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Growth and photosynthetic activity of *Botryococcus braunii* biofilms

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Abstract *Botryococcus braunii* is a green microalga capable of producing large amounts of external long-chain hydrocarbons suitable as a source of biofuel. There have been several studies indicating that cultures of *B. braunii* can reduce the energy and water requirement for mass biofuel production, especially if non-destructive extraction methods for milking hydrocarbons are used. Growing microalgae as a raw material for biofuel using conventional liquid-based cultivation (i.e., raceway ponds) has yet to be shown to be economically successful. An alternative solid grown (biofilm) cultivation method can markedly reduce the energy requirements and costs associated with the harvesting and dewatering process. We evaluated the growth of biofilms of several strains of *B. braunii* (from races A, B, L and S) and found that three of the four tested races successfully grew to stationary phase in 10 weeks with no contamination. Among all races, *B. braunii* BOT22 (race B) reached the highest biomass and lipid yields (3.80 mg dry weight cm^{-2} day^{-1} and 1.11 mg dry weight cm^{-2}). Irrespective of the race, almost all photosynthetic parameters (F_V/F_0, PI_{ABS} and the OJIP curve) showed that the biofilm cultures were more stressed during lag and stationary phases than in logarithmic phase. We also studied the *Botryococcus* biofilm profiles using confocal microscopy and found that this method is suitable for estimating the overall biomass yield when compared with gravimetric measurement. In conclusion, the growth characteristics (biomass and lipid) and photosynthetic performance of all races indicated that *B. braunii* BOT22 is the most promising strain for biofilm cultivation.

Keywords Microalgae · Chlorophyta · Biofuel · Productivity · Hydrocarbon · Quantum yield · OJIP

Introduction

Biofuel has been proposed for many years as a renewable source of energy to reduce the existing demand for fossil fuels (Borowitzka 2013; Borowitzka and Moheimani 2013). Microalgae are claimed to be excellent candidates for biofuel production (Dixon 2013) as they can have high lipid contents (Fon Sing et al. 2013) and can be grown on non-arable land using wastewater (Graham et al. 2009; Dixon 2013). However, the costs of microalgal cultivation and downstream processing to yield the final biofuel product remain high (de Boer et al. 2012). Some of the main reasons for the high cost of production include harvesting-dewatering and extraction of lipids (Grima et al. 2010; Fon Sing et al. 2013). While innovation in downstream processing is required to make the production of microalgae biofuel economically feasible, improvements in cultivation are also necessary. Liquid-based cultivation has been the most common method for the mass culture of microalgae (Borowitzka 2013). The vast amounts of water and nutrients demanded by liquid-based cultivation processes are an issue for microalgal-based commodity products such as biofuels (Borowitzka and Moheimani 2013). An alternative way to cultivate microalgae is to grow them as a biofilm (Berner et al. 2015). Liquid cultures generally have a biomass

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content of between 0.02 and 0.06% solids (Schnurr et al. 2013). However, in a biofilm the organic solids can be as high as 16% (Ozkan et al. 2012). Cultivation of microalgae for biofuel in liquid has three main stages: growth of biomass, biomass harvesting-dewatering and lipid extraction. Biofilm growth can significantly reduce water consumption and dewatering requirements, as well as minimising nutrient use while improving light capture (Liu et al. 2013; Berner et al. 2015). Ozkan et al. (2012) reported that that biofilm cultivation can reduce water usage per kilogramme of algal biomass by 45% and the dewatering energy requirement by 99.7% compared to open pond cultivation. Therefore, the development of large-scale biofilm growth systems for microalgae such as *Botryococcus braunii* can potentially overcome some of the challenges of microalgal biofuel production.

The green microalga *B. braunii* produces significant amounts of long-chain unbranched hydrocarbons (Banerjee et al. 2002; Metzger and Largeau 2005). This species is divided into four races based on the type of hydrocarbon produced (Kawachi et al. 2012). In some cases, especially when cells are in very late stationary phase, the hydrocarbon content of *B. braunii* can be as high as 86% of the organic weight (Brown et al. 1969; Dayananda et al. 2007). These hydrocarbons can be converted to transport fuels due to their similarity to fossil fuel (Hillen et al. 1982). Importantly, *B. braunii* accumulates these hydrocarbons in the extracellular matrix, (Banerjee et al. 2002; Eroglu and Melis 2010) and destructive extraction to harvest the hydrocarbons can potentially be avoided when using this species to produce biofuel if non-destructive hydrocarbon extraction techniques are applied (Moheimani et al. 2013, 2014). By maintaining viable biomass after harvesting, non-destructive hydrocarbon extraction, also known as algal milking, substantially reduces the importance of algal growth rate and increases hydrocarbon productivity (Moheimani et al. 2013, 2014). Chaudry et al. (2015) have shown that non-destructive hydrocarbon production from *B. braunii* consumes 70% less energy and 30% less water compared to conventional wet lipid extraction. This extraction method has been proven as an energetically feasible process for hydrocarbon production from *B. braunii* combined with open pond cultivation, cylindrical sieve rotator filter system dewatering and nanofiltration as the solvent recovery stages (Chaudry et al. 2017). Therefore, non-destructive hydrocarbon extraction from *B. braunii* can be attractive for sustainable biofuel production.

Current knowledge of *B. braunii* biofilm growth and lipid productivity is limited. Therefore, in the present work, we assessed the potential of biofilm growth and the overall lipid productivity of different *B. braunii* races (A, B, L and S). Considering that microalgal biomass productivity is closely correlated with photosynthesis, photosynthetic performance of all strains was also measured.

