www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18:1293-1302(2018) DOI: 10.4194/1303-2712-v18_11_06

Isolation, Identification and Growth Optimisation of Freshwater Microalgae

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Received 04 December 2017
Accepted 15 January 2018

Abstract

Microalgae have been widely used in aquaculture as feedstuff and feed additive. The aim of this research was to isolate and identify the microalgae derived from a pool used to breed soft-shell turtles. Therefore, the biomass and total sugar content under different light and temperature conditions were investigated. Using morphological and molecular identification tools, the microalgae were identified as *Botryococcus* sp. The optimal culture conditions in terms of both the growth and the total sugar content of *Botryococcus* sp. were a culture temperature of 28℃ and an illumination intensity of 65 μmol m^{-2} s^{-1} in BG11 medium; in its stable stage, the biomass of *Botryococcus* sp. could reach 0.86 g/L and its total sugar content, 35.6% (dry weight). Total sugar accumulation was positively correlated with the activities of glucose phosphate isomerase and hexokinase, and *Botryococcus* polysaccharide was composed of L-(+)-rhamnose, D-ribose, D-arabinose, D-xylose, 2-deoxy-D-glucose, D-mannose, D-glucose, D-galactose and N-Acetyl-D-glucosamine.

Keywords: Freshwater microalgae, identification, optimization of growth conditions, polysaccharide synthase, monosaccharide composition.

Introduction

Microalgae (including prokaryotic photosynthetic microorganisms such as cyanobacteria), as the primary producers at the base of most aquatic food chains, are fed to cultures of many aquatic animals like fish, shrimps and molluscs, especially to their early stages. In some coastal environments, the biomass of microphytobenthos can match or even exceed the biomass of bacteria. The use of microalgae in biotechnology has increased in recent years, these organisms being employed in food, cosmetic, aquaculture and pharmaceutical industries (Moreno, 2008). If microalgae are to be suitable for utilisation in aquaculture they should possess certain important characteristics, such as (a) appropriate nutritional value, (b) suitable shape and size for ingestion, (c) a digestible cell wall, making the constituents available, (d) high production rates, (e) suitability for mass cultivation, (f) resistance to fluctuating growth conditions and, of course, (g) non-toxicity. In recent years, microalgae have been widely used in aquaculture as feedstuff and feed additive (Borowitzka, 1997; Spolaore, Cassan, Duran, & Isambert, 2006). Especially in fish farming, microalgae are mainly used as feed for rotifers grown as food for precious fish in the first stage after hatching, but they can also be used directly as fish feed. For instance, a mix of *Chlorella* and rotifers can be fed to *Takifugu rubripes* and other economic fishes in the larval stage (Zhu, Yan, & Chen, 2012; Ma *et al.*, 2014).

The main applications of microalgae for aquaculture are related to their nutritional value, in terms of constituents such as polysaccharides, essential fatty acids, proteins, vitamins and so on. As a bioactive ingredient, polysaccharides are very important for the use of microalgae as feed; e.g. the sulphated polysaccharides isolated from *Porphyridium* have antioxidant effects (Spitz, Bergman, Moppes, Grossman, & Arad, 2005), and the polysaccharides separated and purified from *Chlorella pyrenoidosa* can improve non-specific immunity in mice (Yang, Ying, Sheng, & Hu, 2006). Although polysaccharide profiles are species- and strain-specific, their properties are influenced greatly by environmental conditions. Thus, the specific objectives of this study are (1) the isolation and evaluation of the microalgae based on their morphology, 18S rDNA gene and ITS (Internal

transcriptional spacer) gene analysis; (2) to study the potential of microalgae promising for their biomass and total sugar accumulation in different media, and at different light intensities and temperatures; (3) to analyse the correlation between total sugar accumulation and the activity of polysaccharide synthase, and the monosaccharide composition of the polysaccharides in these microalgae.

Materials and Methods

Sampling of the Microalgae

The microalgae used in our experiments were obtained from Huadan Agricultural Products Co., Ltd., Hangzhou, China. The microalgae were isolated by separating single algal cells from the mixed algae suspension using capillary pipettes and cultivating them in culture dishes. Microalgae that grew successfully were transferred into separate tubes for routine maintenance. After this direct isolation procedure, the remaining cell suspension was inoculated into tubes containing BG11 medium and incubated under fluorescent light with a photoperiod of 12 h before purifying it further using the dilution method (Su, 2015).

Purification and Identification

Purification was performed following the method described by Hutagalung (Hutagalung *et al.*, 2014), as follows. The cell suspension was initiated by diluting to appropriate concentration; aliquots of diluted cell suspension were added to 300 reaction tubes containing BG11 medium in order to purify and cultivate the algal cells of interest. This step was repeated until pure cultures of single cell isolates were obtained. The purified algae were then identified morphologically by their size, form and colour.

