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Bio - Hydrocarbons from Algae

Impacts of temperature, light and salinity on algae growth

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Foreword

An upward trend in the price of petroleum products will become unavoidable after about 2010 when world oil production will have peaked and other more costly supplies are utilised. In this situation, there is a growing interest in renewable energy around the world. *Botrycoccus braunii* (Bb) is a green alga that it has attracted considerable attention for many decades as it produces biotechnologically important compounds in large amounts-mostly various kinds of hydrocarbons. These compounds could be used in the future as an alternative energy source (e.g. fuels). However, the cultivation of Bb for hydrocarbon production has only recently become attractive for economic and environmental reasons.

This study is to better understand and define the operating parameters for the mass production of Bb under controlled conditions. The results are to be used towards identification the feasibility of Bb China strain 1 cultured in Australia for producing hydrocarbons.

The original aims of the study were to**:**

- Determine the environmental tolerance of Bb.
- Demonstration of the successful culture method for Bb.
- Examine the link between oil content of Bb and environmental factors.

This project was funded from RIRDC Core Funds which are provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1200 research publications, forms part of the Environment and Farm Management R&D program, which aims to support innovation in agriculture and the use of frontier technology to meet market demand for accredited sustainable production.

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Tony Byrne
Acting Managing Director
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Financial support for this research project was provided by the Rural Industries Research and Development Corporation and this is acknowledged and greatly appreciated.

Abbreviations

B. braunii	*Botryococcus braunii*
OD	optical density
rTLC	relative total lipid content
G	average generation time
K	proliferous rate
ml	millilitre
μm	micrometre
nm	nanometre
h	hour
W/m^2	Watt per square metre
M	mole
NaCl	sodium chloride
EPS	Exopolysaccharide
RIRDC	Rural Industries Research and Development Corporation
S.E.	Standard Error
ANOVA	Analysis of Variance
SPSS	Statistic Package for Social Sciences

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Executive Summary

Botryococcus braunii is a colonial green alga that is found in lakes and reservoirs in Australia and in other parts of the world. Blooms of this alga resemble a large floating mat on the water surface. This alga contains hydrocarbon up to 75% of dry weight, which can be converted into petrol, diesel or turbine fuel or other liquid or gaseous hydrocarbons. Given that Australia has large areas of available land and brackish water and high average radiant energy influx, there is a great potential to develop a source of biofuel production through cultivation of microalgae.

Five *Botryococcus* strains have been obtained from China, Japan and UK. So far, the research has focused on the physical requirement of *B. braunii* (China strain 1) under intensive culture. We have identified the tolerance range of environmental factors including photoperiod, temperature, light intensity, and salinity and determined the relationship between oil content in the algae and culture conditions.

Firstly, we developed the methods for measuring algal growth and lipid content. Nile red staining was used for lipid measurement, while optical density was used to measure the abundance of *B. braunii* in a spectrophotometer.

Secondly, the photoperiod of 12 h light : 12 h dark was considered the optimum light regime to sustain algal growth. Based on this, temperature, light intensity and salinity experiments were conducted successively. The optimal temperature for China strain 1 was 23 °C under which the algae gained maximum biomass and lipid production. The temperature tolerance was between 20 and 30°C. The suitable range of light intensity for China strain 1 was between 30 and 60 W/m^2.

Finally, six concentrations of NaCl medium (0, 0.15, 0.25, 0.35, 0.5 and 0.7 M) were prepared to examine the salinity tolerance of the algae (0.1 M NaCl = 5.85‰). Algae in 0.15 M NaCl produced maximum biomass and lipid content. The generation time of algal cells was about 2 days. It is possible to culture *B. braunii* in brackish waters in Australia. This is a significant result given the limited availability of freshwater in Australia.

To obtain the maximum biomass and hydrocarbon production in China strain 1, the optimal culture conditions are (1) temperature: 23 °C, (2) light intensity: 30~60 W/m^2 irradiance, (3) photoperiod: 12 h light and 12 h dark, and (4) salinity: 8.775 ‰ medium.

Considering the variation of different *Botryococcus* strains, it will be necessary to select high yielding and commercially viable strains in future to supplement the requirements of energy supply in Australia.

Introduction

Botryococcus braunii is a freshwater green alga (Chlorophyceae). It is able to produce a high level of hydrocarbons in the range of 15-76% dry weight (Metzger *et al.*, 1985; Sawayama *et al*, 1994). *B. braunii* has been considered a renewable source of biofuel for future energy supply. The ability of hydrocarbon production of this alga has been investigated under different growth phase at different culture conditions (Casadevall *et al.*, 1985; Inoue *et al*, 1994).

Casadevall *et al.* (1985) and Villarreal-Rosales *et al*. (1992) demonstrated that hydrocarbon productivity in *B. braunii* varied along the growth cycle with maximum productivity in the exponential and early stationary growth stages. Subsequent studies by Sawayama *et al.* (1992) and Kojima *et al*. (1999) also showed that the hydrocarbon production of this alga was proportional to cell growth. In the stationary stage, unsaponifiable lipids in this alga increased up to 80% of dry weight (Brown *et al.*, 1969), therefore, the algal biomass in the stationary stage is an important premise of hydrocarbon production.

Komárek and Marvan (1992) reported several strains of *B. braunii* growing in a wide spectrum of conditions, which are available for biotechnological exploitation. It is believed that the lipid production varies not only among strains but also environmental conditions (Singh and Kumar, 1994; Pal *et al.*, 1998). The most important environmental factors influencing lipid production are temperature (Lupi *et al*., 1991), photoperiod and light intensity (Brenckmann *et al*., 1985), salinity (Vázquez-Duhalt *et al*., 1991a, b; Derenne *et al.*, 1992) and the level of nitrogen (Largeau *et al*., 1980; Sawayam *et al*., 1992; Singh and Kumar, 1992). The objective of this study is to examine the algal growth and lipid content of *B. braunii* (China strain 1) under various light, temperature and salinity conditions, in an attempt to obtain the optimal culture condition for the maximum biomass and hydrocarbon production.

Materials and methodology

1. Experimental algae

Botryococcus braunii (China strain 1) was obtained from the Institute of Hydrobiology, Wuhan, China. The seed culture was incubated at 25 °C and 10 ml of seed was inoculated to a 100 ml new medium every two weeks. All the cultivations in this study were stirred every 12 h. All the operations in this study are under an anti-bacterial condition.

2. Photoperiod

Four photoperiods (4 h light : 20 h dark, 8 h light : 16 h dark, 12 h light : 12 h dark, and 24 h light : 0 h dark) were used in this study with three replicates, respectively. Each flask contained 150 ml algal cultivation (100 ml medium + 50 ml seed) and aerated with air through a 0.2 μm filter at 25 °C and 30 W/m^2 irradiance. The algal growth was measured by algal optical density (see 6.3). The initial optical density was marked as day 0 (OD_o = 0.011). Then, 4 ml homogenous algal liquid was sampled from each flask twice a week. The same amount of fresh culture medium was added to maintain the volume over time.

