Towards the commercialization of microalgal production: the role of environmental factors in production of triterpenoids from *Botryococcus braunii*

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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My Lord increase me in knowledge

If we knew what we were doing, it would not be called research, would it?

Albert Einstein

DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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ABBREVIATIONS

kg	Kilogram
h	Hour
g	Gram
d	Day
CO_2	Carbon dioxide
%	Percent
±	positive and negative standard deviation from the mean
≥	greater than or equal to
l	Litre
m	Meter
ml	Millilitre
mM	Millimolar
min	minute/minutes
N	Nitrogen
µg	Microgram
µl	Microliter
µm	Micrometer
l^{-1}	per litre
min^{-1}	per minute
s^{-1}	per second
^{0}C	degrees Celsius
OD	Optical density
nm	Nanometre
BG11	Blue green 11 medium
JM	Jaworski's medium
BBM 3N	Bold base medium 3-nitrogen
CG-MS	Gas Chromatography-Mass Spectrometry
RPM	revolutions per minute
B.	Botryococcus
OW	Oil weight
DW	Dry weight
MM	Modified medium
NM	Normal medium
L	Light
D	Dark
PDA	Potato Dextrose Agar
ANOVA	One-way analysis of variance
CCAP	Culture Collection of Algae and Protozoa
MQW	Mill Q water
P	value of 0.05
CF	chlorophyll fluorescence
SS	Small Scale
LS	Large Scale
w/w	Weight
%	Percentage

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STATEMENT OF AUTHORSHIP

Four peer-reviewed papers have been published from this project in international journals, and are presented as Chapters 3-6.

Khalid A. Al-Hothaly (PhD candidate) proposed the gap in knowledge, planned the experimental design, conducted the lab work, analysed and interpreted the results, wrote the manuscript draft and submitted the manuscripts for evaluation.

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SUMMARY

The production of hydrocarbons and biofuels from microalgae is an emerging field that shows great potential for the carbon neutral production of these essential products. The green alga *Botryococcus braunii* is widely recognized as a potential carbon-neutral source of hydrocarbons and biofuels. However a number of issues hamper its commercial development including, low growth rates and biomass production, low hydrocarbon yield, problems associated with the scaling up of production, harvesting of biomass, and recovery of hydrocarbons and/or biofuel. This study addressed these issues using a series of laboratory scale and large scale experiments. Examination of the effects of three growth media on biomass and hydrocarbon production in two *B. braunii* Race B strains, Kossou-4 and Overjuyo-3, previously reported to produce high yields of hydrocarbons including triterpenoids showed that growth of *B. braunii* in Blue green 11 medium (BG11) resulted in significantly higher biomass (dry weight) compared to Jaworski's medium (JM) and Bold base 3-nitrogen medium (BBM-3N) after 15 days for both strains. Oil production was also significantly higher in BG11 whether measured as oil weight or absorbance. Examination of the effects of nutrients (nitrogen and iron) and environmental conditions (temperature, light intensity and photoperiod) on biomass and hydrocarbon production in Kossou-4 and Overjuyo-3 grown in BG11 showed that highest biomass and oil production was obtained at a nitrogen concentration of 750 mg l^{-1}, iron concentration of 6 mg l^{-1}, 25 ^{o}C and at 135 μmol m^{-2} s^{-1} with a photoperiod of 16 h light / 8 h day. Culturing the strains in modified BG11 medium containing optimized nutrients resulted in up to ~10.6 fold increase in biomass from 0.16 g l^{-1} and 0.31 g l^{-1} in normal BG11 medium to 1.74 g l^{-1} and 2.17 g l^{-1} in optimized BG11 media and growth conditions for Kossou-4 and Overjuyo-3 respectively. This was accompanied by ~8-10 fold increase in oil production compared to that in normal BG11 medium, with final oil yields of 264.2 mg l^{-1} (Kossou-4) and 220.6 mg l^{-1} (Overjuyo-3).

Growth of Overjuyo-3 and Kossou-4 in 500 L open tanks with 24 h light (54 μmol photons m^{-2} s^{-1}) at 25°C in modified BG11 medium over 60 day were then carried out. Maximum growth was reached after 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 hydrocarbons (29 % of dry weight) compared to Overjuyo-3 (20% of dry weight).

The final stage investigated the feasibility of harvesting large scale *B. braunii* using co-culture with a fungus to induce flocculation. Co-culture with the fungus *Aspergillus fumigatus* resulted in a flocculation efficiency of over 90% through co-pelletisation with both Overjuyo-3 and Kossou-4. Investigation of the ratio of fungus to algal broth found that 1:40 was the optimal ratio, resulting in large-scale flocculation efficiencies of 97% for Kossou-4 and 98% of Overjuyo-3 after 12 h following inoculation. To determine the effects of co-culture with *A. fumigatus* potential energy yields, pyrolysis was conducted. Proximate analysis showed the proportions of bio-oil, bio-char and bio-gas were very similar for the pure Kossou-4 biomass and the co-cultured pellets resulting from harvest using *A. fumigatus*. A similar result was found for Overjuyo-3 confirming that the co-culture harvest method did not compromise the end product. This research demonstrates for the first time that large-scale production using bio-flocculation for harvesting of high value triterpenoids for commercial application is feasible using *B. braunii* Race B strains Kossou-4 and Overjuyo-3.

CHAPTER ONE

General Introduction and Literature Review

1.0 Introduction

1.1 Global warming and the need for carbon neutral energy sources

Of the main greenhouse gases emitted by human activities, carbon dioxide from fossil fuel use accounts for 57% of global emission with carbon dioxide from deforestation and the decay of biomass accounting for a further 17%. Methane is the second greenhouse gas representing 14% total of global greenhouse gas emissions. Of the industries that contribute to greenhouse emissions, energy supply is the highest at 26% while transport contributed a further 13% (The Intergovernmental Panel on Climate Change, IPCC 2007).

These gases accumulate in the atmosphere (troposphere) enhancing the greenhouse effect and exacerbating global warming (Meng *et al.* 2009). Global carbon emissions from fossil fuels increased by over 16 times in the period 1900 to 2008 (United States Environmental Protection Agency, USEPA 2015).

According to the US National Oceanic & Atmospheric Administration (NOAA), atmospheric carbon dioxide has increased by about 24% since 1958 and has surpassed 400 parts per million (ppm) as of March 2015. Although industrialised countries have emitted most of the anthropogenic greenhouse gases, recently the share from developing countries has surpassed that of the industrialised countries. In the case of emissions from transport, growth has been driven by the road sector, which has increased by 64% since 1990 and in 2013 accounted for about three quarters of transport emissions (International Energy Agency, IEA 2014).

The Kyoto protocol provided a binding agreement for countries to commit to reducing greenhouse emissions. In the period 2008-2012, signatory industrialised countries committed to reducing domestic emission by 5% relative to the 1990 and actual emissions were reduced by 8% in the European Union (EU-15) whereas emissions have increased in other countries such as Australia (International Energy Agency, IEA 2014).

1.2 Renewable energy sources

Environmental concerns relating to global warming have led to increasing attention to the need to replace fossil fuels as the main source of energy with renewable energy sources (Ahmad *et al.* 2011).

Renewable energies are those that are continually replenished by nature and include:

• Energy derived from natural movement (hydroelectric power, wind energy, geothermal energy and marine energy from tidal and/or wave action).

• Energy derived directly from the sun (the various types of solar energy).

• Energy derived *indirectly* from the sun (the photosynthetic energy stored in various types of biomass) (World Bank 2013); (Ellabban *et al.* 2014).

1.3 Bioenergy

The energy stored in biomass as a result of photosynthesis is known as 'bioenergy'. A traditional example is wood burnt for heating and cooking, but modern bioenergy is sourced from dedicated energy crops such as sugarcane. Bioenergy can be 'carbon neutral' – producing no net increase in carbon emissions – because the biomass from which it derives has previously extracted CO_2 from the atmosphere via photosynthesis. Bioenergy thus has a twofold advantage over fossil fuels – being both potentially renewable (if the crops are replanted) and carbon neutral. Like fossil fuels, modern bioenergy can be processed into solid, liquid and gas biofuels, allowing it to be transported to the site of use to produce heat or generate power on demand (Ellabban *et al.* 2014).

Biofuels which are derived from biomass produced from living organisms represent an important source of renewable energy (Chisti 2007). Potentially, such biofuels can help to reduce dependence on fossil fuels, especially in the transport sector, which is the second greatest contributor to greenhouse emissions (Amaro *et al.* 2011; Balat 2011; Chisti 2007).

According to World Bank data, in 2010 the share of renewable energies in terms of total final energy consumption (TFEC) was 18% compared to 79.1% for fossil fuels. Of the renewables, traditional biomass (such as wood for heating and cooking) comprised 9.6% followed by modern biomass (3.7%), hydro-electricity (3.1%) liquid biofuels (0.8%), wind (0.3%) and solar (0.2%), biogas (0.2%), and geothermal (0.2%). Although traditional biomass was the largest share, this has been declining while other renewables have shown a compounded annual growth rate (CAGR) of 4.9% with wind, biofuels, biogas, solar, waste, and geothermal sources showing the highest growth rates, having quadruped over ten years. For biofuels, the CAGR was 11.1% in the period 1990-2010. In 2010-11, liquid biofuels provided about 3.3% of transport fuels with 59% comprising biogasoline and 26% biodiesel (World Bank 2013).

1.4 Biofuels

Biofuels usually divided into primary (or traditional, unprocessed) and secondary (or processed) biofuels. Primary biofuels are derived from the burning of traditional biomass, including firewood, wood chips, pellets, animal waste, forest and crop residues, and are mainly used for heating and cooking. Secondary biofuels (bioethanol, biodiesel and biogas) are produced by processing biomass into liquid or gas forms that can be used in vehicles and various industrial processes (Table 1.1) (Akkerman *et al.* 2002; Dragone *et al.* 2010; Parmar *et al.* 2011).

Table 1.1 Classification of secondary biofuels

Secondary Biofuels			
Type of fuels	**1st generation**	**2nd generation**	**3rd generation**
Bioethanol from fermentation of starch or sugar	• food crops such as wheat, barley, corn, potato; sugarcane, sugar beet	• novel crops such as jatropha, cassava or miscanthus	• microalgae
Biodiesel from transesterification of oils or fats	• rapeseed, soybeans, sunflower, palm, coconut, used cooking oil, animal fats.	• lignocellulosic materials (straw, wood and grass)	• microalgae and seaweeds
Biogas from fermentation	• methane from pig manure and other waste	N/A	• hydrogen from green microalgae and microbes

Modified from (Akkerman *et al.* 2002; Dragone *et al.* 2010; Nigam & Singh 2011).

1.4.1 **Secondary biofuels: 1st generation**

The first generation of (secondary) biofuels involves the use of food crops such as corn, soybean, or sugarcane, for the production of ethanol biofuel (bioethanol) or diesel biofuel (biodiesel). First generation biofuels have the advantages of being non-explosive, biodegradable, and nontoxic. They can be used in conventional engines, are basically free of sulphur and aromatics, and produce no net increase in the release of CO_2. Their price varies depending on the price of the particular feedstock used and the costs of the technology used for conversion (Ellabban *et al.* 2014; Kralova & Sjöblom 2010).

However, a major problem of first generation biofuels is that their raw materials are also used for human consumption, producing a controversy known as 'food versus fuel'. Although these crops have been engineered to produce high yields and are widely produced, there are still not enough crops to supply *both* biofuel and food stocks (Locke & Henley 2014; Souza *et al.* 2015). For edible vegetable oils, the cost of the feedstock can thus amount to 60–80% of the total cost of biofuel production (Borugadda & Goud 2012). In addition, their relative scarcity, their usage of available arable land and the role of crops such as palm oil in stimulating deforestation, further their use in the production of biodiesel (Locke & Henley 2014).

1.4.2 Secondary biofuels: 2nd generation

The second generation of biofuels are produced from non-edible lignocellulosic biomass such as straw, which can be obtained from agricultural residues or grown at low cost on marginal land (Nigam & Singh 2011) or non-food sources of oil (such as jatropha or jojoba); or waste cooking oils or waste animal fats. These sources of biofuel have the advantage of being derived from feedstock that does not compete with human food and can be grown on marginal land (Ahmad *et al.* 2011). Despite these significant advantages they have relatively high production costs. Lignocellulose feedstock requires significant pre-treatment prior to scarification and fermentation (Carere *et al.* 2008; Gao *et al.* 2013), and biological or thermochemical processing that is more technologically complex than for first generation biofuels (Nigam & Singh 2011). Biomass can contain high levels of free fatty acids which require more steps in the biofuel production process. Also, the high amounts of saturated fats in biodiesel can result in poor performance at low temperatures (Borugadda & Goud 2012).

1.4.3 Secondary biofuels: 3rd generation

The third generation of biofuels are derived from microalgae (Ahmad *et al.* 2011; Dragone *et al.* 2010). Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure (Mata *et al.* 2010). Examples of prokaryotic microalgae are Cyanobacteria (Cyanophyceae); examples of eukaryotic microalgae are green algae (Chlorophyta) and diatoms (Bacillariophyta) (Richmond 2008; Richmond & Hu 2013).

Such biofuels represent one of the most important future sources of renewable energy (Chisti 2007), and may potentially help to reduce global dependence on fossil fuels, especially in the transport sector which requires liquid or gas fuels (Amaro *et al.* 2011; Balat 2011; Chisti 2007). Table 1.2 shows the oil yield for a variety of biofuel crops, and the land area required

for cultivation of sufficient stock to produce 50% of the annual transport fuel used in the United States (Chisti 2007).

Table 1.2: Land area required for the production of sufficient biofuel to meet 50% of annual transport fuel need in the United States: source of biofuel, oil yield land area required from cropping

Crops	Oil yield (l ha^{-1})	Land area required (M ha)
Corn	172	1540
Soybean	446	594
Canola	1190	223
Jatropha	1892	140
Coconut	2689	99
Oil palm	5950	45
Microalgae*	58,700	4.5

*Estimated from experimental data, assuming 30 % oil in biomass by dry weight; modified from (Chisti 2007).

Using corn as the source for biodiesel, 1540 million hectares (Mha) of land would be required to produce 50% of the annual transport fuel in the United States. If oil palm was used the land area required would be 45 million hectares. These land requirements are obviously impractical, whereas using microalgae that yield 30% oil, the area required–estimated from experimental data would be 4.5 million hectares. Such estimates have led some researchers to conclude that the use of microalgae to produce oil for conversion into biodiesel may be the only commercially viable form of biodiesel (Chisti 2007). Furthermore, researchers from the Netherlands have proposed that the commercial production of biofuels (biodiesel and biohydrogen) from microalgae can become a reality in the next 10 to 15 years (Amaro *et al.* 2011; Balat 2011).

1.5 Major advantages of microalgae as a source of biofuels

As a source of biofuels, microalgae have a number of advantages in terms of their growth rates, high lipid content, land use and potential for reducing pollution. Microalgae grow faster than conventional oil-producing crops, allowing them to be harvested multiple times per year. For example, compared to conventional oil crops such as rapeseed or soybean, microalgae can produce 15–300 times more oil in the same land area (Dragone *et al.* 2010). This is because certain microalgal species have a doubling period of 1-3 days (Mata *et al.* 2010). The high lipid content of microalgae could provide feedstock for large-scale biodiesel production since some species accumulate large amounts of triglycerides, which are well suited for the production of biodiesel (Amaro *et al.* 2011; Scott *et al.* 2010). Microalgae can be farmed intensively but do not require arable land (Dragone *et al.* 2010), whereas plant-crop sources of biofuel are restricted by requirements of climate and soils (Rupprecht 2009). The use of microalgae for biofuel would thus reduce competition for arable land (Mata *et al.* 2010). Microalgae can grow in water that is not fit for human consumption, and they can easily absorb nutrients from waste water (Mata *et al.* 2010) or CO_2 from waste gases (Yoo *et al.* 2010). Biodiesel produced from microalgal oils release considerably less sulfur dioxide, nitrogen oxides and other contaminants to the atmosphere compared with conventional diesel (Mutanda *et al.* 2011). Burning the oil derived from microalgae makes no net increase in CO_2 levels, the main contributor to global warming. The production of oil by microalgae uses up CO_2 because microalgae are photosynthetic, meaning they produce their hydrocarbons by fixing atmospheric CO_2. Microalgae can also fix CO_2 efficiently from other sources, including industrial exhaust gases, and soluble carbonate salts (Dragone *et al.* 2010). It has been estimated that CO_2 emissions could be reduced by 1.5×10^5 tons/year by using biofuels from microalgae rather than using fossil fuels (Banerjee *et al.* 2002).

1.6 Products from microalgae

Microalgae can produce a diverse range of products including oils (lipids) used in biodiesel, hydrocarbons for bio-gasoline, foodstuffs and cosmetics (Richmond 2008). Considerable research has been undertaken into the capacity for microalgal species to produce lipids and hydrocarbons. Various salt- and fresh-water species of microalgae have substantial oil content ranging from about 20% of dry weight to over 75% (Table 1.3).

Table 1.3: Oil content of different microalgae species

Species	Oil Content (% Dry Weight)	Reference
Chlorella vulgaris	40	(Illman *et al.* 2000)
Chlorella emersonii	63	
Chlorella protothecoides	23	
Chlorella sorokiniana	22	
Chlorella minutissima	57	
Chlorella vulgaris	56.6	(Liu, Z-Y *et al.* 2008)
Chlorella vulgaris	38 ± 2	(Liang *et al.* 2009)
Neochloris oleaabundans	54	(Tornabene *et al.* 1983)
Parietochlori incisa	62	(Solovchenko *et al.* 2009)
Nitzschia laevis	69.1	(Chen, G-Q *et al.* 2008)
Botryococcus braunii	25-75%	(Li *et al.* 2013)

Of these microalgal species, *Chlorella* and *Botryococcus* species have received considerable research attention. *Botryococcus braunii* has been shown to contain up to 75% w/w of oil and has been identified as a promising microalga for the production of biofuels (Chisti 2007; Meng *et al.* 2009). *Botryococcus braunii* also can produce triterpenoid hydrocarbons (triterpenes), which can be converted into high-energy fuels such as jet fuels via catalytic cracking (Okada *et al.* 1995; Tracy *et al.* 2011).

In addition to oils and biofuels, microalgae can produce other commercially valuable products including pigments, polysaccharides, fatty acids and carbohydrates. These compounds represent higher value chemicals which can also be transformed into food additives, cosmetics, animal feeds and vaccines, high-value bioactive compounds, natural dyes and antioxidants and nutraceutical products (Hallmann 2007; Mata *et al.* 2010;

Richmond 2008). For example, the blue-green algae *Spirulina platensis,* which has been used as a food in Central America since ancient times, has been developed into a dietary supplement (Habib *et al.* 2008).

The application of microalgae in the manufacturing of cosmetics is a notable non-fuel use of microalgae that results in the production of high value market products (Yang 2011). The high concentrations of lipids in some microalgal species make them an excellent choice for application in the cosmetics industry (Hallmann 2007). Algal genera with extensive application in the cosmetic industry include *Chlorella*, *Neochloris*, *Parachlorella*, *Bracteacoccus*, *Scenedesmus*, *Anabaena*, *Chlorococcum* and *Ankistrodesmus* (Oilgae 2012).

Botryococcus braunii strains can produce two major types of triterpenoid hydrocarbons, the botryococcenes and squalene (Achitouv *et al.* 2004; Metzger *et al.* 1990). Squalene acts as a biosynthetic precursor of steroids in plants and animals. Besides the production of biofuels, it can be used commercially in cosmetics, as adjuvants and as high-grade lubricants (Eroglu & Melis 2010; Spanova & Daum 2011).

Squalene possesses significant therapeutic properties and pharmaceutical applications. Recent epidemiological studies have demonstrated that squalene is a natural chemopreventive agent, and can inhibit chemically induced skin, lung and colon tumorigenesis in rodents (Reddy & Couvreur 2009; Spanova & Daum 2011). Daily consumption of squalene can reduce the incidence of coronary heart disease and some cancers (Reddy & Couvreur 2009) and in cosmetic (Huang *et al.* 2009). Because of its widespread use in high-end products, the global market for squalene is expected to grow at a CAGR of 10.3% from 2014 to 2019 (Market and Market 2014). Much of this squalene is sourced from sharks, since shark liver oil contains sixty precent squalene by weight (Spanova & Daum 2011). Restrictions on the killing of sharks have led to a lack of raw material availability, and an increasing demand for alternative and renewable sources of squalene (Market and Market 2014).

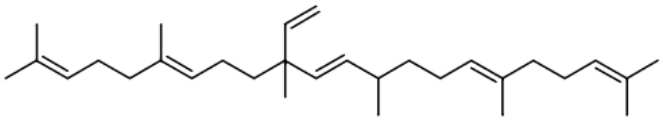
1.7 *Botryococcus braunii*

Botryococcus braunii Kutzing (*Chlorophyta*, *Botryococcaceae*) is a relatively slow growing fresh water microalga that can produce green, orange or rust-coloured algal blooms on the surface of lakes (Brown *et al.* 1969; Wake & Hillen 1980; Wolf *et al.* 1985). *B. braunii* can be found in both temperate and tropical fresh water, and sometimes in brackish water as well (Li & Qin 2005; Murchison 2005). In South Australia, a natural deposit called coorongite, which is rich in hydrocarbons, was found to have been derived from *B. braunii* blooms (Wake & Hillen 1981). *B. braunii* oils and fossils have been found in petroleum, coal deposits and oil shale. This suggests that hydrocarbon products from *B. braunii* once contributed to these reserves (Banerjee *et al.* 2002; Garciano *et al.* 2012; Weiss *et al.* 2012).

