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Productivity and biochemical composition of cyclostat cultures of the marine microalga *Tetraselmis suecica*

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Abstract Cyclostat, light/dark-synchronized cultures of the marine microalga *Tetraselmis suecica* were carried out with six nutrient concentrations: 0.5, 1, 2, 4, 8, and 16 mmol N/l. A renewal of 50% culture volume, equivalent to a dilution rate of 1 day^{-1} was applied. Maximal steady-state cell density (2.5×10^6 cells/ml) and dry-weight productivity (0.2 g l^{-1} day^{-1}) were achieved with 4 mmol N/l. Maximal protein (94.3 pg/cell) and lipid contents (25.4 pg/cell) were achieved with 8 mmol N/l. The high variability reported in chlorophyll cellular content invalidates any estimation of biomass or cell number based on chlorophyll concentration or fluorescence, especially when nutrient concentration is being varied. The total fatty acid cellular content increased with increasing nutrient concentration although the amount of eicosapentaenoic acid decreased. Results indicated that a different desaturation pathway may be present in this species. The change of the nutrient concentration in the cyclostat system is a powerful tool to manipulate the biochemical composition of microalgae regarding protein, lipid, carbohydrates and fatty acid content.

Introduction

Even though a number of different closed photobioreactors have been designed and tested recently for the mass production of microalgae (Spectorova et al. 1981/82; Laing and Jones, 1988; Lee and Bazin 1990; Cohen et al. 1991; Richmond et al. 1993) not many data are available on the biochemical variability generated with different operation regimes in these systems (Caperon and Meyer 1972; Goldman and Peavey 1979; Scott 1980), much of the information available being for cultures with nutrient levels resembling those occurring in natural waters.

In the present work the effect of a wide range of nutrient concentrations on the productivity and biochemical composition of high-dilution-rate cyclostat cultures of the marine microalga *Tetraselmis suecica* was tested.

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Materials and methods

Unialgal cultures of *Tetraselmis suecica* Kylin (Butch) were carried out in 1 l flasks containing 500 ml culture medium. Sterilized sea water (3.5%) was enriched with nutrients (Fábregas et al. 1984) at concentrations of 0.5, 1, 2, 4, 8 and 16 mmol (nitrate) N/l. All the other nutrient components were increased proportionally. Flasks were inoculated with 500000 cells/ml and a daily renewal of 50% of the culture volume was carried out once the culture had reached stationary phase. The resulting growth rate at steady-state (μ) was 1.0 day^{-1}. Cyclostat cultures are different from traditional chemostat cultures in the use of cycles of light/darkness (Chisholm et al. 1975) that cause a synchronous or phased cellular division. As a consequence of using a light/dark photoperiod under a renovation of 50% of the volume ($\mu = 1$ day^{-1}), cultures were completely synchronized, and, therefore, the total microalgal biomass could be considered as an equivalent theoretical unique cell.

Cultures were submitted to aeration supplemented with CO_2 in order to keep the pH below 8. A 12 h/12 h light/dark regime was maintained with a light intensity of 224.6 μmol photon m^{-2} min^{-1}. Dilutions were made during the first hour of the light period with sea water enriched with the corresponding nutrient concentration. Cultures were kept in a continuous regime for 42 days after stabilization without significant variation in steady-state cell density.

Cellular density was established by microscope counting using an improved Neubauer haemocytometer. Biomass was harvested by centrifugation and freeze-dried. All biochemical determinations, except for chlorophyll content, were made on lyophilized biomass. Protein content was derived from nitrogen content using the factor proposed by Gnaiger and Bitterlich (1984). Carbohydrates were measured by the phenol-sulphuric acid method (Kochert 1978) and lipids by the charring method (Marsh and Weinstein 1966). Chlorophylls were extracted at 4° C for 24 h in acetone/methanol (2:1) and calculated from the formulae of Jeffrey and Humphrey (1975). Caloric values were calculated using the conversion figures suggested by the National Research Council (1977).

For fatty acid analysis, lipid extracts were obtained according to the method of Bligh and Dyer (1959) followed by an acid extraction (Dubinsky and Aaronson 1979). The extracts were dried under nitrogen and subjected to methanolysis with 5% HCl in methanol at 85° C for 2.5 h (Sato and Murata 1988). The resultant methyl esters were analysed with a gas chromatograph/mass spectrometer (Fisons Instruments MD-800) using a column Omegawax 250 (Supelco Inc.). Triheptadecanoin was used as the internal standard.

Results

Steady-state density, expressed as the harvesting cellular density, reached a maximum with 4 mmol N/l (Fig. 1). Standard deviations increased with increasing nutrient concentration, as a result of the greater unsteadiness of cultures. For similar steady-state cellular densities, the biomass per unit volume was higher the higher the nutrient concentration, indicating an increase in cellular weight.

