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Hydrocarbon Production by the Yayoi, a New Strain of the Green Microalga *Botryococcus braunii*

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ABSTRACT

The Yayoi, a new strain of the green colonial microalga *Botryococcus braunii*, was cultured with aeration of 2% CO_2-enriched air. The final algal concentration on the basis of dry weight reached 1.9 g/L after 45 d with the minimum biomass doubling time of 3.5 d. Hydrocarbon content ranged from 30.9–40.5% on dry weight basis and was higher in the early culture stages. Hydrocarbon composition and its variation during the culture were typical of the B race of *B. braunii*. Relative percentages of lower botryococcenes, such as C_{30} or C_{32}, increased slightly at the middle stages and then declined with increment of C_{34} components. A C_{34} botryococcene was dominant throughout the culture period.

Index Entries: Green microalga; *Botryococcus braunii*; the Yayoi strain; biomass doubling time; hydrocarbon composition; botryococcenes.

INTRODUCTION

Combustion of fossil fuels has caused various problems to the environment. The greenhouse effect by the CO_2 emission is said to be especially serious, and it is clear that environmentally and economically feasible alternatives to fossil fuels must be identified and developed. One possibility is exploitation of renewable energy sources in which CO_2 is immobilized by the aid of solar energy.

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Botryococcus braunii is a colonial green microalga that produces unusually large amounts of hydrocarbons *(1)*. In this alga, there are at least three races, A, B, and L, which are characterized by distinct types of hydrocarbons. The A race produces linear hydrocarbons, alkadienes, and alkatrienes, of which content is usually 5–20% of the algal dry weight *(2)*. The L race produces a tetraterpene called lycopadiene whose content is <3% *(3)*. The B race produces triterpenoids called botryococcenes *(2,4)*, and their content usually ranges from 25–40% *(2)*. A very large-scale blooming of the B race often happens in the Darwin River Reservoir in Australia *(5)*, and some deposits called Coorongite or Torbanite, which were attributed to fossil *Botryococcus* have been detected *(6,7)*. These suggest the possibility of liquid fuel production by a large-scale culture of the B race of *B. braunii*.

We have recently isolated a few strains of the B race from Japanese waters, and one of them, the Yayoi strain, is most promising from a viewpoint of hydrocarbon content *(8)*. In this article, we describe the growth and hydrocarbon production of the Yayoi strain.

MATERIALS AND METHODS

Algal Strain

The Yayoi strain of *B. braunii* was isolated from a culture tank for water flea at Yayoi Campus, The University of Tokyo. The procedure of isolation was described elsewhere *(8)*.

Culture Conditions

Maintenance cultures were grown at 25°C in 1.5-L Roux flasks containing a modified Chu13 medium *(2,9)* by aerating with filter-sterilized air containing 2% CO_2 at a rate of 150 mL/min under illumination with cool-white fluorescent light of 240 μmol photon m^{-2}/s on a 12 light:12 dark photoperiod. Algal samples were cultured in triplicate for determination of growth curve by inoculating 150 mL of the 50-d maintenance culture (1.30 g/L freeze-dried algal weight) into 1000 mL of newly prepared medium under the same conditions.

Growth Curve

The growth was monitored by measuring freeze-dried algal weight at several stages of culture period: An aliquot (10 mL) from each flask was subjected to sucking filtration on a Whatman GF/C filter, which was previously weighed after desiccating by a vacuum pump for 1 h. The filtrated alga was washed with 10 mL of distilled water three times to remove extraneous mucous matter. The alga together with the filter was freeze-dried and further desiccated by a vacuum pump for 1 h to determine net dry weight of the alga. The biomass doubling time was determined according to the Sorokin's method *(10)*.

