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REVIEW

Botryococcus braunii

An Unusual Hydrocarbon-Producing Alga

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Abstract

The colonial green alga *Botryococcus braunii* has been proposed as a source of renewable liquid fuel principally because of its ability to accumulate large quantities of hydrocarbon and form prodigious natural blooms. A curious feature of the alga is that the type of hydrocarbon produced is apparently related to physiological status. Active state colonies produce unbranched olefins (largely $C_{27:2}$, $C_{29:2}$, $C_{29:3}$, and $C_{31:2}$) that have been reported to comprise up to 32% of the dry weight. In contrast, resting state colonies produce unusual branched olefins (general formula C_nH_{2n-10}; $n = 30$–37) that appear to be of terpenoid origin. This "botryococcene fraction" has been shown to comprise from 27 to 86% of the dry weight in natural collections.

Although these and other considerations may argue on behalf of large-scale cultivation, several problems require further investigation. For example, little is known about bloom formation and maintenance, growth and physiology of resting state colonies, or the mechanism of physiological interconversion. There is also a need to acquire and investigate new isolates, for much experimental work has utilized a handful of active-state isolates long entrained to laboratory culture. Furthermore, currently available data suggest that the conditions required to expedite the alga's typically sluggish growth would increase production costs and likely engender the growth of more competitive phytoplankton.

Present considerations do not point optimistically to the widespread use of *Botryococcus* as a renewable fuel source, although a long-range potential for the production of specific industrial feedstocks may exist.

Index Entries: Algae, hydrocarbon production by; algae, oil production by; *Botryococcus braunii*, as a renewable hydrocarbon source; *Botryococcus braunii*, as a

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renewable liquid fuel source; *Botryococcus braunii*, hydrocarbon production by; *Botryococcus braunii*, oil production by; hydrocarbon biosynthesis; hydrocarbon biomass.

Introduction

The colonial green alga *Botryococcus braunii* is apparently unique among algae in its ability to accumulate substantial quantities of hydrocarbons. Indeed, a freshwater bloom of *Botryococcus* has been reported in which the hydrocarbon fraction comprised 86% of the sample dry weight (*1*), although a more typical range is from about 25 to 40% (*2*). These findings have suggested the possibility of large-scale cultivation of this unusual species as a renewable source of liquid hydrocarbon fuel (*3–8*). This speculation has aroused a certain amount of interest in *Botryococcus*, and although recent investigations have answered many important questions, several enigmas remain to be solved before this organism can be critically appraised as a potential hydrocarbon producer. It is the purpose of this article to summarize our current state of knowledge as well as to indicate important problems that remain.

The Colonies

The irregularly shaped colonies of *Botryococcus* range from about 30 to 500 μm and consist of one to several cell clusters united by a hydrocarbon-rich colonial matrix (Fig. 1). Closer inspection reveals that the typically pyriform cells are embedded within individually secreted matrix cups (Fig. 2) that consist of cell wall components lamellated with hydrocarbon layers (*9*). With each longitudinal cell division a new hydrocarbon "cup" is formed around each cellular progeny. This ongoing process is responsible for colony enlargement and ultimately propagation via fragmentation, the only known means of reproduction.

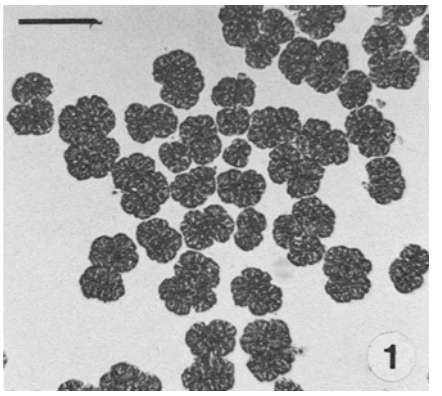

Fig. 1. Light micrograph of resting state colonies. Scale bar = 50 μ*M*.