Maximal productivity per day in cell number, 1240×10^6 cells l^{-1} day^{-1}, dry weight, 0.216 g l^{-1} day^{-1}, and protein weight, 0.096 g l^{-1} day^{-1} was achieved with a nutrient concentration of 4 mmol N/l, although this concentration represented a low conversion rate of nitrate into intracellular nitrogen (59%). The decrease in productivity at higher nutrient concentrations was steeper for dry weight and cell productivity than for protein productivity (Fig.2).

As samples were not washed after harvesting to avoid any alteration of the biochemical composition, the percentages of protein, carbohydrates and lipids were affected by the increase of ash content at nutrient concentrations 8 mmol and 16 mmol N/l. Protein, as a percentage of dry weight, reached a maximum at 4 mmol N/l, ranging between 22.3% and 44%, but when protein per cell is considered, the maximum was achieved with 8 mmol N/l as was the maximum caloric content per cell (Fig. 3). Lipid cellular content also reached a maximum at 8 mmol N/l concentration (25.4 pg/cell). Carbohydrate was the only organic fraction that decreased with increasing nutrient concentration. When expressed as a percentage of the organic fraction (Fig. 4), protein, carbohydrates and lipids reached a plateau at 4 mmol N/l, stabilizing around 60% protein, 20% lipids and 20% carbohydrates. An increase in the organic fraction from 119 pg/cell for 1 mmol N/l to 139 pg/cell in the culture with 16 mmol N/l was recorded. Cellular chlorophyll content increased with nutrient concentration reaching a maximum value of 4.6 pg/cell at the highest nutrient concentration. The ratio C:N (atoms) decreased as the nutrient concentration increased to 4 mmol N/l. This ratio stabilized around 4.4 (Fig. 4). The carbon content was comparatively stable relative to the nitrogen content, the decrease in the C/N ratio being caused almost exclusively by the increase in N content.

The main fatty acids found in *T. suecica* were 16:0, 16:4(*n*–3), 18:0, 18:1(*n*–9) and 18:3(*n*–3). The total

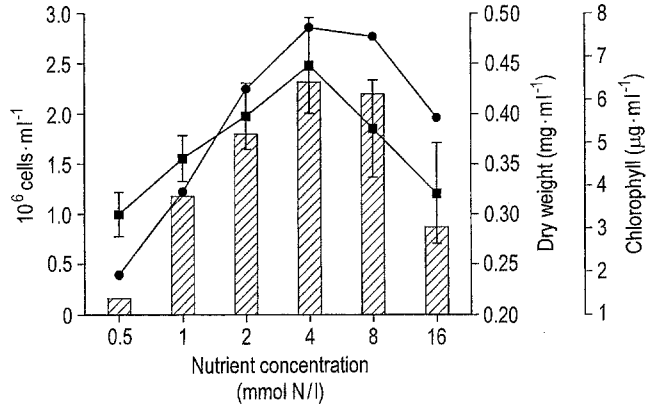

Fig. 1 Steady-state cell density (■), total chlorophyll/ml (●) and dry weight/ml (*bars*) in cyclostat cultures of *T. suecica* with a dilution rate of 50% and different nutrient concentrations. *Vertical bars* standard deviations

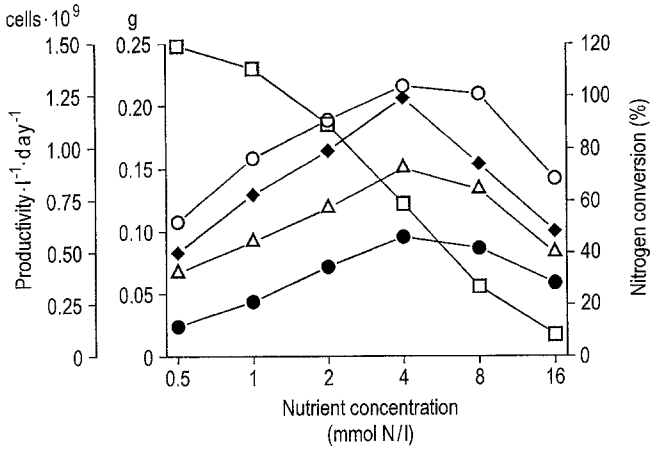

Fig. 2 *T. suecica* cyclostat culture productivity: cell growth (cells l^{-1} day^{-1}; ◆), dry matter (g l^{-1} day^{-1}; ○), organic weight (g l^{-1} day^{-1}; △), protein (g l^{-1} day^{-1}; ●) and nitrogen conversion efficiency (%; □)

