The marine microalga *Chlorella stigmatophora* as a potential source of single cell protein: enhancement of the protein content in response to nutrient enrichment

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SUMMARY

Mass cultures of *Chlorella stigmatophora* were carried out in order to obtain maximum protein production and to study the chemical variations in function of the nutrient concentration. Cultures reached maximum cellular densities of $2.2 \cdot 10^8$ cells/ml, with a growth velocity between 0.49 and 0.55 doublings/day. Carbohydrate content in the stationary phase ranged between 2.23 and 2.74 pg/cell, RNA between 0.78 and 1.36 pg/cell and DNA between 0.013 and 0.016 pg/cell. The maximum value for chlorophyll *a* was 0.13 pg/cell. Maximum protein content was obtained with a nutrient concentration of 16 mM of $NaNO_3$, giving 4.85 pg/cell and a protein concentration of 0.7 g/l. The protein content can be manipulated by changes in the nutrient concentration, showing differences up to a 9.2-fold increase. This characteristic makes *Chlorella stigmatophora* a suitable source of single cell protein.

INTRODUCTION

Microorganisms are useful as food for human consumption, in the production of chemicals and in the bioconversion of solar energy [8,11]. Biomass production of microalgae was focused largely on a few freshwater species. Currently most of the microalgae used as food are from the eukaryotic *Chlorophyta* or are prokaryotic blue-green algae. Almost exclusively these include *Scenedesmus, Chlorella* and *Spirulina* [2,10,19]. Among microalgae, the genus *Chlorella* includes a great number of

freshwater species, while there are only a few marine species; one of these marine species is *Chlorella stigmatophora*. Interest in algal biomass production has now spread to include marine and stuarine species as well [5,6,21]. *C. stigmatophora* has been reported as a good source of single cell protein (SCP) [3]. Mass culture of the organism and improved knowledge of its composition, growth and chemical variability will enable its better use and the obtention of high algal yields.

We report here the response of a mass culture of *C. stigmatophora* to a series of nutrient concentrations, studying its chemical composition during the logarithmic and stationary phases. We estimate its

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biochemical variability, since this variability can affect its nutritive and commercial value when this species is used as SCP.

MATERIALS AND METHODS

The marine microalga *C. stigmatophora* was obtained from The Culture Centre of Algae and Protozoa, Cambridge, U.K. It was cultured in 0.45 μm filtered seawater, autoclaved at 120°C for 60 min and enriched with $NaNO_3$, 2 mM; NaH_2PO_4, 100 μM; $ZnCl_2$, 1 μM; Mn Cl_2, 1 μM; Na_2MoO_4, 1 μM; $CoCl_3$, 0.1 μM; $CuSO_4$, 0.1 μM; ferric citrate, 20 μM; thiamine, 35 μg/l; biotin, 5 μg/l; B_{12}, 3 μg/l; EDTA, 26.4 μM; Tris-HCl, 5 mM. Salinity of the seawater was 35‰ and the initial pH of the cultures was 7.6.

The nutrient solution with the composition given above was the first to be used. From this we followed a geometrical progression, using concentrations corresponding to 4, 8 and 16 mM of $NaNO_3$. Nutrient concentrations are expressed as $NaNO_3$ concentrations, but all the other nutrients were proportionally increased.

Mass cultures were carried out in 10 l flasks containing 9 l of culture medium [5]. An inoculum of $6 \cdot 10^6$ logarithmic phase cells/ml was used. Cultures had air continuously bubbled through them at a rate of 15 l/min.

Cellular density was determined by counting culture aliquots in a Thoma chamber.

Cell volume was calculated by measuring the diameter of a significant number of cells under the microscope and assuming the cell to be spherical.

Chlorophyll *a* was determined spectrophotometrically [7,18]. After obtaining the crude extracts [5], protein was measured by the dye-binding method [1] and carbohydrates by the phenol-sulphuric acid method [12]. Nucleic acids were extracted and determined as described by Kochert [13].

Stationary phases were compared by an overall multivariate one-way analysis of variance and logarithmic phases were compared by an overall multivariate one-way analysis of covariance.

