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SHORT COMMUNICATION

Towards the commercialization of *Botryococcus braunii* for triterpenoid production

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Abstract *Botryococcus braunii* can accumulate unusually high levels of triterpenoid hydrocarbons making it a potential source of high value chemicals. However, its commercial application is hampered by its slow growth and lack of large-scale studies of triterpenoid hydrocarbon production. This study investigated hydrocarbon production in two race B of *B. braunii* strains, Overjuyo-3 and Kossou-4, at 25 °C in 500 L open tanks under artificial lighting in modified BG11 medium over 60 days. Maximum growth was reached by 40 days with Overjuyo-3 producing more biomass (3.05 g L^{-1}) than Kossou-4 (2.55 g L^{-1}). However, Kossou-4 produced more oil (0.75 g L^{-1}) and triterpenoid hydrocarbons (C30–C34; 50 % of oil weight) compared to 0.63 g L^{-1} of oil in Overjuyo-3 with triterpenoid hydrocarbons making up 29 % of oil weight. This research demonstrates for the first time that large-scale production of high value triterpenoid hydrocarbon for commercial application is feasible with Kossou-4 strain.

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Introduction

The colony-forming green algae *Botryococcus braunii* can accumulate unusually high levels of hydrocarbons, in the range of 15–35 % of its dry weight with up to 76 % of the dry weight of the cell material being combustible [1]. *B. braunii* is classified into three chemical races (A, B and L) based on the type of hydrocarbons produced. Race B strains produce two major types of triterpenoid hydrocarbons; botryococcenes and methyl-branched squalene [2]. Up to 30 % of their dry weight can be composed of dimethylated to tetramethylated forms of the C30 to C34 hydrocarbon comprising mainly of botryococcene and squalene [3]. Triterpenoid hydrocarbons (C30–C34) or triterpenes are industrially important. For example, C30 squalene is a biosynthetic precursor of steroids in plants and animals [4] with therapeutic properties as a chemo-preventive agent with tumor-inhibiting properties [5]. Current sources such as sharks' liver and olive oil cannot meet current and future demands as local and international regulations now prohibit or limit the use of using sharks' liver while triterpenes from olive oil and other plant-based sources such as sugarcane, amaranth seed oil are in direct competition with food crops [6].

Microalgae such as *B. braunii* represent a potential source of triterpenes and an alternative to fossil fuels [7]. However, their commercial development has been limited due to their slow growth rates and strain variability in terms of triterpenoid yields [8]. The impacts of different growth conditions, media and nutrients on biomass and hydrocarbon production in different *B. braunii* strains have been investigated under laboratory conditions [8]. However, relatively few studies have examined the effects of these parameters on triterpenoid hydrocarbon production. Only one recent study is known to have investigated triterpenoid hydrocarbon production in multiple *B. braunii* race B strains including Kossou-4 and Overjuyo-3 at laboratory scale. This study reported that these strains produced more than 21 % of their dry weight as C31–C36 hydrocarbons, which mainly comprised of triterpenoids [1]. A number of other *B. braunii* strains have been investigated under large-scale conditions confirming the feasibility of growing *B. braunii* under large-scale conditions, with information on biomass, oil production and hydrocarbon content [1, 9–11]. However, no studies of the large-scale production of Kossou-4 and Overjuyo strains have been reported in the scientific literature. In our earlier laboratory-based experiments, Race B strains Kossou-4 and Overjuyo-3 produced substantial biomass when grown in modified BG11 and environmental conditions successfully producing triterpenes (C30–C34) [1]. To determine whether these growth conditions were suitable for biomass and hydrocarbon production commercially (including specific assays for triterpenes) for both strains were investigated under larger-scale conditions in a 500 L pilot trial. The aim of this study was to assess the feasibility of large-scale cultivation of *B. braunii*, Kossou-4 and Overjuyo-3 grown in open circular tanks under artificial light, determining the yields of the commercially important triterpenoids together with biomass production and oil yield during this process.

Materials and methods

Microalgae source and growth media

The *B. braunii* strains selected in this study were two race B strains, Kossou-4 and Overjuyo-3 [1]. Both strains were obtained from Flinders University and originated from Pierre Metzger's collection [2]. Modified BG11 medium was prepared according to [12] with sodium nitrite concentration ($NaNO_3$) reduced from 1.5 to 0.75 g L^{-1}.

