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The Effect of Media on Biomass and Oil Production in *Botryococcus braunii* Strains Kossou-4 and Overjuyo-3

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Abstract

The green algae *Botryococcus braunii* is widely recognized as a source of oil, including hydrocarbons. However, the slow rate of growth *B. braunii* hampers its commercial development. This study addresses this by examining the effects of three growth media on biomass and oil production in two *B. braunii* Race B strains, Kossou-4 and Overjuyo-3. Growth of *B. braunii* strains in BG11 medium resulted in significantly higher growth (2.3 - 4.2 and 2.9 - 6.0 fold increases in Kossou-4 and Overjuyo-3 respectively) compared to the JM and BBM-3N media after 15 days. A similar trend was obtained when biomass was measured indirectly using optical density (OD) and chlorophyll fluorescence. Oil production was also significantly higher in BG11 whether measured as oil weight or absorbance (ODs at 680 and 750 nm). However, the presence of extracellular oil was shown to increase absorbance values making OD measurements less reliable than dry weight assays. Maximum recovery of oil was recorded when hexane was used as solvent compared to hexane-isopropanol and heptane. These results suggest that BG11 is the best growth medium for these two strains under the conditions of this experiment.

Keywords

***B. braunii*, Race B, Media, Biomass, Oil Production**

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1. Introduction

The potential use of microalgae for the production of biofuels has recently received significant attention [1] because of the search for renewable alternatives to fossil fuel. The advantages of using microalgae as an alternative source of biofuel compared to other algae and plants include a high rate of growth (10 - 50 times faster than terrestrial plants), the fact that they are not food crops and they have a rapid CO_2 fixation rate [2]. Microalgae can also accumulate high concentrations of hydrocarbons and are able to produce both biofuels and valuable co-products [3]. Biofuels that originate from photosynthetic organisms such as microalgae may be carbon-neutral and renewable [4].

The microalga, *Botryococcus braunii* is one of the most widely studied organisms. *B. braunii* is characterized by unusually high hydrocarbon content, thought to reach 86% of the dry weight of the cell. It is a slow growing, colonial, fresh water microalgae [5]. In order to produce biomass and oil from *B. braunii* at a commercial scale, its growth conditions (in terms of medium that enhances growth and biomass production) need to be determined. To maximize biomass production, microalgae require light energy for biomass production and CO_2 fixation, while the culture media must supply both macronutrients and micronutrients to enable the algae to grow [6]. Although some research has been done on oil (hydrocarbon) production by *B. braunii* [7], studies of the effects nutrients and growth media on biomass production are comparatively few [8].

Biomass and oil production in *B. braunii* has been investigated in a number of studies using different media such as Z8, BG11, BBM and CHU 13. However, most studies have been conducted using a single medium. For example, the growth of *B. braunii* and two other species of microalgae in blue green algae (BG11) medium have been studied, with very high biomass and lipid content for *B. braunii* being reported [9]. In a photo-bioreactor, *B. braunii* 765 strain was observed to have good growth in BG11 at 25˚C under continuous light [10]. Apart from BG11, other media have been used for *B. braunii* cultivation [11]. *B. braunii* (SKU: AC-1006 strain) has been cultured in BBM and its modified form (BBM-3N) with higher biomass and lipid production in BBM-3N compared to BBM. *B. braunii* (SKU & AC-1006 strains) also grew well at 25˚C in bold basal medium (BBM) and in BBM with added nitrogen and vitamins *i.e.* BBM-3N medium [11]. Duplicate experiments were conducted for each medium with maximum growth being found in the BBM-3N medium. A comparison of 16 *B. braunii* strains grown in Jaworski's Medium (JM) at 23˚C for 34 days (12 h light; 12 h dark) showed that, out of the 16 Race A, B and L strains evaluated, hydrocarbon content was highest in the three Race B strains, Kossou 4, Overjuyo 3 and Paquemar [12].