3. Temperature

Five levels of temperature were used in this experiment :20, 23, 25, 27 and 30 °C. Four replicates were used in each treatment. At all temperatures, the algae were cultured under the following conditions: aeration, 30 W/m^2 light, and photoperiod of 12 h light : 12 h dark. A volume of 150 ml algal liquid (100 ml medium + 50 ml seed) was incubated in each 250 ml conical flask (OD_o = 0.038). Samples were taken twice a week.

4. Light intensity

Two ranges of light intensity (low and high) were examined under 23 °C and 12 h light : 12 h dark photoperiod. In the first experiment, four light intensities (8, 12, 20 and 30 W/m^2) were included with three replicates at each light level. Each conical flask contained 150 ml algal liquid (OD_o = 0.12) with aeration in each flask. In the second experiment, light intensities included 30, 60, 100, 150, 200 and 300 W/m^2 with three replicates. Each flask contained 150 ml algal liquid (OD_o = 0.008) and 0.05g/L $NaHCO_3$ as carbon resources. The samples were taken twice per week.

5. Salinity

Six levels of salinity were involved: 0, 0.15, 0.25, 0.35, 0.5 and 0.7 M of NaCl (0.1M NaCl is equivalent to 5.85‰) with three replicates. Each flask contained 150 ml algal liquid. The initial optical density was 0.008 unit. The algae were cultured under 23 °C, 60 W/m^2 irradiance with 12 h light : 12 h dark and aeration. The sampling schedule was the same as in the light experiment.

Algal biomass was harvested from each flask at the end of each experiment dried and also weighed to obtain the regressive relationship between the optical density at 680 nm wavelength and the dry weight of this strain.

6. Analysis method

6.1 Proliferous Rate (K)

$$K = \frac{\left(\log OD_t - \log OD_o\right)}{T} \times 3.322$$ (Huang *et al*., 2002a)

OD_t : terminal optical density;
OD_o : initial optical density;
T: days.

6.2 Average Generation Time (G)

$$G(d) = \frac{0.301}{K}$$ (Huang *et al*., 2002b)

6.3 Optical Density (OD)

The cell density was measured with a spectrophotometer (Unicam UV-Vis Spectrometry) using 680 nm wavelength (Lee *et al*., 1998a). The relationship between the algal number and optical density (OD) was developed. In the test, five algal densities were used by diluting the original algal culture with culture medium to 1, 2, 3, 4 and 5 times, named A, B, C, D and E respectively. All of them were cultured for 10 days under the same condition (25 °C, 60 W/m^2 irradiance with 12 h light : 12 h dark photoperiod). Each sample was thoroughly swirled before taking the measurement.

6.4 Relative total lipid content (rTLC)

In this study, the method described by Lee *et al.* (1998b) was used to determine the total lipid content in *B. braunii.* Fluorescence was measured after staining with Nile red on a fluorometer (HITACHI F-4000 Fluorescence Spectrophotometer) using a 490 nm narrow band excitation filter and a 585 nm narrow band emission filter. The relative total lipid content (rTLC) was measured from the reading of fluorescence intensity in 10 seconds after the sample had been placed in the spectrophotometer. The relative fluorescence intensity of Nile red was obtained after subtraction of the autofluorescence of algal cells and the self-fluorescence of Nile red from the gross reading.

7. Statistic analysis

The data in each experiment were analyzed by univariate of general linear model and one-way ANOVA on SPSS. The different letters for each treatment in each Figure and Table indicate significant difference ($P<0.05$).

Results

1. Relationship between the number of algal cells and optical density

The amount of algal cells in each sample was counted using a haemocytometer before taking the optical density reading on a spectrophotometer. A regression equation (1) was obtained from the data analysis.

$$Y = 4\times10^{-6}x + 0.0036, R^2 = 0.9986 \quad (1)$$

x: OD;
Y: The number of algal cells/ ml

It can be seen in Figure 1 that there is a very close linear relationship between algal cells and the OD reading.

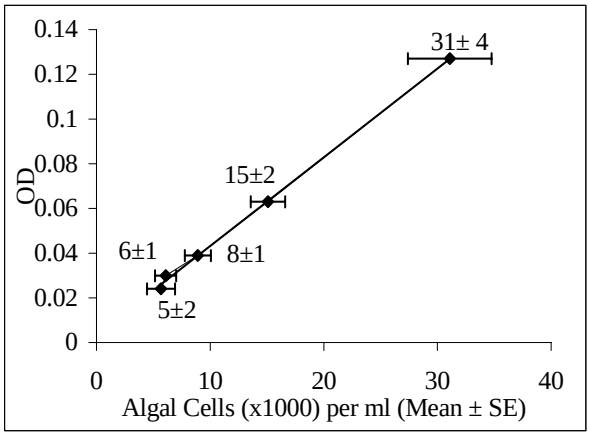


Figure 1. The relationship between OD and the amount of cell (ml).

2. The lipid fluorescence of *B. braunii*

Two *B. braunii* strains (China 1 and China 2) were used for the fluorescence test. China strain 1 was cultured in 8‰ seawater. The algal microstructure after fluorescence stain of China strain 2 is shown in Figure 2.

In Figure 2, the lipid and chloroplast showed different fluorescence under blue light. However, without Nile red staining, the chloroplast autofluorescence of the cell was also reddish (Fig. 3: A and B). The free lipid particles were not observed in the medium until the cells were pressed under a cover slip (Fig. 2: A and F).

China strain 1 had less gelatinous mass than China strain 2. Also many empty and round particles were found among the algal cells of China strain 1 (Fig. 3C). The particle shape and size were different from algal cells, but they still had slightly yellow fluorescence after staining (Fig. 3E). Both China strains 1 and 2 showed a large number of oil drops inside the cell and within the gelatinous matrix, as indicated by the yellow colour under blue light. Based on the observation under the fluorescence microscope, the lipid content in China strain 1 in 8‰ salinity seawater was further tested using the Nile red staining.