A multiple non-linear least-squares regression of

order 4 was applied to the growth curve. The resultant equation was as follows:

$$f(t) = a + bt + ct^2 + dt^3 + et^4$$

where $f(t)$ is cellular density, t is time in days, and a, b, c, d and e are the coefficients of the equation.

From the growth equation we calculated doublings/day:

$$\text{doublings/day} = t_d^{-1} = \frac{\ln f(t_n) - \ln f(t_i)}{\ln 2 \,(t_n - t_i)}$$

where t_i and t_n are the initial and final time of the logarithmic phase, both expressed in days, and t_d is the duplication time.

RESULTS AND DISCUSSION

Culture conditions were established in order to produce maximum biomass to obtain protein. Cultures were illuminated with 11 fluorescent lamps, six under the culture vessels and five beside them, giving 6500 and 5500 lux, respectively, in order to maintain light in saturation. In our growing system the air flow of 15 l/min maintained a transference
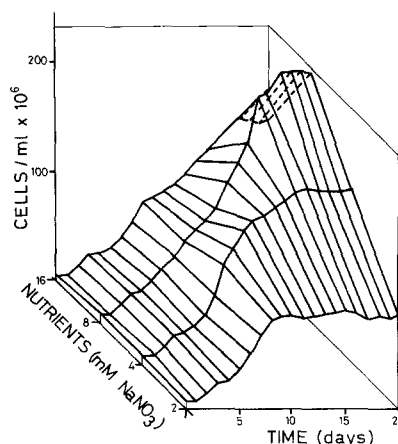

Fig. 1. Growth of *C. stigmatophora* at different nutrient concentrations, expressed as $NaNO_3$ concentrations.
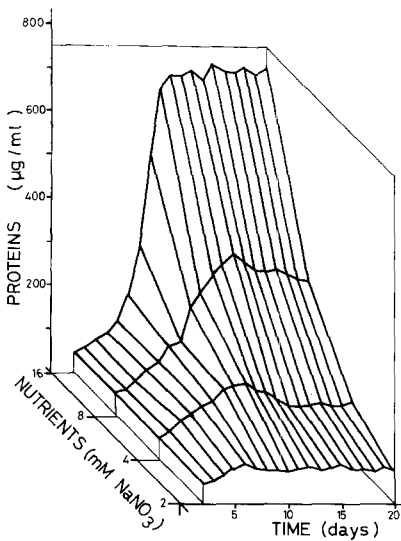
Fig. 2. Protein concentration in mass cultures of *C. stigmato-phora* at different nutrient concentrations, expressed as $NaNO_3$ concentration.

Fig. 4. Carbohydrate concentration in mass cultures of *C. stig-matophora* at different nutrient concentrations, expressed as $NaNO_3$ concentrations.

of CO_2 to the culture medium that kept the pH below 8.4.

Plotting cellular density, protein ($\mu g/ml$), chlorophyll *a* ($\mu g/ml$) and carbohydrates ($\mu g/ml$) against time for each nutrient concentration, we obtained three-dimensional figures (Figs. 1, 2, 3, 4). Statistical treatment of these data is represented in Table 1. From the equation calculated, we can establish the growth kinetics for each culture and pre-
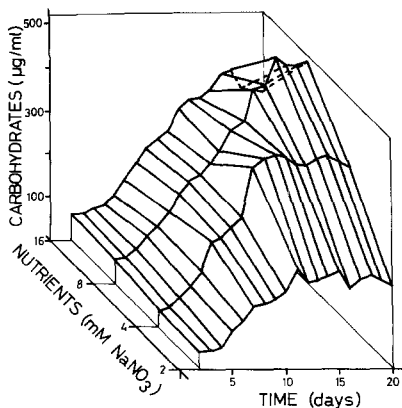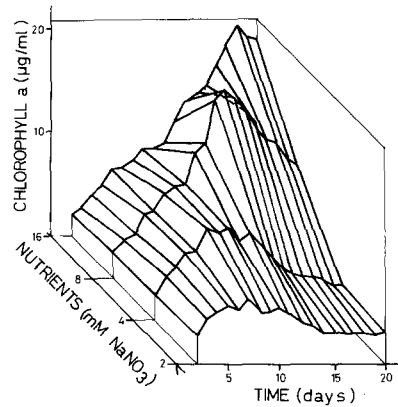
dict the growth of the microalgal population. The values of *a, b, c, d* and *e* are presented in Table 2. From this equation we calculated doublings/day or the inverse of the duplication time (t_d^{-1}). The initial time (t_i) and final time (t_n) of the logarithmic phase were established for each nutrient concentration (Table 1).

