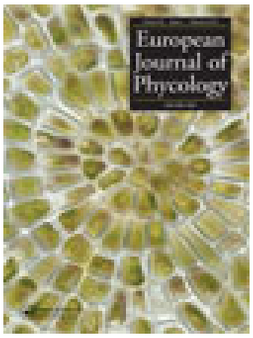

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The effect of nutrients and environmental conditions on biomass and oil production in *Botryococcus braunii* Race B strains

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The green alga *Botryococcus braunii* is widely recognized as a source of non-fossil oil. However, limitations in *Botryococcus* biomass production hamper its commercial exploitation. This study examines the effects of nutrients (nitrogen and iron) and environmental conditions (temperature, light intensity and photoperiod) on biomass and oil production in two *B. braunii* Race B strains, Kossou-4 and Overjuyo-3. The highest biomass and oil production were obtained at a nitrogen concentration of 750 mg l^{-1}, iron concentration of 6 mg l^{-1}, at 25°C and at 135 µmol photons m^{-2} s^{-1} with a photoperiod of 16 h light:8 h darkness. Culturing the strains in Blue-green (BG11) medium containing optimized nutrients under optimal conditions resulted in an up to ~10.6-fold increase in biomass. In Kossou-4 and Overjuyo-3 strains, biomass increased from 1.647 g 10 l^{-1} and 3.137 g 10 l^{-1} respectively in normal BG11 medium to 17.390 g 10 l^{-1} and 21.721 g 10 l^{-1} in optimized BG11 media and growth conditions. This was accompanied by ~8–10.5-fold increase in oil production compared with that in normal BG11 medium. Oil (0.324 g 10 l^{-1} and 0.211 g 10 l^{-1}) was produced in normal BG11 medium in Kossou-4 and Overjuyo-3 strains respectively, compared with 2.642 g 10 l^{-1} (Kossou-4) and 2.206 g 10 l^{-1} (Overjuyo-3) in modified BG11 media under optimized conditions. Therefore, optimization of nutrients and environmental conditions can increase biomass and oil production in the two strains of *B. braunii*.

Key words: *B. braunii*, biomass, culture conditions, nutrients, oil production, Race B

INTRODUCTION

Global warming driven by greenhouse gases such as CO_2 from fossil fuels, chlorofluorocarbons, CH_4 and N_2O is of concern due to its adverse environmental and socio-economic impacts (Hansen *et al.*, 2000; Munday *et al.*, 2012; Scheffran & Battaglini, 2011). Mitigation of the effects of global warming gases requires approaches such as reduction in fuel usage, decarbonization, carbon sequestration (Abbasi & Abbasi, 2011; VijayaVenkataRaman *et al.*, 2012) and the use of alternative renewable and non-polluting fuel sources (Panwar *et al.*, 2011). The need for alternative energy sources has led to increasing interest in biofuel production (Nigam & Singh, 2011). Microalgal biomass is an alternative source for oil from which biodiesel (hydrocarbons), bioethanol and bio-oil can be produced (Borowitzka & Moheimani, 2013; Collet *et al.*, 2014; Sarkar *et al.*, 2014). It has been reported that the green alga *Botryococcus braunii* represents one of the most promising photosynthetic organisms, since it can produce economically useful quantities of hydrocarbons by utilizing artificial or natural light (de la Noue & de Pauw, 1988; Ranga Rao *et al.*, 2012; Khatri *et al.*, 2014). *Botryococcus braunii* can accumulate unusually high levels of hydrocarbons in the range of 11–35% of its dry weight (Metzger & Largeau, 2005; Ranga Rao *et al.*, 2012; Ashokkumar *et al.*, 2015) with up to 76% of the dry weight of the cell material being combustible. For this reason, *B. braunii* is seen as a potential source of renewable biofuels.

Depending upon the type of hydrocarbons found inside its cells, *B. braunii* can be classified into three chemical races (A, B and L) (Metzger *et al.*, 1990; Khatri *et al.*, 2014). Race A contains C_{21} to C_{33} odd numbered n-alkadienes, and mono-, tri, tetra and pentanes while race B produces two types of triterpenes called botryococcenes, C_{30}-C_{37} with a general formula C_nH_{2n+2} as major hydrocarbons and similar amounts of methyl branched squalene. *Botryococcus braunii* belonging to race

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L produce a single C40 isopropenoid hydrocarbon, lycopa-14 (E), 18(E)-diene (Metzger *et al*., 1990). Race B is especially interesting as the squalene produced by this race as a component of microalgal oil is a useful biofuel, similar to fractions found within heavy fuel oil. In addition, while other algae store oils intracellularly, *B. braunii* discharges oils (including hydrocarbons) extracellularly, which may allow biofuel to be continuously acquired from *B. braunii* without cell disruption, a process termed 'milking' (Zhang *et al*., 2013).

Overjuyo-3 and Kossou-4 are two *B. braunii* Race B strains in Pierre Metzger's collection with more than 21% of their dry weight composed of oil (especially C_{31}-C_{36} hydrocarbons), making them suitable for biofuel and bioenergy production (Li *et al*., 2013). Given the depth of understanding of the growth requirement for macronutrients in *B. braunii*, it is surprising that the requirements for trace elements of these two strains are not well known (Song *et al*., 2012). Nutrients such as nitrogen and iron can play critical roles in a variety of metabolic pathways involving the utilization of light, phosphorus and CO_2 for biomass and hydrocarbon production (Raven, 1990; Chen *et al*., 2010). Among the trace elements, iron is essential for photosynthetic electron transport, respiratory electron transport, nitrate and nitrite reduction and detoxification of reactive oxygen species (Liu *et al*., 2008).

