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Assessing the biofuel production potential of *Botryococcus braunii* strains by sensitive rapid qualitative chemotyping using chemometrically-assisted gas chromatography–mass spectrometry

Benjamin Moutel [a], Marion André [b], Delphine Kucma [a], Jack Legrand [a], Dominique Grizeau [a], Jérémy Pruvost [a], Olivier Gonçalves [a,*]

[a] LUNAM Université, Université de Nantes, CNRS, GEPEA, UMR 6144, Bât. CRTT, 37 Boulevard de l'Université, BP 406, F-44602 Saint-Nazaire Cedex, France
[b] Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Avenue de la Terrasse, 91190 Gif-sur-Yvette, France

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ABSTRACT

The microalga *Botryococcus braunii* has been studied for its hydrocarbon biofuel production potential. The range of the lipids it synthesizes cannot be readily profiled, because their main chemical class standards (long chain alkenes and botryococcenes) are at present unavailable.
The aim of this study was to develop a direct, specific GCMS analysis method for the rapid chemotyping of these lipids.
The SIM (Selected Ion Monitoring)-GCMS program we developed discriminates classes and subclasses of the targeted molecules through their main chemical motifs identified with fragment ions of specific *m/z* selected by unsupervised chemometrics.
Our validation results indicate that the SIM mode enhanced their detection by significantly increasing the signal-to-noise ratio *ca.* 20-fold. The profiling results from TL extracts of diverse *B. braunii* strains show that under the culture conditions tested, these strains could be distinguished by their chemical signatures, which also signaled different culture conditions involving various physiological states. We were able to make a rapid estimate of the biofuel potential of these strains, focusing on alkenes and botryococcenes.
The direct injection SIM-GCMS method was successfully applied here for rapid chemotaxonomy, displaying good sensitivity and specificity for new high molecular weight hydrocarbons from *B. braunii*.

1. Introduction

Algal biomass is currently one of the most widely studied bioresources in the search for the perfect sustainable biofuel alternative crop [24]. This interest is notably explained by the remarkable diversity of microalgae, which comprise some 200,000–800,000 different species of which only 35,000 have been described [5]. The diversity of microalgae holds tremendous potential for novel biosynthesized chemical entities. Among the known oleaginous microalgae, the green colonial alga *Botryococcus braunii* has been found to be of interest: under precise environmental conditions (natural or in a photobioreactor) it will accumulate large quantities of hydrocarbons instead of the commonly described triacylglycerol [26]. Based on the type of hydrocarbons they synthesized, *B. braunii* varieties were classified into three chemotypes, noted A, B and L. Chemotype A produced n-alkadienes and n-alkatrienes (C_{25}–C_{31}), chemotype B produced polymethylated unsaturated triterpenes called botryococcenes (C_nH_{2n-10}, $n = 30$–37), and chemotype L produced a single tetraterpene named lycopadiene ($C_{40}H_{78}$) [1].

The structural complexity of those molecules has been assessed and resolved mainly by Metzger and coworkers [14–22]. They developed lengthy preparative chromatographic approaches involving multiple purification steps to obtain analyzable fractions of extracellular or intracellular hydrocarbons. Short contact time with hexane first yielded extracellular hydrocarbons from lyophilized biomass [7], and then suitable protocols [3,6] retrieved intracellular lipids. A combination of different solvent polarity applied directly on dried biomass was also tested by Kojima and Zhang [10], without using a preparative chromatographic approach. In both cases the amount of *B. braunii* biomass needed to perform the experiments was substantial, up to several

Abbreviations: EI, electron impact; FA, fatty acid; (F)AME, (fatty) acid methyl ester; FC, fold change; FS, full scan mode; GC-FID, gas chromatography coupled to a flame ionization detector; GC–MS, gas chromatography coupled to mass spectrometry; HCs, hydrocarbons; HC, hierarchical clustering; HCPC, hierarchical clustering on principal component analysis; ND, not detected; PCA, principal component analysis; TIC, total ion current; TLs, total lipids; TL-HCs, total lipid hydrocarbons; TL-FAs, total lipid fatty acids; TG, triglyceride; NL, neutral lipid; PL, polar lipid; RSD, relative standard deviation; RT, retention time; SIM, selected ion monitoring; SPE, solid phase extraction; SNR, signal-to-noise ratio; WCA, whole cell analytic.