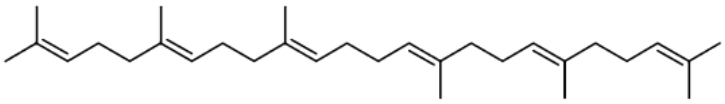
B. braunii produces hydrocarbons by fixing atmospheric CO_2 photosynthetically. *B. braunii* forms colonies which are held together by an extracellular matrix in which 90 to 95% of the liquid hydrocarbon oils are stored (Banerjee *et al.* 2002; Knights *et al.* 1970; Weiss *et al.* 2012). The oil provides buoyancy for the colony keeping it at the surface of the water body (Wolf *et al.* 1985). During the exponential growth phase, the colonies appear green and are heavier than water but as they reach the stationary phase of growth the colour changes to reddish and the colonies rise to the surface layers of the water (Belcher 1968).

Depending upon the type of hydrocarbons found inside its cells, this species can be classified into three chemical races: Race A produces C_{21} to C_{33} odd-numbered n-alkadienes, and mono-, tri, tetra, and pentenes. Race B produces two types of triterpenes as major hydrocarbons, botryococcenes of C_{30}-C_{37} with a general formula $C_n H_{2n+2}$, and similar amounts of methyl branched squalene (Figure 1.1). Race L produces a single C_{40} isopropentoid hydrocarbon, lycopa-14(E), 18(E)-diene (Metzger *et al.* 1990; Okada *et al.* 1995), and recently a Race S has been identified which contains short chain hydrocarbons (Kawachi *et al.* 2012). Of the three races, the oil content has been found to be highest in Race

B (86%) followed by Race A (61%) and Race L (8%) (Banerjee *et al.* 2002). Hydrocarbons produced by Race A and Race L are in many cases cross-linked and used within their cell walls. Hydrocarbons contained in Race B however, are not cross-linked and are found within their extracellular matrix (colony space) making it easier to extract them (Metzger & Largeau 2005).



a. Botryococcene



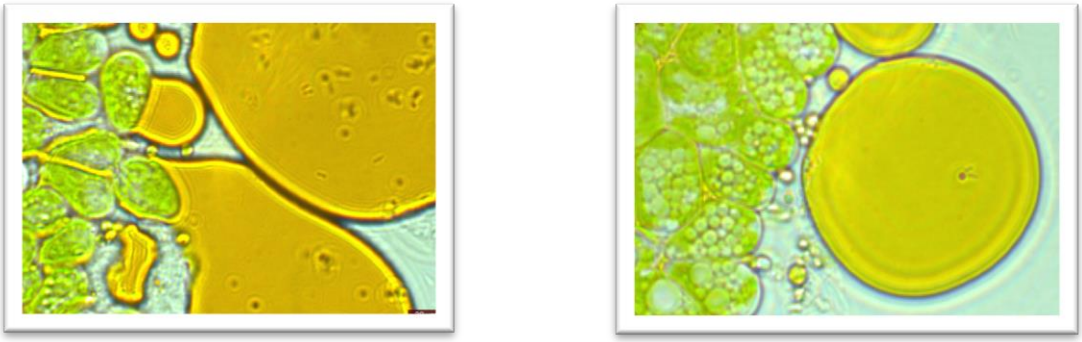
b. Squalene

Figure 1.1:(a Botryococcene and b Squalene). The two main hydrocarbons produced by Race B by *Botryococcus braunii.*

A number of Race B strains of *B. braunii* have been shown to be suitable for biofuel production. Li *et al.* 2013 reported that Ayame 1, Kossou-4, Overjuyo-3 and Paquemar all showed good biofuel production potential, producing greater than 21% of hydrocarbons relative to their dry biomass, with Kossou-4, Overjuyo-3 and Paquemar showing the highest oil contents as a percentage of dry weight (Li *et al.* 2013).

The focus of this study is on two of the high-yielding *B. braunii* Race B strains, Kossou-4 and Overjuyo-3. Kossou-4 was originally collected from Lake Kossou-4 in the Ivory Coast, which was created in 1973 by damming the Bandama River (Metzger *et al.* 1990) and Overjuyo-3 is a Bolivian strain from Lake Overjuyo (Achitouv *et al.* 2004; Metzger &

Largeau 1999; Weiss *et al.* 2012) (Figure 1.2). When mature, Kossou-4 shows a brownish colouring while Overjuyo-3 is green in colour (Metzger *et al.* 1990).



1.2a *B. braunii* strain Kossou-4 (X100 µm) **1.2b *B. braunii* strain Overjuyo-3 (X100 µm)**

Figure 1.2: (a Kossou-4 and b Overjuyo-3): The two strains of *B. braunii* used in study. Both strains are producing oil, which accumulates in the extracellular spaces (Al-Hothaly et al., 20015).

1.8 The Commercial Production of Microalgae

Production of microalgae for commercial end-products involves a number of stages, including growth of the microalgae, harvesting, extraction of oil, and refining of the end-product. Figure 3 illustrates the typical stages in a microalgal production system. The successful growth of algae requires light, a carbon source, water, nutrients and a suitably controlled temperature (van Iersel *et al.* 2009). The algae absorb CO_2 in their growth, are harvested from the culture medium, and the lipids and other products are extracted. These lipids can then be refined and converted into various types of biofuels or other high-end products, and the spent algae can be used for biogas or as animal feed (Figure 1.3) (Pienkos & Darzins 2009).

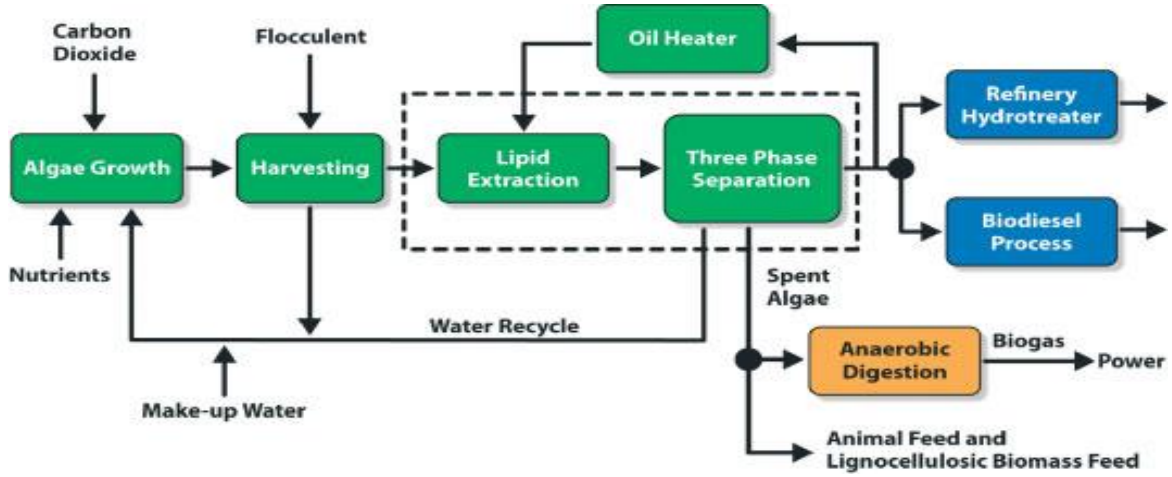


Figure 1.3: Typical Stages in a Model Open-Production System (Pienkos & Darzins 2009)

1.9 Culture systems for microalgae

In research projects, microalgae are usually grown on a small scale in laboratory flasks. In addition, a variety of approaches have been developed and trialled to apply research results to the growth of microalgae on a commercial scale. Culture systems for microalgae can be categorised basically as open production systems, which use open ponds for algal cultivation, or closed systems such as the various types of photobioreactors (Chen *et al.* 2011; Chiaramonti *et al.* 2013; Pienkos & Darzins 2009; Ugwu *et al.* 2008).

1.9.1 Open production systems

Open production systems include natural waters (such as lakes, lagoons, ponds and dams) and artificial ponds or containers (such as large shallow ponds, tanks, circular ponds and raceway ponds) (Pulz 2001; van Iersel *et al.* 2009). Figure 1.4 shows examples of three basic kinds of open production systems currently used for microalgae.

a **b** **c**

Figure 1.4: Examples of Open Production Systems (van Iersel *et al.* 2009).

a. Small pond for *Spirulina* culture

b. Open raceway-type culture ponds

c. Centre-Pivot ponds for the culture of *Chlorella*

The simplest systems are shallow, unstirred ponds, ranging from a few m^2 to 250 ha (Figure 1.4a) (Pulz 2001). A more sophisticated approach is the use of centre pivot ponds in which automatic stirring is provided (Figure 1.4c). The most commonly used are raceway-type production systems (Figure 1.4b) (van Iersel *et al.* 2009).

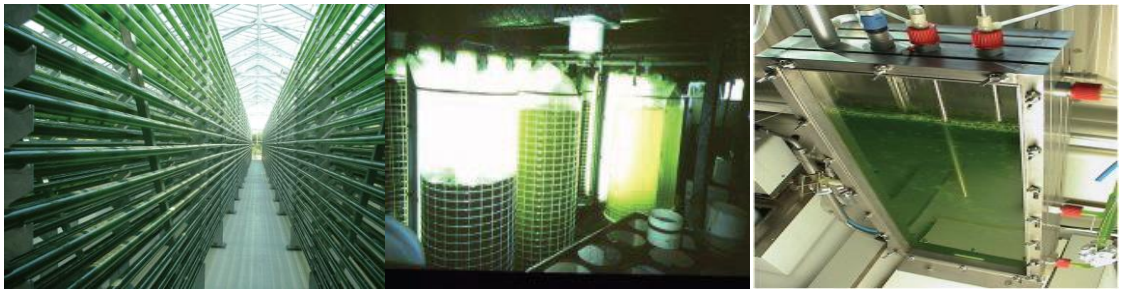
Raceway ponds are open, shallow ring-shaped or oval channels (around 0.2-0.5 m depth) that use a continuous production cycle. Algal broth and nutrients are introduced from the front of a paddle wheel, which is in continuous operation to circulate the broth and nutrients and to prevent sedimentation of cells (Brennan & Owende 2010). Other designs use aeration to circulate the culture broth (Ketheesan & Nirmalakhandan 2012) or use a propeller instead of a paddle wheel (Chiaramonti *et al.* 2013). Open systems remain the most common approaches to the cultivation of microalgae due to their simplicity of construction and relatively low cost (Harun *et al.* 2010; van Iersel *et al.* 2009). However, the yield of microalgal biomass per hectare is relatively low in open systems which have considerable requirement in terms of land area and water (van Iersel *et al.* 2009). A number of factors that limit the productivity of open systems have been identified. The carbon source is limited when CO_2 must diffuse from the atmosphere into the water, light penetration into the pond can be restricted to the surface layers only, and poor mixing of the culture can lead to uneven

distribution of nutrients and access to light. Other issues include flotation and sedimentation of dead algae, evaporation and contamination of the culture by other algal species, algal predators, or parasitic algae (Harun *et al.* 2010; Mata *et al.* 2010; van Iersel *et al.* 2009). A major issue for open systems is the lack of temperature control. Consequently, the pond becomes a function of the local climate, so location can significantly influence productivity (Harun *et al.* 2010).

Measures have been employed to alleviate a number of these limitations. The use of mechanical mixing can improve aeration and circulate both nutrients and algae. The bubbling of air or CO_2 enriched air can also improve the availability of carbon. Temperature control can be achieved when cheap surplus heat is available. The risk of contamination can be limited by selecting algal species and growing conditions that enable the desired species to out-compete others (Harun *et al.* 2010; van Iersel *et al.* 2009).

1.9.2 Closed Production Systems

Closed systems aim to reduce the land and water requirements of cultivation, alleviate other limitation of open systems and improve productivity by culturing algae in containers that enable increased exposure to light, improved gas exchange and greater control of temperature and nutrients (Carvalho *et al.* 2006; van Iersel *et al.* 2009). Pollution and contamination risks with open pond systems can limit their use for the production of high-value food, pharmaceutical or cosmetic products. However, closed systems allow for long-term cultivation of monocultures (Brennan & Owende 2010; Ugwu *et al.* 2008). Various types of closed production systems, referred to as photobioreactors, have been designed. The main types of photobioreactors are tubular, flat panel, and fermenter-type or big bag systems (Carvalho *et al.* 2006; van Iersel *et al.* 2009) (Figure 1.5).



a **b** **c**

Figure 1.5: Examples of close cultivation systems (van Iersel *et al.* 2009).

a. Tubular reactor system

b. 'Big Bag' culture of microalgae

c. Experimental flat photobioreactor

Tubular photobioreactors use an array of straight, transparent tubes that are usually made of plastic or glass material to enable sunlight capture. Arrays can be aligned horizontally (Grima *et al.* 2003; Tredici & Zittelli 1998), vertically (Converti *et al.* 2009), inclined (Ugwu *et al.* 2008; Vunjak-Novakovic *et al.* 2005) or formed into a helix (Hall *et al.* 2003). Tube diameter is generally 0.1 m or less, to minimize dark zones in the culture broth, and tube length is limited due to oxygen accumulation. High yields (in terms of per unit ground area) are produced as a result of stacking the tubes together with the high surface/volume ratio. Compared to open ponds, tubular photo-bioreactors can provide better pH and temperature control, better protection against culture contamination, better mixing, less evaporation loss and higher cell density and productivity (Christenson & Sims 2011; Mata *et al.* 2010). Productivity tends to be higher in vertical than in horizontal tubular systems. It has been estimated that horizontal systems can produce 25-100 tonnes of biomass $ha^{-1}year^{-1}$, while vertical systems could reach 35-155 tonnes ha^{-1} $year^{-1}$ (Slegers *et al.* 2013). Flat panel reactors use large surface areas exposed to illumination and have high densities of photoautotrophic cells (>80 g l^{-1}) (Ugwu *et al.* 2008). They use a flat, transparent vessel only a few centimetres thick. A dense culture flows inside the flat plate. Radiation absorption is effective in the first few millimetres but aeration allows cells to move from dark zones at the

centre/rear of the reactor to the illuminated zone. The location and orientation of the reactor influences productivity since east/west facing systems reduce direct beam radiation on the reactor surface while increasing dispersed radiation, which is more favourable for growth (Richmond 2008; Sierra *et al.* 2008).

Fermenter-Type Reactors consist of tanks that are illuminated internally by fluorescent lamps which are contained in narrow glass or Plexiglas tubes with bubbling being used for aeration. The tanks can be of rigid construction or can be made using big polyethylene bags or sleeves (Carvalho *et al.* 2006; van Iersel *et al.* 2009).

Photobioreactors are considered the most productive systems (Lehr & Posten 2009). Compared to open ponds, tubular photo-bioreactors can provide better pH and temperature control, better protection against culture contamination, better mixing, less evaporation loss and higher cell density and productivity (Mata *et al.* 2010). However, a number of factors can limit productivity in tubular systems. Photosynthetic activity causes oxygen to accumulate inside tubes inhibiting algal growth, so oxygen removal is critical for efficient performance. This effect limits the maximum tube length to 80-100 m. The culture must be returned to a degassing zone and bubbled with air to remove the excess oxygen (Carvalho *et al.* 2006). Since tubes cannot be scaled up indefinitely, large-scale plants use multiple reactor units (Akkerman *et al.* 2002). Another issue is pH, which increases due to the consumption of carbon dioxide by the biomass as the broth moves along the tubes. Carbon dioxide is fed into the degassing zone in response to the culture demand as measured by a pH controller. Also, turbulent flow is required to move the cells homogeneously between illuminated zones at the tube periphery and dark zones at the centre (Chisti 2007; Gordon & Seckbach 2012).

Despite their higher productivity and lower land use, the costs of photobioreactors must be reduced below the cost of the current open systems in order to be competitive (Acién *et al.* 2012). Provided that open pond systems do not compete for agricultural land, their lower cost

and ease of construction, and lower costs of energy and maintenance mean that open systems remain the cheapest method for large scale production of algal biomass (Brennan & Owende 2010; Chiaramonti *et al.* 2013; Chisti 2007; Pienkos & Darzins 2009).

1.10 Environmental conditions required for algal culture

Algae grown for biofuels are autotrophic microalgae, which produce organic compounds through photosynthesis but require inorganic compounds and CO_2 for growth. Irrespective of which culture production system is used for generating microalgal biomass, these organisms require specific environmental and nutrient conditions for optimal microalgal growth, including light, temperature and inorganic nutrients (Brennan & Owende 2010). The following section discusses the influence of these key parameters and discusses their influence on microalgal growth.

1.10.1 Light

In photoautotrophic algae, light is the ultimate source of energy that facilitates the production of algal biomass via photosynthesis. When providing lighting for growing algae, various aspects need to be taken into consideration with regard to light, including the spectral quality, photoperiod, and light intensity (Brennan & Owende 2010; Chen *et al.* 2011). Only light of wavelengths between 400 and 700 nm can be captured by microalgae for photosynthesis. This light is known as photosynthetic active radiation (PAR) and represents 42.3% of the total energy from the light spectrum. The fraction of PAR that is actually fixed as chemical energy during photoautotrophic growth is known as the Photosynthetic Efficiency (PE). The captured energy is used to produce carbohydrates by utilising CO_2 and H_2O molecules (Akkerman *et al.* 2002; Brennan & Owende 2010). In *B. braunii* blue light produced the highest growth and hydrocarbon production, followed by red light (Baba *et al.* 2012).

Under natural conditions, algae experience daily cycles of approximately 12 hours of light and dark, however, photoperiod can be manipulated when artificial lighting is used. Some studies have suggested that different species react differently to different photoperiods. In *Chlorella vulgaris*, 16 hours light and 8 hours dark was found to produce the highest biomass (Khoeyi *et al.* 2012). In a study that compared five species, three *Neochloris* species all grew better under 12 hours light and 12 hours dark whereas *Scenedesmus obliquus* and *B. braunii* grew best and produced maximum biomass at 24 hours illumination (Krzemińska *et al.* 2014). However in another study of *B. braunii*, maximum biomass was obtained with continuous illumination. This was four times the biomass produced with 12 hours light and 12 hours dark but the highest lipid content was with 16 hours light and 8 hours dark (Ruangsomboon 2012).

Light intensity is also a major factor in algal growth. Insufficient light will reduce photosynthesis and consequently slow growth whereas greater light intensity can increase growth. However, there is point of saturation at which photosynthetic efficiency can no longer be increased. Beyond this, further increases in light intensity can inhibit growth (Brenckmann *et al.* 1985; Grobbelaar 2010). Light intensity may also affect the composition of algae. A study of *Chlorella vulgaris* found that the relative concentrations of saturated and unsaturated fatty acids were affected by light intensity (Khoeyi *et al.* 2012) and in *B. braunii* higher light intensities tended to produce higher lipid contents but there was also evidence of photoinhibition of growth and biomass production when the light intensity was highest (Ruangsomboon 2012).

1.10.2 Temperature

Microalgae can grow in a wide range of environments from tropical waters to cold seas and lakes (Mata *et al.* 2010; Morgan-Kiss *et al.* 2006). Under natural conditions, microalgae experience a range of temperatures and tend to be tolerant of lower than optimal temperatures

however they can be less tolerant of overheating (Mata *et al.* 2010). In *B. braunii*, a number of studies have investigated growth at temperatures of 23-25°C (Ambati *et al.* 2010; Li *et al.* 2013; Velichkova *et al.* 2012) but other studies have found that *B. braunii* strains can grow successfully at lower temperatures such as 18°C (Kalacheva *et al.* 2002) but growth and biomass production was higher at temperatures of up to 32°C with inhibition appearing outside the range of 15 - 35°C (Kalacheva *et al.* 2002; Yoshimura *et al.* 2013). Besides affecting growth, temperature has been reported to have effects on algal composition (Guschina & Harwood 2006; Sharma *et al.* 2012). In the case of fatty acids in algal oil, the relative content of saturated FAs tends to increase as temperature increases while the content of unsaturated FAs decreases (Sushchik *et al.* 2003). However, in the case of hydrocarbons in a *B. braunii* Race B strain, hydrocarbon content was found to be stable with productivity being proportional to growth (Yoshimura *et al.* 2013).

1.10.3 Nutrients

Besides the availability of CO_2 and light, microalgae require certain nutrients for growth. These nutrient requirements vary according to species and can be provided by natural or waste water or by a culture medium designed for the growth of the particular alga (Dayananda *et al.* 2005; Mata *et al.* 2010; Órpez *et al.* 2009). Nutrients that can promote the growth of microalgae include sources of nitrogen, phosphorus, iron and micro-nutrients such as manganese, magnesium and molybdenum (Dayananda *et al.* 2005; Órpez *et al.* 2009; Peers & Price 2004; Raven 1990; Song *et al.* 2012; Tanoi *et al.* 2014; Yeesang & Cheirsilp 2011). Conversely, excessive nutrients, in particular nitrogen, may inhibit growth of some species including *B. braunii* (Shen *et al.* 2009).

