Phytochemistry, Vol. 24, No. 10, pp. 2305–2312, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

ALKADIENE- AND BOTRYOCOCCENE-PRODUCING RACES OF WILD STRAINS OF *BOTRYOCOCCUS BRAUNII*

P. METZGER*, C. BERKALOFF†, E. CASADEVALL and A. COUTE‡

Laboratoire de Chimie Bioorganique et Organique Physique, ERA CNRS 685, E.N.S.C.P. 11 Rue P. et M. Curie, 75231 Paris Cedex 05, France; †Laboratoire de Botanique-Cytophysiologie Végétale, LA CNRS 311, E.N.S. 24 Rue Lhomond, 75231 Paris Cedex 05, France; ‡Laboratoire de Cryptogamie, LA CNRS 257, Muséum National d'Histoire Naturelle, 12 Rue Buffon, 75231 Paris Cedex 05, France

(*Revised received* 26 *February* 1985)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; alga; wild strains; growth; botryococcenes; *n*-alkadienes and trienes; chemical variability.

Abstract—Samples of the green colonial alga *Botryococcus braunii*, collected from various localities, were grown in the laboratory and examined for their hydrocarbon content and morphology. Although few differences appeared between the ultrastructures of the samples, the nature of their hydrocarbons, which remains unchanged at any stage of growth, allows the distinction of two physiological races viz algae producing odd-numbered unbranched alkadienes and trienes (C_{25}–C_{31}) (the A race) and those producing polymethylated triterpenes C_nH_{2n-10} (C_{30}–C_{37}), the botryococcenes (the B race). In laboratory culture, the hydrocarbon content of these new strains is very high, from 30 to 60% of the dry biomass. For the two races the greatest hydrocarbon productivity takes place during the active growth phase. The important variability observed in botryococcene distribution could originate both from genetic and environmental factors.

INTRODUCTION

Owing to a noteworthy disposition to accumulate hydrocarbons, up to 76% of dry wt, the green colonial alga *Botryococcus braunii* fills an uncommon position among the plant kingdom [1–5]. Moreover, this alga is able to synthesize two types of hydrocarbons: *n*-alkadienes and trienes, odd-numbered from C_{25}–C_{31} and polymethylated triterpenes of general formula C_nH_{2n-10}, with $30 \leqslant n \leqslant 37$, termed botryococcenes.§

On the basis of observations carried out on samples harvested in nature, some authors suggested that these hydrocarbons could be successively produced at different stages of growth [2]. Unbranched linear hydrocarbons would be produced by green cells during active growth and botryococcenes would originate from orange resting state cells. (The orange aspect of the colonies result probably from a carotenoid accumulation [6], parallel to that of botryococcenes.) To date no experimental evidence has confirmed this assumption. In fact, it was observed that algae from collections‖ do not lead, in laboratory cultures, to the botryococcene-producing orange resting state. Whatever their culture conditions or their physiological state, they produce only alkadienes and trienes [7]. Thus it could be supposed that these collection strains had lost their natural disposition to synthesize botryococcenes. Accordingly the possible existence of two races of *Botryococcus*, each producing only one type of hydrocarbon was not postulated. Recently it was established from samples collected in Australian fresh water lakes [5] that there was not necessarily a close relationship between the colour of the colonies and the hydrocarbon chemical structure, orange coloration being associated in some cases with an alkadienic hydrocarbon content. However, the presence in other samples of low amounts of *n*-alkadienes and trienes jointly with botryococcenes [5] could support the idea that the same cells were able to change their metabolism from the production of one hydrocarbon type to the other. From light microscopy studies, it was also concluded [8, 9] that the colony size or the colour were not suitable criteria to distinguish between different species of *Botryococcus* as was formerly agreed. More recently an electron microscopy study especially focused on the outer wall organization [10] was performed on collection strains and wild samples of *B. braunii*. The outer walls were shown to exhibit two types of organization: either successive well-individualized walls or a rather compact matrix. However, although external walls are the main site of hydrocarbon biosynthesis and accumulation [11], attempts to correlate outer wall organization with hydrocarbon type failed.

The present study was undertaken from new strains grown in the laboratory in order to test, either the capability of one strain to produce the two types of hydrocarbons, or the existence of two *B. braunii* races differing in the type of hydrocarbons they synthesize.

*Author to whom correspondence should be addressed.

§'Botryococcene' was the name formerly given to the first discovered C_{34} compound of the series [1]. Taking into consideration the common structural features of all the afore-mentioned compounds we prefer to name botryococcenes all the hydrocarbons of this family.

