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**Agroproduction of Vicoelastic Biopolymers from
Unicelular Red Algae**

S. Arad, J. Ramus

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AGROPRODUCTION OF VISCOELASTIC BIOPOLYMERS
FROM UNICELLULAR RED ALGAE

Final Report
September 1985 - June 1989

Shoshana (Malis) Arad¹ and Joseph Ramus²
Principal Investigators

¹The Institute for Agriculture & Applied Biology, Ben-Gurion University of the
Negev, Beer-Sheva, Israel

² Duke University, Marine Laboratory, Beaufort, N.C., U.S.A.

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Investigators' Names
(Principal listed first)

Shoshana (Malis) Arad

Joseph Ramus

Investigators' Institutions

The Institutes of Applied Research
Ben-Gurion University of the Negev
Beer-Sheva, Israel

Duke University
Marine Laboratory

Beaufort, N.C., U.S.A.

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Shoshana Arad
Signature
Principal Investigator

[Signature]
Signature
Institution's Authorizing Official

מש. אמיר, פנאל
המח. קה לחוזי פנאל

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c. ABSTRACT

The effect of nitrogen and sulfate starvation on polysaccharide production in the red unicellular algae *Porphyridium* and *Rhodella* were studied using various nutritional regimes. It was found that under nitrogen starvation growth was inhibited, polysaccharide production was enhanced, and the distribution of the polysaccharide between the attached and dissolved fraction was shifted towards the dissolved fraction. Sulfate starvation also caused inhibition of cell growth and enhanced polysaccharide production. The polysaccharide produced under sulfate starvation contained about half of the sulfate content present under standard conditions. Cultivation of various red microalgae outdoors in polyethylene sleeves was compared with that in open ponds with respect to growth and polysaccharide production. It was found that in both growth systems outdoors growth and production was higher under continuous operation than in batch. In continuous cultures the respective daily yields of dry matter and polysaccharides were 17.7 and 7.4 g/m² in sleeves vs. 7.6 and 2.4 g/m² in ponds. It seems to us that polyethylene sleeves have the following advantages over ponds: better light availability as a result of larger surface/volume ratio; a higher rate of heating and cooling; prevention of evaporation; a better mixing system, which prevents oxygen accumulation; operation in small units, thus avoiding collapse of large volumes; relatively simple means of operation; and protection against contamination.

For chemostat-controlled photobioreactor, the per capita polysaccharide copolymer production rate (cellular yield) is the greatest at maximum specific growth rate ($U_{\max} = 1.44/\text{d}$) and highest feedstock concentration (440 μM nitrate-N). Standing cell concentrations are the highest at the lowest volumetric turnover rates, i.e., at zero flow. The strategy for maximum volumetric yield becomes one of balancing standing cell concentration and per capita production rates. The compromise of these two parameters results in maximum volumetric yields at intermediate volumetric turnover rates ($U = 0.6 - 0.9/\text{d}$). For cell populations in exponential phase growth, photosynthetic capacity ($P_{\max}$) is high, and the newly synthesized polysaccharide is diluted by cell replication, thus per capita polysaccharide concentrations remain low. In stationary phase, cell replication ceases, $P_{\max}$ declines, newly synthesized polysaccharide accumulates, and per capita concentration increases. However, the quantitative partitioning of photosynthetically-fixed C remains the same in both replicating and non-replicating cells. Thus, in aged batch cultures per capita and volumetric polysaccharide concentrations are very high, while in continuous cultures the concentrations are considerably lower.

d. OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

1. To optimize production physiology of the viscoelastic biopolymer from *Porphyridium*
2. To scale up outdoors cultivation for production of polysaccharides.

e. BODY OF REPORT (follows page d.)

f. DESCRIPTION OF COOPERATION

The project proposal stated clearly the partitioning of research goals to the Ben-Gurion and Duke labs, respectively. During the project period, the Co-Principal Investigators made reciprocal visits, two each, to the collaborating laboratory. Data was reviewed, and project goals were focussed. Further, the Co-Principal Investigators communicated frequently by mail, telephone, messenger-of-convenience, BITNET and FAX to review data, assess progress, and produce reports.

The Co-Principal Investigators organized and hosted an international workshop entitled "Polysaccharides from Microalgae: a New Agroindustry", 28 October - 1 November 1987, Beaufort, N.C. The workshop was funded jointly by the BARD Fund and the North Carolina Biotechnology Center. A 143-page proceedings was published, summarizing the data offered and the conclusions of the symposium.

g. EVALUATION OF RESEARCH ACHIEVEMENTS

The overall goal of this project was to develop the technology necessary for the agroproduction of viscoelastic biopolymers from unicellular red algae. Efforts were therefore focussed on two specific areas:

- 1) model pilot-scale experiments directed to enhance yield of exocellular polysaccharide copolymers, and
- 2) scale-up and transfer to outdoor cultivation.

Considerable progress was made on both fronts. The first effort determined patterns of C-partitioning during all phases of cell growth and a strategy to satisfy the dual requirements of biomass and turnover. The second effort transferred the technology to relatively large-volume systems in the desert environment.

As the Year 2000 approaches, agriculture is searching for new products and new markets. In both the U.S. and Israel it is being realized that fine biochemicals from microalgae correspond to this need. The development of new polysaccharides from microalgae will help replace the conventional hydrocolloids that are extracted from macroalgae. Macroalgal-based industries are labor intensive, subject to the whims of nature and the erratic economies of developing nations. Cultivation of microalgae for production of hydrocolloids can thus provide a source which has the element of reliability. Moreover, the efforts for developing microalgae cultivation contribute to the emerging field of aquaculture. In order to improve polysaccharide production in red microalgae, C-metabolism and mechanisms of C-partitioning must be understood. The ability to selectively control these processes, by either genetic or physiological manipulations will enable the improvement of exopolysaccharide production. The improvement of polysaccharide yield per unit cell will increase the yield per unit area. The results of the physiological studies are applied to outdoor systems for maximal production.

The development of alternative means for outdoor cultivation of microalgae is crucial for the development of the whole area of biochemical production from microalgae.

The advancement achieved in this research combining physiological results with outdoor cultivation are applied now on large scale in the southern part of Israel.

Ben-Gurion University TeamTechnical staff

Raisa Glozman

Sarah Matyash

Students involved

Yael (Brown) Lerental

Orit (Dahan) Friedman

Avi Rotem

Editing

Inez Mureinik

Dorot Imber

Typing

Seemah Shemesh

Marion Milner

**CONTINUOUS CULTIVATION OF *PORPHYRIDIM* SP.
IN POLYETHYLENE SLEEVES**

**Ephraim Cohen and Shoshana (Malis) Arad
The Institutes for Applied Research,
Ben-Gurion University of the Negev**

MANPOWER

Ms. Sara Matias - technician

Ms. Michal Ucko - M.Sc. student

Ms. Yael (Brown) Lerental - MSc student

This report was edited by Ms. I. Mureinik and typed by Ms. M. Milner.