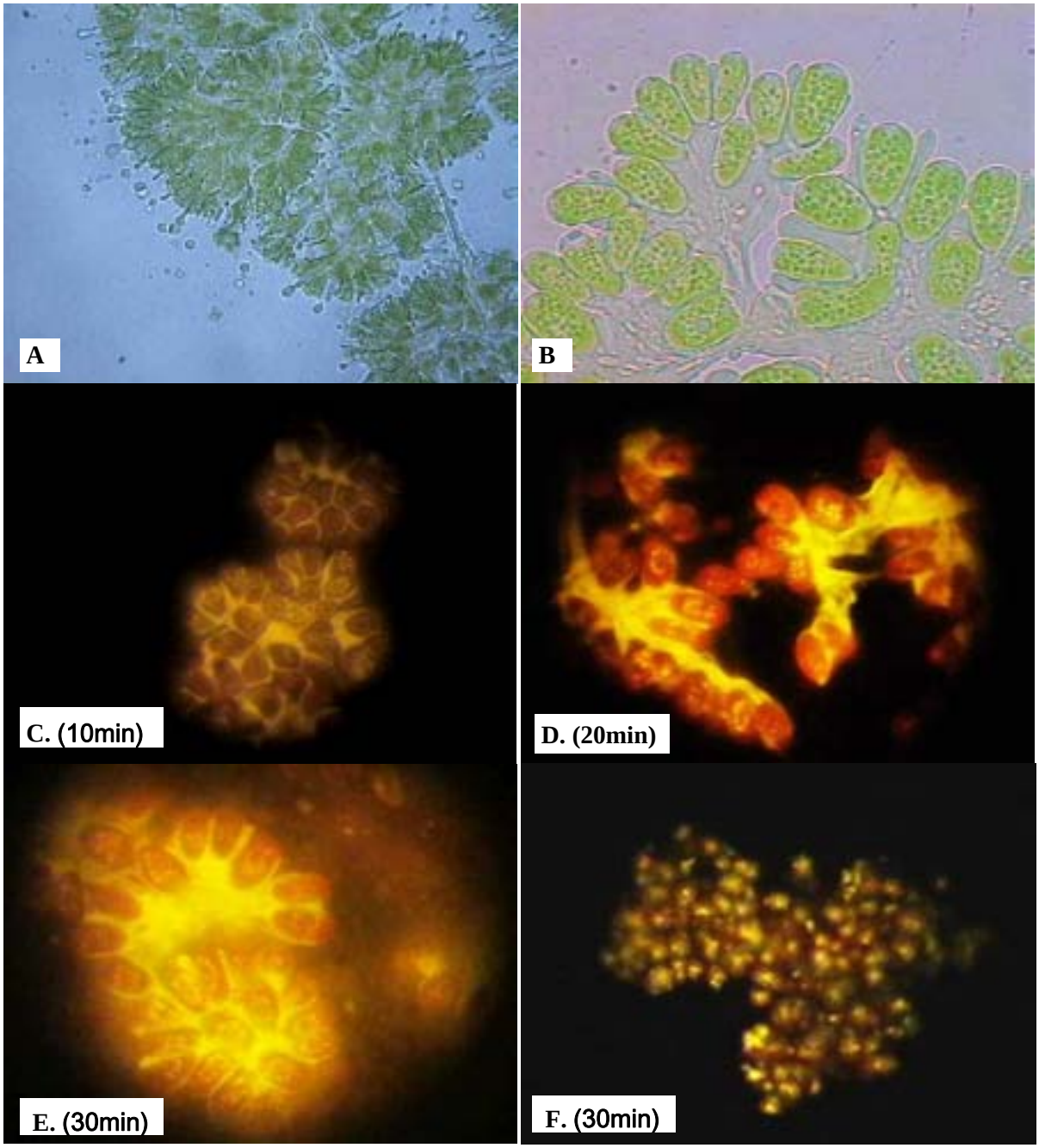


Figure 2. The algal microstructure of *B. braunii* (China strain 2). (A— Cells are embedded in a gelatinous mass containing oil drops extruded from the extracellular pool by pressing; B— there are many oil particles in the algal cells, including nucleus; C— the fluorescence picture of algal cells after 10 min staining. The reddish staining colour is chlorophylls and the yellow colour is lipid after staining; D— the intercellular and extracellular lipid fluorescence become obvious after 20 min staining; E— the lipid fluorescence of algal cells is stronger after 30 min staining; F— the fluorescence picture of an algal colony after 30 min staining)

Figure 4 shows the fluorescence intensity at different times of reading. The peak fluorescence reading occurred at 30 min, but started to fade after 40 min. However, the duration of fluorescence staining was mainly dependent on the size of matrix and light resources. Particularly in the centre of algal colonies of China strain 2, the yellow fluorescence was still seen when stored over night, but the individual cells or the cells at the edge of the colony only appeared reddish. They had been without any yellow intracellular particles.

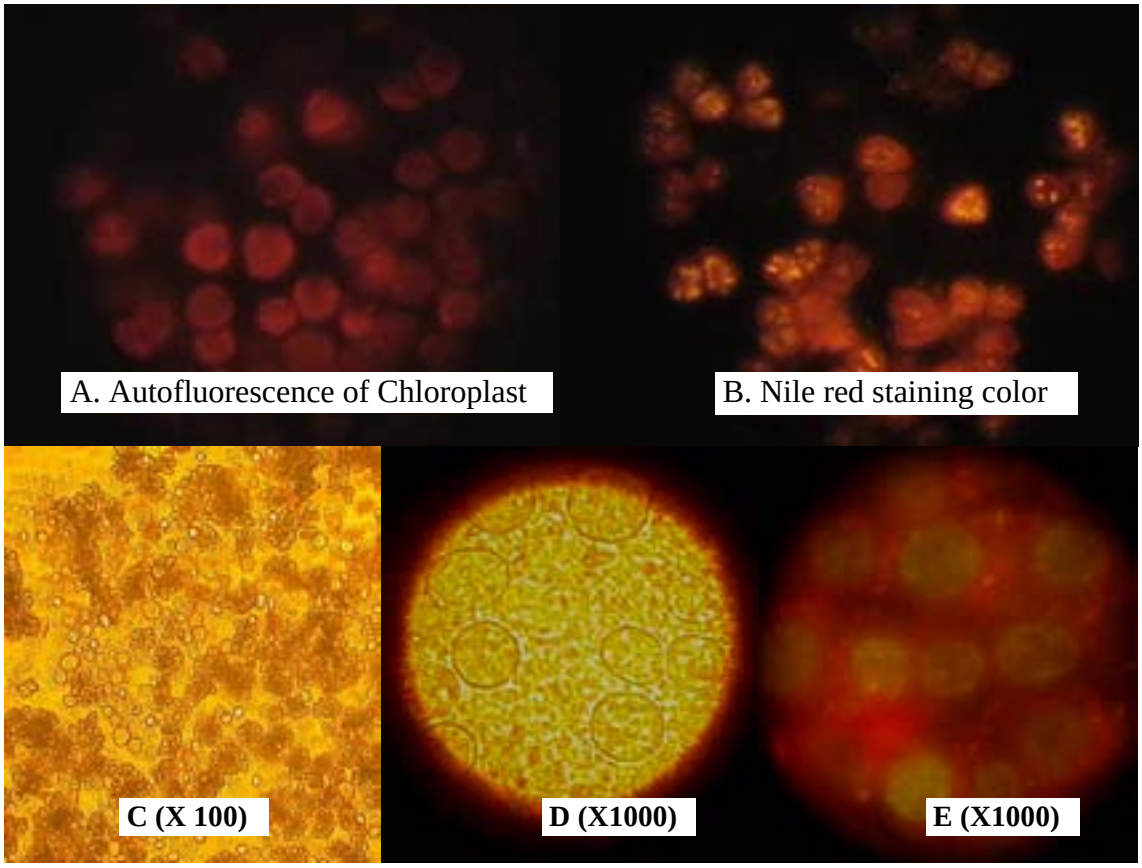


Figure 3. Illustration of China strain 1. (A— Chloroplast autofluorescence without Nile red straining; B— With Nile red staining, the intracellular oil particles display yellow fluorescence and chloroplasts are reddish; C— The morphology of an algal colony, combined with many empty and round particles; D— The particle shape under mixture lights; E— Fluorescence of stained particles.)