Molecular Identification of Algal Species

DNA was extracted from the algal samples using the CTAB method (Li, *et al.*, 2009). The PCR kit was purchased from Takara Bio (Dalian, China). The PCR system (50 µl) contained Premix Taq (25 µl), primer DNA (2 µl, 20 µM), extracted DNA (1 µl) and ddH_2O (22 µl). The ITS gene was amplified by PCR and sequenced using the following two universal primers: (1) (5'-GGAAGTAAAAGTCGTAACAAGG-3'), (2) (5'-TCCTCCGCTTATTGATATGC-3'). Diluted extracted DNA was used to amplify the 18S rRNA gene with two primers, a specific forward primer and a conserved reverse primer, namely 18 S-F (5'-ACCTGGTTGATCCTGCCAGTAG-3') (Medlin, Elwood, Stickel, & Sogin, 1988) and 18 S-R (5'-ACCTTGTTACGACTTCTCCTT- CCTC-3') (White, Bruns, Lee, & Taylor, 1990). Amplification was done by Shanghai Generay Biotechnology Co. Ltd. using these two pairs of primers. The PCR products were purified with the SK1131 kit and sequenced by Meiji Biotech Co. Ltd. (Shanghai, China). The resulting 18S rRNA gene sequences were aligned and a search was carried out for similar sequences with the Basic Local Alignment Search Tool in the GenBank database of the National Center for Biotechnology Information.

Cultivation

BG11 medium, SE medium (Yang, Fei, Peng, & Deng, 2010) and the basic culture medium ($NaNO_3$ 37.5 mg/L, K_2HPO_4 1 mg/L, citric acid 0.16 mg/L, Na_2CO_3 0.5 mg/L, Na_2EDTA 0.026 mg/L, $MgSO_4 \cdot 7H_2O$ 1.875 mg/L, $CaCl_2 \cdot 2H_2O$ 0.68 mg/L, $FeCl_3$ 0.14 mg/L) were used to cultivate the microalgae for screening. The microalgae were cultivated in a 500-ml flask filled with 300 ml of the medium at an ambient temperature of 28°C and illuminated with a light intensity of 65 µmol m^{-2} s^{-1}. The inoculum density was 0.223 g/L. The variation in biomass was measured using a linear relation between dry weight and light absorption, and the variation in total sugar was measured using the phenol sulphuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956).

Monosaccharide Composition of the Microalgal Polysaccharide

The main monosaccharide composition of the microalgal polysaccharide was determined by gas chromatography. Ten mg of sample was hydrolysed with 5 mL of 2 M trifluoroacetic acid at 110°C for 3 h, then 0.6 mL pyridine and 10 mg hydroxylamine hydrochloride were added to the sample. Subsequently, it was incubated at 90°C for 30 min, then cooled down and after adding 0.6 mL acetic oxide, it was incubated at 90°C for another 30 min. A membrane filter was used to filter the supernatant fluid. The gas chromatographic analysis employed the following programme: using an injection temperature of 245°C and a detector temperature of 275°C, the column temperature was programmed to increase from 150°C to 235°C at 10°C/min, and was held at 235°C for 6.5 min. The carrier gas used was nitrogen which was maintained at a flow rate of 1.0 mL/min. L-(+)-rhamnose, D-arabinose, D-galactose, D-glucose, D-mannose, D-xylose, D-ribose, 2-deoxy-D-glucose, L-fucose and N-Acetyl-D-glucosamine were used as standards (Zhu *et al.*, 2013).

Analysis of Polysaccharide Synthase in Microalgae

In this experiment, key enzymes for the accumulation of polysaccharide were selected, namely glucose phosphate isomerase (EC.5.3.1.9-PGI) and hexokinase (EC.2.7.1.1-HK).

Samples (20 mL) of microalgae, taken during the growth stage at 0, 4, 6, 8, 12 and 14 days, were washed with ddH_2O several times, then centrifugated

for 10 min at 3000 rpm. The sediment was collected and ground in liquid nitrogen for 10 min and ground again in an ice bath for another 10 min; an aliquot of 10 mL was then centrifugated again for 10 min at 3000 rpm. The supernatant was collected to determine the enzyme activity, and a curve was drawn of the changing enzyme activity at different growth stages (Herbert, Burkhard, & Schnarrenberger, 1979; Renz, Merlo, & Stitt, 1993).

Determination of Glucose Phosphate Isomerase

The different steps for the determination of GPI are shown in Table 1.