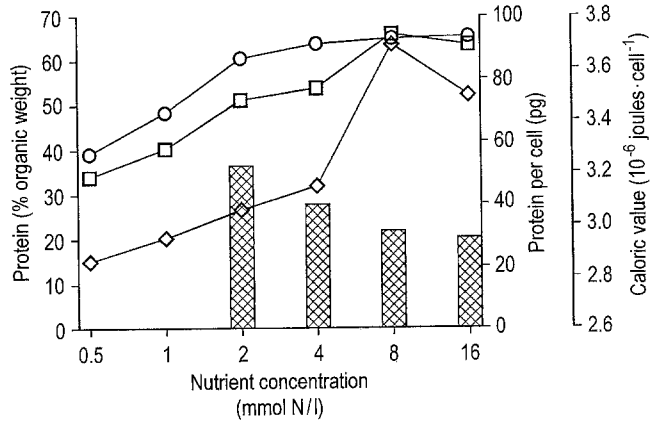

Fig. 3 Protein content as a percentage of organic weight (○) and protein cellular weight (□) in cyclostat cultures of *T. suecica*. Changes in caloric value of cells with nutrient concentration are also shown (◇). *Bars* protein content (pg/cell) in log-phase *T. suecica* grown in mass cultures (Fábregas et al. 1985)

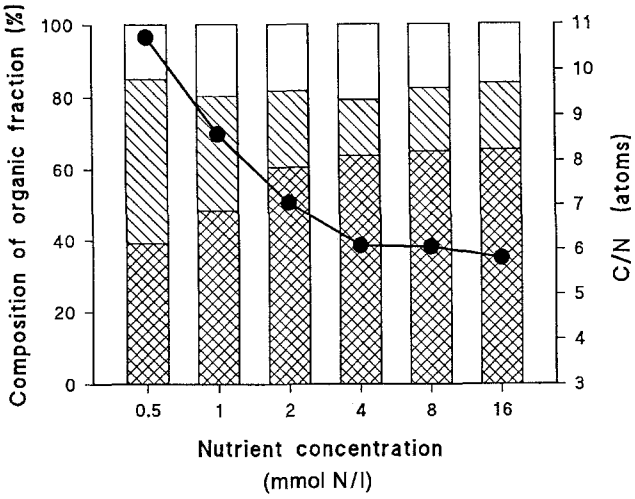

Fig. 4 Gross biochemical composition of *T.suecica* cyclostat cultures as a percentage of the organic fraction. ▩ Protein, ▨ carbohydrates, □ lipids, ● C:N ratio as atoms

fatty acid cellular content increased with increasing nutrient concentration (Table 1) except for the nutrient concentration 4 mmol N/l. The *n*–3 fatty acid content increased up to a nutrient concentration of 2 mmol N/l and decreased at higher concentrations. The increase was mainly due to the increase of 18:3(*n*–3), 18:4(*n*–3) and 16:4(*n*–3), as the cellular content of the long-chain polyunsaturated fatty acids 20:4(*n*–3) and 20:5(*n*–3) decreased continuously with increasing nutrient concentration (Table 1).

Discussion

Maximal productivities of the cyclostat system were similar to the maximal cell productivities obtained in other continuous systems (Laing and Helm 1981; Laing and Jones 1983, 1988). When the cyclostat productivity was compared to the productivity obtained under similar conditions in aerated batch cultures in 10-l flasks, the productivity of cyclostat cultures was higher for nutrient concentrations 2 mmol and 4 mmol N/l and lower for 8 mmol and 16 mmol N/l (Fábregas et al. 1985).

The point at which the protein content of the organic fraction of the biomass was stable indicated the end of nitrogen limitation (Fig. 4) and coincided with the stabilization of the C:N ratio. The gross biochemical composition of the organic fraction of *T. suecica* in cyclostat cultures stabilized at 60% protein, 20% carbohydrates and 20% lipids (Fig. 4). These percentages are similar to the composition expected when all nutrients are in excess and light is the only limiting factor (Goldman 1980). Differences in protein content obtained, when expressed as cellular content or organic fraction percentage, were due to an increase of cellular weight with increasing nutrient concentration.

Nutrient concentration was one of the parameters that regulated the protein content in mass cultures of *T. suecica* during the logarithmic phase (Fábregas et al. 1985), but the effect was opposite to the one reported in cyclostat cultures (Fig. 3). Meanwhile the protein cellular content decreased with increasing nutrient concentration in the logarithmic phase of mass cultures, the maximum being 52.2 pg/cell; the maximum cellular

Table 1 Fatty acid profile of *T. suecica* cyclostat cultures under different nutrient concentrations