Optimization of micronutrient requirements is an important undertaking prior to the establishment of the sustainable production of *B. braunii* on a large scale (Song *et al*., 2012). Consequently, there is a need to determine the optimum nutrient concentrations needed for growth and oil production by these two strains of *B. braunii*. In addition to nutrients, microalgal cell growth rates and oil production are affected by a combination of environmental parameters such as light intensity, photoperiod and temperature (Parmar *et al*., 2011). Microalgae can use light as their source of energy as photoautotrophs (Wahidin *et al*., 2013), although heterotrophic nutrition is also known to occur (Pleissner *et al*., 2013). Temperature has been found to have a major effect on the fatty acid composition of microalgae (Cheng *et al*., 2013). However, the optimal light intensities, photoperiod and environmental temperature that are required for oil production in the two selected strains of *B. braunii* have not been adequately investigated.

Consequently this study aims to determine the optimal nutrients and environmental conditions for increased biomass and oil production in two *B. braunii* strains (Kossou-4 and Overjuyo-3). Unlike previous work published on nutrient optimization of immobilized *B. braunii* (Cheng *et al*., 2013; Cheng *et al*., 2014), this study involves the use of non-immobilized microalgae. The effects of temperature, nitrogen concentrations, iron concentrations, light intensities and photoperiods on the growth and oil production in these strains were evaluated using different assay methods.

MATERIALS AND METHODS

Microalgal 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 oil (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 colour while the Overjuyo-3 strain was from Bolivia and is green in colour (Metzger *et al*., 1990).

Experimental design, medium and culture preparation

Table 1 shows the experimental design used for this study in several experiments each of 60 days duration. Four experiments were carried out sequentially using Blue-green (BG11) medium. The first experiment involved altering only the nitrogen concentration of BG11 medium and growing each of the two strains in this altered medium. The second experiment involved the growth of the strains in BG11 medium with altered iron concentrations and using the nitrogen concentration assessed to be the best for biomass and oil yield from the first experiment. The third experiment involved altering the incubation temperature using the best nitrogen and iron concentrations from experiments 1 and 2 in the modified medium. The final experiment involved the use of varying light intensities and photoperiods (Table 1) in BG11 medium at the best nitrogen and iron concentrations and temperature. The different concentrations and conditions used (Table 1) and the assessment of the effects of all these alterations on biomass and oil production are described below.

The BG11 (pH 7.4) medium used in this study was prepared as previously described (Ge *et al*., 2011). The unmodified BG11 medium contained the following components (mg l^{-1}): $NaNO_3$ (1500), $K_2HPO_4.3H_2O$ (40), $MgSO_4.7H_2O$ (75), $CaCl_2.2H_2O$ (36), $C_6H_8O_7.H_2O$ (6), $Fe(NH_4)_3C_{18}H_{10}O_{14}$ (6), Na_2-EDTA (1), Na_2CO_3 (20), H_3BO_3 (2.86), $MnC_{12}.H_2O$ (1.81), $ZnSO_4.7H_2O$ (0.222), $CuSO_4.5H_2O$ (0.079), $Na_2MoO_4.2H_2O$ (0.39), $Co(NO_3)_2.6H_2O$ (0.049). The pH of the medium was adjusted to 7.4 by adding NaOH and HCl (0.1 N) before autoclaving at 121 ºC for 15 minutes.

Incubation conditions and sampling

Erlenmeyer flasks (1000 ml) containing 600 ml of normal or modified BG11 medium (Table 1) were aseptically inoculated with 0.04 g l^{-1} (dry weight) of Kossou-4 or Overjuyo-3 and incubated on a Ratek incubator shaker (Adelab Scientific) for up to 60 days at 100 rpm and at the desired temperature (20, 25 or 30ºC). Continuous illumination was supplied by cool white fluorescent lamps at the desired light intensities (54, 81 or 135 µmol photons m^{-2} s^{-1}) and

Table 1. Experimental design.

	Culture conditions				
Experimental runs	Nitrogen (mg l^{-1})	Iron (mg l^{-1})	Temperature (ºC)	Light intensity (µmol photons m^{-2} s^{-1})	Photoperiods (h)
1	Variable[a]	Normal	25	54	24
2	Best	Variable[b]	25	54	24
3	Best	Best	Variable[c]	54	24
4	Best	Best	Best	Variable[d]	Variable[e]

Note: All experiments were carried out using BG11 medium for 60 days, sequentially with *B. braunii* Kossou-4 and Overjuyo-3 strains. Sampling was carried out at 10 day intervals. Normal refers to the original nutrient concentration in BG11 medium. Biomass and oil yields analyses carried out for each experimental run. Best refers to the parameter (concentration, temperature or light intensity) that gave the highest biomass and oil yield from the previous experimental run(s).

[a]For experimental run 1, only the nitrogen concentration of BG11 medium was altered. Two altered concentrations, 370 (0.25×) and 750 (0.5×) mg l^{-1} and the normal nitrogen concentration (1×) of 1500 mg l^{-1} were used.

[b]For experimental run 2, the iron concentration was altered. Two altered concentrations, 3 (0.5×), and 9 (1.5×) mg l^{-1} and the normal iron concentration (1×) of 6 mg l^{-1} were used alongside the best nitrogen concentration from run 1.

[c]For experimental run 3, three temperatures, 20, 25 and 30ºC were used alongside the best nitrogen concentration from run 1 and run 2.