‖All the strains available from culture collections—Cambridge (U.K.), Gottingen (F.R.G.), Austin (Texas, U.S.A.) and Thonon (France)—derive from the same sample, isolated by Droop in 1950 from Maddingley Bricks Pits (U.K.).

RESULTS AND DISCUSSION

Isolation and culture of wild algae

It has been reported many times that in fresh water lakes *B. braunii* can give rise to massive blooms [5, 12–14]. In fact their high hydrocarbon contents cause the colonies to float. While the propitious conditions for these blooms remain unknown, it appears that they often occur in recent water bodies like reservoirs or in ephemeral lakes [5].

When sampling was performed during blooms, enough biomass was obtained to perform directly hydrocarbon analysis, isolation of colonies and to bring them into culture. On the other hand, when colonies were in small numbers, relative to the whole phytoplankton of the sampling, only isolation and culture were carried out. In every case, cultures were achieved in two steps. Algae were firstly subjected to purification after growing on Petri dishes and then five to ten colonies were removed and inoculated separately in liquid medium. With three initial samples over ten: Sanguinet and Pareloup (France), Darwin (Australia), cultures failed. The failures encountered with algae originating from Sanguinet and Pareloup were ascribable to fungal contaminations which highly inhibited algal growth. (A study of the incidence of a fungus, *Chytridium marylandicum* [15], on *B. braunii* demonstrated that this fungus derives all the benefit of the close association, hydrocarbons providing nutrients suitable for its growth.) Scanning electron micrographs showed the infection of the Sanguinet sample by a fungus of undetermined species.

For the Australian sample the failure originated from an unicellular alga. Indeed, all isolates developing on an agar medium were contaminated by a green alga growing in close contact with the mucilage of the *B. braunii* colonies. Light microscopic observations showed that the former alga develops at some stage of its growth large spherical green cells *ca* 10 μm in diameter with a parietal chloroplast. When transferred into liquid medium, this alga supplants *B. braunii*. When Brown *et al.* [2] tried to grow botryococcene-producing algae from resting state colonies, they observed the development of large spherical green cells characterized by a very poor hydrocarbon content. These authors considered that they were dealing with a third physiological state of *B. braunii*. The present observations lead us to consider that Brown's isolates, like those from Darwin, were probably not unialgal and that the accompanying algae supplanted *Botryococcus*.

From the seven other collected samples the purification method led to unialgal cultures, with no fungal contaminant, but they were non-axenic. No antibiotic treatment was applied to obtain bacteria-free cultures, in order to discard possible irreversible perturbation in hydrocarbon biosynthesis. Moreover from a comparative study of alkadiene-producing algae (axenic strains and strains associated with bacteria) it was observed that the presence of various bacteria has little effect on hydrocarbon and total biomass yield [7]. Indeed the Morocco sample was the only one from which we isolated bacteria (two species) capable of degrading *n*-alkadienes and trienes. The resulting degradation is very fast when the culture medium is shaken and aerated. Growing the Morocco strain under anaerobic conditions (N_2–CO_2: 99-1) largely decreases this degradation. Attempts to produce axenic cultures from this strain are in progress.

It is important to emphasize that each initial culture arises not from a single cell but from a colony already present in the collected sample and which has increased in size on agar plates. Under these conditions, these new strains cannot be assimilated to clones.

Analyses of collected samples

Five of the ten collected samples, directly allowed a proximate hydrocarbon analysis. Generally, *B. braunii* colonies accounted for 70 to more than 90% of the biomass of the samples. Green colonies were highly predominant, with the exception of a West Indies sample (Paquemar-La Martinique) essentially containing orange colonies. Hydrocarbon levels (24–36% dry wt) were similar to previously quoted values [5], but they did not reach those recorded by Maxwell *et al.* for the Oakmere bloom, 76–86% dry wt [1].

For these samples taken as a whole, 21 botryococcenes, of general formula C_nH_{2n-10} with $31 \leqslant n \leqslant 37$, were identified by GC/MS (Table 1). Low levels of squalene were also detected in all samples but no alkadienes and trienes were observed. The absence of alkadiene and triene-producing populations in these samples, was fortuitous, since *Botryococcus* blooms yielding the latter hydrocarbons conjointly with botryococcenes have already been described [5].

As previously reported for some blooms in Australian fresh-water lakes [5], our results indicate an important variation in the number and relative abundance of botryococcenes in connection with the origin of the alga. For instance, the C_{36} compound *RR*, 1.175 preponderant in the Darwin sample is absent in the Sanguinet one; the latter contains a simple mixture of three botryococcenes absent in all other samples, one C_{31} and two C_{32} isomers. Our observations corroborate also the lack of relation between the colour of the colonies and the type of hydrocarbons produced [5].

