Bioresource Technology 191 (2015) 117–123

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

Khalid A. Al-Hothaly [a,b], Eric M. Adetutu [a], Mohamed Taha [a,d], Daniele Fabbri [c], Chiara Lorenzetti [c], Roberto Conti [c], Brian H. May [a], Sahar S. Shar [a], Reda A. Bayoumi [b], Andrew S. Ball [a,*]

[a] RMIT University, Centre for Environmental Sustainability and Remediation, School of Applied Science, Bundoora, VIC, Australia
[b] Department of Biotechnology, Faculty of Science, Taif University, Saudi Arabia
[c] Laboratory of Environmental Sciences "R.Sartori", C.I.R.S.A., University of Bologna, via S.Alberto 163, Ravenna, Italy
[d] Department of Biochemistry, Faculty of Agriculture, Benha University, Moshtohor, Toukh 13736, Egypt

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*.

ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 28 April 2015
Accepted 29 April 2015
Available online 6 May 2015

Keywords:
Botryococcus braunii
Harvesting
Bioflocculation
Aspergillus fumigatus
Pyrolysis

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

* Corresponding author at: RMIT University, School of Applied Science, Bundoora, VIC 3083, Australia. Tel.: +61 3 99256594.
E-mail address: andy.ball@rmit.edu.au (A.S. Ball).