[d, e]For experimental run 4, three different light intensities, 54, 81 and 135 µmol photons m^{-2} s^{-1} were used with the best concentrations and parameter from runs 1, 2, and 3. Each light intensity was evaluated concurrently at three different photoperiods (24 h of light, 12 h of light:12 h of darkness and 16 h of light:8 h of darkness).

photoperiods (Table 1). LM 37 Luxmeter (Dostman Electronic, Wertheim-Reicholzheim, Germany) with a resolution of 0.01 lx to ~ 10 lx; 0.001 fc to 1 fc and accuracy of ±3% was used to measure the light intensity. All experiments were carried out in triplicate.

Cells for biomass assays were sampled non-destructively by taking ~11 ml of samples from replicate flasks of each microalgal strain from day zero to day 60 at 10 day intervals (in order to allow for sufficient algal growth). Microalgal oil production was also determined, using 40 ml samples from replicate flasks at 10 day intervals. Non-destructive sampling for oil production was however carried out only from day 30 to day 60.

Experimental runs using different culture conditions

Nitrogen concentrations. The first experiment involved changing the nitrate concentrations ($NaNO_3$ component) of BG11 in order to assess the effects of this change on the biomass and oil yield of the two strains. The concentrations used were 370, 750 and 1500 mg l^{-1} which corresponded to 0.25× (quarter strength), 0.5× (half strength) and 1× (normal strength) of $NaNO_3$ component of BG11 medium. All other media components were used in the concentrations described previously (Ge *et al*., 2011). Microalgal cultivation was then carried out in BG11 media at these concentrations at 25°C for up to 60 days in continuous light at 54 µmol photons m^{-2} s^{-1}. Sampling was carried out as described earlier for biomass and oil yield determination.

Iron concentrations. Iron concentration of the BG11 medium was altered for the second experiment according to the best nitrogen concentration (that gave the best biomass and oil yield). Three concentrations, 3, 6 and 9 mg l^{-1} of Fe $(NH_4)_3C_{18}H_{10}O_{14}$ were used. These concentrations corresponded to 0.5×, 1× and 1.5× of the original 1× concentration of 6 mg l^{-1}. All other BG11 media components were unchanged. At the conclusion of the experiment, microalgal biomass and oil yield assays were carried out as described below in the measurements of biomass production section.

Temperature trials. The effects of different temperatures on the biomass and oil production by the two *B. braunii* strains were assessed in the third sequential experiment. Replicate cultures were grown at 20, 25 and 30ºC for up to 60 days in continuous light at 54 µmol photons m^{-2} s^{-1}. These cultures were grown in nitrogen and iron concentrations of BG11 medium previously determined to produce highest biomass and oil yields. All other media components were unchanged and sampling for biomass and oil yield assays were carried out as previously described.

Light intensity and photoperiod trials. The effects of different light intensities and photoperiods on the growth and oil production by the two microalgal strains were evaluated in the fourth experiment. This was carried out in BG11 medium with nitrogen and iron concentrations and temperature assessed to produce the highest biomass and oil yields. All other media components remained unchanged. Microalgae were cultured at three different light intensities (54, 81 and 135 µmol photons m^{-2} s^{-1}). Additionally, at each light intensity, three photoperiods were used (24 h of light, 12 h of light:12 h of darkness and 16 h of light:8 h of darkness). Sampling for biomass and oil yield assays were carried out as previously described.

Measurements of biomass production

Replicate samples obtained from these sequential experiments were subjected to five different biomass assays: cell count, optical density (at 680 nm and 750 nm), dry weight and chlorophyll fluorescence.

Cell count

Cells were counted using the Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's guidelines. A selected sample (10 µl) was vigorously mixed with 10 µl of Trypan blue stain (BioRad, Australia) by continuous pipetting. An aliquot (10 µl) was added to the disposable slides and loaded into the chamber ports of the cell counting

chamber. Counts were recorded for live (i.e. stained) and dead cells. Growth was measured as total live cell count per ml as previously described (Madigan, 2005).

Optical densities (ODs)

Optical density provides a measure of algal growth; higher absorbance values indicate greater growth (Griffiths *et al*., 2011). Optical density was assessed using a POLARstar Omega Microtitre (BMG Labtech) plate reader. An aliquot (200 µl) of algal suspension was added to each selected well (in replicates) in a 96-well microtitre plate. Before taking the individual reading, the plate was shaken for 30 s continuously. Light absorbance was measured at wavelengths of 680 nm and 750 nm.

Chlorophyll fluorescence (CF)

A POLARstar Omega microtitre plate reader was also used to measure chlorophyll fluorescence. Culture solutions (200 µl) were taken and placed in 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 Mohsenpour *et al*. (2012).

Dry weight (DW)

An aliquot (10 ml) of each sample solution was taken and 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 to constant weight at 65°C. 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 in a constant aliquot (10 ml), which was then expressed as percentage dry weight values as previously described (Zhu & Lee, 1997).

Measurement of oil production

Oil was extracted from *B. braunii* strains Kossou-4 and Overjuyo-3 as previously described (Sawayama *et al*., 1992). Briefly, algal dry weight was measured gravimetrically in a freeze-dried sample. Freeze-dried algal cells in 50 ml n-hexane were sonicated in a Soniclean bath for 30 min and finally the upper layer was filtered and then transferred into a pre-weighed Agilent glass tube. To determine the amount of oil produced, the n-hexane was evaporated at room temperature in a fume hood, the glass tube reweighed and the difference represented the amount of oil extracted from the sample. Oil from the algae was analysed 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 Auto sampler 7890A (Agilent Technologies Inc., Forest Hill, Australia).