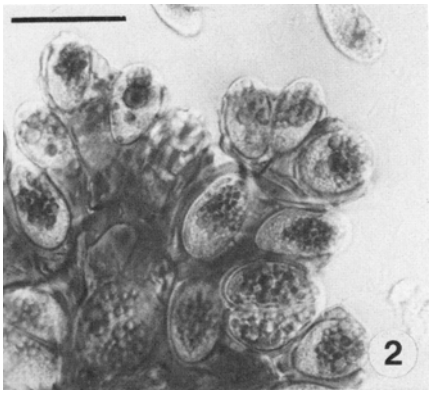

Fig. 2. Compressed portion of resting state colony showing cells embedded within oily cups of the colonial matrix. Scale bar = 20 μM.

Electron microscopy has shown cells in various physiological states to be similar to certain other ostensibly related algae (*10–13*). Cells are delimited by a two-layered cell wall; a relatively thick inner polysaccharidic wall and a thin outer wall, or trilamellar structure (TLS). The protoplast contains a parietal cup-shaped chloroplast with a basal pyrenoid, a more or less centrally located nucleus, an anteriorly located and directed Golgi apparatus, and an otherwise normal complement of cell components (Fig. 3). What appear to be functionally coherent associations of ribosomes, endoplasmic reticulum, and mitochondria have been observed at the cell periphery, but their relation to hydrocarbon secretion, if any, is unknown (*13*). The Golgi apparatus, on the other hand, appears to be primarily responsible for the production of the polysaccharidic mucilage that typically ensheaths the colony (*11, 13*).

It was formerly believed that the oil (once thought to consist of fatty acids) was produced and stored within cells and then excreted into the matrix (*9, 14–16*). An investigation of active state colonies has shown that intracellular (5% of total) and extracellular (i.e., matrix) hydrocarbon pools do exist, but that little or no transport occurs from the former to the latter (*17*). Instead, this finding and others indicate that at least the terminal steps of extracellular hydrocarbon biosynthesis occur within the TLS (*17*), which is also the primary site of hydrocarbon accumulation (*12*). It has recently been shown that the sporopollenin-like TLS is formed via oxidative polymerization of the dienic hydrocarbons typically produced by active state colonies (*18*). The composition and function of the TLS in the other important physiological state (i.e., the resting state) is unknown.

Natural History

Botryococcus is widely distributed in temperate and tropical regions where it occurs primarily in fresh, though occasionally brackish and salt waters. Colonies present in substantial numbers often appear as a green to orange, red or brown