Although nutrients are important for growth, it has been reported that nutrient limitation and other stress conditions can increase the total oil content of some algal species (Mata *et al.* 2010; Roleda *et al.* 2013; Sharma *et al.* 2012). For example, in *C. vulgaris*, lipid production

was found to increase from 18% to 40% under conditions of nitrogen stress but in other species the response to nutrient stress was less pronounced (Illman *et al.* 2000; Praveenkumar *et al.* 2012). In one *Botryococcus. braunii* strain, nitrogen deficiency has also been shown to favour lipid accumulation but it also resulted in lower biomass production (Qin 2010). In a different *B. braunii* strain, both the nitrogen and iron concentrations in the growth medium affected lipid content but showed only small effects on biomass production (Ruangsomboon 2012). However, in both studies, increasing salinity had negative effects on growth and salt stress did not increase lipid content (Qin 2010; Ruangsomboon 2012).

1.11 Harvesting and drying of algal biomass

A major obstacle to the commercial production microalgae is the fact that harvesting methods are not economically feasible because they require too much energy or require the use of chemicals. For successful commercial production of *B. braunii* strains, an efficient and economical method for microalgal harvest must be developed, to allow the subsequent conversion of the biomass into biofuels or other products (Leite *et al.* 2013; Pragya *et al.* 2013).

Microalgae are very small organisms (1–20 μm), suspended in liquid from which they must be removed (Lam & Lee 2012). Harvesting is thus energy intensive and costly. Estimates of the cost of harvesting include 20–30 % of the costs of micro-algal biomass (Mata *et al.* 2010) and up to 50% of the total cost of biofuel (biodiesel) production (Wrede *et al.* 2014).

Currently there is no single established method for the harvesting of microalgae and a variety of approaches are in use (Table 1.4) (Milledge & Heaven 2013). Sedimentation and flotation can both be used to separate the algal biomass from the broth using gravity. These methods are inexpensive but are very slow (Pragya *et al.* 2013). Centrifugation is a rapid and highly efficient harvesting method which is used in the laboratory setting, but is too energy intensive and costly for ‘low value, high demand products’ (Leite *et al.* 2013), making it economically

unfeasible for biofuel on a commercial scale (Pragya *et al.* 2013).

Filtration methods are frequently used but the passage of the algal broth through the filters can be slow and issues with clogging can lead to higher costs. Different filtration methods involve the use of different sized filters, exerting pressure on the solution, or using a filter aid to allow for easier harvest of smaller particles. Larger microalgae such as *Coelastrum proboscideum* and *Spirulina platensis* are easier to filter but the filters fail to recover microalgal species which approach bacterial dimensions (Grima *et al.* 2003). Filtration methods can produce efficient harvest but filters need to be replaced frequently due to fouling. Replacing filters and running machines and pressure pumps adds to the cost of production and this becomes uneconomical on a larger scale (Grima *et al.* 2003; Pragya *et al.* 2013). In addition filtration approaches can be unsuitable for certain algal species, especially when there is high extracellular oil content (Leite *et al.* 2013; Milledge & Heaven 2013). Flocculation involves the addition to the algal broth of a compound or substance that causes the algae to form clumps, pellets or pellet-like compounds called flocs, which sink to the bottom and are easily harvested. This process has long been used to produce potable water (Leite *et al.* 2013). Both chemicals and natural products have been investigated (Milledge & Heaven 2013). Chemical flocculation involves adding a chemical to cause the microalgae to form flocs. The problems associated with the various chemicals used in flocculation are cost and the use of certain chemicals can make the biomass unsuitable for certain end uses.

Table 1.4: Comparison of microalgae harvesting methods: Advantages, disadvantages, and cost.

Methods	Advantages	Disadvantages	Effectiveness (% of solids)	Cost
Centrifugation	Can handle most algal types with rapid and efficient cell harvesting	High capital and operational costs	10-22	High
Filtration	Wide variety of filter and membrane types available	Highly dependent on algal species; best suited to large algal cells. Clogging or fouling is an issue	2-27	Low

Ultrafiltration	Can handle delicate cells	High capital and operational costs	1.5-4	High
Sedimentation	Low cost–potential for use as a first stage to reduce energy input and cost of subsequent stages	Algal-species specific, best suited to dense non-motile cells. Separation can be slow. Low final concentration	60	Low
Chemical flocculation	Wide range of flocculants available; price varies although can be low cost;	Requires removal of flocculants; chemical contamination	85 - 95	Low
Flotation	Can be more rapid than sedimentation. Possibility to combine with gaseous transfer	Algal species specific. High capital and operational cost	7	Low

Modified from (Milledge & Heaven 2013)

Alum, ferric chloride and other metal salts are used as flocculating agents in industries such as mining and for wastewater treatment. However, the metals in the biomass residue limit the end-uses of the algae (Vandamme *et al.* 2013).

The use of organic flocculants such as chitosan can be effective as flocculating agents but are species dependent, and the results are also dependent on culture pH and temperature (Uduman *et al.* 2010). Another approach is electro-flocculation. This uses electric currents to dissolve sacrificial metals to supply the ions needed for flocculation (Uduman *et al.* 2010). A recent investigation of the economics of electro-flocculation suggests it could be cost effective (Lee *et al.* 2013).

Bio-flocculation is an innovative approach to flocculation using fungi. Certain species of filamentous fungi can spontaneously form pellets when grown in a solution or can be induced to form pellets by chemical means. These pellets sink to the bottom of the solution and are easily harvested. When such fungi are inoculated into a microalgal broth, the fungi can entrap microalgal cells into the filamentous mass as a co-culture and flocculate the microalgae (Gultom & Hu 2013; Vandamme *et al.* 2013).

There are limited reports of the use of bio-flocculation for microalgal harvest in the literature. The oleaginous fungus *Cunninghamella echinulata* has been reported to co-flocculate with *Chlorella vulgaris* (Xie *et al.* 2013). *Aspergillus* species have been successfully used for co-

pelletization and harvesting of *Chlorella vulgaris* from different sources including treated wastewater (Zhang & Hu 2012; Zhou *et al.* 2013). A recent study demonstrated the feasibility of using the fungus *Aspergillus fumigatus* to flocculate a range of microalgal species used for waste water treatment (Wrede *et al.* 2014). The mechanism responsible for this bio-flocculation appears to be the neutralization of negative charges on the algal surface (which prevent self-flocculation) by the positively charged fungi. Moreover, this approach to bio-flocculation tends to enhance biomass and lipid production (Wrede *et al.* 2014).

The efficiency of the harvesting approach and the extent to which de-watering of the algal broth is achieved has important economic considerations since the remaining water may need to be extracted prior to further processing (Uduman *et al.* 2010). Sun drying is the most economical approach to removing any remaining water, but this requires a long drying time and a large drying area. Spray and freeze drying and heat drying are rapid drying methods, but these are substantially more expensive and they can damage algal pigments or lipids (Brennan & Owende 2010; Grima *et al.* 2003).

1.12 Oil extraction from microalgae

Following harvesting and drying the next step is the extraction of oil from the biomass. Lysing refers to the process of breaking down the cells of algae to release oil. The walls of algae cell are quite hard to break down and mechanical methods of cell disruption (lysing) are energy-intensive (Farag 2012). Lee *et al.* (2013) compared microwaves, sonication, beadbeating, autoclaving and osmotic pressure methods in *Botryococcus sp.*, *Chlorella vulgaris*, and *Scenedesmus sp.* They reported the amount of lipids extracted differed according to the method and species with bead-beating and microwave methods being be the most efficient for the *Botryococcus sp.* (Lee *et al.* 2010). However, the need for cell disruption is considerably reduced in *B braunii* since much of its lipids are extracellular (Lee *et al.* 2013).

Following lysing, conventional methods to oil extraction include oil expeller/press, solvent extraction and supercritical fluid extraction (Farag 2012). Researchers have noted that oil extraction on the industrial scale is energy intensive and costly and remains a challenge for the biofuel industry (Neto *et al.* 2013). The most common method of extracting oil from biomass following lysis is the use of chemical solvents. These can be toxic, need special handling and can be a source of pollution. In order to limit costs the solvent should be recovered and reused, However, there remains no established method for extraction oil from microalgae on an industrial scale (Farag 2012; Neto *et al.* 2013).

1.13 Energy extraction from biomass by pyrolysis

Another challenge for the commercial production of biofuels from micro-algae is the approach to conversion of algal biomass into energy. A conventional approach involves the production of biomass followed by cell disruption, drying and the extraction of lipids using solvents, removal of the solvent and then the conversion of the lipids into biodiesel using processes such as micro-emulsification, pyrolysis and catalytic cracking, and transesterification. Another approach is the direct conversion of biomass into fuel products via thermal or chemical methods. The main thermal methods include gasification, thermo-chemical liquefaction, pyrolysis and direct combustion (Pragya *et al.* 2013).

Pyrolysis involves the thermal degradation of the biomass in the absence of oxygen to produce gaseous, liquid and solid products. Of these, the gaseous and liquid products are useful in biofuel production while the solid product (bio-char) is mostly composed of carbon. An advantage of pyrolysis over other methods of converting biomass into energy is it can directly produce a liquid fuel (Bridgwater *et al.* 1999). There are three main types of pyrolysis. Slow pyrolysis uses the lowest temperature (400 ºC) for more than 20 mins, fast pyrolysis uses 500 ºC for 10 to 20 seconds, while flash pyrolysis uses the same temperature

but only for about one second (Amin 2009; Marcilla *et al.* 2013). The set-up of a pyrolysis system is presented in Figure 1.6.

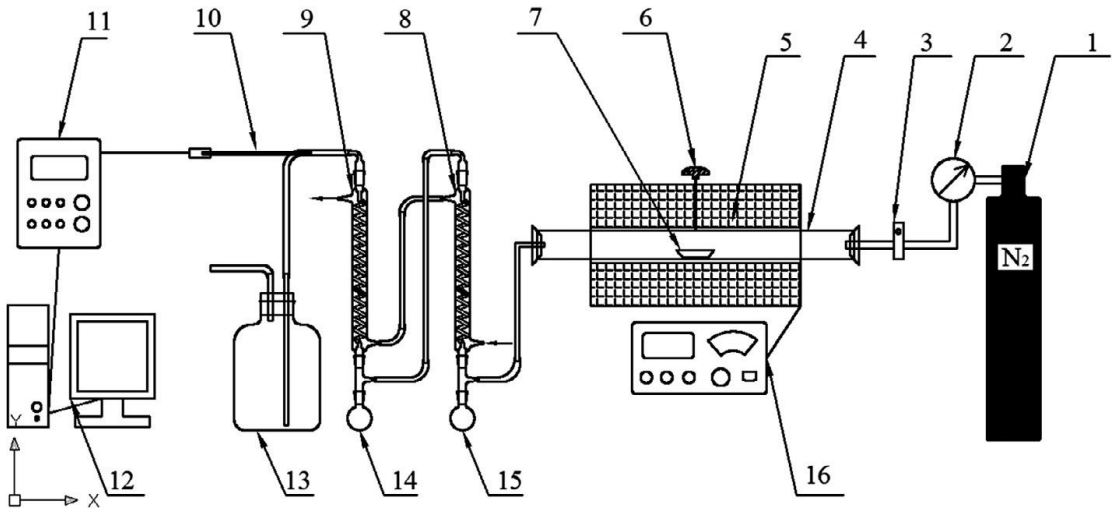


Figure: 1.6 Schematic diagram of fast pyrolysis experimental system:

(1) nitrogen bottle; (2) pressure reducing valve; (3) float flowmeter; (4) quartz tube reactor; (5) tube furnace; (6) thermocouple; (7) crucible and samples; (8) the first condenser; (9) the second condenser; (10) acquisition sensor; (11) the flue gas analyzer; (12) the data acquisition system; (13) gas collecting bottle; (14) the second liquid collecting bottle; (15) the first liquid collecting bottle; (16) the temperature controller from (Hu *et al.* 2013).

In the case of microalgae, pyrolysis does not require the prior extraction of oil from the biomass and oils from biomass pyrolysis can be upgraded to liquid hydrocarbon fuels through hydro-treating and/or hydro-cracking processes (Lange 2007). Various types of pyrolysis have been investigated as methods for extracting energy from biomass in a number of microalgal species (Chaiwong *et al.* 2013; Kebelmann *et al.* 2013; Pan *et al.* 2010; Wang *et al.* 2013).

Pyrolysis has also been used for energy extraction and analysis of *B. braunii* strains (Watanabe *et al.* 2014). (Garciano *et al.* 2012) analysed a coorongite specimen, which is a natural deposit derived mainly from *B. braunii*, and compared this to a freeze dried *B. braunii* sample (Garciano *et al.* 2012). Liu *et al* 2012 conducted thermogravimetric analysis (TGA) of *B. braunii* and *Hapalosiphon sp.* which showed that more energy could be recovered from

the *B. braunii* (Liu *et al.* 2012). Although pyrolysis is an effective way of directly converting oil-rich algal biomass into liquid and gaseous biofuels, it is not a suitable method for extracting specific hydrocarbons such as triterpenes since these are destroyed by the heat involved in pyrolysis.

1.14 Rationale for this research

B. braunii race B strains produce high levels of hydrocarbons, in particular the high value triterpenes, bottyrococcene and squalene, which makes race B strains a commercially attractive proposition (Qin 2005).

However, *B. braunii* is relatively slow growing. The doubling time of *B. braunii* is usually 6-7 days under natural conditions, which is longer than other microalgae but this can be halved with more suitable conditions (Borowitzka & Moheimani 2013; Qin 2005; Wake & Hillen 1980; Wolf *et al.* 1985). However, the optimal parameters for growth in race B strains, Kossou-4 and Overjuyo-3 have not yet been fully determined. So, this project investigated the effects of different growth media, lighting, temperature, and the nutrients nitrogen and iron on biomass, oil and triterpenoid hydrocarbon production.

To establish the commercial relevance of this research, this project also investigated the feasibility of scaling-up the growth conditions that were shown to produce higher biomass, oil and triterpenoid hydrocarbons at the laboratory scale to larger scale production on open tanks.

The harvesting of algal biomass in large scale production systems is a major contributor to costs (Vandamme *et al.* 2013; Xie *et al.* 2013). Therefore, this study investigated the feasibility of harvesting the biomass produced by these *B. braunii* strains using a novel, low-cost approach to bio-flocculation.

1.15 Aims of the project

- To assess the potential of two Race *B strains* of *B. braunii* to produce triterpenoid hydrocarbons when grown in a range of growth media.
- To assess the factors affecting biomass, oil and triterpenoid production by these *B. braunii* strains.
- To investigate biomass, oil and triterpenoid production in these two strains when grown on a large scale.

1.16 Specific research questions

1.16.1 Chapter 3

Which of three growth media produces the highest growth in terms of biomass of Kossou-4 and Overjuyo-3?

Which of three growth media produces the highest production of oil in Kossou-4 and Overjuyo-3?

Which of three solvents extracts the most oil from Kossou-4 and Overjuyo-3?

1.16.2 Chapter 4

Which concentrations of iron and nitrogen in the growth medium results in the highest production of biomass, oil and triterpenoids in Kossou-4 and Overjuyo-3?

Which temperature conditions result in the highest production of biomass, oil and triterpenoids in Kossou-4 and Overjuyo-3?

Which light intensities and photoperiods result in the highest production of biomass, oil and triterpenoids in Kossou-4 and Overjuyo-3?

What are the optimal conditions for the growth of Kossou-4 and Overjuyo-3 for production of biomass, oil and triterpenoids?

1.16.3 Chapter 5

Can the cultivation of Kossou-4 and Overjuyo-3 be successfully scaled up and grown for an extended period?

Can Kossou-4 and Overjuyo-3 be cultivated in large open tanks without contamination?

What are the yields of biomass, oil and triterpenoids from Kossou-4 and Overjuyo-3 cultivated in large open tanks?

1.16.4 Chapter 6

Which species of fungi are suitable for bio-flocculation of Kossou-4 and Overjuyo-3?

Is it feasible to harvest Kossou-4 and Overjuyo-3 on a large scale by bio-flocculation using fungi?

Following pyrolysis, what is the composition and energy content of biomass produced by large scale cultivation of Kossou-4 and Overjuyo-3?

Following pyrolysis, what is the composition and energy content of biomass produced by large-scale cultivation of Kossou-4 and Overjuyo-3 after harvest by bio-flocculation?

CHAPTER TWO

General Materials and Methods

2.0 General Materials and Methods

This chapter reports on the materials and methods used routinely throughout experiments relating to Chapters 3, 4, 5 and 6, including the microalgae used, the growth media, apparatus and procedures. Specific methodologies for the key experiments are described within the Methods section of the publication found in each research chapter.

2.1 Materials

2.1.1 Chemical and media

All chemicals were of analytical grade and were purchased from Sigma-Aldrich or Merck unless mentioned in the text.

2.1.2 Microalgal Strains

Two strains of *Botryococcus. 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 (Li *et al.* 2013). 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 (Metzger *et al.* 1990) (Figure 2.1). In this project microalgae were cultured in small and large scale. Erlenmeyer Flasks (250 to 1000 ml) were used for growing microalgae for small-scale experiments while 500 l capacity fiberglass tanks were used for large-scale studies.

2.1.3 Fungal strains

The five fungal isolates used in this study were obtained from Professor A. S. Ball's collection (RMIT University, Melbourne, Australia). The fungi used were *Aspergillus*

fumigatus, *Phanerochaete chrysosporium*, *Trametes versicolor*, *Iprex lacteus*, and *Pleurotus ostreatus*.

2.1.4 Media

Three growth media, (i) Blue Green medium (BG11) (ii) Bold base medium (BBM-3N) and (iii) Jaworski's medium (JM) were used in this study. All prepared media were sterilized at 121oC for 15 min.

2.1.4.1 BG11 medium

BG11 medium was used for the growth of the two strains of *Botryococcus. braunii*, Kossou-4 and Overjuyo-3, and was prepared as described previously (Ge *et al.* 2011), with the media containing the following: 1.5g $NaNO_3$, 0.04 g K_2HPO_4, 0.075 g $MgSO_4.7H_2O$, 0.036 g $CaCl_2$. $2H_2O$, 0.006 g citric acid ($C_6H_8O_7$), 0.006 g ferric ammonium citrate ($C_6H_8O_7Fe_3$) 0.001 g disodium EDTA salt (ethylenediaminetetraacetic acid disodium), 0.02 g Na_2CO_3, and 1.0 ml trace metal mix A5 in 1 l of distilled water. The composition of the trace metal mix A5 was as follows: 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.222 g $ZnSO_4.7H_2O$, 0.39 g $NaMoO_4.2H_2O$, 0.079 g $CuSO_4.4H_2O$, and 49.4 mg $Co(NO_3)2.6H_2O$ in 1 l of distilled water.

2.1.4.2 JM medium

JM medium was used for the growth of *B. brauni* strains Kossou-4 and Overjuyo-3 and was prepared as described by Thompson *et al.* (1988) with the following composition per l: 4.0 g $Ca(NO_3)2.4H_2O$, 2.48 g KH_2PO_4, 10.0 g $MgSO_4.7H_2O$, 3.18 g $NaHCO_3$, 0.45 g EDTAFeNa (Ethylenediaminetetraacetic acid, ferric), 0.45 g $EDTANa_2$,(ethylenediaminetetraacetic acid disodium) 0.496 g H_3BO_3, 0.278 g $MnCl_2.4H_2O$, 0.20 g $(NH_4)6Mo_7O_{24}.4H_2O$, 0.008 g cyanocobalamin, 0.008 g thiamine HCl, 0.008 g biotin, 16.0 g $NaNO_3$ and 7.2 g Na $HPO_4.12H_2O$ (Thompson *et al.* 1988).

2.1.4.3 BBM 3N medium

BBM 3N medium was used for the growth of *B. braunii* strains Kossou-4 and Overjuyo-3 and was prepared as described by (Velichkova *et al.* 2012), using the following: 10.0 g $NaNO_3$, 1.0 g l $CaCl_2$, 3.0 g l $MgSO_4.7H_2O$, 3.0 g K_2HPO_4, 7.0 g KH_2PO_4, 0.750 g $EDTANa_2$,(ethylenediaminetetraacetic acid disodium), 1.0 g NaCl, 0.06 mg vitamin B12, 5 g garden soil (dry) , 1 mg $CaCO_3$, 200 mL in 1 l of distilled water; DAS vitamin cocktail 85 ml in glass-distilled water. To this, 0.9 g tricene was added. The pH was adjusted to 8.0 with 2 M NaOH, then the following was added: thiamine HCl 67 mg, d-biotin 1 mg, cyanocobalamin (Vitamin B_{12}), pantothenate (vitamin B_5) 2 mg, PABA (p-aminobenzoic acid) 4 mg. The final volume was brought to 100 ml with glass-distilled water, and sterilized by passing through a 0.45 micrometer filter. The P-IV metal solution was then added. P-IV contains (per l): 97.0 mg $FeCl_3.6H_2O$; 41.0 mg $MnCl_2.4H_2O$; 5.0 mg $ZnCl_2$; 2.0 mg $CoCl_2.6H_2O$; 4.0 mg $NaMoO_4$. Media (500 ml) were inoculated into 2000 ml flasks. The final pH of the media was adjusted to 7.4 and this pH was maintained throughout the study by adding HCl and NaOH respectively.

2.1.5 Fungal growth medium

Fungal were grown on freshly prepared sterile Potato Dextrose Agar (PDA) plates. The capability to form pellets (pelletization; ball-like) of the selected fungal species was assessed in Potato Dextrose Broth (PDB) in 100 ml flasks inoculated with 1 cm^2 fungal plug and incubated at 28°C for three to five days on an orbital shaker (Ratek Australia) at 150 rpm.