Few reports evaluating the growth efficiency of *B. braunii* in different media are available. The growth of *B. braunii* LB 572 and SAG 30.81 in four media—bold basal medium (BBM), bold basal with ammonium carbonate (BBMa), BG11 and modified Chu 13 medium for 6 weeks has been evaluated [13]. The investigators found the highest biomass production in BG11 medium for both strains and both strains also produced high hydrocarbon (oil) yields in BG11 [13]. However, [2] suggested that CHU 13 medium was better for *B. braunii* growth than either BG11 or BBM media. Another study [14] compared the growth of *B. braunii* in four autotrophic media (CHU13, Z8, BBM, and BG11) at 25˚C and found that the highest biomass was produced in BG11 medium. Reports on other microalgae (for example *Ankistrodesmus falcatus*) have shown better growth in BBM compared to BG11 medium [15]. However, there are no reports comparing the growth of *B. braunii* in BG11 medium to the growth in JM medium despite the fact that both media have been used to culture the microalgae [9] [12]. Therefore in this study, we will assess biomass and oil production in two high hydrocarbon-yielding strains of *B. braunii*, Kossou-4 and Overjuyo-3 when grown in the three different media, JM, BG11 and BBM3N in order to determine the best medium for the growth of these strains.

Previous studies of *B. braunii* have used dry weight (DW) as the primary measure of biomass [10]. In addition, changes in optical density (OD) have been used as a measure of growth. This method has the advantage of being less time-consuming than measuring microalgal dry weight. The following OD wavelengths have been used in previous studies: 550 nm for *B. braunii* SKU, AC-1006 [11], 750 nm for *B. braunii* BOT-22 [16], 680 nm for *B. braunii* FACHB-357 231 [17] and 750/680 nm for *Chlorella vulgaris* [18]. Other methods that have been used for measuring biomass include chlorophyll content [10] [11] [19]. For *B. braunii* Kossou 4 and Overjuyo 3, the only published study is that of [12] which focussed on hydrocarbon yield. Therefore, it is important to determine the best method for measuring the biomass of these two strains.

A number of methods have been reported for the solvent extraction of oil from microalgal biomass. Chloroform-methanol is a frequently used solvent system [20], but the carcinogenic effects of chloroform are of con-

cern (NLM-Toxnet http://toxnet.nlm.nih.gov National Library of Medicine, USA). Hexane is commonly used due its low cost and good extraction efficiency [21] while heptane has also been used [22] as well as hexane-isopropanol 2:3 (v/v) [20]. In order to investigate which solvent was the best for extracting oil from the two *B. braunii* strains, the less toxic solvents—isopropanol, hexane and heptane were selected for use in this study (NLM-Toxnet http://toxnet.nlm.nih.gov National Library of Medicine, USA (accessed 13082014).

The specific aims of this study are to i) investigate the ability of two strains of *Botryococcus braunii* Race B to grow in three different growth media, ii) compare five methods for measuring biomass in the two *B. braunii* strains, and iii) compare three methods for extracting oil from biomass produced by the two *B. braunii* strains. Based on the results obtained, the best of these media, growth assays and solvents for oil extraction for commercial production of the *B. braunii* strains will be determined.

2. Materials and Methods

2.1. Microalgae Source

Two race B strains of *B. braunii* were selected for use in this study. The two strains selected have been classified into race B, with members of this race being known for their high level of hydrocarbon production. Both strains were obtained from Flinders University and originated from Pierre Metzger's collection. The Kossou-4 strain was originally from the Ivory Coast and shows a brownish colouring while Overjuyo-3 was from Bolivia and is green in colour [23].

2.2. Apparatus

A POLARstar Omega (BMG TABTECH) plate reader was used to measure optical density and chlorophyll fluorescence. A Ratek incubator shaker was used to provide continuous shaking of cultures. To observe biomass and oil production, fluorescence microscopy was carried out using a Leica DM 2500 microscope equipped with a Leica DFC 310 FX camera. Magnification was 100×; excitation was at 543 nm and emission 555 - 650 nm.

2.3. Media and Culture Preparation

Three growth media, i) Blue Green medium (BG11) ii) Bold base medium (BBM-3N) and iii) Jaworski's medium (JM) were used. BG11 were prepared according to [10]. BBM-3N medium was prepared according the recipe provided on the Culture Collection of Algae and Protozoa (CCAP) website: (http://www.ccap.ac.uk/media/documents/3N_BBM_V_000.pdf) and JM medium was prepared according to [24]. The components of each medium are shown in **Table A1**.

2.4. Experimental Design

Six hundred millilitres of each of the medium were added to Erlenmeyer flasks (2000 mL). For Kossou-4 and Overjuyo-3, the experiments were conducted in replicates for each medium. Flasks were inoculated with an aliquot (6 mL) of Kossou-4 or Overjuyo-3, which corresponded to 0.04 $g \cdot L^{-1}$ (dry weight) of microalgal culture.

