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GROWTH AND PRODUCTION OF CELL CONSTITUENTS IN BATCH CULTURES OF *BOTRYOCOCCUS SUDETICUS*

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Key Word Index—*Botryococcus sudeticus*; Botryococceae; lipid production; protein and carbohydrate contents; mass production rates.

Abstract—The growth of, and the production of neutral lipids, carbohydrates and proteins by, the alga *Botryococcus sudeticus* in batch culture is described. The algal mass contains, during the stationary phase of growth, about 4.5% protein, 7.5% carbohydrate and 22.0% neutral lipid on a dry weight basis. Some physiological characteristics of this species are discussed.

INTRODUCTION

The freshwater alga *Botryococcus sudeticus* is often confused with *B. braunii* [1]. Indeed, the strain used in this study is classified under *B. braunii* species in the Cambridge collection.

B. sudeticus (Cambridge strain of *B. braunii*) produces very small amounts of hydrocarbons [2–5] in comparison to *B. braunii* strains [6–10]. This species is described by Lemmerman [10] who noted that the cells accumulate oil. Chodat [11] re-examined *B. sudeticus* and designated the alga as *Botryosphaera sudetica* in order to distinguish it from *Botryococcus* Kützing.

Information on the physiological properties of *B. sudeticus* is lacking. In this study data concerning the development of this strain are presented, along with some physiological notes.

RESULTS AND DISCUSSION

Protein, carbohydrate and lipid contents during growth

The pH of the medium increased during the first 2 weeks of the exponential phase of growth. When growth slowed down, the pH decreased and stabilized at about 8.1 (Fig. 1). The increase of pH can be linked to exponential growth because a new growth rate and new increase of pH were observed (data not shown) when the medium was replaced by fresh medium (pH 7.5) during the stationary phase.

The dry weight (Fig. 1) and protein (Fig. 2) curves were of the expected type and fitted the Gompertz growth curve [12]. Protein reached its maximal value at the fourth week and preceded increases in dry weight, which reached its maximal value after the tenth week. This dephasing in time could be due to the production of extracellular compounds, such as lipids and matrix compounds.

In general, it is known that the generation time of colonial matrix algae cannot be estimated easily because, as in the case of *B. sudeticus*, the cells are embedded in mucilage, and the numbers of cells in the colonies are not homogeneous. Dry weight measurements thus include not only the weight of cells but also those of mucilage and lipids confined to the matrix. Therefore, neither the number of colonies nor the dry weight are necessarily correlated with the number of cells.

During growth, *B. sudeticus* produced insignificant amounts of extracellular protein. Therefore, the total protein content could be used as a parameter for estimating the generation time. In this work, we found an initial generation time of about 2.2 days (53 hr) on a dry weight basis and about 1 day (25 hr) on a protein basis.

The kinetics of carbohydrate production did not fit a classic curve (Fig. 2) since it showed two phases. The first occurred between inoculation and week 4 (exponential

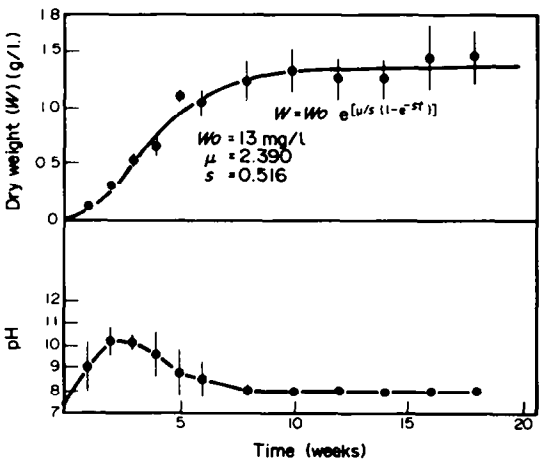

Fig. 1. Kinetics of dry weight production of batch cultures of *B. sudeticus* and changes in the pH of the medium. The dry weight curve fitted the Gompertz growth curve [12], where W_0 is the initial weight (inoculated), μ is the specific growth rate at $t = 0$, and s is a parameter that describes empirically the progressive reduction in the specific growth rate which may be ascribed to senescence.

