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Improvement of growth rate and cell productivity by aeration rate in cultures of the marine microalga *Dunaliella tertiolecta*

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Abstract

The effect of different aeration rates and CO_2 supply regimes on mass cultures of the marine microalga Dunaiella tertiolecta was studied. Eight aeration rates in the range 0-6.51 litres of air min^{-1} litre of $culture^{-1}$ were tested. The results were compared to those obtained in non-aerated cultures into which pure CO_2 was introduced and to cultures into which air enriched with CO_2 was bubbled. The growth rate and final cellular density of D. tertiolecta in a sea-water-based medium increased with the aeration rate in the culture vessel. The maximal cellular density was 12.46 x 10^6 cells ml^{-1} under an air flow rate of 6.51 litres min^{-1} litre of $culture^{-1}$, but evaporation and salinity increased sharply at this high aeration rate. The final cell density was proportional to the air flow rate and CO_2 following the range (figures being litres of air min^{-1} litre of culture $^{-1}$): 6.51 = 3.72 > 1.86 > CO_2 = (0.93 + CO_2) > 0.93 > 0.46 > 0.23 > 0.11 > 0 (Mann-Whitney test $p < 0.05$). When D. tertiolecta was grown under a CO_2 supply within the optimal pH levels but without aeration the cultures reached a cell density of 7 x 10^6 cells ml^{-1}. D. tertiolecta growth rate was inversely proportional to pH, the upper boundary for maximal growth rate being pH 9.2. Oxygen in the culture media produced by the photosynthetic activity of the microalgae did not inhibit growth. The dissolved CO_2 concentration in seawater was the limiting factor for D. tertiolecta growth. At a pH value

of 8.3, D. tertiolecta was not able to take up carbon in the form of carbonates dissolved in seawater.

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Author keywords

Aeration; Carbonates; CO_2; Dunaliella tertiolecta; Mass culture; Microalgae; pH

Indexed keywords

Engineering controlled terms: Algae; Carbon dioxide; Growth kinetics; Marine biology; pH effects; Productivity; Seawater; Substrates; Water aeration

Engineering uncontrolled terms: Aeration rate; Dunaliella tertiolecta

Engineering main heading: Plant cell culture

EMTREE drug terms: carbon; carbon dioxide; carbonic acid derivative; oxygen; sea water; sodium chloride

EMTREE medical terms: aeration; airflow; article; cell culture; cell density; cell growth; culture medium; growth rate; nonhuman; ph; photosynthesis; phytoplankton; priority journal; productivity

INTRODUCTION

Species of the genus *Dunaliella* are being commercially cultured for various purposes. Glycerol, β-carotene and protein-enriched biomass can be produced from the genus *Dunaliella* (Gibbs & Duffus, 1976; BenAmotz & Avron, 1978, 1980; Ben-Amotz *et aI., 1982;* Borowitzka, 1991). *Dunaliella tertiolecta* can also be used as a source of SCP (Fabregas & Herrero, 1985) and as a mineral supplement in fish feed (Fabregas & Herrero, 1986). One of the most characteristic features of this species is the lack of a rigid cell wall (Oliveira *et aI.,* 1980) which makes its digestion easier. Recently its use as a health food and its anti-cancer effect have been studied (Mokady, 1992; Fujii *etal., 1993).*

One of the factors limiting growth-rate in cultures of *D. tertiolecta* is pH. When the source of nitrogen is NaNO) the pH level in the medium increases as a consequence of microalgal metabolic activity. One of the ways of keeping the pH within an optimal range for maximal growth rate is the sparging of pure CO_2 or CO_2 and air (air by itself contains 0'03% of CO_2) at different rates. The supply of CO2 is one of the main items in the calculation of costs in mass cultures of microalgae. **In** the present work the optimization of air flow without any CO_2 supplementation was investigated in order to achieve maximal growth rate and final cell density.

METHODS

The marine microalga *Dunaliella tertiolecta* was grown in sea-water previously filtered through fibre glass *(GF/* C), autoclaved and enriched with the commercial inorganic nutrient Algal-1 (Nutrición Avanzada SA. Avda Cortes 8. Fuentes Nuevas. Ponferrada. Leon. Spain) (Herrero *et aI.,* 1991) in which the source of nitrogen was 2 mM $NaNO_3$. The initial pH of the cultures was 8'1 and the initial salinity was 3'5%.