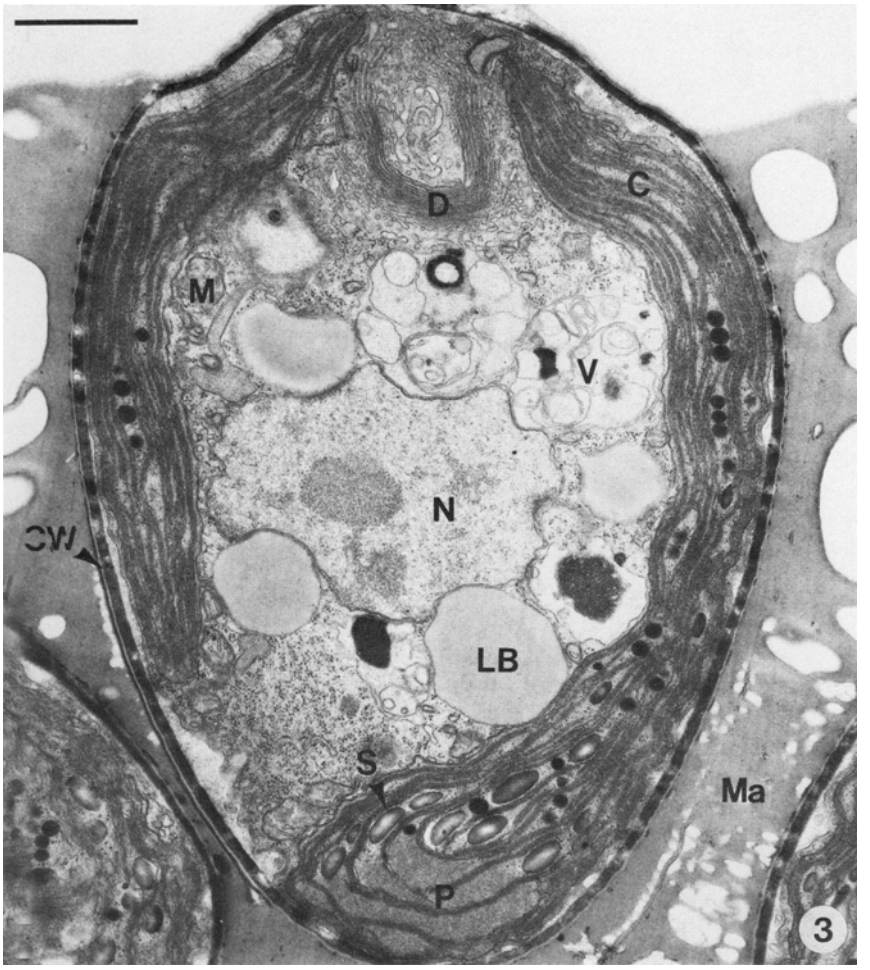

Fig. 3. Transmission electron micrograph of a resting state cell. Abbreviations: C = chloroplast; CW = cell wall; D = dictyosome; LB = lipid body; Ma = matrix; M = mitochondrion; N = nucleus; P = pyrenoid; S = starch; V = vacuole. Note double layered cell wall (arrow). The thin outer component is the TLS. Scale bar = 1 μM.

floating scum on the surface of undisturbed waters; this characteristic buoyancy results from the reduction of specific gravity by accumulated oil (*19*). *Botryococcus* is occasionally a major component of the algal flora and may form extensive and enduring blooms. Indeed, a relatively recent bloom in the 40 hectare Darwin River Reservoir in Australia was estimated to contain at least 1500 tons (dry weight) of *Botryococcus* assayed at 30% oil (*4*).

The alga's ability to form extensive blooms is not restricted to the present day. Fossilized remains morphologically indistinguishable from extant colonies have contributed to the formation of oil-rich deposits spanning from the Ordivician period to the present (*20*). Some of these, including coorongite, torbanite, and the boghead coals, are the subject of an extensive and sometimes confusing literature that has been largely compiled and reviewed by Cane (*21*). The hydrocarbon con-

tent of certain localized deposits is considerable: An Australian torbanite (a Permian oil-shale) mined during the 1940s was assayed at approximately 500 L oil/ton shale (*22*). More recently, a derivative of an hydrocarbon considered uniquely attributable to *Botyrococcus* has been discovered to comprise a remarkable 1.4% of a Sumatran petroleum—the highest level of a complex fossil biological marker ever reported in a crude oil (*23*).

The initial stages in the formation of these deposits may be observed today. Factors including wind and evaporation may result in the accumulation of floating colonies along or upon the shoreline to such an extent that oily mats are formed (*26*). Subsequent oxidation of hydrocarbons may result in the formation of a rubbery material that is apparently quite similar from locality to locality (*21*).Coorongite (named for the Coorong district of South Australia), the best characterized of these substances, is generally regarded as the "peat stage" in the diagenesis of torbanite and related deposits (*24*, others).

The formation of extensive and enduring natural blooms may argue in favor of large-scale cultivation, the ecology of bloom formation is little understood. Swale (*25*) observed bloom development in Oak Mere, England, during a period when phyto–planktonic competitors had been decimated by zooplankton grazing. He consequently suggested that minimal competition may be necessary to foster bloom initiation and maintenance in such a slow-growing alga. This is interesting because subsequent bloom decline was correlated with an increase in both numbers and varieties of the normally occurring planktonic algae. The production of growth inhibitors by this resurgent population was invoked as a possible cause of the decline, although no additional evidence was cited.