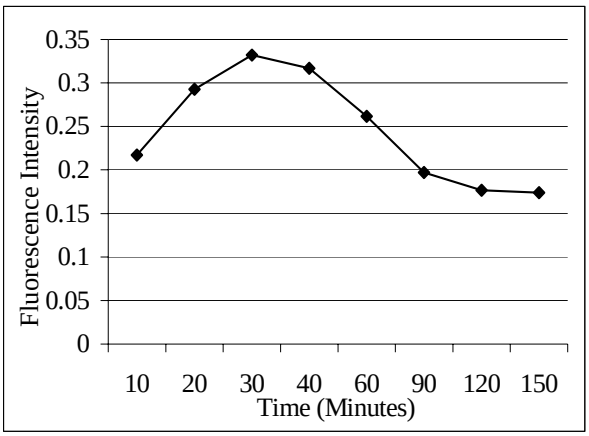


Figure 4. Fluorescence intensity at different reading time.

3. Photoperiod experiment

In Figure 5, algal growth was faster under the photoperiod of 24 and 12 h light than that under 8 and 4 h light per day ($P<0.05$). The algal biomass under 12 h light still showed a tendency of increasing after 56 days. The algal growth under 12 and 24 h light became much faster than that under 8 and 4 h after 16 days. One month later, algal growth under 8 h light was faster than that under 4 h. Compared with other treatments, 4 h light was inadequate to support the algal growth. Although there was no difference between 4 h and 8 h daily light cycles ($P>0.05$), the latter biomass was nearly two times as high as the former. There was also no significant difference in biomass between 12 and 24 h light cycles ($P>0.05$).

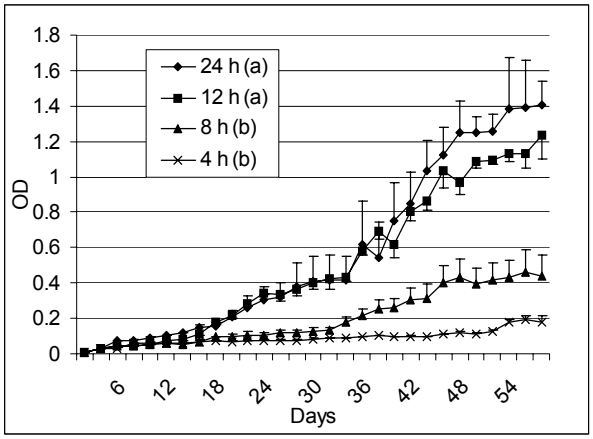


Figure 5. Impact of different photoperiods on algal growth. Different letters indicate significant difference ($P<0.05$). Error bar represents standard error ($n = 3$).

In Table 1, the proliferous rate (K) was consistent with algal growth (Fig. 5). When the light period increased from 4 to 24 h daily, the K value increased, while the generation time (G) decreased. The G and K values under 4 h light were significantly different from those under 24 and 12 h light ($P<0.05$), but did not differ from those under 8 h light ($P>0.05$).

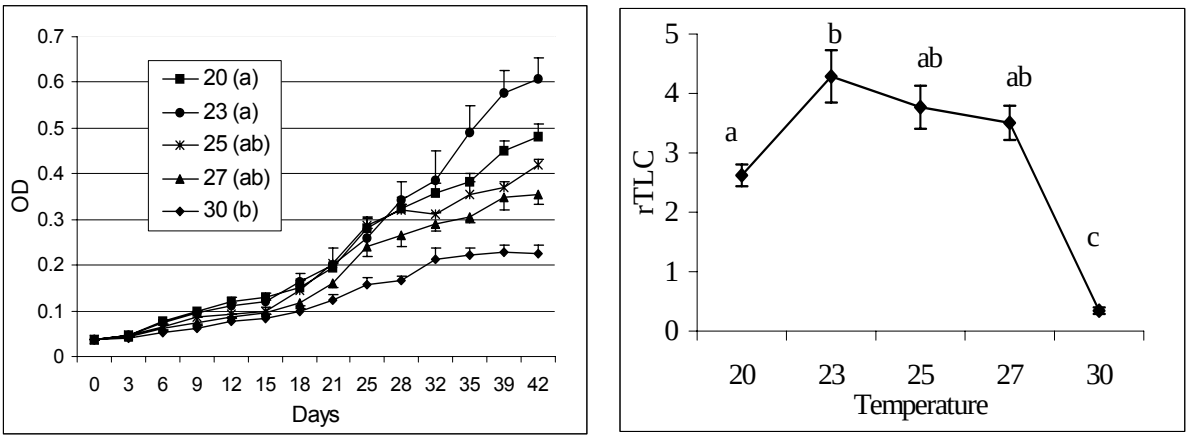
Table 1. The average generation time (G) and proliferous rate (K) of the photoperiod experiment. (Mean ± S.E, $n = 3$). Different letters indicate significant difference ($P<0.05$).

Photoperiod	G	K
24 h	2.41 ± 0.05 (a)	0.125 ± 0.002 (a)
12 h	2.49 ± 0.06 (a)	0.121 ± 0.003 (a)
8 h	3.33 ± 0.35 (ab)	0.092 ± 0.001 (b)
4 h	4.27 ± 0.26 (b)	0.071 ± 0.005 (b)

After 56 days, the algal colour changed from green to brown under 24 h light. Many tiny air bulbs were suspended on the surface and on flask walls. In contrast, algal culture under other light regimes showed fresh green. But most of algal cells under 4 h light were slightly brown and loosely flocculated on the bottom of flasks.

4. Temperature experiment

In Figure 6 (A), all algal abundance varied among the temperature treatments. After 42 days, the algal growth rate at 20 and 23 °C was significantly higher than that at 30 °C ($P<0.05$). Algal growth rates at 25 and 27 °C were not significantly different ($P>0.05$). Among the growth curves, the algae did not show an obviously exponential phase below 30 °C and turned into the stationary phase at an early stage (Fig. 6A). However, the algae at 20, 23 and 25 °C still showed a tendency to continuous growth, but the growth rate dropped. Based on the final biomass (bar in Fig. 6B), the suitable temperatures for the growth of *B. braunii* should be in a sequence of 23°C>20°C>25°C>27°C>30°C.