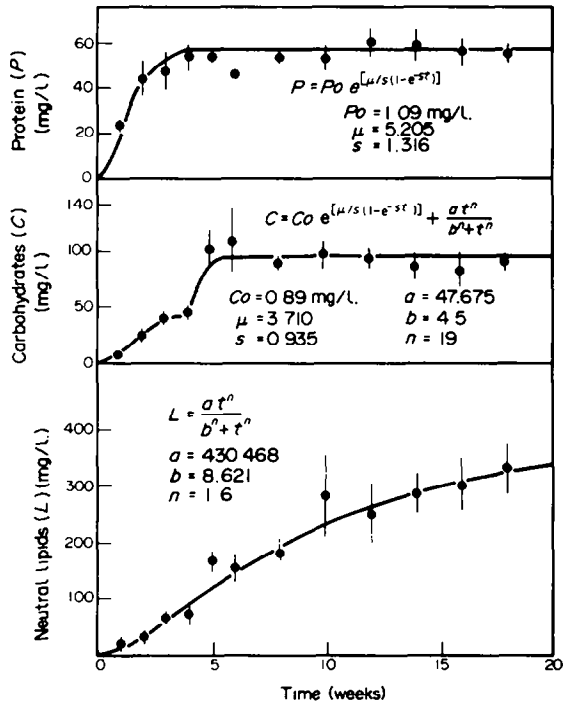

Fig. 2. Kinetics of production of protein, carbohydrates and neutral lipids during growth of *B. sudeticus*. The neutral lipids fitted the threshold response curve [12], where a is a constant (maximal value of L), b is the inflection-point constant (time of inflection), and n is a positive number, usually taken to be an integer. The protein fitted the Gompertz curve (see legend of Fig. 1).

phase of growth on a dry weight basis) with a leveling off of carbohydrate accumulation during the third and fourth weeks. The second occurred during the fourth week of culturing with stabilization after 5 weeks. TLC analysis of total carbohydrates from the first and second phases showed several differences in the composition (Table 1). In the first phase significant amounts of carbohydrates with low M_rs could be detected. Low M_r carbohydrates were, however, of minor importance during the second phase. This result could be due to a change in carbohydrate anabolism. During the first phase carbohydrate synthesis might principally result in the production of cell constitutive carbohydrates, whereas with longer growth periods the production of extracellular polysaccharides which form part of the colonial matrix or mucilage might predominate.

Neutral lipid accumulation (Fig. 2) followed different kinetics as compared to those of dry weight and protein. Neutral lipid production continued during the stationary phase of growth. The method employed for lipid determination extracted all the lipids except the phospholipids, which were precipitated in cold acetone, and the glycolipids, which were retained on the alumina column. TLC confirmed that only neutral lipids were present in the extracts after removal of the fatty acids.

The neutral lipids (Table 2) showed a very low content of hydrocarbon (*ca* 0.9% of dry weight). The components of the hydrocarbon fraction were identified by GC/MS

Table 1. Carbohydrate distribution in *B. sudeticus* during the first and second phases of growth

Carbohydrate R_f	Densitometer response (% total)* From 1 to 3 weeks of culture	From 5 to 7 weeks of culture
0.94	46.9–50.6	<0.09
0.84	10.8–13.2	7.2–8.9
0.81	13.6–14.6	20.5–24.3
0.61	1.3–1.4	<0.09
0.53	18.0–20.7	66.8–72.3
0.47	3.2–6.3	<0.09

*Sum of all spots, except spot of R_f 0.0.