Inoculation of tanks and culture conditions

Two circular fiberglass tanks (height 83 cm, diameter 143 cm, capacity 500 L) were used, one for each strain. Five liters of the modified BG11 medium concentrate was added to 495 L of fresh water in each tank. An aliquot (2.5 L) of Kossou-4 which corresponded to 0.4 g L^{-1} (dry weight) of microalgae culture was added to the tank as inoculum (Supplementary figure 1a). An equivalent aliquot of Overjuyo-3 was added to the other tank. The aliquot was prepared by inoculating 5 L of modified BG11 medium with 0.04 mg of culture. Culture standardization was carried out by using dry weight assay as described by [13]. The inoculated cultures were incubated in the tanks for 60 days with air supply provided by continuous bubbling of air up into the tank from appropriate angles at an airflow rate of 18.1747 m^3 min^{-1} to ensure complete mixing of tank content. Light intensity was 54 μmol photons m^{-2} s^{-1}. Temperature was maintained by a thermostat in the range 24.8–25.5 °C [14].

Measurements of biomass production

Aliquots (10 mL) of each sample solution were taken (three replicates) and filtered using a Millipore Filter (45 μm, 47 mm) of predetermined weight via a standard vacuum pump every 10 days. The dry weight of the microalgal biomass was expressed as percentage dry weight values [13].

Oil extraction and analysis

To extract oil from *B. braunii,* strains Kossou-4 and Overjuyo-3 the method described by Sawayama et al. was used [15]. Replicate samples were evaluated and the oil contents measured gravimetrically. The oil composition was analyzed by using gas chromatography–mass spectrometry (GC/MS) using an Agilent Technologies 5975C mass spectrometer inert XLE/CI MSD with Triple Axis Detection equipped with an Agilent Technologies 7890A GC system gas Chromatograph and a 7683B Autosampler 7890A (Agilent Technologies Inc., Forest Hill, Australia) [1]. Appropriate hydrocarbon standards were prepared and used for calibration and peak identification. Triterpenoid hydrocarbons were identified as between C30–34 fractions. Total oil and triterpenoid hydrocarbon contents were expressed as both gram per liter and percentage of dry weight (Supplementary figure 1b).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the differences in the levels of growth at different time points (three replicate samples) for each strain of *B. braunii*. A p value of 0.05 or less was considered as the statistically significant value. ANOVA tests were conducted for each measurement of biomass.

Results

Microalgae growth and biomass estimation

Maximum growth, as assessed by dry weight measurements, was reached after 40 days with Overjuyo-3 producing more biomass (3.05 g L^{-1}) than Kossou-4 (2.55 g L^{-1}) (Fig. 1). Both strains grew successfully in the open tanks over the 60 days until the final harvest was carried out.

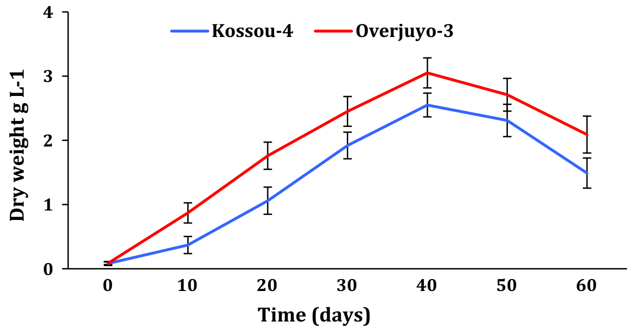

Fig. 1 Growth as assessed by dry weight (g L^{-1}) of *B. braunii* strains Kossou-4 (*blue line*) and Overjuyo-3 (*red line*) under large-scale 500 L cultivation in modified BG11 medium over 60 days. Results shown are the means of three replicates with standard errors shown (color figure online)

Contamination was monitored by microscopy at 10-day intervals with no substantial bacterial contamination being observed. After day 40, the dry weight measurements of both strains decreased although this decrease was not statistically significant ($p > 0.05$). Statistically analyses with ANOVA in both strains showed significant increases in biomass between days 10, 20 and 40 ($p < 0.05$).

Oil weight (g L^{-1})

At day 40, the total oil extracted by *n*-hexane from Kossou-4 culture grown in the modified BG11 medium in the circular tank was 0.751 g L^{-1} (±0.023) representing 29 % of the total dry cell weight (Fig. 2a). For Overjuyo-3 at day 40, the total oil extracted by *n*-hexane was slightly lower at 0.631 g L^{-1} (±0.162) which corresponded to 20 % of its biomass (Fig. 2a, b). ANOVA for oil weight values at day 40 compared to days 10 and 20 only showed significant increases for both Kossou-4 and Overjuyo-3 ($p < 0.01$).