Culture Conditions

Inoculated culture media were incubated on a Ratek incubator shaker, which was set to a rotation frequency of 100 rpm at 25°C for 15 days. Continuous white fluorescent light illumination at intensity of 54 µmol *photons* $m^{-2} \cdot s^{-1}$ was provided.

2.5. Measurements of Biomass Production

Sampling was carried at 3-day intervals, with replicate samples being subject to four different assays. The assays were as follows: optical density at a wavelength of at 680 and 750 nm, chlorophyll fluorescence at 430 nm and dry weight.

2.5.1. Optical Density (OD)

Optical density provides a measure of algal growth. Optical density was determined using a POLAR star Omega Microtitre plate reader. Algal suspension (200 µL) was added to each selected well (in replicates) in a 96 well

microtitre plate. Before taking the individual reading, the plate was shaken continuously for 30 s. Light absorbance was measured at a wavelength of 680 and 750 nm. Higher absorbance values indicate greater growth [18].

2.5.2. Chlorophyll Fluorescence

A POLARstar Omega (BMG TABTECH) microtitre plate reader was also used to measure chlorophyll fluorescence. Culture solutions (200 µL) were taken and inoculated in replicates into selected wells in a 96 well black microtitre plate. The plate was shaken for 30 s before fluorescence was read at 430 nm. Higher fluorescence values indicate greater growth according to [25].

2.5.3. Dry Weight (DW)

An aliquot (100 mL) of each algal solution was filtered using a MILLIPORE Filter (45 µm, 47 mm) of predetermined weight via a standard vacuum pump. The filter paper-culture complex was weighed before and after drying at 65˚C until a constant weight was attained. The weight of the filter paper was deducted from the total weight of samples (before and after drying) to determine the dry weight of the microalgal biomass**,** which was then expressed as percentage dry weight values according to [26].

2.6. Oil Extraction

2.6.1. Hexane

To extract oil from *B. braunii*, strains Kossou-4 and Overjuyo-3, the method described by [27] was used. Briefly, algal dry weight was measured gravimetrically in a freeze-dried sample. N-hexane (10 mL, Sigma-Aldrich; Australia) was added to freeze-dried algal cells before being put in a sonication bath for 5 min to disrupt the cells. Finally the upper layer was transferred into a pre-weighed Agilent glass tube. To determine the amount of oil produced, the n-hexane was removed by evaporation with pure N_2 gas in a fume hood. Replicate samples were evaluated and the oil contents measured gravimetrically.

2.6.2. Hexane-Isopropanol

The protocol used for solvent extraction of oil in the two strains was based the method of [20] which involved the use of a mixture of hexane/isopropanol (3:2) (Sigma-Aldrich; Australia). Freeze dried algal biomass were mixed with solvent (10 mL) and incubated overnight [20]. The samples were placed in a water bath for sonication and the rest of the procedure as described in Section 2.6.1 was followed.

2.6.3. Heptane

Heptane (Sigma-Aldrich; Australia) was used to extract oil from the two strains of *B. braunii*. Day 15 cultures were harvested and freeze dried. Heptane (10 mL) was added to the cells and samples placed in a water bath for sonication and the rest of the procedure as described in Section 2.6.1 was followed [28] [29].

2.6.4. Absorbance by Extracted Oil

In order to assess the effects of oil on absorbance values determined by OD measurements, the absorbance values of 200 µL of extracted oil were determined at 680 and 750 nm. Oil samples extracted by hexane from cultures grown in the three media were used.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the differences between the levels of growth between media at different time points 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. Post-hoc multiple comparisons were conducted using Tukey's HSD. Data analysis was conducted using IBM SPSS 21 for Windows (SPSS Inc., USA).

3. Results

3.1. Microalgal Growth

Both strains grew in each of the media under the growth conditions of 25˚C temperature, under continuous light

and agitation at 100 rpm (**Figure 1**). **Figure 1(a)**, **Figure 1(c)** and **Figure 1(e)** shows the growth of Kossou-4 in BG11, JM and BBM 3N media respectively. **Figure 1(b)**, **Figure 1(d)** and **Figure 1(f)** shows the growth of Overjuyo-3 strain in BG11, JM and BBM 3N media respectively.

