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Studies on the Nutritional Requirements of the Oil-Producing Alga *Botryococcus braunii*

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ABSTRACT

Studies were carried out on the ability of the algae *Botryococcus braunii* to grow in the presence of possible carbon sources. Sources examined included compounds for C_1–C_6, as well as the disaccharides sucrose and lactose. Dividing times were decreased from an average of over 1 wk to less than 3 d by addition of the appropriate carbon sources.

Examination of the oils produced in the presence and absence of exogenous carbon indicated no differences. However, the total biomass produced in the presence of a usable carbon source exceeded that produced in the carbon's absence.

Index Entries: Sugar; oil; *Botryococcus braunii*; algae; carbon; *botrycoccus braunii*, growth on carbon; dividing times. Nutritional requirements, of *B. braunii;* oil-producing alga, nutritional requirements of; alga, nutritional requirements of the oil-producing *B. braunii; Botryococcus braunii,* nutriitional requirements of the oil-producing alga.

INTRODUCTION

The extraction and use of oils from plants, such as sunflower, peanut, and corn, are well known. These oils, generally used in cooking and in various foods, have a value of approximately 34¢/lb. Recently, there

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has been much discussion of using these oils as fuels. This being the case, it may be of value to examine additional sources of biologically produced oil, both for food applications and possibly as a fuel.

One relatively obscure source of oil is the microalga, *Botryococcus braunii,* in which the hydrocarbon content can reach a major portion of the total plant mass.

Coorongite is a naturally occurring material found on the shoreline of dried-up lakes in many locations of Australia, Mosambique, and even Turkestan. This material is black in color, but upon close examination it is obviously of biological origin. This was proven by the work of Ward (*1–2*) and Thiessen (*3*).

Similar materials were found in the UK, Europe, and the United States during this period and given various names. However, one fact emerged. These materials were of biological origin and were in fact produced by what appeared to be a microalga.

Later, the mineral Torbanite was found to be related to Coorongite, and is probably the final stage of this material's mineralization process. For details of these studies, see the excellent review of Hillen and Warren (*4*). The final outcome of these studies are as follows: An alga (*B. braunii*) produces an oil. These algae dry into a peat-type mat, becoming Coorongite, which eventually mineralizes into Torbanite.

Torbanite can be found in layers as thick as 600 mm (2 ft). It is also believed that these layers of Torbanite (containing 60–90% oil by weight) are the result of a single annual growth of an alga that is believed to be identical to the modern day *Botryococcus*. This being the case, it is apparent that this alga is capable of phenomenal growth.

A relatively recent "bloom" of *B. braunii* was recorded in the Darwin River Reservoir in Australia (*5*). This reservoir has a surface area of 4000 ha and an average depth of 6 m. The bloom was estimated to contain at least 3000 t of algae.

Boytrycoccus braunii is a ubiquitous freshwater alga found in most temperate and tropical areas of the world. The alga is a colonial member of the *Chlorophyceae,* the colonies being roughly spherical and resembling rosettes. Depending on the conditions, larger colonies may sectionalize, being held together by connecting strands. Individual cells are pear-shaped, but will change to rounded shape under special conditions. The alga has a high oil content. Reports range from an average of 30–40% to 90% on a dry weight basis. Wild examples have always been found to have higher oil content than cultured samples. Most cultured samples average 25–30% oil, whereas wild samples average 75% oil. The types and quantities of oils reported in the literature are given in Table 1.

The alga is found in different states. The green "exponential" state covers the period when the culture is rapidly dividing with time. In this state the oil content may be up to 20% of the dry algal mass. The oils are generally linear, unsaturated hydrocarbons. At this stage the algae is denser than water and sits on the bottom of a culture flask.

TABLE 1
Hydrocarbons Common to *Botryococcus braunii*

	% Composition of hydrocarbon[a]		
Hydrocarbon formula	1	2	3
$C_{17}H_{34}$	1.52	—	—
$C_{23}H_{46}$	0.14	—	—
$C_{25}H_{46}$	0.10	—	—
$C_{25}H_{48}$	0.65	—	—
$C_{27}H_{52}$	11.10	—	7.2
$C_{28}H_{54}$	0.65	—	—
$C_{29}H_{54}$	5.54	—	23.0
$C_{29}H_{56}$	50.40	—	32.6
$C_{31}H_{60}$	27.90	—	25.1
$C_{33}H_{62}$	2.0	—	—
$C_{34}H_{58}$	—	83.5	—
$isoC_{34}H_{58}$	—	8.2	—
Other oils	—	8.3	12.1
Total	100	100	100
% Hydrocarbon	0.3%	75%	17%
Source	Lab 1–3 week culture	Wild UK	Lab, 16 wk

[a]Three different experimenters data on algae grown under different conditions.