Following over a 18 month period the composition of samples regularly collected in La Manzo (Martinique) continuously showed a very high hydrocarbon content (36–43% dry wt) Table 2. Variations in botryococcenes distribution were also observed. Although the same hydrocarbons are always present and the C_{34} compound, *RR*, 0.814, largely predominated, the percentage of C_{34}, *RR*, 0.870, and C_{33}, *RR*, 0.877, compounds fluctuated between 7.4–30.6% and 5.4–39.8%, respectively, while the combined C_{36} isomers varied between 4.7 and 20.5%. As shown later, the isolation from this lake of two distinct botryococcene-producing algal populations is consistent with such chemical variations.

Hydrocarbon analyses and growth of cultivated strains

GC/MS analyses of hydrocarbons were performed on several subcultures, each established from a single colony. The daughter subcultures grown from a given sample, afforded *ca* the same hydrocarbon composition, with the exception of La Manzo. As shown below, this latter sample consisted of at least of two strains (Table 3), exhibiting different botyococcene compositions.

Considering the type of hydrocarbons produced, the algae were separated into two categories: those yielding botryococcenes (Table 3) and those yielding unbranched alkadienes and trienes (Table 4). For the whole subcultures tested, no variations in the hydrocarbon type were

Table 1. GC/MS analysis of botryococcenes from collected *Botryococcus braunii* samples

C_nH_{2n-10} *n*	Relative retention/ squalene	Grographic origin and date of the sampling				
		Australia	French West Indies			France
		Darwin November 1981	Guadeloupe Chateaubrun May 1983	Martinique La Manzo May 1982	Martinique Paquemar May 1982	Sanguinet August 1983
31	0.770					19.1
34	0.775		2.4			
34	0.781			2.3	3.9	
33	0.799	0.8				
34	0.814	8.3	4.3	35.2	71.6	
34	0.847	19.0	8.1	4.3	8.8	
34	0.850			1.6	2.0	
32	0.870					17.6
34	0.870		29.8	7.4		
32	0.887					61.1
33	0.887		36.3	39.8		
34	0.894	2.4		1.6		
34	0.912	15.2				
34	0.963	3.4				
35	1.011	2.6				
36	1.131		4.7	2.7		
36	1.175	34.8	1.2	1.6	5.8	
36	1.219	0.9			5.0	
37	1.263	10.3	2.4	0.4		
37	1.277	0.4				
37	1.296	0.6				
n.i.*		1.2	10.7	3.1	2.9	2.2
% dry wt		29.2	24.1	35.8	28.2	34.5

*n.i.: Compounds not identified due to poor resolution of GC peaks; their R_ts are different from those of alkadienes and trienes.

Table 2. Evolutions of the botryococcene composition and content in *Botryococcus braunii* samples collected in a West Indian fresh water lake (La Martinique–La Manzo)

C_nH_{2n-10} *n*	Relative retention/ squalene	May 1982	January 1983	February 1983	March 1983	April 1983	June 1983	November 1983
34	0.781	2.3	3.8	4.2	3.8	3.8	2.5	3.2
34	0.814	35.2	43.6	38.3	31.1	34.0	25.8	34.4
34	0.847	4.3	2.8	1.8	1.1	1.3	1.9	2.1
34	0.850	1.6	1.4	1.2	—	1.3	1.2	1.1
34	0.870	7.4	18.5	24.0	30.6	23.7	21.4	22.7
33	0.887	39.8	9.5	5.4	7.1	6.4	31.4	14.9
34	0.894	1.6	3.8	4.2	4.4	3.2	1.9	5.7
36*		4.3	12.8	16.8	15.8	20.5	10.7	11.0
37*		0.4	1.9	1.8	2.7	3.2	1.9	1.1
n.i.†		3.1	1.9	2.3	3.3	2.6	1.2	3.8
% dry wt		35.8	38.4	36.7	43.2	38.8	41.3	39.8

*Data concern the total C_{36} and C_{37} isomers.
†See footnote Table 1.

observed, neither during growth nor during the resting state. Thus the hypothesis relating the type of hydrocarbons produced by *Botryococcus* to the physiological state of the cells is not confirmed. Therefore it must be accepted that two races of *B. braunii* exist, each synthesizing a well-defined type of hydrocarbons, whatever their physiological state.