2.2 Small scale microalgae culture

Erlenmeyer flasks (1000 ml) containing 600 ml of normal or modified BG11 medium 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 30 ºC). Continuous illumination was supplied by Cool White Fluorescent Lamps at the desired light intensities 4000, 6000 and 10,000 lux (54, 81 or 135 µmol *photons* m^{-2} s^{-1}) and photoperiods used were: (a) 4000 Lux for (i) 24 h L, (ii) 16 h L / 8 h D and (iii) 12 h L / 12 h D; (b) 6000 Lux for (i) 24 h L, (ii) 16 h L / 8 h D and (iii) 12 h L / 12 h D; and (c) 10000 Lux for (i) 24 h L, (ii) 16 h L / 8 h D and (iii) 12 h L / 12 h D. Non-destructive sampling was carried out for biomass assay (day 0 to day 60) and hydrocarbon production (day 30 to day 60) as earlier described. A light meter (LM 37 Luxmeter, Dostman Electronic, and Wertheim-Reicholzheim, Germany) was used to measure the light intensity. All experiments were carried out in triplicate.

2.3 Large-scale microalgal culture

Ten litres of the modified BG11 medium was prepared as described in Section 2.1.1, with the exception that the sodium nitrate ($NaNO_3$) concentration was reduced from 1.5g l^{-1} to 0.75 g l^{-1}. This medium concentrate was added to 490 l of fresh water in each tank. Two circular fibre glass tanks (height 83 cm, diameter 143 cm, capacity 500 l) were used for each strain. An aliquot (2.5 l) of Kossou-4 which corresponded to 0.04 g l^{-1} (dry weight) of microalgae culture was added to one tank . An equivalent aliquot of Overjuyo-3 was added to the second tank. The inoculated cultures were incubated in the tanks for 60 days with continuous air supply. Aeration was provided by continuous bubbling of air up from the center of the tank at an airflow rate of 18.2-m^3 min^{-1} with illumination from a cool fluorescence lamp at 54 µmol m^{-2} s^{-1}. Temperature was maintained by a thermostat in the range 24.8 to 25.5 ºC.

2.4 Culture illumination and photoperiod

Lighting for culturing microalgae was supplied by cool white fluorescent tubes placed at a distance of 60 cm from the flasks. The light intensity used in this study ranged from 4000 Lux (54 μmol $m^{-2}s^{-1}$) to 10,000 Lux (135 μmol $m^{-2}s^{-1}$). The light source was regulated with an adjustable timer that could be set to different photoperiod of light and dark. The photoperiods used were 24 h continuous light, 16 h of light and 8 h of darkness and 12 h of light and 12 h of darkness.

2.5 Microscopy

To assess biomass and oil production, 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. 100X magnifications were used for all photographs.

2.6 Biomass measurement

Four different methods were used for biomass measurement in this project. These were: 1. direct cell count; 2. optical density assays at two wavelengths 680 and 750 nm; 3. chlorophyll fluorescence; and 4. dry weight.

2.6.1 Cell count

The numbers of cells in samples were counted using the Countess® Automated Cell Counter (Invitrogen) according to the manufacturer's guidelines. Each sample (10 µl) was vigorously mixed with 10 µl of Trypan blue stain by continuous pipetting. An aliquot (10 µl) was added to the disposable slides for use in the counting chamber. The slides were 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 according to (Madigan 2005).

2.6.2 Optical Densities (ODs)

Optical density provides a measure of the growth of the algae. Optical density was measured using a POLAR star Omega microtitre plate reader. An algae 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 for 30 s continuously. Light absorbance was measured at wavelengths of 680 nm and 750 nm. Higher absorbance values indicate greater growth (Griffiths *et al.* 2011).

2.6.3 Chlorophyll Fluorescence (CF)

A POLARstar Omega (BMG TABTECH) microtitre plate reader was also used to measure chlorophyll fluorescence at 430 nm. 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 (Mohsenpour *et al.* 2012).

2.6.4 Dry weight (DW)

An aliquot (100 ml) of each sample solution was taken and filtered using a MILLIPORE paper filter (45 µm) of predetermined weight via a standard vacuum pump. The filter paper-culture complex was weighed before and after drying at 65ºC 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 in a constant aliquot (100 ml), which was then expressed as percentage dry weight values (Zhu & Lee 1997).

2.7 Fungal cultures

Fungal isolates were grown on freshly prepared sterile Potato Dextrose Agar (PDA) plates. The capability to form pellets (pelletization; ball-like) of the selected fungal species was assessed in Potato Dextrose Broth (PDB) in 100 ml flasks inoculated with 1 cm^2 fungal plug

and incubated at 28 °C for three to five days on an orbital shaker (Ratek Australia) at 150 rpm. This experiment was carried out in triplicate. The extent of pellet formation was then visually assessed. The best pellet forming candidate (*Aspergillus fumigatus*) was selected for use for harvesting microalgal biomass in large-scale studies.

2.7.1 Harvesting of Algal Biomass

Harvesting of microalgal biomass was carried out using two different methods. The first method involved the use of centrifuges (Avanti J-25I, USA) while the second method involved the use of *Aspergillus fumigatus* for the bio-flocculation of microalgal culture. Harvesting of microalgae using centrifugation (Avanti J-25I, USA) involved taking a known amount of microalgal culture, centrifuging at 10,000 × g for 10 min at 4 ^{o}C. The supernatant was then discarded and the microalgal pellet removed and used for further analysis. Harvesting of microalgae with fungus involved the use of a pre-determined optimum fungus: The impact of the fungal: microalgae application was assessed using 2 l flasks with different fungus: microalgal broth ratios. The selected fungal candidate was grown in Potato Dextrose Broth for up to 72 hours. Microalgal broths were prepared individually for both strains of *B. braunii* by inoculating BG11 media with 0.04 g l^{-1} of inoculum and incubating the medium for 72 hours at 54 μmol m^{-2} s^{-1} of continuous light from a cool fluorescence lamp. Six different ratios of fungal: algal broth as were used. These were 1:1, 1:10, 1:20, 1:30, 1:40 and 1:50. The ODs at 680 and 750 nm of the supernatant was measured at 4 hourly intervals over 120 hours after the addition of the fungus to the algae to determine the amount of algae remaining in the supernatant as the co-culture developed. Control experiments were composed of only the microalgae without any addition of fungi.

2.7.2 Drying of algal biomass

The harvested biomass was dried in an oven (Heraeus Instruments) at 65^{o}C for at least 24 hours or until a constant weight was obtained. For large-scale studies, the harvested biomass was freeze-dried (Member of the Panasonic Group, Australian) before being stored at -80^{o}C.

2.7.3 Sonication

A sonication bath (Adelab Scientific) was used to disrupt algal cells to release oil. Sonication was for 5 minutes in all experiments.

2.7.4 Filtration

Filtration of oil after sonication was carried out (0.45 µm, NYL) to remove any biomass from the oil extraction (Zhu & Lee 1997).

2.8 Oil Extraction

Oil was extracted from harvested microalgae using three different solvents; N-hexane, hexane-isopropanol and heptane (Eroglu *et al.* 2011; Moheimani *et al.* 2013; Ryckebosch *et al.* 2012; Sawayama *et al.* 1992). Solvent (10 mL) was added to the freeze-dried algal cells of Kossou-4 were ~2188 mg l^{-1} from BG11, 966.4 mg l^{-1} from JM and 517.8 mg l^{-1} from BBM 3N. For Overjuyo-3 the dry weight values were ~2529.2 mg l^{-1} from BG11, 870.2 mg l^{-1} from JM and 415.8 mg l^{-1} from BBM 3N and incubated overnight (Ryckebosch *et al.* 2012) at room temperature. The samples were then placed 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 solvent was removed by evaporation with pure N_2 gas in a fume hood. Replicate samples were evaluated and the oil content measured gravimetrically.

2.9 Analysis of extracted oil

The composition of the oil extracted from the microalgae was analyzed 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) (Li *et al.* 2013). The capillary column was an Alltech EC-5 (15 m x 0.25 mm). Splitless injection was used with a split time of 60 s. Injection port temperature was 300°C. The carrier gas was helium in constant flow mode at 1.8 ml/min. Injection volume was 1 µl. The GC temperature program was: initial temperature of 40°C for 4 min, ramp rate of 20℃/min, final temperature of 350°C for 4 min. The GC/MS interface temperature was set to 280°C. The FID temperature was 325°C. For full scan mass spectra, the mass spectrometer was scanned from m/z 35 to m/z 550. Electron ionization (EI) was used with the electron energy set to 70 eves. The source temperature was 230°C. The MS quadruple was set to 150°C. Appropriate hydrocarbon standards were prepared and used for calibration and peak identification. Triterpenoid hydrocarbons were identified as C32-34 fractions. Total oil and triterpenoid hydrocarbon contents were expressed as both g l^{-1} and percentage of dry weight.

2.10 Chemical analysis

The harvested biomass was dried in the oven at 65^{o}C for at least 24 hours or until a constant weight was obtained. Elemental analysis (HCNS) was determined by combustion using a Thermo Scientific Flash 2000 series analyzer. Ash was determined as the residual mass left after exposure at 600°C for 5 hours. From the ash content, the content of oxygen was calculated with the high heating value (HHV) being calculated by the Dulong formula from these values (Torri *et al.* 2011).

2.11 Pyrolysis

The dried biomass was divided into three subsamples that were utilised in the pyrolysis experiments. Analytical pyrolysis (Py-GC-MS) was conducted on a small aliquot of dried biomass (about 5 mg) spiked with an internal standard (1.0 ug *o*-isoeugenol) at 600°C for 10 seconds with a heated platinum filament CDS 5250 pyroprobe interfaced to a Varian 3400 GC-Saturn 2000 MS. GC-MS conditions were described in (Conti *et al.* 2014).

Preparative pyrolysis was performed with 4.0 g of dried biomass deposited onto a sliding quartz boat inserted into a horizontal fixed bed quartz reactor (length 650 mm, internal diameter 37 mm) heated at 460 °C (measured internally by a thermocouple) for 15 minutes under a constant flux of nitrogen at 1000 ml min^{-1} (Torri *et al.* 2011). The liquid fractions were collected in two cold traps, the first one immersed into an ice/salt bath (trap 1, - 15 °C) and second one into a dry ice bath (trap 2, - 50°C). The exit of the second trap was connected to a sorbent trap filled with 3.0 g XAD-2 Amberlite (trap 3). The liquid collected in trap 1 was an emulsion (organic and aqueous phase not separable), the oil collected in trap 2 was solubilised in cyclohexane:acetone 2:1 v/v and analysed by GC-MS.

The quartz boat, the cold traps and the sorbent trap were weighed before and after each pyrolysis in order to calculate the weight of solid fraction (char) and liquid fractions (liquid fraction in trap 1, oil fraction in trap 2 and volatiles in trap 3). The obtained weights were used to calculate the yields of each pyrolysis fraction. It was assumed that the components sorbed in trap 3 could be condensed under more efficient trapping conditions; therefore this fraction was included in the liquid fraction. GC-MS analysis was performed with a gas chromatograph Agilent HP 6850, connected to a mass spectrometer quadrupole Agilent HP 5975, equipped with a capillar non-polar column HP-5MS (stationary phase poly[5% difenil/95% dimethyl]siloxane, 30 m x 0.25 mm i.d., 0.25 μm film thickness), using helium as gas carrier (constant pressure 33 cm /s, linear velocity at 200 °C). The thermal program

was: 50°C for 5 min, then 325°C at 10°C/min, hold for 7.5 min. Samples (1 µl) were injected in splitless conditions (1 min, then 1:50 split until end of analysis) at injector temperature 280°C. The mass spectrometer operates in the electronic ionization mode (70 eV) in full-scan acquisition, range m/z 29-600.

2.12 Statistical analysis

Analysis of variance (ANOVA) was used to determine the differences between the levels of growth at different time points for each strain of *B. braunii*. A P value of 0.05 or less was considered as statistically significant. 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) and MiniTab 17.

CHAPTER THREE

The effect of media on biomass and oil production in *Botryococcus braunii* strains Kossou-4 and Overjuyo-3

STATEMENT OF AUTHORSHIP

School of Applied Sciences

Chapter Declaration for Thesis with Publications

The following declaration needs to be made for each paper included in the thesis.
Chapter [Chapter 3] is represented by the following paper:
Paper name **[**The Effect of Media on Biomass and Oil Production in Botryococcus braunii Strains Kossou-4 and Overjuyo-3**]**
Author List **[**Khalid A. Al-Hothaly, Aidyn Mouradov, Abdulatif A. Mansur, Brian H. May, Andrew S. Ball1, Eric M. Adetutu**]**
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Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

Nature of contribution	Extent of contribution (%)
wrote the first draft, collected all of the data, conducted most of the analysis, etc etc]	79.5

The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

Co-author name	Nature of contribution	Extent of Contribution (%)	Signature
Aidyn Mouradov	Contributed to manuscript evaluation	1.5	Signed
Abdulatif A. Mansur	Contributed to manuscript evaluation	1.5	Signed
Brian H. May	Contributed to manuscript evaluation	2.5	Signed
Eric M. Adetutu	Contributed to manuscript evaluation	5	Signed
Andrew S. Ball1	Contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation	10	Signed

Candidate's Signature September / 2015

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Primary Supervisor's Signature September / 2015

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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 ($p < 0.05$).

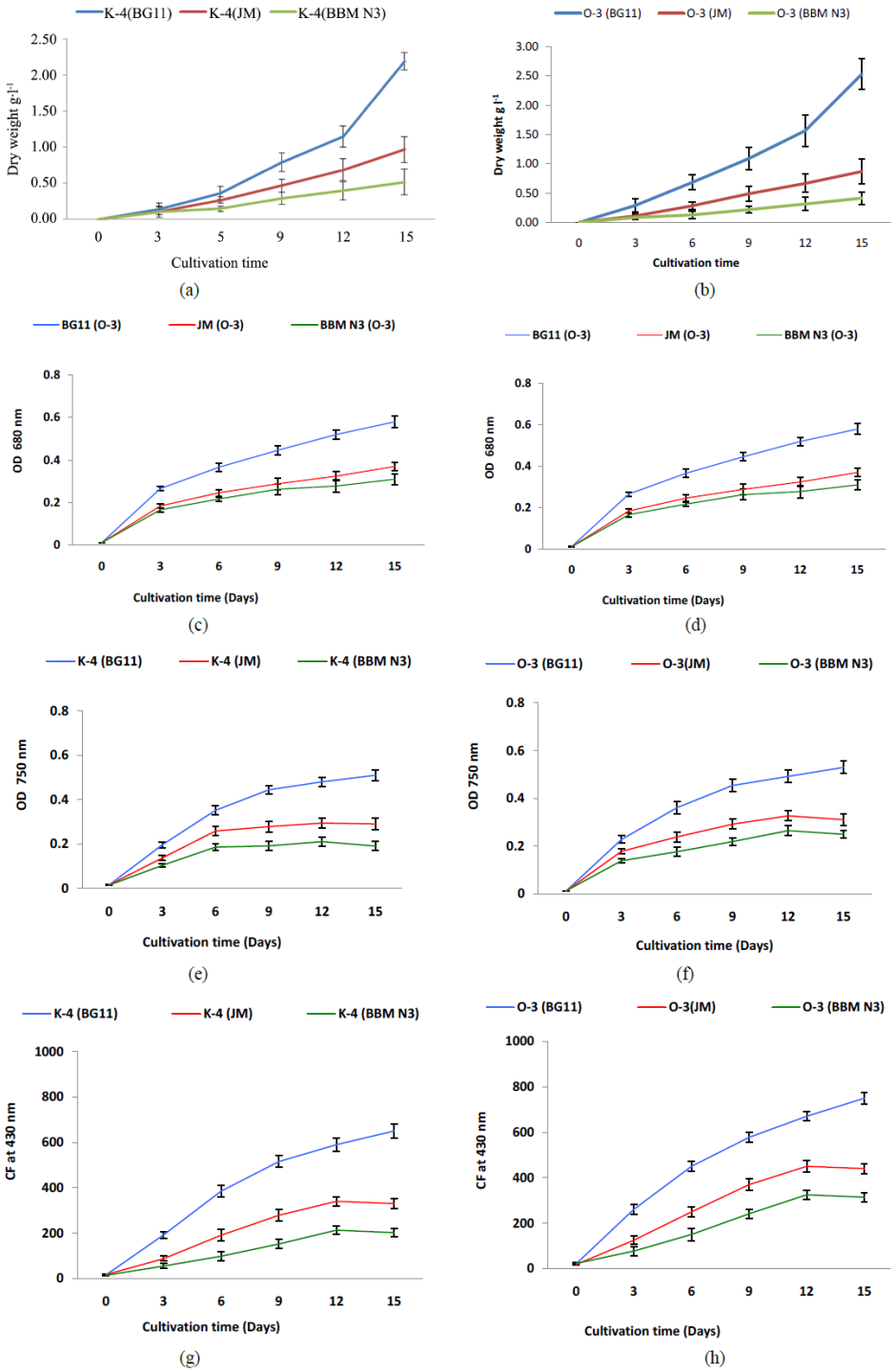

Figure 1. Effects of growth media on the dry weight (a) and (b), ODs at 680 (c) and (d) and 750 nm (e) and (f) and chlorophyll fluorescence (g) and (h) (at 430 nm) of *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3) over 15 days.

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 Over-juyo-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.

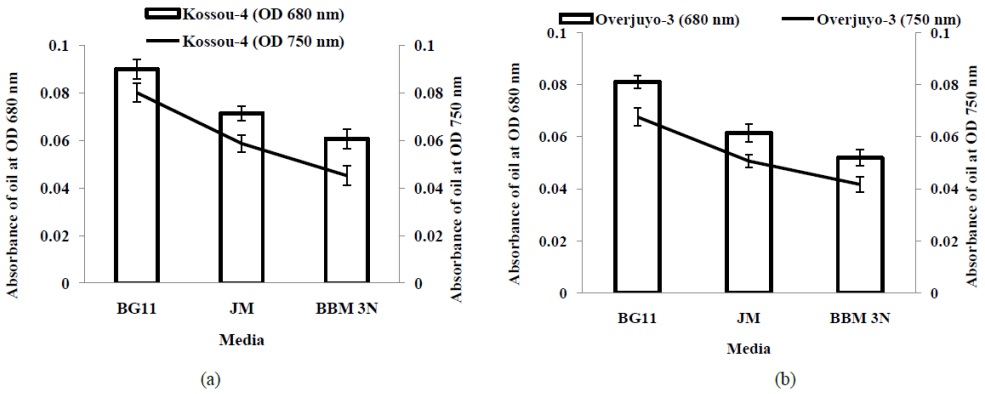

Figure 2. Absorbance values of extracted oil in different media at ODs of 680 and 750 nm (a) & (b) in *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3).

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	-

CHAPTER FOUR

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

STATEMENT OF AUTHORSHIP

School of Applied Sciences

Chapter Declaration for Thesis with Publications

The following declaration needs to be made for each paper included in the thesis.
Chapter [Chapter 4] is represented by the following paper:
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Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

Nature of contribution	Extent of contribution (%)
wrote the first draft, collected all of the data, conducted most of the analysis, etc etc]	81.5

The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

Co-author name	Nature of contribution	Extent of Contribution (%)	Signature
Mohamed Taha	Contributed to manuscript evaluation	1.5	Signed
Brian H May	Contributed to manuscript evaluation	2.5	Signed
Stella Stylianou	Contributed to manuscript evaluation	1.5	Signed
Eric M Adetutu	Contributed to manuscript evaluation	3	Signed
Andrew S.Ball	Contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation	10	Signed

Candidate's Signature September / 2015

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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.

Experimental runs	Culture conditions				
	Nitrogen (mg l^{-1})	Iron (mg l^{-1})	Temperature (°C)	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 30°C 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 30°C 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

Addendum: For GC/MS analysis, see Supplementary figure 1b, p 151.

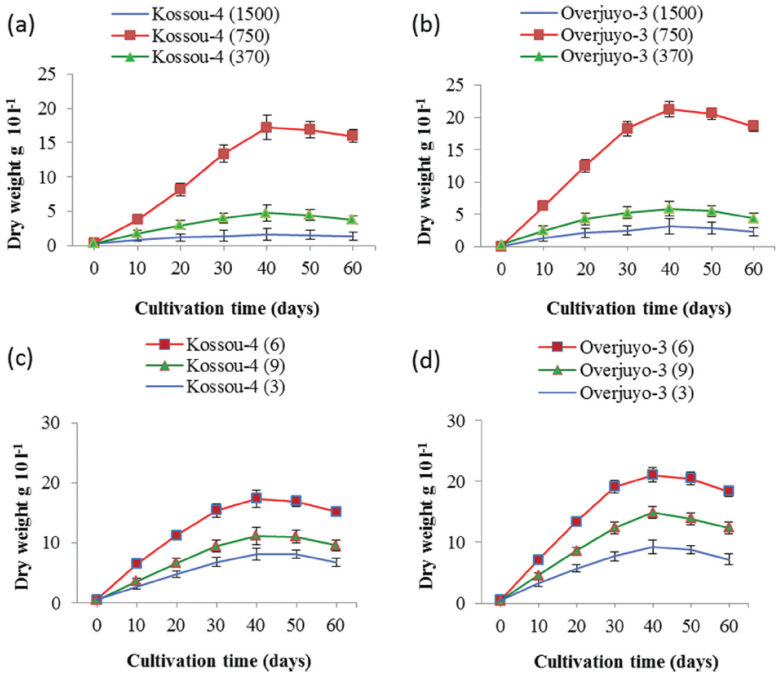

Fig. 1. The effects of nitrogen (a, b) and iron concentrations (c, d) (mg l^{-1}) on the dry weight (g l^{-1}) of *B. braunii* strains Kossou-4 (a, c) and Overjuyo-3 (b, d) respectively. Error bars shown on the graphs represent standard deviation of replicate (n = 3) samples.