Fluorescence microscopy

To assess biomass growth and detect oil production under 100× magnification, fluorescence microscopy analysis was carried out using a Leica DM 2500 microscope equipped with a Leica DFC 310 FX camera. Excitation was at 543 nm and emission 555–650 nm.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether there were significant increases in biomass and oil production at different time points for each factor being optimized for each strain of *B. braunii*. A full factorial design with two factors (seven levels and three levels) was used. Three replications of the full factorial were conducted. The collected data for each experiment were analysed using multivariate ANOVA by fitting a model including the main effects of the factors and their interactions. Reference was made to day 40 data as biomass and oil yield were highest at this time-frame.

RESULTS

Effects of different nitrogen concentrations

The results of microalgal growth in different nitrogen concentrations are shown in Fig. 1a and b. For both strains, Kossou-4 and Overjuyo-3, the best nitrogen concentration for growth or increased biomass was found to be 750 mg l^{-1} (0.5×), although Overjuyo-3 had a higher biomass yield of 21.24 g 10 l^{-1} compared with 17.23 g 10 l^{-1} for Kossou-4 at day 40. The original nitrogen concentration of 1500 mg l^{-1} (1×) found in BG11 medium led to the lowest biomass yield in both strains. Therefore, a reduction in the initial nitrogen concentration to 750 mg l^{-1} caused a significant increase in biomass in both strains at day 40 compared with other concentrations ($P < 0.05$) as estimated by dry weight measurements. Comparison with the results obtained using OD 680 nm, 750 nm and chlorophyll fluorescence confirmed that all these methodologies had similar trends as observed with dry weight (data not shown). Therefore, the data obtained by dry weight assay were used throughout this report. The highest recovery of oil (2.549 g 10 l^{-1} for Kossou-4 and 2.143 g 10 l^{-1} for Overjuyo-3) was observed in cultures inoculated into medium containing 750 mg l^{-1} nitrogen. At a nitrogen concentration of 1500 mg l^{-1}, significantly lower oil recovery was observed (0.324 g 10 l^{-1} for Kossou-4 and 0.211 g 10 l^{-1} for Overjuyo-3) (Table 2). The optimum nitrogen concentration for the two *B. braunii* strains, Kossou-4 and Overjuyo-4, used in this study has not been previously reported in scientific literature to the authors' best knowledge.

Effects of different iron concentrations

In terms of the influence of iron, an iron concentration of 6 mg l^{-1} (1×) resulted in significantly greater biomass ($P < 0.05$) as compared with 0.5× (3 mg l^{-1}) or

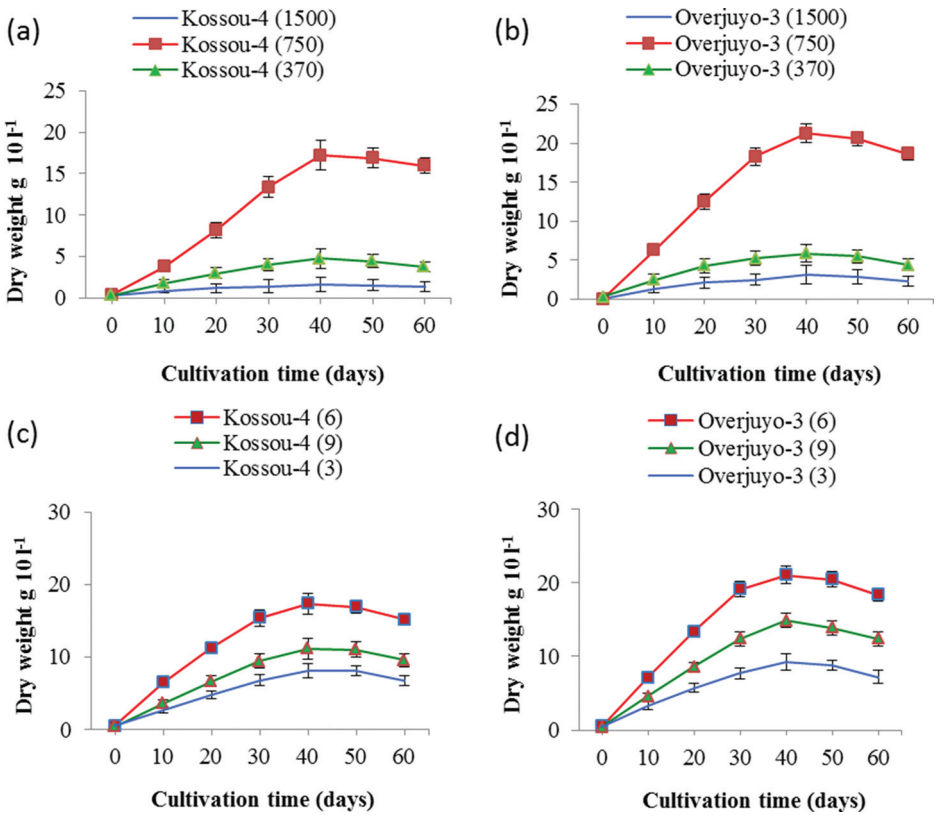

Fig. 1. The effects of nitrogen (a, b) and iron concentrations (c, d) (mg l^{-1}) on the dry weight (g l^{-1}) of *B. braunii* strains Kossou-4 (a, c) and Overjuyo-3 (b, d) respectively. Error bars shown on the graphs represent standard deviation of replicate (n = 3) samples.