ABSTRACT

Large-scale outdoor cultivation of algae in open ponds suffers from low productivity due to low light availability, evaporation, temperature fluctuations, and contamination. Hence, there is a tendency to use closed systems rather than open ponds. The polyethylene sleeve is one such closed system. When the red unicellular alga *Porphyridium* was grown continuously in polyethylene sleeves, it produced two to three times more biomass and polysaccharide per volume unit than when grown in open ponds. These results encourage the continuation of this research towards scaling up of the system.

Key words: Red algae, *Porphyridium*, continuous culture, open ponds, polyethylene sleeves, bioreactor, polysaccharide

BACKGROUND

Large-scale cultivation of microalgae is usually performed outdoors in open ponds - raceways - that are stirred with paddle wheels. In recent years it has become evident that there are a number of disadvantages inherent in this method:

1. Limited light availability to the cells has been demonstrated in various types of pond at different geographic locations (Goldman 1979). At only a few centimeters below the surface of the ponds the water is almost dark, so that photosynthesis is inhibited, whereas at the surface high light intensity causes photoinhibition (Powels 1984).
2. Various algal species have different optimal temperatures for growth and production (Richmond 1986). Temperature fluctuations, especially in the desert significantly affect growth and production. During the summer growth is inhibited at midday because of the high pond temperatures that can reach 40°C. At night there is a loss of biomass due to respiration. In the winter, night temperatures are low, and the temperature increase during the day is slow. Thus, during the effective photosynthesis hours temperature becomes the limiting factor.
3. Evaporation in ponds in hot areas that are suitable for algal growth can reach 2500 mm per year (Richmond 1986). This high evaporation not only results in high water losses, but also causes the cultures to be exposed to increasing salinities which can inhibit growth.
4. Contamination by bacteria, fungi, protozoa or undesired algae is one of the main problems in outdoor algal cultivation. The contaminating micro-organisms can cause rapid collapse of large volumes of the ponds and affect product quality (Canter & Lund 1968).

These problems and others that have arisen in recent years have resulted in a tendency to change from open to closed systems. A variety of closed systems have been tried out all over the world (Chaumont et al. 1987, Dicorato 1980). We have

recently begun operating a closed system composed of polyethylene sleeves for outdoor cultivation of the alga *Porphyridium* sp. grown in batch culture (Cohen & Arad 1989). Our system has the following advantages over open ponds:

1. higher growth and production
 2. higher light availability
 3. prevention of evaporation
 4. improved heating and cooling rates so that cultivation can be continued throughout the year
 5. improved maintenance of contamination-free conditions
 6. prevention of large losses of algal cultures due to collapse of the cultures (this is achieved by separation of the culture into small units)
 7. simplicity of operation
- And the disadvantages are: ...*

The results obtained so far indicate that the development and scaling-up of the system is certainly worthwhile. This is currently under way in our laboratory.

In this report, we describe the continuous cultivation of *Porphyridium* sp. outdoors in order to determine potential for growth and production.

MATERIALS & METHODS

Microalga and Growth Conditions

Porphyridium sp. (UTEX 637) obtained from the culture collection of the University of Texas was grown in artificial seawater (ASW) prepared according to Jones et al. (1963) When cultures grown indoors as previously described (Adda et al. 1986) reached a maximum density, they were transferred to outdoor ponds or to polyethylene sleeves at a density of 4×10^6 cells ml⁻¹.

Outdoor Cultivation

Closed system. Each closed system consisted of a 150-cm-long transparent polyethylene sleeve 0.2 mm thick and 32 cm wide. The bottom of the sleeve was sealed in a cone shape by heat welding to prevent cell settling. The sleeve was then hung on an iron frame by means of an iron tube. Each sleeve was filled with 25 liters of medium and inoculated with $4-5 \times 10^6$ cells ml⁻¹. In the summer the temperature in the sleeves was prevented from rising beyond 28-30°C by automatic spraying with water when the temperature began to rise above that value. The cultures were mixed by an air stream containing 3-4% CO₂ pumped into the sleeve via glass tubing.

Open ponds. Plastic containers, each with a capacity of 100 liters (10 cm depth, 10- m² surface area), were used. Mixing was achieved by means of a paddle wheel, and CO₂ was supplied via porous tubing laid at the bottom of the containers.

Measurements

Growth. Cells were counted with a hemocytometer using a Zeiss microscope. For the measurement of biomass, a cell sample was centrifuged, washed twice with water at pH 4.0, and filtered through a 0.45-μm filter. The filtrate was then oven dried (70°C) for 24 h and weighed.

Light intensity. Light was measured with a Li-Cor instrument with a spheric sensor (model SPH Quantum SR no. SPWA 0710) immersed to a depth of 10 cm in the cultures grown in the sleeves or in the ponds.

Polysaccharide. Determination of cell-wall polysaccharide was performed as previously described (Adda et al. 1986).

RESULTS

The experiments were carried out during summer of 1989. Changes in temperature and pH during growth in the sleeves and in the ponds are presented in Table 1. The pH was maintained between 6-7. The minimum temperature in the sleeves was higher by about 10°C than that of the ponds. The maximum temperature in the ponds reached up to 39°C, whereas that in the sleeves reached 34°C due to the cooling system (with spraying). The conductivity was also measured (not shown), but did not undergo any changes during growth.

The growth pattern, including dilutions, is presented in Fig. 1 and 2 for the sleeves and ponds, respectively. During the experiment 14 dilutions were carried out in the sleeves as opposed to only 9 in the ponds. The biomass per volume unit was not constant in the various dilutions since dilutions were determined according to cell number.

Polysaccharide production - soluble, bound, and total - is presented in Fig. 3, 4, and 5, respectively. It is noteworthy, that under the conditions of this experiment the ratio between soluble and bound polysaccharide is about 1:1, whereas in other experiments carried out both indoors and outdoors the soluble polysaccharide comprised only about 20% of the total polysaccharide.

The daily production of biomass and polysaccharides is presented in Table 2. In the sleeves operated continuously biomass production was about 17.7 g m⁻² day⁻¹ and polysaccharide production 7.4 g m⁻² day⁻¹, which is about 41% of the biomass. These values are 2.3 and 3 times higher than those obtained in the ponds for biomass and polysaccharides, respectively.

DISCUSSION

In order to compare biomass and polysaccharide production in sleeves and in ponds, accumulated biomass during growth was divided by days of growth and was calculated to 100 liters, i.e. the volume of a culture grown in a 1-m² pond. This calculation is quite legitimate, since four 25-liter sleeves can be hung over an area of 1 m². At this volume, continuous biomass and polysaccharide production were two to three times higher in sleeves than in ponds. The higher production in the sleeves is due to the greater number of dilutions as compared with the ponds, i.e., to a higher growth rate.