The cultures were grown in 6 litre flasks containing 4·3 litres of medium at 18°C under a light intensity of 81·04 µE m^{-2} S^{-1} and a Ijght periodicity of 12:12 h light: darkness.

The initial inoculum density was 5 x 10^5 cells ml^{-1} from an exponentially growing culture.

Seven different flows of air were applied to the cultures: 0·11, 0·23, 0.46, 0·93,1·86, 3·72, 6·51 litres of air min^{-1} litre of $culture^{-1}$. Two controls were set up as follows: one without aeration (named as air flow 0) and another with no aeration to which different quantities of CO_2 were added by sparging twice a day in order to keep the pH in the range 7·2-7·9. A third control was aerated at 0·93 litres of air min^{-l} litre of culture-I with an intermittent supply of CO_2 to keep the pH in this same range. Two replicates were set for each condition. The diameter of the aeration line through which air and CO_2 were bubbled was 6 mm.

The cell density was determined daily using an improved Neubauer haemacytometer. The pH was measured using a portable Radiometer model 80 and an Ingold U455 pH electrode. The dissolved oxygen was measured with an Orion oxygen electrode (Model

97-08) and the total salinity with an Atago S/Mill refractometer.

Dissolved O_2, salinity (%) and pH were measured daily in the cultures. The pH and oxygen levels were measured between 1·3 and 2 h from the beginning of the light periods. In the cultures under pure CO_2 the pH was also measured after 5 -6 h of light period and CO_2 was added if necessary. The salinity was corrected with distilled water when values reached 4·0%. Distilled water was added slowly to avoid the formation ofstrong salinity gradients.

The growth rates were calculated from linear regression analyses of average cell density values during the exponential phase using the method proposed by Guillard (1973). Final cell densities were compared using the non-parametric statistics (Mann-Whitney test).

RESULTS

The salinity in aerated cultures was increased by evaporation during the 12 days of culture. Aerations between 0 and 0·23 litre min^{-1} litre of $culture^{-1}$ caused a small increase in salinity, while aeration rates in the range 0.46-6.51 litre min^{-1} litre of $culture^{-1}$ had higher increases in salinity (Table I). In the cultures with aeration rates higher than 0·93 litre min^{-1} of $culture^{-1}$, salinity exceeded 4·0% requiring the addition of distilled water to avoid the introduction of important variations from the initial conditions of the

culture and to allow a correct calculation of cell density (Table 1), although at these aeration rates the stationary phase was reached before the maximum level of salinity.

In the present experiments, the highest amount of oxygen that remained dissolved in the culture was 14·7 ppm recorded in the culture with only CO_2 supplementation (Table 2), and this was caused by the absence of aeration. Oxygen concentration in the aerated cultures ranged between 5 and 9 ppm. Cultures under aeration flows of 0·11-0·93 litre min^{-1} litre of $culture^{-1}$ had higher O_2 values than those with aerations in the range 1·86-6·51 litres min^{-1} litre of $culture^{-1}$. The culture under 6·51 litre min^{-1} litre of $culture^{-1}$ had, in general, the lowest oxygen values throughout the culture period (Table 2). In the nonaerated culture the oxygen level decreased due to the absence of photosynthetic activity.

The final cell density was proportional to the airflow rate and CO_2 following the range (the figures show litres of air min^{-1} litre of $culture^{-1}$): 6·51 = 3·72 > 1·86 > CO_2 = (0·93 + CO_2) > 0·93 > 0·46 > 0·23 > 0·11 > 0 (Mann-Whitney test p< 0·05) (Fig. 1). Non-aerated cultures showed negligible growth. The control culture submitted to CO, additions without air (in order to keep the pH between 7·2 and 7·9) reached a final cell density of 7 x 106 cells ml^{-1}, higher than those reached in cultures under aeration flows of 0- 0·93 litres min^{-1} litre of $culture^{-1}$, but lower than in the cultures with aeration flows between 1·86 and 6·51. No significant difference (p= 0·82) in stationary-phase cell densities was recorded between the cultures under 0·93 litre min^{-1} litre of $culture^{-1}$ plus CO_2 and cultures to which only CO_2 was added. Maximal final densities were 11 ·32 x 10^6 cells ml^{-1} and 12,46 x 10^6 , achieved with aeration rates of 3·72 and 6'51 litres min^{-1} litre of $culture^{-1}$ respectively (Fig. I).