Materials and methods

Microalgae culture source and liquid culture maintenance

Four strains of *B. braunii,* one from each race, were used in this study. *Botryococcus braunii* strains BOT22 (race B), BOT84 (race L) and BOT7 (race S) were obtained from the Algae Biomass and Energy System R&D Center (ABES), University of Tsukuba, Tsukuba, Japan. *Botryococcus braunii* UTEX2441 (race A) was obtained from the University of Texas culture collection. All cultures were maintained in modified AF-6 medium (Watanabe et al. 2000) and were grown at 25 ± 2 °C under 50 ± 5 μmol photons m^{-2} s^{-1} provided by natural cool white fluorescent lights with a 12:12 day/night cycle.

Biofilm cultivation

To grow *B. braunii* strains in biofilm, choosing the right nutrient delivery method is essential. Preliminary studies showed that use of a sponge for maintaining constant wetness and medium supply is more effective than agar or adding a liquid medium directly to the biofilm (unpublished data, see online resource 1). In this study, filtering the liquid *B. braunii* culture on to an inoculum attachment surface (2.834 cm^2) was chosen as the inoculation technique (Liu et al. 2013). Various types of material were used to identify the best attachment surface for the *B. braunii* biofilm. Of the five tested materials [glass microfibre (GF/C), cellulose nitrate (CN) and cellulose ester filter paper; toilet hand towels and Harris™ coffee filter paper], *B. braunii* grew best on cellulose nitrate filter papers (see online resource 2).

Botryococcus braunii were grown in biofilm using CN membrane filters with a 0.45 μm pore size and a diameter of 25 mm. The initial liquid stock culture for all strains for inoculation was grown in the same condition for 4 weeks. Five millilitre of the *B. braunii* liquid culture were filtered through the CN filters and these were then placed inside six-well cell culture plates which covered with the lid on top of 35 mm × 10 mm (diameter×depth) polyvinyl alcohol (PVA) sponges (media preserver). To start, 8 mL of AF-6 medium was added into each well containing a PVA sponge. One CN membrane filter was placed on the top of each wet sponge. Membrane filters were incubated overnight before being weighed as a blank filter. Pre-weighed membrane filters were inoculated by filtering 5 mL of *B. braunii* stock culture for all strains. We chose 5 mL as the preliminary results indicated this to be the best amount for inoculation of CN filters

(unpublished data). The membrane filters were incubated overnight before being weighed (day 1 of the trials). The biofilm cultures were grown for 10 weeks under an irradiance of 75 ± 2 μmol photons m^{-2} s^{-1} using natural cool white fluorescent lights with a 12:12 day/night cycle, at 25 ± 2 °C. The medium was changed every week only for the first 4 weeks. This provided the biofilm cultures with the same nutrients as 32 mL of the liquid AF-6 medium. To counter evaporative loss, after 4 weeks, sterile deionised water was added to the sponges. Choosing samples for biochemical, photosynthetic activity and biofilm structure analysis was by using a table of random numbers. Filters were photographed weekly using a digital camera for monitoring visual changes in the biofilm cultures.

Analytical measurements

Growth measurement A method for measuring biofilm growth non-destructively was developed by adapting several different weight conversion methods previously used by Gates et al. (1982) and Ricciardi and Bourget (1998). Five different volumes (1, 3, 5, 7 and 10 mL) of *B. braunii* cultures were filtered onto different pre-weighed GF/C filters (each volume had six replicates). The wet weights of the filter papers with algae were measured three times, every 2 h and once after 24 h. The dry weights were determined following Moheimani et al. (2013) after samples were dried overnight at 60 °C. Linear regression with the model forced through the origin was performed using Microsoft Excel to determine the correlation between dry weight and wet weight. This analysis resulted in equations for converting wet weight to dry weight, which were applied to allow non-destructive measurements of growth for each race of *B. braunii*. Wet weights were measured weekly using a calibrated five-digit Mettler-Toledo AB135-S balance.

Biofilm structure analysis A fresh sample of algal biofilm was used for confocal microscopy. The biofilm was stained with BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a–diaza-s-indacene, Life Technologies Molecular Probes) to highlight lipid content (Brennan et al. 2012; Cirulis et al. 2012; Govender et al. 2012). *Botryococcus braunii* cells were identified via autofluorescence of chlorophyll content. The BODIPY stain was freshly prepared just prior to observation (Govender et al. 2012) by adding 100 μg mL^{-1} BODIPY 505/515 in 2% dimethyl sulfoxide (DMSO), then diluting to 0.75 μg mL^{-1} in 2% DMSO, and storing in a dark bottle to avoid light exposure. One drop of the stain was placed on the sample, followed by 4 min incubation in the dark before viewing.

The three-dimensional structure if the biofilms was viewed with a Nikon C2+ multispectral laser scanning confocal microscope using 20× objectives (Lawrence et al. 1998; Neu et al. 2004) and laser excitation at 640.0 nm for observing chlorophyll content and 488.0 nm for observing the BODIPY-stained lipid. Images were taken and processed by the Nikon Imaging Software (NIS) Elements Advanced Research package and processed into three-dimensional composite images approximately 632 μm (L) × 632 μm (W) with various biofilm depths (36–192 μm). Quantitative analyses of the distribution of algal cells and lipid within the biofilm matrix were carried out using COMSTAT software (Heydorn et al. 2000).