A B

Figure 6. A: The impact of temperature on algal growth over time, and B: the impact of temperature on lipid production (rTLC). Error bar represents standard error (n = 4). Different letters indicate significant difference ($P<0.05$).

In Figure 6 (B), the curve of total lipid content (rTLC) peaked at 23 °C. Subsequently, the rTLC became relatively stable when the temperature increased from 23 to 27 °C. The rTLC at 25 and 27 °C was not different from that at 20 and 23 °C ($P>0.05$). But, it dropped drastically when temperature increased to 30 °C ($P<0.05$). The value of rTLC correlated with the biomass in each treatment (Fig. 6B).

In Table 2, the G and K values at 30 °C were significant different from other temperatures ($P<0.05$). However, the algal average generation time (G) in all treatments was less than 5 days. The shortest generation time (3.18 ± 0.09 days) and highest proliferous rate (0.095 ± 0.003) occurred at 23 °C.

Table 2. The average generation time (G) and proliferous rate (K) of the temperature experiment. (Mean ± S.E, n = 4). Different letters in each column indicate significant difference ($P<0.05$).

Treatments	G	K
20 °C	3.47 ± 0.09 (ab)	0.087 ± 0.002 (ab)
23 °C	3.18 ± 0.09 (a)	0.095 ± 0.003 (a)
25 °C	3.65 ± 0.04 (bc)	0.082 ± 0.001 (ab)
27 °C	3.94 ± 0.10 (c)	0.077 ± 0.002 (b)
30 °C	4.97 ± 0.21 (d)	0.061 ± 0.003 (c)

After 42 days, the algal colour under 30 °C was brown and most of cells inclined to flocculate on the bottom (Fig. 7A). Moreover it was easy to find air bubbles on the surface when stirring the liquid. Compared with 30 °C, the algal color under lower temperatures was fresh green and the intercellular particles were clearly observed (Fig. 7B). Furthermore, the cells were distributed evenly if the flask was stirred. Otherwise, most of cells sank on the bottom without aeration.

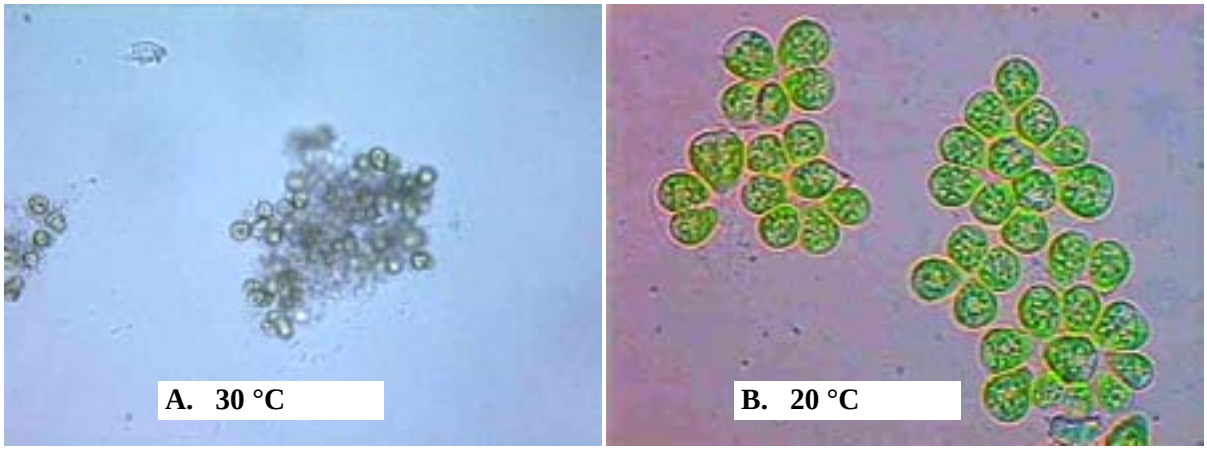
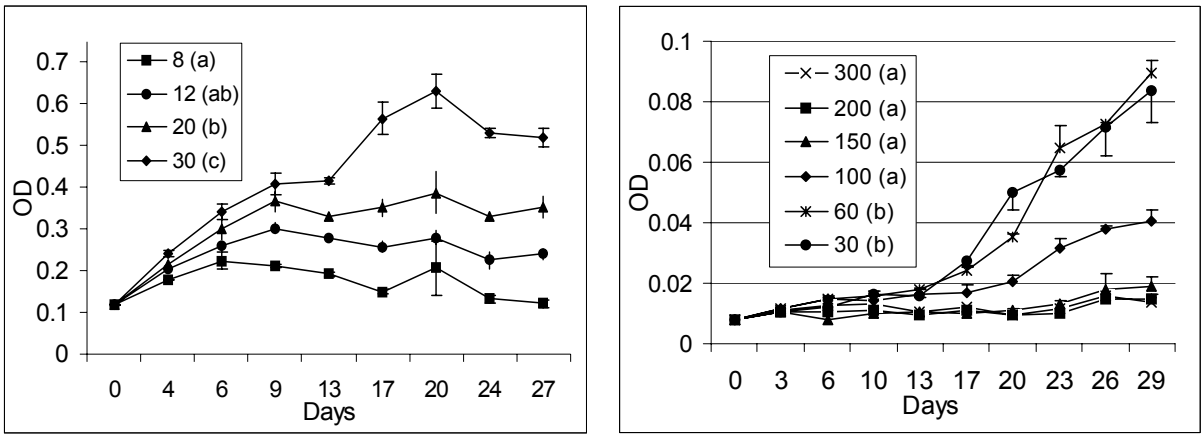


Figure 7. Morphological character of algal cells under 30 °C (A) and 20 °C (B).

5. Light intensity experiment

In the experiment of low light range, the algal biomass under 30 W/m^2 was significant different from other treatments ($P<0.05$, Fig. 8A). After 27 days, most of algal cells died and the colour of the cultivation turned slightly brown under 8 and 12 W/m^2, which was different from that under 20 and 30 W/m^2. Algal cells under 8, 12 and 20 W/m^2 agglutinated together rather than distributing evenly after stirring, especially under 8 W/m^2. In Table 3, under 30 W/m^2, the algal generation time (G) was shortest (3.85 ± 0.12 days), but the algal proliferous rate (K) was greatest (0.078 ± 0.002). The G and K values were significantly impacted with decreasing light intensity ($P<0.05$), especially under 8 W/m^2.



A B

Figure 8. The impact of light intensity on algal growth. (A) Low light range; (B) High light range. Different letters indicate a significant treatment difference ($P<0.05$). Error bar represents standard error (n = 3)

In the experiment of high light range (Fig. 8B), the 30 and 60 W/m^2 treatments were more suitable to algal culture than high light intensities ($P<0.05$). Under high light intensity (>150 W/m^2), algal cells were bounded by filamentous bacteria in few flasks, and a thin green layer was developed on the bottom. At 300 W/m^2, most algal cells died resulting in some brown flocculation on the bottom.

