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Effect of Nitrogen on Polysaccharide Production in a *Porphyridium* sp.

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Porphyridium **cultures grown on either nitrate or ammonium as the nitrogen source showed similar patterns of growth and cell wall polysaccharide production. The effect of nitrogen on growth and cell wall polysaccharide production was studied by applying three regimens of supply: batch mode, in which nitrate was supplied at the beginning of the experiment and became depleted at day 6; continual mode, in which nitrate was added daily; and deficient mode, in which the cells were cultured in a nitrate-free medium. Growth was similar in the batch- and continual-mode cultures, whereas it was totally inhibited in the deficient-mode culture. Polysaccharide content (per volume) was highest in the batch-mode culture and lowest in the deficient-mode culture. However, polysaccharide production per cell was similar in the continual- and deficient-mode cultures, the highest value being found in the batch-mode culture. In addition to its effect on polysaccharide content, nitrogen affected the polysaccharide distribution between soluble and bound polysaccharides. In the deficient-mode culture, most of the cell wall polysaccharide was dissolved in the medium.**

The polysaccharide of the marine unicellular red alga *Porphyridium* sp. has recently gained attention owing to its special properties, as expressed by its resistance to various stresses; i.e., the viscosity of the polysaccharide remains constant over a wide range of pHs, temperatures, and salinities (3, 5, 11). These properties make it suitable for various commercial applications in which xanthan gum is traditionally used. The algal cells are encapsulated in an envelope of sulfated polysaccharide, the external part of which becomes dissolved in the medium (4, 10). Production of the cell wall polysaccharide is enhanced during the stationary phase of growth, as has also been found in other algae producing extracellular polysaccharides, e.g., *Chlamydomonas mexicana* (8).

Environmental conditions are known to affect partitioning of the various photosynthates; e.g., in immobilized *Porphyridium cruentum* cells depletion of nitrate from the medium increased the production of the soluble polysaccharide (13). Enhanced cell wall polysaccharide production in response to nitrate starvation was also shown in free *Porphyridium* sp. cells (1). Similarly, carbohydrate accumulation under nitrate starvation conditions was shown in a *Chlorella* sp. (12) and in some marine algae (14). In *Porphyridium* sp. strain UTEX 637, carbohydrate accumulation seems to take place at the expense of protein production (3).

The general purpose of our research is to optimize the production of cell wall polysaccharide in this *Porphyridium* sp. In this study, the effect of the source of nitrogen and its mode of supply on polysaccharide production and on the distribution of polysaccharides between the cell and the medium of this *Porphyridium* sp. was studied.

MATERIALS AND METHODS

Porphyridium sp. strain UTEX 637 from the Culture Collection of Algae at the University of Texas, Austin, was grown in batch culture under sterile conditions. The cells were cultured in 1-liter columns 6 cm in diameter at 24 ± 1°C in artificial seawater by the method of Jones et al. (7). The cultures were illuminated continuously with fluorescent cool-white lamps at an irradiance of 150 microeinsteins m^{-2} s^{-1}. The medium was aerated with sterile air containing 3% CO_2.

Nitrogen nutrition. The nitrogen source was either ammonium or nitrate. Ammonium was supplied as either NH_4HCO_3 (10 mM) or NH_4NO_3 (5 mM). Nitrate was supplied in the form of the potassium salt as follows: (i) addition of nitrate (10 mM KNO_3) at the beginning of the experiment only (batch); (ii) daily supply of nitrate to restore the concentration to its original level (continual); (iii) no addition of nitrate to the medium (deficient).

Cell number. Cells were counted with a cell counter (Analysis Instrument, Stockholm, Sweden). During growth, 5- to 30-ml samples were withdrawn aseptically and centrifuged at 20,000 × *g*. The pellet was used for protein and carbohydrate determinations, while the supernatant was used for determination of nitrate, ammonium, and soluble cell wall polysaccharide.