Photosynthesis measurement Photosynthesis measurements were performed directly on *B. braunii* biofilms using a Handy-PEA chlorophyll fluorometer (Hansatech Instruments, UK) paired with PEA Plus V1.10 software. This instrument has a high-intensity LED array (3 lamps centred on 650 nm) with an NIR short-pass filter and allows measurement of the so called *fast phase* of the fluorescence induction curve and estimation of the OJIP parameters. Saturation pulse measurements performed on dark-adapted samples allowed derivation of the photosynthetic parameters. The maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS-II related to the dark adaptation state (F_V/F_0) was one of the parameters that was measured, instead of measuring the more usual maximum quantum yield of PS-II photochemistry (F_V/F_M). This parameter was used because it is a more sensitive parameter than F_V/F_M for indicating the maximum efficiency of photochemical processes in PS-II (Roháček 2002). The other parameters measured were the photosynthesis performance index (PI_{ABS}), the light absorption flux for PS-II antenna chlorophyll (ABS/RC), and energy dissipation at the level of antenna chlorophyll at Time 0 in PS-II (DI_0/RC), all which are the key parameters for the OJIP parameters according to Dao and Beardall (2016). These derivations were performed as described in Cosgrove and Borowitzka (2010) and Strasser et al. (2000) (see the details in online resource 3). Due to the low growth rate of *B. braunii* in the biofilms, the aforementioned photosynthetic measurements (F_V/F_0, PI_{ABS}, ABS/RC, and DI_0/RC) were measured every 2 weeks. However, the fast phase-fluorescence induction curves were only analysed for three different weeks to represent the chlorophyll fluorescence transient changes in each growth phase, which were the lag phase (week 0), the logarithmic phase (week 6), and the stationary phase (week 10). Along with the fast phase fluorescence induction curve analysis, the relative variable fluorescence at 2 ms (V_J) was also measured to express the connection between PS-II units (Force et al. 2003).

Biofilms were dark-adapted for 20 min prior to measurement to allow re-oxidation of all PS-II reaction centres and estimation of the minimum fluorescence yield. Measurements (n = 4) were performed by exposing the samples to a 1.2 s

pulse of high intensity light (3500 µmol photon m^{-2} s^{-1}) to saturate the photosystems and create maximal fluorescence yield. Handy-PEA provided fluorescence values every 10 µs to 1 ms between 0 and 1.2 s measurement times in logarithmic time sequence. The emitted fluorescence from the sample was recorded and digitised by the Handy-PEA. The data were analysed and displayed graphically using SigmaPlot Version 13 software.

Statistical analysis

One way ANOVA and Tukey's HSD post hoc tests were used to determine the significant difference in the photosynthetic activity during the growth period. The statistical analysis was conducted for each *B. braunii* race.

Results

Four strains, one from each race of *B. braunii,* were grown for 10 weeks as a biofilm (Fig. 1). As *B. braunii* UTEX2441 (race A) cultures were heavily contaminated by fungi from week 5, we stopped recording growth measurements for this strain (Figs. 1 and 2). *Botryococcus braunii* UTEX2441 biofilm was re-grown one more time. However, the culture was again contaminated after week 5. The other three *B. braunii* strains grew well as biofilms, with no contamination between weeks 0 (lag phase) and 10 (stationary phase) (Fig. 2). Furthermore, no contamination was observed in the biofilms up to week 27. Before becoming contaminated, *B. braunii* UTEX2441 showed growth between weeks 1 and 5 (Fig. 2). The other three strains has a lag phase of three to 5 weeks before entering the logarithmic phase, which was characterised by a substantial increase in biofilm wet weight (Fig. 2).

Botryococcus braunii BOT22 (race B), BOT84 (race L) and BOT7 (race S) reached stationary phase in week 8 (Fig. 2), and the culture colour changed from green to yellowish due to accumulation of carotenoids (Fig. 3). Growth was gravimetrically measured as wet weigh, and this was converted to dry weight using the equations shown in (able 1. Biomass and lipid yields, as well as the productivities of each strain, are summarised in Table 2. Based on the wet weight measurement, *B. braunii* BOT 22 achieved the highest biomass yield (107.24 ± 7.86 mg wet weight cm^{-2}) and productivity (40.61 ± 25.10 mg wet weight cm^{-2} day^{-1}), compared to *B. braunii* BOT7 and BOT84. *Botryococcus braunii* BOT22 also showed the highest biomass productivity (3.36 ± 2.35 mg dry weight cm^{-2} day^{-1}) in the logarithmic phase compared to the other strains. *Botryococcus braunii* BOT22 achieved the highest lipid yield, which was 1.11 ± 0.08 mg cm^{-2} in the stationary phase (Table 2) while both *B. braunii* BOT7 and BOT84 yielded 0.83 ± 0.08 mg cm^{-2} and 0.83 ± 0.03 mg cm^{-2}, respectively. However, among the tested strains the highest lipid content (26.6%) was achieved in the culture of *B. braunii* BOT7 (Table 2).

Biofilm thickness for all strains increased over time, ranging from 62 to 92 µm from the initial thickness between weeks 0 and 10 (Figs. 3 and 4). The area under the curve for the cell and lipid distributions of *B. braunii* are presented in each graph (Fig. 4). Both the cell and the lipid distribution areas increased and reached their peak between 20 and 60 µm from the biofilm surface, and then gradually decreased in the deeper

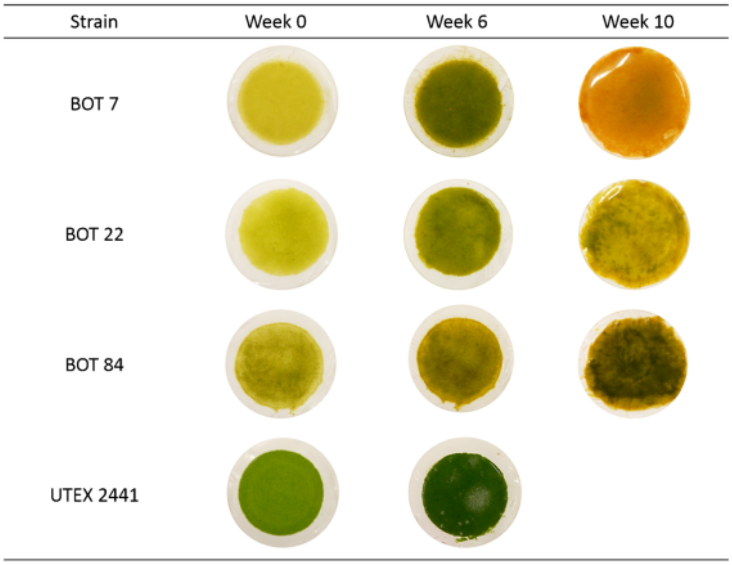


Fig. 1 *Botryococcus braunii* biofilm cultures during the lag phase (week 0), logarithmic phase (week 6) and stationary phase (week 10). Each culture is 25 mm in diameter