Table 3. The average generation time (G) and proliferous rate (K) of algal liquid under low range of light intensity (Mean ± S. E, n = 3). Different letters in each column indicate significant difference (P<0.05).

Intensity (W/m^2)	G	K
30	3.85 ± 0.12 (a)	0.078 ± 0.002 (a)
20	5.30 ± 0.34 (a)	0.057 ± 0.004 (a)
12	8.26 ± 0.72 (b)	0.037 ± 0.003 (a)
8	54.43 ± 33.49 (c)	0.003 ± 0.002 (b)

Table 4. The average generation time (G) and proliferous rate (K) of algal liquid under high range of light intensity (Mean ± S. E, n = 3). Different letters in each column indicate significant difference (P<0.05).

Intensity (W/m^2)	G	K
30	2.61 ± 0.15 (a)	0.116 ± 0.006 (a)
60	2.51 ± 0.05 (a)	0.120 ± 0.002 (a)
100	3.77 ± 0.22 (a)	0.080 ± 0.005 (b)
150	7.59 ± 1.14 (ab)	0.042 ± 0.007 (c)
200	10.56± 2.19 (ab)	0.031 ± 0.005 (c)
300	13.79 ± 3.38 (b)	0.025 ± 0.008 (c)

In Table 4, significant treatment effects were observed on both G and K values (P<0.05). When light intensity increased from 60 to 300 W/m^2, the K value gradually decreased, while the G value increased. Algae took less than 4 days to double the population size when the light intensity was 30~100 W/m^2. Compared with the low light experiment (Tab. 3), the highest K and shortest G occurred at 60 W/m^2.

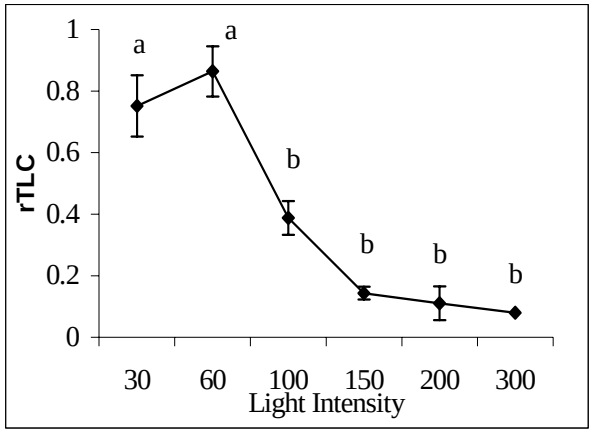
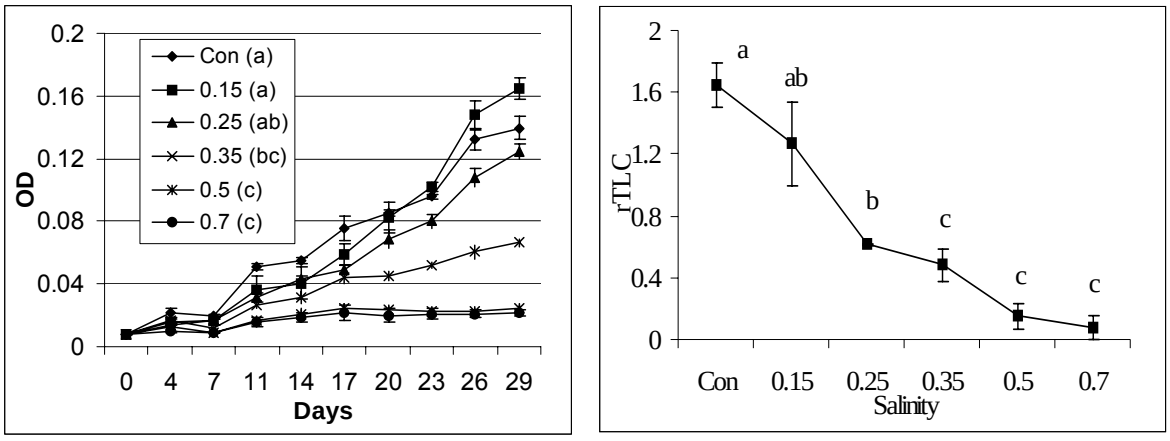


Figure 9. Relative total lipid content (rTLC) under various light intensities (n=3). Different letters indicate significant difference (P<0.05). Error bar represents standard error (n=3)

Total lipid content at 30 and 60 W/m^2 was significantly higher than that at other light intensity (P<0.05, Fig. 9). However, lipid content did not differ when the light intensity was above 100 W/m^2 (P>0.05). Lipid content decreased sharply when light intensity increased from 60 to 150 W/m^2, but levelled when the light intensity was between 150 and 300 W/m^2, which was consistent with the final algal abundance (Fig. 8).

6. Salinity experiment

After 29 days, algal cells in 0.7 M NaCl could barely survive but hardly gained any growth. Algal cells in 0.5 M NaCl were green, but the growth nearly stopped (Fig. 10A). When salinity decreased under 0.35 M, obvious algal growth was observed. There was no significant difference among control, 0.15 and 0.25 M of NaCl treatments ($P<0.05$). The algal abundance in 0.15 M reached the same level as control in 20 days and exceeded the control thereafter.



A B

Figure 10. The impact of salinity on the algal growth over time (A); and the mean lipid production (rTLC) of *B. braunii* at different salinities (B). Different letters indicate significant treatment difference ($P<0.05$). Error bar represents standard error (n = 3).

In Figure 10 (B), the lipid content decreased sharply when salinity was increasing and levelled between 0.35 and 0.7 M NaCl ($P<0.05$). In comparison, the *B. braunii* in freshwater produced the greatest amount of lipid, but it was no different from that in 0.15 M medium ($P>0.05$).

Table 5. The average generation time (G) and proliferous rate (K) of *B. braunii* under various salinities (Mean ± S. E., n = 3). Different letters in each column indicate significant difference ($P<0.05$).

Salinity (M)	G	K
Control	2.12 ± 0.04 (a)	0.142 ± 0.003 (a)
0.15	2.00 ± 0.02 (a)	0.150 ± 0.002 (a)
0.25	2.21 ± 0.03 (a)	0.137 ± 0.002 (a)
0.35	2.86 ± 0.06 (a)	0.105 ± 0.002 (b)
0.5	5.44 ± 0.33 (b)	0.056 ± 0.004 (c)
0.7	6.11 ± 0.49 (b)	0.050 ± 0.003 (c)

In Table 5, the impact of salinity on algal growth was shown by the G and K values. In the salinities of 0.5 and 0.7 M NaCl, the generation time (G) was higher while the proliferous rate (K) was lower than any other salinities ($P<0.05$). Under 0.15 M NaCl, the G value was smallest (2.00 ± 0.02 days) and K value was largest (0.150 ± 0.002). Judging by their morphology, the cell division was much more active in control, 0.15, 0.25 and 0.35 M NaCl than that in 0.5 and 0.7 M NaCl. Although a few cell divisions could be found under 0.5 M NaCl, the intracellular oil particles were much less in 0.5 and 0.7 M NaCl than other salinities (Fig. 11). Some flocculation occurred in 0.7 NaCl medium.