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

			Kossou-4	Overjuyo-3
Treatments	Culture condition		Oil weight (g 10 l^{-1})	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).

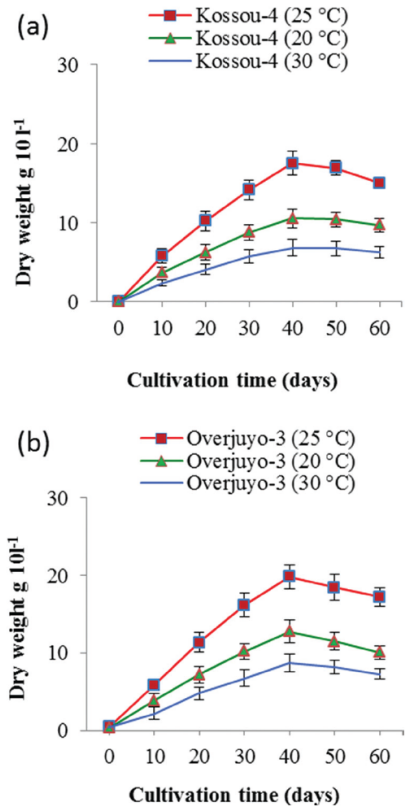

Fig. 2. The effects of temperature on the dry weight (g l^{-1}) of *B. braunii* strains (a) Kossou-4 and (b) Overjuyo-3 (n = 3). Error bars shown on the graphs represent standard deviation of replicate samples.

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

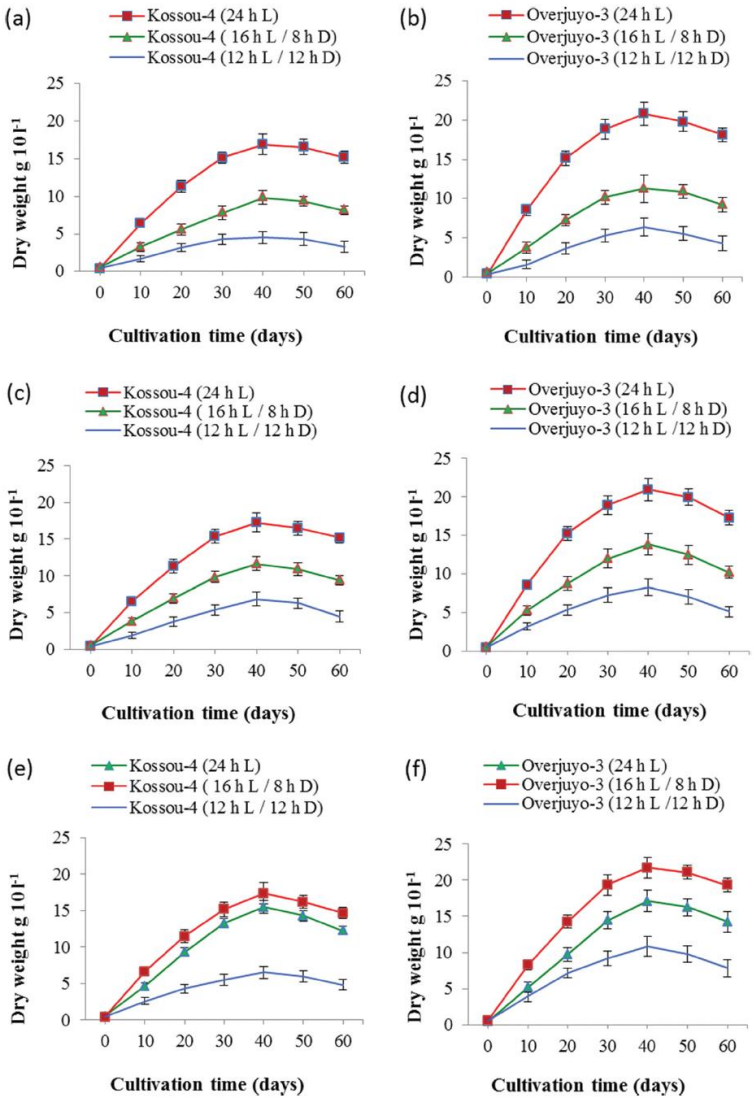

Fig. 3. The effects of photoperiod and light intensity at 54 (a, b), 81 (c, d) and 135 (e, f) µmol photons m^{-2} s^{-1} on the dry weight (g l^{-1}) of *B. braunii* strains Kossou-4 and Overjuyo-3 (n = 3). Error bars shown on the graphs represent standard deviation of replicate samples. L refers to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

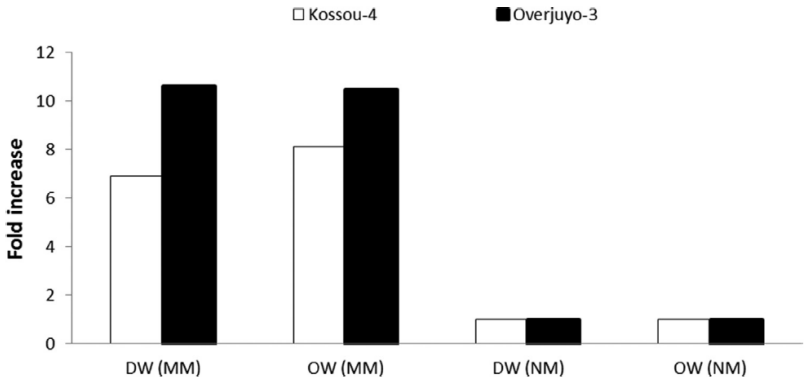

Fig. 4. Comparison of growth and oil production in normal and optimized media for Kossou-4 and Overjuyo-3 assessed by dry weight assay. OW, Oil weight; DW, dry weight; NM, normal BG11 medium; MM, modified BG11 medium.
Note: For NM (nitrogen and iron concentrations of 1500 and 6 mg l^{-1} respectively), 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.

ACKNOWLEDGEMENTS

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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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CHAPTER FIVE

Towards the commercialization of *Botryococcus braunii* for triterpenoid production

STATEMENT OF AUTHORSHIP

School of Applied Sciences

Chapter Declaration for Thesis with Publications

The following declaration needs to be made for each paper included in the thesis.
Chapter [Chapter 5] is represented by the following paper:
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Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

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wrote the first draft, collected all of the data, conducted most of the analysis, etc etc]	82

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Andrew S. Ball	Contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation	10	Signed

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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.

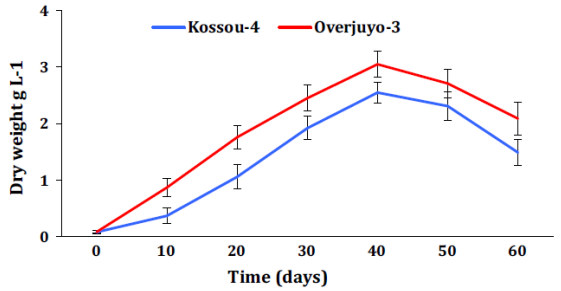

Fig. 1 Growth as assessed by dry weight (g L^{-1}) of *B. braunii* strains Kossou-4 (*blue line*) and Overjuyo-3 (*red line*) under large-scale 500 L cultivation in modified BG11 medium over 60 days. Results shown are the means of three replicates with standard errors shown (color figure online)

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 ($p < 0.05$).

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

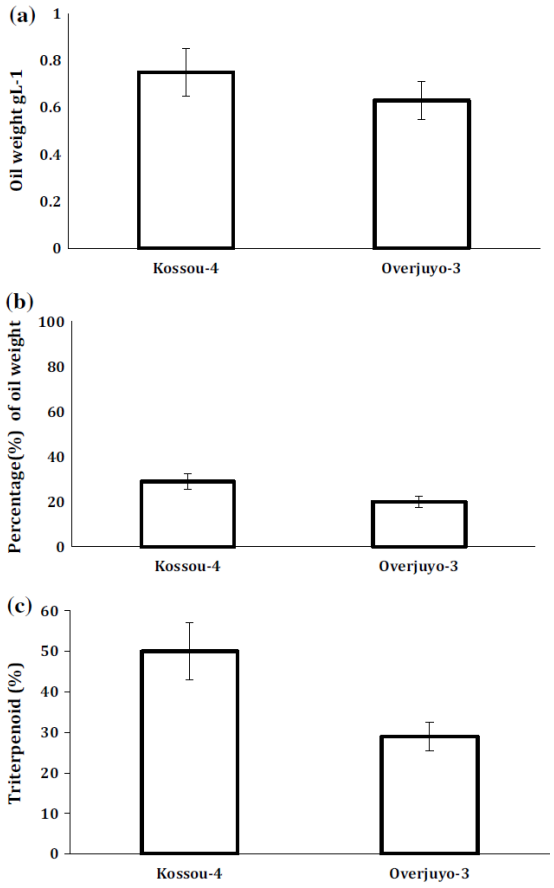

Fig. 2 Oil weight (g L^{-1}) (**a**) and expressed as a percentage (%) (**b**) in *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3 (O-3) grown under large-scale 500 L cultivation in modified BG11 medium after 40 days. **c** % triterpenoid in oil from *B. braunii* strains Kossou-4 (K-4) and Overjuyo-3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

[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.

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CHAPTER SIX

Bio-harvesting and pyrolysis of the microalgae *Botryococcus braunii*

STATEMENT OF AUTHORSHIP

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Chapter Declaration for Thesis with Publications

The following declaration needs to be made for each paper included in the thesis.
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Author List [Khalid A. Al-Hothaly, Eric M. Adetutu , Mohamed Taha, Daniele Fabbri , Chiara Lorenzetti , Roberto Conti , Brian H. May , Sahar S. Shar , Reda A. Bayoumi , Andrew S. Ball
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Declaration by candidate

I declare that I wrote the initial draft of this manuscript, and my overall contribution to this paper is detailed below:

Nature of contribution	Extent of contribution (%)
wrote the first draft, collected all of the data, conducted most of the analysis, etc etc]	79

The following co-authors contributed to the work. The undersigned declare that the contributions of the candidate and co-authors are correctly attributed below.

Co-author name	Nature of contribution	Extent of Contribution (%)	Signature
Eric M. Adetutu	Contributed to manuscript evaluation	2.5	Signed
Mohamed Taha	Contributed to manuscript evaluation	1.5	Signed
Daniele Fabbri	Contributed to manuscript evaluation	3	Signed
Chiara Lorenzetti	Contributed to manuscript evaluation	2	Signed
Roberto Conti	Contributed to manuscript evaluation	1.5	Signed
Brian H. May	Contributed to manuscript evaluation	2.5	Signed
Sahar S. Shar	Contributed to manuscript evaluation	1.5	Signed
Reda A. Bayoumi	Contributed to manuscript evaluation	1.5	Signed
Andrew S. Ball	Contributed in the planning of the experimental design, supervision of research, manuscript preparation and evaluation	5	Signed

Candidate's Signature September / 2015

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Primary Supervisor's Signature September / 2015

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Bio-harvesting and pyrolysis of the microalgae *Botryococcus braunii*

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HIGHLIGHTS

- 500 l-scale cultivation of *B. braunii* strains Kossou-4 and Overjuyo-3.
- Screening of fungal isolates led to selection of bioflocculation candidate.
- *Aspergillus fumigatus* successfully used to harvest up to 98% of biomass.
- Ultimate and pyrolysis analyses showed no impairment of feedstock value by fungus.
- First time *A. fumigatus* used for economical and efficient harvesting of *B. braunii*.

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ABSTRACT

The microalgae *Botryococcus braunii* is widely recognized as a potentially important biofuel-feedstock whose commercial exploitation is limited by difficulties with its cultivation and harvesting. In this study, two *B. braunii* strains, Kossou-4 and Overjuyo-3 were successfully cultured at a 500 l-scale for 60-days. Harvesting by bio-flocculation with *Aspergillus fumigatus* at an optimum ratio of 1:40 of fungus to microalgal culture resulted in up to 98% recovery of biomass in the two strains. Ultimate analysis (C, N, H, S, ash, high heating value) and pyrolysis (analytical and preparative pyrolysis and GC–MS assays) showed that co-harvesting with fungi did not cause any impairment of the feedstock value of the microalgal biomass. This work represents the first report on the successful culturing and harvesting of these strains at a 500 l-scale using bio-flocculation. The use of *A. fumigatus* represents an efficient and economical method for the harvest of *B. braunii* for biofuel production.

1. Introduction

Global warming, arising from the use of fossil fuels is a world-wide environmental problem. Mitigation of the effects of global warming gases such as CO_2 requires the use of alternative renewable and non-polluting fuel sources. This need for alternative energy sources has led to increasing interest in biofuel (oil) production. One alternative fuel source is microalgal biomass from which biodiesel, bioethanol and bio-oil can be produced (Sarkar et al., 2015). The potential use of microalgae for the production of biofuels has therefore received significant attention (Costa et al., 2007).

The green alga, *Botryococcus braunii* is believed to represent one of the most promising sources of biofuels for the production of economic quantities of hydrocarbons by utilizing artificial or natural light (de la Noue and de Pauw, 1988). *B. braunii* is characterized by unusually high hydrocarbon content, reported to reach up to 75% of the dry weight of the cell (Banerjee et al., 2002). It is a slow growing, colonial, fresh water microalga (Brown et al., 1969). *B. braunii* represents a potential source of valuable hydrocarbons such as triterpenes and an alternative to fossil fuels (Chisti, 2007). Although race B strains of *B. braunii* are known to produce triterpenoids and other useful products, their commercial development is limited due to their slow growth rates and strain variability (Qin, 2010).

Hydrocarbons from microalgae need to be separated from the biomass using an appropriate solvent extraction method before being upgraded into hydrocarbons with lower degrees of

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unsaturation and shorter chain length (e.g. C_{10}–C_{20} diesel fuel vs. C_{30}–C_{36} of botryococcenes) for their utilization in current energetic systems. This is carried out through pyrolysis. Pyrolysis is a thermochemical process whereby hydrocarbons are thermally cracked and converted into more readily extractable oil. With respect to *B. braunii*, the pyrolytic behaviour of hydrocarbons obtained from the microalgae has been previously investigated on an analytical scale (Liu et al., 2012; Nguyen et al., 2003). Preparative thermochemical experiments on *B. braunii* with the recovery of bio-oil were reported for hydrothermal liquefaction (Sawayama et al., 1999) and steam pyrolysis (Watanabe et al., 2014), while studies on catalytic upgrading were conducted on the extracted oil or single hydrocarbons (Tran et al., 2010; Yamamoto et al., 2014).

To develop the commercial potential of this organism, strain selection and growth media must be optimized. 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 (Al-Hothaly et al., 2014; Gim et al., 2014; Kalacheva et al., 2002; Krzemińska et al., 2014; Leite et al., 2013; Qin, 2005). Previous work by the authors has demonstrated the benefits of selecting the right medium and solvent for hydrocarbon extraction and optimizing culture conditions for maximum biomass and oil yield (Al-Hothaly et al., 2014). The microalgae were also cultured at the 500 l-scale without any significant impairment of biomass and oil yield. However following cultivation, the method of microalgae harvest is crucial.

For successful commercial use of *B. braunii* strains, an efficient and economical method for microalgal harvest must be developed for the subsequent conversion of the biomass into biofuels (Leite et al., 2013; Pragya et al., 2013). Harvesting of microalgae is energy intensive and estimated to account for up to 50% of the total cost of biofuel (biodiesel) production (Wrede et al., 2014). Methods of harvesting microalgae include sun drying, thermal drying, spray drying (Brennan and Owende, 2010), centrifugation, filtration, sedimentation, flocculation and flotation. Sun drying is the most economical approach, but requires a long drying time and large drying area (Brennan and Owende, 2010). Thermal, spray and freeze drying are all rapid drying methods, but are substantially more expensive or can damage algal pigments or lipids (Brennan and Owende, 2010; Widjaja et al., 2009). Centrifugation and filtration are the more commonly used harvesting methods but they also tend to be energy intensive and costly (Leite et al., 2013) while sedimentation and flotation are inexpensive but are species specific (Zhou et al., 2012). Flocculation methods have also been investigated using chemicals and natural products (Lam and Lee, 2012; Milledge and Heaven, 2013) with some success.

One innovative approach to the harvest of microalgae by flocculation involves the use of fungi. Certain species of filamentous fungi can spontaneously form pellets when grown in a solution or can be induced to form pellets by chemical means. These pellets sink to the bottom of the solution and are easily harvested. When such fungi are inoculated into a microalgal broth, the fungi can entrap microalgal cells into the filamentous mass as a co-culture and flocculate the microalgae (Gultom and Hu, 2013). There are limited reports of the use of bio-flocculation for microalgal harvest in the literature. *Aspergillus* species have been successfully used for co-pelletization and harvesting of *Chlorella vulgaris* from different sources including treated wastewater (Zhang and Hu, 2012; Zhou et al., 2012). A recent study by Wrede et al. (2014) demonstrated the feasibility of using the fungus *Aspergillus fumigatus* to flocculate a range of microalgal species used for waste water treatment (Wrede et al., 2014). The mechanism responsible for this bio-flocculation appears to be the neutralization of negative charges on the algal surface by the positively charged fungi. Moreover, this approach to bio-flocculation tends to enhance biomass and lipid production (Wrede et al., 2014).

However, questions remain on the suitability of bio-flocculation as a method for microalgal harvest. For example, the few reported studies have all been carried out at a laboratory scale. While it is possible that bio-flocculation using fungi may be applicable to the harvest of microalgae for biofuel production at commercial or large scale, there is no report of this in the literature to best of the authors' knowledge. With regards to one of the most promising source of biofuel, *B. braunii*, nothing is known about any fungus that can be used for its harvesting or even whether bio-flocculation is possible with this microalgael. It is also unclear whether any developed bio-flocculation method for *B. braunii* can be used for commercial purposes. Therefore, this study aims to (i) assess selected fungal species for their suitability for harvesting *B. braunii* via co-culture induced flocculation and (ii) investigate the suitability of co-culture flocculation harvesting method in 500 l tanks in two *B. braunii* strains. In addition, (iii) the possible influence on the harvesting procedure on the pyrolytic behaviour of the biomass was investigated by analytical and preparative pyrolysis.

2. Methods

2.1. Microalgae source

The *B. braunii* strains selected for use in this study were two race B strains, Kossou-4 and Overjuyo-3 which are known for their high level of hydrocarbon production (Li et al., 2013). Both strains were obtained from Flinders University and originated from Pierre Metzger's collection. Kossou-4 was originally from the Ivory Coast and shows a brownish coloring while Overjuyo-3 was from Bolivia and is green in color (Metzger et al., 1990).

2.2. Selection of fungal candidates

Five fungal isolates used in this study were collected from Professor Ball's collection (RMIT University, Melbourne, Australia). Two of the fungal strains used, *A. fumigatus* and *Phanerochaete chrysosporium*, have been reported to successfully co-pelletize with *C. vulgaris* (Zhang and Hu, 2012). For the three remaining species, *Trametes versicolor*, *Iprex lacteus* and *Pleurotus ostreatus* co-pelletization data were either unavailable or unknown. These fungal isolates were grown on freshly prepared sterile Potato Dextrose Agar (PDA) plates containing 0.015 g l^{-1} tetracycline at 28 °C. The ability to form pellets (ball-like forms) by the selected fungal species was assessed in Potato Dextrose Broth (PDB) in 100 ml flasks inoculated with 1 cm^2 fungal plug. The flasks were incubated at 28 °C for three to 5 days on an orbital shaker (Ratek, Australia) at 150 rpm. This experiment was carried out in triplicate. The extent of pellet formation was then visually assessed. The best pellet-forming candidate was selected for further investigation. The fungal candidate was maintained on sterile PDA and maintained at 4 °C.

2.3. Co-pelletization of fungus and microalgae

To assess whether a co-culture of *B. braunii* with the selected fungal candidate would result in harvesting of microalgae biomass, 20 ml of this candidate was added to 400 ml of algal broth and agitated at 100 rpm for 12 h at 25 °C. The fungus: algal broth was visually assessed for pellet formation and the disappearance of green algal coloration (Wrede et al., 2014).

2.4. Determination of fungal: microalgae application ratio

The impact of the fungal application at different concentrations on harvesting efficiency was assessed using 2 l flasks using different fungus: microalgal broth ratios. The selected fungal candidate was grown in PDB for up to 72 h to generate sufficient fungal biomass for experimental use. Microalgal broths were prepared individually for the two race B strains of *B. braunii* (Kossou-4 and Overjuyo-3) by inoculating the BG11 media with 0.04 g l^{-1} of the desired inoculum. The inoculated media were incubated for 72 h at 54 μmol m^{-2} s^{-1} of continuous light from a cool fluorescence lamp to generate the required microalgal biomass. Six different ratios of fungal: algal broth were used; 20/20 ml (1:1), 20/200 ml (1:10), 20/400 ml (1:20), 20/600 ml (1:30), 20/800 ml (1:40), 20/1000 ml (1:50). The ODs at 680 and 750 nm of the supernatant was measured at 4 hourly intervals over 120 h to determine the amount of algae remaining in the supernatant as the co-culture developed. Control experiments were composed of only the microalgae without any addition of fungi.

