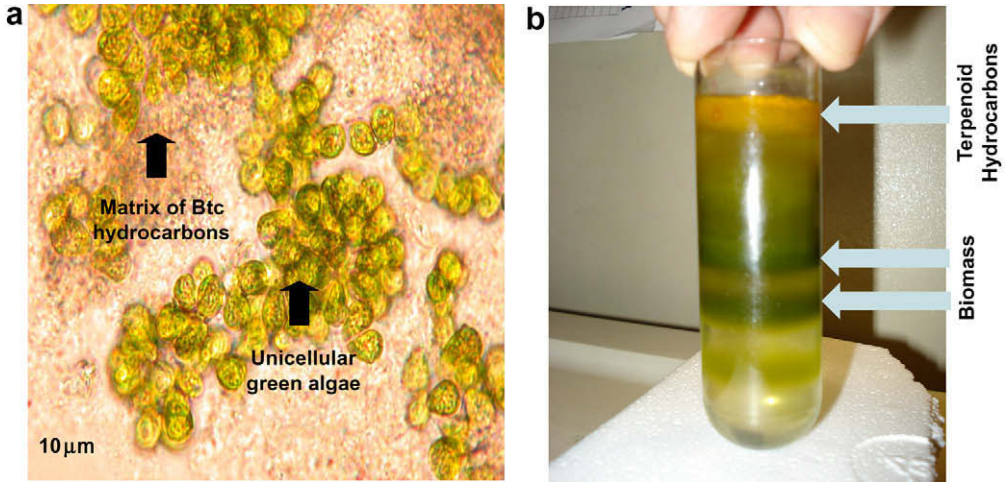

Fig. 5. (a) Microscopic observation of a dispersed *B. braunii* var. Showa micro-colony, showing the grape-seed-like green cells and the yellowish-orange botryococcene-carotenoid matrix (Btc). Nile red staining suggested the yellowish-orange matrix to be highly fluorescent, consistent with a highly hydrophobic environment. (b) Aqueous density equilibrium partition of extracellular botryococcene/carotenoid from the *B. braunii* var. Showa biomass, based on sucrose gradient density equilibrium centrifugation. Micro-colonies were mechanically disrupted prior to the sucrose density centrifugation. A discontinuous 10–80% (w/v), sucrose gradient having a concentration increment step of 10% was employed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

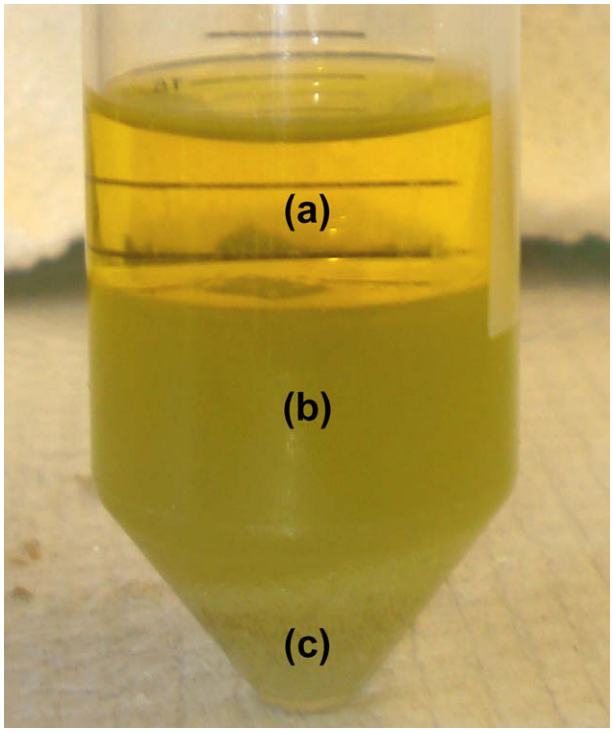

Fig. 6. Aqueous-organic two-phase partition of the botryococcene-carotenoid-containing heptane upper phase (a) from the *B. braunii* var. Showa biomass lower phase (b). Also shown are the glass beads used for the mechanical disruption of the micro-colonies, resting in the bottom of the conical Falcon centrifuge tube (c). Vortexing of the 1 g wet packed cell biomass with 10 mL heptane in the presence of glass beads was followed by addition of 10 mL of growth medium, causing separation of the organic (a) from the aqueous (b) phase.

provided from the results of Figs. 1b and 2b, a [Btc]/[Car] = 200:1 - mol:mol ratio was determined in the Showa extract ([Car]:[Btc] = 0.5:100 mol:mol).