The orange "resting" state covers the period during which the oil content may rise to greater than 75% of the dry mass. The major component at this stage is botryococcene, $C_{34}H_{58}$. High concentrations of this hydrocarbon have only been observed in the wild. During this period cell division stops and photosynthetic metabolism is diverted to oil production.

Nutritional requirements for this organism are unknown. However, it is generally recognized that both phosphorus and nitrogen are required. Doubling times have been reported to range from a low of 7 d to as great as 25 d. The oil may be extracted by a simple filter press, solvent extraction, or destructive distillation.

Estimated production costs for this alga in 1972 ranged between \$0.11 and 0.27/kg. If one could obtain 50% of the dry weight as oil, then the oil would cost \$0.22–0.54/kg. Assuming 10% inflation in the preceding 13 y would bring the cost/kg to approximately \$1.00–2.00/kg. One bbl of oil at 55 gal would weigh approximately 450 lb, or 200 kg. At \$40.00/bbl of oil, the cost of oil today is approximately \$0.20/kg or 5–10 times less than oil produced via the algae. This would seem to indicate that, at least at this time, Botryococcus is not a good choice for fuel oil. It

also assumes that the cost of algae production has more than doubled since 1972.

What about as an edible oil? Plant oils average 30–60¢/lb or $1.50/kg. This is in the ballpark for the algal oil. Since the initial cost figures were carried out assuming a doubling time greater than 1 wk, decreasing doubling time could decrease cost. In order to do this it is important to know more about the organism's growth requirements.

METHODS AND MATERIALS

We have initiated a program to better understand the growth requirements for this organism. In addition, we have attempted to relate the effects of modified growth conditions to oil production.

Culture of the Algae

A slant of *B. braunii* was purchased from the Texas Culture Collection and initially grown on agar enriched with soil extract in the light. Samples were transferred to Chu 10 media (*6*), enriched four times over that described. Cultures were grown in unshaken flasks under constant fluorescent light. Each day the growing samples were slightly shaken and returned to their static position.

Microscopy

We have examined the algae using scanning electron microscopy (SEM). The cells were fixed with gluturaldehyde and treated with 1% OsO_4, followed by dehydration.

Growth Experiments

Studies were carried out under fluorescent lighting. All measurements of growth were carried out via turbidmetry (*7*), using the Beer–Lambert equation for a bacterial system:

$$-\log I/I_o = I1/I = W/W_{10}$$

where

I = unscattered light
I_o = incident light
W_{10} = concentration of algae that gives a 10-fold decrease of light intensity
$\text{Log } I/I_o$ = adsorbance (A) when plotted so that data should yield a straight line.
$A = K \times W$ where K is the slope, A has replaced log I_o/I, and W is $1/W_{10}$

In order to determine if this data also holds for *B. braunii,* we monitored chlorophyl content vs adsorbance at 450 nm. If the two are directly proportional we would expect a straight line relationship.

The experiment was carried out by growing the algae in 30 mL capped Cortex™ centrifuge tubes in Chu 10-4X (four times) media. The tubes were read weekly by first vortexing to suspend the cells. The tubes were then placed directly into a Coleman Jr. spectrophotometer and read at 450 nm. After taking the reading they were centrifuged at 1500*g* for 20 min and the supernatant removed. To these tubes was added 1 mL boiling water, after which the tubes were placed in boiling water for 2 min, quenched in ice, and centrifuged at 4°C at 15,000*g* for 10 min. To this is added 2 mL methanol in the dark. The tubes are allowed to stand in the dark for 5 min. Each tube was then read at 650 and 665 nm in the Perkins-Elmer Model 124 dual-beam spectrophotometer. This data was plotted vs the optical density reading at 450 nm. The results of these experiments, collected over 4 wk of growth, clearly showed the expected relationship. Keeping this in mind, all remaining data were collected as OD measurements.

Effect of $NaHCO_3$

A series of experiments was carried out to determine whether $NaHCO_3$ was still necessary, even in the presence of a usable organic carbon source. Another series of experiments was also carried out, using 0–0.8% dextrose. However, in these experiments, $NaHCO_3$ was eliminated from the Chu 10-4X media. A similar series was run in the presence of added $NaHCO_3$.