After a lag phase of 4–5 days the cells entered into exponential growth, which lasted between 4 and 12 days. Optimal nutrient concentration to produce a maximum cellular density was 8 mM of $NaNO_3$, which gave $225 \cdot 10^6$ cells/ml (Fig. 1). Statistically this value is significantly higher ($P < 0.001$) than those obtained with the remaining nutrient concentrations. Cellular density increased as nutrient concentration increased to 8 mM of $NaNO_3$; the highest nutrient concentration produced a decrease in the cellular density. A nutrient concentration of 2 mM of $NaNO_3$ is commonly used for culturing marine microalgae [15].

The maximum growth velocity of *C. stigmato-phora* cultures in the logarithmic phase was between 0.49 and 0.55 doublings/day (Table 1). The growth velocities were very similar in all the cultures and similar to those obtained in cultures of *Tetraselmis suecica* and *Isochrysis galbana* under the same conditions [5,6].

Maximum cellular volumes were obtained at 2 and 16 mM of $NaNO_3$ with mean values of 20 and

Fig. 3. Chlorophyll *a* concentration in mass cultures of *C. stig-matophora* at different nutrient concentrations, expressed as $NaNO_3$ concentrations.

Table 1

Statistical analysis of the growth curves of *C. stigmatophora* at different nutrient concentrations in mass culture

Each value corresponding to a nutrient concentration is only compared with the succeeding concentration ($P < 0.001$).

	Growth phase	P	Nutrient concentration[a] (mM)			
			2	4	8	16
10^6 Cell/ml	Stationary[b]	0.001	86 ± 2.5	<149 ± 8.5	<225 ± 10.4	>127 ± 27.2
Time interval, days ($t_n - t_i$)			8–4	10–4	15–4	8–5
Doublings/day	Logarithmic[c]		0.55	0.53	0.49	0.53
Protein (μg/ml)	Stationary	0.001	76 ± 4.6	<127 ± 6.4	<323 ± 12.8	<701 ± 10.6
Chlorophyll *a* (μg/ml)	Stationary	0.001	2.2 ± 0.3	< 3.8 ± 0.6	< 11.2 ± 1.9	< 17.5 ± 2.6
Carbohydrates (μg/ml)	Stationary	0.001	204 ± 13	<379 ± 10	<511 ± 8.2	>348 ± 22.0
Volume (μm^3)	Stationary	0.001	20 ± 2.2	> 16 ± 1.5	= 16.5 ± 1.6	< 23.1 ± 4.0
Efficiency (%)	Stationary		49	49	53	51

[a] Expressed as $NaNO_3$ concentration.
[b] One-way analysis of variance (ANOVA).
[c] One-way analysis of covariance (ANCOVA).

23 μm^3, respectively (Table 1). Cellular volumes were significantly lower with 4 and 8 mM of $NaNO_3$.

At the end of the culture period the protein content had reached maximum values of 701 μg/ml and 4.85 pg/cell with a nutrient concentration of 16 mM $NaNO_3$ (Tables 1 and 3). This means a protein concentration of 0.7 g/l of culture, which constitutes an important quantity for using this marine microalga as a SCP source. The protein content of the cultures increased in proportion to nutrient concentration

and differences as great as a 9.2-fold increase can be observed. These differences in the protein content are more acute than those found in other microalgae such as *Dunaliella salina* grown in different culture media, nitrate or glutamine [14], or *T. suecica* and *I. galbana* with different nutrient concentrations [5,6]. Maximum protein content and maximum cellular density occurred at different nutrient concentrations, but changes in the protein content are not necessarily related to cellular density in the culture medium because the biochemical composi-