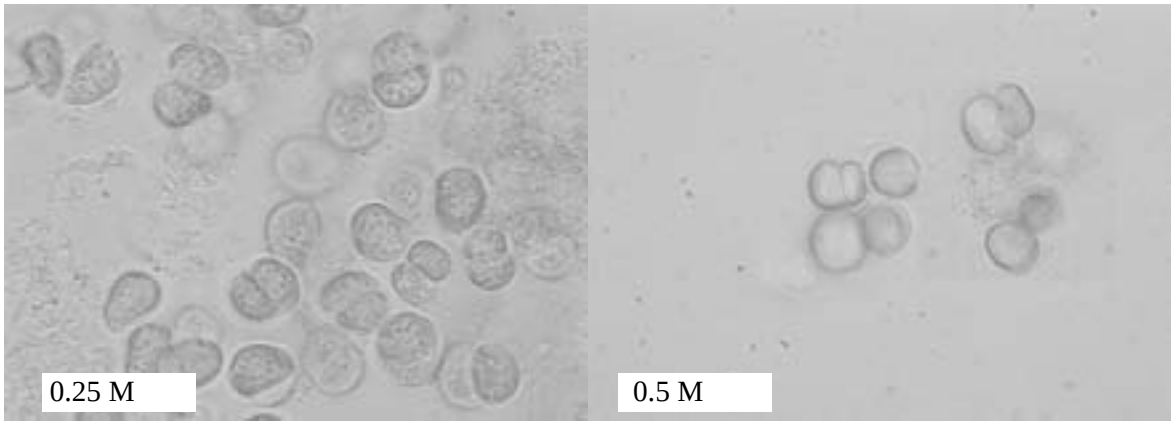


Figure 11. The morphology of algal cells in 0.25 and 0.5 M NaCl.

7. Regression relationship between algal absorbency and dry weight

When each experiment was terminated, the algal weight in each flask was dried in 70 °C for 48 h. Paired with each individual optical density, a regression equation (2) was developed to predict dry weight from OD the reading under laboratory condition:

$$W(g/L) = 1.594 \times OD_{680} + 0.0526, R^2 = 0.8926 \quad (2)$$

The R-squared value is 0.8926, which means there is a significant linear relationship between the algal dry weight and the OD reading.

Discussion

1. Lipid fluorescence

Since the biomass of *B. braunii* available for lipid analysis in this study was small, the Nile red fluorescence method was used to measure the content of lipid droplets. A study on *Boekelovia* stained with Nile red demonstrated that the amount of yellow fluorescence was correlated with lipid droplets in the algal cells, suggesting that fluorescence could be used as a tool to screen algal cells for lipid content (Jean, 1986-1987). Lee *et al.* (1998b) reported that the Nile red staining method was as effective as the gravimetric method commonly used for lipid determination. Although the Nile red staining cannot detect the structure of hydrocarbon compounds, it is a simple, fast and sensitive method for measuring total lipids in algae.

Because Nile red can permeate the algal cell walls, therefore, all lipid droplets can be stained and emit yellow fluorescence (Fig. 2, 3). The fluorescent distribution corresponded to the distinct sites of hydrocarbon accumulation in *B. braunii* (Largeau *et al*., 1980; Casadevall *et al*., 1985; Wolf *et al*., 1985; Zhang *et al*., 1998). In comparison with Figures 2 and 3, China strain 2 had more mucous matrix fluorescence than China strain 1. This may explain why China strain 1 inclined to sink without aeration due to the lack of extracellular lipid.

Because Nile red is excited at a wavelength of 490 nm, and emits yellow light at 585 nm (Lee *et al.*, 1998b), the autofluorescence of chloroplast was not stained by Nile red (Fig. 3: A and B), therefore, the fluorometer can detect the amount of total lipids in each treatment (Fig. 4). This study showed that the fluorescence level peaked after about 30 minutes from the time of staining with Nile red (Fig. 2, 4), though previous reports indicated that the fluorescence intensity was stable between 10 and 45 minutes after staining (Jean, 1986-1987; Lee *et al.*, 1998b). All readings in this study were taken at 30 minutes after staining.

2. Impact of external conditions

Although *B. braunii* is considered an important energy-fixing organism in providing hydrocarbons for fuel, the hydrocarbon production kinetics vary depending on the external growth conditions (Vázquez-Duhalt, *et al.*, 1991a, b; Kojima *et al.*, 1999) such as availability of nitrogen and phosphate (Largeau *et al*., 1980; Sawayam *et al*., 1992, 1995; Singh and Kumar, 1992; Lupi *et al.*, 1994, Jun et *al.*, 2003; Suling *et al*., 2004), light intensity (Brenckmann *et al.*, 1985), pH (Lee *et al.*, 1998a), temperature (Lupi *et al*., 1991), and bacterial abundance (Lupi *et al*., 1991). As environmental factors, temperature, light intensity and salinity are particularly important in regulating algal growth. In our study, the optical density of algae was used as an indicator to test the response of algal growth to environmental factors.

2. 1. Temperature

The optimal temperature for the growth of China strain 1 was 23 °C, though many studies have shown that the optimal temperature for majority of *B. braunii* strains was about 25 °C (Casadevall *et al*., 1985; Fernandes *et al*., 1989; Lupi *et al*., 1991; Vladislav *et al.*, 1994). We found that *B. braunii* could tolerate 30 °C, but the algal growth was slowest (K value is 0.061 ± 0.003, $P<0.05$) and turned into the stationary phase in an early stage. The result was in accordance with a previous study that the optimum temperature for various strains of *B. braunii* could fluctuate from 20 to 28 °C (Nicoara *et al*., 1988). It seemed that China strain 1 is a relatively cold-favouring strain.

In this study, the biomass and lipid content of *B. braunii* declined when temperature increased above 23 °C (Fig. 6B). It was different from the report of Oliveira *et al*. (1999) that high temperature enhanced the production of lipids and carbohydrates in *Spirulena maxima* and *Spirulena platensis*. It

seems that the response of chemical composition in microalgae to temperature is species specific (Thompson *et al*., 1992).

