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Optimal Renewal Rate and Nutrient Concentration for the Production of the Marine Microalga *Phaeodactylum tricornutum* in Semicontinuous Cultures

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A factorial experiment comprising six nutrient concentrations and six renewal rates was set to optimize semicontinuous cultures of *Phaeodactylum tricornutum*. Maximal cell density, 85×10^6 cells ml^{-1}, was obtained with 8 and 16 mmol of N per liter and a renewal rate of 10%. Protein stabilized at about 60% of the organic fraction for nitrogen-sufficient cultures. Both lipids and carbohydrates were stored as energetic reserves.

Microalgae are of increasing interest as sources of many substances of commercial value, but the development of microalgal production systems requires the solution of many physiological and bioengineering problems. Although a number of works have dealt in the past with the effect of continuous-culture conditions on microalgal physiology (8, 12), most of these works were carried out with nutrient concentrations much lower than those required for cost-effective mass production of microalgae.

A factorial experimental design was set to establish the influence of nutrient concentration, from very limiting levels (0.5 mmol of N per liter) to nutrient-saturated conditions (16 mmol of N per liter) and renewal rates in the range from 10 to 60% of culture volume, on the productivity and biochemical composition of semicontinuous, light-dark-synchronized cultures of the marine microalga *Phaeodactylum tricornutum* Bohlin, which is one of the best microbial sources of the polyunsaturated fatty acid eicosapentaenoic acid (20).

Unialgal cultures of *P. tricornutum* were made in 80-ml tubular units under previously described conditions (4), being maintained under a semicontinuous regimen with nutrient concentrations of 0.5, 1, 2, 4, 8, and 16 mmol of N per liter, expressed as a function of the concentration of nitrogen in the renewal medium (4). For each nutrient concentration, six renewal rates were applied, with a daily renewal of 10, 20, 30, 40, 50, and 60% of the volume of the cultures, equivalent to growth rates (k) of 0.2, 0.4, 0.6, 0.8, 1, and 1.2 divisions day^{-1}, respectively. Cell density was measured in the outflow of cultures by microscope counting with a Neubauer hemacytometer. At steady state, microalgal biomass was harvested by centrifugation and freeze-dried for biochemical analysis. C, H, and N contents were determined with an elemental analyzer (Perkin-Elmer). Protein content was derived from the nitrogen content by using the factor proposed by Gnaiger and Bitterlich (7). Carbohydrates were measured by the phenol-sulfuric acid method (10), and total lipids were measured by the charring method (13).

Steady-state cell density decreased with increasing renewal rates for all nutrient concentrations tested (Fig. 1), but the decrease could only be considered linear for nutrient concentrations of 2 and 4 mmol of N per liter ($r^2 = 0.94$ and 0.98, respectively). A one-way analysis of variance (ANOVA) (Duncan test, $P < 0.05$; data normality previously tested with the Kolmogorov-Smirnov test, $P < 0.05$) revealed no significant differences between cell densities obtained with 8 and 16 mmol of N per liter except for renewal rates of 40 and 60%. Statistical analysis demonstrated that for nutrient-limited cultures (0.5 to 4 mmol of N per liter), the influence of the factor nutrient concentration on steady-state cell density is higher than the influence of the renewal rate (two-way ANOVA, $F < 0.0001$, $R^2 = 0.742$; nutrient concentration [NC], eta = beta = 0.68; renewal rate [RR], eta = beta = 0.52), but the amount of variance explained by both factors was equal when the whole range of nutrient concentrations was considered (two-way ANOVA, $F < 0.0001$, $R^2 = 0.71$; NC, eta = beta = 0.58; RR, eta = beta = 0.61). Maximal steady-state cell density, 85×10^6 cells ml^{-1}, equivalent to 3 mg (dry weight) ml^{-1} and 2 to 2.3 mg (organic weight) ml^{-1}, was obtained with nutrient concentrations of 8 and 16 mmol of N per liter and a renewal rate of 10%. For renewal rates higher than 10%, the cell densities obtained with 8 and 16 mmol of N per liter were lower than the densities obtained with 4 mmol of N per liter (Fig. 1; Duncan test, $P < 0.05$). This decrease in the steady-state cell densities obtained when the nutrient concentration was increased above 4 mmol of N per liter indicates that cell division should be inhibited to some extent by the high concentration of nutrients in the medium. On the other hand, although cell numbers are lower, cellular organic weight was higher for the highest nutrient concentrations (3), indicating that different factors are affecting cellular division and growth measured as cellular weight. A similar phenomenon of inhibition of cell division, possibly derived from an effect of excess substrate, was found for *Tetraselmis suecica* with high nutrient concentrations and renewal rates (5).

Although the maximal growth rate expected was 1 division day^{-1} because of the synchronizing effect of the application of light-dark cycles on cell division, it was possible to maintain a growth rate of 1.2 divisions day^{-1} (renewal rate of 60%), but the stabilization of cell density under such conditions was very low. Several authors have observed better growth under continuous light than under light-dark cycles in batch cultures (9, 21), but maximal growth rates obtained in continuous cultures under continuous illumination are not much higher than the maximal growth rate maintained in the semicontinuous system: 1.5 divisions day^{-1} (6), 1.3 divisions day^{-1} (18), and 1 division day^{-1} (14).