Investigations of the Darwin River Reservoir suggested that minimal algal competition, oligohaline conditions, absence of shoreline algae, loss of fish, nutrient input from below the thermocline and depth of the reservoir combined with the low phototrophic zone contributed to the bloom observed (*26*). But as the authors noted, *Botryococcus* has been discovered in bodies of water differing considerably with respect to dimensions, major ions, salinity, pH and temperature (*2*, *25*, *27–31*, others). For example, the Darwin bloom developed under warm (32°C), oligohaline, and somewhat alkaline (pH 7.5–8.5) conditions, whereas the Oak Mere bloom grew in a cooler (20–25°C), eutrophic, acidic environment (pH 4.5–5.0). Thus, with the possible exception of reduced phytoplanktonic competition, no factor(s) can be confidentally identified as being necessary for sustained bloom formation.

Physiology and Hydrocarbon Production

Brown et al. (*1*) originally described three apparently distinct and interconvertible physiological states for *Botryococcus:* (1) green "active state" colonies containing a complex mixture of hydrocarbons of general formulas C_nH_{2n-2} and C_nH_{2n-4}; (2) brown "resting state" colonies containing a high concentration of a nearly pure (90%) hydrocarbon botryococcene; (3) large green cells showing very little synthesis of hydrocarbons. Recent investigations have indicated the need to mod-

ify and amend certain aspects of this system, although it appears to be fundamentally accurate. The ensuing discussion considers each physiological state within the broader context of what is presently known.

The primary hydrocarbons of the active state are now known to include straight-chain mono-, di-, and triunsaturated C_{17}–C_{33} compounds that may comprise from 0.1 to 32% of the sample dry weight (*32–34*). Three of the major constituents have been identified as heptacosa-1,18-diene ($C_{27}H_{52}$), nonacosa-1,20-diene ($C_{29}H_{56}$) and hentriaconta-1,22-diene ($C_{31}H_{60}$) (*35*). Incorporation experiments with a variety of radiolabeled fatty acids, coupled with studies of fatty acid composition and hydrocarbon structure, have consistently implicated oleic acid as the precursor to these compounds (*36, 37*). This would explain the consistent presence of a double bond in the cis-9,10 position. Additional experiments utilizing [1-^{14}C; 9,10-^{3}H]-oleic acid as a precursor have shown that biosynthesis takes place through an elongation–decarboxylation mechanism (*36, 37*).

The terms "active" and "resting" are presently meaningful only within the species since even active state colonies grow slowly in comparison to many other algae. Largeau et al. (*5*) reported a mean generation time of about 1 week in unshaken and unaerated active-state batch cultures grown on a standard inorganic growth medium at 20°C. This time was reduced to approximately 2.5 d by shaking, aerating with 1% CO_2 (via air lift), and elevating the temperature to 26°C. These growth parameters increased hydrocarbon production from 0.011 to 0.084 g/L/d. A still greater increase (0.148 g/L/d) was obtained under the latter growth regimen by doubling the initial nitrate concentration. This result is of particular interest since increases in the rate of lipid production in many algae have been demonstrated to occur under the opposite circumstance, i.e., nitrogen depletion or reduced initial nitrogen concentration (*38–42,* others).

Resting state colonies, which may be various shades of brown, red, or orange, owe their coloration to carotenoids primarily localized within the colonial matrix. The degree of pigmentation may be sufficient to obscure the cells; this phenomenon was largely responsible for the taxonomic uncertainties that existed until chlorophyll analysis unequivocally placed *Botryococcus* among the green algae (*43*). Colony color is not, however, always a reliable indicator of physiological status. Green colonies collected from several localities in Australia were discovered to contain branched hydrocarbons of the botryococcene type (*26*), although closer inspection revealed that the green cells obscured the orange colonial matrices. On the other hand, green colonies with hyaline matrices collected from Lake Michigan contained a predominance of straight-chain hydrocarbons, while their red-matrixed counterparts contained botryococcenes (*44*). The reverse situation (i.e., straight-chain compounds in carotenoid-rich colonies) has not been verified (*2*). These observations suggest that the color of the colonial matrix may be a more dependable indicator of the class of hydrocarbon present than overall macroscopic appearance.