Dry weight. A cell sample was washed twice with water at pH 4, filtered through a preweighed 0.45-μm-pore-size filter, centrifuged, oven dried at 70°C for 24 h, and weighed.

Photosynthetic potential. A cell sample was centrifuged, diluted with 5 ml of fresh medium containing $NaHCO_3$ (0.5 mg ml^{-1}) to a concentration of 10^7 cells ml^{-1}, and illuminated at a light intensity of 140 microeinsteins m^{-2} s^{-1}. The oxygen evolution rate was measured with an oxygen electrode (Rank Brothers).

Protein. Total cell protein was determined in the alkaline hydrolysate (0.5 N NaOH) of the cells by the technique of Lowry et al. (9).

Polysaccharide. To determine polysaccharide content, the method of Adda et al. (1) was used. For the bound fraction (which also includes starch and soluble sugars), the harvested cells were hydrolyzed in sulfuric acid (1 N) in a boiling water bath for 1 h. The cell debris was removed by centrifugation, and total sugars were determined in the supernatant by means of the phenol-sulfuric acid method (6). For the soluble fraction, the sugar content was analyzed in the cell-free medium by the phenol-sulfuric acid method. Total polysaccharide is thus the sum of the polysaccharide contents of the bound and the soluble fractions.

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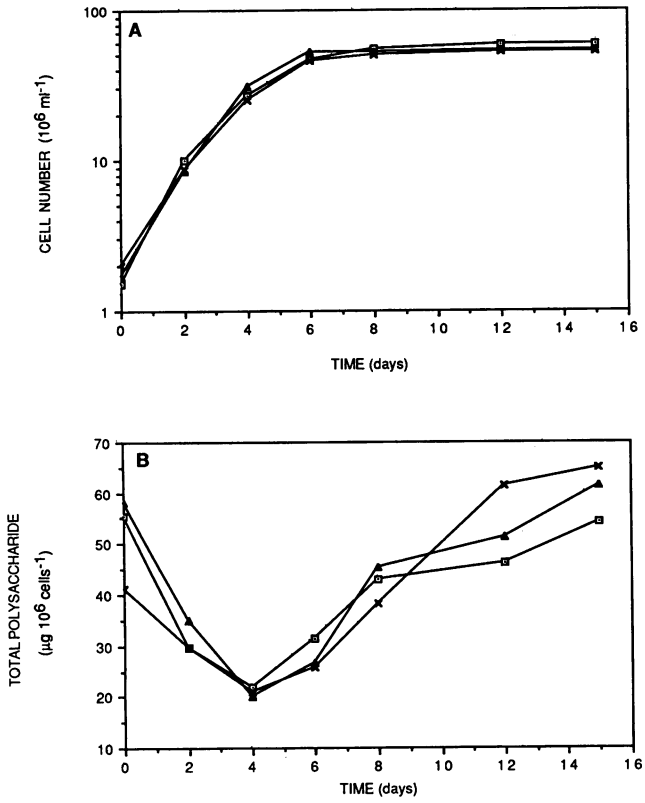

FIG. 1. Effect of nitrogen source on the growth (A) and polysaccharide production (B) of *Porphyridium* sp. strain UTEX 637. Total polysaccharide is the sum of the polysaccharide contents of the bound and soluble fractions. Symbols: ⊡, NH_4HCO_3; ▲, NH_4NO_3; ×, KNO_3.