Hydrocarbon Analysis

The algal samples collected to monitor the growth were also used for analyses of hydrocarbon content and composition. The alga-sticking filter was cut into small pieces and extracted by sonication with 10 mL of acetone to which a known amount of squalene was added as an internal standard. The extract was centrifuged at 1000 *g* at 15°C for 10 min. The precipitate was repeatedly extracted with acetone until the supernatant became colorless. The supernatants were combined and concentrated under reduced pressure. The residue was dissolved in *n*-hexane and subjected to column chromatography on Wakogel C-300 (5 × 1 cm). Hydrocarbons were completely eluted with 20 mL of *n*-hexane. They were analyzed by gas chromatography (GC) and GC-electron impact mass spectrometry (GC-EIMS) using a Shimadzu 9A gas chromatograph with an FID detector and a Shimadzu QP1100 mass spectrometer with a Shimadzu 14B gas chromatograph, respectively. Both GC and GC-EIMS were carried out using a fused silica capillary column (DB-1, 60 m × 0.25 mm, film thickness 0.25 mm). The column temperature was held at 200°C for 1 min after injection and raised to 250°C at a rate of 8°C/min, and then to 280°C at a rate of 0.6°C/min. Individual hydrocarbons were identified by direct GC-MS analyses and/or comparison of retention times with those of authentic botryococcenes. Total hydrocarbon content and relative percentages of individual hydrocarbons were determined by comparison of peak areas with that of the internal standard.

RESULTS

Growth Curve

The growth curve of the Yayoi strain is shown in Fig. 1. A lag phase was observed from time 0 to d 3, and the growth decelerated after d 24. The final algal concentration reached 1.9 g/L. A minimum mass doubling time of 3.5 d was obtained during d 3–6. Initially, the color of the culture solution was brilliant green, but finally it changed to moss green. The culture solution became viscous as the culture time proceeded owing to the release of mucous matter from the cells.

Hydrocarbon Content

The variation of hydrocarbon production is also shown in Fig. 1. The hydrocarbon production changed in a similar tendency to algal dry biomass. The initial hydrocarbon content was 36.5% of the algal dry matter. It reached a maximum of 40.5% at d 3 and then gradually declined to 30.9% at d 45. The hydrocarbon production also decelerated after d 24.

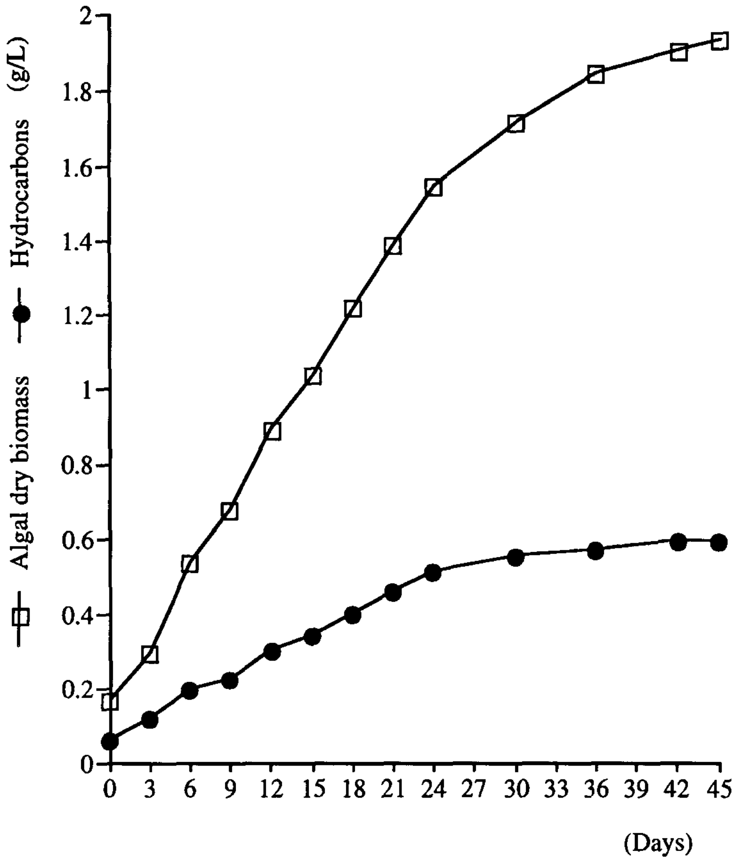

Fig. 1. Growth curve and hydrocarbon production of the Yayoi strain.