Each solution was added to the tube according to the order in Table 1 at 30°C, and the rate of absorption at 340 nm was measured every 30 s for 3 min. The enzyme's activity was calculated using the following formula (Herbert, *et al*., 1979):

$$\Delta A_{340\text{nm}/\text{min}} = \Delta A_{340\text{nmsample}/\text{min}} - \Delta A_{340\text{nmblank}/\text{min}}$$

$$\text{PGI activity (U/mg)} = \frac{\Delta A_{340\text{nm/min}}}{6.22 \times \text{mg PGI/mL reaction solution}}$$

Determination of Hexokinase

The different steps for the determination of hexokinase (HK) are shown in Table 2.

Each solution was added to the tube according to the order in Table 2 at 25°C; the rate of absorption was measured and the enzyme's activity was calculated as described in Section 2.6.1 (Renz, Merlo, & Stitt, 1993).

Statistical Analysis

Data were expressed as means ±standard deviations of six replicated determinations. T-test were applied for determining significant difference at $P<0.05$, $P<0.01$ and $P<0.001$ between the results.

Results

Morphological and Molecular Identification of the Microalgae

The microalgae were successfully isolated from miscellaneous other bacteria. In Figure 1, 1 and 2 are microalgae contaminated by bacteria and fungi, respectively, and 3 and 4 are microalgae with a green

Table 1. The determination steps of PGI activity

Step	Blank control tube (mL)	Sample tube (mL)
1. Potassium phosphate buffer solution (0.1 M pH6.8)	0.5	0.5
2. Magnesium chloride solution (0.05 M)	0.1	0.1
3. $NADP^+$ solution (4 mM)	0.04	0.04
4. glucose 6-phosphate dehydrogenase solution (40 U/ml)	0.1	0.1
5. Sample solution (pure water in the blank control tube)	0.16	0.16
6. fructose-6-phosphate solution (50 mM)	0.1	0.1

Table 2. The determination steps of hexokinase activity

Step	Blank control tube (mL)	Sample tube (mL)
1. triethanolamine buffe solution (0.1 M pH7.6)	1.20	1.10
2. enzyme substrate (10% β-D-glucose solution)	1.20	1.20
3. Magnesium chloride solution (0.1 M)	0.2	0.2
4. ATP (0.08 M)	0.1	0.1
5. $NADP^+$ solution (0.013 M)	0.2	0.2
6. glucose 6-phosphate dehydrogenase solution (15 U/ml)	0.1	0.1
7. Sample solution		0.1

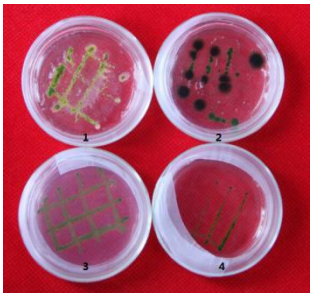

Figure1. The non-purified microalgae and the third-generation of microalgae streak culture.

colour and a single algae colony obtained after separation and purification. The morphological analyses showed that the green microalgae colonies clustered into a group and were composed of 2–20 cells with a round shape. The colonies ranged from 4 to 15 μm in diameter, depending on their growth phase. These microalgae had the distinct characteristics of *Botryococcus* sp. which is a family of *Choricystis, Trebouxiophyceae, Chlorophyta*.

To further verify the taxonomic position of the microalgae, molecular phylogenetic analyses were made; the results are shown in Figure 2, Figure 3, Figure 4. The partial 18S rRNA sequence from the microalgae was determined; the 18S rDNA and the

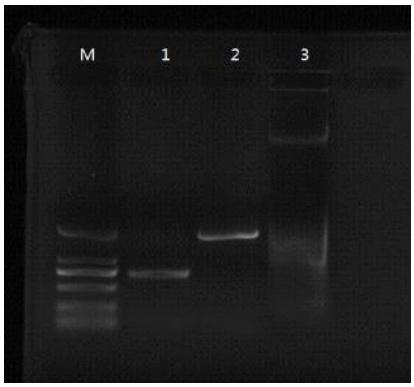

Figure2. Micro-algae PCR product and genome DNA.
Note: M: 2 kb DNA marker; 1: ITS rDNA PCR product; 2: 18S rDNA PCR product; 3: Micro-algae genome DNA.