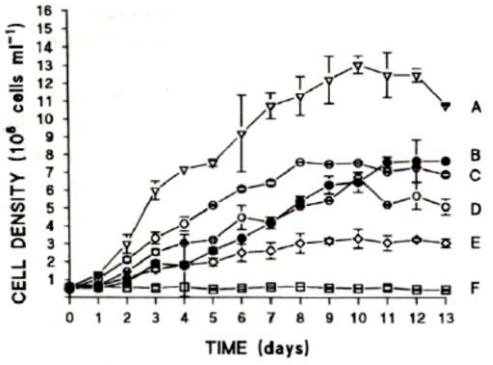

Fig. 1. *Dunaliella tertiolecta* growth curves under different aeration and CO_2 supply regimes. For clarity only the curves for some of the conditions tested are drawn, the other curves were of similar shape and fell between the curves shown. The figures are expressed as litres of air min^{-1} litre of $culture^{-1}$. A, 6·51; B, CO_2; C, (CO_2 + 0·93); D, 0·93; E, 0·23; J, 0. Error bars represent standard deviations.

An increase in the aeration rate caused a decrease in the pH and a proportional increase in the growth rate. The higher the aeration rates applied, the more CO_2 would be transferred to the culture medium from the air, resulting in a decrease in the pH and a subsequent increase in growth rate (Table 3). Increasing cell density produced linear increases in pH during exponential growth in the aerated cultures (r^2 > 0·9 except for the aeration rate 0·93 litre min^{-1} litre of $culture^{-1}$ for which r^2 = 0'7) (Fig. 2).

The maximal growth rate increased with aeration flow following a logistic curve, a maximum being reached at an aeration rate about 3·7 litres min^{-1} litre of $culture^{-1}$ (Fig. 3). The maximal growth rate of the culture under pure CO, during the logarithmic phase was 0·94 day^{-1}, higher than the growth rate of the culture to which a mixture of air and CO, was bubbled: 0·82 day^{1}, and similar to the culture with air alone supplied at a rate of 0'93 litre min^{-1} litre of $culture^{-1}$.

DISCUSSION

In cultures with high cell densities the oxygen produced by photosynthetic activity may reach concentrations as high as 35 ppm (Richmond, 1983). High concentrations of dissolved oxygen could be toxic for the cells thus resulting in a decrease in photosynthetic activity. However, the results in the cultures grown under pure CO_2 supply, in which the oxygen concentration was very high (14 ppm) but the final density was 7 x 10^6 cells ml^{-1}, were similar to those achieved in cultures with aeration rates of 0,93-1,86 litre min^{-1} litre of $culture^{-1}$ (Fig. 1) where the dissolved oxygen was about 7 -8 ppm. This indicates that oxygen overload in the former cultures was not a limiting factor. Moreover, despite the high oxygen levels produced by microalgal growth during the first 3 days of the cultures under CO_2 supply without air (8'2-14'7 ppm) the cultures doubled their cell density every 24 h (growth rate 0·94 $days^{-1}$), indicating again that the dissolved oxygen in the medium did not act as an important limiting factor for growth.

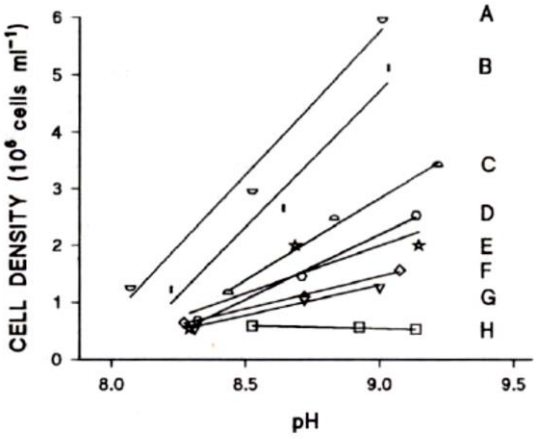

Fig. 2. Relationship between cell density, pH value and aeration rate during maximal growth-rate period in *D. tertiolecta* cultures (days 1–3). Linear regression lines are drawn ($r^2 > 0{\cdot}9$ except for aeration 0·93 litres of air min^{-1} litre of $culture^{-1}$, $r^2 = 0{\cdot}71$). Aeration rate is expressed as litres of air min^{-1} litre of $culture^{-1}$: A, 6·51; B, 3·72; C, 1·86; D, 0·93; E, 0·46; F, 0·23; G, 0·11; H, 0.