Continuous operation of growth and production results in higher productivity as compared with the batch mode of operation (not shown in this report). The values obtained in this study for biomass and polysaccharide production in both sleeves and ponds were higher than those obtained previously for batch cultures (Cohen & Arad 1989).

The temperatures in the sleeves do not fluctuate as much as those in the ponds because of the water spraying system. The minimum night temperatures in the sleeves being higher than those in the ponds might cause enhanced losses in biomass due to respiration. Thus, it is possible that during the day the differences in productivities between sleeves and ponds are even greater than the 24-h values reported above.

We still can not pinpoint the main factor which makes the sleeve superior to the pond. We are now developing a data acquisition system for continuous measurements of light inside the cultures photosynthesis, and growth in the sleeves and ponds. These data might give us a clue to this issue. The identification of the

main factor will enable us to design and construct improved system for outdoor algal cultivation.

Although the results presented here encourage us to continue the development of this system, its scaling-up operation is the real challenge. This is now under way in our laboratory.

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Table 1. Growth conditions of *Porphyridium* sp. grown continuously in sleeves or pond

Culture age (days after inoculation)	Sleeves			Ponds		
	pH	Temperature		pH	Temperature	
		Noon	Morning		Noon	Morning
2	6.64	32	33	6.02	39	23
3	6.62	32	32	6.26	36	23
5	6.51	32	30	6.17	35	22
6	6.47	29	31	6.24	35	25
7	6.47	30	31	6.16	38	23
8	6.66	-	32	6.26	-	24
9	6.80	-	32	6.43	-	23
10	6.64	-	-	6.69	-	-
12	6.75	33	35	7.07	39	23
13	6.80	-	34	6.84	-	24
14	6.64	33	32	6.68	38	25
16	6.77	-	33	6.70	36	-
17	6.41	-	-	6.73	-	-
19	6.66	33	-	7.03	-	-
20	6.64	33	32	7.10	36	21
21	6.88	33	34	7.01	37	26
22	6.83	33	34	6.37	-	22
23	6.62	33	32	6.19	37	126
24	6.63	33	32	6.24	-	24
26	6.41	-	32	6.03	-	26
27-	33	33	-	-	-	25
28	6.91	32	33	6.75	-	26
29	6.31	33	33	6.58	-	22
30	6.53	-	32	6.50	-	25
31	6.59	-	33	-	-	24
33	6.74	-	-	-	-	-
34	6.74	-	-	-	-	-

Temperature, pH and conductivity were measured in the morning (0830-0930) when light intensity outdoors was 2000-3000 $\mu\text{E m}^{-2} \text{s}^{-1}$. The temperature at midday was taken between 1200-1300.

Table 2. Biomass and polysaccharide production in continuous growth of *Porphyridium* sp.

	Ponds		Sleeves	
	Harvested material (g)	Productivity (g m ⁻² day ⁻¹)	Harvested material (g)	Productivity (g m ⁻² day ⁻¹)
Biomass	228.0	7.6	460.0	17.7
Total polysaccharide	71.6	2.4	193.0	7.4

The calculation of per m² basis in the sleeves was done assuming 4 sleeves are hung in this area.

Fig. 1. Continuous growth of *Porphyridium* sp. in sleeves

— growth; - - - - dilution

Fig. 2. Continuous growth of *Porphyridium* in ponds

Fig. 3. Soluble polysaccharide production in *Porphyridium* sp. grown continuously in sleeves (a) or ponds (b)

Fig. 4. Bound polysaccharide production from *Porphyridium* sp. grown continuously in sleeves (a) or ponds (b)

Fig. 5. Total polysaccharide production from *Porphyridium* sp. grown continuously in a sleeves (a) or ponds (b)

**EFFECT OF NITRATE AND SULFATE STARVATION
ON POLYSACCHARIDE PRODUCTION IN *RHODELLA RETICULATA***

Yael (Brown) Lerental* and Shoshana (Malis) Arad

The Institutes for Applied Research,

Ben-Gurion University of the Negev

*M.Sc. student at the Department of Biology, Ben-Gurion University.

ABSTRACT

The red unicellular alga *Rhodella reticulata* was cultivated under various modes of nitrate nutrition. Growth patterns were similar in the batch and continuous regimes, whereas growth was totally inhibited in a nitrate-deficient medium. The cell-wall polysaccharide content and its distribution between bound and dissolved fractions was affected by the mode of nitrate nutrition. The highest polysaccharide content per cell was obtained in the nitrogen-deficient culture, which also exhibited the highest ratio of dissolved to bound fractions. Photosynthesis decreased sharply during the stationary phase of the batch culture and was very low throughout the experiment in the nitrate-deficient cells. Similarly, sulfate starvation affected both polysaccharide production and its distribution between the bound and dissolved fractions. The highest polysaccharide content was found under sulfate deficiency to cells that exhibited lowest photosynthetic potential.

Key index words: cell wall, carbon partitioning, nitrate, red algae (Rhodophyta), *Rhodella*, cell-wall polysaccharides, sulfate

BACKGROUND

The unicellular red algae belong to the order Porphyridiales which includes about eight genera, the best known of which are *Porphyridium*, *Rhodella* and *Rhodorus*. The cells are encapsulated in a sulfated polysaccharide, the external part of which becomes dissolved in the medium. Most of the research on the sulfated cell-wall polysaccharide has been confined to various species of *Porphyridium* (Ramus 1972 and 1986, Percival & Foyle 1979), although some work has also been done on *Rhodella maculata* (Evans et al. 1974, Fareed & Percival 1977). Another species of *Rhodella*, *R. reticulata*, was recently characterized by Deason et al. (1983), but its polysaccharide production and chemical composition have not been studied. However, in a preliminary investigation in our laboratory its rheological properties were found to be of industrial interest.

The influence of environmental conditions on polysaccharide production has been demonstrated in many algal systems (Kroen & Raynburn 1984, Spoer & Milner 1949, Thomas et al. 1984). Recently, interest was focused on the effect of nitrate on the production of polysaccharide in *Porphyridium* (Thepenier et al. 1985, Adda et al. 1986). The effect of the nitrate and sulfate starvation on *Porphyridium* has already been investigated (Köst et al. 1984; Wanner & Köst 1984, Arad et al. 1988). In this study the effect of nitrate and sulfate starvation on growth and polysaccharide production in the unicellular red alga *R. reticulata* was investigated.

MATERIALS & METHODS

Alga and growth conditions.