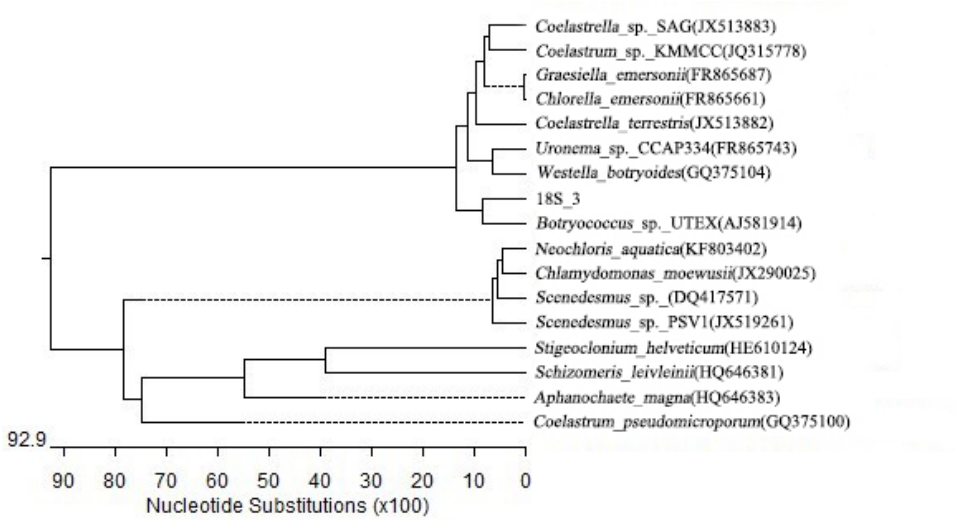

Figure3. Phylogram inferred from 18S rDNA partial gene sequences.

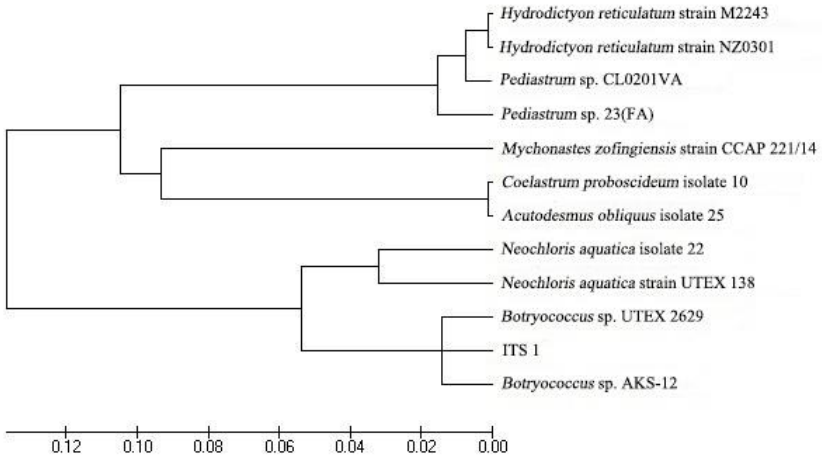

Figure4. Phylogram inferred from ITS rDNA partial gene sequences.

ITS sequences of the microalgae confirmed their identification as *Botryococcus* sp. The partial 18S rRNA sequence(1543b) and the ITS sequence(625b) from the microalgae were 98.82% and 96.45% identical to other *Botryococcus* species tested in NCBI using DNAMAN software, i.e. *Botryococcus* sp. UTEX 2629 (AJ581914.1) (Senousy, Gordon, & Ethan, 2004).

Growth properties of *Botryococcus* sp. in Different Culture Media

The growth of *Botryococcus* sp. was the fastest in BG11 medium, and the dry weight was highest at day 15, i.e. 0.92 g/L. The maximum dry weight of *Botryococcus* sp. was only 0.83 g/L and 0.56 g/L in SE medium and in basic culture medium, respectively, at days 3, 6, 9, 12 and 15 (Figure 5). Therefore, BG11 medium was the most suitable for cultivating *Botryococcus* sp.

Growth and Total Sugar Accumulation of *Botryococcus* sp. at Different Culturing Temperatures and Illumination Intensities

When the culture temperature was 28°C, the biomass of *Botryococcus* sp. was the highest at an illumination intensity of 65 μmol m^{-2} s^{-1}, namely 0.86 g/L, but its highest biomass was only 0.83 g/L and 0.56 g/L at illumination intensities of 43 μmol m^{-2} s^{-1} and 86 μmol m^{-2} s^{-1}, respectively (Figure 6). By the same token, when the culture temperature was set at 33°C, the biomass of *Botryococcus* sp. was again highest at an illumination intensity of 65 μmol m^{-2} s^{-1}, attaining 0.80 g/L. The highest biomass was only 0.70 g/L and 0.63 g/L at illumination intensities of 43 μmol m^{-2} s^{-1} and 86 μmol m^{-2} s^{-1} respectively (Figure 7). The biomass of *Botryococcus* sp. at 28°C was higher than at 33°C under the same light conditions, so the culture temperature of 28°C and an illumination intensity of 65 μmol m^{-2} s^{-1} are the most suitable for the growth of *Botryococcus* sp.