Maximal productivity in cell number, 12.2×10^9 to $12.7 \times$

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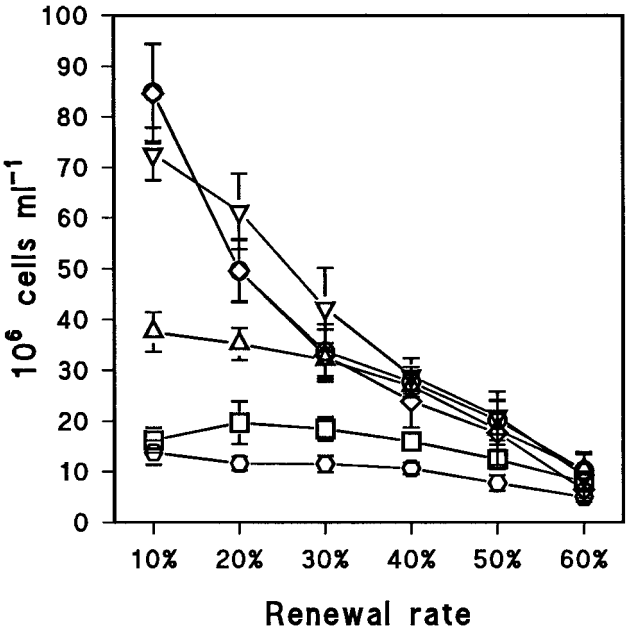

FIG. 1. Steady-state cell density in semicontinuous cultures of the marine diatom *P. tricornutum* with different nutrient concentrations and renewal rates. N concentrations (millimoles per liter): ○, 0.5; □, 1; △, 2; ▽, 4; ○, 8; ◇, 16.

10^9 cells per liter per day, was obtained with 4 mmol of N per liter and renewal rates of 20 and 30%, but maximal productivity of organic weight, 0.23 g $liter^{-1}$ day^{-1}, was obtained with 16 mmol of N per liter and renewal rates of 10 and 20%. Productivity results reported in the semicontinuous system, 12.7×10^9 cells $liter^{-1}$ day^{-1} and 0.35 g (dry weight) $liter^{-1}$ day^{-1}, equivalent to 0.206 g (organic weight) $liter^{-1}$ day^{-1}, are similar to maximal results obtained by other authors (19, 21) but lower than results reported recently for outdoor tubular reactors, 2.57 g $liter^{-1}$ day^{-1} (15). This high productivity was the result of the very high biomass concentration that can be obtained with high solar irradiances and not the result of increasing growth rates, as maximal productivity was obtained with a dilution rate of 0.36 day^{-1} (15).

The protein content of the biomass, expressed as a percentage of the organic fraction (protein plus carbohydrate plus lipid), increased with increasing renewal rate and nutrient concentration (Fig. 2A). Minimal values were 25 to 30% of the organic fraction. The protein percentage stabilized at about 60% of the organic fraction under non-nitrogen-limited conditions, although a slight increase, from 49 to 59% of the organic fraction, was still recorded with increasing renewal rates for cultures with 16 mmol of N per liter (Fig. 2A) that were not nitrogen limited for any of the renewal rates tested. Most of the variance of protein percentages was explained by the factor renewal rate (two-way ANOVA, $F < 0.0001$, $R^2 = 0.93$; NC, eta = beta = 0.18; RR, eta = beta = 0.95), although this effect was clearer for nutrient-limited cultures (less than 4 mmol of N per liter). Despite the stability of the protein percentage in the organic fraction under nitrogen-sufficient conditions, a clear decrease in protein cellular content was recorded with increasing renewal rates as a result of the decrease in cellular weight with increasing renewal rates (3). Although cell densities are equal for 8 and 16 mmol of N per liter and a dilution rate of 10% (Fig. 1), protein cellular content is considerably higher in the latter, indicating that nitrogen incorporation is still active when cell division is already blocked. The

