Growth and biochemical variability of the marine microalga *Chlorella stigmatophora* in batch cultures with different salinities and nutrient gradient concentration

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Abstract

Chlorella stigmatophora was cultured under 56 different combinations of nutrient-salinity concentrations, ranging from 1 to 64 mM $NaNO_3$ and from 0 to 35%. salinity. Optimal growth conditions for obtaining maximum cellular densities were between 1 and 8 mM $NaNO_3$ for any salinity, with a maximum growth rate at the logarithmic phase of 0·51 doublings d^{-1}. Production over the 15 days as measured by chlorophyll *a* estimation were maximal between 5 and 20%. and 8–32 mM $NaNO_3$. Maximum protein per ml occurred at 8 and 16 mM $NaNO_3$ for all the salinities, whereas maximum protein/cell contents were obtained at 16 and 32 mM $NaNO_3$. Protein content per ml and per cell was not affected by salinity. The nitrate/protein transformation rate is related to nitrate concentration. Maximum rates were obtained at 1 mM $NaNO_3$, with values between 92% and 100%. Nutrient concentration produced changes in the biomass production and biochemical composition of this marine microalga, with wide variations in the chlorophyll *a* and protein content per ml (up to 700% and 500%, respectively).

This microalga shows an important capacity to adapt to changes in salinity, ranging from freshwater (0%.) to oceanic sea-water (35%.). The significance of these results for

the possible utilization of *Ch. stigmatophora* as source of Single Cell Protein (SCP) is discussed.

Micro-organisms are potentially useful as food for human consumption, in the production of chemicals, in aquaculture and in the bioconversion of solar energy (Goldman, 1979; Kharatyan, 1978). Of such microorganisms, a few freshwater species of microalgae have been used as food (Hwang, Wang & Lii, 1980). 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* Butcher.

Salinity, nutrient concentration, light, temperature and carbon source are considered to be the most important factors influencing the growth of marine microalgae in culture. High nutrient concentrations usually lead to maximum production of total biomass and are used in aquaculture, or for industrial applications. Batch cultures are a helpful tool for establishing, in part or completely, optimal conditions for subsequent mass culture (Fabregas *et al.,* 1984; Fabregas *et al.,* 1985a).

We report here the growth of cultures of the marine microalga *Chlorella stigmatophora* and its protein and chlorophyll a content when cultivated at different salinities and levels of nutrients.

Materials and methods

Chlorella stigmatophora was obtained from "The Culture Centre of Algae and Protozoa", Cambridge, England. It was grown in sea-water filtered through a 0.45-µm Millipore filter, autoclaved at 120°C for 20 min and enriched with $NaNO_3$, 2 mM; NaH_2PO_4, 100 µM; $ZnCl_2$, 1 µM, $MnCl_2$, 1 µM; Na_2MoO_4, 1 µM; $CoCI_3$, 0.1 µM; $CuSO_4$, 0.1 µM; ferric citrate, 20 µM; thiamine, 35 µg l^{-1}; biotin, 5 µg l^{-1}; B_{12}, 3 µg l^{-1}; EDTA, 26-4 mM; Tris-HCl, 15 mM; pH, 7'6.

Salinities of 35, 30, 25, 20, 15, 10, 5 and 0‰ were used. The salinity of the sea-water (35‰) was reduced by the addition of appropriate volumes of fresh distilled water prior to medium preparation.

The first nutrient concentration utilized was the one whose composition was the half of that given above and which corresponds to $NaNO_3$, 1 mM. From this we followed a geometrical progression, using concentrations corresponding to 2, 4, 8, 16, 32 and 64

mM $NaNO_3$. Nutrient concentrations are expressed as NaNO3 concentrations, but all the other nutrients were increased proportionally.

Cells were cultured in Kimax screw-capped test tubes (15 x 2.5 cm) with 40 ml of medium. All cultures were grown in a controlled environmental incubator (New Brunswick) at 15°C and 3900 lx from fluorescent lamps (Phillips TL 20W/55). A 12:12 light-dark regime was maintained in order to obtain synchronous cultures and an inoculum of 2 x 10^5 logarithmic phase cells was used. pH was below 8-6 in all the cultures (Fig. 1).