For a time it appeared that botryococcene ($C_{34}H_{58}$) and its isomer isobotryococcene were the only hydrocarbons produced by resting state colonies (*45*). Subsequently, however, resting-state colonies obtained from several localities have collectively revealed at least 17 additional compounds whose partial charac-

terizations indicate their placement within the botryococcene family (*22, 44, 46*). Gas chromatography–mass spectrometry has shown them to possess the general formula C_nH_{2n-10} where $n = 30$–37. It is of interest that colonies collected from different localities may possess qualitatively distinctive hydrocarbon fractions that may remain similar over time (*2, 44*). For example, the hydrocarbon fraction of resting state colonies collected from Lake Michigan remained virtually identical from sample to sample over a 3-yr period (*44*, unpublished data). Wake and Hillen (*2*) have suggested a possible genetic basis for this variation, but note that environmental influences on hydrocarbon content are unknown.

Botryococcene itself (Fig. 4) is the only compound for which a structure has been proposed (*47*), although work on isobotryococcene and a C_{36} compound has been undertaken (L. V. Wake, personal communication). It has been proposed that botryococcene is synthesized by tail-to-tail linkage of dimethylated C_{15}-units to yield the saturated quaternary carbon; such a precedent exists in isodigeranyl (*48*). The peculiar methylation pattern, on the other hand, bears comparison to the side chain of cyclolaudenol (*49*). Botryococcene and related compounds are assumed to be of terpenoid origin although no biosynthetic studies have been published.

Relatively little is known about resting state physiology and hydrocarbon production largely because of difficulties encountered in isolating and growing viable cultures from natural collections. Similarly, attempts to induce the conversion of at least one active-state isolate to the resting state via manipulation of physicochemical factors have failed (*44*). Brown et al. (*1*) elicited limited synthesis of botryococcene (identified by GLC) in active state cultures subjected to high light intensity and nitrogen deficiency, although a complete transition was not achieved. Thus, unfortunately, no solid hydrocarbon productivity data is available for the typically hydrocarbon-rich resting state.

The few experiments conducted with resting state cultures have either lacked supporting hydrocarbon data or were of a cursory nature. Belcher, for instance, induced red floating colonies from a natural isolate to turn green, sink, and gradually revert to the red form (*19*). These static batch cultures grew exponentially for the first 5 weeks (mean generation time ca. 1 week), entered stationary phase, and continued a slow increase in dry weight for 3 months thereafter. After 16 weeks the reverted red cultures had achieved an average density of 250 mg/L, of which 22% was unsaponifiable (probably hydrocarbon) and 12% saponifiable lipid. It is likely that the red inoculum colonies used in this study were in the resting state, but it is by no means certain that the observed color transitions reflected a switchover in hydrocarbon biosynthesis. The slow mean generation time during exponential growth agrees with estimates reported for static active state cultures (*5, 19*) and a natural population (24 d) (*25*). Insufficient data is available for estimation of the rate of lipid production during the exponential phase of growth.

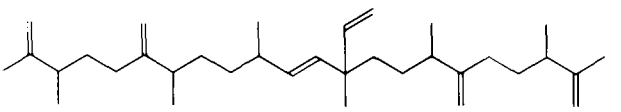

Fig. 4. Proposed structure for botryococcene (after Cox et al., 1973).

