*Ann. Bot. Fennici* 47: 109–112 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 30 April 2010 

# The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunaliella* sp.

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*Received 28 Aug. 2008, revised version received 22 Apr. 2009, accepted 23 Apr. 2009*

Becerra-Dórame, M. J., López-Elías, J. A., Enríquez-Ocaña, F., Huerta-Aldaz, N., Voltolina, D., Osuna-López, I. & Izaguirre-Fierro, G. 2010: The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunaliella* sp. — *Ann. Bot. Fennici* 47: 109–112.

The final concentrations of non-axenic outdoor mass cultures of *Dunaliella* sp. grown for two or three days in f/2 and 2f media were found to be dependent on the initial cell concentration, since the cultures started with $80 \times 10^3$ cells ml$^{-1}$ gave better yields than those started with $40 \times 10^3$ cells ml$^{-1}$. There was a correlation between nutrient availability and cell yields: the two and three days-old cultures started with $40 \times 10^3$ cells ml$^{-1}$ and grown in medium f/2 had lower concentrations than those grown in 2f medium, whereas in cultures with $80 \times 10^3$ cells ml$^{-1}$ inoculum the yield was significantly higher only after three days. The lowest dry biomass yields after two and three days were with the inoculum of $40 \times 10^3$ cells ml$^{-1}$ and medium f/2.

Key words: biomass production, culture medium, *Dunaliella* sp., microalgae cultures

## Introduction

The most common microalgae used in Mexican shrimp and oyster hatcheries are *Chaetoceros* spp., *Isochrysis* spp., *Tetraselmis* spp. and *Dunaliella* spp., which are grown using the traditional multi-step procedure. Their mass cultures are routinely kept outdoors, because of their wide tolerance to the local environmental conditions (Malagrino *et al*. 1999, López-Elías *et al*. 2004).

The growth media are based on the traditional formulation f (Guillard & Ryther 1962), but there are wide differences in the design and volume of culture systems as well as in the production routines, which could explain the wide range and the highly variable final cell and biomass yields (López-Elías *et al*. 2003).

The aim of this study was to evaluate the effect of initial cell and nutrient concentrations on the growth rate and on the cell and biomass production of *Dunaliella* sp. cultures, grown outdoors for 48 and 72 hours.

## Material and methods

*Dunaliella* sp. is a local strain which is used in some Mexican hatcheries as a complement to other food sources, mostly for oyster broodstock and for the larval stages of bivalve mollusks and penaeid shrimp (López-Elías *et al*. 2004)

Two experiments were run at the DICTUS Experimental Unit under the late summer (October) conditions of Bahía Kino, Sonora (28°50´N, 111°56´W). In each, triplicate outdoor cultures for each treatment were maintained during three days in 250-l transparent fiberglass cylinders with continuous aeration.

The experimental treatments were: initial concentrations of $40 \times 10^3$ and $80 \times 10^3$ cells ml$^{-1}$ and nutrient concentrations equivalent to 50% and 200% those of the original Guillard and Ryther's (1962) medium f (f/2 and 2f). The cultures used as inocula were mixtures of three 16-l cultures in late exponential phase, grown indoors during the previous steps (0.25, 2 and 16 l), with the respective experimental media.

Water temperature and pH were measured every 6 h. Light was measured at 2-h intervals close to the surface of each culture from dawn to sunset, using a portable luxmeter. The readings, converted to $\mu$mol m$^{-2}$ s$^{-1}$ (54.4 lux = 1 $\mu$mol m$^{-2}$ s$^{-1}$; Morel & Smith 1974), were used to calculate by integration the total irradiance received in one day by each culture.

Triplicate samples were obtained after 48 and 72 h, to determine the cell concentration and the respective total and organic biomass of each culture. Cell counts were performed in at least six 9-mm$^2$ grids of a 0.1-mm-deep haemacytometer or until the coefficient of variation was < 10%. Dry total and organic biomass was determined using triplicate 100 ml samples concentrated on pre-weighed 47 mm Whatman GF-C glass fiber filters, washed with 5–6 ml of 3% ammonium formate, and dried at 65–70 °C until constant weight (DW). The inorganic content (AW) was obtained after ashing for 12 h at 480 °C in a muffle furnace, and the organic biomass (AFW) was calculated as AFW = DW – AW.

After $\log_2$-transformation, the total number of cell divisions of each culture (accumulated growth rate = $\Sigma\mu$) was calculated from the respective initial cell concentration ($N_0$) and that determined after two and three days ($N_t$), using the traditional equation: $\Sigma\mu = \log_2(N_t/N_0)$.

All the data were normally distributed and homoscedastic (Lilliefors's and Bartlett's tests, respectively). Therefore, the total number of cell divisions, the mean cell concentrations and the biomass yields after two and three days were compared with two-way repeated ANOVA and Scheffé multiple-comparison tests (Zar 1984). Differences were considered significant at $\alpha = 0.05$

## Results

During the two experiments, mean daily temperatures and total irradiances ranged from 26.1 to 32.0 °C and from 67.6 to 94.4 mol m$^{-2}$, respectively, and there were no differences between treatments. The global mean values calculated for both experiments were 30.1 ± 0.8 °C and 85.28 ± 3.80 mol m$^{-2}$ d$^{-1}$, respectively.

However, there were significant differences in the mean values of the total number of cell divisions and of the cell and dry biomass yields. After 48 h, the combination of low cell and low nutrient concentrations gave the lowest yields, as well as the lowest number of cell divisions. In spite of their high growth rate, the concentrations of the cultures started with $40 \times 10^3$ cells ml$^{-1}$ and medium 2f were lower than those obtained with both media and the high initial inoculum. The lowest biomass yields were with $40 \times 10^3$ cells ml$^{-1}$ and medium f/2, and there were no significant differences among the rest of the treatments.