http://dx.doi.org/10.1016/j.biortech.2015.04.113
0960-8524/

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

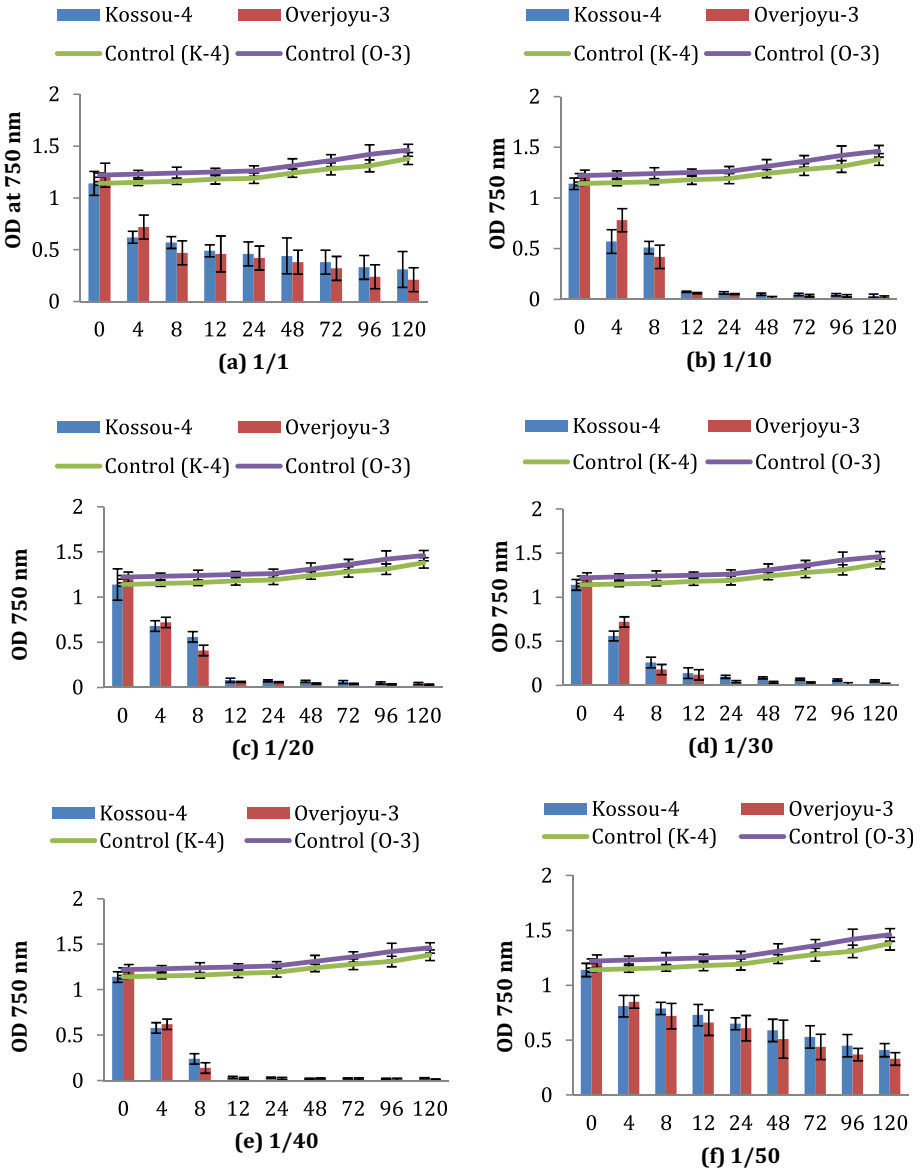

Fig. 1. Bio-flocculation of *B. braunii* strains Kossou-4 and Overjuyo-3 (as assessed by OD_{750} analysis of culture supernatants) using different *Aspergillus fumigatus*/*B. braunii* ratios together with control (non-flocculated cultures of microalgae). Optical density assessment at 750 nm at microalgae: 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

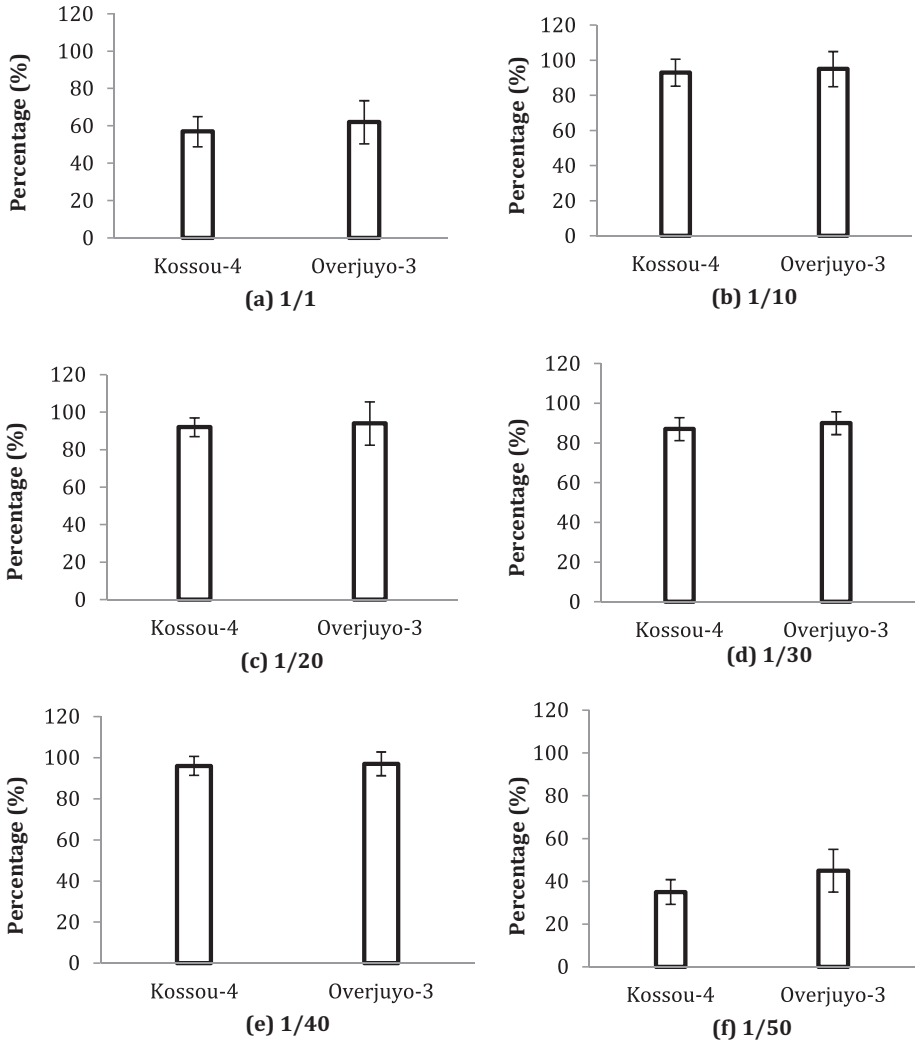

Fig. 2. Percentage of biomass removed by different ratios of *Aspergillus fumigatus* to *B. braunii*; 1/1 (a), (b) 1/10, 1/20 (c), 1/30 (d), 1/40 (e) and 1/50 (f). Algal abundance was measured using OD at 750 nm.