Table 2. Neutral lipid composition of Cambridge strain of *B. braunii*

Compound		Relative amounts (% total neutral lipids)
Free fatty acids		
Hexadecatrienoic	16:3	0.05–0.15
Hexadecadienoic	16:2	0.06–0.09
Palmitoleic	16:1	0.06–0.08
Palmitic	16:0	0.44–0.53
Linolenic	18:3	0.02–0.04
Linoleic	18:2	0.12–0.18
Oleic	18:1	0.77–0.92
Stearic	18:0	0.02–0.05
Cadoleic	20:1	0.01–0.02
	Total	0.51–3.22
Hydrocarbons		
Heptadecene	$C_{17}H_{34}$	0.07–0.19
Heptadecane	$C_{17}H_{36}$	0.65–0.99
Docosene	$C_{22}H_{46}$	0.10–0.46
Squalene	$C_{30}H_{50}$	2.90–3.52
β-Carotene	$C_{40}H_{56}$	0.09–0.57
	Total	4.16–4.72
Sterols (esters)		
Fungisterol	$C_{28}H_{48}O$	0.43–1.68
Chondrillasterol	$C_{29}H_{48}O$	0.47–2.69
Dihydrochondrillasterol	$C_{29}H_{50}O$	0.49–1.25
	Total	1.51–5.59
Triglycerides		
Hexadecatrienoic	16:3	2.50–4.65
Hexadecadienoic	16:2	2.63–5.03
Palmitoleic	16:1	2.72–3.73
Palmitic	16:0	14.80–19.10
Linolenic	18:3	0.07–1.56
Linoleic	18.2	2.14–7.27
Oleic	18:1	45.06–50.73
Stearic	18:0	0.28–0.39
Gadoleic	20:1	0.77–1.31
	Total	84.43–92.12
Alcohols (phytol)	$C_{20}H_{40}O$	0.17–0.28

(except β-carotene, which was assayed spectrophotometrically). The main hydrocarbons were heptadecane, heptadecene, docosene and squalene, with squalene as the major component. The sterol compounds present occurred as esters of fatty acid with a range of chain length and a degree of unsaturation similar to those of the fatty acyl residues of the glycerides. The mass spectra of three major sterols showed that they were probably fungisterol (5α-ergost-7-en-3β-ol), chondrillasterol (24*S*-24-ethyl-5α-cholest-7,22-dien-3β-ol) and 22-dihydrochondrillasterol (24*S*-24-ethyl-5α-cholest-7-en-3β-ol). This fraction constituted from 1.5 to 5.6% of total neutral lipids. The triglyceride fraction was quantitatively the most important. The fatty acids of the triglycerides consisted principally of C_{16} and C_{18} fatty acids, both saturated and mono-, di- or tri-unsaturated. Oleic, palmitic and linoleic acids were predominant. The fatty alcohol content was *ca* 0.2% of total neutral lipids, and consisted mainly of phytol, which may have been accumulated as a result of chlorophyll degradation. Finally, the free fatty acid fraction, previously separated by column chromatography, showed the same range of chain lengths and degree of unsaturation as the fatty acids of the triglycerides (Table 2).

Several species of algae have been found to accumulate triglycerides, sometimes up to 80% of their dry weight [13, 14]. *B. sudeticus* produced triglycerides characterized by a relatively high content of unsaturated fatty acids and a low content of polyunsaturated fatty acids. The amount of phospholipids found in the total lipid extract (chloroform–methanol extraction) was about 4.6% of total lipids (1.85 ± 0.09 μg of P/g of lipids). This fact may be of potential significance in human nutrition.

Composition of algal mass and pigments

At the beginning of growth (1 week), the proteins represented 19.4% of the dry biomass but their contribution diminished rapidly at 5 weeks (4.9%). Apparently most protein had been synthetized during the first few days of growth. Carbohydrates represented about 7% of the dry weight during the first 4 weeks of growth, but between the fourth and fifth week the content of carbohydrates increased rapidly from 6.7 to 9.4%. These results support the proposition of the appearance of a second system for the synthesis of carbohydrates after the fourth week. Finally, the neutral lipid content increased from low values to *ca* 23% of the dry weight after 18 weeks of growth.

Analysis of the acetone extracts by lipophilic TLC showed the presence of chlorophyll a, chlorophyll b, β-carotene, lutein, antheraxanthin, violaxanthin and neoxanthin. This pigment distribution is typical for the Chlorophyceae. The total amount of chlorophylls increased up to 4 weeks then diminished slowly (Fig. 3). In batch cultures, when temperature and light intensity are kept constant, it is impossible to decide if the changes in chlorophyll content are due to exhaustion of the nutrient medium, to senescence of a part of the algal population or to both exhaustion and senescence.

The cessation of chlorophyll production might well be due to exhaustion of medium nutrients, and their disappearance to photo-oxidation and/or chlorophyll turnover and degradation. Carotenes accumulate with similar kinetics to chlorophylls; however, after a small drop, they reach a stable, final value (Fig. 3). Apparently carotenogenesis was not stimulated by nutrient deficiency.