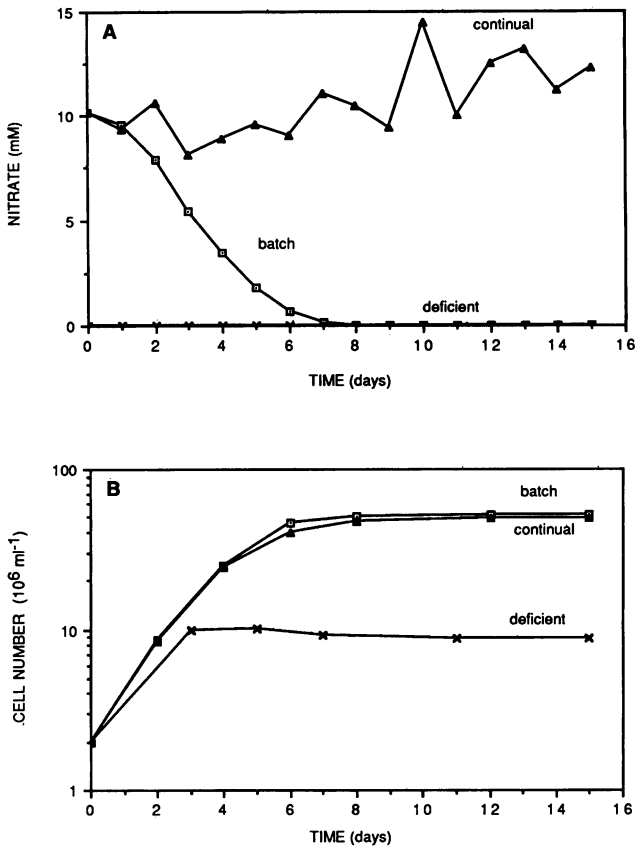

FIG. 2. Effect of the mode of nitrogen supply on the nitrate content in the growth medium (A) and on the growth of *Porphyridium* sp. strain UTEX 637 (B).

Starch. Starch content was measured in cell samples (10^7 cells) after freezing the cells in liquid nitrogen and thawing them three times, adding 0.06 N K_2HPO_4, boiling the suspension for 20 min, and centrifuging it (10 min at 6,000 × *g*). Starch content in the supernatant was measured by using the I-KI method with potato starch (Merck) as a standard.

Nitrate content. The nitrate content of the medium was determined by the method of Greenberg et al. (2).

The results of one of three similar experiments are presented in each of the figures.

RESULTS

Porphyridium sp. strain UTEX 637 grown in batch culture responded in the same way to nitrate and to the two ammonium salts with respect to both the growth pattern (Fig. 1A) and the total cell wall polysaccharide content (Fig. 1B).

As expected, the nitrate content decreased rapidly in the medium of the batch-mode cultures, approaching zero on day 6, whereas in the cultures supplied with KNO_3 daily it stayed at a high level with daily fluctuations. In the nitrate-free medium, the nitrate content was obviously essentially zero (Fig. 2A). Despite the difference in nitrogen content in the medium, the growth, as determined by cell number, was similar in the batch-mode and continual-mode cultures, being logarithmic until about day 6 of growth and then levelling off. Growth of cultures in the nitrate-deficient medium was almost completely inhibited (Fig. 2B).

Rates of photosynthesis were found to decrease over time in all cultures (Table 1). In the batch-mode and continual-mode cultures photosynthetic rates were similar in the logarithmic phase, with batch-mode cultures showing decreasing rates later in the stationary phase of growth. The deficient-mode cultures had the lowest rates of photosynthesis.

The influence of the mode of nitrate application on growth was less marked than that on the protein level, which was lower in the batch-mode culture than in the continual-mode culture. In the continual-mode culture protein content continued to increase throughout the experimental period (12 days), whereas in the batch-mode culture protein content peaked at the end of the logarithmic phase of growth and

TABLE 1. Rates of photosynthesis of *Porphyridium* sp. strain UTEX 637 during growth as affected by the mode of nitrate supply

Day	Rate with the following mode of nitrate supply[a]		
	Batch	Continual	Deficient
0	0.116	0.116	0.108
3	0.118	0.117	0.016
5	0.104	0.082	0.008
7	0.108	0.113	0.008
10	0.086	0.098	0.008
12	0.063	0.110	0.008

[a] Milligrams of O_2 per liter per minute per 10^6 cells.