Effect of Light

In the presence of added organic carbon the question still remained whether the organism was acting as a true heterotroph in utilizing the organic carbon or whether it still required its photosynthetic apparatus for growth and maintenance.

To settle this question a series of experiments, similar to those previously described, was carried out. In these experiments 0 and 0.4% dextrose in the presence and absence of $NaHCO_3$ was used with cultures grown in the dark. Samples were exposed to light only when examined.

Determination of Optimum Nitrogen and Phosphorus Concentrations

Using the light scattering method, we examined the effects of the addition of $Ca(NO_3)_2$ and/or K_2HPO_4 to the modified Chu 10 media. Salt concentrations tested ranged from 0 to 34.2 mg/100 mL media for the $Ca(NO_3)_2$ and 0–20 mg/100 mL K_2HPO_4. All studies were carried out in media containing 0.5% dextrose in the light.

Effects of Carbon Compounds

After determining that the optimal nitrogen and phosphorus concentrations were 34.2 and 2.25 mg/100 ML, respectively, these concentrations were used on all studies. Carbon compounds were tested in the range of 0–1% (w/v). All tests were carried out for a minimum of 25 d. The compounds examined are given in Table 2.

TABLE 2
Summary of Results

Compound	Concentrations at maximum growth rate, %	Minimum doubling[a] time, d
C_6		
D-Fructose	0.4	5.0
D-Glucose	0.5	5.0
D-Galactose	0.4	5.0
D-Mannose	0.4	2.0
6-Deoxymannose	No Increase	—
D-Fucose	No Increase	—
2-Desoxyglucose	No Increase	—
D-Tagatose	No Increase	—
D-Allose	No Increase	—
2-Glucose	No Increase	—
L-Sorbose	No Increase	—
L-Galactose	—	—
Mannitol	—	—
L-Mannose	—	—
C_5		
D-Xylose	No Increase	—
D-Ribose	No Increase	—
D-Arabinose	No Increase	—
D-Lylose	No Increase	—
L-Arabitol	No Increase	—
C_4		
Maleic acid	—	—
C_3		
D,L-Lactic acid	0.4	17.0
C_2		
Sodium acetate	No increase	—

(*continued on next page*)

TABLE 2 (*continued*)

Compound	Concentrations at maximum growth rate, %	Minimum doubling[a] time, d
Glycine	No increase	—
Disacchrides		
Sucrose	1.0	12.5
Maltose	Slight possible increase	—
Lactose	No increase	—
Others		
90% Hydrolyzed cheese whey	0.3	5.0

[a]The average doubling time of controls (n = 24) was 18.5 ± 4.2 d.

Hydrolyzed Cheese Whey

Acid cheese whey previously hydrolyzed with *beta*-galactosidase to 90% glucose and galactose was examined as a possible cheap carbon source. The total weight was determined and the whey tested over a 0–1.0% w/v range.

Eleven Liter Fermentation

Large 15-L carboys were used for growing large quantities of algae. The algae were grown, using 0.3% (w/v) hydrolyzed cheese whey in the modified Chu 10 media at optimal nitrogen and phosphorus concentrations. Cultures were seeded from 4-L seed cultures by addition of 2 L of cells previously grown for a minimum of 4 wk. Cells were harvested from the 15-L jars after 16–30 d.

Oil Extraction

The cells were collected by centrifugation and lyophilized overnight. They were then placed in a capped vial in acetone in a sonic washer for 30 min and centrifuged and the acetone collected. This was repeated five times. The fractions were collected and evaporated to dryness. The oils were brought up into *n*-hexane and passed on to a dry neutral alunina column. The oils were eluted with *n*-hexane, evaporated to dryness, and brought up into chloroform. The chloroform preparation was examined by gas chromatography. In some cases an additional fraction was collected after the *n*-hexane elution by passing a mixture of *n*-hexane–acetone (10:1) over the column.

RESULTS

Figures 1 and 2 show the external structure of a "rosette" or cell mass. The cells appear surrounded by a sheath of mucous like material.

Figure 3 indicates the location of Na, Ca, and Fe in the cell and its surrounding membranes. Na appears present in the cell and Fe in the sheath area.

Figure 4 shows the effect of pectinase treatment on the rosettes. The photos indicate that the thick membranous material is removed. No major differences are discernable between the cellulase- and pectinase-treated cells. This data would indicate that the thick sheath consists of cellulosic material over which resides a mucous-like material that remains after cellulose treatment.