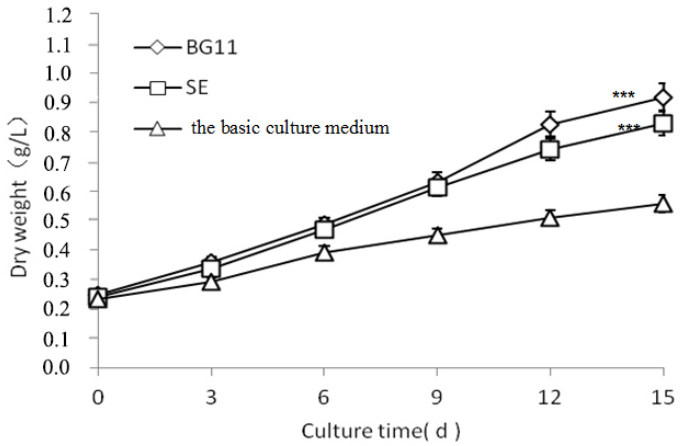

Figure 5. Biomass of *Botryococcus* sp. in three different culture media. Values represents mean ± S.D. n=6/group. *P<0.05; **P<0.01, ***P<0.001 compared to the basic culture medium.

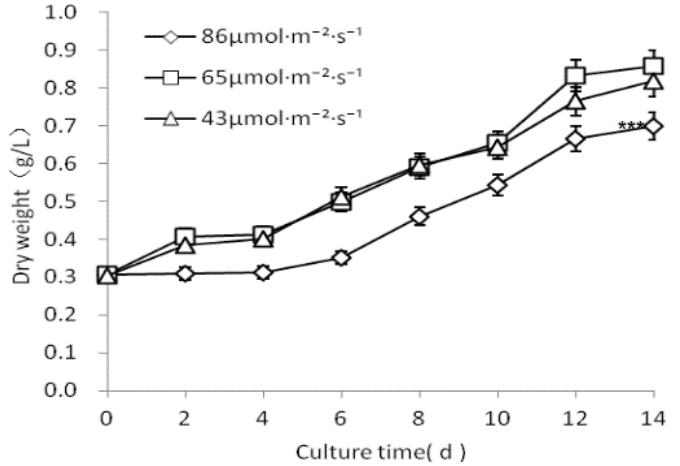

Figure6. Biomass of *Botryococcus* sp. under different illumination intensity in 28°C .Values represents mean±S.D. n=6/group.*P<0.05;**P<0.01, ***P<0.001 compared to 65 μmol m^{-2} s^{-1}.

The total sugar content of *Botryococcus* sp. was the highest at these optimal culturing conditions, namely 32.7% (0.28 g/L). It reached only 21.7% (0.18 g/L) and 17.8% (0.10 g/L) at illumination intensities of 43 and 86 μmol m^{-2} s^{-1}, respectively (Figure 8). Similarly, when the culture temperature was 33°C, the total sugar content of *Botryococcus* sp. was also highest at an illumination intensity of 65 μmol m^{-2} s^{-1}, namely 35.6% (0.28 g/L), while it only reached 22.2% (0.15 g/L) and 20.6% (0.13 g/L) at illumination intensities of 43 and 86 μmol m^{-2} s^{-1}, respectively (Figure 9). The total sugar content of *Botryococcus* sp. at 28°C was as much as at 33°C at an illumination intensity of 65 μmol m^{-2} s^{-1}, while its biomass at 28°C was higher. Thus, a temperature of 28°C and an illumination intensity of 65 μmol m^{-2} s^{-1} are the optimal culture conditions for both the growth and the total sugar content of *Botryococcus* sp.

Analysis of the Monosaccharide Composition of *Botryococcus* Polysaccharide

The retention times for the standard monosaccharide mix are shown in Table 3, and the gas chromatograms of the sample are shown in Figure 10.

Based on the standard equation for mixed monosaccharides, *Botryococcus* polysaccharides are composed of L-(+)-rhamnose, D-ribose, D-arabinose, D-xylose, 2-deoxy-D-glucose, D-mannose, D-glucose, D-galactose and N-Acetyl-D-glucosamine. Their approximate mole ratio was rhamnose: ribose: arabinose: xylose: deoxyglucose: mannose: glucose: galactose: glucosamine = 0.26: 0.62: 0.21: 0.11: 0.08: 0.18: 1.00: 0.42: 0.17.

Correlation Between the Total Sugar Content and the Activity of Polysaccharide Synthase in Microalgae

The biomass and total sugar content correlation curves of *Botryococcus* and the activity of GPI and HK at its different growth stages are shown in Figures 11 and 12, respectively.