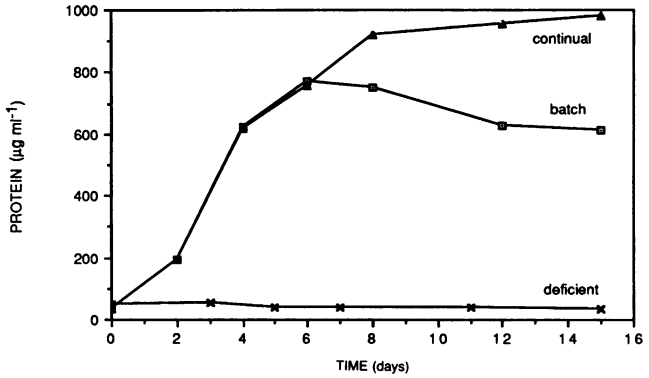

FIG. 3. Effect of the mode of nitrogen supply on the protein content of *Porphyridium* sp. strain UTEX 637.

then declined (Fig. 3). As expected, in the deficient-mode culture protein content was very low.

With respect to the cell wall polysaccharide, the amount in both the bound and the soluble fractions, on a culture volume basis (Fig. 4A and B), increased somewhat during the logarithmic phase of growth and even more so during the stationary phase in both the batch-mode and the continual-mode cultures. In both treatments, the bound fraction was much larger than the soluble fraction. Thus, nitrate supply affected the polysaccharide distribution between bound and soluble fractions. The pattern of increase of the soluble fraction was similar in the batch-mode and continual-mode cultures, whereas the bound fraction was relatively lower in the batch-mode culture. In the deficient-mode culture both fractions were very small; the bound fraction did not increase at all during the experiment, and the soluble fraction, which constituted the new polysaccharide produced, increased slightly during the stationary phase. In all the cultures, the ratio of soluble to bound fractions decreased over time (from day 0 to day 15) from 3.7 to 0.16 and 0.21 in the batch- and continual-mode cultures, respectively, and from 1.20 to 0.39 in the deficient-mode culture.

When total cell wall polysaccharide was expressed on a per cell basis, a different pattern was obtained (Fig. 4C). The highest amount was found in the batch-mode culture, whereas the other two cultures had lesser, similar amounts.

Starch content (Table 2) was relatively low, i.e., 5.8% of the bound polysaccharide in the batch and continual treatments on day 3, decreasing to 1.2 and 1.6% in the two treatments, respectively, on day 7. The decrease over time may stem from the fact that the cells used for initiation of the culture were taken from the stationary phase. The highest starch content was in the nitrogen-deficient cells, i.e., 8.2% of the bound polysaccharide on day 3, increasing to 16.2% on day 7.

DISCUSSION

The stationary phase of growth begins when certain factors, e.g., nutrients, becoming limiting. When nitrate was added daily to the medium, it did not prevent the onset of the stationary phase. Thus, it seems that some determinants other than nitrogen were the limiting factors that caused termination of the logarithmic phase under these conditions.

When nitrogen was depleted from the medium from the time of inoculation, growth and photosynthesis practically stopped, but both starch and cell wall polysaccharide synthesis continued. Since protein content did not change, it