Figure 5 is a beautiful cross-section of the cells in the mucous material surrounded by a cell wall. The oil vacuoles are visable within the cells themselves.

Based on the EM data, the following description has been pieced together. The cells appear pear-shaped, with the apical end toward the center of the rosette. They are surrounded by a gelatinous material (possibly polysaccahride in nature) that appears to end in at least two membranes that surround the thicker sheath membrane around each cell. The sheath itself appears to be relatively thick—on the order of 0.1–0.2 μm vs the outer membranes, which are no more than 0.01–0.02 μm thick.

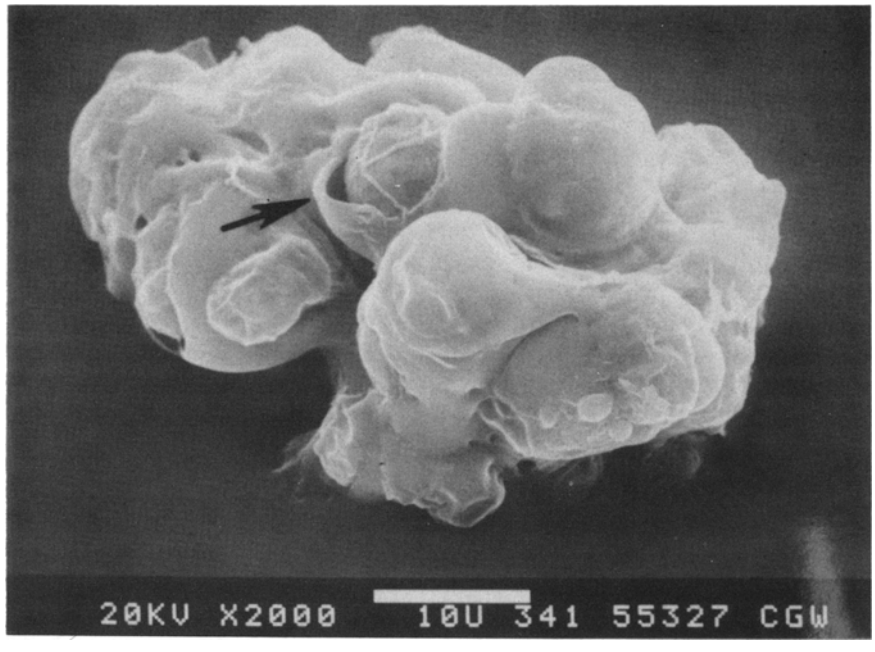

Fig. 1. A rosette showing the sheath surrounding the cells. Arrow points to a break in the sheath.

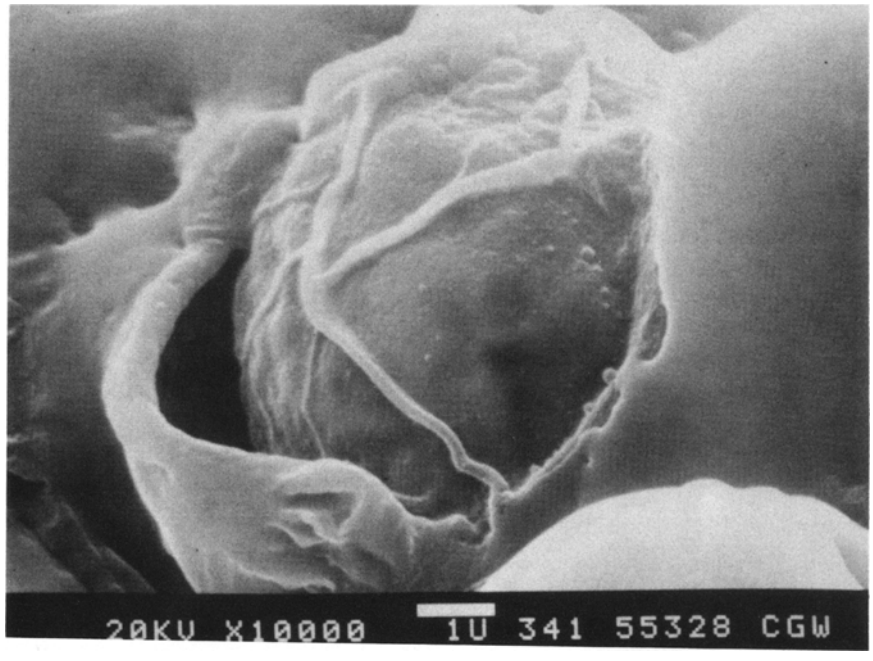

Fig. 2. A close up of the break in the sheath; note the thickness of the sheath shown here.