Until recently [16; F. R. Wolf, personal communication], no cultures of botryococcene-producing algae were reported. From a biotechnological point of view, the

Table 3. GC/MS analyses of botryococcenes extracted from cultivated *Botryococcus braunii* strains* originating from the French West Indies

C_nH_{2n-10} n	Relative retention/ squalene	Geographic origin and date of the sampling: Martinique La Manzo May 1982 MLM1†	MLM2	Martinique Paquemar May 1982 MP1	Guadeloupe Chateaubrun May 1983 GC1
30	0.760		0.5		
34	0.781		5.2	1.9	4.0
34	0.814		66.5	35.5	66.7
34	0.847	3.1	7.5	14.0	10.0
34	0.850		1.4		2.0
34	0.870	10.9	0.7	39.3	3.3
33	0.887	85.9		9.3	2.7
35	1.011				0.6
36	1.109		3.8		2.7
36	1.131		3.8		4.7
36	1.175		5.3		
36	1.219		2.3		2.7
37	1.263		2.3		0.6
37	1.277		0.7		
% dry wt		32.0	37.6	38.1	37.2

*Under standard conditions (see Experimental); two month old cultures.
†Abbreviation of geographic origin.

Table 4. GC/MS analyses of alkadienes and trienes extracted from cultivated *Botryococcus braunii* strains* and comparison with a culture collection strain

C_nH_{2n-2} C_nH_{2n-4}	France (Morvan) Chaumeçon	Crescent	Lingoult	Morocco (Atlas) Oukaimden Culture 1†	Culture 2*	Culture collection Austin, Texas
$C_{23}H_{44}$	0.7	0.8	0.7	—	—	—
$C_{25}H_{48}$	2.6	2.0	1.6	—	—	1.0
$C_{27}H_{52}$	19.5	16.9	12.2	21.7	9.7	11.0
$C_{27}H_{50}$	2.2	1.2	4.6	2.9	1.1	—
$C_{29}H_{56}$	61.8	65.4	56.8	68.1	75.9	62.0
$C_{29}H_{54}$	3.7	2.4	11.7	4.3	6.3	1.0
$C_{31}H_{60}$	9.6	10.5	12.4	3.0	7.0	25.0
$C_{31}H_{58}$	—	0.8	—	—	—	—
% dry wt	61.0	40.1	32.4	42.3	19.2‡	20.0

*Air lift batch cultures: air–CO_2 (99:1).
†Culture 1: bubbled with N_2–CO_2 (99:1).
‡Shaking and aeration promote hydrocarbon degradation by contaminating bacteria.

culture of two races of *B. braunii* increases the potential use of these algae as renewable hydrocarbon sources. In this prospect the high hydrocarbon contents noticed for all the new cultivated strains are very promising (Tables 3 and 4).

It is well documented that many cosmopolitan plant species can exhibit some chemical variations in one or more classes of their constituents [17]. However for alkadiene-producing strains (A strains), the screening, up to now, has not shown important variation in the composition of hydrocarbon mixtures. The C_{29} compound is always preponderant in the four new strains,* as it was in the collection strains, whether the medium was aerated by

*The origin of the alkadienic wild strains, studied here, is restricted to two geographical areas, one in France (Morvan), the other in Morocco (Atlas). The existence of larger variations in hydrocarbon composition for strains collected in other countries cannot be excluded.

an air-lift system (1% CO_2) or not. In sharp contrast hydrocarbon levels were greatly affected by culture conditions; air supplied with 1% CO_2 increased the hydrocarbon contents from 5% in the unaerated conditions to 20–61% depending on the origin of the strain.

In contrast, botryococcene-producing strains (B strains) exhibited a large variation in the number and in the relative percentage of their hydrocarbons (Table 3). Among the botryococcenes identified in the samples collected from nature, 13 have been again obtained under our culture conditions. In addition to these, the $C_{30}H_{50}$ component [16] was also detected. Comparison of the data listed in Table 3, leads to the recognition of three different strains, MLM_1, MP_1 and MLM_2 (or GC_1), the latter two showing a rather similar hydrocarbon distribution. Repeated analyses of these strains during one year showed an almost invariant hydrocarbon composition when they were cultivated under standard conditions (unaerated cultures, see Experimental). The comparison of the hydrocarbons for natural and laboratory cultivated algae of a same strain is of interest in connection with the origin of the chemical variations. The average compositions of the hydrocarbon mixtures produced by strains MLM_1 and MLM_2 taken together, is very close to the composition of the hydrocarbon content of the mother sample (May 1982, Table 1). Each of these two strains synthesizes either the C_{33} compound, RR_t 0.887, (86% MLM_1 mixture) or the C_{34} compound, RR_t 0.814, (66.5% MLM_2 mixture), when these two hydrocarbons were in equal proportions in the mother sample. From this result we are inclined to consider that at least two different botryococcene-producing populations coexist in the La Manzo lake, exhibiting different predominance periods.