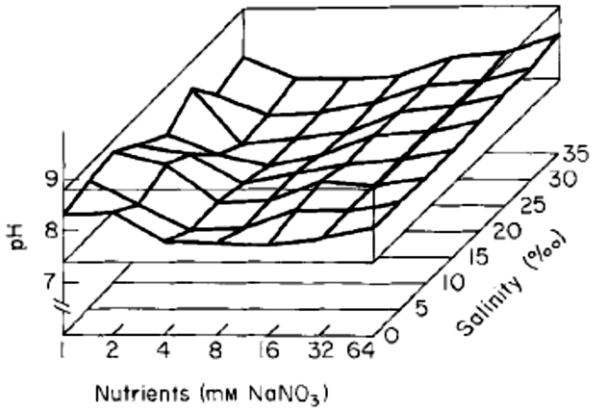

FIG. 1. pH values at the stationary phase in cultures of *Chlorella stigmatophora* at different salinities and nutrient concentrations.

The kinetics of the cultures were established by transmittance measurements. transmittance of the cultures was determined with a Coleman II 6/20 spectrophotometer reading at 530 nm and values were expressed as (100-T). Transmittance, expressed as (100-T), was plotted against time and against salinity for each nutrient concentration, obtaining three dimensional figures (Fig. 2). Stationary phases, corresponding to maximum biomass production, were compared by an overall multivariate oneway analysis of variance (ANOVA). Statistical treatment of these data is represented in Table I.

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

Chlorophylls were extracted in acetonemethanol 2 : 1 for 48 h from the stationary-phase cells. The extracts were filtered through a Fluoropore Millipore filter for clarification (Fabregas, Herrero & Veiga, 1984) and absorbances of the pigment extract at specific wavelengths were recorded. The concentration of chlorophyll a was spectrophotometrically determined by the formula of Parsons & Strickland, 1965).

Protein was measured in the stationary phase by the dye-binding method (Bradford, 1976).