Hydrocarbon Composition

Nine main peaks were observed in gas chromatograms of the hydrocarbons in every stage examined during the culture period. Each peak was composed of a single hydrocarbon, except one peak in which a C_{31} and a C_{33} botryococcenes were coeluted. Therefore, the Yayoi strain contained at least 10 hydrocarbons, namely one C_{30} **1**, two C_{31} **2**, **3**, one C_{32} **9**, four C_{33} **4**, **6**, **7**, **10**, and two C_{34} **5**, **8** botryococcenes. The hydrocarbons **1**, **5**, **6**, **8**, **9**, and **10** were isolated, but **2** and **3** were obtained as a mixture by HPLC, and their chemical structures were elucidated by combination of various spectrometries. The hydrocarbons **4** and **7** could not be isolated because of their limited amounts. The hydrocarbons **5** and **6** were found to be novel botryococcenes. Structural elucidation of them will be described elsewhere. Trace amounts of a cyclic C_{32} botryococcene called meijicoccene **11**, which was originally isolated from the Berkeley (Showa) strain *(11,12)*, and a squalene-related compound, such as tetramethylsqualene **12** *(13)*, were also detected at the initial and final stages of the culture period. Structures of known hydrocarbons from the Yayoi strain are shown in Fig. 2.

1
2*
3*
8
9
10
11
12

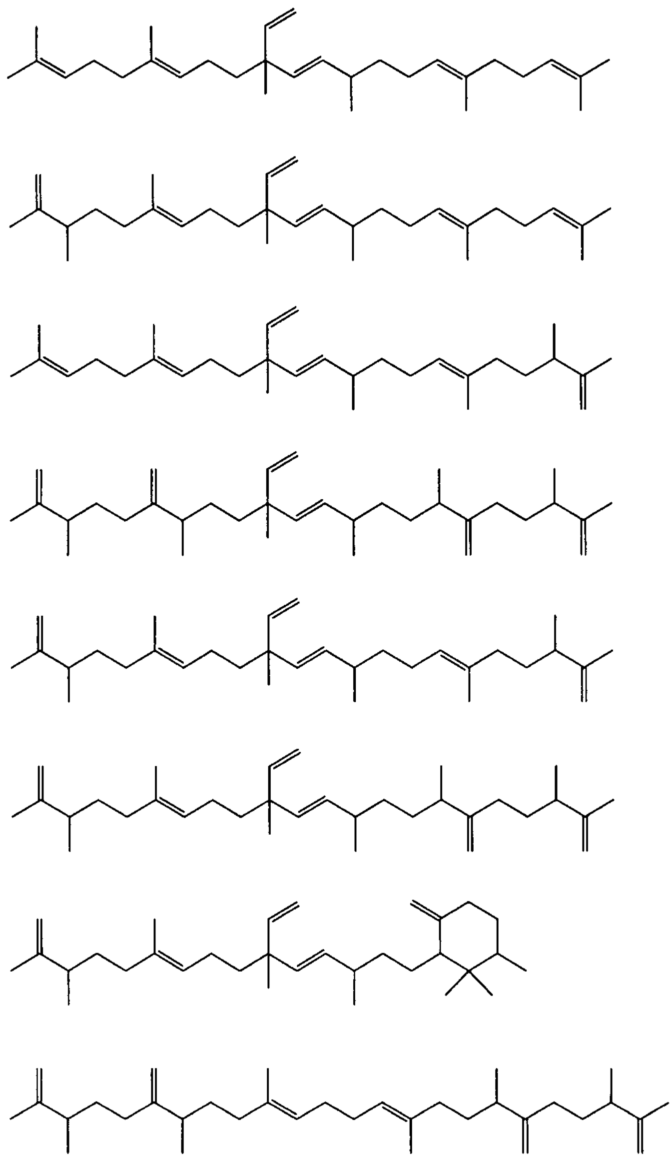

Fig. 2. Structures of identified hydrocarbons from the Yayoi strain. The compound numbers with asterisks may be interchanged.

Relative percentages of the hydrocarbons at various stages are shown in Table 1. Throughout the culture period, **8** was predominating. The percentage of **8** declined to 68.6% with increase of **9** and **10** until d 21, and then gradually recovered to 75.9% with the decrease of **9** and **10** until d 45. The sum of C_{34} and C_{33} hydrocarbons occupied as high as 85.7–91.9% of total hydrocarbons. Proportions of lower botryococcenes, such as **1, 2,** or **9,** became higher at the middle period of the culture.