Rhodella reticulata (LB 2320) from the culture collection of the University of Texas, Austin, was grown under sterile conditions in batch culture in a brackish-water medium according to Schlosser (1982). The cells were grown in 1-L columns, 6 cm in diameter, at $24 \pm 1^\circ\text{C}$ under continuous illumination supplied by fluorescent cool-white lamps at an irradiance of $150 \text{ mE} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The medium was aerated with sterile air containing 2-3% CO_2 .

Nitrogen nutrition. The nitrogen source comprised nitrate supplied in the following regime a) batch - KNO_3 (7 mM) was supplied to the medium only at the onset of the culture; b) continuous - KNO_3 was added daily to maintain the original nitrate level; c) deficient - for comparison one culture was grown in a medium free of nitrate.

Sulphate nutrition.

Prior to the experiments *R. reticulata* cells were cultivated for three cycles (5 days each) in medium containing 1/10 of the sulfate of the original medium. After that the cells were cultivated in regular medium (3.3 g/l $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$ - control), partial, (0.32 g/l Na_2SO_4 and 4.51 g/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 1/100 sulfate that of the original medium (0.031 g/l Na_2SO_4 and 5.12 g/l MgCl_2 - deficient).

Nitrate content in the medium. Nitrate was determined as described by Greenberg et al. (1980).

Cell number. Cells were counted with a hemocytometer using a light microscope. At certain intervals throughout the growth period 5- to 30-ml samples were centrifuged at $20,000 \times g$. The pellet was used for biomass, protein, and sugar determinations, while the supernatant was used for nitrate and dissolved cell-wall polysaccharide determinations.

Dry weight. A cell sample was washed twice with water at pH 4, filtered through a 0.45-mm 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 ($0.5 \text{ mg}\cdot\text{ml}^{-1}$) to a cell concentration of $10\cdot 10^6 \text{ cells}\cdot\text{ml}^{-1}$, and illuminated at a light intensity of $150 \text{ mE}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. 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, using the technique of Lowry et al. (1951).

Cell wall polysaccharide. Bound fraction: The cells were hydrolyzed in sulfuric acid (1 N) over a boiling water bath for 1 h. The cell debris was removed by centrifugation, and the sugars were determined in the supernatant, by means of the phenol sulfuric acid method (Hellebust and Craigie 1978). Dissolved fraction: Sugar content was analyzed in the cell-free medium by the phenol sulfuric acid method. Total cell-wall polysaccharide is thus the sum of the bound and the dissolved fractions.

The results depicted in the figures are representative results from one of three similar experiments.

RESULTS

Nitrogen starvation

The effect of nitrogen supply on growth and polysaccharide production was studied by comparing the three regimes -- a batch mode, in which nitrogen became progressively depleted; a continuous mode, involving daily application of nitrate; and a nitrogen-deficient system. The nitrogen content of the medium during growth is shown in Figure 1.

The effect of the nitrogen regime on growth, as expressed by the cell number is shown in Fig. 2A. Batch and continuous cultures showed similar growth patterns, with a doubling time of about one day, while in the nitrogen-deficient system growth was totally inhibited. From the pattern of changes in dry weight (Fig. 2B), it can be seen that biomass continued to increase in the batch and continuous modes even after cell division had ceased. As expected, the protein content on culture volume basis was the highest in the continuous mode and the lowest in the nitrate-deficient cells (Fig. 3A). However, on per cell basis it was the highest in the nitrate-deficient culture and the lowest in the batch-mode culture (Fig. 3B).

Photosynthetic potential decreased throughout the experiment in all treatments (Table 1). Both the continuous and the batch regimes exhibited high initial values; however, in the continuous regime photosynthetic potential decreased slowly during the stationary phase, whereas in the batch culture it declined more rapidly

with time. Photosynthetic potential in the nitrate-deficient culture was very low from the beginning.

The concentration of dissolved and bound polysaccharides is shown in Fig. 4A+B. In the batch and continuous regimes the dissolved fraction was much smaller than the bound fraction, and it increased mainly during the stationary phase. In the nitrate-deficient culture the bound fraction did not change during the experiment while the dissolved fraction increased significantly, resulting in an increase in the ratio dissolved/bound fractions (Table 2). When total polysaccharides are described on a per cell basis (Fig. 4C), the picture changes, the highest content corresponding to the nitrate-deficient culture. The batch and continual modes produced similar, relatively low values per cell.

Sulfate deficiency

After three cycles of sulfate starvation (5 days each) *R. reticulata* cells were cultivated in three types of medium: control - usual sulfate concentration, partial - 1/10 of the usual sulfate concentration and deficient - 1/100 of the regular sulfate concentration. Growth (Fig. 5) photosynthesis (Table 3) and polysaccharides (Fig. 6) were followed under these conditions.

From Fig. 5A it can be seen that the growth pattern in 1/10 sulfate was identical to that of the control. Although sulfate deficiency did not affect the growth rate, the maximal cell concentration in the sulfate-deficient culture was 8×10^6 cells/ml vs. 20×10^6 cells/ml in the control and partial treatments.

The dry weight pattern for all the three treatments was similar. Since in the deficient treatment maximal cell concentration was lower, the biomass produced per cell was higher than in the control and partial treatments. Polysaccharide production under sulfate starvation is shown in Fig. 6. The pattern of the dissolved fraction was similar for all the three treatments but the bound fraction in the deficient treatment was significantly lower, than the control and partial treatments. When total polysaccharide (bound + dissolved) produced was calculated per cell, (Fig. 6C) a different picture emerged, i.e. the deficient treatment produced the highest amount of polysaccharide per cell, the value being higher than that of the control and partial treatments at day 10.

DISCUSSION

Under our experimental conditions the cell-wall polysaccharide yield per cell of *R. reticulata* was higher than that for *Porphyridium* sp. (Arad et al. 1988), as was its photosynthetic potential. However, since the maximum number of cells produced by *R. reticulata* is smaller, the quantity of polysaccharide produced per ml is similar in the two algae. Thus, at a certain point during growth *R. reticulata* stops dividing at an earlier stage, dedicating its newly fixed carbon to polysaccharide production, whereas *Porphyridium* sp. continues to direct its energy towards protein synthesis. In addition, under sulfate deficiency, although growth rate is similar the cells enter stationary phase earlier and the culture reaches lower maximal concentration. The polysaccharide produced under these conditions is the highest. It is possible that since the cell concentration in *R. reticulata* during the stationary phase is lower than that of *Porphyridium* sp. under sulfate deficiency, light availability to the cells is higher which results in enhanced polysaccharide production.

The exhaustion of nitrate from the medium alone is not the reason that the culture enters the stationary phase, as can be seen from the comparison between the batch and continuous mode of nitrate nutrition. Thus, the factor that induces the cessation in cell division is not nitrate. Light limitation to the algal cells might be one factor, or deficiency of one of the nutrients.

