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EFFECTS OF GLUCOSE AND LIGHT ON CELLULOSE CONTENT OF *CHLORELLA PYRENOIDOSA*

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Key Word Index—*Chlorella pyrenoidosa*; Chlorophyta; unicellular algae; cellulose biosynthesis; cell wall insoluble polysaccharides; cultural conditions.

Abstract—Cultures of *Chlorella pyrenoidosa* (strain 395) were grown under photoautotrophic, mixotrophic, and heterotrophic conditions. Cellulose, protein, and cellulose/protein ratios were determined. Cellulose content per cell was correlated to the average cell size for each batch culture. Whereas mixotrophic cells had the highest amount of cellulose, the heterotrophic cells had the lowest and the photoautotrophic cells an intermediate amount. The photoautotrophic cells contained the highest amount of protein as compared to the heterotrophic cells which had the lowest amount and the mixotrophic cells which were intermediate. Comparison of the cellulose/protein ratios revealed a significant difference ($p < 0.05$) in the ratios of the photoautotrophic and mixotrophic cells and between the ratios of the mixotrophic and heterotrophic cells. The difference in the ratios of the photoautotrophic and heterotrophic cells however, was not significant. Correlation of the cellulose content per cell to its surface area indicated that more cellulose was found in the mixotrophic than in the photoautotrophic or heterotrophic cells.

INTRODUCTION

Chlorella pyrenoidosa, which is a unicellular green alga with photoautotrophic, mixotrophic, and heterotrophic modes of nutrition, has a cell wall composed of cellulose (and hemicelluloses) as the major structural polysaccharides. Cellulose is synthesized under all three conditions. It is not known if the amount of cellulose is constant or whether the organism responds to the cultural conditions imposed upon it by regulating the amount of the cellulose it synthesizes based on the availability of organic and or inorganic carbon sources.

RESULTS AND DISCUSSION

Table 1 shows the amounts of cellulose (% dry wt of the algal crop) in cultures grown under the three growth conditions. While the cellulose content of mixotrophic cultures were the highest, the heterotrophic cultures contained the lowest amount. In order to examine the possibility that the variation in cellulose content may have been a reflection of a change in the overall synthesis of the structural polymers, the protein content of the cultures was measured and compared with the cellulose produced (Table 1). Protein contents of the photoautotrophic cultures were the highest, whereas the heterotrophic cultures had the lowest amount of protein. Under the three conditions, the ratios of cellulose/protein did not remain constant.

The observed difference in the amount of cellulose formed by the cells grown under the three growth conditions may have also been due to the fact that each population (batch culture) contained cells of different sizes and hence possibly different cellulose contents. To examine this possibility, the cell size profile for each batch culture was determined and the average cell size and surface area/cell were calculated. The average amount of cellulose was then correlated with the average surface area per cell (Table 2). The data demonstrate that mixotrophically grown cells contained the highest amount of cellulose/unit surface area (cf. Table 1), whereas the heterotrophic cells had the lowest amount.

An increased cellulose content can be explained if two pathways for the synthesis of cellulose are assumed. One,

Table 1. Comparisons of the cellulose and protein contents and cellulose/protein ratios in cultures of *C. pyrenoidosa* grown photoautotrophically, mixotrophically, and heterotrophically

Growth condition	% Cellulose*	% Protein‡	Cellulose/protein
Photoautotrophy	1·09 ± 0·16 (9)†	45 ± 6·09 (6)†	0·02 (4)†
Mixotrophy	2·46 ± 0·18 (20)†	24·1 ± 1·33 (13)†	0·09 (6)
Heterotrophy	0·89 ± 0·09 (13)†	17·4 ± 2·04 (8)†	0·05 (5)

* Percentages of the dry wt of the algal crop. Values are means ± S.E. Figures in parentheses indicated numbers of determinations. † Significant difference ($P < 0.05$) as determined by the student "t" test. Hydrolysis of the alkali insoluble materials was incomplete and was corrected for 94% hydrolysis. ‡ Calculated from the Kjeldahl nitrogen content × 6·25.