3.2. Biomass Estimation

3.2.1. Dry Weight

By day 15, the dry weight of Kossou-4 grown in the BG11 medium was the highest, reaching ~2.19 $g \cdot L^{-1}$ compared with 0.97 $g \cdot L^{-1}$ in JM medium and 0.52 $g \cdot L^{-1}$ in BBM 3N medium (**Figure 1(a)**). ANOVA for dry weight $mg \cdot L^{-1}$ values at day 15 for Kossou-4 showed a significant difference between media. Tukey's HSD showed significant differences between BG11 and JM ($p < 0.01$), between BG11 and BBM-N3 ($p < 0.01$) and between JM and BBM-N3 ($p < 0.05$).

At day 15, the dry weight of Overjuyo-3 grown in the BG11 medium reached ~2.53 $g \cdot L^{-1}$ compared with 0.87 $g \cdot L^{-1}$ in the JM medium and 0.42 $g \cdot L^{-1}$ in BBM 3N medium (**Figure 1(b)**). ANOVA for dry weight ($mg \cdot L^{-1}$) at day 15 for Overjuyo-3 showed a significant difference between media.

3.2.2. Optical Density (OD at 680 nm)

Growth measurement at an optical density of 680 nm, indicated that Kossou-4's growth was greater in the BG11 medium than in other media. **Figure 1(c)**, shows the sharp rise in the growth of Kossou-4 in BG11 medium at this O.D (680 nm) while the growth in the other two media were found to be similar but lower than was observed in BG11. There were significantly different results between all three media at day 15. The OD values at 680 nm in BG11 (0.56 nm) were 1.7-fold higher than in JM (0.33 nm) and 2.1 fold higher than in BBM-N3 medium (0.26 nm).

At day 15, Overjuyo-3 grown in BG11 medium reached an OD value of 0.58 (1.6 and 1.9 fold higher than in JM and BBM-N3 respectively) compared with 0.37 in JM medium and 0.31 in BBM 3N medium (**Figure 1(d)**). The ANOVA for Optical density at 680 nm at Day 15 for Overjuyo-3 showed significant differences between media. Tukey's HSD showed significant differences between BG11 and JM ($p < 0.05$), between BG11 and BBM-N3 ($p < 0.05$) and between JM and BBM-N3 ($p < 0.05$).

3.2.3. Optical Density (OD at 750 nm)

When measured at an OD of 750 nm, there was a gradual increase in algal growth of Kossou-4 over a 15-day time-frame. Growth in the BG11 medium was higher than in the other two media (JM and BBM). At day 15, both Kossou-4 (**Figure 1(e)**) and Overjuyo-3 (**Figure 1(f)**) showed a similar trend with the highest growth in BG11, followed by JM and BBM-N3 media with significant differences being observed ($p < 0.05$).

3.2.4. Chlorophyll Fluorescence (CF at 430 nm)

The chlorophyll fluorescence readings of Kossou-4 samples are shown in **Figure 1(g)**. The samples showed steady increases in CF measured at 430 nm over the 15 days of growth. At day 15, the chlorophyll fluorescence (CF) of Kossou-4 culture grown in the BG11 medium reached 650 nm compared with 330 nm in the JM medium and 202 nm in the BBM 3N medium (**Figure 1(g)**). Tukey's HSD showed significant differences between growth in BG11 and JM ($p < 0.01$), between BG11 and BBM-N3 $p < 0.01$) and between JM and BBM-N3 ($p < 0.01$). A similar trend was observed in Overjuyo-3 (**Figure 1(h)**).

3.3. Oil Extraction Efficiencies of Solvents (Dry Weight)

3.3.1. Hexane

At day 15, the total oil extracted by n-hexane from Kossou-4 cultures grown in the BG11 medium was 762.3 $mg \cdot L^{-1}$ (0.76 $g \cdot L^{-1}$) compared with 395.6 $mg \cdot L^{-1}$ (0.40 $g \cdot L^{-1}$) of oil extracted in the JM medium and 287.3 $mg \cdot L^{-1}$ (0.29 $g \cdot L^{-1}$) oil extracted in the BBM 3N medium (**Table 1**). At day 15, the total oil extracted by n-hexane from Overjuyo-3 cultures grown in the BG11 medium was slightly lower; 644.5 $mg \cdot L^{-1}$ (0.65 $g \cdot L^{-1}$) (BG11), 382.4 $mg \cdot L^{-1}$ (0.38 $g \cdot L^{-1}$) (JM) and 268.6 $mg \cdot L^{-1}$ (0.27 $g \cdot L^{-1}$) (BBM-N3) (**Table 1**). There were significant differences between the amount of oil recovered from microalgae grown in different media. Tukey's HSD showed significant differences between BG11 and JM ($p < 0.05$), between BG11 and BBM-N3 ($p < 0.05$) and between JM and BBM-N3 media ($p < 0.05$).