Despite the study of *B. braunii* being conducted for decades, reports of temperature tolerance are rare. Lupi *et al*. (1991) found that the growth of *B. braunii* was up to 32 °C, and stabilised growth was not observed above 33 °C, showing that 32 °C was the maximum temperature for growth. Lupi *et al* (1991) also reported that a relatively high specific growth rate occurred at 20 $^{\circ}C$, but resulting in a very low production of the soluble exopolysaccharide (EPS). This study did not measure the production of EPS, but the range of temperature tolerance was similar to the previous study (Lupi *et al*. 1991). However, we noticed that the viscosity of cultivation under 30 °C was high because of production of exopolysaccharides (EPS). The increase of viscosity is an indication of the production of EPS (Vladislay *et al*., 1994; Suling *et al*., 2004). Although we did not quantify the EPS production, the increased viscosity of the medium was noticed in this study. The change of viscosity in *B. braunii* culture has been reported in other studies (Casadevall *et al*., 1985; Fernandes *et al*., 1989; Lupi *et al*., 1991).

2. 2. Light

2.2.1 Photoperiod

Photoperiod has been reported to affect the fatty acid production in marine microalgae (Ohta *et al*., 1993). A decade ago, Vladislay *et al*. (1994) reported that photoperiod could be one of the factors that triggered hydrocarbon production, but the impact of photoperiod on the growth of *B. braunii* is not clear yet. This study shows that no significant difference in algal growth between 12 and 24 h light daily ($P>0.05$), but a low photoperiod of 4 and 8 h light could not sustain the algal growth (Fig. 5). Algal cells under 24 h light daily showed brown colour at the end of cultivation compared with 12 h light. Therefore, we consider that the 12 h light and 12 h dark cycle is a better light regime for *B. braunii*, not only to stimulate algal growth in a long term, but also to run this project in an outdoor environment.

2.2.2 Light intensity

Chirac *et al.* (1985) reported that lower illumination could reduce hydrocarbon production. In this study, we found that China strain 1 could survive from 30 to 100 W/m^2. China strain 1 has a narrow range of light intensity for growth compared with the report by Vladislay *et al*. (1994) who found that 15 and 180 W/m^2 could stimulate growth with doubling time of 5 and 2.5 days, respectively. In this study, growth was inhibited when light intensity was less than 30 W/m^2 while photoinhibition occurred when light intensity was above 100 W/m^2. The lipid content decreased drastically when light intensity was above 100 W/m^2, not only due to low biomass, but also fewer hydrocarbon droplets. The optimal light intensity for China 1 strain was between 30 and 60 W/m^2 which was similar to other studies (Lagreau *et al.*, 1980; Casadevall *et al*., 1985).

In Australia, the average irradiance is 600-800 W/m^2. However, in the report by Roman *et al*. (2001), solar radiation above the water surface declined from 1380 to 3.57 W/m^2 for UV-A and from 13.1 to 0.026 W/m^2 for UV-B, respectively. UV-B radiation could not be detected at a depth greater than 0.25 m. Therefore, it is reasonable to believe that *B. braunii* can adapt to and grow well in the Australian environment.

2. 3. Salinity

B. braunii could not only survive in freshwater, but also adapt to large salinity variations (Derenne *et al*., 1992). At present, the highest salinity that *Botryococcus* sp. can survive is 3 M NaCl (Vázquez-Duhalt *et al*., 1991a). But *B. braunii* has shown a maximum growth in 0.25 M of NaCl media (Vázquez-Duhalt *et al.*, 1991b). In this study, the maximum biomass and lipid production were obtained in the 0.15 M NaCl medium. Although the greatest mean lipid content occurred in control, there was no different between them (Fig. 10B, $P<0.05$). This alga failed to grow if salinity was higher than 0.5 M of NaCl (Fig. 10A). High algal mortality occurred in 0.7 M of NaCl. But Ben-Amotz *et al*. (1985) found that low contents of protein, carbohydrates and pigments were found in cells grown in 0.5 M NaCl, while the lipid content in cells was higher than that in the cells grown without NaCl (cf. Vázquez-Duhalt and Arredondo-Vega, 1990). In Figure 11, the amount of intercellular particles and cell division were obviously reduced when salinity was up to 0.5 M NaCl. This agrees with the description by Vázquez-Duhalt *et al*. (1991a) that increased saline stress does not result in lipid accumulation inside the cells. The phenomenon of flocculation in this experiment might be due to the death of cells or to some liberation of substance from the cells for protection.

Our study showed that the level of salinity significantly influenced both algal growth and lipid content ($P<0.05$). A moderate increase in salinity (<0.25 M of NaCl) could stimulate algal growth and lipid content. A recent study has shown that the lipid composition, especially the polar lipids, can be changed by salinity (Vázquez-Duhalt *et al*., 1991a). Therefore, the salinity manipulation may be used as a tool to yield algal biomass containing desired lipid composition (Vázquez-Duhalt *et al*., 1991b).

2. 4. Alga growth

B. braunii is considered a slow-growing alga with production of valuable and energy-rich compounds (mostly hydrocarbon) (Vladislav *et al*., 1994). But recent studies indicated that the growth rate could be improved by manipulating culture conditions. Despite Swale (1968) reporting that generation time of *B. braunii* was about one week, the generation time could be reduced to 2 days in the laboratory conditions (Largeau *et al.*, 1980). In this study, the shortest average generation time was 3.18 ± 0.09 days in the temperature experiment, but the generation time was further reduced to 2.51 ± 0.05 days under 23°C and 60 W/m^2. In the salinity experiment, under the optimal culture condition (23 °C and 60 W/m^2 irradiance with 12 h daily light), the average generation time was 2.00 ± 0.02 days with 0.15 M NaCl.

In addition, our study showed that the lipid content depended on final biomass. Casadevall *et al.* (1985) claimed that hydrocarbon productivity varied during the growth cycle of *B. braunii* with maximum productivity during the exponential and early stationary stages of growth. Therefore, the biomass on exponential and early stationary stages is closely related to the lipid content.

Based on the data analyses under different ranges of temperature, light and salinity we intent to conclude that the optimal culture condition for maximum biomass of China strain 1 is to use the SE culture medium with 23 °C, 60 W/m^2 for 12 h daily light and 0.15 M of NaCl.

3. Future development

In Australia, the desert regions and some brackish waters could be attractive areas in which to locate microalgal-based biodiesel production facilities. This, in part, dictates the required strain characteristics. These characteristics included the ability of the algal strains to grow rapidly and have high lipid productivity when growing under fluctuating light intensity, temperature, and in saline waters indigenous to the area in which the commercial production facility is located.

In this report, we have documented our study on *B. braunii* (China strain 1). In addition, we have collected more strains from China, Japan and UK, but these strains are yet to be fully studied. Considering the production of biofuels from the current stain of *B. braunii* is not cost-effective at present, it is necessary to select strains with fast growth and high hydrocarbon production. For example, if the growth rate of *Botryococcus* sp could be increased to the values obtained from *Chlorella* and *Scenedesmus*, hydrocarbon yield could be 10-15 times higher (Reinhard Vachofen, 1982). The knowledge of the biochemistry and physiology of lipid synthesis, combined with basic studies on microalgal molecular biology and genetic engineering to develop *B. braunii* strains with optimal properties of growth and lipid production, may lead to great improvement and enhance the commercial viability of this alga as an optimum hydrocarbon source.

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