Table 2. Oil production by day 40 in *B braunii* Kossou-4 and Overjuyo-3 strains grown under different culture conditions (n = 3).

Treatments	Culture condition		Kossou-4 Oil weight (g 10 l^{-1})	Overjuyo-3 Oil weight (g 10 l^{-1})
[a]Nitrogen concentration mg l^{-1}	370		0.778 (±0.020)	0.571 (±0.031)
	750		2.549 (±0.030)	2.143 (±0.040)
	1500		0.324 (±0.035)	0.211 (±0.031)
[b]Iron concentration mg l^{-1}	3		0.454 (±0.015)	0.351 (±0.035)
	6		2.597 (±0.025)	1.954 (±0.040)
	9		1.142 (±0.020)	0.734 (±0.042)
[*c]Temperature ºC	20		0.772 (±0.030)	0.614 (±0.040)
	25		2.678 (±0.031)	2.130 (±0.252)
	30		0.425 (±0.041)	0.897 (±0.042)
[d]Light intensity (µmol photons m^{-2} s^{-1}) and Photoperiod (h)	54	12 h L/12 h D	0.442 (±0.033)	0.384 (±0.024)
		16 h L/8 h D	0.884 (±0.021)	0.624 (±0.035)
		24 h L	2.561 (±0.040)	2.138 (±0.027)
	81	12 h L/12 h D	0.607 (±0.022)	0.431 (±0.031)
		16 h L/8 h D	0. 917 (±0.034)	0.711 (±0.040)
		24 h L	2.596 (±0.041)	2.182 (±0.026)
	135	12 h L/12 h D	0.903 (±0.044)	0.694 (±0.036)
		16 h L/8 h D	2.642 (±0.041)	2.206 (±0.034)
		24 h L	2.248 (±0.037)	2.039 (±0.025)

Note: Standard deviation of replicate samples shown. L refers to incubation under light conditions, D refers to incubations carried out in the darkness
[a]Standard BG11 components used. Only N_2 concentration varied. Culture grown using standard conditions (25ºC, at 54 µmol photons m^{-2} s^{-1} (24 h continuous light) for 60 days).
[b]Medium used contained optimized N_2 concentration (750 mg l^{-1}). All other medium component concentrations were the same as in the standard BG11 medium. Culture grown using standard conditions (25ºC, at 54 µmol photons m^{-2} s^{-1} (24 h continuous light) for 60 days).
[c]Medium used contained optimized N_2 (750 mg l^{-1}) and Fe (6 mg l^{-1}) concentrations. All other medium component concentrations were the same as in the standard BG11 medium. Culture grown at 54 µmol photons m^{-2} s^{-1} (24 h continuous light) for 60 days).
[d]Medium used contained optimized N_2 (750 mg l^{-1}) and Fe (6 mg l^{-1}) concentrations and incubated at 25ºC. All other medium component concentrations were the same as in the standard BG11 medium.

1.5× (9 mg l^{-1}) (Fig. 1c and d) as assessed using dry weight assays. As observed in the nitrogen concentration optimizations, Overjuyo-3 had a higher biomass yield of 21.07 g 10 l^{-1} compared with 17.32 g 10 l^{-1} for Kossou-4 at day 40 (Fig. 1c and d). In terms of oil extraction (Table 2), for the three iron concentrations, the oil weight was highest at 1× (1.954 g 10 l^{-1}), while the lowest weight of 0.351 g 10 l^{-1} was recorded at 0.5× in Overjuyo-3. The same trend was observed in Kossou-4.

Effects of different temperatures

Using optimized nitrogen and iron concentrations, the effect of temperature at 20, 25 and 30°C on biomass production and oil recovery was assessed (Fig. 2). For both strains of *B. braunii*, dry weight was highest at 25°C in Overjuyo-3 and lowest at 30°C in Kossou-4. Oil recovery followed a similar trend, being highest at 25°C in Kossou-4 and lowest at 30°C (also in Kossou-4). The same oil weight trend was observed in Overjuyo-3 (Table 2).

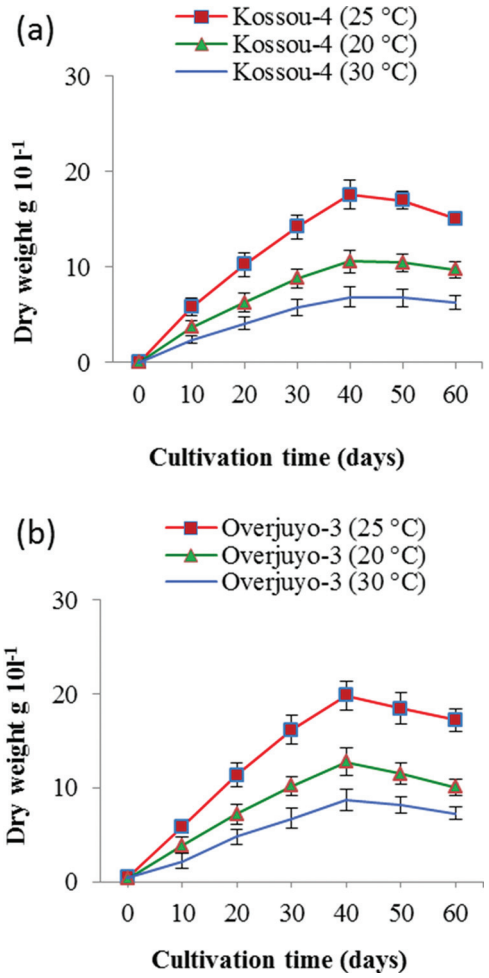

Fig. 2. The effects of temperature on the dry weight (g l^{-1}) of *B. braunii* strains (a) Kossou-4 and (b) Overjuyo-3 (n = 3). Error bars shown on the graphs represent standard deviation of replicate samples.