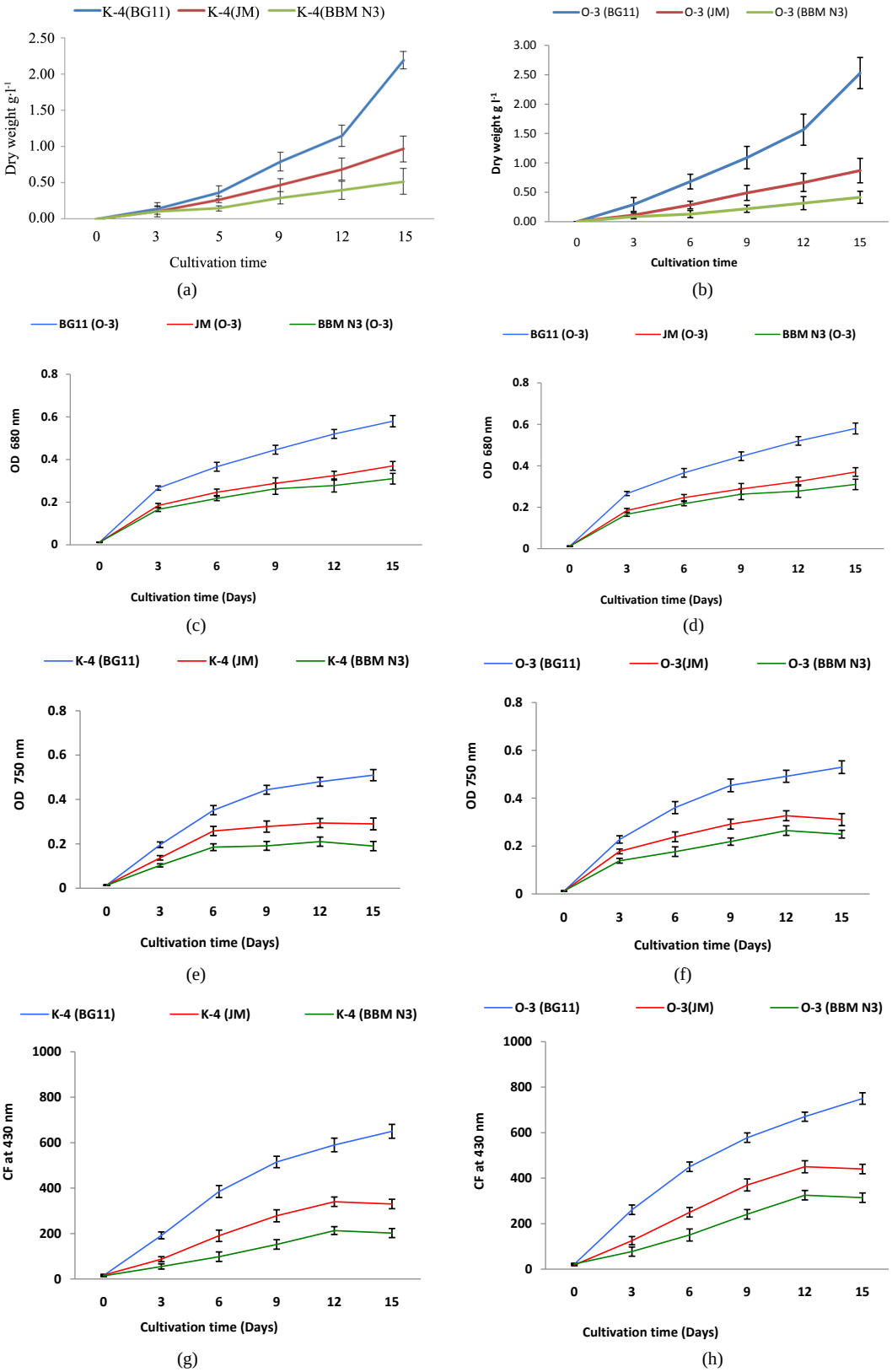

Figure 1. Effects of growth media on the dry weight (a) and (b), ODs at 680 (c) and (d) and 750 nm (e) and (f) and chlorophyll fluorescence (g) and (h) (at 430 nm) of *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3) over 15 days.