As shown in Figure 11, the increases in biomass and total sugar content of *Botryococcus* can basically be displayed as S-shaped growth curves. In the early growth phase, the biomass increases slowly, i.e. from

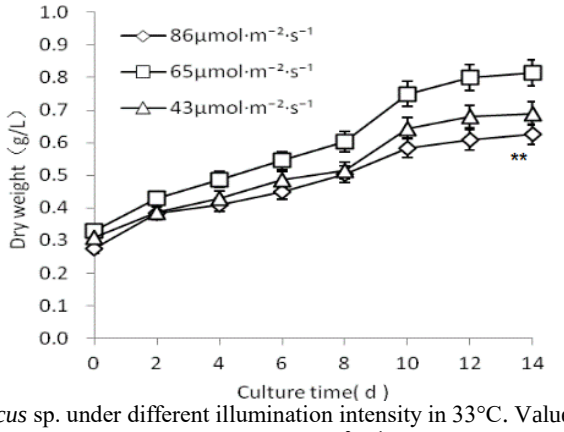

Figure 7. Biomass of *Botryococcus* sp. under different illumination intensity in 33°C. Values represents mean±S.D. n=6/group.*P<0.05;**P<0.01, ***P<0.001 compared to 65 μmol m^{-2} s^{-1}.

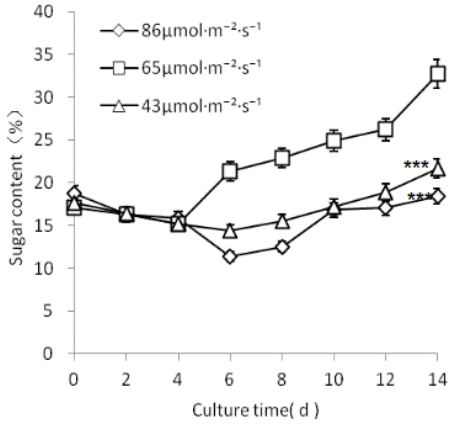

Figure 8. The total sugar content of *Botryococcus* sp. under different illumination intensity in 28°C.Values represents mean±S.D. n=6/group.*P<0.05; **P<0.01, ***P<0.001 compared to 65 μmol m^{-2} s^{-1}.

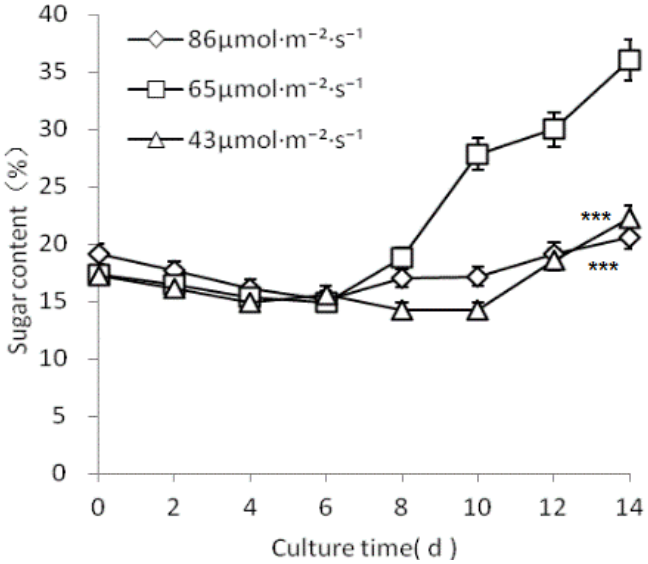

Figure 9. The total sugar content of *Botryococcus* sp. under different illumination intensity in 33°C. Values represents mean±S.D. n=6/group. *P<0.05; **P<0.01,***P<0.001 compared to 65 μmol m^{-2} s^{-1}.

Table 3. Mixed monosaccharide standard equation of regression

Monosaccharides	Reserve time/min	Equation of regression	R^2
L-(+)-Rhamnose	10.90	y = 188842x - 14979	0.9433
D-ribose	11.01	y = 159094x - 45984	0.9747
D-arabinose	11.23	y = 218098x - 16460	0.7319
2-Deoxy-D-glucose	12.96	y = 102706x - 800.47	0.9846
D-mannose	13.85	y = 162696x - 8209.9	0.9165
D-glucose	14.03	y = 139417x - 4995	0.8942
D-galactose	14.36	y = 120725x - 2441.2	0.9141
AC-D-N-amino glucose	16.15	y = 31336x - 382.78	0.9155
L-fucose	11.15	y = 171384x - 36853	0.9865
D-xylose	11.44	y = 155605x - 6357.2	0.9263