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.

Acknowledgements

The authors are grateful for the provision of PhD scholarship to Khalid Al-Hothaly by the Kingdom of Saudi Arabia.

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.

References

Al-Hothaly, K.A., Mouradov, A., Mansur, A.A., May, B.H., Ball, A.S., Adetutu, E.M., 2014. The effect of media on biomass and oil production in *Botryococcus braunii* strains Kossou-4 and Overjuyo-3. Int. J. Clean Coal Energ. 4, 11–22.

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U., 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol. 22, 245–279.

Brennan, L., Owende, P., 2010. Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sust. Energ. Rev. 14, 557–577.

Brown, A., Knights, B., Conway, E., 1969. Hydrocarbon content and its relationship to physiological state in the green alga *Botryococcus braunii*. Phytochemistry 8, 543–547.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Conti, R., Rombolà, A.G., Modelli, A., Torri, C., Fabbri, D., 2014. Evaluation of the thermal and environmental stability of switchgrass biochars by Py–GC–MS. J. Anal. Appl. Pyrolysis 110, 239–247.
Costa, M.H., Yanagi, S.N., Souza, P.J., Ribeiro, A., Rocha, E.J., 2007. Climate change in Amazonia caused by soybean cropland expansion, as compared to caused by pastureland expansion. Geophys. Res. Lett. 34 (L07706), 1–4.
de la Noue, J., de Pauw, N., 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol. Adv. 6, 725–770.
Ge, Y., Liu, J., Tian, G., 2011. Growth characteristics of *Botryococcus braunii_ 765* under high CO_2 concentration in photobioreactor. Bioresour. Technol. 102, 130–134.
Gim, G.H., Kim, J.K., Kim, H.S., Kathiravan, M.N., Yang, H., Jeong, S.-H., Kim, S.W., 2014. Comparison of biomass production and total lipid content of freshwater green microalgae cultivated under various culture conditions. Bioprocess Biosys. Eng. 37, 99–106.
Grima, E.M., Belarbi, E.-H., Fernández, F.A., Medina, A.R., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491–515.
Gultom, S.O., Hu, B., 2013. Review of microalgae harvesting via co-pelletization with filamentous fungus. Energies 6, 5921–5939.
Kalacheva, G., Zhila, N., Volova, T., Gladyshev, M., 2002. The effect of temperature on the lipid composition of the green alga *Botryococcus*. Microbiol 71, 286–293.
Krzemińska, I., Pawlik-Skowrońska, B., Trzcińska, M., Tys, J., 2014. Erratum to: influence of photoperiods on the growth rate and biomass productivity of green microalgae. Bioprocess Biosys. Eng. 37, 2137.
Lam, M.K., Lee, K.T., 2012. Microalgae biofuels: a critical review of issues, problems and the way forward. Biotechnol. Adv. 30, 673–690.
Leite, G.B., Abdelaziz, A.E., Hallenbeck, P.C., 2013. Algal biofuels: challenges and opportunities. Bioresour. Technol. 145, 134–141.
Li, Y., Moore, R., Qin, J., Scott, A., Ball, A., 2013. Extractable liquid, its energy and hydrocarbon content in the green alga *Botryococcus braunii*. Biomass Bioenerg. 52, 103–112.
Liu, Y.-Q., Lim, L.R., Wang, J., Yan, R., Mahakhant, A., 2012. Investigation on pyrolysis of microalgae *Botryococcus braunii* and *Hapalosiphon* sp. Ind. Eng. Chem. Res. 51, 10320–10326.