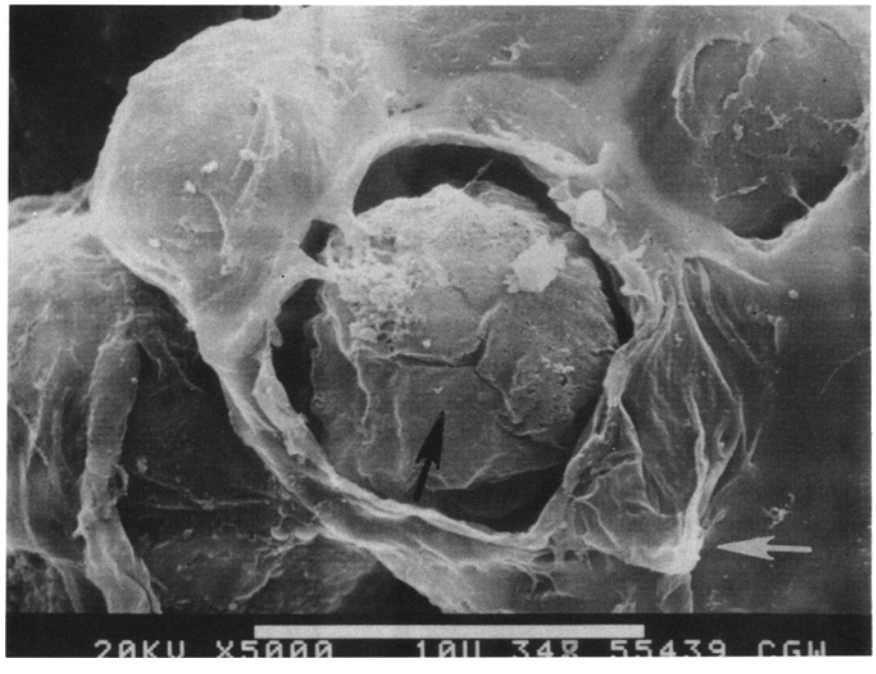

Fig. 3. A close up showing a break in the sheath that was analyzed via α-RED analysis on the sheath and the exposed area. Analysis revealed Na present where the black arrows indicate and calcium and iron present where the white arrow indicates.

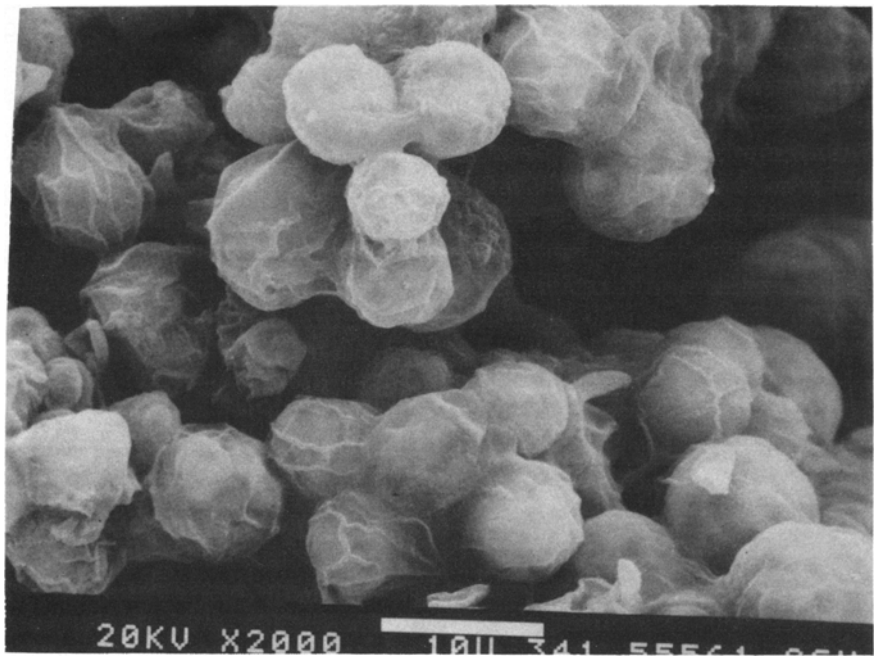

Fig. 4. Cells were treated with 1% pectinase for 3 min to remove the sheath. Results obtained with cellulase were identical as determined by SEM.