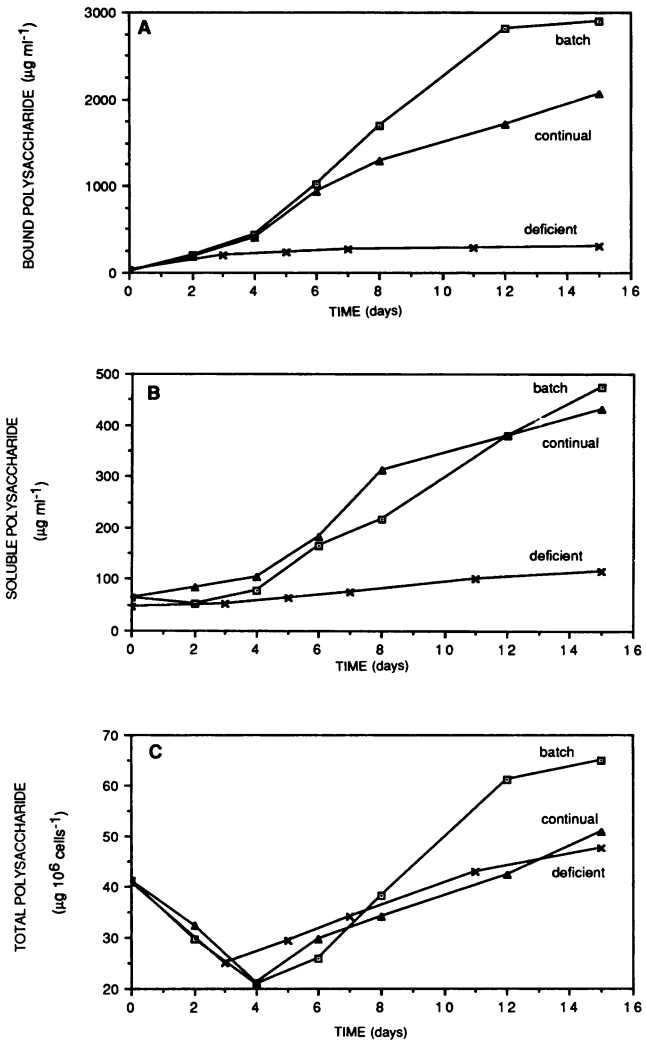

FIG. 4. Effect of the mode of nitrogen supply on *Porphyridium* sp. strain UTEX 637 cell wall polysaccharide. Shown are the bound (A) and soluble (B) polysaccharide on a per volume basis and the total polysaccharide content (sum of the polysaccharide contents of the bound and soluble fractions) (C) on a per cell basis.

seems that polysaccharide synthesis depends either on preexisting enzymes having a slow turnover or on newly synthesized enzymes, although the amount of them is very small. Almost all of the cell wall polysaccharides produced were released into the medium. Whether the composition of the polysaccharide produced under conditions of nitrogen limitation is identical to that produced under conditions of

TABLE 2. Starch content in *Porphyridium* sp. strain UTEX 637 during growth as affected by the mode of nitrate supply

Day	% of the bound polysaccharide[a] with the following mode of nitrate supply		
	Batch	Continual	Deficient
3	5.8	5.8	8.2
7	1.2	1.6	10.3
12	4.1	5.2	16.2

[a] The amount of the bound polysaccharide at day 3 was 14.5, 14.3, and 27.0 mg 10^6 cells^{-1} in the batch-mode, continual-mode, and deficient-mode treatments, respectively.

adequate nitrogen supply is currently under investigation in our laboratory.

Despite the low metabolism of the nitrogen-deficient cells, they direct most of their energy to synthesizing cell wall polysaccharides, which are then released into the medium. This fact might indicate the importance of the soluble fraction for the survival of the cells. Its role, however, still needs to be elucidated. The effect of nitrogen deficiency described above is not limited to the marine *Porphyridium* species studied here; it has also been found in the freshwater species *P. aerugineum* (data not shown).

Maximum polysaccharide production (per milliliter) was obtained in batch culture. Hence, for maximal polysaccharide production a batch mode of operation is recommended. Under these conditions, only about 20 to 30% of the total polysaccharide produced by *Porphyridium* sp. strain UTEX 637 was dissolved in the medium. Thus, when a very pure polysaccharide is required, nitrate limitation is recommended. The production process of choice may thus be dictated by our ability to control the distribution of the polysaccharide fractions. It is worth noting that the relative amounts of the polysaccharide fractions constitute an important factor in choosing the method of extraction. For collection of the soluble fraction only, in which case very pure polysaccharide is required, separation between cells and supernatant is the first step. In contrast, for collection of both fractions together this separation step is not needed. The cells containing the bound polysaccharide are precipitated from the medium together with the soluble polysaccharide, followed by extraction of total polysaccharides.

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