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Table 2. Comparison of the cellulose content/unit surface area in cultures of *C. pyrenoidosa* grown photoautotrophically, mixotrophically, and heterotrophically

Growth condition	Calculated cellulose/cell* (mg × 10^{-12})	Calculated cellulose/unit surface area (mg × 10^{-12})
Photoautotrophy	112 ± 28 (9)	8·94 ± 1·88 (9)†
Mixotrophy	282 ± 67 (13)	21·11 ± 3·62 (13)†
Heterotrophy	29 ± 3 (13)	3·85 ± 0·57 (13)†

* Cellulose/cell was calculated from the percentages of cellulose content of lyophilized samples, cell number and cell wt. The surface area/cell μm^2 was derived from the average cell volume which was determined with the Coulter Counter Model B and was calculated as described in the Experimental.
† Significantly different from each other ($P < 0·05$) as determined by the student's "t" test.

a photosynthetic pathway, in which the generated CO_2 from the metabolism of glucose is reassimilated in the presence of light for the synthesis of monosaccharides, some of which may then be utilized for the polymerization into cellulose. A second pathway may involve direct utilization of the exogenous glucose into cellulose. Thus, a common glucose pool for the synthesis of cellulose may be assumed, similar to that suggested by Schramm *et al.* [1] for *A. xylinum*. Therefore, in the heterotrophic cultures the first pathway is eliminated whereas in the photoautotrophic cultures the second is. Studies of Neish [2] in wheat indicated that C-1 as well as other carbons of the cellulose monomer became labeled when radioactive glucose, ribose, allose, or sedoheptulose were used as substrates. Neish suggested that this may be due to the formation of labeled CO_2 followed by reassimilation under photosynthetic conditions. If this is the case with *Chlorella*, then the observed increase under mixotrophy could be accounted for by increased synthesis via both pathways.

The low cellulose content of the heterotrophic cells (Table 1) may be caused by the cells relying on exogenous glucose as a source of energy and as a precursor for the synthesis of all of their biological materials including cellulose. This could compel the cells to expend a minimal amount of energy for cellulose synthesis but a large portion of energy for maintenance. A higher amount of glucose uptake by these cells (data not included) seems to support this possibility. With respect to the amount of cellulose our heterotrophic cells are in contrast to those of Prince [3] who reported thickening of cell walls as assessed by staining cells for cellulose.

Under photoautotrophy, 1·09% (0·16 S.E.) cellulose was obtained. Northcote *et al.* [4a] have reported 2·1% cellulose. However, these authors had grown the cultures under sunlight and measured cellulose gravimetrically. Also, their cellulose fraction was found to contain other sugars in addition to glucose. In comparison with other algae, [4–9] *Chlorella* appears to have a lower cellulose content. In our study, the method of Northcote *et al.* [4a,b], was used with slight modification to extract cellulose. The majority of our cellulose samples contained only glucose. However, a few samples gave, upon hydrolysis, spots with R_f values different from those of glucose, ribose, arabinose, xylose, or cellobiose. The possibility of the oxidation of glucose monomers in the presence of strong acid is likely. Kjeldahl nitrogen determination of cellulose indicated the presence of only a small amount (0·61%) of nitrogen. Contamination with cellular proteins and the presence of aminosugars cannot be excluded.

While it has been shown that *Chlorella* cells have different metabolic activities at different stages of their life cycle [5,6], it is also known that the size of cells also varies. We thought it is more practical and far superior to relate a property as a function of cell size rather than cell age [7]. Despite several important advantages, the disadvantage of our experimental model to calculate cellulose per cell lay in the fact that this alga divides into 2, 4, 8 or more daughter cells. Such cells may remain within the mother cells or attached to one another after they are released. If so, such clusters may possibly be counted as one cell in the Coulter counter. Thus, the interpretation of the reported values for cellulose per cell must take this point into consideration. The ideal situation would be to use a synchronously dividing culture. Unfortunately, our preliminary attempts to achieve a perfectly homogeneous population of the mass cultures failed. However, the cell size in our batch cultures ranged from 3–6 μm in diamater. The higher values of cellulose per unit surface area in mixotrophic cells (Table 2) may not necessarily reflect an increase in cell wall thickness, for the increase in cellulose may have been accomplished by a similar increase in hemicellulose. A second possibility is that the observed increase in cellulose per unit surface area may result from the cellulose fibers becoming tightly packed. Thus, in the heterotrophic cells with less cellulose per μm^2 there may be less packing than in cells with more cellulose. This could occur without any thinning of the cell wall in the heterotrophic cells. Griffiths and Griffiths [8] have examined the heterotrophic cells of *C. vulgaris* with an electron microscope and reported no thinning of the cell wall. However, Prince [3] observed that heterotrophic cells of *Chlorella* had thicker cell walls than photoautotrophic cells. Considering that the stain used by Prince [3] is now considered non-specific for cellulose and that the observations made with the light microscope were nonquantative, the implications of the latter report are questionable.