As was previously shown for *Porphyridium* sp. nitrate influences not only the quantity of polysaccharide produced but also its distribution between the cell wall and the medium (bound or soluble fractions). The ability of nitrogen-deficient cells to synthesize large amounts of cell-wall polysaccharide and to excrete it into the medium while their general metabolism is inhibited is of particular interest and warrants further exploration. It seems to us that such an investment from the cells must have a special importance to the cell. One explanation is that the cells have to get rid of the potential accumulated in them and thus excrete polysaccharides to the medium.

The rate of production of exocellular polysaccharides in unicellular red algae is probably controlled at two main levels - by the rate of carbon dioxide uptake, or at the level of partitioning i.e. distribution of fixed-C. The second possibility, i.e., that cellwall production is controlled at the level of partitioning and not at the level of photosynthesis, as was previously suggested (Kroen & Ramus 1988), is supported by some of our results: a) Rate of Photosynthetic declines during the stationary phase, whereas cell polysaccharide production is enhanced at that stage. b) In cultures subjected to nitrogen limitation and sulfate deficiency photosynthesis is the lowest, whereas exopolysaccharide production per cell is the highest.

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Table 1. Influence of the mode of nitrate supply on the photosynthetic potential of *Rhodella reticulata* during growth.

Day	Photosynthetic potential in following mode of nitrate supply: mg O ₂ ·L ⁻¹ ·min ⁻¹ ·10 ⁻⁶ cells		
	Batch	Continuous	Deficient
1	0.59	0.67	0.21
4	0.42	0.46	0.13
8	0.27	0.53	0.08
10	0.19	0.50	0.07
12	0.09	0.37	-

Table 2. Ratio between dissolved and bound polysaccharide in *Rhodella reticulata*

D	Ratio of dissolved to bound polysaccharide in the following mode of nitrate supply:		
	Batch	Continuous	Deficient
0	0.87	0.87	0.87
2	0.70	0.49	0.24
4	0.50	0.26	0.09
6	0.34	0.34	0.24
9	0.10	0.21	0.35
12	0.19	0.23	0.59

Table 3. Influence of sulfate starvation on the photosynthetic potential of *Rhodella reticulata* during growth

Day	Photosynthetic potential in following sulfate treatments mg O ₂ L ⁻¹ min 10 ⁻⁶ cells		
	Control	Partial (1/10)	Deficient
0	0.060	0.086	0.077
3	0.208	0.199	0.228
5	0.385	0.180	0.166
7	0.225	0.165	0.095
10	0.047	0.039	0.010

Fig. 1. Nitrate content in the growth medium of *Rhodella reticulata* grown under various nitrate regimes.

Fig. 2. Effect of the mode of nitrogen supply on the growth of *Rhodella reticulata*

Fig. 3. Effect of the mode of nitrogen supply on the protein content of *Rhodella reticulata*

Fig. 4. Effect of the mode of nitrogen supply on the cell-wall polysaccharide (bound dissolved and total) of *Rhodella reticulata*. Polysaccharide content is presented on per volume basis. Total polysaccharides is the sum of the bound and dissolved fractions and presented on per cell basis.

Fig. 5. Effect of sulfate on the growth of *Rhodella reticulata*.

R. reticulata was cultivated as described. After 3 cycles of starvation (5 days each) the cells were cultivated in 3 sulfate concentrations containing regular sulfate content (control) 1/10 - partial 1/100 deficiency.

During growth cultures were sampled and cell concentration and biomass determined. The results are average of 5 different experiments.

Fig. 6. Effect of sulphate concentration on polysaccharide production in *R. reticulata*. Cells of *R. reticulata* were grown as described in Fig. 5, during growth the cultures were sampled, centrifuged and polysaccharide determined in the supernatant (dissolved (A) and in the cells - Bound (B). Total polysaccharides was calculated from the dissolved and bound fractions (C).

**EFFECT OF NITROGEN ON POLYSACCHARIDE PRODUCTION
IN *PORPHYRIDIUM* SP.**

Shoshana (Malis) Arad, Orit (Dahan) Friedman*

and Avi Rotem**

**The Institutes for Applied Research,
Ben-Gurion University of the Negev**

*M.Sc. student at the Department of Biology, Ben-Gurion University of the Negev

**Ph.D. student at the Department of Chemical Engineering, Ben-Gurion University
of the Negev

ABSTRACT

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 regimes 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 modes of nutrition, whereas it was totally inhibited in the deficient-mode culture. Polysaccharide content (per volume) was the highest in the batch-mode culture and the lowest in the nitrogen-deficient 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 distribution between dissolved and bound polysaccharides. In the nitrate-deficient medium most of the cell-wall polysaccharide was dissolved in the medium.

Key words: biomass; cell-wall polysaccharides; growth; photosynthesis;
Porphyridium sp.; red algae (Rhodophyta)

INTRODUCTION

The polysaccharide of the marine unicellular red alga *Porphyridium* has recently gained attention due 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 pH, temperatures and salinities (2, 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 (3, 10). The 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* (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 was shown in *Chlorella* (12) and in some marine algae (14). In *Porphyridium* carbohydrate accumulation seems to take place at the expense of protein production (2).

The general purpose of our research is to optimize the production of cell wall polysaccharide in *Porphyridium*. 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 in *Porphyridium* sp. was studied.

MATERIALS & METHODS

Porphyridium sp. (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 $24 \pm 1^\circ\text{C}$ in artificial sea water according to Jones et al. (7). The cultures were illuminated continuously with fluorescent cool-white lamps at an irradiance of $150 \text{ mE m}^{-2} \text{ s}^{-1}$. 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 as the potassium salt, as follows: a) addition of nitrate (10 mM KNO_3) only at the beginning of the experiment (batch); b) daily supply of nitrate to restore the concentration to its original level (continual); c) 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 $20,000 \times g$. 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-mm 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 cell concentration of $10^7 \text{ cells ml}^{-1}$ and

illuminated at light intensity of $140 \text{ mE m}^{-2} \text{ s}^{-1}$. 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 means of the technique of Lowry et al. (9).

Polysaccharide. 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 bound and the soluble fractions.

Starch. Starch content was measured in cell samples (10×10^6 cells) after freezing the cells in liquid nitrogen and thawing three times, addition of $0.06 \text{ NK}_2\text{HPO}_4$, boiling the suspension for 20 min, and centrifugation (10 min at $6,000 \times g$). Starch was measured in the supernatant using the I-KI method with potato starch (Merck) as a standard.

Nitrate content in the medium. Nitrate was determined as described by Greenberg et al. (4).