Growth and production rates

The dry weight and production of cell constituents fitted the Gompertz or threshold response models [12] (see Figs 1 and 2). Production and growth rates (Fig. 4) were calculated from the derivative of the mathematical models. The rate of dry weight production had a maximal value at the third week of culture and was preceded by the protein rate (maximal value at the first week), and followed by the neutral lipid and carbohydrate rates. The carbohydrate rate showed a double threshold, one with a maximal value which was at *ca* 1.5 weeks and another at 4.5 weeks. As we mentioned above, the first step might be due to the synthesis of intracellular carbohydrates which coincides with the rate of protein synthesis, and the second step might be due to extracellular carbohydrate production (mucilage polysaccharides). The rate of neutral lipid production reached its maximum 3.4 weeks after inoculation. Thereafter the rate of neutral lipid synthesis continued at about 50% of its maximal capacity, whereas the rate of dry weight production had slowed down to zero.

In conclusion, we interpret the results obtained so far with respect to the channelling of the metabolically

Table 3. Composition of dry algal mass at different ages of culture

Age (weeks)	Amount (% dry wt)		
	Protein	Carbohydrates	Neutral lipids
2	13.82 (±2.62)	7.67 (±1.56)	9.39 (±3.19)
4	8.28 (±1.09)	6.73 (±0.97)	11.11 (±3.00)
6	4.45 (±0.14)	10.54 (±2.84)	14.90 (±2.57)
8	4.44 (±0.22)	7.35 (±0.53)	14.91 (±1.82)
10	4.08 (±0.43)	7.42 (±0.88)	21.56 (±5.57)
12	4.88 (±0.51)	7.60 (±0.73)	20.26 (±4.27)
14	4.76 (±0.57)	7.00 (±0.96)	23.19 (±2.82)
16	3.95 (±0.41)	5.82 (±1.13)	21.08 (±3.20)
18	3.89 (±0.33)	6.28 (±0.56)	23.09 (±3.22)

The standard deviation (in parentheses) was calculated from five values obtained in independent replicate experiments.

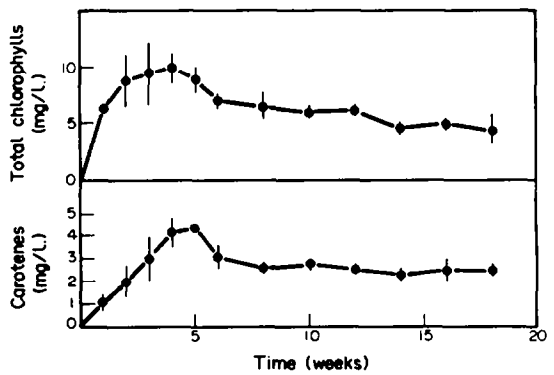

Fig. 3. Pigment production during the growth of *B. sudeticus*.

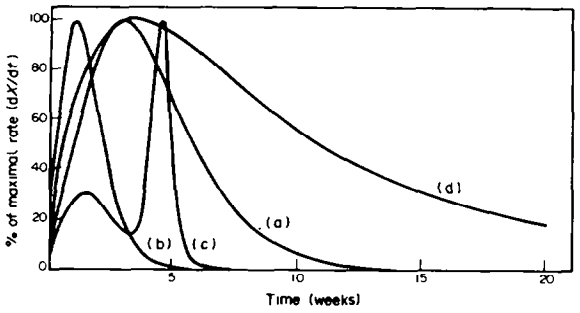

Fig. 4. Growth and production rates from derivative of the mathematical models (see Figs 1 and 2). (a) Dry weight rate (dW/dt) with a maximal value of 253.77 mg/week at week 3. (b) Protein rate (dP/dt) with a maximal value of 27.47 mg/week at week 1. (c) Carbohydrate rate (dC/dt) with a maximal value of 52.96 mg/week at 4.5 weeks. (d) Neutral lipid rate (dL/dt) with a maximal value of 30.43 mg/week at 3.4 weeks of culture.

produced energy into different cell compounds as follows: During the exponential phase of growth, most of metabolic energy is used for synthesis of cell constituents, especially protein and intracellular carbohydrate. However, when the exponential phase changes over to the stationary phase (slackening of growth), the energy is used predominantly for the synthesis of extracellular compounds (lipids and carbohydrates). It should be kept in mind that lipid production still continued after 20 weeks of culture.