The chlorophyll and total carotenoid content of the micro-colonies was also measured, following the methanol extraction and spectrophotometric quantitation method of Lichtenthaler and Buschmann (2001). Total chlorophyll ($a + b$) was found to be 5 ± 1 mg per g dcw (0.5% w/dcw), and the Chl a/Chl b ratio of the micro-colonies was 2.2:1 (Table 1). This Chl content is similar to that reported by Singh and Kumar (1992), who measured the Chl a content of *B. braunii* under optimal and nitrogen-deficient conditions in batch cultures to be 0.7% and 0.4% of dry cell weight, respectively. Total carotenoid content of the Showa cultures was 2.5 ± 1 mg per g dcw (0.25% w/dcw), translating into a Chl/Car ratio around 2:1 (w/w). This carotenoid content includes both extracellular carotenoids, associated with the botryococcene fraction, and thylakoid membrane-bound carotenoids, associated with the photosynthetic apparatus.

Table 1
Total pigment and extracellular terpenoid hydrocarbon content of *B. braunii* var. Showa.

Parameter measured	Amount measured
Chl ($a + b$)	5 ± 1 mg g^{-1} dcw
Chl a/Chl b	2.2 (±0.2):1
Total carotenoids	2.5 ± 1 mg g^{-1} dcw
Botryococcene hydrocarbons	320 ± 30 mg g^{-1} dcw
Extracellular carotenoids	2 ± 0.2 mg g^{-1} dcw

Application of the molecular extinction coefficients of botryococcene and β-carotene in heptane (Figs. 1b and 2b, respectively) provided a direct and convenient way for the quantitative measurement of the amount of these extracellular hydrocarbons, isolated from the *B. braunii* micro-colonies by the glass bead method. Botryococcene (Btc) and carotenoid (Car) extracted from Showa cultures were calculated on the basis of the following equations:

$$[\text{Btc}] = [(A_{190}/\varepsilon_{190}) \times \text{MW}_{\text{Btc}} \times \text{V}]/m_{\text{dcw}} \quad (1)$$

$$[\text{Car}] = [(A_{450}/\varepsilon_{450}) \times \text{MW}_{\text{Car}} \times \text{V}]/m_{\text{dcw}} \quad (2)$$

where [Btc] and [Car] are given in μg per g dcw. Also:

A	absorbance
ε	molar extinction coefficient for botryococcene (190 nm) and carotene (450 nm)
MW_{Btc}	molecular weight of botryoccocene (411 g/mol),
MW_{Car}	molecular weight of β-carotene (537 g/mol),
V	volume of heptane used for extraction (mL),
m_{dcw}	amount of biomass that was subjected to extraction (gram dry cell weight)

Kinetics of botryococcene extraction of control samples (Fig. 8, circles) and samples incubated at 100 °C for 10 min (Fig. 8, triangles), as a function of vortexing time in the presence of heptane

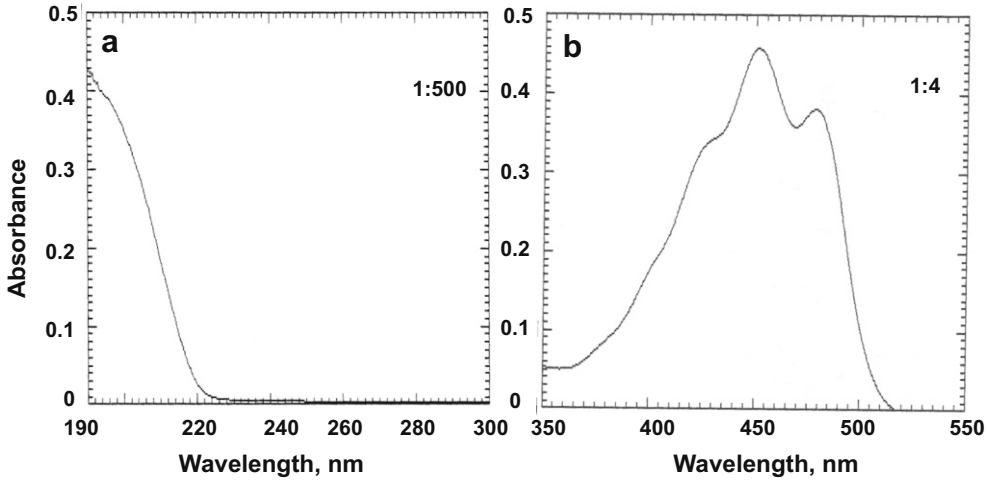

Fig. 7. Absorbance spectra of the *B. braunii* var. Showa heptane extract after vortexing of the micro-colonies with glass beads. Seen are two distinct absorbance bands: (a) in the UV-C (~190 nm) region, attributed to triterpenoid botryococcene hydrocarbons (dilution scale = 1:500); and (b) in the blue (380–520 nm) region of the spectrum, attributed to extracellular carotenoids (dilution scale = 1:4), respectively.