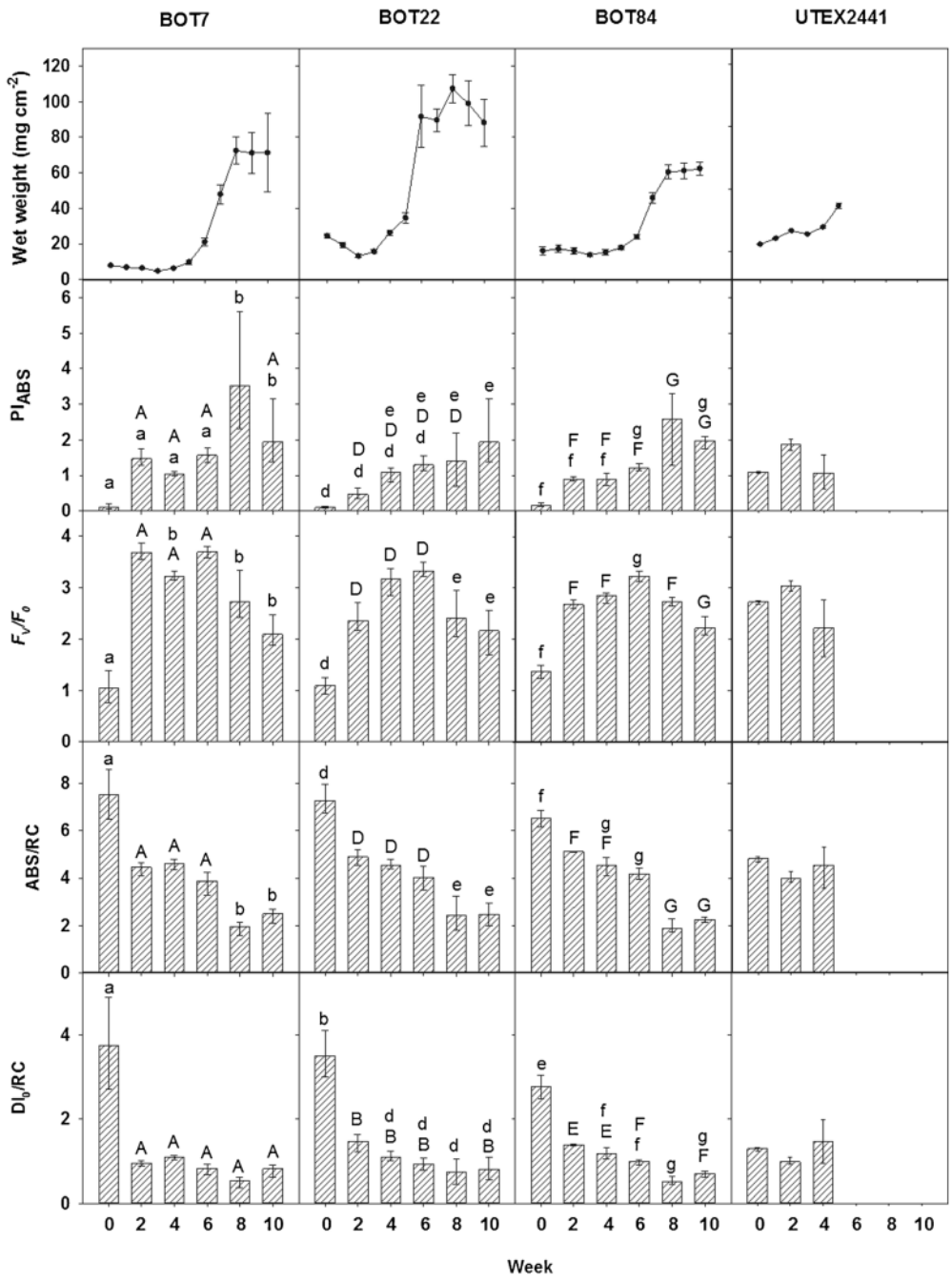


Fig. 2 Growth and photosynthetic activity of all *Botryococcus braunii* biofilm cultures during the growth period. The growth curve based on the wet weight (*error bars* = standard deviation); photosynthesis performance index (PI_{ABS}), maximum photochemical efficiency (F_V/F_0) (*dark adapted*); the light absorption flux for PSII antenna chlorophyll (ABS/RC); the dissipation at the level of antenna chlorophyll at time 0 in PS-II (DI_0/RC). The *same letter above each bar*, indicates no significant difference (one way ANOVA, $P > 0.05$, $n = 4$). For the photosynthetic activity measurements, the *error bars* indicate the range

layers of the biofilm (Fig. 4), even though the biofilm thickness increased during the growth period. Based on the area under the curve measurements, the cell and lipid distribution areas of *B. braunii* BOT7 and BOT22 showed a similar