Table 1

GC and GC-EIMS Analyses of Hydrocarbons Extracted from the Yayoi Strain with Passage of the Culture Time

Compound No.	Relative retention time / squalene	Relative percentage													
		Time(days)	0	3	6	9	12	15	18	21	24	30	36	42	45
C_{30} (**1**)	0.768		0.27	0.35	0.43	0.51	0.46	0.38	0.38	2.06	1.63	1.37	1.37	0.84	0.82
C_{31} (**2**)	0.830		0.66	0.60	0.68	0.76	0.76	0.77	0.64	1.55	1.19	0.91	0.88	0.67	0.65
C_{31} (**3**)+C_{33}(**4**)	0.838		1.53	1.15	1.58	1.73	2.10	2.27	2.26	2.94	3.16	3.40	3.34	3.36	3.35
C_{34} (**5**)	0.862		6.16	6.01	5.90	5.94	5.85	5.84	5.72	5.62	6.22	6.18	5.94	6.24	6.42
C_{33} (**6**)	0.875		2.16	2.18	2.13	2.19	2.07	2.05	2.14	2.57	2.41	2.45	2.39	2.19	2.16
C_{33} (**7**)	0.880		0.48	0.51	0.53	0.54	0.62	0.70	0.59	0.76	0.59	0.47	0.54	0.50	0.49
C_{34} (**8**)	0.892		75.76	75.71	74.61	73.62	72.36	72.01	72.53	68.61	70.82	72.76	74.07	75.86	75.87
C_{32} (**9**)	0.901		9.15	9.51	9.82	10.07	10.51	10.52	10.36	10.66	9.24	8.21	7.49	6.64	6.59
C_{33} (**10**)	0.909		3.83	3.98	4.32	4.64	5.27	5.46	5.38	5.23	4.74	4.25	3.98	3.70	3.65
C_{32} (**11**)	0.922		trace*										trace*	trace*	trace*
C_{34} (**12**)	1.080		trace*										trace*	trace*	trace*

* <0.1%

DISCUSSION

As far as the hydrocarbon content is concerned, the Yayoi strain is comparable to other B race strains, but its growth is fairly slow. Generally, the growth of *B. braunii* has been found to be very slow in the static culture in contrast to other green microalgae. It is known, however, that the supply of CO_2-enriched air can enhance the growth of this alga. When 1% CO_2-enriched air was supplied, a strain of the A race showed the biomass doubling time of 2.3 d *(14)*, and the La Manzo strain belonging to the B race showed a doubling time of 2.2 d during the exponential growth phase *(2)*. The Berkeley strain, which is one of the B race, had a doubling time of 40 h when aerated with 0.3% CO_2-enriched air *(15)*. The Yayoi strain also showed no significant increase in biomass under a static condition like other strains, but its growth was slow even though 2% CO_2-enriched air was supplied.

The hydrocarbon composition of the Yayoi strain also reflected its slow growth. In this connection, the hydrocarbon composition of the B race is known to depend not only on genetic characters of the strains, but also on culture conditions. The alga cultured under growth-enhancing conditions has a tendency to produce low-molecular botryococcene homologs like C_{31} or C_{32}. The supply of CO_2-enriched air especially makes lower botryococcenes dominant *(16)*. The Berkeley strain which contained a C_{34} component (isobotryococcene) dominantly in the ambient air culture accumulated lower botryococcene homologs when CO_2-enriched air was supplied *(15)*. The Berkeley strain cultured in our laboratory under the same conditions as the Yayoi strain showed a hydrocarbon composition in which higher botryococcene homologs were dominant *(8)*. Therefore, the slow growth of the Yayoi strain in this experiment might be owing not to its genetic factors, but to the culture conditions, especially the supply of CO_2. The CO_2 concentration of 2% could be too high. Further studies are clearly needed on the effect of CO_2 concentration on the growth rate of the Yayoi strain.

There is a report on the growth of the UTEX strain of *B. braunii* to which various carbon sources were supplied *(17)*: The doubling time was reduced from over 1 wk to 2 d by addition of D-mannose as a carbon source. Thus, it should be necessary to survey suitable carbon sources to enhance the growth of the Yayoi strain.

In the Yayoi strain, the proportion of lower botryococcenes in the whole hydrocarbons became larger at first and then declined. This suggests that higher botryococcenes were synthesized by methylation of lower homologs similarly to other B races *(16,18)*. The hydrocarbon content of the Yayoi strain was high in the early stages of the culture and then decreased like other strains. Therefore, it may be said that no significant differences exist in the hydrocarbon production between the Yayoi strain and other B race strains. The deceleration of hydrocarbon production in the later part of the culture period observed in the Yayoi strain may be counterbalanced by the production of the mucous matter. Therefore, this strain should be harvested at the end of the linear growth phase for an efficient hydrocarbon production, because filtration of biomass becomes harder by increase of viscosity of culture solution when the alga reaches the stationary phase.

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