Effects of different light intensities and photoperiods

In Kossou-4 for the three photoperiods at a light intensity of 54 µmol photons m^{-2} s^{-1} at 25°C, dry weight measurements were highest with continuous light (16.91 g 10 l^{-1}), and lowest at 12 h light/12 darkness (4.53 g 10 l^{-1}) (Fig. 3a). Similarly, for oil weight, maximum production was highest at 24 h light, followed by 16 h light/8 h darkness and lowest at 12 h light/12 darkness in Kossou-4 (Table 2). For *B. braunii* Overjuyo-3 (Fig. 3b) at 25°C, dry weight was also highest under continuous light (20.81 g 10 l^{-1}) and lowest at 12 h light/12 darkness (6.43 g l^{-1}). Oil weight was again highest at 24 h light (2.14 g 10 l^{-1}) and lowest at 12 h light/12 darkness (0.38 g 10 l^{-1}) (Table 2). At an increased light intensity (81 µmol photons m^{-2} s^{-1}), similar results were obtained for both strains over the same photoperiods (Fig. 3c and d). No significant changes in either biomass production or oil extracted were observed between studies at these two light levels (Fig. 3) ($P > 0.05$). However, significant differences in the results were observed at the three photoperiods at a light intensity of 135 µmol photons m^{-2} s^{-1} (Fig. 3 e, f). The dry weight measurements were highest at 16 h light/8 h darkness (17.39 g 10 l^{-1}), and lowest at 12 h light/12 darkness (6.51 g 10 l^{-1}) in Kossou-4. Similarly, oil weight was highest at 16 h light/8 h darkness (2.64 g 10 l^{-1}) and lowest at 12 h light/12 darkness (0.90 g 10 l^{-1}) in Kossou-4 (Table 2). In Overjuyo-3, the same trend was observed, although the dry weight was higher at 16 h light/8 h darkness than for Kossou (Fig. 3f) but with lower oil weight (Table 2).

DISCUSSION

Botryococcus braunii is an alga that shows considerable potential as a renewable source of hydrocarbons (derived from algal oil) for use in biofuels (Metzger *et al*., 1990; Cheng *et al*., 2014). In order to realize this potential it is essential to investigate which *B. braunii* strains produce high yields of biomass and oil (hydrocarbons). From a commercial perspective media and cultivation conditions are crucial for optimal yield. This study has investigated each of these aspects in the two selected strains.

Nitrogen concentrations

With regards to nitrogen concentration, this study is the first (to the best of the author's knowledge) to determine that the optimum nitrogen concentration for *B. braunii* Kossou-4 and Overjuyo-3 strains was 750 mg l^{-1}. This corresponded to half the concentration found in normal BG11 medium. Similar work on BG11 optimization for the cultivation of other *B. braunii* strains (Tran *et al*., 2010) focused on other nutrients such as potassium (phosphate) and