In microalgal cultures, the pH is one of the main variables to be controlled in order to achieve the maximal growth rate. The metabolic activity of microalgae increases the pH of the medium when the source of nitrogen is nitrate. On the other hand, in aerated cultures, the CO, present at 0'033% in air (Warburg, 1919) tends to decrease the pH through the buffer system H_2CO_3- CO_2. An equilibrium between the increase of H derived from algal growth and CO, input should be pursued in order to maintain a constant pH during culture growth. In another marine microalgal species, *Tetraselmis suecica,* the specific growth rate is increased proportionally with CO_2 (Molina *et al.,* 1990). *D. tertiolecta* growth was influenced by CO_2 concentration, provided by the different aeration rates, in the same way as has been described for the microalga *Chlorella vulgaris* (Markl, 1977).

Taking into account the total amount of CO_2 contained in the bubbled air, more than three times as much CO_2 was bubbled into the culture under an air flow of 0·93 litre min- I litre of culture- I than into the culture with pure CO_2 during the culture period to achieve even lower final cell densities in the former (Table 4), which demonstrated that the efficiency of CO_2 transfer from air was lower than the efficiency of the addition of pure CO_2.

All the cultures reached a pH value of 9·2 by the third day of culture, even though the growth rates, and therefore the cell densities, during this period were different for each culture and proportional to the aeration. During this period maximal growth rate was recorded for all cultures, decreasing once a pH value of 9·2 was reached, except for an aeration rate of 1·86 for which maximal growth rate was recorded between days o and

2. A similar high pH boundary of 9·3 was also described by Goldman *et al.* (1982). The growth rate and final cell density of *D. teniolecta* cultures depended on the pH of the culture. There was a linear relation between the pH increase and the increase in microalgal growth during the early logarithmic phase of growth (Fig. 2).

Despite the increase in maximal growth rate being linear up to an air flow of 0·93 litre min^{-1} litre of $culture^{-1}$ (r^2 = 0'99), when the final density is considered the linear relationship was retained only up to about 0-46 litre min^{-1} litre of $culture^{-1}$ (r^2 = 0'89) (Fig. 3). No statistically significant difference was recorded in the final cell density for aeration rates of 3·72 and 6·51 litres min^{-1} litre of $culture^{-1}$ (Mann-Whitney test, p= 0'2), although the values for the latter aeration rate were slightly higher. A higher standard deviation for stationary-phase values of the cultures under the highest air flow may indicate a lower capacity of the cells to survive under starvation conditions, probably due to the lower content of storage substances.

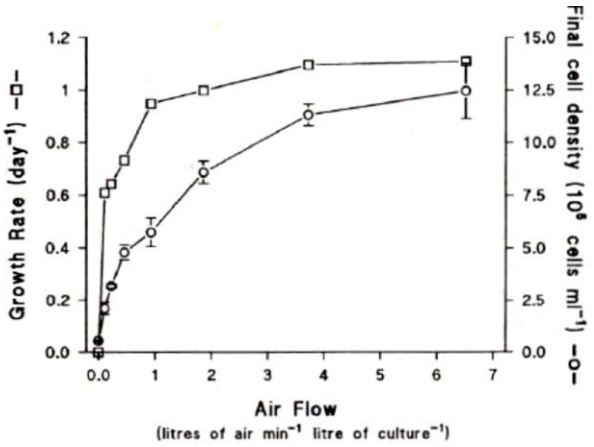

Fig. 3. *Dunaliella tertiolecta* maximal growth rate and final cell density obtained with different aeration rates. Growth rates were calculated as the slopes of linear regression fitting of data ($r^2 > 0{\cdot}99$) on increase in cell density from days 1 to 3 for all cultures except for air flow 1·86 litres of air min^{-1} litre of $culture^{-1}$, for which maximal growth rate was recorded between days 0 and 2. Error bars represent standard deviations.

The final cell density increased up to an aeration rate of 3·72 litres although the culture seemed to be CO_2 saturated at an aeration rate around 0·93 litre min^{-1} litre of $culture^{-1}$, as in the culture under pure CO_2 the final cell density was between those obtained with 0·93 and 1·86 litres min^{-1} litre of $culture^{-1}$, and there was no significant difference in the final cell density between the culture with the CO_2 supply withoutair and the one with 0·93 litre min^{-1} litre of $culture^{-1}$ supplemented with CO_2 , It is therefore possible that the growth of *D. tertiolecta* did not depend on pH and CO_2 concentration alone. It would be logical to suppose that turbulence as a result of air flux, producing different movement regimes of the cells within the media under an equal light intensity, could be an

important factor. The effect of turbulence and the importance of the flashing effect have been studied recently (Grobbelaar, 1991). It may be possible that there would be a different internal pool of limiting inorganic nutrient for each aeration regime. In this way, the higher the aeration the lower the intracellular pool needed for division resulting in a higher number of cells and/or a change of the cellular weight or volume.