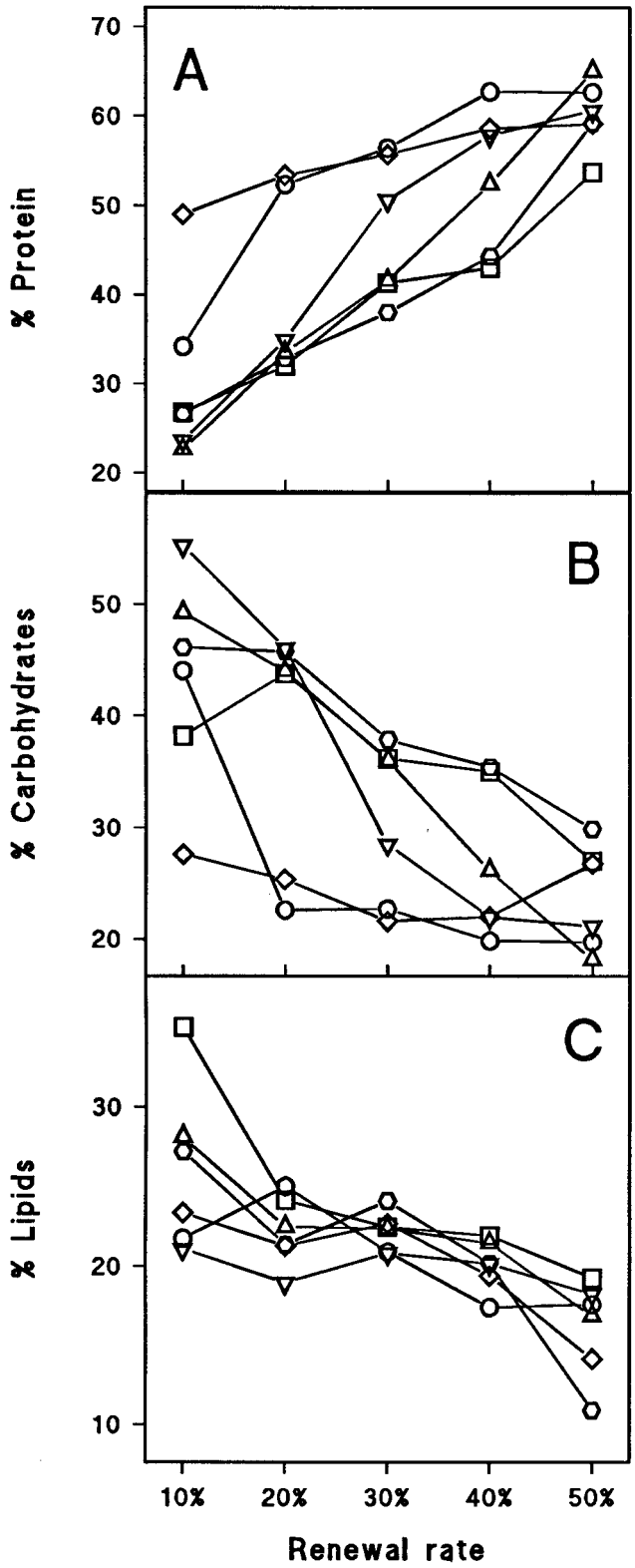

FIG. 2. Composition of the organic fraction of semicontinuous cultures of the marine diatom *P. tricornutum*. (A) Protein. (B) Carbohydrate. (C) Lipid. N concentrations (millimoles per liter): ○, 0.5; □, 1; △, 2; ▽, 4; ○, 8; ◇, 16.

uncoupling between cell division and protein synthesis under conditions of cell division arrest has been reported previously (11).

Both lipid and carbohydrate percentages decreased as a result of the increase in nitrogen availability caused by increasing renewal rates (Fig. 2B and C). Most of the variance of lipid percentages was explained by the factor renewal rate (two-way ANOVA, $F < 0.0001$, $R^2 = 0.61$; RR, eta = beta = 0.72; NC, eta = beta = 0.39) either under nutrient-limited conditions or for the whole range of nutrient concentrations tested. On the other hand, for carbohydrate, although the renewal rate is the main factor responsible for the variance under nutrient-limited conditions (ANOVA, $F < 0.0001$, $R^2 = 0.82$; RR, eta = beta = 0.88; NC, eta = beta = 0.20), when the nutrient concentration in the range from 0.5 to 16 mmol of N per liter is considered, both factors explain equally the variance obtained (ANOVA, $F < 0.0001$, $R^2 = 0.75$; RR, eta = beta = 0.69; NC, eta = beta = 0.52). This means that the lipid percentage is still affected by the renewal rate under nutrient-saturated conditions, while carbohydrate remains stable. As indicated by the high degree of fatty acid insaturation found in the nutrient-saturated cultures maintained with 16 mmol of N per liter (16), it seems that the light limitation present in the cultures with low renewal rates, a result of self-shading with such high cellular densities (85×10^6 cells ml^{-1} for a renewal rate of 10%), would force an increase in the bulk of structural, insaturated lipids (1). Therefore, under such conditions, the lipid fraction would be reduced as a response to higher light availability caused by the lower cell densities obtained with increasing renewal rates.

Higher lipid and carbohydrate levels with low growth rates have been reported for *P. tricornutum* by other authors (2, 19), but in batch cultures, only lipid increased in response to nitrogen deficiency (9). As pointed out by other authors, the type of fraction accumulated is not only dependent on genus but also changes depending on the culture system for each species (17). Our results suggest that the relative importance of both storage fractions also depends on the level of nutrient limitation.

Cultures with 8 mmol of N per liter and a renewal rate of 20% and 16 mmol of N per liter and a renewal rate of 10%, presenting maximal biomass productivity, high protein and lipid percentages, and nitrogen transformation rates near maximum, should be considered optimum for the production of *P. tricornutum* under the conditions studied. These cultures also presented the maximum production of eicosapentaenoic acid (16). The wide range of biochemical profiles generated highlights the importance of the correct manipulation of operational variables to control the productivity and biochemical composition of continuous microalgal cultures.

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