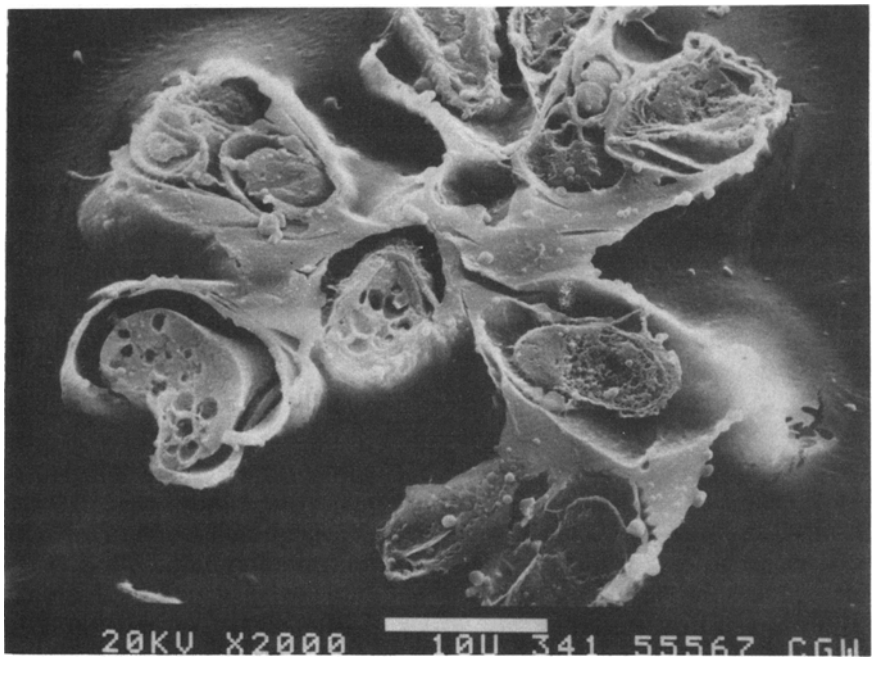

Fig. 5. A micrograph showing the membranous structures surrounding the cells. One can also see the oil vacuoles within the cells. The pear shape of the cells are also discernable.

Within the sheath is an open area that may be an artifact or might have contained oil, as described by Largeau (*8*).

Largeau indicates that the oil is found in vacuoles within the cell and in an external pool outside the cell membrane. These micrographs would indicate that this external pool lies between the cell membrane and the cell sheath. The interstices of the cell show only the oil vacuoles and what may be plastids that contain chlorophyll. The micrographs do not show a well-defined chloroplast structure. His studies also appear to indicate that the plastid are throughout the cell and not in an ordered array, as in higher plants.

Effect of $NaHCO_3$

To determine whether $NaHCO_3$ was still necessary in addition to the glucose, a series of experiments was carried out, as previously described, using 0–0.8% dextrose. However, this time the $NaHCO_3$ was eliminated from the Chu 10-4x media. The results are similar to the data obtained only in the presence of added $NaHCO_3$, indicating that when dextrose is available as a carbon source, inorganic carbon is unnecessary.

Effect of Light

The results of these experiments indicated that no growth occurred in the dark during the length of the experiment. This data indicate that photosynthesis is still necessary for growth, even when dextrose is used a substitute for $NaHCO_3$ as a carbon source.

Optimal Nitrogen and Phosphorus Concentrations

Results of these studies indicated that there is indeed an optimum concentration for both calcium and phosphorous. For the remaining studies the optimum concentrations of these compounds were used throughout. These concentrations were 34.2 mg/100 mL $Ca(NO_3)_2$ and 1.25 mg/100 mL K_2HPO_4.

Effects of Carbon Compounds

A summary of the results is given in Table 2. The only compounds that increased the rate of growth were D-fructose, D-glucose, D-mannose, D-galactose, and D,L-lactic acid. A sample of 90% hydrolyzed cheese whey containing both glucose and galactose was also found to greatly enhance growth rate. Figure 6 represents an example of one compound causing an increased growth rate. The example shown is for growth on mannose. Each sugar tested that had an effect showed an optimum sugar concentration. In the case of dextrose, the optimum was found at a concentration of 0.5%.