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

RESULTS

Porphyridium sp. 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) 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 the 6th day, 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 the 6th day 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 with 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 protein level, which was lower in the batch-mode culture than in the continual-mode culture. In the continual-mode culture protein 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 then

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

With respect to the cell-wall polysaccharide, the content of both the bound and the soluble fractions, on a culture volume basis (Fig. 4A, B), increased somewhat during the logarithmic phase of growth and even more so during the stationary phase in both the batch- and the continuous-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- 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 not increasing at all during the experiment, and the soluble fraction, which constituted the new polysaccharide produced, increasing slightly during the stationary phase. In all the cultures the ratio of soluble to bound fractions decreased with 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 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 with 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 start of the stationary phase of growth is determined by 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 continued to be produced. Since protein content did not change, it seems that polysaccharide synthesis depends either on pre-existing enzymes having a slow turnover or on newly synthesized enzymes, although their content is very small. Almost all of the cell-wall polysaccharides produced was released into the medium. Whether the composition of the polysaccharide produced under nitrogen limitation is identical to that produced under conditions of 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, needs still to be elucidated. The effect of nitrogen deficiency described above is not limited to the marine species of

Porphyridium studied here: it has also been found in the fresh water species *P. aerugineum* (not shown).

Maximum polysaccharide production (per ml) was obtained in batch culture. Hence, for maximal polysaccharide production a batch mode of operation is recommended. Under these conditions, only about 20-30% of the total polysaccharide produced by *Porphyridium* sp. 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 when choosing the method of extraction. For collection of the soluble fraction only, when 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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TABLE 1. Rates of photosynthesis of *Porphyridium* sp. during growth as affected by the mode of nitrate supply

Mode of nitrate supply			
Day	Batch	Continual	Deficient
mg O ₂ liter ⁻¹ min ⁻¹ 10 ⁻⁶ cells			
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

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

Mode of nitrate supply			
Day	Batch	Continual	Deficient
% of the bound polysaccharide ^a			
3	5.8	5.8	8.2
7	1.2	1.6	10.3
12	4.1	5.2	16.2

^aThe content of the bound polysaccharide at day 3 was 14.5, 14.3 and 27.0 mg 10⁻⁶ cells in the batch, continual and deficient treatments, respectively.

FIG. 1. Effect of nitrogen source on the growth (A) and polysaccharide production (B) of *Porphyridium* sp. Total polysaccharide is the sum of the bound and soluble fractions. $\square$ - NH_4HCO_3 ; $\blacktriangle$ - NH_4NO_3 ; $\times$ - KNO_3 .

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

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

FIG. 4. Effect of the mode of nitrogen supply on *Porphyridium* sp. cell-wall polysaccharide. Bound (A) and soluble (B) polysaccharide on per volume basis. Total polysaccharide content (sum of the bound and soluble fractions) (C) on per cell basis.

Exopolymer Yield Optimization for Porphyridium in Chemostat-Controlled Photobioreactor

J. Ramus, B.E. Kenney & Mary C. Jones
Botany Department & Marine Laboratory
Duke University
Beaufort, NC 28516

ABSTRACT

The per capita polysaccharide copolymer production rate (cellular yield) is greatest at maximum specific growth rate ($U_{max} = 1.44/d$) and highest feedstock concentration (440 μM nitrate-N). On an absolute scale, more photosynthetically-fixed carbon is partitioned to polysaccharide copolymer when the cells are at peak growth rates and N-sufficiency. Standing cell concentrations are highest at the lowest volumetric turnover rates, near zero flow. Per capita production rates are highest at the highest volumetric turnover rates, just short of washout. The strategy for maximum volumetric yield becomes one of balancing standing cell stock (concentration) and per capita production rates. The compromise of these two parameters results in maximum volumetric yields at intermediate volumetric turnover rates (0.6 - 0.9/d), i.e. intermediate specific growth rates.

INTRODUCTION

The cost-effective production of value-added biochemicals is dependent on the sum of rate-limiting conversions, conceptually presented as follows:

limiting feedstock $\rightarrow$ catalyst $\rightarrow$ product
(nitrate-N) (cells) (copolymer)

In this specific case, the product is the exocellular copolymer (primarily polysaccharide) of the catalyst, namely the cells of the rhodophyte microalga Porphyridium sp., a photoautotroph. The copolymer and its fluid dynamic properties are described elsewhere (Ramus, 1986; Ramus et al., 1989; Ramus & Kenney, 1989). The limiting feedstock here is the essential nutrient nitrogen, supplied as the inorganic anion nitrate. Insofar as the cells are grown in aqueous medium, and the product concentration is relatively dilute, product recovery is dependent on dewatering procedures.

Given here is that polymer shall be produced in a photobioreactor operated in continuous mode. Operation in batch mode has been considered in detail elsewhere (Adda, et al. 1986; Arad, et al. 1985; Ramus 1986), and forms a basis for comparison. The question is the rate of supply of limiting feedstock (NO_3^-) to the catalyst (cell suspension) such that product (exocellular copolymer) yield is optimized.

An underlying assumption in the determination of yield enhancement strategies is the chemical partitioning of finite quantities of photosynthetically-fixed carbon to various "sinks", which are conceptualized as being in competition. Those sinks include the major classes of cellular chemical constituents as protein, carbohydrate, lipid, nucleic acid, or specific subclasses of these constituents as light-harvesting pigment proteins (LHPPs), ribulose biphosphate carboxylase/oxygenase (RuBisCO), exocellular polysaccharide, starch, storage lipid and RNAs. The "conventional wisdom" (e.g. Ramus, 1986) is that for cells in N-nutrient sufficiency, most carbon is partitioned to N-rich macromolecules as new protein, primarily LHPPs and RuBisCO, and hence to new photosynthetic macromolecules and hence cell production. For cells in N-nutrient deficiency, carbon can no longer be partitioned to new protein, and so long as the cells continue to fix carbon, it must be partitioned to C-rich sinks as storage carbohydrates, storage lipids and secondary metabolites as exocellular polysaccharides. However no new cells are produced, and insofar as cells are the source of exocellular copolymer, yield is limited by standing biomass.

Photobioreactors operating in continuous mode under N-nutrient chemostat control are N-nutrient limited, i.e., growth rate is limited by the feedstock supply rate. Nevertheless, cells are N-nutrient sufficient, or at least poised delicately between sufficiency and deficiency, i.e., their carbon conversion efficiency is limited. To reiterate, the point in question is at what rate does feedstock supply produce the greatest exopolymer (exopolysaccharide) yield. Or rephrased, at what point is cell yield balanced by the chemical partitioning of carbon to exocellular polysaccharide such that total yield is optimized? We seek to provide an answer to that question.