3.3.2. Hexane-Isopropanol

At day 15, the total oil weight extracted with hexane and isopropanol from Kossou-4 cultures grown in the BG11 and other medium was lower than the total oil recovered from hexane extractions; BG11 (528.5 mg·L^{-1} or 0.53 g·L^{-1}), JM (361.4 mg·L^{-1} or 0.36 g·L^{-1}) and BBM 3N (235.6 mg·L^{-1} or 0.24 g·L^{-1}) (**Table 1**). Statistical analyses (Tukey's HSD) showed significant differences between BG11 and JM ($p < 0.05$), between BG11 and BBM-N3 ($p < 0.05$) and between JM and BBM-N3 media ($p < 0.05$). The same trend was observed in Overjuyo-3 cultures with a lower amount of total oil recovered compared to hexane with the weight of recovered oil being significantly different between media ($P < 0.05$) (**Table 1**).

3.3.3. Heptane

At day 15, the total oil extracted by n-heptane from Kossou-4 cultures grown in the BG11 and other media was the lowest amongst the three solvents tested; BG11 (345.5 mg·L^{-1} or 0.35 g·L^{-1}), JM (222.3 mg·L^{-1} or 0.22 g·L^{-1}) and BBN 3N (176.1 mg·L^{-1} or 0.18 g·L^{-1}) (**Table 1**). Tukey's HSD showed significant differences between the three different media and a similar trend was observed in Overjuyo-3 cultures.

3.4. Measurement of the Absorbance by Extracted Oil

The effects of extracted oil at day 15 (no algal biomass) on absorbance reading were assessed. The absorbance of oil extracted from Kossou-4 cultures grown in the BG11 medium reached 0.090 at 680 and 0.080 at 750 nm compared with 0.072 at 680 and 0.059 at 750 nm in the JM medium and 0.060 at 680 and 0.0450 at 750 nm in the BBM 3N medium (**Figure 2(a)**). The absorbance values of oil extracted Overjuyo-3 cultures grown in the three media are shown in **Figure 2(b)**. The results showed that the presence of oil (without any biomass) in the media caused a detectable increase in optical density readings.

4. Discussion

In previous studies, *B. braunii* strains had been cultured in a range of media and were reported to have grown successfully in JM [24], BBM-N3 (http://www.ccap.ac.uk/media/documents/3N_BBM_V_000.pdf) and BG11 media [10]. However there is a gap in knowledge regarding the media which is optimal for biomass yield for *B. braunii* race B strains Kossou-4 and Overjuyo-3. This study fills this gap by showing that microalgal cultures in BG11 resulted in the highest biomass yield (assessed by dry weight) amongst the three media tested. The same trend was observed in biomass assay through OD, and chlorophyll fluorescence for both strains. There are many reports on the growth of *B. braunii* strains in different but single medium [11] [13] [14] [30] although none of

Table 1. Extraction of oil produced by *B. braunii* strains Kossou-4 and Overjuyo-3 cultures in different media using different solvents.

Extraction by different solvents	Media	Kossou-4	Overjuyo-3
		Oil extraction mg·L^{-1}	Oil extraction mg·L^{-1}
Hexane	BG11	762.3 (±0.23)	644.5 (±0.21)
	JM	395.6 (±0.11)	382.4 (±0.21)
	BBM 3N	287.3 (±0.06)	268.6 (±0.21)
Hexane & Isopropanol	BG11	528.5 (±0.15)	530.9 (±0.35)
	JM	361.4 (±0.25)	239.4 (±0.35)
	BBM 3N	235.6 (±0.05)	221.5 (±0.35)
Heptane	BG11	345.5 (±0.12)	318.3 (±0.20)
	JM	222.3 (±0.12)	206.5 (±0.20)
	BBM 3N	176.1 (±0.12)	167.2 (±0.20)

Note: Day 15 cultures used, (n = 3) and standard deviations shown.