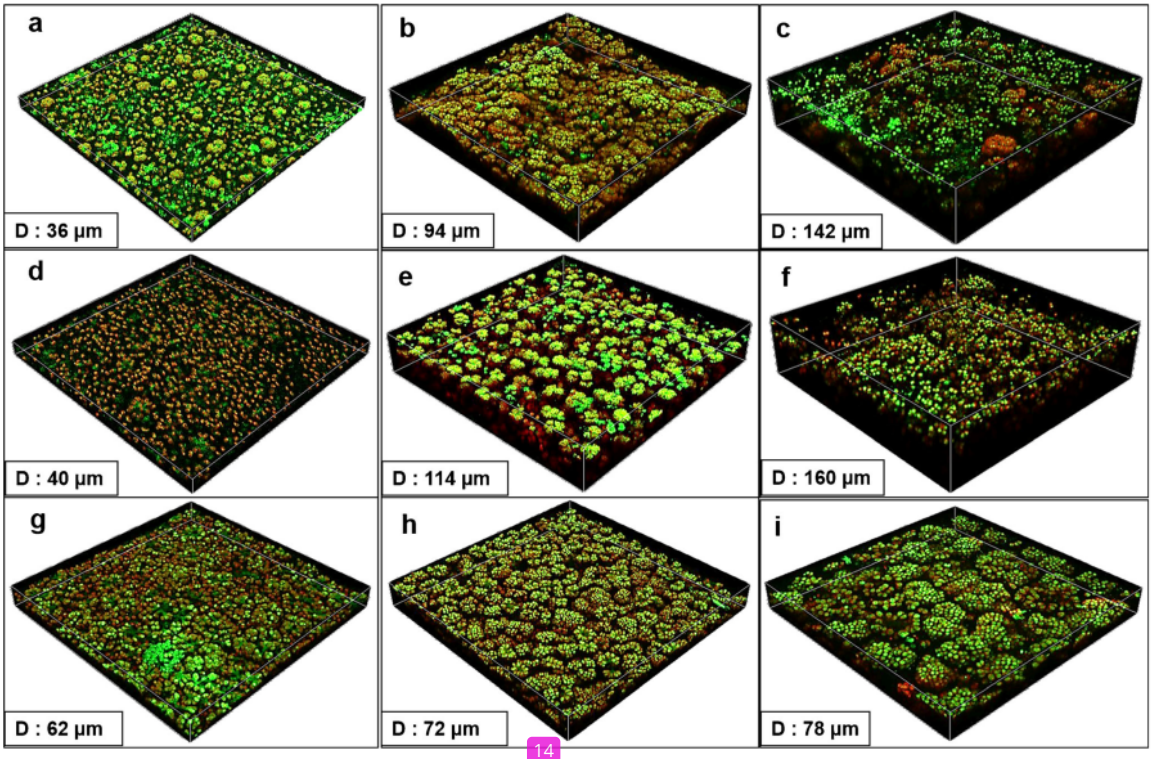


Fig. 3 Confocal microscope images of *Botryococcus braunii* BOT 7 (**a**, **b**, **c**), BOT 22 (**d**, **e**, **f**), and BOT 84 (**g**, **h**, **i**) in the lag (week 0) (**a**, **d**, **g**), logarithmic (week 6) (**b**, **e**, **h**) and stationary (week 10) (**c**, **f**, **i**) phase

pattern, increasing from between lag phase and log phase and then decreasing by the beginning of the stationary phase (Fig. 4). On the other hand, both the cell and lipid distribution areas of *B. braunii* BOT84, which had a bigger initial cell distribution in the lag phase, increased through the log and stationary phases of growth.

The confocal microscopy analysis of algal cell distribution showed a similar trend to the dry weight measurement results. The highest productivity was achieved in the biofilm of *B. braunii* BOT22. The ratio of total area that covered by the lipid to the cells (the lipid ratio of the cells) decreased during the logarithmic phase and increased in the stationary phase in all three *B. braunii* strains. However, in *B. braunii* BOT22 this ratio increased greatly compared to the other strains from the logarithmic to the stationary phase.

Table 1 The equations for converting wet weight (x) into dry weight (y) for all *B. braunii* strains

Strain	Equation	R^2
UTEX2441	$y = 0.17x$	0.85
BOT7	$y = 0.04x$	0.93
BOT22	$y = 0.09x$	0.93
BOT84	$y = 0.22x$	0.68

Chlorophyll fluorescence parameters were used to rapidly and non-destructively investigate the relationship between biofilm growth and photosynthetic activity in the *B. braunii* strains (Fig. 2). Although the growth curves showed that most *B. braunii* strains remained in lag phase until week 5 the photosynthetic activity parameters (PI_{ABS}, F_V/F_0, ABS/RC and DI_0/RC) showed increases from week 2. Lower PI_{ABS} and F_V/F_0 were found in week 0 than in week 2, indicating that *B. braunii* strains were stressed at the beginning of the experiment (Fig. 2). The ABS/RC ($F(2.81) = 74.764$ (BOT7); 56.147 (BOT22); 185.363 (BOT84), $P < 0.05$) and DI_0/RC ($F(2.81) = 37.688$ (BOT7); 49.380 (BOT22); 120.786 (BOT84), $P < 0.05$) decreased significantly from week 0 to week 2 as the algae began to acclimate to the new environment (Fig. 2). The PI_{ABS} values for each of the remaining strains remained steady for *B. braunii* BOT7 and increased for *B. braunii* BOT22 and BOT84 when they entered the logarithmic phase between weeks 2 and 6. The same phenomenon was observed for F_V/F_0, indicating that photosynthetic activity improved when cells began entering logarithmic phase in week 6. The PI_{ABS} and F_V/F_0 values of *B. braunii* UTEX 2441 also increased in week 2 but then decreased at week 4 due to the

Table 2 Biomass yield, productivity, lipid yield of *B. braunii* biofilm

Strain	Race	Yield (mg cm^{-2})		Productivity* (mg cm^{-2} day^{-1})		Lipid yield (mg dry weight cm^{-2})
		Wet weight	Estimated dry weight**	Wet weight	Estimated dry weight**	
BOT7	S	72.44 ± 7.68	3.12 ± 0.33	9.76 ± 1.63	0.42 ± 0.07	0.83 ± 0.08
BOT22	B	107.24 ± 7.68	10.04 ± 0.74	40.61 ± 25.10	3.80 ± 2.35	1.11 ± 0.08
BOT84	L	62.31 ± 3.62	13.60 ± 0.79	4.53 ± 0.77	0.99 ± 0.17	0.83 ± 0.03

*Productivity was calculated during the logarithmic phase (weeks 5–6 for BOT2, weeks 5–8 for BOT84 and BOT7)

**Based on the equations on Table 1

(data are means ± standard deviation, $n = 6$)