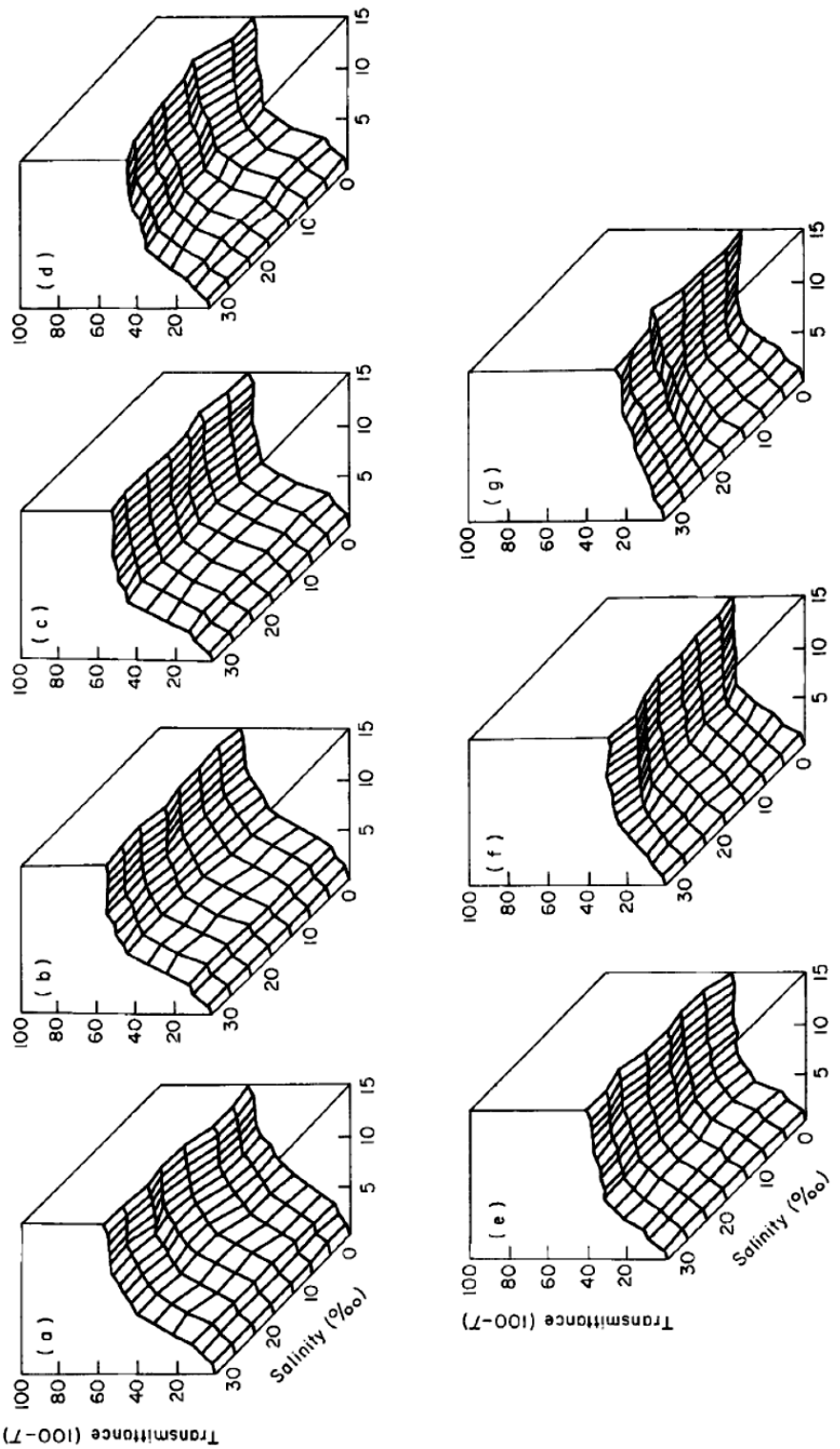

FIG. 2. Growth of *Chlorella stigmatophora* at different salinities and nutrient concentrations, expressed as $NaNO_3$ concentrations. Transmittance at 530 nm is represented as $100 - T$ values. (a), nutrient concentration 1 mM $NaNO_3$; (b) 2 mM; (c) 4 mM; (d) 8 mM; (e) 16 mM; (f) 32 mM; (g) 64 mM.

Results

There is a relationship between transmittance of *Chlorella stigmatophora* cultures, measured at 530 nm in screw-capped test tubes (15 x 2.5 cm) and cellular density. This relationship fitted to a linear equation $y = 0.84\ x+9.07$, with a correlation coefficient of 0-97, for the optimum growth interval, where y = (100- T) and *x* = cellular density. This optimum interval was between 1 and 8 mM $NaNO_3$ for all the salinities. For the remaining nutrient concentrations another linear regression was established: $y = 1.02\ x+10.92$, with a correlation coefficient of 0.99. These relationships were only calculated for the stationary phases.

Optimal conditions for obtaining a maximum cellular density in the stationary phase were 1 to 8 mM $NaNO_3$ for all the salinities (Fig. 3), with cellular densities between 40 x 10^6 and 57 x 10*6 Ch. stigmatophora* cells per ml. Therefore, this marine microalga shows an important capacity for adaptation to changes in salinity conditions, ranging from 0%° (fresh water) to 35‰ (oceanic seawater) (Fig. 2).

Ch. stigmatophora growth was only affected by salinity when the cells were exposed to the highest nutrient concentrations (32 and 64 mM $NaNO_3$) (Fig. 2F, G). Under such conditions, the cellular density of the cultures decreased as the salinity increased.

Salinity and nutrient concentrations have no effect on the growth rate of *Ch. stigmatophora* in synchronous cultures. Maximum growth rate in the logarithmic phase was 0-51 doublings d^{-1} in all the conditions, except for 64 mM $NaNO_3$ whichreduced the maximum rate to 0.38 doublings d^{-1} at all salinities.

Maximum values of chlorophyll a occurred at 5-15‰ salinity and 8-32mM $NaNO_3$ (Fig. 4), with a maximum 5.14 µg ml^{-1}. Chlorophyll a content in the cultures is not directly related to salinity or nutrient concentrations used.

Maximum concentrations of chlorophyll a per cell were obtained at 16 and 32mM $NaNO_3$ for all the salinities (Fig. 4B). Maximum value was 0.23 pg per cell.

When the nutrient concentration increased the total protein content of the cultures and the protein per cell increased (Fig. 5), whereas salinity produced no effect. Maximum protein concentrations per ml of culture were found at 8 and 16 mM $NaNO_3$, whereas maximum contents of protein per ml were obtained at 16 and 32 mM $NaNO_3$ for any salinity, with values between 5.64 and 7.82 pg per cell.

The efficiency of nitrate-N/protein-N transformation expressed as the ratio between nitrogen added in nitrate form to the culture medium and the protein nitrogen produced per culture was established. Maximum efficiencies were found at 1 mM $NaNO_3$ for any salinity (Fig. 6), with values between 92% and 100%. With these culture conditions a more efficient transformation of nitrate in microalgal biomass occurred.