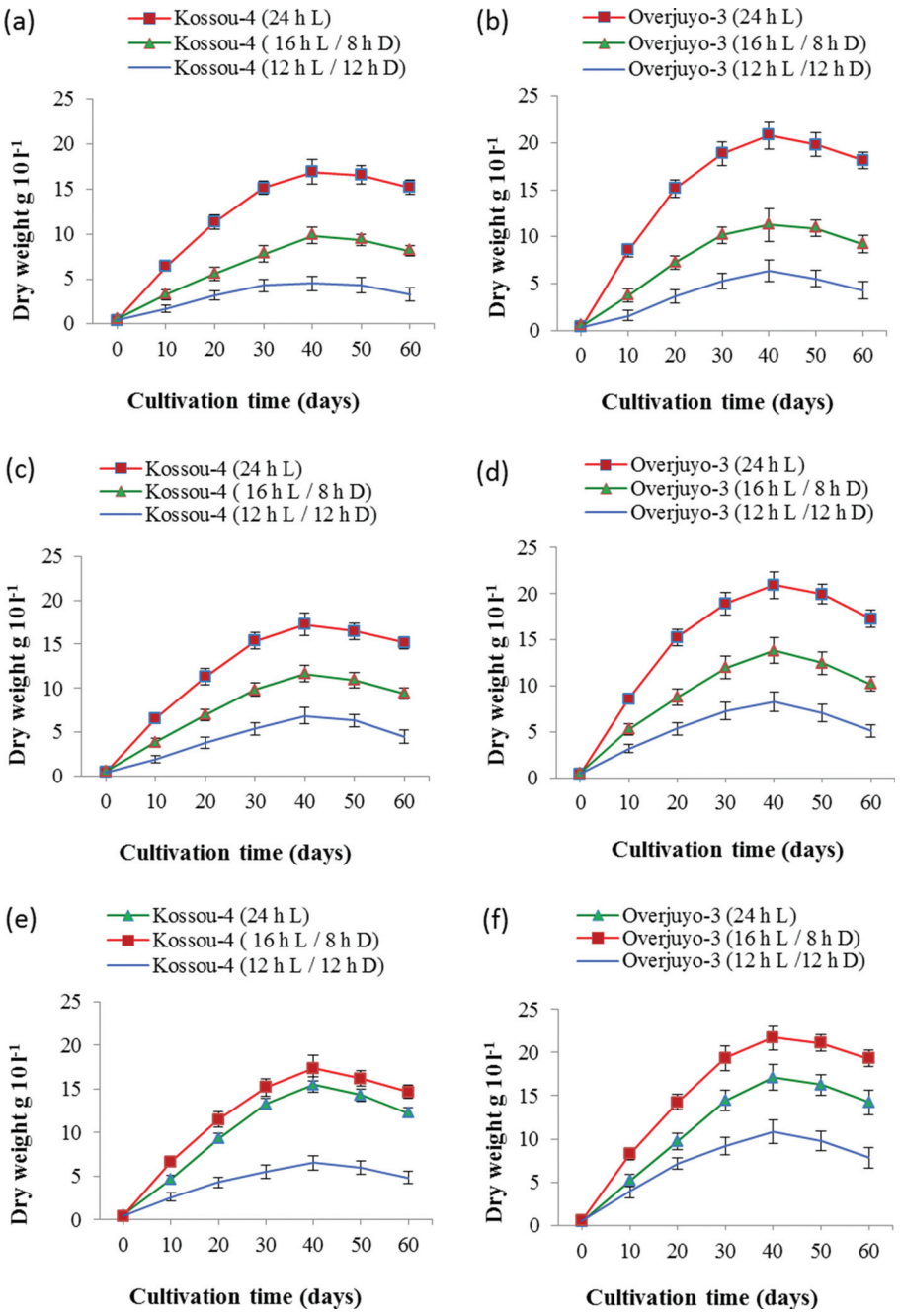

Fig. 3. The effects of photoperiod and light intensity at 54 (a, b), 81 (c, d) and 135 (e, f) µmol photons m^{-2} s^{-1} on the dry weight (g l^{-1}) of *B. braunii* strains Kossou-4 and Overjuyo-3 (n = 3). Error bars shown on the graphs represent standard deviation of replicate samples. L refers to light, D refers to darkness.

magnesium (sulphate) at different nitrogen concentrations (750, 1500 and 2250 mg l^{-1}) with the optimal concentration for nitrogen not being reported. Nitrogen is a very important nutrient for the growth of *B. braunii*. Recent work (Yeesang & Cheirsilp, 2011) on the growth of four *B. braunii* strains (not Kossou-4 or Overjuyo-3) in Chu-13 medium showed better growth under nitrogen rich (with addition of KNO_3) conditions than under nitrogen poor conditions (no KNO_3). Studies of the growth of other *B. braunii* races have shown the benefits of the optimization of nitrogen concentration on biomass yield. For example in the growth of *B. braunii* strain UC58 in Chu-13, the nitrogen concentration in the culture medium was observed to be optimal at 8 mM rather than at either 0.5 mM or 2.0 mM (Lupi *et al.*, 1994). This indicated that higher nitrogen was beneficial for *B. braunii* growth but the nitrogen source used was potassium nitrate rather than sodium nitrate, which is used in BG11 medium. The optimal nitrogen concentration for the growth of the two *B. braunii* strains used in this study was substantially higher than those reported for the optimum growth of other microalgae (Cai *et al.*, 2013). For example, Cai *et al.* (2013) investigated the effects of six nitrogen concentrations (0, 7.5, 15, 37.5, 75 and 150 mg l^{-1}) on the growth of

the unicellular green microalga *Platymonas subcordiformis* over 11 days and found that dry weight was greatest at the highest nitrogen concentration (Cai *et al*., 2013). Therefore, higher nitrogen concentrations could be better for *Botryococcus* growth, although this may be species dependent.

Iron concentrations

Although there is no available information on the *B. braunii* strains used in this study, the effect of iron has been evaluated in another *B. braunii* race B strain BOT-22 grown in modified Chu medium at 25°C (Tanoi *et al*., 2014) and tested with modified media containing 0.1 mg l^{-1} of iron (III) citrate hydrate or 20 mg l^{-1} of iron (III) citrate hydrate for one month. Growth was very slow in the low-iron medium compared with that of the iron-rich culture and iron affected cell size and shape; the addition of glucose further enhanced growth. In our experiment, low growth was found with low iron concentrations but the iron source was different, as was the strain, so it is difficult to compare results. However, this study did show that the iron content of the normal BG11 medium was optimal for the growth and oil production of the two test strains.

Temperature effects

Studies of other *B. braunii strains* (Showa strains) in modified Chu 13 medium reported stable growth at temperatures ranging from 15–30°C, with maximum growth rate observed at 30°C. Hydrocarbon production was also observed to have increased with growth rate (Yoshimura *et al*., 2013). Another report (Kalacheva *et al*., 2002) on other *Botryococcus* strains observed that the optimum growth temperature was 30°C. In *B. braunii* (strain No LB 807/1 Droop 1950 H-25) grown in Prat medium for 14 days under 14 hours light, biomass production was higher at 32°C than at 18 and 25°C (Kalacheva *et al*., 2002). In contrast, in this study the optimum growth temperature for Kossou-4 and Overjuyo-3 strains of *B braunii* was found to be 25°C, with optimum oil production at this temperature. This could be due to the strain differences but could also be due to other effects such as the media and lighting used. In this study, growth was lower at 30°C than at 25°C in both strains. In addition, oil production was best at 25°C but the amount of oil was directly proportional to the level of growth at all temperatures.