Triterpenoid hydrocarbon production

Total triterpenoid hydrocarbon (C30–C34) production in the oil extracted at day 40 from the large-scale studies with Kossou-4 cultures reached 50 % of oil dry weight (Fig. 2c). For Overjuyo-3, the oil at day 40 showed a total triterpenoid production of 29 % of dry weight (Fig. 2c). ANOVA for triterpenoid hydrocarbon values showed a significant difference between Kossou-4 and Overjuyo-3 at day 40 ($p < 0.05$).

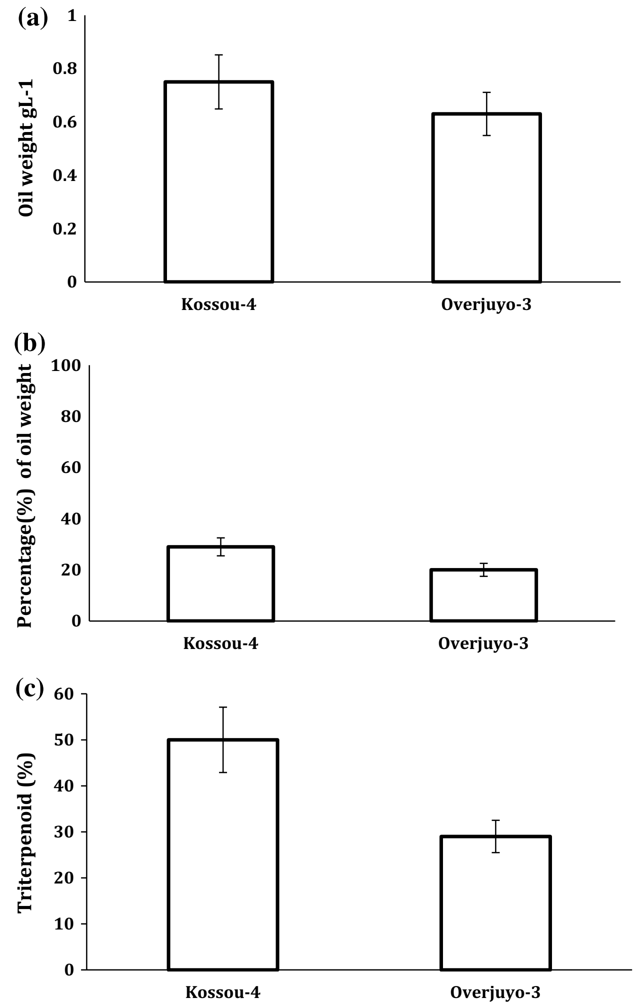

Fig. 2 Oil weight (g L^{-1}) (**a**) and expressed as a percentage (%) (**b**) in *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3) grown under large-scale 500 L cultivation in modified BG11 medium after 40 days. **c** % triterpenoid in oil from *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3)

Discussion

B. braunii is an algae species that shows considerable potential as source of high value chemicals such as triterpenoid hydrocarbons (composed mainly of squalene and botryococcene) [2, 16]. To realize this potential, it is essential to investigate whether *B. braunii* strains can produce high yields of biomass and triterpenoid hydrocarbons under large-scale conditions. This has not been reported in literature up till date. This study investigated some of these aspects (biomass assay method and large-scale cultivation) in two of the strains that show commercial potential; Kossou-4 and Overjuyo-3 [1].

Large-scale growth (500 L) in a modified BG11 medium for up to 60 days revealed that maximum dry weight was obtained at day 40 for both Kossou-4 (2.55 g L^{-1}) and Overjuyo-3 (3.01 g L^{-1}) strains. The biomass at day 15 was 1.5 g L^{-1} for Overjuyo-3 and 0.9 g L^{-1} for Kossou-4 and 3.05 and 2.55 g L^{-1} for Overjuyo-3 and Kossou-4, respectively, at day 40. For Overjuyo-3, this was higher than the values reported by Dayananda et al. [10] and Ashokkumar and Rengasamy

[9, 10]. At day 25, the estimates were 2.26 g L^{-1} for Overjuyo-3 and 1.49 g L^{-1} for Kossou-4 which in Overjuyo-3 was higher than 2 g L^{-1} in Ranga Rao et al. [11]. Contamination was not an issue even with cultivation in open tanks for 60 days. Large-scale studies have been reported for other *B. braunii* strains (and not for the two strains used in this study) by different authors [9–11]; however, these studies were conducted over shorter periods and did not evaluate the production of high value triterpenoids which is the focus of this study. Triterpenoid hydrocarbon yield in Overjuyo-3 was 29.5 %, higher than the 24.97 % reported by Li et al. in laboratory-scale studies [1]. The reasons for these increases could be due to the differences in growth conditions, growth media, and culture cultivation time.