TABLE I. Statistical analysis of the growth curves of *Chlorella stigmatophora* (represented as $100-T$) at different salinities and nutrient concentrations

Nutrient concentration* (mM)	Salinities (‰)							
	0	5	10	15	20	25	30	35
1	$47{\cdot}75\pm4{\cdot}43$ =	$45{\cdot}00\pm4{\cdot}95$ =	$46{\cdot}56\pm4{\cdot}93$ =	$52{\cdot}56\pm4{\cdot}45$ =	$54{\cdot}78\pm4{\cdot}82$ =	$50{\cdot}67\pm4{\cdot}39$ =	$54{\cdot}56\pm4{\cdot}00$ =	$53{\cdot}00\pm4{\cdot}50$ =
2	$52{\cdot}22\pm5{\cdot}02$ =	$53{\cdot}67\pm4{\cdot}56$ =	$53{\cdot}33\pm4{\cdot}69$ =	$56{\cdot}00\pm3{\cdot}77$ =	$53{\cdot}33\pm2{\cdot}45$ =	$56{\cdot}44\pm2{\cdot}60$ =	$54{\cdot}33\pm2{\cdot}96$ =	$53{\cdot}22\pm3{\cdot}11$ =
4	$50{\cdot}89\pm1{\cdot}62$ ⩾	$51{\cdot}89\pm1{\cdot}76$ =	$54{\cdot}89\pm2{\cdot}20$ =	$52{\cdot}78\pm1{\cdot}30$ =	$50{\cdot}64\pm4{\cdot}84$ =	$51{\cdot}64\pm4{\cdot}63$ =	$54{\cdot}11\pm2{\cdot}37$ ⩾	$51{\cdot}44\pm2{\cdot}60$ ⩾
8	$46{\cdot}60\pm1{\cdot}84$ ⩾	$47{\cdot}27\pm4{\cdot}61$ ⩾	$52{\cdot}62\pm5{\cdot}73$ ⩾	$48{\cdot}82\pm5{\cdot}56$ ⩾	$49{\cdot}91\pm5{\cdot}15$ ⩾	$46{\cdot}45\pm5{\cdot}34$ =	$46{\cdot}40\pm4{\cdot}74$ ⩾	$41{\cdot}56\pm3{\cdot}88$ =
16	$35{\cdot}27\pm2{\cdot}41$ =	$37{\cdot}64\pm2{\cdot}87$ ⩾	$38{\cdot}09\pm3{\cdot}33$ ⩾	$35{\cdot}45\pm2{\cdot}73$ ⩾	$36{\cdot}55\pm3{\cdot}47$ ⩾	$38{\cdot}78\pm3{\cdot}83$ ⩾	$35{\cdot}45\pm4{\cdot}34$ ⩾	$36{\cdot}30\pm3{\cdot}16$ ⩾
32	$35{\cdot}55\pm2{\cdot}42$ ⩾	$31{\cdot}00\pm0{\cdot}87$ =	$32{\cdot}56\pm1{\cdot}01$ =	$30{\cdot}56\pm1{\cdot}01$ =	$31{\cdot}00\pm2{\cdot}68$ =	$29{\cdot}56\pm1{\cdot}01$ ⩾	$21{\cdot}82\pm1{\cdot}66$ ⩾	$27{\cdot}90\pm1{\cdot}91$ ⩾
64	$33{\cdot}20\pm1{\cdot}40$ ⩾	$27{\cdot}64\pm2{\cdot}01$ =	$28{\cdot}75\pm3{\cdot}93$ =	$27{\cdot}54\pm4{\cdot}72$ =	$26{\cdot}46\pm7{\cdot}63$ =	$18{\cdot}62\pm5{\cdot}85$ =	$20{\cdot}92\pm6{\cdot}42$ =	$16{\cdot}85\pm6{\cdot}00$

Values are expressed as mean ±S.D. Each value corresponding to a salinity and a nutrient concentration is compared by a one-way analysis of variance (ANOVA) with both the following salinity and nutrient concentration.
* Expressed as $NaNO_3$ concentration.

Discussion

Culture conditions were aimed at obtaining maximum biomass production. In general, batch cultures are a helpful tool for establishing, in part or completely, the optimum parameters for a subsequent mass culture (Fabregas *et al.,* 1984; Fabregas *et al.,* 1985a, 1985b). When batch cultures are used, some of these parameters can become limiting.