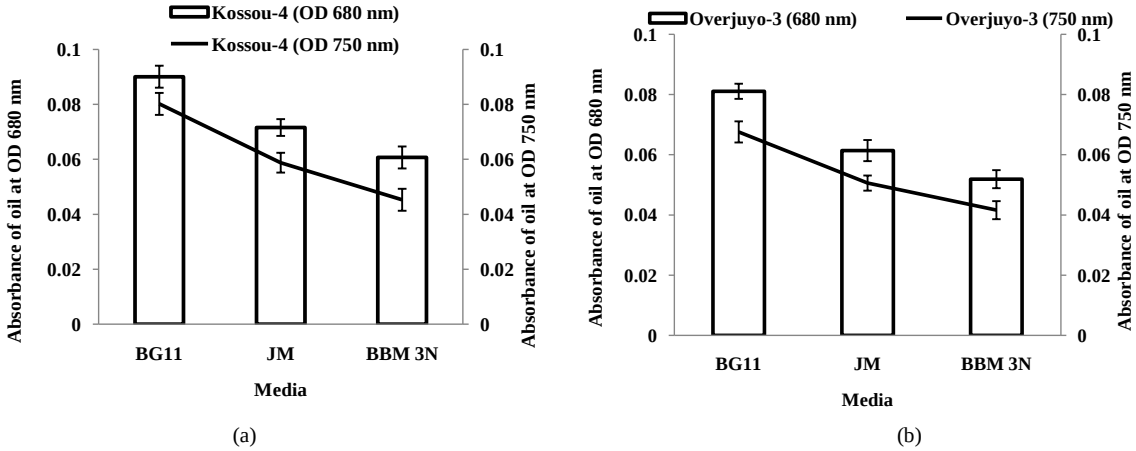

Figure 2. Absorbance values of extracted oil in different media at ODs of 680 and 750 nm (a) & (b) in *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3).

these studies compared all the three media used in this study or used the same strains. One key difference between BG11 and the other media tested was the relatively high content of sodium nitrite (1.5 $g \cdot L^{-1}$) in BG11 and this may have contributed to the enhanced microalgal growth observed in this medium [13]. In addition, BG11 was richer in key nutrients that favoured the growth of these strains, which were absent in the other media. A detailed analysis of the BG11 media components showed that it was rich in some trace elements and these were absent in JM and BBM 3N media. For example, $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$ and ($C_6H_8FeNO_7$) are present in BG11 but absent in the other media (**Table A1**).

With regards to optical density measurements, previous studies have measured OD at different wavelengths but these studies did not use *B. braunii strains* Kossou-4 and Overjuyo-3. For example, four wavelengths (438, 540, 678 and 750 nm) have been used to measure microalgal growth and similar patterns of change were found for each OD in each of the algae tested [31]. In general, higher OD readings were found at the lower wavelengths. In our study, the readings were also taken at 680 nm rather than at 750 nm alone for both strains. Griffiths *et al.* (2011) also used OD of 680 and 750 to investigate the effects of the pigment content of *Chlorella vulgaris* on estimates of growth and reported that OD 750 readings were much less affected by changes in pigment than were readings at 680 nm [18]. In our experiment, only Kossou-4 produced pigments and there was no evidence of the OD 680 readings being affected, but there was only a little pigmentation in the Kossou-4 since the experiment was conducted during the early growth stages. Therefore it is possible that an effect of pigmentation of Kossou-4 on OD could arise with a longer duration of the experiment and consequently greater pigmentation. This issue should be considered when using OD as an estimate of biomass in Kossou-4.

Comparing dry weight assays with OD assays, different growth patterns were observed in the first six days. While dry weight assays showed very little growth from day 0 to day 6, OD assays at 680 and 750 nm appear to show that most microalgal growth (>60% of total growth) had occurred within this time frame. The same trend was observed in CF assays for both strains. Therefore, optical density measurements appear to over-estimate microalgal biomass for these two *Botryococcus braunii* strains compared to dry weight assays. OD based assays are relatively easier to carry out than dry weight based assays and have been used in many studies for estimating the biomass of microalgae [11]. **Figure 2** shows that there were absorbance readings when only extracted microalgal oil (no biomass) was present in the culture medium. Therefore, the reason for this over estimation appears to be related to the presence of oil in the culture. Given that both Kossou-4 and Overjuyo-3 were producing oil extracellularly during the OD based biomass assay, it was possible that absorbance associated with the oil contributed to the overestimation of biomass by optical density based methods. Therefore, OD based assay methods may not be appropriate for estimating biomass in oil producing microalgae. If the research questions were focussed on only changes in the biomass of microalgae that does not produce hydrocarbons (oil), then OD measurements would be faster and more appropriate. However, if other assays are planned (such as as says for total oil, squalene and botryococcene production) in addition to biomass assay, dry weight based assays would be more appropriate.

With respect to oil (hydrocarbon) production, the highest concentration was observed in both strains in BG11

medium, irrespective of the solvent used or assay method. Although Overjuyo-3 showed the higher biomass, oil production was higher in Kossou-4 in all measurements, although the differences were not substantial. This was similar to the result by another group of investigators [12] which showed a slightly lower hydrocarbon content for Overjuyo-3 compared to Kossou-4. The reason for this unclear, however it might be related to the genetic make-up of the Kossou-4 strains.