Note: Y refers to peak area; x is the concentration of monosaccharide mmol/mL

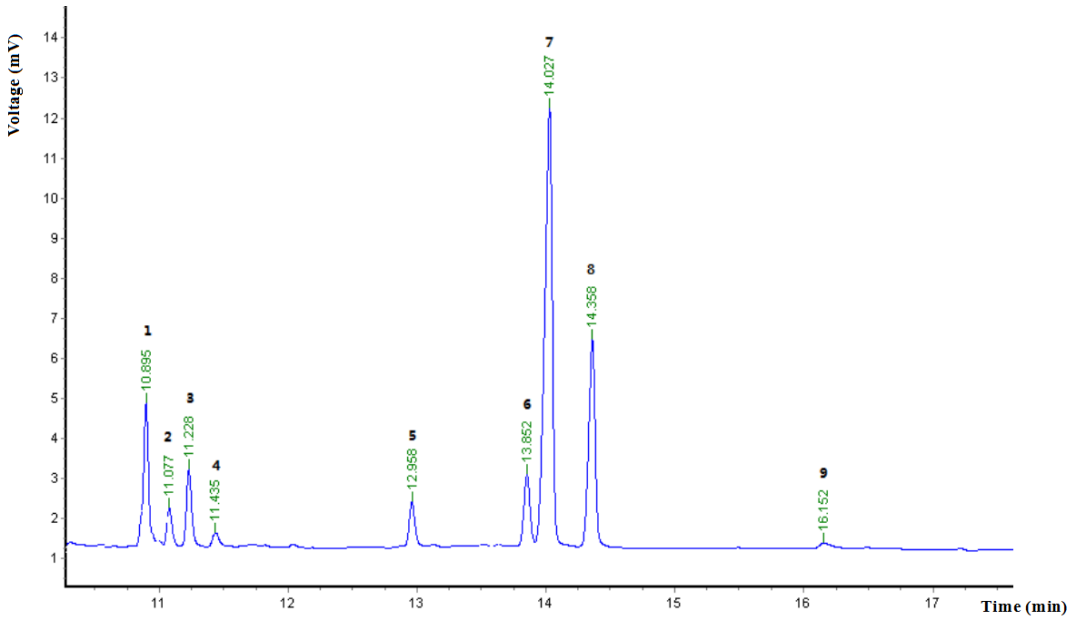

Figure 10. Gas chromatogram of the monosaccharide composition of *Botryococcus* polysaccharides.

a start of 0.2 g/L to 0.26 g/L on the fourth day. From the fifth day onward, the biomass increases rapidly, reaching 0.64 g/L on day eight. After that, the biomass increases more slowly again, and at the end of the experiment, the biomass had increased to 0.78 g/L. From the beginning of the experiment to the second day, the total sugar content showed a slight decline, and then it began to rise again slowly. From day four to day ten, the total sugar content increases rapidly, from 17.3% to 29.7%. At the end of the experiment, the total sugar content had reached 31.54%.

Figure 12 shows that the trend in the change of GPI and HK's enzyme activity is basically similar. The enzyme activity of GPI and HK at first increases and then decreases, the highest values being found on day eight. The change is more pronounced for GPI: its enzyme activity increases slowly at first, from a start of 0.69 U/g to 0.86 U/g on day four, rising rapidly to 1.49 U/g on day eight and then decreasing rapidly to 0.87 U/g at the end of the experiment. The enzyme activity of HK displays a steady upward trend from the beginning of the experiment to day eight, from 0.26 U/g to 0.68 U/g. After that, the enzyme activity of HK decreases rapidly, to 0.78 U/g at the end of the experiment.

Discussions

Microalgae are at the base of the entire aquatic food chain, and can also be used directly as fish feed, supporting the production of renewable fishery resources. Besides providing nutrients, microalgae may potentially be used for the treatment of waste effluents (Cantrell, Ducey, Ro, & Hunt, 2008). Thus, our recent studies have focused on microalgae from a pool for breeding soft-shell turtles; they were successfully isolated and the optimal conditions for maximising their biomass and total sugar yield were identified. Based on morphological and molecular identification criteria, these microalgae were shown to have the distinct characteristics of *Botryococcus* sp. It should be noted, however, that when this isolate was cross-checked with the reference database, the similarity uncovered by the query was only 98.82%. In order to ensure precise identification, a query coverage of minimally 99% should be reached.

Culture media were screened to select the optimal content of elements and their ratio in the medium. Studies have indicated that the amount of

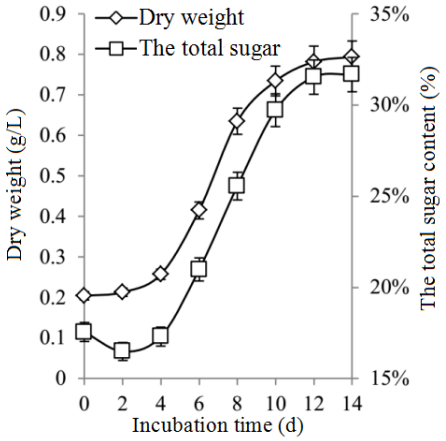

Figure 11. The correlation curves of *Botryococcus* in biomass and the total sugar content.