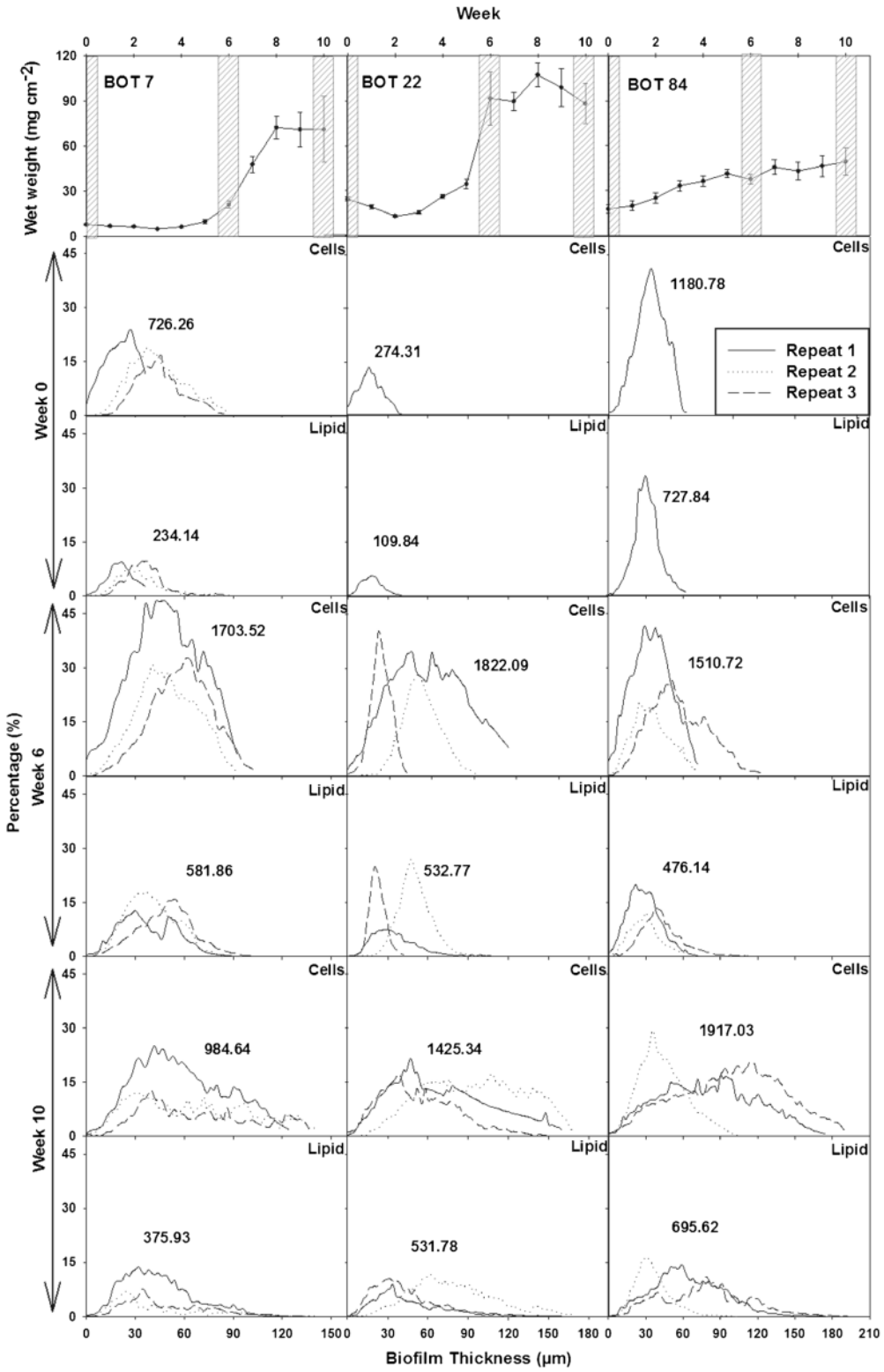
contamination load (Fig. 2). Among the strains that survived the 10 weeks and reached stationary phase (week 8), the PI_{ABS} and F_V/F_0 values were observed to decrease, except for the PI_{ABS} value of *B. braunii* BOT22, which was still increasing. Within each strain, the recorded ABS/RC did not change significantly in the logarithmic phase until the culture reached stationary phase. Similarly, the DI_0/RC values showed no significant change during logarithmic and stationary phases, except for *B. braunii* BOT84.

The fast phase of fluorescence induction curves (OJIP), which were measured in three different growth phases, revealed similar patterns to those of the other photosynthetic activity parameters (Fig. 5). In the lag phase (week 0), the biofilm was apparently under stress, as indicated by the appearance of a large inflexion on the curve (Fig. 5) and also the higher V_J compared to other growth phase (Fig. 6). Photosynthetic activity improved in the logarithmic phase (week 6), which was characterised by reduced inflexion and V_J. (Fig. 5). There were no obvious inflexions on the curve for *B. braunii* BOT7, BOT22 and BOT84 in the stationary phase compared to the previous growth phase, while the V_J values were still decreased in this phase.