2.5. l-scale microalgal culture

Ten liters of the modified BG11 medium was prepared as previously described (Ge et al., 2011; Zhila et al., 2005), with sodium nitrate ($NaNO_3$) reduced from 1.5 g l^{-1} to 0.75 g l^{-1}. This medium concentrate was added to 490 l of fresh water in each tank. Two circular fiber glass tanks (height 83 cm, diameter 143 cm, capacity 500 l) were used for each strain. An aliquot (2.5 l) of Kossou-4 which corresponded to 0.04 g l^{-1} (dry weight) of microalgae culture was added to one tank. An equivalent aliquot of Overjuyo-3 was added to the other tank. The inoculated cultures were incubated in the tanks for 60 days with continuous air supply. Aeration was provided by continuous bubbling of air up from the center of the tank at an air flow rate of 18.2 m^3 min^{-1} with illumination from a cool fluorescence lamp at 54 μmol m^{-2} s^{-1}. Temperature was maintained by a thermostat in the range 24.8–25.5 °C (Brennan and Owende, 2010).

2.6. Harvesting of microalgae with fungus

Based on the results obtained from Section 2.4, the optimum ratio was used for harvesting the two *Botryococcus* strains. Harvesting of the 500 l cultures was achieved over a 12 h period. For *B. braunii* Kossou-4 and Overjuyo-3, the 60 day cultures were divided into two 250 l aliquots. Microalgae in one aliquot were harvested with the fungal candidate at the selected ratio by bio-flocculation with continuous agitation with air. The remaining 250 l of each culture was harvested without fungi by the use of a centrifuge (Grima et al., 2003) to generate biomass that would be used as control samples for subsequent analysis.

2.7. Chemical analysis

The harvested biomass was dried in the oven at 65 °C for at least 24 h or until a constant weight was obtained. Elemental analysis (HCNS) was determined by combustion using a Thermo Scientific Flash 2000 series analyzer. Ash was determined as the residual mass left after exposure at 600 °C for 5 h. From the ash content, the content of oxygen was calculated with the high heating value (HHV) being calculated by the Dulong formula from these values (Torri et al., 2011).

2.8. Pyrolysis

The dried biomass was divided into three subsamples that were utilised in the pyrolysis experiments. Analytical pyrolysis (Py–GC–MS) was conducted on a small aliquot of dried biomass (about 5 mg) spiked with an internal standard (1.0 μg *o*-isoeugenol) at 600 °C for 10 s with a heated platinum filament CDS 5250 pyroprobe interfaced to a Varian 3400 GC-Saturn 2000 MS. The GC–MS conditions used were as previously described (Conti et al., 2014).

Preparative pyrolysis was performed with 4.0 g of dried biomass deposited onto a sliding quartz boat inserted into a horizontal fixed bed quartz reactor(length 650 mm, internal diameter 37 mm) heated at 460 °C (measured internally by a thermocouple) for 15 min under a constant flux of nitrogen at 1000 ml min^{-1} (Torri et al., 2011). The liquid fractions were collected in two cold traps, the first one immersed into an ice/salt bath (trap 1, −15 °C) and the second immersed into a dry ice bath (trap 2, −50 °C). The exit of the second trap was connected to a sorbent trap filled with 3.0 g XAD-2 Amberlite (trap 3). The liquid collected in trap 1 was an emulsion (organic and aqueous phase not separable), the oil collected in trap 2 was solubilised in cyclohexane:acetone (2:1 v/v) and analysed by GC–MS.

The quartz boat, the cold traps and the sorbent trap were weighed before and after each pyrolysis in order to calculate the weight of solid fraction (char) and liquid fractions (liquid fraction in trap 1, oil fraction in trap 2 and volatiles in trap 3). The obtained weights were used to calculate the yields of each pyrolysis fraction. It was assumed that the components sorbed in trap 3 could be condensed under more efficient trapping conditions; therefore this fraction was included in the liquid fraction.

GC–MS analysis was performed with a gas chromatograph Agilent HP 6850, connected to a mass spectrometer quadrupole Agilent HP 5975, equipped with a capillary non-polar column HP-5MS (stationary phase poly [5% difenil/95% dimethyl] siloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness), using helium as gas carrier (constant pressure 33 cm/s, linear velocity at 200 °C). The thermal program was: 50 °C for 5 min, then 325 °C at 10 °C/min, hold for 7.5 min. Samples (1 μl) were injected in splitless conditions (1 min, then 1:50 split until end of analysis) at injector temperature 280 °C. The mass spectrometer operates in the electronic ionization mode (70 eV) in full-scan acquisition, range m/z 29–600.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the differences in the levels of growth at different time points (3 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.

3. Results and discussion

3.1. Fungi-microalgae pelletization

Five species of fungi were assessed for their pelletization abilities. These were *T. versicolor*, *I. lacteus*, *P. chrysosporium*, *P. ostreatus* and *A. fumigatus*. Of these, *A. fumigatus* produced the highest amount of pellets after 72 h growth (data not shown) (Wrede et al., 2014). *T. versicolor*, *P. chrysosporium* and *P. ostreatus* all produced fewer pellets which were deemed to be too low in concentration for potential commercial application. Only *I. lacteus* did not produce any pellet. *A. fumigatus* was therefore selected for use in subsequent investigations. Visual assessment of the *A. fumigatus*: algal broth showed successful pelletization, with the pellets composed of algae and *B. braunii* strains. The green coloration of the broth was also substantially reduced after 12 h of incubation.

Members of the genus *Aspergillus* have been successfully used for harvesting microalgae. For example, *Aspergillus niger* has been

used for harvesting the algae *C. vulgaris* (Zhang and Hu, 2012). Similarly, Zhou et al. (2012) found that *Aspergillus* sp. UMN F01 and F02 were successfully used for the bio-flocculation of *C. vulgaris* (Zhou et al., 2012) in BG11 medium. *Aspergillus oryzae* has also been successfully used to harvest *C. vulgaris* under laboratory based conditions (Zhou et al., 2012). Wrede et al. (2014) reported that *A. fumigatus* was successfully used for harvesting 10 out of 11 different microalgal strains including *C. vulgaris* with up to 90% flocculation after 24 h (Wrede et al., 2014). Therefore, in most studies *C. vulgaris* has been the target microalgae. This present study is therefore the first to demonstrate that *A. fumigatus* can be successfully used for co-pelletization of *B. braunii*. In this study, *A. fumigatus* produced 97–98% flocculation of the *B. braunii* strains within 8~12 h at the laboratory scale (data not shown). While the mechanism of fungal–algal pelletization remains unclear, it is believed that algae surfaces are negatively charged due to the presence of some functional groups which attract positively charged fungal mycelia leading to attachment (Wrede et al., 2014).

3.2. Optimization of harvest efficiency

Optimization is crucial to making effective use of the fungus being used for harvesting for efficiency and cost saving purposes. Therefore in this study different ratios were assessed with a view of getting the best ratio that involved the lowest use of fungal biomass with the highest algal biomass. This is especially important for any commercial exploitation of this fungus-microalgae interaction for harvesting microalgae.

Fig. 1 shows the results for the two strains at the six different tested ratios and controls at 750 nm while Fig. 2 summarizes the results by showing the percentage flocculation efficiencies for the two strains at each ratio. At the ratio of 1:1, harvest was slow and incomplete by 120 h (57% Kossou-4; 62% Overjuyo-3), while the control treatment showed slow growth over the 120 h (Fig. 2a). In contrast, at a *A. fumigatus*/*B. braunii* ratio of 1:10, the harvest was largely completed by 12 h with 93% of Kossou-4 and 95% of Overjuyo-3 being harvested (Figs. 1 and 2b). A similar trend

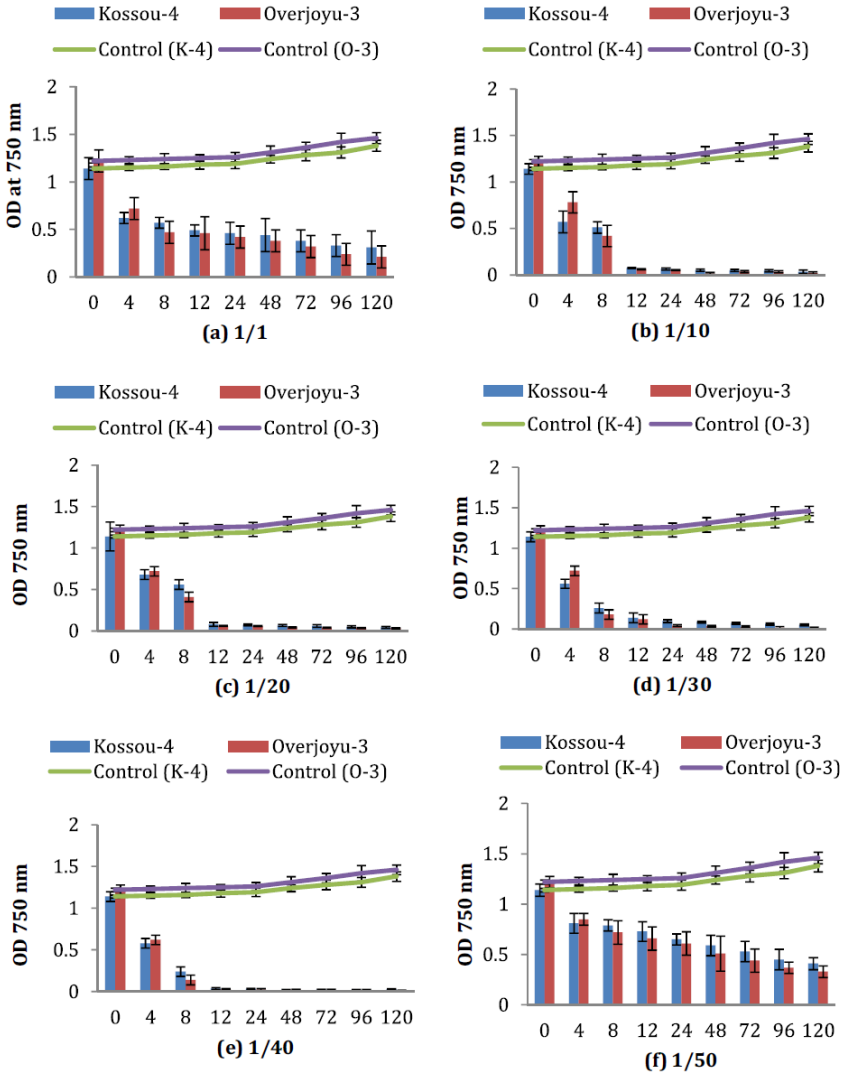

Fig. 1. Bio-flocculation of *B. braunii* strains Kossou-4 and Overjuyo-3 (as assessed by OD_{750} analysis of culture supernatants) using different *Aspergillus fumigatus*/*B. braunii* ratios together with control (non-flocculated cultures of microalgae). Optical density assessment at 750 nm at microalgae: fungal ratios of 1/1 (a), 1/10 (b), 1/20 (c), 1/30 (d), 1/40 (e) and 1/50 (f) in BG11 medium. $N = 3$ and control samples consist of only microalgae.

was observed at a ratio of 1:20 (92–94% of algae harvested), 1:30 (87–90% of algae harvested) and 1:40 (96–97% of algae harvested) (Figs. 1 and 2c, d and e). However at a ratio 1:50, the harvest was slower and efficiency declined to 35% for Kossou-4 and 45% for Overjuyo-3 (Figs. 1 and 2f). In terms of efficient use of fungus, a ratio of 1:40 was selected as the optimal ratio for harvesting from 500 l-scale studies as it resulted in the highest harvesting percentage (Fig. 2e). OD measurements at 680 nm also confirmed that the ratio of 1/40 was the best for harvesting (data not shown). The successful co-pelletization at a 1:40 ratio demonstrated that this is a feasible approach with a relatively small (and therefore economic) volume of fungi resulting in an efficient flocculation of microalgae. Since there was little difference between the results for the two strains, it appears likely that this approach could be applied to other *B. braunii* strains.

3.3. 500 l-scale microalgae culture

Successful harvesting of the 500 l-scale cultures was achieved at the 1:40 ratio over 12 h, for *B. braunii* Overjuyo-3 resulting in the harvesting of 0.75 kg of dry biomass from 250 l of culture (3.0 g l^{-1}), representing approximately 98% of the estimated total *B. braunii* Overjuyo-3 biomass produced, harvested with *A. fumigatus*. Another 250 l of culture from Overjuyo-3 was harvested by centrifugation alone (without fungus) resulting in 0.76 kg of dry biomass. The same trend was observed with Kossou-4 with a 1:40 ratio over 12 h with 0.64 kg of dry biomass from 250 l of culture (2.6 g l^{-1}) (representing 97% of total *B. braunii* Kossou-4 biomass) being harvested with *A. fumigatus*. The remaining 250 l of culture from Kossou-4 was harvested by centrifuging alone (0.65 kg of dry biomass). This study therefore has shown that *A. fumigatus* can be successfully used to co-harvest the two *B. braunii* strains in 500 l-scale studies. This is the first time this has been carried out for *B. braunii* strains.

3.4. Chemical characteristics of harvested biomass

Given that *B. braunii* strains are likely to be used for biofuel production, it is important not only to have an efficient and economical harvesting method but also to make sure that the process of harvesting did not significantly alter the microalgae composition. Therefore, analysis of the elemental compositions of the harvested microalgae was carried out. The results reported in Table 1 indicate that the elemental composition was very similar for all the biomass samples and were not significantly different when the biomass was obtained with or without fungal bioflocculation ($P \geqslant 0.05$). The calculated mean values for carbon, hydrogen and nitrogen of the microalgal biomass were 44, 6.5 and 7.0% d.w., respectively, with

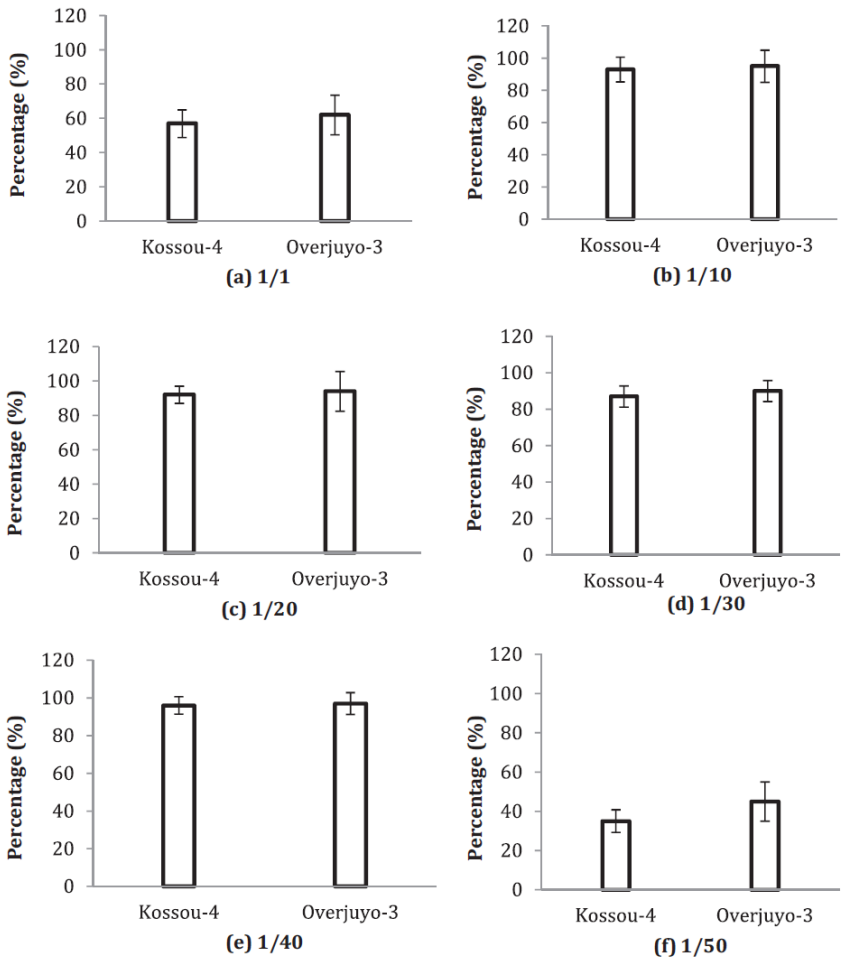

Fig. 2. Percentage of biomass removed by different ratios of *Aspergillus fumigatus* to *B. braunii*; 1/1 (a), (b) 1/10, 1/20 (c), 1/30 (d), 1/40 (e) and 1/50 (f). Algal abundance was measured using OD at 750 nm.

a relative standard deviation (RSD) below 2%, corresponding to the experimental data variability. The values of H/C molar ratios were 1.8 for the biomass samples. From the ash content of 10.5 ± 0.6%, the content of oxygen was calculated to be 42.3 ± 0.7%; from these values the high heating value (HHV) calculated by the Dulong formula (Torri et al., 2011) was calculated to be 19 MJ kg^{-1} (data not shown).

Altogether, these data indicate that the presence of fungi did not substantially alter the composition of the major elements of the microalgae strains. From the pollutant perspective, the percentage of sulphur was fairly low in the biomass for both strains (0.2% wt/wt; Table 1), with and without fungus. Liu et al. (2012) and Watanabe et al. (2014) both reported low sulphur content (0.18%) in biomass from *B. braunii*. The direct use of fuel with low sulphur content is an important positive feature in reducing its environmental impact. However, the presence of nitrogen at 7% may pose problems due to the possible formation of nitrous oxides (NOx). From an energetic perspective, the values of C and HHV placed the harvested biomass in the range of other microalgal species but somewhat lower than other values reported for another *B. braunii* strain (Watanabe et al., 2014). This discrepancy might be due to a higher content of ash and a lower level of hydrocarbons (20%) of the cultivated strains.

3.5. Pyrolysis of the harvested biomass

The pyrolytic behavior of the *B. braunii* biomass harvested with and without fungi was investigated by analytical Py–GC–MS and preparative pyrolysis (fixed bed reactor). The molecular distribution of the pyrolysate evolved by Py–GC–MS provided preliminary indications on the possible composition of the bio-oil. The molecular pattern of the pyrolysates of the four samples was quite similar, principally characterized by the thermal degradation products of proteins (e.g. indole, phenols), carbohydrates (e.g. furanmethanol, hydroxycyclopentenone) and lipids, in particular phytadienes from chlorophyll. The pyrograms were similar to that reported in the literature from Py–GC–MS of fresh *B. braunii* (Nguyen et al., 2003). The estimates made on the yields of selected pyrolysis products was affected by a rather low precision (RSD in the 4–35% range), probably due to the analysis of a small amount of a highly heterogeneous sample; however, no significant differences were be observed among the four biomass samples (on average the yield was 6.2 μg mg^{-1} with 24% RSD).

Preparative pyrolysis of biomass was conducted at 460 °C, close to the maximum weight loss reported for *B. braunii* (Liu et al., 2012). The yields of the pyrolysis fractions of the two strains obtained with and without fungal flocculation are reported in Table 2 with statistical analyses showing no significant differences between these values ($P \geqslant 0.05$). Most of the products were condensed in trap 1 (salt/ice bath) in the form of an aqueous emulsion with an inter-sample average yield of 34.8% (3.6% RSD vs.1–6% RSD from intra-sample replicate analyses). A minor fraction was condensed in trap 2 (dry ice), while the presence of products in trap 3 (sorbent) indicated that the cold trapping was not quantitative.

Table 1
Mean values and standard deviation (±sd) of nitrogen (N), carbon (C), hydrogen (H) and sulphur (S) (% weight) of the two strains of *B. braunii* collected with and without flocculation with the fungus *Aspergillus fumigatus*. H/C atomic ratios are also reported.

	N	±sd	C	±sd	H	±sd	S	±sd	H/C
K4	6.96	0.10	44.96	0.84	6.64	0.12	0.24	0.04	1.77
K4 + fungi	6.89	0.10	43.52	0.47	6.55	0.33	0.15	0.14	1.81
O3	7.09	0.06	43.68	0.52	6.54	0.09	0.26	0.03	1.80
O3 + fungi	6.99	0.12	43.79	0.57	6.39	0.22	0.20	0.05	1.75

Note: K4 refers to *B. braunii* Kossou-4 while O3 refers to *B. braunii* Overjuyo-3.

Table 2
Yields (weight% from original biomass) of the fractions collected from the pyrolysis of two strains of *B. braunii* at 475 °C with and without flocculation with fungi *Aspergillus fumigatus*.

	Kossou-4		Kossou-4 + *A. fumigatus*		Overjuyo-3		Overjuyo-3 + *A. fumigatus*	
	Mean	±sd	Mean	±sd	Mean	±sd	Mean	±sd
Trap 1	33.2	1.7	36.3	2.3	34.9	1.7	34.7	0.43
Trap 2	8.7	5.1	4.8	0.76	3.42	0.29	3.75	0.25
Trap 3	8.4	2.1	9.7	5.0	13.3	0.4	12.0	0.3
Total liquid	50	6	51	5	52	2	51	1
Char	28.8	0.3	28.5	0.3	29.3	0.1	29.6	0.3

Mean and sd (standard deviation) from three replicates.