EXPERIMENTAL

Algal culture. The alga was obtained from the Cambridge University collection (*Botryococcus braunii* Kutz No. LB 807/1; Droop 1950) and rendered axenic in our laboratory. It was grown in the following medium [wt (or vol)/l distilled H_2O]: KNO_3, 200 mg; K_2HPO_4, 40 mg; $MgSO_4 \cdot 7H_2O$, 30 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 30 mg; iron citrate, 0.5 mg; trace elements (in μg: Zn, 0.7; Cu, 0.2; Co, 0.2 and Mo, 0.2) and soil extract, 15 ml. The pH was adjusted to 7.5 with NaOH before autoclaving.

The alga was cultured at 23° and 3000 lx (16 hr illumination per day with daylight Sylvania tubes) in stirred 150 ml conical flasks. All expts were repeated 5 times. The cultures were tested for the presence of free bacteria by using 0.2% glucose agar medium.

Dry weight determination. The dry wt of algal mass was determined by filtration of 10–20 ml of culture through a tared Whatman 1.2 μ WCN filter. The filter with algae was then dried at 70° for 24 hr, cooled and weighed.

Lipid extraction and analysis. The culture (30 ml) was centrifuged at 20 000 *g* for 10 min. The algal pellet was then ground in a mortar with 3 g of fine granular Quartz and Me_2CO. After centrifugation at 6000 *g* for 5 min, the pellet was washed (× 6) with Me_2CO until free of lipid. The Me_2CO extracts were combined and, after measurement of its chlorophyll content by Arnon's method [15], the resultant soln was evaporated under vacuum at 50°. The dry residue was chromatographed on neutral alumina eluted with Et_2O. The Et_2O eluate, after estimation of its total carotene content (A 450 nm), was dried under N_2 and weighed. It was then fractionated on a KOH–silica gel column and the free fatty acids were separated according to the method of McCarthy and Duthie [16]. The fatty acid fraction was dried, weighed and then the acids were converted into their methyl esters (10% BF_3–MeOH) for analysis by GC/MS.

The neutral fraction was separated into hydrocarbons and other neutral lipid fractions on an alumina column successively eluted with hexane and Et_2O. The Et_2O eluate was fractionated by prep. TLC [silica gel, petrol (40–60°)–Et_2O–HOAc, 90:10:1], to give sterol esters (R_f 0.86–0.95), triglycerides (R_f 0.4–0.7) and long-chain alcohols (R_f 0.14–0.17). The compounds were located as follows. (a) general detection: 5% H_2SO_4 in EtOH at 180°; (b) sterol and sterol esters: $FeCl_3$ reagent [17]; (c) unsaturated molecules: OsO_4; (d) esters: method of Skidmore and Eternman [18]; (e) aldehydes and plasmalogens: 2,4-dinitrophenylhydrazine and Schiff reagent [19]; (f) fatty acids: bromocresol green indicator.

The hydrocarbon fraction was directly analysed by GC/MS, while the fatty alcohol and saponified sterol fractions were analysed after the preparation of trimethylsilyl (TMS) ether derivatives. Finally, the glyceride fraction was saponified and the fatty acids obtained were converted to methyl esters (10% BF_3–MeOH) and analysed by GC/MS.

GC/MS was performed on a Finnigan 9610, equipped with a 20 m × 0.32 mm column, type WCOT, OV-73 and hydrocarbon FID. The oven temp. was held for 1.2 min at 60° after injection (splitless) and was subsequently raised to 150° at 10°/min and then to 280° at 4°/min, at which temp. it was held for 30 min.

Protein and carbohydrate determinations. The lipid-free algal pellet (see above) was resuspended in 2 ml 0.5% NaOH and, after centrifugation, the pellet was washed (× 6) with H_2O. The supernatants were recovered and combined. The total carbohydrate content was estimated with anthrone reagent [20] and its composition was determined by TLC (silica gel, *n*-BuOH–MeOH–H_2O, 10:6:3). The carbohydrates were detected by spraying an acid–ethanolic soln of thymol and heating to 120° for 10 min. The TLC plates were quantified by densitometry using a Vernon photometer–integrator–enregisteur.

The protein content was estimated according to Lowry *et al.* [21] and the pigment distribution was determined by lipophilic TLC [22].

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