MATERIALS AND METHODS

The species used in this study was the rhodophyte unicell Porphyridium sp. (University of Texas Culture Collection #637). Cells were grown in aqueous suspension in a photobioreactor, chemostat-controlled continuous mode. Eight "chemostats" were operated simultaneously, each at an independent flow rate to 1.44 volume turnovers/d, i.e., to 1.44 dilutions/d. Each chemostat was a Pyrex culture tube (6 X 50 cm) which had a surface area:volume aspect of 0.67, and a working volume of 1l. Light was supplied continuously by 20W "cool white" fluorescent lamps. The irradiance of light arriving to the center of water-filled tubes was 300 umoles quanta/m exp 2/s. Temperature control was provided by water jackets connected to a circulating water bath. A Lauda K2R chiller and a thermostated heater produced a very stable and precise water temperature (25C). The chemostats were sparged through Gelman bacterial air vent filters with oil-free air from a diaphragm pump. Sampling spurs below the air filters allowed cell suspensions to be sampled without contamination.

Cells were suspended in offshore heat-sterilized seawater diluted to 80% with distilled water. The seawater was supplemented with "f" enrichments (Guillard & Ryther 1962) to either of three N-NO₃ concentrations, specifically 88 μ M, 176 μ M and 440 μ M. The eight different flow rates combined with the three different feedstock nitrate concentrations gave a wide range of nitrogen loading rates (volume turnover rate X concentration). Independent volume turnover rates were maintained with an eight-channel peristaltic pump. The cell populations were allowed to reach equilibrium ($dN/dt = 0$, where N is the cell concentration) as a condition for sampling. Upon attainment of equilibrium, the suspensions were sampled at three successive one-day intervals, and these were taken to be replicate samples for purposes of numerical analyses. Thus, in the scatter diagrams which are presented in the RESULTS section (Figs. 1 - 18), each data point presented represents a chemostat at equilibrium cell population dynamic.

Cell concentrations were determined with a Neubauer-ruled 0.1 mm deep haemocytometer viewed through a compound microscope. Carbon (C) and nitrogen (N) content of cells were determined from washed and lyophilized material on a Carlo Erba 111B elemental analyzer.

The exopolymer released by the microalgae was partitioned to the cell-free medium by centrifugation, yielding the "free" polymer fraction. The centrifuged pellet yielded the "bound" polymer fraction. Both were analyzed for polysaccharide concentration by the phenol-sulfuric acid assay (PSAA) (Dubois, et al. 1956) as glucose equivalents.

RESULTS

A. Chemical Composition.

Gross chemical compositions were determined from random samples of cell suspensions (Table 1). C:N atom ratios were roughly twice those of Redfield ratios (6.63) for algal unicells (phytoplankton), although this is expected for cells which deposit large quantities of exopolysaccharides to their surfaces.

TABLE I. Gross chemical composition of cells, given as mean values from n samples.

dry weight	52.6 pg / cell	n = 17
%C	25.2	n = 46
%N	1.89	n = 46
C:N (atom)	15.6	n = 46

B. Feedstock to Catalyst Conversion.

Of particular interest to this discussion is the efficiency with which inorganic nitrogen is converted to cell biomass, i.e., feedstock to catalyst conversion efficiency. Peak cell and biomass production occurs at N-loading rates (feedstock concentration X chemostat turnover rate) of approximately 4 mg N/l/d, yielding approximately 40×10^8 and 210 mg dry biomass/l/d (Figs. 1 & 2). Thus approximately 50 mg of dry biomass is produced for each mg of N feedstock at peak rates, a number (approx. 2%) consistent with the cellular content of N (Table I). Beyond loading rates of 4 mg N/l/d, dry biomass production falls precipitously, a consequence of chemostat turnover rates exceeding the intrinsic maximum growth rate (U_{max}) for the species. Nitrogen conversion efficiency at peak production, i.e., feedstock-N to cellular-N, is near 100%, expected for N-limited chemostat operation. Cellular C:N atom ratio at peak production is 15.51, also consistent with mean composition (Table I).

C. Feedstock to Product Conversion.

Product, in this case exocellular polysaccharide, is deposited to the cell surface, encapsulates the cell, and solubilizes to the aqueous phase. Thus, there occurs two polymer fractions, designated "bound" (encapsulating the cell) and "free" (solubilized).

Total (bound + free) and free saccharide per cell are greatest at the lowest N-loading rates, decline rapidly as N-loading rates increase, then level off at 15 pg/cell and 5 pg/cell, respectively (Figs. 3 & 4). The ratio of bound : free saccharide polymer is fairly consistent at 2.

Volumetric saccharide yield also peaks at a loading rate of 4 mg N/l/d, yielding mean values of 60 mg total and 20 mg free saccharide polymer/l/d (Figs. 5 & 6). Beyond that loading rate, chemostat washout rapidly diminishes the yield.

The per capita total saccharide yield (pg/cell/d) (Fig. 7) is an exponential function of N-loading rate, being highest at the highest loading rates. Mean values reach near 25 pg/cell/d. The per capita free saccharide yield (Fig. 8) is somewhat more difficult to interpret insofar as it is a function of both polymer synthesis and solubilization from the cell surface. However, free saccharide yield can be as high as 11 pg/cell/d at N-loading rates of 2.5 mg/l/d.

D. Standing Stocks and Yield as a Function of Turnover Rate.

Standing cell concentrations vary as a function of chemostat volumetric turnover rate and feedstock (nitrate) concentration (Fig. 9). The maximum sustainable specific growth rate

(equivalent to the volumetric turnover rate) for this species was 1.44/d, or 2.08 cell divisions/d. Lower specific growth rates resulted in higher standing cell concentrations, and the effect was enhanced as feedstock concentration increased. Cell concentrations attained 12×10^9 cells/l.

Volumetric cell yield (cells/l/d) (turnover rate $\times$ standing cell concentration) was greatest at intermediate volumetric turnover rates (0.6 - 0.9 /d) (Fig. 10). Feedstock concentration had a salutary effect on cell yield, the greatest yields (60×10^7 cells/l/d) occurring at the highest feedstock concentrations.

Standing total saccharide concentrations (Fig. 11) (mg/l) increased with decreasing volumetric turnover rate and increasing feedstock concentration, reaching a maximum of about 200 mg/l. Standing free saccharide concentrations (Fig. 12) followed the same pattern, reaching a maximum of about 75 mg/l at 440 uM N.

Total volumetric saccharide yield (mg/l/d) was greatest at intermediate turnover rates (0.4 - 0.6/d), and increased with increasing feedstock concentration (Fig. 13). Maximum yields were near 100 mg/l/d. Free saccharide yields follow the same pattern, with maximum values of 30 mg/l/d (Fig. 14).

The per capita concentration of total saccharide (pg/cell) is independent of specific growth rate (1/d), except at the lowest feedstock concentrations where the quantity per cell increases at the lowest growth rates (Fig. 15). The mean concentrations hover at near 15 pg/cell. The per capita concentration of free saccharide follow the same pattern, and the mean values hover at near 5 pg/cell (Fig. 16).

