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# Development of a screening procedure for the characterization of *Botryococcus braunii* strains for biofuel application

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ABSTRACT

An integrated screening approach was developed to assess the potential of the *Botryococcus* genus for the production of lipids for biofuel application (hydrocarbons and triacylglycerols). The strategy developed in this study permitted to rigorously measure mandatory parameters for the determination of strain performance – *i.e.* growth rate and oil content. For that purpose, mini-photobioreactors run in parallel were used together with an exhaustive lipid class analysis. The quantitative productivity measurements indicated that the ten screened strains presented very different patterns in the conditions of PBR culture, firstly run in batch, and secondly in continuous mode. Indeed, with the applied setup, only four strains presented biomass productivities close to that previously measured for the reference strain 807/1 (between 74 and 307 mg $L^{-1}$ $day^{-1}$ of biomass for the tested strains). HC productivity obtained under continuous light (~14 mg $L^{-1}$ $day^{-1}$) was close to that measured for the strain 807/1 (~20 mg $L^{-1}$ $day^{-1}$), confirming *B. braunii* tested strains potential for biofuel application. The analysis of the lipid chemodiversity revealed moreover that this genus should be further investigated for its polar lipids, as it suggests potential pharmaceutical applications such as production of antitumorals or immunosuppressors.



## 1. Introduction

Microalgae are the most studied bioresources in the search for sustainable biofuel production [1]. Within the remarkable diversity of microalgae, *Botryococcus braunii* holds a very unique place and has been studied specifically as a new source for jet fuel since the 1980s [2]. It produces very large amounts of polyaldehydic or polyetheric compounds (polymers that mainly constitute the outer cell wall of the microalgae) and hydrocarbons (HC), constituting up to 10% and 60% of their dry weight (DW) respectively [3]. The main industrial potential of *B. braunii* relies on its ability to directly produce HC by photosynthesis. These lipids fit jet fuel specificities, as they do not contain oxygen and are formed of long-chain alkenes (with terminal double bonds, of major interest to the olefin chemists) or botryococcenes (with original methyl squalene-like compounds). HC accumulate in large quantities within the original structural organization of *B. braunii* biomass and are thus easily convertible in jet fuels in contrast to more usual lipids such as

**Symbols**

| | |
|---|---|
| $C_x$ | Biomass DW concentration |
| $P_{HC}$ | Hydrocarbon productivity |
| $P_{TAG}$ | Triacylglycerol productivity |
| $P_x$ | Biomass DW productivity |
| $t_d$ | Doubling time |
| $\mu_{max}$ | Maximum specific growth rate |

*Abbreviations:* CL, cardiolipin; DGDG, digalactosyldiacylglycerol; DIC, dissolved inorganic carbon; DW, dry weight; EOSS-PBR, efficient overproducing strain screening photobioreactor; FA, fatty acid; GC-FID, gas chromatography coupled to a flame ionization detector; HC, hydrocarbon; HPTLC, high-performance thin layer chromatography; LPC, lysophosphatidylcholine; MTCS, medium high-throughput cultivation screening; MGDG, monogalactosyldiacylglycerol; NL, neutral lipids; OD, optical density; ONL, other neutral lipids; PBR, photobioreactor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFD, photon flux density; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, polar lipid; PS, phosphatidylserine; RSD, relative standard deviation; SM, sphingomyelin; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TL, total lipids.

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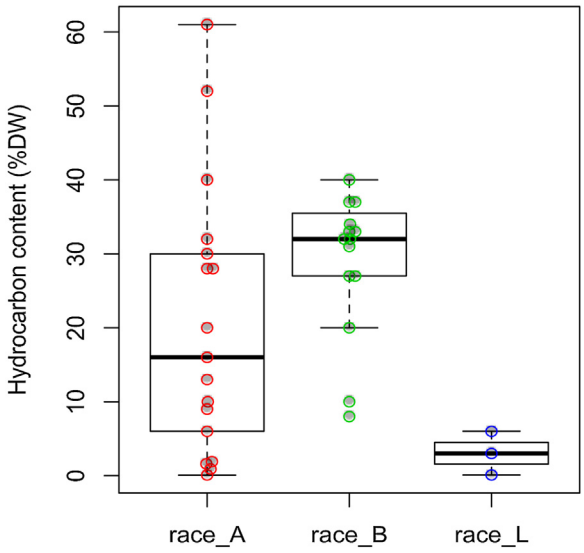


**Fig. 1.** Variability of the hydrocarbon content of *Botryococcus braunii*. Data from 35 measurements obtained for different strains of the three races of *B. braunii*, A, B, and L, and different culture conditions were used for the graphical summary [2,5,6,7,61–64]. Data are expressed as dry weight percentage (DW%).

triacylglycerols (TAG) which require further expensive chemical modifications.