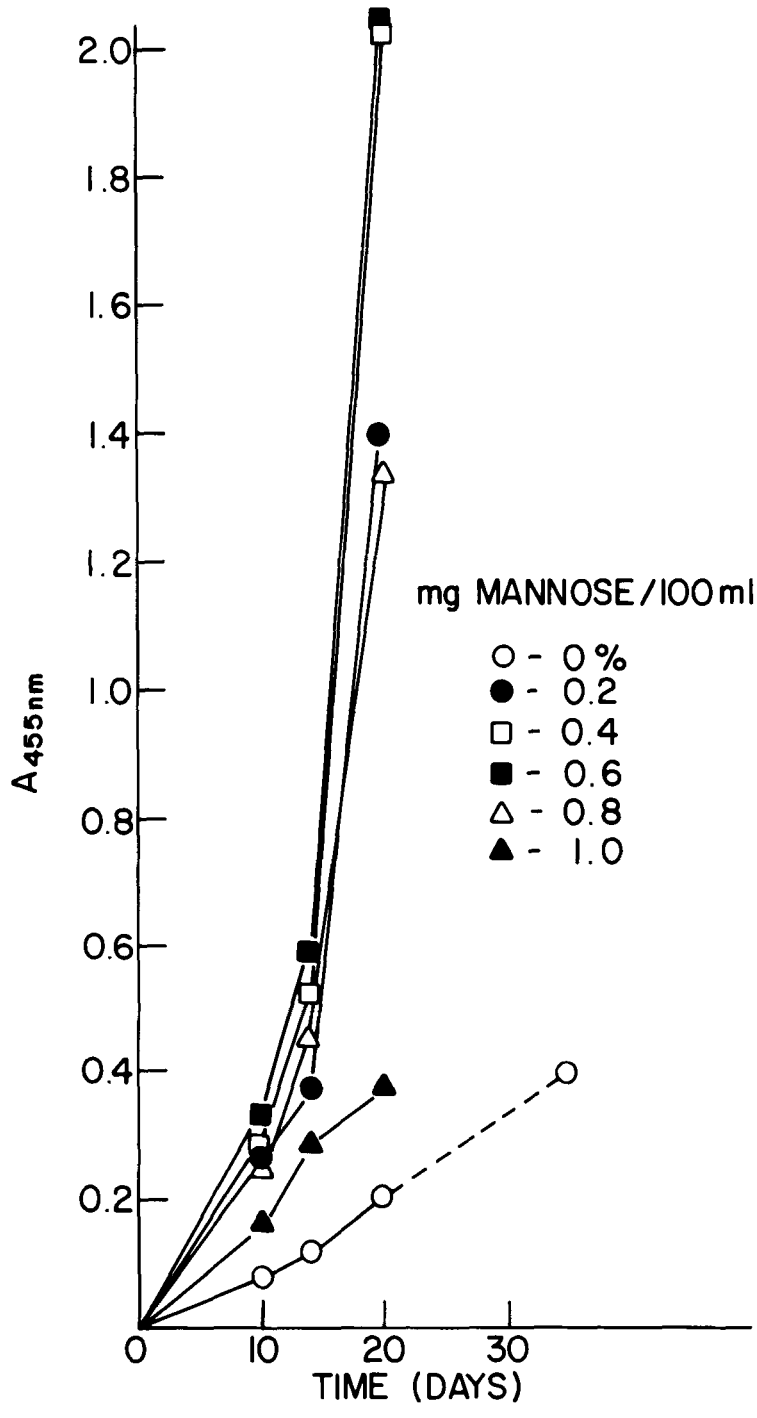

Fig. 6. The growth curves for cells grown on D-mannose over a 30-d period. Highest growth rates occurred between 0.4–0.6% D-mannose.

Oil Production

Since we as yet have little understanding of the growth conditions for the alga, we are not yet in a position to make comparison vs the oil found in the wild. We have tested a sample of oil from two observed stages of this alga. The green stage (exponential growth) and the white stage (a stage where the cell appears to decrease in size and content). The results of two runs made with the two different algal preparations indicated no differences in the concentration of several of the larger carbon containing compounds in the white cells, specifically C_{27}, C_{29}, and C_{31}, based on using *n*-paraffin standards.