Metzger, P., Allard, B., Casadevall, E., Berkaloff, C., Couté, A., 1990. Structure and chemistry of a new chemical race of *Botryococcus braunii* (*chlorophyceae*) that produces lycopadiene, a tetraterpenoid hydrocarbon. J. Phycol. 26, 258–266.
Milledge, J.J., Heaven, S., 2013. A review of the harvesting of micro-algae for biofuel production. Rev. Environ. Sci. Biotechnol. 12, 165–178.
Nguyen, R.T., Harvey, H.R., Zang, X., van Heemst, J.D., Hetényi, M., Hatcher, P.G., 2003. Preservation of algaenan and proteinaceous material during the oxic decay of *Botryococcus braunii* as revealed by pyrolysis-gas chromatography/mass spectrometry and 13C NMR spectroscopy. Org. Geochem. 34, 483–497.
Pragya, N., Pandey, K.K., Sahoo, P., 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renew. Sust. Energ. Rev. 24, 159–171.
Qin, J., 2005. Bio-hydrocarbons from algae: impacts of temperature, light and salinity on algae growth, a report for the Australian Rural Industries Research and Development Corporation. Rirdc 1741511240, 1–18.
Qin, J., 2010. Hydrocarbons from algae. In: Handbook of Hydrocarbon and Lipid Microbiology. Springer, pp. 2817–2826.
Sarkar, O., Agarwal, M., Kumar, A.N., Mohan, S.V., 2015. Retrofitting hetrotrophically cultivated algae biomass as pyrolytic feedstock for biogas, bio-char and bio-oil production encompassing biorefinery. Bioresour. Technol. 178, 132–138.
Sawayama, S., Minowa, T., Yokoyama, S.-Y., 1999. Possibility of renewable energy production and CO_2 mitigation by thermochemical liquefaction of microalgae. Biomass Bioenerg. 17, 33–39.
Torri, C., Samorì, C., Adamiano, A., Fabbri, D., Faraloni, C., Torzillo, G., 2011. Preliminary investigation on the production of fuels and bio-char from *Chlamydomonas reinhardtii* biomass residue after bio-hydrogen production. Bioresour. Technol. 102, 8707–8713.
Tran, N., Bartlett, J., Kannangara, G., Milev, A., Volk, H., Wilson, M., 2010. Catalytic upgrading of biorefinery oil from micro-algae. Fuel 89, 265–274.
Watanabe, H., Li, D., Nakagawa, Y., Tomishige, K., Kaya, K., Watanabe, M.M., 2014. Characterization of oil-extracted residue biomass of *Botryococcus braunii* as a biofuel feedstock and its pyrolytic behavior. Appl. Energy 132, 475–484.
Widjaja, A., Chien, C.-C., Ju, Y.-H., 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J. Taiw. Inst. Chem. Eng. 40, 13–20.
Wrede, D., Taha, M., Miranda, A.F., Kadali, K., Stevenson, T., Ball, A.S., Mouradov, A., 2014. Co-cultivation of fungal and microalgal cells as an efficient system for harvesting microalgal cells, lipid production and wastewater treatment. PloS one 9, 11–22.
Yamamoto, S., Mandokoro, Y., Nagano, S., Nagakubo, M., Atsumi, K., Watanabe, M.M., 2014. Catalytic conversion of *Botryococcus braunii* oil to diesel fuel under mild reaction conditions. J. Appl. Phycol. 26, 55–64.
Zhang, J., Hu, B., 2012. A novel method to harvest microalgae via co-culture of filamentous fungi to form cell pellets. Bioresour. Technol. 114, 529–535.
Zhila, N., Kalacheva, G., Volova, T., 2005. Effect of nitrogen limitation on the growth and lipid composition of the green alga *Botryococcus braunii Kutz IPPAS H*-252. Russ. J. Plant Physiol. 52, 311–319.
Zhou, W., Cheng, Y., Li, Y., Wan, Y., Liu, Y., Lin, X., Ruan, R., 2012. Novel fungal pelletization-assisted technology for algae harvesting and wastewater treatment. Appl. Biochem. Biotechnol. 167, 214–228.