Observations in our study seem to give support to the statement made by Herbert [9] that "changing the external environment, as by placing a cell in a different medium, will inevitably cause a complex shift in the concentration of all components of the internal environment, which in turn will react upon the rates of synthesis of the various large molecules, decreasing some rates and increasing other, thus altering the ratios of their steady state concentration; in other words, changing the chemical composition of the cell".

The evidence presented indicates that the amount of cellulose varies in response to the cultural conditions imposed upon the cells. A similar variation was also observed in the protein contents. Based on these findings, therefore, certain predictions can be made with regard to the chemical composition of the cells under specified growth conditions.

EXPERIMENTAL

Stock culture. Axenic cultures of *C. pyrenoidosa* strain 395 were obtained from the Indiana University culture collection of algae (Starr [10]) and maintained on Bristol's mineral salts agar, as modified by Bold, and supplemented with Trelease's

trace elements [10] at pH 7 and incubated under a dark-light cycle of 12 hr each at an intensity of 100 lx at 20°.

Growth conditions. (*a*) *Photoautotrophy.* Mass cultures were grown bacteria free in 2 l. flasks containing the above medium. 4–5 day old liquid cultures were used as inocula. Cultures were aerated with 5% CO_2 in air, at a flow rate of 0·1 SCFH, which was dried and filtered through Millipore filters (0·45 μm) before entering the flasks. Illumination was provided by 4 20 W white fluorescent lamps, placed on the sides and above the flasks, to give a 100 lx intensity. Cultures were stirred constantly, and examined periodically for bacterial contamination. At the end of the incubation period, samples were taken for cell count and wt determinations. The cultures were harvested and centrifuged at 14600 *g* for 10 min. The packed cells were washed and lyophilized. (*b*) *Mixotrophy and Heterotrophy.* Culture medium was supplemented with 1% glucose prefiltered through a 0·22 μm Millipore filter. Mixotrophic cultures were incubated in the light and heterotrophic cultures in the dark.

Nitrogen determination. N contents of lyophilized cells were measured in duplicate or triplicate using the Kjeldahl method. The % recovery was tested with urea and $(NH_4)_2SO_4$ solns and was found to be 98–99%. Samples of cellulose were also tested for their N contents.

Cellulose extraction and hydrolysis. Considering the available techniques for the extraction and assay of cellulose [2–5,11–27] we defined cellulose as that fraction which is insoluble in boiling NaOH soln but soluble in a 72% w/w H_2SO_4 which upon hydrolysis gives a similar absorption spectrum to that of glucose. To extract cellulose, lyophilized samples were refluxed in MeOH and Me_2CO for 6 hr. Depigmented cells were refluxed in 3% NaOH at 100° for 6 hr. Insoluble material was washed until neutral and then treated with 6 N NaOH at room temp. for 6 hr [4]. Insoluble material ("α-cellulose") was washed until neutral and then dried. Cellulose was hydrolyzed according to ref. [29] with slight modification in that the H_2SO_4 was dil to 2% instead of 1% and reflux time increased to 24 hr. Purified wood cellulose was used as a reference. The effect of time on the degree of hydrolysis was also studied. Because after 24 hr as much as 94% of the cellulose was hydrolyzed, this period was used for the hydrolysis of cellulose of algae. The cellulose hydrolysates were neutralized and tested for the presence of reducing sugars using Bial's orcinol reagent [30]. Glucose was used as a standard. Standard curves were prepared for each individual expt.

Cell number, size, and wt determinations. Cell number and size distributions of batch cultures were measured with a Coulter counter [31] and an automatic model plotter. A 70 μm aperture was used. Optimum "pulse" patterns were obtained at reciprocal aperture current and amplification settings of 1 and 1. A 0·85% soln of NaCl, filtered through a 0·22 μm Millipore filter, was used as the diluent. Duplicate cell counts were taken and corrected for both background and "coincidence loss". Precision of counts was 1%. For cell size determination, the instrument was calibrated with red blood cells (4C Hematology Reference Control) having an average vol of 87 μm^3. The calibration factor (vol equiv. for each threshold division) was 1·95. Hence, vol of 7·8–195 μm^3 (corresponding to diameters of 2·4–7·1 μm) could be measured at a threshold setting of 4–100. Assuming cells were perfect spheres, the vol and corresponding diameters were calculated for each "window" with the use of a computer program. From the size distribution histograms, the average surface area per cell was also calculated using a computer program.

For cell wt determinations, known aliquots of cultures were aseptically filtered through predried and tared glass fiber disks (grade 984H, size 4·25 cm). Disks were washed and dried at 60° for 3 days and then weighed. Weight per cell was calculated from this and the knowledge of cell numbers in each aliquot.

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