Table 2

Values of the coefficients *a*, *b*, *c*, *d* and *e* of the growth equation $f(t) = a + bt + ct^2 + dt^3 + et^4$ calculated by a multiple non-linear least-squares regression

	Nutrient concentration (mM)			
	2	4	8	16
a	−97.7117	−86.976	+32.7486	+ 9.09947
b	+41.333	+22.1502	− 4.03336	+24.5803
c	− 3.17156	+ 0.69485	+ 0.833387	− 3.66664
d	+ 0.096455	− 0.113279	+ 0.052788	+ 0.310898
e	− 0.000921	+ 0.002656	− 0.002939	− 0.008866
S.D.	3.36	3.62	7.52	6.73

Table 3

Statistical analysis of the cellular content of C. *stigmatophora* grown at different nutrient concentrations in mass culture ($P < 0.001$)

	Growth phase	P	Nutrient concentration[a] (mM)			
			2	4	8	16
Protein	Stationary[b]	0.001	0.88 ± 0.05	= 0.88 ± 0.15	< 1.43 ± 0.11	< 4.85 ± 0.43
(pg/cell)	Logarithmic[c]	0.001	1.98 ± 0.54	= 2.57 ± 0.55	= 2.65 ± 0.24	= 4.57 ± 2.18
Chlorophyll *a*	Stationary	0.001	0.03 ± 0.01	= 0.03 ± 0.01	= 0.05 ± 0.01	< 0.12 ± 0.005
(pg/cell)	Logarithmic	0.001	0.11 ± 0.05	= 0.12 ± 0.05	= 0.12 ± 0.02	= 0.13 ± 0.04
Carbohydrates	Stationary	0.001	2.28 ± 0.26	= 2.53 ± 0.19	= 2.23 ± 0.04	= 2.74 ± 0.48
(pg/cell)	Logarithmic	0.001	2.03 ± 0.25	= 2.59 ± 0.48	= 2.57 ± 0.24	= 2.59 ± 0.19
RNA	Stationary	0.001	1.01 ± 0.06	> 0.78 ± 0.05	< 0.92 ± 0.08	< 1.36 ± 0.23
(pg/cell)	Logarithmic	0.001	1.09 ± 0.12	= 1.37 ± 0.41	= 1.42 ± 0.32	= 1.90 ± 0.10
DNA	Stationary	0.001	0.016 ± 0.005	= 0.014 ± 0.005	= 0.013 ± 0.004	= 0.014 ± 0.007
(pg/cell)	Logarithmic	0.001	0.016 ± 0.005	= 0.018 ± 0.007	= 0.017 ± 0.008	= 0.020 ± 0.006
Protein/carbo-	Stationary		0.38	0.35	0.64	1.77
hydrate ratio	Logarithmic		0.97	0.99	1.03	1.76
Protein/chloro-	Stationary		27.50	26.65	27.50	37.30
phyll *a* ratio	Logarithmic		18.00	21.41	22.08	34.36
Protein/RNA	Stationary		1.02	1.13	1.54	2.55
	Logarithmic		1.82	1.88	1.87	2.40
DNA/RNA	Stationary		0.015	0.017	0.014	0.010
	Logarithmic		0.014	0.013	0.011	0.010

[a] Expressed as $NaNO_3$ concentration.
[b] One-way analysis of variance (ANOVA).
[c] One-way analysis of covariance (ANCOVA).

tion of microalgal cells may change within more or less narrow limits depending on environmental action [5,6,20]. During the logarithmic phase the protein/cell ratio was independent of the nutrient concentration (Table 3). Differences in the protein/cell ratio appear when the cultures entered into stationary phase. In this phase, protein/cell content was similar for the cultures with lower nutrient concentrations, increasing with higher concentrations, mainly with 16 mM of $NaNO_3$. This nutrient concentration also produced the maximum cellular volume.