Table 3. pH changes in *D. tertiolecta* cultures grown with different aeration rates and pure CO_2 without air supply

	CO_2	Air flow (litres of air min^{-1} litre of $culture^{-1}$)							
		0	0·11	0·23	0·46	0·93	1·86	3·72	6·51
		pH							
Day									
0	8·44	8·39	8·50	8·52	8·52	8·51	8·38	8·19	8·14
1	8·56	8·54	8·31	8·27	8·29	8·32	8·44	8·22	8·07
2	7·80	8·93	8·73	8·72	8·69	8·71	8·83	8·64	8·53
3	8·79	9·14	9·00	9·08	9·15	9·14	9·22	9·03	9·01
4	8·31	9·11	8·78	8·83	8·85	8·74	8·83	8·75	8·70
5	8·00	9·08	8·66	8·81	8·80	8·75	8·78	8·71	8·66
6	8·39	9·18	9·00	9·05	9·13	9·11	9·04	8·86	8·87
7	7·41	9·09	8·89	8·89	8·74	8·62	8·57	8·47	8·42
8	8·18	9·18	8·99	9·10	9·09	9·07	8·90	8·71	8·61
9	7·67	9·16	8·93	9·04	8·97	8·85	8·78	8·62	8·38
10	8·10	9·23	8·95	9·04	8·96	8·93	8·80	8·69	8·42
11	8·00	9·20	8·94	8·99	8·92	8·93	8·75	8·40	8·40
12	7·56	9·19	8·90	8·82	8·81	8·70	8·63	8·43	8·14

D. tertiolecta adapts proportionally and readily to changes in the availability of dissolved inorganic carbon by the induction of high affinity/high capacity CO_2-concentrating systems (CCMS) (Miyachi *el al.,* 1985; Aizawa *el al.,* 1986; Coleman, 1991). CO_2 is converted from HCO_3 via carbonic anhydrase (CA) located at the cell surface (Aizawa & Miyachi, 1984; Aizawa *el al.,* 1986). Although the ability of *Dunaliella* to take up bicarbonate has been described (Burns & Beardall, 1987), 14C incorporation studies have demonstrated that the microalga appeared to utilize free carbon dioxide during the mid-exponential phase (Mukerji *el al.,* 1977). Our results agree with the latter, as the non-aerated culture showed no increase in cell density. The level of dissolved carbonates in sea-water is high enough to support growth, which indicates that limitation is caused by the absence of dissolved CO_2 It can be concluded that at a pH of 8·3 the microalga cannot utilize carbonates as a source of carbon. It could have been possible that the CA activity was inhibited at high pH, thus limiting growth rate. The optimal aeration rate would be the one defining the highest growth rate and highest final cell density with the lowest air flow and evaporation. No culture fulfilled all these conditions, but an equilibrium among these characteristics can be established by choosing an aeration rate of 0·93 litre min^{-1} litre of $culture^{-1}$ (Table 4).

Table 4. (A) Air supplied per day and litre of culture ($\times 10^3$). (B) Days to reach the stationary phase. (C) Total volume of air ($\times 10^3$) supplied per litre of culture during the period of growth (from items A and B). (D) CO_2 supply per litre of culture (litres/day) (0·33% of CO_2 in the air is considered for the calculations in aerated cultures). (E) Total volume of CO_2 (litres) supplied during the period of growth (from items B and E). (F) Final cell density (cells/ml $\times 10^6$)

	CO_2	Air flow (litres of air min^{-1} litre of $culture^{-1}$)							
		0	0·11	0·23	0·46	0·93	1·86	3·72	6·51
A	0	0	0·158	0·331	0·662	1·339	2·678	5·356	9·374
B	11	0	4	10	11	10	10	9	8
C	0	0	0·633	3·312	7·286	13·392	26·781	18·211	74·995
D	0·125	0	0·047	0·099	0·198	0·401	0·803	1·607	2·812
E	1·381	0	0·190	0·993	2·185	4·017	8·035	14·463	22·499
F	7·0	0·55	2·11	3·18	4·79	5·75	8·59	11·32	12·46

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