A second series of experiments was run to determine whether diffrences in oil components occur in the presence and absence of dextrose. For these studies the following samples were grown and extracted:

(a) 0.4% dextrose − $NaHCO_3$
(b) 0.4% dextrose + $NaHCO_3$
(c) No dextrose + $NaHCO_3$

Samples were weighted before extraction and oils produced compared on a total oil/mg dry-state basis. All results indicated 25–30% oil by weight. No differences were observed in total oil/mg of cells. However, the total biomass produced in the presence of dextrose was 5–7 times greater than the 0% dextrose control.

Eleven Liter Fermentations

Fifteen liter carboys were seeded with 2 L of 30-d grown cells. Both the seed cultures and the 11 L cultures contained 0.3% whey in Chu 10-4X media. Cells were grown for an additional 16–30 d, harvested, and extracted, as previously described.

Lipid yields averaged 30–35% of the dry cell mass on the 11-L cultures. The oils were taken as two fractions. The first fraction was clear and contained C_{18}–C_{34} oils. The second fraction, eluted with hexane: acetone (10:1) was yellow to orange in color and by IR measurements was found to contain what appeared to be esters of long chain alcohols and phthalic acid.

Sampling the second fraction for odor indicated a rather astringent but flowery odor, with an underlying odor of musk. A valuable essence used in the perfume industry.

Several batches were grown for different periods of time. Although we have no solid data to support our feelings at this time, it may be that younger cultures are higher in the odor containing compounds than the older cultures. Further studies must be carried out to optimize the production of the potentially highly desirable products.

DISCUSSION

The SEMs show that the sheath is made up of mostly cellulosic material that can be digested away, leaving intact, but single, cells. The sheath apparently contains some mucopolysaccharide-like material susceptible to pectinase attack. Similarly, these appear to be some small quantity of protein in the sheath that, when digested, still leave the sheath intact, but with a somewhat thinner appearance.

The cell wall of *B. braunii* possesses an internal fibrillar layer and an external trilaminar sheath. The material between the fibrillar layer and the trilaminar sheath appears to be lipid, as indicated by the Sudan Red staining. The Periodate Acid Schiff Stain (PAS) was used to indicate that polysaccharide material is also present. This is most likely associated with the fibrillar material and the cell wall.

Transmission election microscopy of the cells showed some refringent material in the cell interior, which is rather homogenous looking and appears lipid in nature. The oil vacuoles appear associated with gland-like structures (*10*) that may be responsible for the oil production. The whole cell appears to be surrounded by additional sheaths that appear to be associated with the surrounding mucus-like material that holds the cells together as colonies. The apical end of the cell does not appear to have oil droplets, but does have oil associated with the membrane structures. The remaining features look like what one would expect. A well-defined nucleus, numerous starch granules, and the expected thylocoids.

In Largeau's (*8*) studies, he observed the same trilaminar structure we found. However, he also noted that each cell was usually surrounded by three or more layers (up to six) of membrane, with rarely more than one cell encompassed on the same mmembrane. He also observed that "vacuoles are most likely glands and are definitely associated with the oil-containing areas of the cell." He made no statement concerning the nucleus, its location, size, or shape. We found the nucleus only after sectioning the cell down toward the apical end.

Growth experiments carried out with a variety of C_2–C_6 compounds only turned up a few that could act as carbon sources, but only in the continued presence of light.

It is known that sugars, including fructose, glucose, mannose, and galactose, are found in plants associated with either metabolism or formation of one of the many nucleotides found in plants (*9*). It is also known that mannose 1,6-diphosphate can be converted to fructose 1,6-diphosphate, which then feeds directly into the Calvin cycle. Similarly, the other sugars probably can feed directly into the algaes metabolic processes as well, once they are inside the cell. It is not surprising then that the sugars get in, since it is well known that chlorella can utilize glucose or acetate as carbon sources (*10*).

Why and how the mannose produced the fastest doubling time is unknown at this time. In fact, unless one were to use labeled sugars, it is still possible that the sugars did not enter the cells at all, but caused their observed effect by some other, at the moment obscure, mechanism. Over the time this project was carried out (about 18 mo), we never encountered the production of the "red" form of the alga, which has been described in the wild. Attempts to use nitrogen or phosphorus starvation yielded very slow growth or "white" algae. These were generally higher in oil than the "green" alga, but not in the 75–90% w/w range described in the literature.

It is obvious from the studies that much remains to be learned regarding the nutritional requirements of this organism and how these and environmental factors effect growth and oil development.

Oil content of the cells grown on the various carbon sources still yielded cells that contained the 25–40 w/w range of oil, as normally found under laboratory conditions.

ACKNOWLEDGMENTS

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