* Corresponding author.
E-mail address: olivier.goncalves@univ-nantes.fr (O. Gonçalves).

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grams of material. In most cases analysis was performed on very aged biomass known to accumulate larger amounts of hydrocarbons. The significantly long generation time of this microalga, i.e. 2–4 days, can be seen as an important limiting step for such studies.

Recent investigations making use of lipidomic-related techniques permitted the hydrocarbon phenotyping of two *B. braunii* races (A and B) using pyrolysis–gas chromatography–mass spectrometry [2]. Very small quantities of material were used (~1 mg of dried biomass), and no complex sample preparation was needed to achieve chemotyping of these races. The main chemical features of races A and B were recorded on the hydrocarbon pyrograms [8], with mass signatures associated with botryococcenes, nonacosadiene, eicosadiene, phytadiene, etc. However, the proposed structural assignment of the fragments obtained after mass spectrometry analysis relied on an in silico semi-automatic annotation pipeline and not on experimental fragmentation pattern data. This constraint is due to the absence of easily producible or commercially available standards for botryococcenes, alkadienes and trienes. Although such a chemotyping strategy was quite straightforward and parsimonious in terms of sample preparation, it implies destructive analysis, which caused numerous chemical rearrangements, generating chemical structures that could not always be easily elucidated [9]. The simpler sample preparation procedure was offset by additional difficulties in mass spectral data interpretation and structural resolution.

In the work of Mjøs [22], a very interesting strategy was highlighted for fatty acid structure prediction, especially applicable when reference standards were not available. The results of this study indicated that by means of chemometrics it was possible to solve the fatty acid methyl ester (FAME) structure of marine lipids from tiny differences in the relative abundance of all or some of the fragment ions characterized by their m/z values in a low-resolution mass spectrum. By working in a very narrow mass range ($m/z = 50$–110) the author was able to assess structural differences such as numbers of double bonds, distinguishing polyenes from dienes, monoenes and saturated compounds. In addition, this approach revealed the existence of a subset of fragment ions suitable for selected ion monitoring (SIM) with predictivity close to that obtained in full scan mode. Adapting such a strategy for the structural characterization of the new lipids from *B. braunii* and especially its hydrocarbons had never been attempted before. It should surmount the absence of standards normally required for developing an assay method. Such an approach should make lipid analysis possible on an affordable low-resolution mass spectrometry system for chemical profiling, potentially enhanced for both identification and sensitivity. Concerning this last, the results of Mjøs [23] also suggest the possibility of remedying the signal loss typically observed for the hydrocarbon family by developing a very sensitive SIM mode [25].

In this work we explored the potential of such an approach, focusing on the development of a sensitive, easy-to-use qualitative analytical method for the hydrocarbon chemotyping of *B. braunii* races A and B. A gas chromatography–mass spectrometry profiling method was set up using chemometrics for optimized fragment ion selection and data interpretation. Our aim here was to propose an affordable analytical method that would make up for the lack of chemical standards and a sensitive technique for assessing targeted hydrocarbon diversity at early stages of the microalgal culture. Efforts were also made to use the biological material sparingly. The profiling method developed was applied in real conditions for the qualitative chemotyping of *B. braunii* races A and B. Results and perspectives emerging from this study are described and discussed.

2. Materials and methods

2.1. Chemicals and hydrocarbon standards

Analytical grade solvents were purchased from VWR International. Butylated hydroxytoluene (BHT), boron trifluoride (BF_3, 14% by weight in methanol), glyceryl triheptadecanoate (TAGs17:0), the 37 FAME components and tricosane were obtained from Sigma Aldrich.