In addition, for a given strain, the composition of the botryococcene mixture is sensitive to the growth conditions. While the MLM_1 strain produced, under standard conditions, essentially a C_{33} hydrocarbon (Table 3), it disappeared almost completely and was replaced by C_{30}, when the culture medium was aerated by air supplied with 1% CO_2. Thus it appears that at least two factors, genetical and physicochemical, are responsible for the chemical variations observed for the B race.

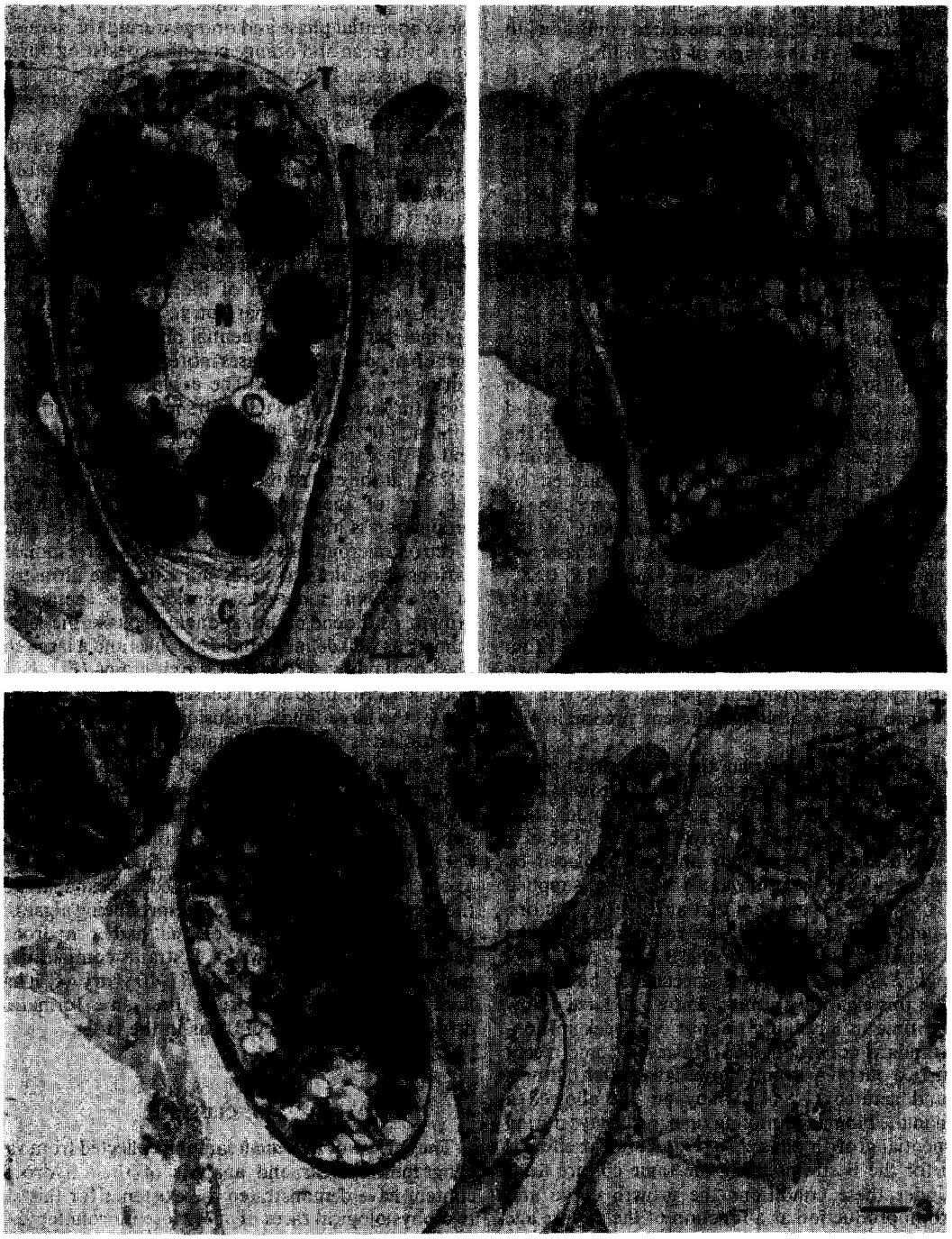
Although *B. braunii* develops spectacular blooms in some lakes, this alga was formerly known to have a slow generation time in laboratory, about one week in non-aerated standard conditions [6]. However, some recent studies carried out on A strains showed that batch cultures shaken and aerated (1% CO_2) by air lift allowed a reduction in the biomass doubling time to 2.3 days during the exponential growth phase [18, 19]. A similar effect is observed for the B strains using the same culture technique. Under these conditions the growth curve and hydrocarbon production as a function of the culture age were set up. Three phases can be distinguished on the growth curve (Fig. 4 La Manzo strain). An exponential growth phase up to the seventh day; the mean doubling time in biomass is 2.2 days, a linear phase, due to the progressive decrease in nutrient level, up to 13 days and a deceleration phase up to 33 days. The slow increase in biomass during this period is accompanied by an increase in medium viscosity. Such a phenomenon has also been noticed for one A strain [19]; it can be related to the dissolution in the medium of fibrillar exocellular polysaccharides revealed when colonies are observed by transmission electron microscopy [10]. Biomass decrease was not initiated after one month of culture.