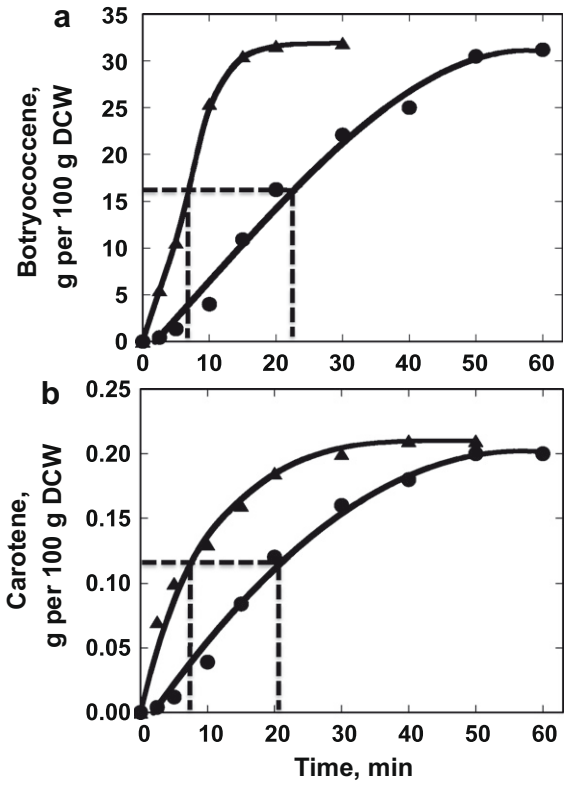

Fig. 8. Amounts of botryococcene (a), and carotenoid (b) extracted from wet *B. braunii* var. Showa biomass in control samples (solid circles) and samples incubated at 100 °C for 10 min (solid triangles), as a function of vortexing time in the presence of heptane and glass beads.

and glass beads are reported. Increasing amounts of botryococcene were extracted from the micro-colonies as a function of vortexing time, reaching 0.32 g Btc per g dcw (32% w/dcw, Fig. 8a). Heating the samples to 100 °C for 10 min prior to vortexing enhanced the efficiency of Btc extraction and shortened the time needed for extraction of these hydrocarbons by the factor of about 3.5× (Fig. 8a). Qualitatively similar results were obtained with respect to the extraction of extracellular carotenoid from the micro-colonies (Fig. 8b), reaching 0.0022 g Car per g dcw (0.22% w/dcw). Heating of the samples to 100 °C for 10 min prior to vortexing enhanced the efficiency of Car extraction and shortened the time needed for extraction of these hydrocarbons by the factor of about 3.3×, consistent with the results obtained in the extraction of Btc. Table 1 summarizes the total pigment and extracellular terpenoid hydrocarbon content of the Showa cultures, as reported in this work.

Results of Btc and Car content, based on the spectrophotometric absorbance analysis, are consistent with gravimetric measurements of extracts from the Showa strain (Eroglu and Melis, 2009) and also with results in the literature. For example, Wolf et al. (1985) reported that Showa accumulates 24–29% of its dry biomass in the form botryococcene hydrocarbons. Yamaguchi et al. (1987) measured 34 g hydrocarbons per 100 g dcw from the "Berkeley" strain, i.e., Showa. Nonomura (1988) reported a greater Btc hydrocarbon content in Showa (about 30% w/dcw) than in other strains of *B. braunii* (1.5–20%). Okada et al. (1995) estimated that the B-race of *B. braunii* micro-colonies accumulate hydrocarbons in the range of 10–38% of dry cell weight. The presence of a carotenoid that co-extracts with botryococcene hydrocarbons from *B. braunii* cultures has also been reported in the literature. Thomas et al. (1984) reported carotenoid formation ranging between 0.22% and 0.48% w/dcw in *B. braunii* UTEX-572. Rao et al. (2007) estimated the content of extractable carotenoid pigments to be about 0.25% w/dcw in *B. braunii* UTEX-572. It should be pointed out that carotenoid accumulation relative to biomass might depend on the "age" of the culture. For example, cells in the stationary phase, having a brownish coloration, might contain greater relative amounts of this pigment than actively growing cells that usually appear to be green (Largeau et al., 1980). It was also reported that carotenoids covalently bound to botryococcenes might form the extracellular matrix in some of the Botryococci species (Okada et al., 1997). Such modified extracellular carotenoids were termed "botryoxanthins". The association of one carotenoid with each botrycoccene implied stoichiometric parity between botryococcenes and botryoxanthins. However, it is evident from our results that botryococcene hydrocarbons far outnumber any such carotenoids in the extracts of *B. braunii* var. Showa.

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