With regards to total oil production, Kossou-4 produced 29 % (w/w) of oil compared to 20 % (w/w) for Overjuyo-3 in this study. These values are broadly consistent with those reported in small-scale studies confirming the feasibility of growing these strains under large-scale conditions while maintaining high yields of oil and high value triterpenoids. Compared to other large-scale studies, the yields from this study were substantially higher. In terms of hydrocarbons, Dayananda et al. reported a hydrocarbon content of 0.0063 g L^{-1} in 18 days [10]. In Ashokkumar and Rengasamy, the dry biomass contained 11 % hydrocarbons but data were only available for C15–C17 saturated hydrocarbons which are typical of Race A, whereas the strains in the present study mainly contained C30–C34 hydrocarbons [9]. Ranga Rao et al. (2012) reported a total hydrocarbon (w/w) content of 28 % with ≥C30 content being 20.32–28.41 % of total hydrocarbon content. This was slightly lower than the ~29 % of oil content and ~50 % of C30–C34 (of oil content) produced by Kossou-4 in the present study [11].

Conclusions

Botryococcus braunii strains, Overjuyo-3 and Kossou-4 were cultured at 25 °C in 500 L open tanks under artificial lighting in modified BG11 medium over 60 days. Greater oil production occurred in Kossou-4 (0.75 g L^{-1}), with 50 % triterpenoid hydrocarbons compared to 0.63 g L^{-1}, with 29 % triterpenoid hydrocarbons) for Overjuyo-3. This research demonstrates that large-scale production of high value triterpenoid hydrocarbon for commercial application is feasible with Kossou-4 strain.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests The authors declare that they have no competing interests exist.

References

1. Li Y, Moore R, Qin J, Scott A, Ball A (2013) Extractable liquid, its energy and hydrocarbon content in the green alga *Botryococcus braunii*. Biomass Bioenergy 52:103–112
2. Metzger P, Allard B, Casadevall E, Berkaloff C, Couté A (1990) Structure and chemistry of a new chemical race of *Botryococcus braunii* (chlorophyceae) that produces lycopasiene, a tetraterpenoid hydrocarbon. J Phycol 26:258–266
3. Niehaus TD, Okada S, Devarenne TP, Watt DS, Sviripa V, Chappell J (2011) Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii*. Proc Natl Acad Sci 108:12260–12265
4. Eroglu E, Melis A (2010) Extracellular terpenoid hydrocarbon extraction and quantitation from the green microalgae *Botryococcus braunii* var Showa. Bioresour Technol 101:2359–2366
5. Reddy LH, Couvreur P (2009) Squalene: A natural triterpene for use in disease management and therapy. Adv Drug Deliv Rev 61:1412–1426
6. He H-P, Cai Y, Sun M, Corke H (2002) Extraction and purification of squalene from Amaranthus grain. J Agric Food Chem 50:368–372
7. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306
8. Qin JG (2010) Hydrocarbons from algae. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin Heidelberg, pp 2817–2826
9. Ashokkumar V, Rengasamy R (2012) Mass culture of *Botryococcus braunii* Kutz. under open raceway pond for biofuel production. Bioresour Technol 104:394–399
10. Dayananda C, Kumudha A, Sarada R, Ravishankar G (2010) Isolation, characterization and outdoor cultivation of green microalgae *Botryococcus* sp. Sci Res Essays 5:2497–2505
11. Ranga Rao A, Ravishankar G, Sarada R (2012) Cultivation of green alga *Botryococcus braunii* in raceway, circular ponds under outdoor conditions and its growth, hydrocarbon production. Bioresour Technol 123:528–533
12. Ge Y, Liu J, Tian G (2011) Growth characteristics of *Botryococcus braunii* 765 under high CO_2 concentration in photobioreactor. Bioresour Technol 102:130–134
13. Zhu C, Lee Y (1997) Determination of biomass dry weight of marine microalgae. J Appl Phycol 9:189–194
14. Brennan L, Owende P (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sustain Energy Rev 14:557–577
15. Sawayama S, Minowa T, Dote Y, Yokoyama S (1992) Growth of the hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. Appl Microbiol Biotechnol 38:135–138
16. Cheng P, Ji B, Gao L, Zhang W, Wang J, Liu T (2013) The growth, lipid and hydrocarbon production of *Botryococcus braunii* with attached cultivation. Bioresour Technol 138:95–100