Fatty acid	Fatty acid content (pg/cell) with a nutrient concentration of:					
	0.5 mmol N/l	1 mmol N/l	2 mmol N/l	4 mmol N/l	8 mmol N/l	16 mmol N/l
12:0	0.15	0.14	1.02	0.61	1.07	1.51
14:0	0.32	0.40	0.79	0.56	0.84	1.06
16:0	3.53	3.64	3.83	2.66	3.94	4.20
16:1 (*n*-9)	0.15	0.16	0.16	0.16	0.20	0.21
16:1 (*n*-7)	0.10	0.13	0.17	0.09	0.16	0.19
16:2 (*n*-6)	0.06	0.06	0.06	0.05	0.07	0.07
16:3 (*n*-3)	0.17	0.20	0.22	0.13	0.17	0.08
16:4 (*n*-3)	1.08	1.26	1.43	1.07	1.25	0.69
18:0	1.37	1.28	1.65	0.97	1.68	2.29
18:1 (*n*-9)	1.56	1.35	1.40	1.17	1.43	1.40
18:1 (*n*-7)	0.40	0.35	0.31	0.18	0.30	0.47
18:2 (*n*-6)	0.64	0.48	0.61	0.46	0.67	0.97
18:3 (*n*-3)	1.26	1.34	1.40	1.25	1.37	0.70
18:4 (*n*-3)	0.41	0.45	0.52	0.46	0.56	0.32
20:1 (*n*-9)	0.33	0.35	0.43	0.34	0.31	0.17
20:4 (*n*-6)	0.04	0.04	0.03	0.03	0.03	0.04
20:4 (*n*-3)	0.13	0.09	0.12	0.07	0.09	0.08
20:5 (*n*-3)	0.44	0.36	0.34	0.25	0.27	0.13
Total	12.14	12.07	14.49	10.52	14.42	14.57
Saturated	5.37	5.46	7.28	4.81	7.53	9.06
Total (*n*-3)	3.49	3.70	4.04	3.24	3.71	2.00

protein content in the cyclostat cultures was 94.3 pg/cell, obtained with the highest nutrient concentrations. Growth rate in microalgae does not depend on the current environmental nutrient concentration, but upon the nutrient supply during the period preceding the determination of growth rate (Caperon and Meyer 1972). Predictably this same effect was reflected in the biochemical composition of the microalgae in the cyclostat system. A mechanism of adaptation to a high nitrogen concentration in the medium must be involved in the high protein content of cells growing in continuous cultures, with an uncoupling between nitrogen absorption and growth measured as cellular division or carbon fixation (Caperon 1968; Dortch et al. 1991; McCarthy and Goldman 1979).

Changes in cellular protein content were produced at the expense of carbohydrate content, lipid being only slightly affected by nutrient concentration at this high dilution rate (Fig. 4). Carbohydrates were the only fraction used for energy storage in response to nutrient stress. Similar results have been previously described for *T. suecica* (Thomas et al. 1984).

Steady-state cell density and chlorophyll content per unit volume did not follow the same pattern, as almost the same chlorophyll concentration was present in the cultures with 4 mmol and 8 mmol N/l, which had cellular densities significantly different (Mann-Whitney test, $P<0.01$) (Fig. 1). Corroborating previous reports on bath cultures (Davidson et al. 1992), the high variability reported in chlorophyll cellular content for cyclostat cultures invalidated any estimation of biomass or cell number based on absorbance or chlorophyll content, especially when the nutrient concentration was being varied.

As the chlorophyll cellular content increased linearly with nutrient concentration and the carbon content was stable, a lower photosynthetic efficiency in the cultures with higher nutrient concentration could be deduced. The high energetic requirements for NO_3^- incorporation and reduction could be also responsible for the low photosynthetic performance of cultures with high nutrient concentrations (Smith et al. 1992) and could also explain the decrease of steady-state cell density in those cultures, although an effect of inhibition of enzymatic activity by an excess of substrate may also be involved.

In cyclostat cultures, although the chlorophyll content per cell reached a maximum with the highest nutrient concentration, the amount of *n*–3 polyunsaturated fatty acids, usually located in membrane-associated polar lipids (Arao et al. 1987; Sukenik and Wahnon 1991; Sukenik et al. 1989), did not increase proportionally, and the cellular content of 20:5 *n*–3 decreased continuously with increasing nutrient concentration. On the other hand, a positive correlation between polar lipids and chlorophyll content has been reported for several microalgae (Sukenik et al. 1989, Parrish and Wangersky 1987). Acid extraction increases the amount of polar lipids extracted (Dubinksy and Aaronson 1979) but, surprisingly, in *T. suecica* this type of extraction increased the amount of saturated fatty acids extracted with respect to the standard extraction, mainly 12:0, 14:0 and 18:0, usually located in the neutral lipid fraction, and to a lesser extent the amount of 18:4 extracted. Despite the fact that a detailed study of the distribution of fatty acids among lipid classes in *T. suecica* is required, results suggest that a different desaturation pathway may be present in this species.

A wide range of variation in the biochemical composition of *T. suecica*, regarding protein, carbohydrates, lipids and fatty acids, has been recorded in cyclostat cultures when the nutrient concentration was changed, representing an interesting potential for nutritional applications or the production of fine chemicals.

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