During the stationary phase, transmittance measurements can be transformed into cellular densities, since it is generally accepted that a relation exists between optical density and cellular density (Fabregas *et al.,* 1984; Lyon & Woo, 1980).This relationship can be fitted to a power curve considering the two growth phases (Fabregas *et al.,* 1984), but a linear relationship can be obtained from the stationary phase (Fabregas *et al.,* 1985a). This kind of linear relationship was obtained for *Ch. stigmatophora* under optimum and non-optimum conditions.

The characteristic of this microalga for adaptation to changes in salinity conditions (ranging from 0‰ to 35‰) has not been observed in other marine microalgae under the same conditions (Fabregas *et at.,* 1984; Fabregas *et at.,* 1985).

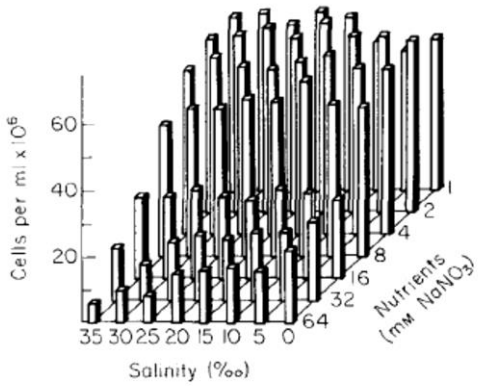

FIG. 3. Maximum cellular densities of *Chlorella stigmatophora* in stationary phase at different salinities and nutrient concentrations.

At high nutrient concentrations, the first limiting factor for microalgal growth usually becomes the carbon source, which is not included in the culture medium because it is supplied in seawater. In batch cultures without aeration, carbon is not supplied as CO_2. Carbon dioxide is closely related to the pH of the cultures, and because CO_2 is not added, pH can appear as a limiting factor. *Chlorella stigmatophora* growth generates strong alkalinity in the cultures. Since the uptake of $NaNO_3$ during photosynthesis generates a strong base (Brewer & Goldman, 1976; Goldman *et al.,* 1972) in our growing systems the culture medium was buffered with Tris (Guillard & Ryther, 1962; McLachlan & Gorham, 1962; Pintner & Provasoli, 1958; Sorge & McLaughlin, 1970) which maintained the pH below 8.6 (Fig. 1).

Samples for protein and chlorophyll a determinations were always collected at the same time of the light period because protein concentration varies depending on the moment in the light period at which the sample is taken (Van Liere *et al.,* 1979). The results showed a great deal of variability in chlorophyll *a* content per cell caused by the salinity and the nutrient concentration, and differences of 700% or more could be observed. Variations in the protein content can range up to 500%. These variations in protein are mainly caused by the nutrient concentration. *Ch. stigmatophora* has been suggested as Single Cell Protein (Fabregas & Herrero, 1985). Differences in the protein content such as those shown here must be taken into account when *Ch. stigmatophora* is going to be used as SCP.

Changes in the protein content are not related to cellular density, because the biochemical composition of microalgal cells may change within more or less narrow limits depending on culture conditions (Fabregas *et al.,* 1985b; Stross & Pemrick, 1974).

These data are in general agreement with those of other authors which indicate that the nutrient supply influences the chlorophyll a and protein content of unialgal cultures, with important variations in the biochemical composition of microalgal cells as a function of the nutrient concentration in the culture medium (Fabregas *et al.,* 1984; Myklestad, 1974).

The maximum transformation of inorganic nitrogen in microalgal biomass occurred at 1 mM $NaNO_3$ for any salinity. With higher nutrient concentrations the most important limiting factor is the carbon source, since in seawater inorganic carbon concentration is about 2 mM (Burris, 1977).

Great variability in the protein and chlorophyll a content of *Ch. stigmatophora* has been shown as a result of the culture conditions. This information can be used for producing cells with protein and chlorophyll a contents that can be previously determined as a function of the culture conditions, mainly the nutrient concentration, with wide variations (500% for the protein content and 700% for the chlorophyll a content). This variability can be useful when *Ch.stigmatophora* is used for producing biomass with a composition previously established.

On the other hand, *Ch: stigmatophora* can be a good source of Single Cell Protein (Fabregas & Herrero, 1985) and its nitrate- N/protein-N efficiency is independent of salinity and reached values of about 100%, clearly higher than those of other microalgae such as *Tetraselmis suecica* (Kylin) Butcher (64%) (Fabregas *et al.,* 1984) or *lsochrysis galbana* Parke (84%) (Fabregas *et al.,* 1985a), under the same culture conditions.

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