The precision of yields in traps 2 and 3 was not high due to the low amount of collected material, but the overall yield of the liquid fraction obtained by summing the contribution of the three traps was 51% (RSD 1%). Again, no significant differences were observed when the biomass was harvested in the presence of *A. fumigatus*.

The chemical composition of the oil collected in the second trap was investigated by GC–MS. The GC–MS traces were characterized by the dominant presence of hydrocarbons, that included phytadienes, phytene and pristene from the thermal cracking of chlorophyll, while an unresolved complex mixture (UCM) in the range of C_{15}–C_{30} peaked by *n*-alkenes/*n*-alkanes probably derived from the thermal cracking of triterpenoids and algaenans (Nguyen et al., 2003).

4. Conclusion

This study has shown that *A. fumigatus* can be used to harvest two *B. braunii* strains Kossou-4 and Overjuyo-3. The optimum co-harvesting ratio was found to be 1:40 (fungus to microalgal culture) and was used to harvest 0.6–0.75 kg of the strains in 500 l-scale studies. Ultimate analysis and pyrolysis experiments showed that co-harvesting with fungi did not significantly change the C, H, N, S and ash content, HHV, bio-char and bio-oil content (feedstock value) of the harvested biomass. Therefore, the use of *A. fumigatus* offers an efficient and economical method for harvesting of *B. braunii* for biofuel production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.04.113.

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CHAPTER SEVEN

General Discussion

7.0 General Discussion

The colonial, fresh water microalgae *Botryococcus braunii* has received considerable research attention since it produces hydrocarbons by fixing atmospheric CO_2 photosynthetically (Chen *et al.* 2011), accumulating unusually high amounts of hydrocarbons (11-35% of its dry weight) (Ashokkumar & Rengasamy 2012; Metzger & Largeau 2005; Rao *et al.* 2012). This organism therefore holds considerable potential as a renewable source of petroleum substitutes (Banerjee *et al.* 2002; Metzger & Largeau 2005). Of the three races of *B. braunii* (A, B and L), the highest oil content is produced by Race B (approx. 86%) (Banerjee *et al.* 2002). Race B strains also produce triterpenoid hydrocarbons (triterpenes) including botryococcene and squalene (Metzger *et al.* 1985) which are high-value products that can be used as biofuels as well as in a range of other products (Richmond 2008).

A number of Race B strains of *B. braunii* have been shown to have high hydrocarbon content which makes them suitable for biofuel production. Li *et al* 2013 reported that Ayame 1, Kossou-4, Overjuyo-3 and Paquemar all showed good biofuel production potential, producing greater than 21% of hydrocarbons relative to their dry biomass, with Kossou-4, Overjuyo-3 and Paquemar showing the highest oil content as a percentage of dry weight (Li *et al.* 2013). In this project the strains Kossou-4 and Overjuyo-3 were selected for investigation and their potential for large scale production of hydrocarbons, and in particular triterpenes, was assessed to provide directions for their future large-scale commercial production. This project aimed firstly to assess the production of biomass, oil and triterpenes in the two race B strains of *B. braunii*, Koussou-4 and Overjuyo-3, when grown in a range of media and under a range of conditions; and secondly, to assess the potential of these two *B. braunii* strains to produce biomass and triterpenes when grown on a large scale under optimised conditions. Chapter three reported and discussed the results of a series of experiments which investigated the effects of different growth conditions on oil and

hydrocarbon production. This chapter also compared different solvent conditions for the extraction of oil from algal biomass (Al-Hothaly *et al.* 2015a). Chapter four investigated the effects of different nutrient, temperature and lighting conditions on biomass, oil and hydrocarbon production in order to select conditions suitable for growing the two *B. braunii* strains under large-scale conditions (Al-Hothaly *et al.,* (2015b); In Press, DOI: 10.1080/09670262.2015.1071875). Chapter five investigated the large-scale growth of these two *B. braunii* strains under optimised conditions and determined the yields of biomass, oil and triterpenes. In addition, this chapter investigated a novel approach to the large-scale harvesting of algal biomass using flocculation induced by fungus (Al-Hothaly *et al.* 2015c).

7.1 Effect of Media on Biomass and Oil Production

B. braunii is a relatively slow-growing microalga (Al-Hothaly *et al.* 2015a) so optimisation of its growth is an important issue for its commercial production. Important determinants of growth include the nutrients contained in the growth medium, temperature, and light. Previous studies have investigated the growth and biomass production of *B. braunii* strains in a diversity of growth media including: blue-green algae medium (BG11) (Rani *et al.* 2011); bold basal medium (BBM) and in BBM with added nitrogen and vitamins i.e. BBM-3N medium (Velichkova *et al.* 2012); Jaworski's Medium (JM) (Li *et al.* 2013); CHU13 (Furuhashi *et al.* 2013).

One study compared the growth of *B. braunii* in four autotrophic growth media (CHU13, Z8, BBM, and BG11) at 25^{o}C and found that the highest biomass was produced in Z8 and BG11 highest biomass yield (Ambati *et al.* 2010).

However, data from studies that directly compared the effect of different growth media on the Koussou-4 and Overjuyo-3 strains were not available. Consequently, a series of experiments were conducted to determine the effects of three of the commonly used growth media (BG11,

JM and BBM-3N) on growth and biomass production in these two *B. braunii* strains in order to select which of these growth media was the most suitable for further research. In these experiments, in addition to the direct measurement of the dry weight of biomass, multiple indirect measures of algal growth using optical density (at OD 680 nm and OD 750 nm), chlorophyll fluorescence (CF) at 430 nm and cell count were used to determine which of these measures was the most suitable for use in subsequent experiments (Myers *et al.* 2013; Sakamoto *et al.* 2012; Zhang, *et al.* 2013).

In the experiments, both *B. braunii* strains grew successfully in all three media at 25^{o}C. After 15 days, growth and biomass production were higher in BG11 for the Kossou-4 and Overjuyo-3 strains (Al-Hothaly *et al.* 2015a). Biomass production was greatest in BG11 and was about three times that produced in JM, which was the second most productive medium. Previous studies have successfully used all three media to grow *B. braunii* but did not make direct comparisons between these three media (Ambati *et al.* 2010; Li *et al.* 2013; Rani *et al.* 2011; Velichkova *et al.* 2012).

These findings are of importance to researchers of *B. braunii* since they show that growth in BG11 resulted in considerably greater growth in both Race B strains; consequently, it is likely that this result is relevant to other high hydrocarbon-yielding Race B strains; further work is required to confirm this. In addition, although BG11 was the most productive of the three media compared, we did not investigate growth in CHU13 or Z8. Therefore, further research into the effect of growth media on these *B. braunii* strains is still needed.

The results obtained using indirect measures of growth based on OD (680 and 750 nm) and CF values were consistent with the dry weight of biomass at each measurement point for both strains (Velichkova *et al.* 2012). However, OD measurements appeared to consistently overestimate growth relative to measures of dry weight. In contrast, cell count using Countess (Madigan 2005) did not produce consistent measures of growth. This was probably due to the

effect of colony formation in these strains, which led to fluctuations in the estimates of cell number from time-point to time-point. Therefore this approach to growth measurement cannot be recommended.

The determination that OD 680 and 750 nm provide good approximations of biomass is an important result which has implications for commercial production. Although the direct measure of dry weight is the most accurate measure of biomass production, this requires the removal of samples of algal biomass followed by the slow process of drying, filtering and weighing. In contrast, OD provides a quick, convenient and reliable alternative which could be used in the large-scale commercial setting.

It has been proposed that the pigmentation found in some *B. braunii* strains may affect the accuracy of optical density measures (Belcher 1968; Griffiths *et al.* 2011; Wolf *et al.* 1985). This possibility was not substantiated in the two strains investigated in this study. Whether this factor limits the usefulness of optical measures of growth in other *B. braunii* strains remains to be investigated.

7.2 Efficiency of different solvents in extracting oil from *B. braunii* biomass

In order to assess the impact of the three growth media on oil production and to obtain accurate measures of total oil production, it was necessary to optimise the oil extraction process. A number of different solvents have been used for oil extraction including chloroform-methanol (Ryckebosch *et al.* 2012), hexane (Mercer & Armenta 2011) heptane (Eroglu & Melis 2010) and hexane-isopropanol (Ryckebosch *et al.* 2012); however, it was not apparent from the literature which solvents were the most efficient for the extraction of oil from *B. braunii* biomass.

Since chloroform-methanol is highly toxic it was not considered appropriate to commercial-scale production and was not investigated. When the other three solvents–hexane, hexane-isopropanol (ratio of 3:2) and heptane–were compared, the oil weight at day 15 was

consistently higher when hexane was used for both Kossou-4 and Overjuyo-3 in all three media, while heptane yielded the lowest oil content.

The results for oil production were consistent with those for biomass for all three solvents, with the most oil being produced in BG11 (Al-Hothaly *et al.* 2015a).

These results made an important contribution to the data available to commercial producers since they demonstrate that the popular solvent hexane, which has low toxicity, can effectively extract oil from these *B. braunii* strains, recovering 1.6 times more oil than hexane-isopropanol, the second best of the three solvents. Since hexane showed the best efficiency, is relatively low cost and is safe (Eroglu & Melis 2010; Mercer & Armenta 2011; Ryckebosch *et al.* 2012), it was selected for use in subsequent experiments. Although this study showed that hexane was the best of the three solvents tested, this does not necessarily mean that it is the best possible solvent or the best approach to oil extraction so further research is still needed to investigate methods for the commercial extraction of oil from *B. braunii* stains.

7.3 Effect of nutrients and environmental conditions on biomass and oil production

On the basis of the results from Chapter 3, BG 11 was selected as the growth medium for further experiments which investigated the effects of modification of the nitrogen and iron content of BG11 as well as the effects of temperature conditions, light intensity and photo-period on both strains grown over an extended period of 60 days. The principal aim of this series of experiments was to select optimal culture and growth conditions for Overjuyo-3 and Kossou-4 in terms of nitrogen and iron concentration in BG11, temperature, light intensity and photoperiod.

7.3.1 Effects of nitrogen and iron

Optimization of nutrient requirements is an important undertaking prior to the establishment of the sustainable production of *B. braunii* on a large scale (Song *et al.* 2012). Nitrogen is essential for the growth of algae and low nitrogen can lead to low chlorophyll and reduced overall growth (da Silva *et al.* 2009; Velichkova *et al.* 2012). In contrast, nitrogen deficiency can favour the accumulation of lipids while increased nitrogen concentration can lower the lipid content of microalgae (Li *et al.* 2008; Velichkova *et al.* 2012). In a previous study the level of oleic acid (an important component of biodiesel) in *B. braunii*, was reported to be higher, at 86% of the total fatty acids, under N-limited conditions (0.04 mM nitrate) compared to 69% under N-sufficient conditions (3.66 mM nitrate) (Choi *et al.* 2011). Given these reports on the effects of nitrogen, it was important to determine whether variation in the nitrogen content of BG11 had effects on growth and the production of biomass and oil.

When the effect of three different concentrations of sodium nitrate ($NaNO_3$) in BG11 medium was assessed, it was evident that maximum growth was reached at or around 40 days under continuous lighting for both strains, after which growth slowly declined to 60 days. A nitrate concentration of 1500 mg l^{-1}, which is that used in normal BG11, was found to produce considerably less growth than the two lower concentrations (750 and 370 mg l^{-1}), of which 750 mg l^{-1} was found to produce significantly higher growth in terms of OD and CF measurements and dry weight of biomass. The same pattern of results was evident for both Kossou-4 and Overjuyo-3 (Al-Hothaly *et al.* 2015a).

Previous work published on nutrient optimization investigated the effects of different nitrogen sources and concentrations on *B. braunii* and found that potassium nitrate and sodium nitrate were the best nitrogen sources. However, these experiments used immobilized *B. braunii* (SAG 807-1 strain) grown in CHU 13 medium (Cheng *et al.* 2013; Cheng *et al.* 2014), so these results are difficult to compare with those of the experiments reported in Chapter 4.

Iron is one of the mineral components that must be supplied in the substrate for algal cells (Frac *et al.* 2010) and changes in the availability of iron affect the growth of algal cultures (Liu *et al.* 2008). Some algal species require higher iron concentrations than others (Sutak *et al.* 2012). In a study on *B. braunii*, cultures with increased iron showed larger cell sizes, larger colonies and overall increases in growth (Tanoi *et al.* 2014). Therefore it appeared likely that changes in the availability of iron in BG11 may affect the growth and biomass production of Kossou-4 and Overjuyo-3.

The concentrations of iron, as Fe $(NH_4)_3C_{18}H_{10}O_{14}$ were manipulated in BG11 modified to contain 750 mg l^{-1} of nitrogen based on the results of the previous experiment on the optimisation of nitrogen. The optimal iron concentration for the growth of both *B. braunii* strains was found to be 6 mg l^{-1} which is the concentration used in normal BG11. Growth was significantly lower at iron concentrations of 3 mg l^{-1} (lowest) and 9 mg l^{-1}.

In an earlier study, growth of *B. braunii* race B strain BOT-22 was also found to be very slow in the low-iron medium compared to a higher iron medium but this experiment used modified CHU medium and the iron source was iron (III) citrate hydrate (Tanoi *et al.* 2014) so it is not possible to make direct comparisons with the results found in Chapter 4.

7.3.2 Effects of temperature

Temperature has been found to have a major effect on the fatty acid (FA) composition of microalgae (Guschina & Harwood 2006; Morgan-Kiss *et al.* 2006). A general trend towards increased proportions of saturated FAs with increasing temperature, and conversely, increased unsaturated FAs with decreasing temperature, has been observed in many microalgae and cyanobacteria (Renaud *et al.* 2002; Sato *et al.* 2003). In *Chlorella vulgaris* and *B. braunii*, as temperature increased the relative content of saturated FAs increased while the content of unsaturated FAs decreased (Sushchik *et al.* 2003).

Velichkova *et al*. (2012) found that *B. braunii* (SKU & AC-1006 strains) grew well at 25^{o}C in BBM and BBM-3N. Ambati *et al*. (2010) also used 25^{o}C when comparing the growth of *B. braunii* in four different media (CHU13, Z8, BBM, and BG11) and found that the highest biomass was produced in Z8 and BG11. When Li *et al*. (2013) compared 16 *B. braunii* strains, including Kossou-4 in Jaworski's Medium (JM) at 23^{o}C they found good growth and hydrocarbon content. Consequently, the temperature selected for the experiments reported in Chapter 4 was 25^{o}C. However, a study of *B. braunii* Showa strain in modified CHU 13 medium reported stable growth at temperatures ranging from 15–30°C, with maximum growth at 30 °C (Yoshimura *et al.* 2013). Another *B. braunii* strain (No LB 807/1 Droop 1950 H-25), grown in Prat medium, showed higher biomass production at 32^{o}C than at 18 and 25^{o}C (Kalacheva *et al.* 2002). Therefore it was important to investigate the effect of different temperatures on the two *B. braunii* strains used in the present study.

Using a modified BG11 that contained reduced nitrogen (750 mg l^{-1}) and normal iron (as determined by the previous experiments), the effect of three temperatures (20 ºC, 25 ºC and 30ºC) on growth under continuous lighting was investigated. For both strains the highest temperature produced the lowest growth while 25ºC, which is the temperate that was used in the previous experiments in this study, was found to be optimal for growth. For oil production, 25ºC was also found to be optimal. In addition, the level of oil was directly proportional to the level of growth at all three temperatures. Consequently, 25ºC was selected for subsequent experiments including the experiments on large scale production (Al-Hothaly *et al.* 2015c).

7.3.3 Effects of photoperiod and light intensity

In addition to temperature, microalgal cell growth rates and hydrocarbon production are affected by light intensity and photoperiod (Kitaya 2008; Parmar *et al.* 2011). Microalgae use light as their source of energy for synthesizing cell protoplasm (Wahidin *et al.* 2013).

However, when the microalgae are exposed to light above the saturation limit, the growth of microalgae becomes inhibited (Xue *et al.* 2011). On the other hand, available light varies greatly with the depth and the density of the microalgal culture. If the microalgae are cultured at higher depth and higher cell concentrations, the light intensity must be increased to penetrate through the culture (Wahidin *et al.* 2013). An insufficient amount of light can lower the growth rate. In contrast, under natural conditions the intensity of sunlight can be in excess of that required for saturation and may be high enough to inhibit growth during much of the day. Optimization of light conditions is therefore one of the most important factors in the mass cultivation of photosynthetic organisms (Ugwu *et al.* 2008). Thus, light regime analysis is important in order to produce optimal microalgal growth (Cheirsilp & Torpee 2012).

Richardson *et al*. (1983) reported that microalgae which were cultivated at various light intensities and light and dark cycles exhibited important changes in their general chemical composition, pigment content and photosynthetic activity (Richardson *et al.* 1983). To date however, the optimum light intensities, photoperiod and environmental temperature that are required for hydrocarbon production in the two selected strains of *B. braunii* have not been adequately investigated.

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). In a *B. braunii* strain, Brenckmann *et al.* (1985) investigated the effects of four light intensities (with continuous illumination) on biomass and hydrocarbon production for 20 days and found the highest intensity initially stimulated growth followed by inhibition, while the two medium levels produced normal exponential growth and the lowest light intensity produced slow but stable growth (Brenckmann *et al.* 1985). Similar results were found for hydrocarbon production after 20 days (Al-Hothaly *et al.*, 2015b; DOI: 10.1080/09670262.2015.1071875).

A study that investigated the effects of continuous illumination versus 12 hours light/ 12 hours dark on growth and biomass production in *Neochloris conjuncta*, *Neochloris terrestris*, *Neochloris texensis*, *Botryococcus. braunii* (SAG 30.81) and *Scenedesmus obliquus*, found that *B. braunii* and *S. obliquus* grew better under continuous illumination (at 60 μmol m^{-2} s^{-1} for 60 days at 24 ^{o}C), whereas the *Neochloris* species grew better with 12 hours light/ 12 hours dark (Krzemińska *et al.* 2014).

To examine the effects of light on growth, biomass and hydrocarbon production in Kossou-4 and Overjuyo-3, a series of experiments were conducted at a temperature of 25^{o}C in BG11 modified according to the results of the previous experiments (see Chapter 4). These experiments compared three light intensity conditions (54, 81 and 135 μmol m^{-2} s^{-1}) and three photoperiods (light/darkness of 24 /0, 16/8, 12/12 hours).

These experiments found that *B. braunii* does not need a period of dark to grow successfully. The results demonstrated that 24 hours of light at 54 μmol m^{-2} s^{-1} produced significant higher growth, biomass and oil production in both strains than at shorter photoperiods (Al-Hothaly *et al.* 2015c).

For each of the above experimental conditions, oil was extracted from samples of algal biomass at day 40 which was the point of maximum growth in both strains. Maximum oil weight was obtained under the conditions that produced the highest biomass. For both strains grown in BG11 modified with 750 mg l^{-1} nitrogen and normal iron, and cultured at 25 ºC, the highest oil content was obtained under the maximum light intensity condition of 135 μmol m^{-2} s^{-1} and a photoperiod of 16 h light / 8 h darkness. However, at 24 hours illumination there was a decline in growth. At the lower light intensities of 54 μmol m^{-2} s^{-1} and 81 μmol m^{-2} s^{-1}, 24 hours illumination produced the highest yields. In terms of actual yield, the increases in oil production achieved by increases in light intensity above 54 μmol m^{-2} s^{-1} and 24 hours were relatively small.

This result was similar to those found in earlier experiments (Brenckmann *et al.* 1985; Krzemińska *et al.* 2014). *B. braunii* grew well under continuous illumination but at the highest light intensity there was evidence of photo-inhibition. This result was also consistent with data from a study using a bubble column photobioreactor, in which a high light intensity (10 k lux, which is equivalent to 135 μmol m^{-2} s^{-1}) produced photo-inhibition in a Race B strain of *B. braunii* grown in CHU 13, but following partial shading (which reduced the photoperiod), the algae adapted to this high level of illumination. This study also found that hydrocarbon production was proportional to biomass production irrespective of illumination (Kojima & Zhang 1999).

This series of experiments demonstrated that BG11 medium can be optimised for these *B. braunii* strains by reducing the nitrogen content to 750 mg l^{-1} while retaining the same concentration of iron. Moreover, it was evident that the optimised BG11 produced approximately a ten-fold increase in biomass and oil weight in Overjuyo-3 and a seven-fold increase in biomass, combined with an eight-fold increase in oil in Kossou-4, when these strains were cultured under 135 μmol m^{-2} s^{-1} and a photoperiod of 16 h light / 8 h darkness at a constant temperature of 25ºC. This is the first time (to my knowledge) that this combination of conditions has been investigated. While it shows that this combination of conditions resulted in considerable increases in biomass and oil production, not all possible growth parameters were investigated. Therefore it remains possible that illumination intensity could be further refined.

It appears that 135 μmol m^{-2} s^{-1} is optimal for biomass and hydrocarbon production, but this was obtained at 16 h light / 8 h darkness. In contrast, at 24 hours light the oil yield declined which suggests that illumination at 135 μmol m^{-2} s^{-1} may be too intense. Consequently, further studies could investigate other light intensities to determine whether an intermediate

light intensity between 81 and 135 μmol m^{-2} s^{-1} could produce a maximal yield at 24 hours illumination.