Discussion

This study clearly showed that it is possible to grow certain strains of *B. braunii* (three races: B, L and S) as a biofilm with no contamination from the lag phase through to the stationary phase. Our study also indicated that the ability of *B. braunii* to grow in a biofilm is strain-specific. Our *B. braunii* race A cultures became contaminated, and their growth was not sustainable. Interestingly, Metzger and Casadevall (1989) showed that *B. braunii* races B and L are more resistant to contamination due to their high terpenoid content acting as an antimicrobial agent. Although in this study, *B. braunii* race A was less resistant to contamination, several other studies have used this race in their experiments. However, those studies were conducted over shorter periods than this study. Compared to *B. braunii* liquid cultures grown in open raceway ponds, this study potentially achieved higher biomass productivity. The biomass productivity was higher than the 3.42 mg cm^{-2} day^{-1} recorded by Ashokkumar and Rengasamy (2012) and the 0.75 mg cm^{-2} day^{-1} recorded by Zhang (2013). Ozkan et al. (2012) successfully grew *B. braunii* (LB 572; race A) a biofilm for 5 weeks with 0.071 ± 0.06 mg DW cm^{-2} day^{-1} biomass productivity (2750 cm^2 *B. braunii* biofilm at 55 µmol photons m^{-2} s^{-1} resulted in 2.49 ± 0.21 mg DW cm^{-2} biomass yield and with 27 ± 2% lipid content). Cheng et al. (2013) grew *B. braunii* biofilm in attached cultivation photobioreactors. The attached photobioreactor produced 0.65 mg DW cm^{-2} day^{-1} biomass productivity (= 6.20 mg DW cm^{-2} biomass yield with 42.5% lipid content on 800 cm^{-2} surface area under 100 µmol photons m^{-2} s^{-1}). Our results indicated a five- and 53-fold higher biomass yield and productivity compared to those reported by Ozkan et al. (2012). *Bortyococcus braunii* BOT22 also showed a two- and sixfolds higher biomass yield and productivity compared to Cheng et al. (2013). However, the *B. braunii* lipid yield achieved in current study was lower than in the Cheng et al. (2013) study. For generating the biofilm, we used sponges that were submerged in a liquid medium similar to the two layer system of Shi et al. (2007), while in other similar studies the medium flowed over the biofilm (Berner et al. 2015). Liquid flow can be critical factors in algal biofilm development (Berner et al. 2015). The static growth method used in our study possibly had a negative influence on the overall lipid production of *B. braunii*.

The multi-layer vertical *B. braunii* biofilm culture used by Cheng et al. (2013) yielded 4.91 mg DW cm^{-2} day^{-1} productivity (56 mg DW cm^{-2} biomass yield and 51.6% lipid content). These figures are significantly higher than the results obtained in single-layer horizontal biofilms.



◀ **Fig. 4** **a** Growth curve of *B. braunii* strains based on confocal microscope observation time; comparison of *B. braunii* BOT7, BOT22 and BOT84 biofilm thickness; cells and lipid distribution; and the average of area under the curve in: **b** the lag (week 0); **c** logarithmic (week 6); **d** and stationary (week 10) phase. The number of repetitions in measurement was based on sample availability, ranging between 1 and 3

Liu et al. (2013) also used a multi-layer vertical biofilm culture with *Scenedesmus obiliquus*, resulting in 7.09 mg DW cm^{-2} day^{-1} biomass productivity (80 mg DW cm^{-2} biomass yield and 47% lipid content).

Confocal microscopy also showed that the increased biofilm thickness during the growth period did not increase the cell distribution area in the biofilm. Since the confocal microscope captured the presence of algal cells through chlorophyll autofluorescence (Satpati and Pal 2015), the colour change in the stationary phase biofilm cultures may also reflect the cell distributions observed by confocal microscopy. Colour changes in algal colonies are closely related to the physiological state of the organism (Metzger et al. 1985). *Botryococcus braunii* cells dominated the top layer of biofilm (20–60 μm from the surface) which indicated that in the biofilms, *B. braunii* cells grew on top of each other towards the light (phototropic positive). Therefore, the older cells and colonies dominated the bottom layer. Increasing biofilm thickness can affect algal cells due to light or nutrient limitations in the deeper regions (Berner et al. 2015). As a result of this environmental stress (lack of nutrients) in the stationary phase, the *B. braunii* colour changed from green to yellowish due to an accumulation of carotenoids (Metzger et al. 1985).

Confocal imaging and the profile analysis also indicated that most of lipids were concentrated in the top layer (between the 20–60 μm depth range of the biofilm). This could potentially be very advantageous, especially if the aim is to extract the oils non-destructively. This means that if solvents are to be used for milking, they can reach the hydrocarbons (the product of interest) first, which may result in fewer detrimental effects on the *B. braunii* cultures. Obviously, the proposed milking method will need to be tested on *B. braunii* cultures grown in biofilm.

Measuring photosynthetic activity is one of the methods to monitor microalgal growth (Consalvey et al. 2005). To the best our knowledge, this is the first detailed study of photosynthetic activity in *B. braunii* biofilms. *Botryococcus braunii* BOT7, BOT22 and BOT84 showed a similar pattern for all photosynthetic parameters measured. At the start of the experiments, the algal biofilms were stressed because the algae had been moved to a new environment with less water, which changed the nutrient availability and had a higher light intensity (Ozkan et al. 2012; Schnurr et al. 2013; Berner et al. 2015). The reduced water availability and higher light intensity in the biofilm cultivation system resulted in low PI_{ABS} and F_V/F_0. PI_{ABS} values, which represent the vitality of photosynthetic organisms and cover three primary steps in photosynthetic activity: absorbing light energy, trapping this energy and converting the energy via electron transport (Strauss et al. 2006). The photochemical efficiency (F_V/F_0) represents the ability of the algae to use light for photosynthesis and shows the effect of stressful conditions on an organism's photosynthetic apparatus (Cosgrove and Borowitzka 2010; Dao and Beardall 2016). The low PI_{ABS} and F_V/F_0 values indicate that the algae were under stress due to their new environment. The higher irradiance received by the biofilm system compared to liquid cultures also resulted in a high absorbance of photons per reaction centre (ABS/RC), which reflected the apparent antenna size of the photosynthesis organelle (Demetriou et al. 2007). This phenomenon was also observed by Lu and Vonshak (1999) in their study of photoinhibition caused by high sun irradiance in outdoor