After three days, the highest mean cell yield was with the combination of high initial nutrient and cell concentrations and the lowest total and organic biomass yields were those of the cultures started in f/2 medium with $40 \times 10^3$ cells ml$^{-1}$ (Table 1).

## Discussion

The growth rates of the cultures started with the low inoculum indicate that, at least for this species, the nutrient concentrations of medium f/2 might be limiting even for the first stages of growth, since after 48 they gave significantly lower cell and biomass yields than those in 2f medium. However, there were no nutrient-related differences between media for the cultures started with $80 \times 10^3$ cells ml$^{-1}$, possibly due to light limitation caused by the higher initial cell number.

With both initial cell concentrations, the number of cell divisions calculated between days two and three for the cultures with medium f/2 were 0.64 and 0.66, respectively, in comparison with the 0.84 and 1.04 divisions, respectively, of the cultures in medium 2f, showing that during the last 24 h the nutrient concentration of medium f/2 was the variable limiting growth.

Non-axenic outdoor cultures are subject to bacterial contamination, which might result in reduced microalgae production due to competition for nutrients (Rothaupt & Giide 1992) or to the presence of allelopathic substances inhibiting growth (Berland *et al*. 1972), although there are also examples of competitive exclusion of bacteria by microalgae (Risgaard-Petersen *et al*. 2004). However, the most common bacteria-microalgae interaction is a symbiotic relationship, in which bacteria benefit from phytoplankton products such as exudates, whereas phytoplankton growth is enhanced by remineralized nutrients and other growth factors (Cole 1982).

For this reason, the presence of bacteria is generally considered beneficial for microalgae cultures (Berland *et al*. 1970, Grossart 1999, de-Bashan *et al*. 2004). This seems confirmed by some of our previous experiments, which showed higher biomass and cell production outdoors than indoors (Gallegos-Simental *et al*. 2002, López-Elías *et al*. 2005) although the outdoor cultures, which were kept under the natural day/night photoperiod, had lower bacterial concentrations than those kept under continuous lighting indoors, which were contaminated by nocturnal flying insects (Huerta-Aldaz 1997).

Previous results in several Mexican commercial hatcheries indicate that the nutrient sources of microalgae growth media may vary widely, from commercial fertilizers to technical, and in some cases analytical-grade reagents, but that the most common main nutrient concentrations (N, P and Si) are those of medium f/2 (López-Elías *et al*. 2003, 2005).

According to our results, these nutrient concentrations may be limiting, although they may allow cell and biomass harvests similar to those obtained with a richer medium, provided that the initial cell concentration is high and that the cells have internal cell reserves sufficient to prevent nutrient-limited growth.

**Table 1.** Final cell concentrations (cells $\times 10^3$ ml$^{-1}$), number of cell divisions, and total dry and organic biomass yields (TW and AFW, in g l$^{-1}$) of outdoor *Dunaliella* sp. cultures grown in 2f and f/2 medium and started with $40 \times 10^3$ and $80 \times 10^3$ cells ml$^{-1}$. Different letters indicate significant ($\alpha = 0.05$) differences (two-way repeated ANOVA and Scheffé multiple-comparison tests: a ≤ ab ≤ b and a < b < c < d).

| Inoculum | Medium | Cells $\times 10^3$ ml$^{-1}$ | Cell divisions | TW (g l$^{-1}$) | AFW (g l$^{-1}$) |
|---|---|---|---|---|---|
| Day 2 | | | | | |
| $40 \times 10^3$ cells ml$^{-1}$ | f/2 | $151.5 \pm 16.8^{a}$ | $1.91 \pm 0.16^{a}$ | $0.066 \pm 0.009^{a}$ | $0.043 \pm 0.011^{a}$ |
| $40 \times 10^3$ cells ml$^{-1}$ | 2f | $272.9 \pm 57.6^{b}$ | $2.74 \pm 0.29^{c}$ | $0.092 \pm 0.006^{b}$ | $0.064 \pm 0.004^{b}$ |
| $80 \times 10^3$ cells ml$^{-1}$ | f/2 | $457.4 \pm 72.2^{c}$ | $2.50 \pm 0.23^{bc}$ | $0.092 \pm 0.008^{b}$ | $0.061 \pm 0.011^{b}$ |
| $80 \times 10^3$ cells ml$^{-1}$ | 2f | $385.7 \pm 24.9^{c}$ | $2.27 \pm 0.10^{b}$ | $0.099 \pm 0.009^{b}$ | $0.070 \pm 0.009^{b}$ |
| Day 3 | | | | | |
| $40 \times 10^3$ cells ml$^{-1}$ | f/2 | $238.0 \pm 18.0^{a}$ | $2.57 \pm 0.11^{a}$ | $0.082 \pm 0.003^{a}$ | $0.059 \pm 0.003^{a}$ |
| $40 \times 10^3$ cells ml$^{-1}$ | 2f | $480.1 \pm 57.0^{b}$ | $3.58 \pm 0.17^{c}$ | $0.101 \pm 0.009^{b}$ | $0.066 \pm 0.006^{ab}$ |
| $80 \times 10^3$ cells ml$^{-1}$ | f/2 | $707.0 \pm 14.0^{c}$ | $3.14 \pm 0.03^{b}$ | $0.115 \pm 0.014^{b}$ | $0.075 \pm 0.006^{b}$ |
| $80 \times 10^3$ cells ml$^{-1}$ | 2f | $796.0 \pm 54.0^{d}$ | $3.31 \pm 0.10^{c}$ | $0.116 \pm 0.013^{b}$ | $0.073 \pm 0.010^{b}$ |

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