From the commercial perspective, using a high level of illumination would be energy intensive and may be uneconomic to implement. The yields obtained at the lower illuminations and 24 hours light were only slightly lower than at 135 μmol m^{-2} s^{-1} (16 h light / 8 h darkness). Also, there was very little difference between oil yields when the illumination was 54 and 81 μmol m^{-2} s^{-1} for 24 hours. Consequently, in terms of benefit relative to energy use, the combination of 54 μmol m^{-2} s^{-1} and 24 hours photoperiod would appear to be the most economically attractive of the conditions tested in the experiments. However, this study did not attempt a cost-benefit analysis of different lighting regimens in these two strains. This is a question that deserves further research attention in order to evaluate the economics of *B. braunii* production under artificial illumination.

There is also scope for further research into the optimisation of the components of BG11 beyond those of nitrogen and iron. Variation of the amounts of phosphorus and trace elements could also be considered (Carvalho *et al.* 2006; Chernikova *et al.* 2006; Peers & Price 2004; Raven 1990). Another issue is the cost of growth media, so research into the cost/benefits of different media will be of commercial importance.

7.4 The large-scale production of *Botryococcus braunii*

B. braunii Race B strains can accumulate high levels of hydrocarbons of which the triterpenoid hydrocarbons (triterpenes), the botryococcenes and methyl-branched squalene, make up large proportions. These triterpenes are of high commercial value and can be used as biofuels and in a range of industrial processes (Borowitzka & Moheimani 2013; Li *et al.* 2013; Qin 2010).

To investigate the feasibility of growing the two *B. braunii* strains Kossou-4 and Overjuyo-3 for the commercial production of triterpenes, large scale cultures were undertaken for 60 days

in 500 litre open tanks. The growth medium used was BG11 modified with reduced nitrogen as was determined to be optimal in the previous experiments. Inoculants (2.5 l) of each *B. braunii* strain were added to the 500 l tanks which were housed in a building and maintained in the temperature range 24.8 to 25.5 ºC. Aeration was provided by continuous bubbling. The illumination parameters chosen were 54 μmol m^{-2} s^{-1} and 24 hours photoperiod rather than the 135 μmol m^{-2} s^{-1} and photoperiod of 16 h light / 8 h darkness that was reported to produce the highest oil yield in the previous experiments. There were three reasons for this selection. Firstly, the difference between oil production under light intensity of 54 μmol m^{-2} s^{-1} and that under 135 μmol m^{-2} s^{-1} was very small. Secondly, 135 μmol m^{-2} s^{-1} produces a considerable amount of heat, which is likely to affect the temperature of the tanks. Thirdly, 135 μmol m^{-2} s^{-1} uses more electricity. Consequently, it did not appear to be justified to use the higher light intensity for a large-scale experiment.

Maximum growth was obtained at 40 days for both strains followed by slight declines. There was no evidence of contamination by bacteria or fungi. At day 40, Overjuyo-3 produced more biomass than Kossou-4 but the oil weight was higher for Kossou-4 (0.75 g l^{-1}) than for Overjuyo-3 (0.63 g l^{-1}). As a percentage of biomass, Kossou-4 produced 29 % of oil compared with 20% for Overjuyo-3. As in the previous small scale experiments, maximum growth and biomass production was reached at 40 days (Al-Hothaly et al., (2015b); In Press, DOI: 10.1080/09670262.2015.1071875). When the content of triterpenoid hydrocarbons in the oil was assessed using GC-MS, these were found to mainly comprise squalene and botryococcene. At day 40, the oil produced by Overjuyo-3 contained 29% of total triterpenoids while the oil produced by Kossou-4 was 50% triterpenoid hydrocarbons. This compares favourably with the results reported by Li et al. (2013) for the same two strains cultured at the laboratory scale. Although Li et al. (2013) did not report data for squalene and botryococcene specifically, the data for C32-C34 hydrocarbons in oil (Kossou-4 21.12%;

Overjuyo-3, 24.97%) is comparable, since this fraction is likely to have been largely composed of these triterpenoids (Li *et al.* 2013).

Previous studies have reported the large scale culture of other *B. braunii* strains. Dayananda et al. (2010) grew *B. braunii* Mahabali strain in 1000 l outdoor raceway ponds in Tamil Nadu, India for two weeks and measured biomass and total hydrocarbon yield but did not report data for specific hydrocarbons (Dayananda *et al.* 2010). Ranga Rao et al. (2012) grew *B. braunii* LB-572 and N-836 strains in outdoor raceway and circular ponds and reported on hydrocarbon content and profile but did not provide data on squalene or botryococcene (Rao *et al.* 2012). Ashokkumar and Regasamy (2012) grew *B. braunii* AP-105 strain in an outdoor raceway pond and reported on biomass and hydrocarbon profile but this strain mainly produced lower C15–C17 hydrocarbons rather than squalene and botryococcene (Ashokkumar & Rengasamy 2012).

These studies were conducted over considerably shorter periods than in the present study and all used open raceway ponds and natural light. In terms of biomass production, the authors noted that this was lower in large scale cultures than at the laboratory level. One reason for the lower productivity is outdoor raceways ponds are subject to variable environmental conditions (Ashokkumar & Rengasamy 2012; Dayananda *et al.* 2010; Rao *et al.* 2012).

In contrast, the present study found higher biomass and oil production in the large scale culture compared to the small scale experiments even though the same growth medium and culture conditions were used and time period was the same. A number of factors may have led to this result. Although the medium and culture conditions were very similar, the large scale tanks were well aerated since they were open to the air and there was continuous bubbling of air through the growth medium, which also resulted in thorough mixing of the algal broth. In contrast, the small scale cultures were contained in flasks and covered with gauze. Although cultures were shaken using an electronic shaker to mix the culture and

promote aeration, bubbling of air was not used (Al-Hothaly *et al.* 2015a). The effects of these differences on growth conditions could be investigated in future experiments. It may be interesting to investigate whether air high in CO_2 bubbled through the culture would promote growth.

Although some previous studies have reported the large scale culture of other *B. braunii* strains, these were conducted over considerably shorter periods, using other growth media, and under quite different growth conditions compared with this study. In addition, the previous large scale studies did not report on triterpene production (Ashokkumar & Rengasamy 2012; Dayananda *et al.* 2010; Rao *et al.* 2012). Consequently, this study is the first to report on the large scale production of high value triterpenoid hydrocarbons under conditions that could be used in a commercial setting. Future studies could investigate whether further scaling up is feasible and undertake an analysis of the economics of triterpenoid production using the approaches developed in this study.

In addition, researchers could investigate the effects of natural light at different intensities and the effects of natural changes in temperature on growth and oil production. Such research is important to assess the feasibility of large scale outdoor production of these *B. braunii* strains.

7.5 Bio-harvesting and pyrolysis of *Botryococcus braunii*

The challenges of harvesting microalgal biomass from growth media have received considerable attention (Leite *et al.* 2013; Pragya *et al.* 2013). Conventional methods such as centrifugation and filtration require equipment and can be costly (Brennan & Owende 2010), whereas the more recently developed method of bio-flocculation with fungi appears to be more cost effective (Wrede *et al.* 2014). However, this method had not previously been explored in *B. braunii*. Consequently, a series of experiments were conducted to investigate the feasibility of bio-flocculation using a number of species of fungi. These determined that

Aspergillus fumigatus could co-pelletize with both *B. braunii* strains, resulting in the flocculation of the majority of the biomass. To optimise this approach, experiments were conducted to determine the optimal ratio of fungus to algae and found that the ratio of 1:40 resulted in the flocculation of 98% of Overjuyo-3 and 97% of Kossou-4 biomass after 12 hours. At this ratio, it would be feasible to produce sufficient *Aspergillus fumigatus* to bio-flocculate *B. braunii* on a large scale. The biomass yield using this method of bio-flocculation was similar to that from centrifugation, producing 7.512 kg of Overjuyo-3 and 6.377 kg, each from 250 l of algal broth (Al-Hothaly *et al.* 2015c).

To determine whether the fungi significantly affected the composition of the harvested biomass, ultimate analysis of the elemental compositions of the harvested biomass was carried out. This determined that there was little difference in the composition of biomass harvested by centrifugation and that harvested using this method of bio-flocculation (Al-Hothaly *et al.* 2015c).

These experiments demonstrated for the first time that it would be feasible use the approach to bio-flocculation developed in this study to harvest *B. braunii* at a large scale and this method is of comparable efficiency to the use of a centrifuge. Moreover, the use of bio-flocculation had no appreciable effect on the composition of the harvested biomass. However, it remains possible that bio-flocculation may not be appropriate for some end uses of the algal biomass, such as its use in foods or pharmaceuticals. This aspect requires further investigation.

Future experiments could further investigate the use of bio-flocculation under larger scale conditions and the feasibility of using this approach in continuous or semi-continuous production systems.

7.6 Implications for commercialisation and for future research

This study demonstrates that it is feasible to grow the *B. braunii* strains Kossou-4 and Overjuyo-3 on a large scale in open tanks. Over the 40 days required for these slow-growing microalgae to reach maximum growth, the algae grew successfully under artificial light and there were no apparent issues with contamination. The resultant oil extracted from the biomass contained high levels of triterpenoid hydrocarbons so these two strains, in particular Kossou-4, would appear suitable candidates for the commercial production of these high value hydrocarbons.

One limitation of this project was the range of components of BG 11 that were investigated. There remains further scope for optimisation of growth media in terms of maximising oil production and in terms of minimising cost. Similarly, illumination schedules could be further investigated to determine the cost/benefit of different illumination schedules in terms of increased oil production.

Large-scale production was undertaken in open circular tanks with air bubbling but this tank design may not be optimal. Further research could examine the effects of tank depth on light penetration; other methods of aeration; and the effects of turbulence on colony formation. Each of these factors may impact on the amount of biomass obtained and consequently on the yields of oil and triterpenes.

This project did not examine the outdoor production of these strains under natural light conditions. The experiments indicated that *B. braunii* grew well under 24 hours illumination so it appears likely that when grown outdoors, growth could be slower and maximum biomass may not be obtained at 40 days. On the other hand, when grown in regions with suitable temperature and light conditions, outdoor biomass production may be commercially attractive. These questions require further research.

The approach to bioflocculation developed in this study offers a viable alternative to centrifugation. Bioflocculation using fungi has the potential to lower the cost of harvesting but this approach could be further developed to improve efficiency. Although, this approach did not appear to affect the end product, it is also important to undertake further research to determine whether this approach is applicable for the production of oil that is intended for human use.

7.7 Economic considerations

Currently, the production of microalgae biodiesel is not sufficiently cost effective for it to compete with diesel from fossil fuels, however research into production systems is underway with the aim of developing economically viable approaches to the large-scale production of biodiesel from microalgae. Aspects of production where efficiencies in both technology and cost can be obtained include: plant and equipment used in production systems, temperature control, water use, contamination, harvesting, drying, and oil extraction (Chisti 2007; Mata et al 2010; Parmar 2011; Pienkos and Darzins 2009). In particular, the conventional processes used for harvesting and biomass recovery can account for 20–30% of the production cost (Mata et al 2010). The approach to bio-flocculation investigated in this study has the potential to reduce these costs (Al Hothaly et al 2015d).

In the large-scale experiments used in this study, the cost of plant and equipment used for growing the micro-algae were relatively low compared to those for a photo-bioreactor but these costs could be higher than for open race-way ponds since the tanks were housed in a large shed. Open systems such as race-way ponds are considered to be relatively inexpensive production systems, but they are subject to the effects of variation in light and temperature as well as contamination (Harun et al. 2010). By housing the tanks in a shed we were able to control these factors and it is likely that these aspects, together with the use of air bubbling, contributed to the relatively high biomass produced when compared to other studies that grew

B. braunii in open systems (Dayananda et al. 2010, Ashokkumar & Rengasamy 2012). Future studies could consider comparing the relative costs of production using open raceways versus indoor tanks.

As Parmar et al (2011) have noted, the economics of biofuel production from microalgae can be improved when multiple revenue streams can be achieved by co-production of a variety of products. In the case of the B. braunii Race B strains used in this study, their commercial potential is not limited to economics of biodiesel since they produce high-value triterpenes which have uses in multiple industries (Al Hothaly et al 2015c).

7.8 Conclusions

The production of hydrocarbons and biofuels from microalgae is an emerging field that shows great potential for the carbon neutral production of these essential products. The green algae *Botryococcus braunii* is known to produce oil with a high content of hydrocarbons but there is variation in the oil content and composition of the hydrocarbons amongst different algal strains. Two *B. braunii* strains Kossou-4 and Overjuyo-3 had been previously identified as promising for the commercial production of hydrocarbons but this was based on laboratory scale experiments and the feasibility of growing these microalgae on a large scale had not been investigated.

Previous large scale experiments using other algal species have reported relatively low biomass production and contamination of the culture with other organisms, issues which compromised commercial viability. In this study a range of questions relating to the large scale commercial cultivation of these microalgae were investigated. These included the optimisation of the growth medium to maximise biomass production; assessment of the effects of media and other cultivation conditions on hydrocarbon production and the type of hydrocarbons produced; the feasibility of scaling up laboratory scale investigations to a large

scale which approximates a commercial operation; assessment of biomass production and contamination in large scale production; development of an efficient approach to harvesting algal biomass; and assessment of the biomass produced from large scale production.

The experiments conducted in this study determined that the optimal growth medium for both *B. braunii* strains at both the laboratory scale and the large scale is BG 11 modified to reduce the nitrogen content to 750 mg/l. Also, the optimal temperature for biomass production was 25°C. As regards illumination, the use of high intensity produces the highest oil yields but the gains were small relative to the increase in energy usage.

In terms of growth period, maximum biomass was produced by 40 days at both the small and large scale for both strains. Increased biomass was also associated with increased oil weight.

A new approach to harvesting using bio-flocculation with *Aspergillus fumigatus* was developed which proved to recover a similar proportion of biomass to the more energy intensive process of centrifugation. Moreover, this approach had little effect on the composition of algal biomass which was largely comprised of the triterpenoids squalene and botrycoccene.

The results obtained for each component of this study have progressed knowledge of microalgal culture and made an original contribution towards the commercial production of hydrocarbons using *B. braunii*.

8.0 References

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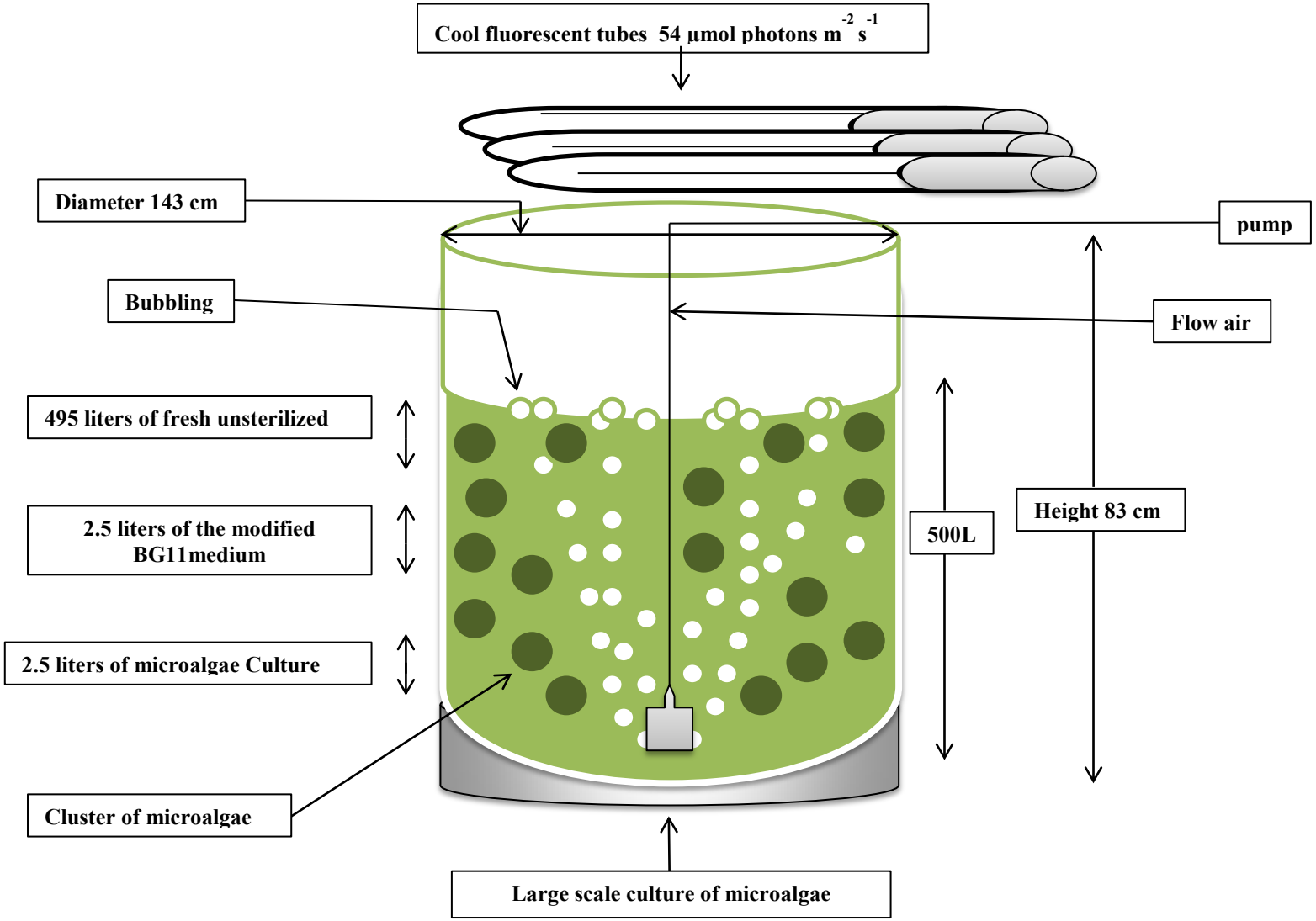
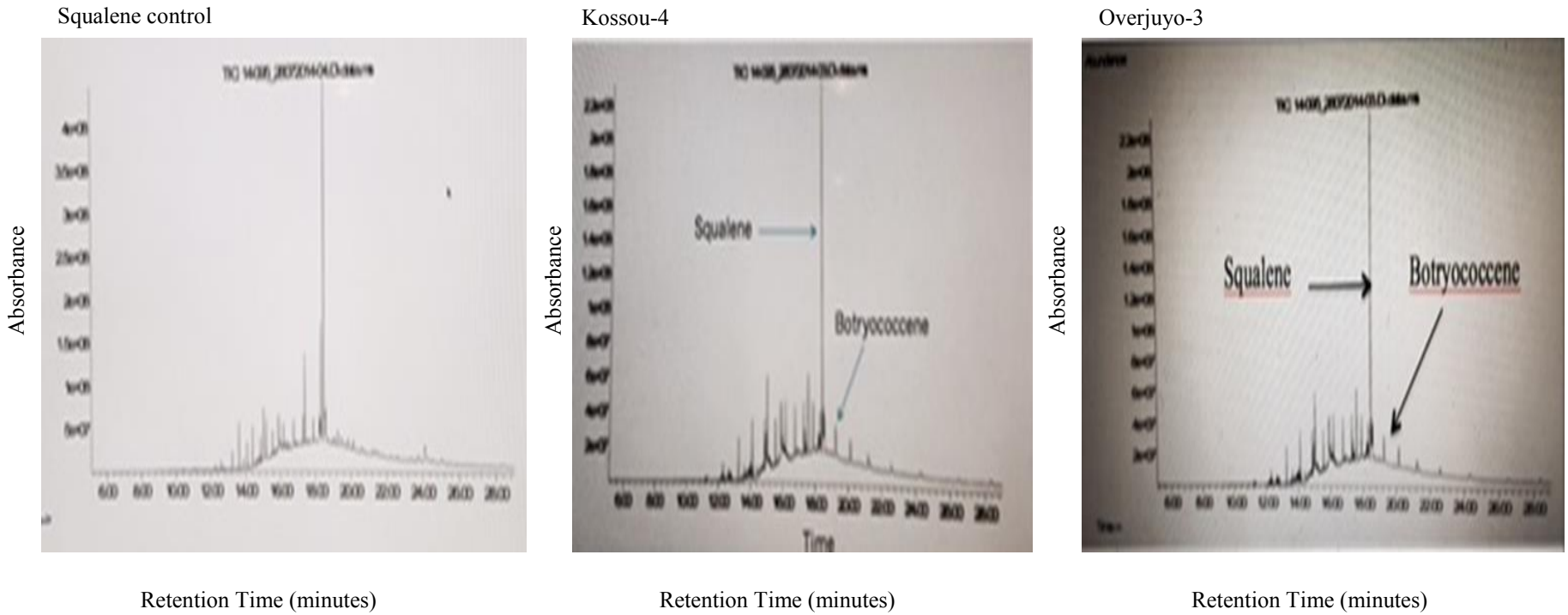
Appendix:



Supplementary Figure 1a. Schematic diagram of the large scale tank (500 L) used for growing Kossou-4 and Overjuyo-3 individually.



Supplementary figure 1b. The Gas Chromatography-Mass Spectrometry (GC/MS) results of Squalene and Botryococcene traces (Triterpenoid hydrocarbon fractions) from squalene (standard), Kossou-4 and Overjuyo-3. These results analyzed using GC/MS, an Agilent Technologies 5975C mass spectrometer inert XLE/CI MSD with Triple Axis Detection equipped with an Agilent Technologies 7890A GC system.