Photoperiods and light intensities

When microalgae are exposed to light above the saturation limit, their growth becomes inhibited. On the other hand, light intensity plays an important role and the requirement varies greatly with culture depth and density of the microalgae. If the microalgae are cultured at greater depth and cell concentrations, the light intensity must be increased to penetrate through the culture (Wahidin *et al*., 2013). In nature, the intensity is well above saturation and may be high enough to inhibit growth during much of the day. An insufficient amount of light might however lower the growth rate. Optimization of light conditions is therefore one of the most important factors in establishing cost and energy-efficient mass cultivation of photosynthetic organisms (Ugwu *et al*., 2008).

In this study, the optimum photoperiod at 54 and 81 µmol photons m^{-2} s^{-1} for the highest biomass and oil production was 24 h in the two strains. However at 135 µmol photons m^{-2} s^{-1}, 16 h light/8 h darkness was the optimum photoperiod. The highest biomass and oil production was reported at this photoperiod and light intensity in both strains, suggesting that these were their ideal culture conditions. In all photoperiod and light intensity experiments, Kossou-4 produced more hydrocarbon than Overjuyo-3 (although the latter had greater biomass under the same conditions) indicating that it was able to better utilize the conditions for oil production than Overjuyo-3. The reason for this is not clear but may be related to the genetics of the microalgae. Varying reports have been published concerning the effects of the optimization of light intensity on other *B. braunii* strains. A two-fold increase in hydrocarbon (oil) productivity and biomass under an optimized photoperiod and light intensity has been reported (Brenckmann *et al*., 1985). Additionally, different light intensities and wavelengths have been reported to change the nature of lipid metabolism in microalgae resulting in altered lipid profile (Harwood, 1998).

The *Botryococcus* strains used in this study produced greater biomass yields in modified BG11 medium under optimized environmental conditions compared with growth in the normal BG11 medium, although higher biomass-producing *Botryococcus* strains (30–35 g 10 l^{-1}) have been reported (Zhila *et al*., 2005; Ge *et al*. 2011). In contrast, the strains used in this study had biomass yields of between 17.39 to 21.72 g 10 l^{-1} which are somewhat lower. However, optimization led to a 10-fold increase in biomass yield compared with growth in the unmodified medium (Figure 4). It is therefore possible that greater yield can be obtained when other key nutrients (apart from nitrogen and iron) are optimized. This study demonstrates that optimization of nutrient and environmental conditions are critical to obtain excellent biomass yield specifically in *Botryococcus* strains Kossou-4 and Overjuyo-3 cultured in BG11 medium.

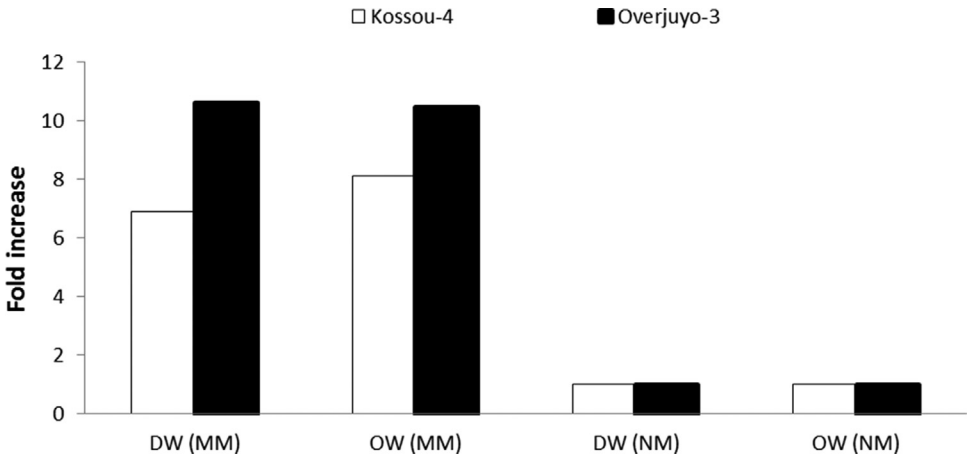

Fig. 4. Comparison of growth and oil production in normal and optimized media for Kossou-4 and Overjuyo-3 assessed by dry weight assay. OW, Oil weight; DW, dry weight; NM, normal BG11 medium; MM, modified BG11 medium.
Note: For NM (nitrogen and iron concentrations of 1500 and 6 mg l^{-1} respectively), values obtained from day 40 cultures grown under 24 h of light at 54 µmol photons m^{-2} s^{-1} at 25ºC. For MM (nitrogen and iron concentrations of 750 and 6 mg l^{-1} respectively), values obtained from day 40 cultures grown under 16 h of light and 8 h of darkness at 135 µmol photons m^{-2} s^{-1} at 25ºC.

CONCLUSIONS

This study has determined the optimum nitrogen (750 mg l^{-1}) and iron (6 mg l^{-1}) concentrations in BG11 for improved biomass and oil production by the *B. braunii* strains Kossou-4 and Overjuyo-3. The optimum temperature (25°C), photoperiod and light intensity (16 h light/8 h darkness at 135 µmol photons m^{-2} s^{-1}) needed for improved biomass (6.9- to 10.6-fold increase) and oil production (8.1- to 10.5-fold increase) was determined. This represents the first reported study of the optimum conditions for growth and oil production for these strains, demonstrating the benefits of optimizing nutrient and environmental conditions in increasing biomass and oil production in *B. braunii* strains for potential commercial activities.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

AUTHOR CONTRIBUTIONS

K. A Al-hothaly, A.Ball and E. Adetutu performed the experiments. K. A Al-hothaly conceived and designed the experiments. All authors analysed the data, and wrote the manuscript.

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