Substitution standards were used for the development of the analytical methods, since the major hydrocarbons synthesized by *B. braunii* species were not commercially or academically available. Linear or branched alkadienes or trienes (race A), botryococcenes (race B), and lycopadiene (race L) were no longer available (Chiron AS®, Trondheim, Norway). The n-alkane standard mixture (C_{10}–C_{40}, all even), squalane, squalene and phytol, were respectively chosen as substitutes for these compounds, because they contain the major structural elements found in the hydrocarbons synthesized by *B. braunii* (Table 1). A substitution standard mixture was made up of FAME concentration set at 1.6 $\mu g \cdot \mu L^{-1}$, and all other compounds between 20 and 90 $ng \cdot \mu L^{-1}$ (n-alkane all even: 20 $ng \cdot \mu L^{-1}$, squalane: 50 $ng \cdot \mu L^{-}$, squalene: 70 $ng \cdot \mu L^{-1}$ and phytol 90 $ng \cdot \mu L^{-1}$).

2.2. Strains and culture conditions

Ten strains of *B. braunii* were purchased from international algae banks: Algoban (Caen, France), CCA (Scotland), CCALA (Czech Republic), and SAG (Germany). Table 2 gives their features and the codes used in this work.

The algal cells were cultured in 250 mL conical flasks under constant stirring at 75 rpm, constant pH 7.5 (maintained using 4% CO_2-enriched air bubbled at 20 $mL \cdot min^{-1}$) and constant illumination at approximately 80 μmol $m^2\ s^{-1}$ at 25 °C using modified Chu 13 medium, which contains 3 times higher concentrations of inorganic macronutrients and trace metals than the original Chu 13 medium [11].

2.3. Analytical methods used on the culture medium

All analyses were performed in triplicate. Culture turbidity was measured spectrophotometrically at 750 nm. The algal dry weight (DW) measurements were determined by filtration through a pre-dried, preweighed 0.2 μm glass-fiber filter (Whatman GF/F) with demineralized water used as washing buffer. The filter was dried for 24 h at 105 °C, cooled in a desiccator and then re-weighed to within 0.01 mg on an XA 105 analytical balance (Mettler Toledo). For each particle concentration, microalgal cells were counted using a hemocytometer (Malassez device) with an optical microscope (Zeiss).

2.4. Development of the chemotyping method

2.4.1. B. braunii biomass harvesting protocol

All the *B. braunii* cell cultures were run to obtain a final biomass concentration of ~0.5 $g \cdot L^{-1}$, which corresponded to very early stages in our batch cultivation protocol, i.e. ~2 days for usual cultivation duration in the range of 7–10 days in our conditions. Approx. 50 mL samples of fresh culture were taken from each replicate flask, and the whole volume was frozen at −80 °C. The frozen cells in their culture media were then lyophilized for 12 h at 4 °C (RP2V, SGD Sérail, Argenteuil, France) and stored at −20 °C before undergoing the total lipid (TL) extraction protocol. This biomass sampling protocol was chosen because it avoided any leakage of the targeted metabolites during the cell harvesting procedure. It thus ensured acquisition of a faithful image of the compounds present in the analyzed matrix.

2.4.2. TL extraction protocol

The TL extraction protocol was developed for lyophilized *B. braunii* biomass as follows. The glassware was systematically heated for 6 h at 450 °C to prevent organic pollution. Aliquots of 50 mg of lyophilized biomass sample were placed in glass vials in triplicate, and assayed for TL extraction. This consisted in extracting all the TLs, i.e. including the targeted hydrocarbons, irrespective of their cellular localization, using a chloroform/methanol mixture (2/1 v/v) as the single extraction solvent. It was adapted from the whole cell analytic (WCA) approach developed by Meier et al. [13].