Growth experiments with axenic resting state cultures were conducted by Brown et al. (*1*), but these were of a nonquantitative, generalized nature. It was shown that within a week after inoculation of resting state colonies into fresh medium botryococcene had largely disappeared and the characteristic active-state hydrocarbons had been formed. The fate of the botryococcenes during this time was not explained. Experiments with active state cultures have indicated that matrix hydrocarbons are not capable of being catabolized, although this finding may not extend to resting state colonies. Belcher (*19*) noted the possibility that the hydrocarbons might undergo gradual oxidation that would prevent efficient extraction by the usual methods employed. In any case, attempts to convert these cultures back to the resting state were not reported, which is unfortunate because the hydrocarbon content of the inoculum ranged up to 86% of the dry weight—the highest figure reported in the literature.

Resting state colonies grown under certain circumstances gave rise to large, green hydrocarbon-deficient cells that characterize a third physiological state. These cells could be induced to assume typical colonial morphology only with great difficulty under circumstances that were not elaborated (*1*). This condition has not since been reported in the laboratory and has never been observed in nature, although identification of such cells from natural collections would be difficult. The significance of this physiological state in the alga's life cycle, if any, remains unknown.

Discussion

Botryococcus possesses several attributes that suggest its potential as a renewable hydrocarbon source:

—It has the ability to accumulate hydrocarbon at levels unknown among algae and higher plants; most algae, for instance, usually contain no more than about 0.1% hydrocarbon on a dry weight basis (*50*). There are also data that indicate that other components of the lipid fraction may be worth evaluating (*51*).

—High hydrocarbon content would not only reduce processing costs, but perhaps harvesting costs as well. Hillen and Wake (*4*) have suggested that the alga could be initially concentrated by taking advantage of its tendency to float and accumulate on the leeward side of a body of water. Thus, the alga-rich water could be drawn off at advantageous times and pumped over prepared areas for drying. Although this method is clearly speculative, the growth characteristics of the alga suggest the possibility of avoiding a costly harvesting process such as centrifugation.

—Preliminary studies indicate the suitability of botryococcenes as a feedstock for hydrocracking to transport fuels (*52*). For example, a hydrocracked distillate derived from the Darwin River Reservoir bloom yielded the following fractions: petroleum, 67%; aviation turbine fuel, 15%; diesel fuel, 15%; residual oil, 3%. Although these analyses were conducted with limited quantities, no particular difficulties were envisioned for large-scale production.

—Populations occasionally develop into prodigious and enduring blooms under a variety of environmental conditions. Though the factors responsible for bloom formation are not understood, at least the potential for "cultivation" is indicated.

—There are indications throughout the literature that *Botryococcus* is able to withstand circumstances that many other algae could not tolerate. For instance, Dubinsky et al. have grown cultures in media with osmotic potentials ranging up to that of seawater (*51*). It has even been suggested that oil could be mechanically removed, thus permitting the colonies to be recycled (*5*). Once dead, however, colonies typically remain largely impervious to physical and biological degradation (*25*). The durability of both living and dead colonies could ease certain restrictions (e.g., time constraints in harvesting) generally imposed by land crops.

—Colonies do not appear particularly vulnerable to zooplankton grazing, presumably owing to their relatively large size (*25*). Only one such report was encountered in the literature (*28*), although there is much room for study. It may be possible to exploit this fact as a means of controlling competing phytoplankton during large-scale cultivation.

Although these attributes and others may argue on behalf of cultivation, the bottom line is how much oil can be generated per unit volume over a given period of time. Hillen and Wake (*4*) estimated a yield of 35 tons of hydrocarbon/ha/yr based on their observations of a natural bloom and growth rates of other algae. This figure is contingent, however, on inducing growth rates comparable to those obtained with rapidly growing algae such as *Chlorella* and *Scenedesmus* (*53*), even though there is no present evidence to suggest that *Botryococcus* is capable of such growth. This figure also presupposes that the rate of hydrocarbon production would match this increase in growth. Largeau et al. (*5*) have projected a yield of 60 tons/ha/yr in a facility with a depth of 0.2*M* aerated with CO_2-enriched air.This estimate was extrapolated from laboratory-grown active-state batch cultures under the unproven assumption that data derived from such cultures would translate on a per unit volume basis to a large-scale facility.