The protein/carbohydrate ratio ranged between 0.33 and 1.77 in the stationary phase and between 0.97 and 1.76 in the logarithmic phase (Table 3). These ratios were similar to those found for other microalgae [9,16,17].

Protein/chlorophyll *a* ratios ranged between 26.65 and 37.30 in the stationary phase and between 18.00 and 34.36 in the logarithmic phase (Table 3). These ratios are similar to those obtained for *T. suecica* and *I. galbana* under similar conditions [5,6].
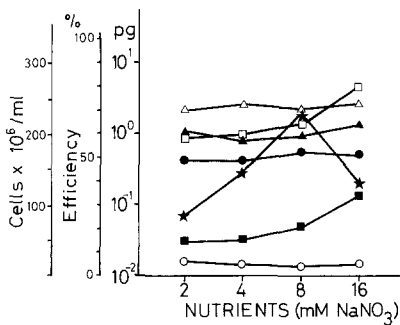
The protein/RNA ratio was practically constant

Fig. 5. Cellular density and chemical composition of *C. stigmatophora* in the stationary phase, at different nutrient concentrations. (★) cells/ml; (□) protein/cell; (△) carbohydrates/cell; (■) chlorophyll *a*/cell; (▲) RNA/cell; (○) DNA/cell; (●) efficiency (nitrate-N/protein-N transformation rate).

during the logarithmic phase, whereas this ratio increased with the nutrient concentration in the stationary phase.

The efficiency of nitrogen transformation was independent of the nutrient concentration, with values between 49 and 53% (Fig. 5). We established this efficiency as the ratio between the nitrogen added in nitrate form to the culture medium and the protein nitrogen produced per culture. This means that the inorganic nitrogen is not entirely transformed into protein nitrogen by the cells. Protein content per cell and per ml increased proportionally to nutrient concentration in the stationary phase and this may be due to the inclusion in the culture medium of a limiting factor for the growth or for the protein synthesis. A more exact adjustment of the composition of the culture medium would allow better cellular metabolism, providing improved nutrient utilization. This would diminish the costs of production and produce better yields. By altering the culture medium composition of mass cultures of *C. stigmatophora* it may be possible not only to obtain high algal yields, but also to transfer this food efficiently to higher components of the food chain so as to maximize the conversion of dissolved nutrients into biomass of commercially important marine food crops.

Chlorophyll *a*/ml increased with the nutrient concentration and the maximum value of 17.5 μg/ml occurred at 16 mM of NaNO$_3$. Chlorophyll *a*/cell was constant in the logarithmic phase for all

the nutrient concentrations and in the stationary phase for 2, 4 and 8 mM NaNO$_3$, showing a maximum value of 0.13 pg/cell at 16 mM NaNO$_3$ (Tables 1 and 3).

Maximum carbohydrate concentration in the stationary phase occurred at 8 mM of NaNO$_3$, being 511.6 μg/ml. This nutrient concentration gave also the maximum values of biomass (cells/ml). The carbohydrate/cell ratio was constant in both phases and at all nutrient concentrations, with values between 2.03 and 2.74 pg/cell (Table 3).

RNA/cell concentrations were constant in the logarithmic phase, and in the stationary phase they ranged between 0.78 and 1.36 pg/cell, tending to increase with the nutrient concentration.

DNA/cell concentrations were constant at all the nutrient concentrations assayed and in both growth phases. DNA contents of *C. stigmatophora* cells ranged from 0.013 to 0.020 pg/cell.

It has been shown that the protein content of *C. stigmatophora* cultures can be manipulated by changes in the nutrient concentration, showing differences of up to a 9.2-fold increase in the stationary phase. Chlorophyll *a*, carbohydrate and RNA content also varies, but differences are lower. *C. stigmatophora* has been suggested as a mineral source in fish diets [4]. The protein of *C. stigmatophora* is of good biological value and because of this it has been suggested as a potential source of SCP [3]. The protein concentration obtained (0.7 g/l) and the possibility of manipulating its chemical composition enhance these possibilities, making *C. stigmatophora* a potentially interesting source of SCP, including a marine species of this genus among the *Chlorella* sources of protein.

ACKNOWLEDGEMENTS

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