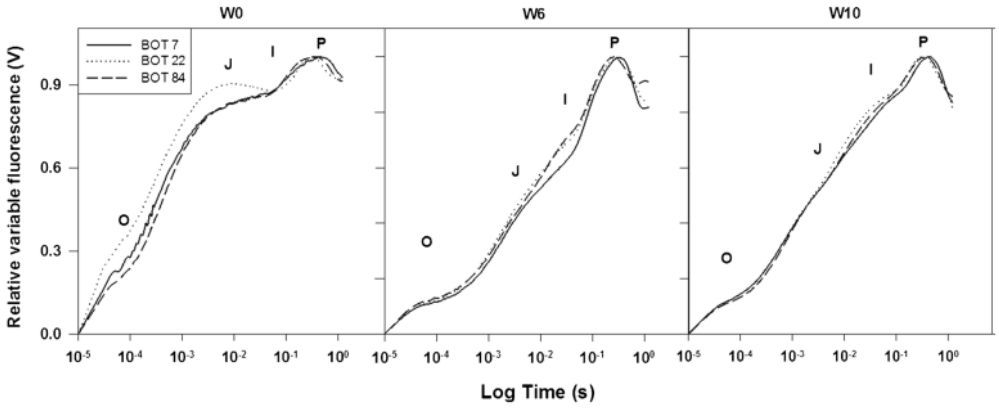


Fig. 5 Fluorescence induction curve for *B. braunii* BOT7, BOT22 and BOT84 in the lag (week 0), logarithmic (week 6) and stationary (week 10) phase

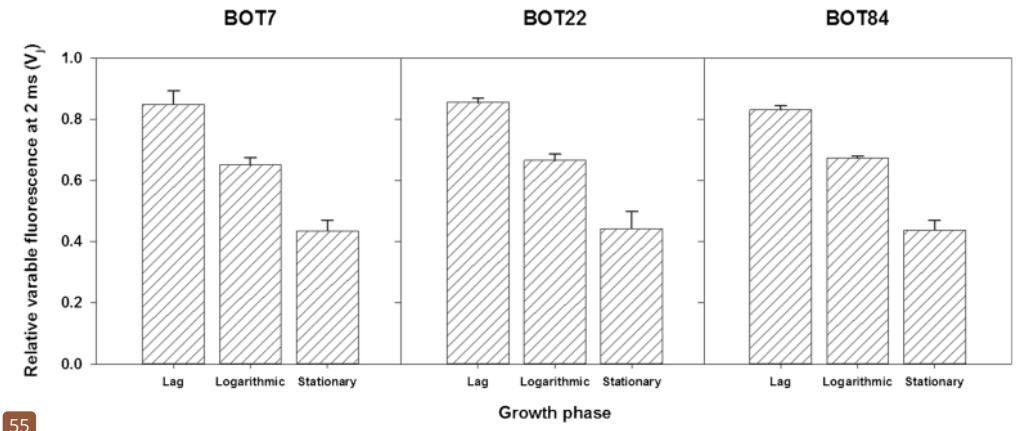


Fig. 6 Relative variable fluorescence at 2 ms (V_J) of *B. braunii* BOT7, BOT22 and BOT84 in the lag (week 0), logarithmic (week 6) and stationary (week 10) phase

Spirulina platensis cultures. The high value of ABS/RC could result from inactivation of the reaction centres (Strasser et al. 1999). At this stage, the low F_V/F_0 and the high ABS/RC values seem to be a consequence of the high DI_0/RC (Demetriou et al. 2007).

The photosynthetic activity of all *B. braunii* biofilms improved greatly when the cultures reached logarithmic phase. The fluorescence induction curve had a more proportional shape and lower V_J compared to those from the previous phases. The high value of V_J illustrated the actual proportion of closed reaction centres at 2 ms, resulting in less connectivity in the PS-II unit (Force et al. 2003). Other parameters, such as PI_{ABS} and F_V/F_0, were also improved in the logarithmic phase, while ABS/RC and DI_0/RC decreased. The increases in PI_{ABS} and F_V/F_0 indicated that the algal vitality and ability to utilise light for photosynthetic activity was improving (Maxwell and Johnson 2000; Cosgrove and Borowitzka 2010). In the logarithmic phase, *B. braunii* had acclimated to the new environment, and the cells were dividing at a constant rate (Becker 1994; Basanti and Gualtieri 2014). The stationary phase begins when the algae increase their biomass during growth and the loss during the degradation process reaches an equilibrium, and one or more nutrients are depleted (Becker 1994; Basanti and Gualtieri 2014). Our study showed that a significant decrease of F_V/F_0 can be an indicator variable showing that the algae had entered stationary phase. At this stage, although the ability of *B. braunii* to utilise light for photosynthesis has reduced, the photosynthetic activity at the reaction centres was still effective (characterised by a decrease of ABS/RC and DI_0/RC). This is a consequence of the high value of PI_{ABS}, which is directly affected by the absorption of light energy and the trapping of the excited energy (Strauss et al. 2006). Further investigations should be conducted to determine the photosynthetic apparatus that is directly affected when the algae enter the stationary phase. A decrease in V_J also occurred in the stationary phase, which indicated that the PS-II units in the photosynthesis system were still well connected (Force et al. 2003).

In conclusion, our study found that *B. braunii* has the potential to be grown as a biofilm. We also found that photosynthetic activity measurements can be used as a rapid and non-destructive way to analyse overall stress in *B. braunii* cultures during the different phases of growth. Furthermore, a visible sign in the photosynthetic parameters that shows that the algae have entered stationary phase may be an advantage in indicating the appropriate time for the lipid extraction process. However, this method still requires some improvements. Among the strains tested here, *B. braunii* BOT22 showed the highest biomass yield and lipid productivity on biofilm, which makes this strain a very good candidate for potential advanced studies. It should be noted that our long-term aim is to milk these biofilms for their hydrocarbons. The fact that *B. braunii* hydrocarbons remain in the top layers of the biofilm makes *B. braunii* biofilm cultures a very good candidate for the milking process. However, further larger scale growth studies, particularly under outdoor conditions, are necessary to indicate the reliability of *B. braunii* cultures as a promising raw material for biofuel production.

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