Colour of the colonies appeared closely related to the physiological state of the algae; exclusively green during the exponential phase and orange during the deceleration one with green and orange colonies coexisting during the linear phase. Inoculating orange colonies in a fresh medium results in recovery of the green pigmentation within 24 to 48 hr.

Comparison of the variation of total biomass and of its hydrocarbon content as a function of the culture age shows that hydrocarbons are produced essentially during the first two phases, a very low production being observed along the deceleration. Semi-logarithmic representation of the biomass and the hydrocarbons is more expressive (Fig. 5). The parallelism of the straight lines until the seventh day indicates that botryococcene productivity is optimal during the exponential phase. Thereafter hydrocarbon production increases more slowly and finally stops during the third phase. The evolution of hydrocarbon levels (in percent dry wt, Fig. 4) shows also that the highest content, 40% in the culture conditions, is reached at the end of the exponential phase. This level decreases slowly to 25% at the end of the culture revealing that as soon as the end of the exponential phase, the hydrocarbon production is lower than that of other cell constituents. Electron micrographs of some samples corresponding to various physiological states show that the ultrastructure of *B. braunii* undergoes considerable change during growth. At the end of the exponential phase, the cells show numerous well defined lipidic globules and a large chloroplast containing some small starch grains (Fig. 1). When botryococcene production ceases, at the end of the linear phase, the large lipidic inclusions are confluent and the chloroplast is invaded by starch (Fig. 2). The successive TLS, closely appressed, form an hydrocarbon-rich compact matrix. After one month (Fig. 3) the chloroplast is no longer obvious, lipidic inclusions have taken a granular appearance in some cells, while in others the presence of a fibrillar material can be noticed. Such structural alterations, the fibrillar material excepted, appear analogous to those observed in some alkadiene-producing algae during growth [7, 19]. In spite of the higher hydrocarbon contents (25–40% dry wt) of B strains comparatively to that of some collection A strains (5–20% dry wt), it has not been observed in the former the same formation of droplets distending the TLS as in the latter.

CONCLUSION

The culture of *B. braunii* samples collected from various geographical areas and analysis of their hydrocarbon content have demonstrated the existence for this alga of two physiological races of very close morphology. Up to now, the hydrocarbon chemical structure appears the only criterion to distinguish each race unequivocally. The hydrocarbon nature in a given strain is unchanged, whatever the physiological state, straight chain alkadienes and trienes for the A race, polymethylated triterpenes–botryococcenes for the B race. Under the culture conditions tested, only B strains show an orange colour at the end of the growth period, associated with nutrient depletion of the medium. It was also observed that all the new collected algae have in culture higher hydrocarbon contents than those of collection origin and that the alkadiene-producing algae are, from a productivity point



Figs 1–3. Transmission electron micrographs of botryococcene-producing algae. Ultrastructure of La Manzo strain at different growth stages. Fig. 1. A cell from a culture at the end of the exponential phase. The chloroplast contains numerous thylakoids regularly arranged parallel to the cell surface with very few starch granules. The cytoplasm is filled with lipid globules. Fig. 2. A cell at the end of linear phase. The chloroplast contains numerous starch granules but the thylakoids are still well developed. The lipid inclusions are more or less confluent. Fig. 3. In the stationary phase, many cells appear rather degenerate. The chloroplast is entirely invaded by starch. The cytoplasm contains voluminous inclusions with a granular (g) or fibrillar (f) structure. N, nucleus; C, chloroplast; L, lipid droplet; s, starch; t, thylakoid; T, TLS; W, hydrocarbon rich matrix of the external wall. Scale bar 1 μm.