Although such speculation has been and remains tempting, it is nonetheless premature in view of the basic questions that remain. The amenability of *Botryococcus* to outdoor culture has yet to be demonstrated. Ecological data is sparse. Fundamental knowledge concerning regulation of growth and hydrocarbon production in the different physiological states is lacking. Out of these myriad questions, however, a few are particularly critical in assessing *Botryococcus* as an hydrocarbon producer. For example:

—What degree of genetic variation occurs among populations in nature? Presently available data is largely based upon experiments performed with active-state isolates currently available from culture collections. It is difficult to draw confident conclusions from an isolate that has been maintained on a laboratory shelf in artificial medium over a period of many years. The existence of diversity in nature is suggested not only by hydrocarbon data, but morphological (*9*) and culture studies as well. Belcher, for instance, noted that clones isolated from different localities and grown under similar conditions in the laboratory varied considerably in the ratio of lipid to carotenoid (*19*). Thus, the investigation of new isolates is indispensible to the further evaluation of the alga's potential.

—What are the physiological characteristics of the hydrocarbon-rich resting-state colonies? The rate at which these colonies are able to produce botryococcenes is unknown, making a direct comparison to active-state colonies impossible. This breach of data presents a major obstacle to the proper assessment of the alga as an oil producer.

—What factor(s) control the interconversion between the active and resting state? It is uncertain whether the active state precedes the resting state during bloom formation, or whether the latter is the perenniating form under most circumstances. This information, coupled with resting-state data, could profoundly affect cultivation strategy. At minimum it is essential to elucidate the conditions required to maintain a population in the desired physiological state, as unpredictable partial or complete conversions could obviously have enormous impact on the type and amount of hydrocarbon product. Apart from practical considerations, this metabolic switchover presents an intrinsically interesting problem in the regulation of gene expression.

—What factors are responsible for the typically slow rate of growth? Unfortunately, very little is known about cellular physiology and metabolism, although existing evidence indicates that the photosynthetic rate (as measured by O_2 evolution/mg chlorophyll) in active state colonies is comparable to that of other green algae (*5*). This indicates the possibility that growth is subject to constraints imposed by the alga's unusual morphology. Belcher (*19*) has suggested that sluggish growth may be attributable to the hindrance of cellular gas exchange to the cells by the colonial matrix and the alga's peculiar ability to direct metabolism into metabolically expensive lipids. The reduction of light reaching the chloroplast of resting-state cells caused by abundant matrix carotenoid was also cited as a potential impediment to growth, although the question of cause and effect arises. It may be possible to better understand growth and physiology by studying single cell cultures derived from intact colonies (*34*).

Though the factors responsible for slow growth remain unclear, existing data indicate that the highest attainable rates of growth, density and hydrocarbon production will require aeration with CO_2-enriched air, an adequately enriched inorganic growth medium and perhaps mechanical agitation as well— the very circumstances that favor the growth of much more rapidly growing algal species. This prospective difficulty must weigh heavily in the consideration of large-scale production.

—Does hydrocarbon oxidation and/or polymerization occur to any extent in living colonies, and, if so, to what extent under various conditions? Belcher's (*19*) analyses could not account, on the average, for about 30% of the sample dry weight. This led to the speculation that some of the "lipoid material" had oxidized and had consequently become resistant to conventional extraction with organic solvents. Electron microscopic observation of certain active-state cultures extracted with hexane seem to verify this fact (personal observations). This phenomenon could have substantial bearing on any study that evaluates hydrocarbon production.

Obviously this list of questions and considerations could be expanded, but it is not intended here to exhaustively examine *Botryococcus* as either a biological or potential economic entity. In many respects currently available data do not point

favorably to the widespread use of *Botryococcus*-derived fuel anytime in the near future; perhaps it would be more realistic to evaluate the alga's long-range potential as a source of specific industrial feedstocks. In any event, the need for sustained basic research is a clear prerequisite to the further consideration of *Botryococcus* as an oil producer.

Acknowledgments

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