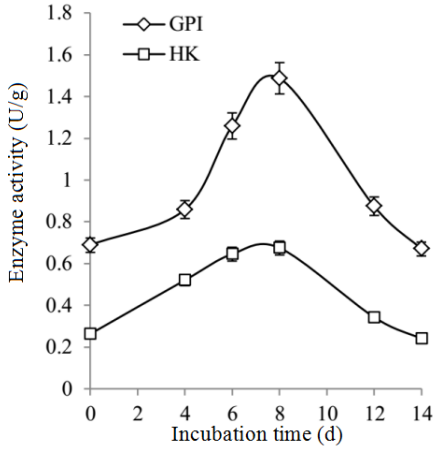

Figure 12. The enzyme activities in different growth stages of *Botryococcus*.

Cu, Mo, B and other trace elements in the medium influences the growth and accumulation of material in *Dunaliella* (Guo & Yang, 2013; Yang and Guo, 2013a, b). In our study, the basic culture medium lacked these trace elements, so the biomass accumulated by *Botryococcus* sp. was lower than in the other media tested. BG11 culture medium is more suitable for the growth of *Botryococcus* sp., because it contains 36 mg/L of Ca^{2+} in addition to other trace elements. Zhao, Ding, Lu, and Li (2014) also found that specifically the Ca^{2+} content determined the growth of *Scenedesmus obliquus*.

Temperature and light are important factors affecting the growth of microalgae. The temperature can change the photosynthetic and respiration intensity of microalgae by affecting the activity of the related enzymes. While light energy is the driving force behind the photochemical reaction in the process of photosynthesis, high light intensities can only be sustained when the generated heat can be dissipated and the energy generated by low light intensities is not enough to drive the photosynthesis (Zhang, Zeng, Wang, Wu, & Ren, 2007). In order to obtain higher biomass and polysaccharide yields, we experimentally determined the optimal temperature and illumination conditions for the cultivation of *Botryococcus* sp. The results showed that the most suitable light intensity was 3000 lux, and the optimum growth temperature was 28°C. At the same time, the total sugar content was found in general to show a trend to decrease at first and then increase again, the upward trend appearing from days six to eight. It can be speculated that the total sugar in *Botryococcus* sp. was consumed in the first stage while it adapted to the growth conditions, only starting to synthesise more sugar after entering the fast growing period.

GPI and HK were selected to study the relation between enzyme activity and the synthesis of sugar. GPI and HK are ubiquitous in plants, and are important for the processes of sugar biosynthesis and metabolism. In this experiment, the enzyme activities of GPI and HK were highest from day six to day ten, and were correlated with the accumulation of sugar and biomass. The correlation coefficient (R^2) of the total sugar content of *Botryococcus* and GPI was 0.9759, and for HK, R^2 was 0.9188. It can be concluded that GPI and HK are key enzymes in the synthesis of sugar in *Botryococcus*.

Our study of *Botryococcus* shows that it can efficiently harvest the energy of light available in the water in aquaculture and provide a new way to make fish breeding profitable.

Conclusions

As primary producers, microalgae, which have high photosynthetic efficiency, are at the base of most aquatic food chains. A species of microalgae was successfully isolated and, using the 18S ribosomal RNA gene and the internal transcribed spacer, identified from a soft-shell turtle breeding pool, namely *Botryococcus* sp. The optimal culture conditions for *Botryococcus* sp. in terms of both growth rate and total sugar content are a culture temperature of 28°C and an illumination intensity of 65 μmol m^{-2} s^{-1} in BG11 medium. In the stable stage, the biomass of *Botryococcus* sp. may attain 0.86 g/L and its total sugar content can reach 35.6% (dry weight). *Botryococcus* polysaccharide is a heteropolysaccharide composed of L-(+)-rhamnose, D-ribose, D-arabinose, D-xylose, 2-deoxy-D-glucose, D-mannose, D-glucose, D-galactose and N-Acetyl-D-glucosamine, at a mole ratio of approximately 0.26: 0.62: 0.21: 0.11: 0.08: 0.18: 1.00: 0.42: 0.17. The accumulation of total sugar is positively correlated with the activities of GPI and HK, and the correlation coefficient (R^2) of the total sugar content and GPI was 0.9759, and for HK, 0.9188.

Acknowledgements

This work was supported by financial support of the programme for the 'Twelfth Five-Year' National Science and Technology Project in Rural Areas (No. 2013BAD10B02), and a grant from the Key Laboratory of Marine Food Quality and Hazard Controlling Technology of Zhejiang Province.

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