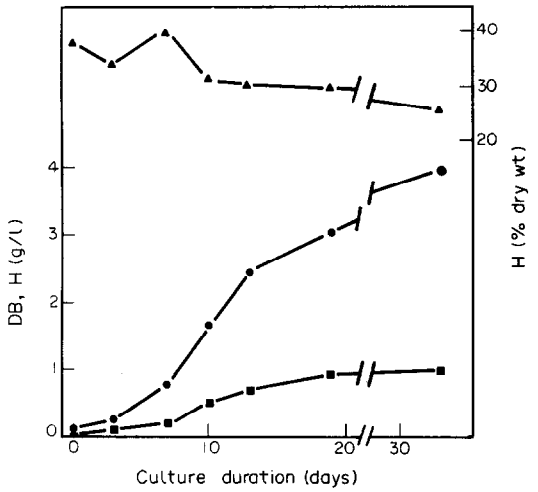


Fig. 4. Growth curve of La Manzo strain in air-lift batch culture. Hydrocarbon production and content. ■, H: hydrocarbons (g/l.); ●, DB: dry biomass (g/l.); ▲, hydrocarbons, percentage dry wt.

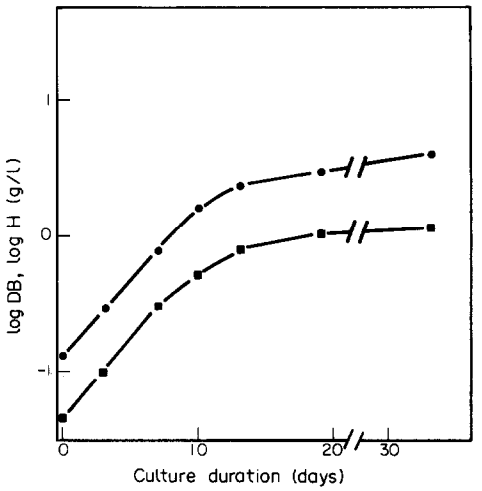


Fig. 5. Semi-log representation of total biomass and hydrocarbon production. ● and ■: as for Fig. 4.

of view, more sensitive to culture conditions than the botryococcene-producing algae.

From our screening, it appears also that the algae of the B race exhibit a very large chemical variability. The rather stable composition of the botryococcene mixture, produced by a given strain in well defined medium conditions, leads us to attribute this variability to genetic factors and so to expect the existence of distinct populations in the botryococcene-producing algae. Physicochemical factors can also play a 'reversible' role in this variability. On the other hand this variability affords the possibility of selecting highly productive clones for large scale hydrocarbon production.

EXPERIMENTAL

Origin of the samples. The collections were made at the following sites and dates. France: Morvan region. Small pool near Lingoult (28-9-81 and 1-7-82), pH 6.9, H_2O temp 15°. Two barrier lakes, Chaumecon (28-9-81 and 1-7-82), pH 6.1, H_2O temp 15.5°; Crescent (28-9-81 and 1-7-82), pH 7, H_2O temp 15.5°. Arcachon region. Large lake of Sanguinet (31-8-82), pH 6.8, H_2O temp 24°. Rodez region. Barrier lake of Pareloup (21-9-82), pH 6.7, H_2O temp 19°. French West Indies: Guadeloupe, small pool near Chateaubrun (1-5-83), pH 5.8, H_2O temp 32°. Martinique; large barrier lake of La Manzo (8-5-82), pH 8.6, H_2O temp 34°. Small pool near Paquemar (8-5-82), pH 8.6, H_2O temp 32°. Morocco: small pool in Oukaimden valley (21-4-83), pH 6.6, H_2O temp 15°.

Isolation and culture conditions. Cultures were carried out using a modified CHU 13 medium, as previously described [4], except that Fe was introduced as FeNa EDTA (10 mg/l.) and that no citric acid was added. Immediately on receipt in the laboratory, and depending on the available biomass wt, each sample or a part was subjected to purification via inoculation of agar medium (12 g/l.). When *B. braunii* accounted for a low amount of the whole phytoplankton, some colonies were collected from the sample with the help of a micropipette under a binocular microscope, before inoculation. After two months of growth on agar plate, unialgal *B. braunii* colonies were removed and inoculated into liquid medium. Two culture conditions were used. Standard conditions: conical vessels containing 100 ml of inoculated medium were shaken on a rotating plate. Growth was carried out at 22° and under a light-dark cycle (14 hr illumination per day; 100 $\mu E/m^2 \cdot s^1$). Batch air-lift conditions: cylindrical tubes containing 700 ml of medium were aerated by sterile air containing 1% CO_2 at a rate of 20 l./hr for each l. of medium. Growth was performed at 25°, under continuous illumination (470 $\mu E/m^2 \cdot s^1$).