Of particular interest to this discussion is the per capita yield (pg/cell/d) of saccharide polymer as a function of specific growth rate (1/d). Per capita yield increases with specific growth rate, especially as feedstock concentration increases. Maximum total saccharide yields are near 25 pg/cell/d (Fig. 17), and maximum free saccharide yields are near 8 pg/cell/d (Fig. 18).

E. Calibration

Comparison to batch (0.0 volume turnovers/d) culture was used as an internal calibration for this series of chemostat-controlled continuous cultures. The data (Table II) are given for elapsed times of approximately 21 days, and are not included with the chemostat data at the 0.0/d abscissa point because the cultures are not of comparable age.

TABLE II. Accumulated (~21 days) polysaccharide copolymer in batch cultures of specified initial nitrate-N concentrations. Data given as mean value + 1 S.D. (n).

<u>nitrate-N</u>	<u>fraction</u>	<u>wppm</u>	<u>pg/cell</u>
88 uM	total	130.7 + 23.7 (3)	92.3 + 10.1 (3)
	free	45.3 + 4.7 (3)	33.0 + 1.0 (3)
176 uM	total	241.2 + 26.5 (5)	78.8 + 15.6 (5)
	free	74.8 + 23.7 (5)	24.8 + 33.0 (5)
440 uM	total	392.5 + 149.2 (6)	96.3 + 39.8 (6)
	free	138.8 + 26.5 (6)	38.3 + 21.7 (6)
880 uM	total	417.7 + 40.6 (6)	102.1 + 18.8 (9)
	free	169.0 + 29.8 (6)	41.2 + 7.1 (9)

DISCUSSION

The salient feature of this study is the fact that the per capita polysaccharide production rate (cellular yield) is greatest at maximum specific growth rate (U_{max}) and highest feedstock concentration (Fig. 17). High performance is also seen as a linear function of N-loading (Fig. 7). This finding is in direct contradiction to the "conventional wisdom", as outlined in the introductory paragraphs of this article. On an absolute scale, more photosynthetically-fixed carbon (C) is partitioned to polysaccharide copolymer when the cells are at peak growth rates and N-sufficiency.

With this observation in hand, the strategy for maximum volumetric yield becomes one of balancing standing cell stock (concentration) and per capita production rate, i.e. catalyst concentration and product formation rate. Standing cell concentrations are highest at the lowest volumetric turnover rates (Fig. 9), just short of zero flow rates or zero volumetric turnovers/d. Per capita production rates are highest at the highest volumetric turnover rates, just short of washout (Fig. 17). The compromise of these two parameters results in maximum volumetric yields at intermediate volumetric turnover rates (0.6 - 0.9/d) (Fig. 13), or growth rates of 0.87 - 1.30 cell divisions/d, or 0.42 - 0.63 U_{max} , respectively. This, of course, requires that nitrate feedstock concentrations be as high as practicable, in this case 440 uM. Nitrate feedstock concentrations of 880 uM were attempted, but resulted in erratic data for the cellular parameters tested.

On the basis of this data, a scaled-up photobioreactor production facility operated in N-limited continuous mode (chemostat control) of 1×10^6 l could expect a daily yield of 30 Kg of free polysaccharide and 100 Kg of total polysaccharide. The former would require dewatering of relatively dilute solutions of the product, i.e. 25 - 50 wppm. The latter would require extraction and dewatering of product concentrations ranging from 75 -150 wppm. A batch culture of equivalent volume with an initial nitrate-N concentration of 440 μ M would produce about 400 Kg of total polysaccharide and 140 Kg of free polysaccharide (Table II) in 21 days. Lower and higher initial nitrate-N concentrations would result in lower and higher yields, respectively (Table II). In the same period of time, the continuous culture with a feedstock nitrate-N concentration of 440 μ M would produce 2100 Kg of total polymer and 630 Kg of free polymer.

Recently, Kroen and Ramus (1990) determined that the partitioning of photosynthetically-fixed C was similar in N-sufficient replicating cells and N-deficient non-replicating cells. The determinations were made with radioisotopic (14 C) pulse-chase and dilution techniques, followed by cell fractionations. During batch culture, the invariate observation was that per capita polysaccharide concentrations remained low as long as the cell population was in exponential phase growth, then increased as the population entered stationary phase. The observation was interpreted as differential C-partitioning favoring polysaccharide synthesis when cells ceased to replicate. The new findings are contrary to this notion.

For cell populations in exponential phase growth, photosynthetic capacity (P_{max}) remains high, and newly synthesized polysaccharide is diluted by cell replication, thus per capita polysaccharide concentrations remain low. Upon entering stationary phase, cell replication ceases, P_{max} declines precipitously, newly synthesized polysaccharide accumulates and the per capita concentration increases. However, the quantitative partitioning of photosynthetically-fixed C remains the same in both replicating and non-replicating cells. These observations are recapitulated in Fig. 15. Thus, in aged batch cultures, per capita and volumetric polysaccharide concentrations are very high, while in continuous cultures the concentrations are considerably lower.

The debate then becomes one of batch versus continuous mode photobioreactors for commercial production. Over a finite period of time, the batch mode produces a single volume of high polysaccharide copolymer concentration. The continuous mode produces many volumes of relatively low polysaccharide copolymer concentrations in the same period of time. The resolution of the debate becomes one of specific goals and production technology.

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FIGURE CAPTIONS

Fig. 1. Volumetric cell production rate (cells/l/d) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 2. Volumetric dry biomass production rate (mg/l/d) as a function of nitrate-N loading rate (mg N /l/d).

Fig. 3. Per capita total polysaccharide copolymer (pg/cell) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 4. Per capita free polysaccharide copolymer (pg/cell) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 5. Volumetric total polysaccharide copolymer production rate (mg/l/d) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 6. Volumetric free polysaccharide copolymer production rate (mg/l/d) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 7. Per capita total polysaccharide copolymer production rate (pg/cell/d) as a function of nitrate-N loading rate (mg N/l/d).

Fig. 8. Per capita free polysaccharide copolymer production rate (pg/cell/d) as a function of nitrate-N loading rate (mg/l/d).

Fig. 9. Volumetric cell concentration (cells/l) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 10. Volumetric cell production rate (cells/l/d) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 11. Volumetric total polysaccharide copolymer (mg/l) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 12. Volumetric free polysaccharide copolymer (mg/l) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 13. Volumetric total polysaccharide copolymer production rate (mg/l/d) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 14. Volumetric free polysaccharide copolymer production rate (mg/l/d) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 15. Per capita total polysaccharide copolymer (pg/cell) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 16. Per capita free polysaccharide copolymer (pg/cell) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 17. Per capita total polysaccharide copolymer production rate (pg/cell/d) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

Fig. 18. Per capita free polysaccharide copolymer production rate (pg/cell/d) as a function of chemostat volumetric turnover rate (1/l/d) = specific growth rate (1/d).

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