Previous studies had involved the use of a variety of solvents and solvent combinations for extracting oil from microalgae. These include chloroform-methanol [20], hexane [21], hexane-isopropanol [20] and heptane [22] solvents; however comparisons between solvent systems for oil extraction from *B. braunii* are not available. Chloroform was not used because of its high toxicity. Of the three lower toxicity solvents evaluated in this experiment, hexane yielded the highest oil content for both strains in all media while heptane yielded the least content. This suggests that hexane should be the preferred solvent for extraction when assessing oil production in these *B. braunii* strains; higher oil production was obtained with n-hexane according to [32]. Moreover, hexane-based processes have been in commercial operation for a long time. For such processes oil yields in excess of 95% can be achieved with a solvent recovery of over 95%.

5. Conclusion

These experiments have shown that biomass production is highly influenced by the type of the medium used for culturing both Kossou-4 and Overjuyo-3 strains of *B. braunii*. The BG11 medium produced significantly higher growth compared to the other media after 15 days for both strains but a different biomass trend was observed when Kossou-4 and Overjuyo-3 biomass were measured indirectly using optical density in the first 6 days. Overestimation of biomass by OD measurements was associated with the presence of the oil in growth media. Dry weight assays were therefore more accurate than OD measurements for biomass estimation. Oil production was also significantly higher in BG11 medium. The same result was obtained when three different solvents were used to extract oil. Of these, hexane extracted the highest oil by weight. These results suggest that BG11 is the best growth medium while hexane is the best solvent for oil extraction for these two strains under the conditions of this experiment.

6. Highlights

- Study carried out on B. braunii race B strains, Kossou-4 and Overjuyo-3;
- Different media (BG11, JM and BBM-3N) were used to assess biomass yield;
- Efficiencies of different solvents for oil extraction was assessed;
- Growth measurement at ODs (680 and 750 nm) affected by oil production;
- Biomass and oil production were highest in BG11;
- Overjuyo-3 produced higher biomass than Kosssou-4;
- Kossou-4 had higher oil production than Overjuyo-3;
- Hexane extraction released the highest oil concentrations in both strains.

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Appendix

Table A1. Composition of the different autotrophic culture media for *B. braunii strains* Kossou-4 and Overjuyo-3.

Composition	BG11 ($g \cdot L^{-1}$)	JM ($g \cdot L^{-1}$)	BBM 3N ($g \cdot L^{-1}$)
$NaNO_3$	1.5	8.0	25.0
$MgSO_4 \cdot 7H_2O$	0.075	5.0[1]	7.5
$CaC_{l2} \cdot 2H_2O$	0.036	-	2.5
KH_2PO_4	-	-	17.5
$NaHCO_3$	-	1.59	-
$K_2HPO_4 \cdot 3H_2O$	0.040	1.24	7.5
$Na_2HPO_4 \cdot 12H_2O$	-	3.6	-
NaCl	-	-	2.5
$Ca(NO_3)_2 \cdot 4H_2O$	-	2.0	-
H_3BO_3	0.00286 Trace element	0.248 Trace element	-
$MnCl_2 \cdot 4H_2O$	-	0.139 Trace element	-
$MnSO_4 \cdot H_2O$	0.00181 Trace element	-	-
$ZnSO_4 \cdot 7H_2O$	0.000222 Trace element	-	-
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	-	0.1	-
$Na_2MoO_4 \cdot 2H_2O$	0.00039 Trace element	-	0.004
$CuSO_4 \cdot 5H_2O$	0.000079 Trace element	-	-
$Co(NO_3)_2 \cdot 6H2O$	0.00049 Trace element	-	-
Ferric ammonium citrate ($C_6H_8FeNO_7$)	0.006	-	-
Citric acid ($C_6H_8O_7$)	0.006	-	-
$FeCl_3 \cdot 6H_2O$	-	-	0.097 Trace element
$MnCl_2 \cdot 4H_2O$	-	-	0.041 Trace element
$ZnCl_2$	-	-	0.005 Trace element
$CoCl_2 \cdot 6H_2O$	-	-	0.002 Trace element
Thiamine-HCl (Vitamin B1)	-	0.004	0.12
Vitamin B12	-	0.004	0.1
EDTA-Na_2	0.006	0.225	-
EDTA-Fe-Na	-	0.225	-
Biotin	-	0.004	-