Strains of *B. braunii* were classified in three different races (A, B and L) according to the type of HC they synthesize. Race A strains produce *n*-alkadienes and *n*-alkatrienes ($C_{25}$–$C_{31}$). Race B strains produce polymethylated unsaturated triterpenes called botryococcenes ($C_nH_{2n-10}$, $n$ = 30–37). Race L strains produce a single tetraterpene, named lycopadiene ($C_{40}H_{78}$) [4]. HC content of *Botryococcus* species was generally found high but was also extremely variable depending upon culture conditions, species and strains. HC content can vary from 2.6 [5] to c.a. 60% DW [6]. Fig. 1 summarizes the data extracted from 35 published measurements of HC content in strains of the three known races. It also clearly indicates the potential to produce HC for the strains of the races A and B with a median of respectively 16% DW and 33% DW. Race L strains were clearly less productive for HC, with a median HC content of 3%DW for an extracted oil composed of a single tetraterpene (lycopadiene) [7]. Other important features of *B. braunii* are its low growth rate, with a minimum generation time of 2 days [4] and its specific colonial organization in which cells are embedded in a mucilaginous sheath. This low growth rate, combined to the risk of rapid cellular degeneration of *B. braunii*, results in a strong instability if the culture conditions are not controlled [6,8].

*B. braunii* is thus a complex biological model, with high variability of physiological behaviors [9] that needs to be taken into account for the optimization of its cultivation [10]. Growing conditions could be optimized for *B. braunii*, as already done for other microalgae models, using medium- and high-throughput cultivation screening approach (MTCS) through response surface methodology [11–13]. Nevertheless, it still makes strains characterization laborious, particularly for the study of their biofuel potential. To answer such a challenge, cultivations in rigorously controlled conditions need to be also performed. The culture in photobioreactors (PBR) instead of microplates or flasks should allow to more reliably monitor and control parameters that greatly influence the photosynthetic metabolism of the microalgae (incident photon fluxes, carbon supply, pH, temperature, etc.), generating therefore experimental conditions suitable for scale-up perspectives [14,15]. However, in such a process context, a MTCS approach could not reasonably be performed simultaneously, since it will add too much variables to be taken into account for PBR conditions optimization.

In the majority of the studies on *B. braunii* cultivated in PBR [6,10,14,16–21], airlift PBR with tubular geometry were used in batch mode with fixed cultivation conditions. It led to reach up to 30% of HC in DW and biomass concentration up to 6 g $L^{-1}$. The use of the batch mode was mainly justified by the fact that *B. braunii* HC biosynthesis was related to its photosynthetic growth. When compared to the methodologies usually employed for the cultivation of the TAG-accumulating strains, for which nitrogen starvation is needed to induce lipid accumulation [22], it highlighted another interest of exploiting HC from *B. braunii i.e.* the simplicity of their production mode. Even though batch culture could still be used, since HC and biomass productions were simultaneous, continuous culture appeared to be also a suitable cultivation mode for *B. braunii*. Continuous culture in the general case of microalgae, gives also another particular benefit by reducing the light attenuation which progressively increases in batch culture with the biomass density augmentation. Moreover, the physiological responses associated to the photosynthetic process could evolve with the batch culture age, biasing therefore the analysis of results. In continuous mode, light attenuation is constant since the cell population is maintained at a fixed concentration [23]. The microalga metabolism is therefore maintained in a stationary state, permitting the robust measurement of parameters such as productivities for well-defined conditions of light absorption in the culture volume. Moreover long-term experiment can be conducted, permitting the strain to fully adapt to the culture conditions, here in the case of PBR culture [24]. However, in the latest studies involving well controlled cultivation experiments of *B. braunii,* comparison was especially difficult since the experiments were conducted in various conditions (batch, continuous, immobilized, etc.) and time scale [18,25–31]. Such heterogeneity of the experimental production conditions constitutes a real bottleneck that needs to be overcome for an unbiased (as possible as it could be) measurement of the strain process performance parameters.

In this article a novel methodology developed for the rigorous screening of *B. braunii* strains for their biofuel potential measurement is presented. It has to be considered as being complementary of a MTCS approach since it is designed from a process point of view. Indeed, this strategy was elaborated to allow the selection of the best strains in terms of culture robustness and biofuel molecular precursor productivity (namely TAG and HC) in highly controlled culture conditions. For that purpose, parallel mini PBR systems (efficient overproducing strain screening–PBR or EOSS-PBR) run in continuous mode with standardized cultivation medium were exploited in order to obtain comparable and unbiased productivity parameters (biomass and lipids of interest) [24]. To embrace the chemical potential of the tested strains, this was combined with an exhaustive high-throughput lipid class profiling methodology adapted to *B. braunii*, using high-performance thin layer chromatography (HPTLC). Results and perspectives emerging from this study are described and discussed in this manuscript.

## 2. Material and methods

### 2.1. Chemicals and standards

HPLC-grade solvents were purchased from VWR International. Butylated hydroxytoluene (BHT) was obtained from Sigma–Aldrich. All the lipid class standards (Table S1) and the chemicals used for HPTLC were commercially available, and obtained from Sigma–Aldrich, except glycolipids, which were supplied by Larodan. HPTLC glass plates (20 × 20 mm) pre-coated

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