Hydrocarbon analysis. After concn and drying of the samples or of the cultures under vacuum at 50°, materials were extracted × 2 with hexane for one hr at room temp. The crude extracts were combined, concd under vacuum and purified by CC on alumina (activity II) with hexane as eluent. GC/MS analyses were performed on a fused silica SE 52 column, 25 m × 0.31 mm, film thickness 0.19 μm, temp prog from 220° to 260° at 2°/min. R_ts were determined relative to squalene. Quantitative determinations were performed by adding int. standards before GC analyses; squalene for botryococcenes and *n*-tricosane for alkadienes.

Growth curve. The buoyancy of the colonies excluding the use of spectrophotometric methods, biomass concentrations were determined from dry wt measurements (filtration of aliquots on Millipore prefilters and drying at 80° for one day). The mean biomass doubling time during the exponential phase was determined from the slope of the linear part of the graph $\Delta \log_2$ biomass $= f$ (time).

Electron microscopy. Fixation, embedding and dehydration of samples for TEM were carried out as previously described [4]. Fixation and handling of cells for SEM have already been described [20].

Acknowledgements—We are grateful to Dr. Hillen of the Aeronautical Research Laboratories, Melbourne for a sample of Darwin River Reservoir, and to Dr. Sellier and Miss Mauroy of the Laboratoire de Spectrometrie de Masse, E.N.S.C. Paris for GC/MS analyses. This work was supported by the CNRS as a part of the 'Programme Interdisciplinaire de Recherche sur les Sciences pour l'Energie et les Matières Premières'.

REFERENCES

1. Maxwell, J. R., Douglas, A. G., Eglinton, G. and McCormick, A. (1968) *Phytochemistry* 7, 2157.

2. Brown, A. C., Knights, B. A. and Conway, E. (1969) *Phytochemistry* **8**, 543.
3. Gelpi, E., Schneider, H., Mann, J. and Oro, J. (1970) *Phytochemistry* **9**, 603.
4. Largeau, C., Casadevall, E., Berkaloff, C. and Dhamelincourt, P. (1980) *Phytochemistry* **19**, 1043.
5. Wake, L. V. and Hillen, L. W. (1981) *Aust. J. Mar. Freshwater Res.* **32**, 353.
6. Belcher, J. H. (1968) *Arch. Mikrobiol.* **61**, 335.
7. Casadevall, E., Chirac, C., Largeau, C. and Metzger, P., *J. Phycol.* (in press).
8. Blackburn, K. B. (1936) *Trans. R. Soc. Edinburgh* **58**, 841.
9. Temperley, B. N. (1936) *Trans. R. Soc. Edinburgh* **58**, 855.
10. Berkaloff, C., Coute, A., Casadevall, E., Rousseau, B., Metzger, P. and Chirac, C. (1984) *J. Phycol.* **20**, 377.
11. Largeau, C., Casadevall, E. and Berkaloff, C. (1980) *Phytochemistry* **19**, 1081.
12. Swale, E. M. F. (1968) *Br. Phycol. Bull.* **3**, 441.
13. Wake, L. V. and Hillen, L. W. (1980) *Biotechn. Bioeng.* **22**, 1637.
14. Aaranson, S., Berner, T., Gold, K., Kushner, L., Patni, N. J., Replak, A. and Rubin, D. (1983) *J. Plank. Res.* **5**, 693.
15. Masters, M. J. (1971) *Can. J. Botany* **49**, 1479.
16. Metzger, P. and Casadevall, E. (1983) *Tetrahedron Letters* **24**, 4013.
17. Swain, T. (1973) *International Symposium on Chemistry in Evolution and Systematics* 1972—*Strasbourg*. Butterworth, London.
18. Largeau, C., Casadevall, E., Dif, D. and Berkaloff, C. (1980) *Energy from Biomass*, 1*st E.C. Conference*, p. 653. Appl. Sci. Publishers, London.
19. Casadevall, E., Dif, D., Largeau, C., Gudin, C., Chaumont, D. and Descanti, O. (1985) *Biotechn. Bioeng.* **27**, 286.
20. Coute, A. and Tell, G. (1981) *Beih. Z. Nova Hedwigia* **68**, 1.