Biomass **12** (1987) 37–47

Lipid and Biomass Production by the Halotolerant Microalga *Nannochloropsis salina*

Sammy Boussiba, Avigad Vonshak, Zvi Cohen, Yael Avissar and Amos Richmond

Algal Biotechnology Laboratory, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede-Boqer Campus, 84993, Israel

(Received 20 June 1986; revised version accepted 6 January 1987)

ABSTRACT

The effect of environmental factors on cell-lipid content, on the growth rate and on the overall productivity of Nannochloropsis salina *was tested in the laboratory and in outdoor cultures. Under optimum conditions in the laboratory, the maximum growth rate ($\mu_{max.}$) was 0·030 h^{-1}, which corresponds to a doubling time of 23 h. Cellular lipid content was affected by the phase of growth and the temperature, but not by nitrogen starvation, pH or the source of sea water. The most important factor affecting the output rate of biomass was the cell concentration. The maximum biomass productivity obtained in outdoor ponds was 24·5 $g \cdot m^{-2} \cdot day^{-1}$, and the lipid production rate was 4·0 $g\ m^{-2} \cdot day^{-1}$.*

Key words: Nannochloropsis salina, steady-state growth, algal biomass, lipid production.

INTRODUCTION

The potential of cultured algae to provide food and chemicals has prompted increasing interest in these microorganisms, and production of microalgal biomass has been studied intensively.[1–4] Since the worldwide energy shortage in the early 1970s microalgae have also been studied as a possible source of energy,[5,6] but, in most researches, the effects of environmental factors such as temperature, light intensity and medium composition on lipid content were tested mainly under laboratory conditions.[5,7,8]

In some of the algal species studied lipid accumulation was enhanced under nitrogen-deficient conditions. The highest concentration of lipids

Biomass 0144-4565/87/$03.50 —

in *Nannochloropsis salina,* up to 70% of dry weight, occurred when it was deprived of nitrogen for nine days.[5] In *Chlorella,* changes in the level of nitrogen in the medium significantly altered the proportions of saturated and unsaturated fatty acids.[9] High light intensity and low temperature had opposite effects on total cell-lipid, depending on the species.[9] In *Botryococcus braunii* (a freshwater organism), lipids (mainly hydrocarbons) accumulated up to 75% of the dry weight during the stationary phase of culture growth.[10]

In the experiments presented here, we examined the effects of environmental factors on the overall rates of biomass and lipid production by the halotolerant microalga *Nannochloropsis salina.* Special emphasis was placed on outdoor cultures, so as to evaluate their potential as a source of oil.

MATERIALS AND METHODS

Organism and growth media

The crysophyte *Nannochloropsis salina* (GSB STICHO) was obtained from the Solar Energy Research Institute (SERI) Culture Collection in Golden, Colorado, USA, and was cultivated in artificial sea water (ASW) or enriched sea water (ESW). The components of the ASW medium (in $g.l^{-1}$) were: NaCl (27), $MgSO_4.7H_2O$ (6·6), $CaCl_2.2H_2O$ (1·5), KNO_3 (1·0), KH_2PO_4 (0·070), $FeCl_3.6H_2O$ (0·014), Na_2EDTA (0·019) and 1 $ml.l^{-1}$ of a microelement solution containing (in mg l^{-1}):$ZnCl_2$ (40), H_3BO_3 (600), $CaCl_2$ (1·5), $CuCl_2$ $2H_2O$ (40), $MnCl_2$ (400) and $(NH_4)_6Mo_7O_{24}.4H_2O$ (370). The ESW medium consisted of filtered Mediterranean sea water supplemented with KNO_3, KH_2PO_4, $FeCl_3$, Na_2EDTA and microelements at the same concentrations as in ASW.

Growth conditions

Laboratory cultures

Algae were cultivated in ASW either in 100 ml of medium in 250 ml flasks, placed on a gyratory shaker under constant illumination of 75 $\mu E.m^{-2}.s^{-1}$ at a constant temperature of 27°C; or in 500 ml glass tubes, into which either air or 1·5% CO_2 in air was bubbled, and which were placed in a water bath at a constant temperature of 28°C. The light intensity at the surface of the vessels was 175 $\mu E.m^{-2}.s^{-1}$. Illumination was provided by 20-W cool white fluorescent lamps. The effect of tem-

perature on growth was studied in a temperature block maintaining a temperature gradient of 20°–35°C with 1·5°C increments between adjacent test tubes. The light intensity at the bottom surface of the tubes was 110 $\mu E.m^{-2}.s^{-1}$.

Outdoor cultures

The algae were cultivated in 1 m^2 or 2·5 m^2 ponds that contained 120 or 300 l, respectively, to a depth of 12 cm. Agitation was achieved by a paddle-wheel that maintained a water velocity of ~ 20 $cm.s^{-1}$. The pH was maintained within a range of 7·0–7·5 by bubbling in CO_2 at a rate of 40–50 ml min^{-1}. The climatic conditions were as described previously.[11] During the winter the ponds were covered with polyethylene sheeting.

To test the effects of pH, medium composition, nitrogen starvation and cell concentration, cultures were maintained at steady state at a given cell concentration for at least 20 days. Unless otherwise stated, the cultures were diluted whenever the chlorophyll concentration reached 10–15% over the pre-set value.

Nitrogen starvation

In the laboratory, cultures in the mid-logarithmic phase (cultivated in glass tubes into which 1·5% CO_2 in air was bubbled) were centrifuged, washed once and resuspended in nitrogen-free medium. Cell concentration, pH, temperature and light intensity were kept constant during the starvation.

In outdoor cultures, algae were separated from the culture by centrifugation, and resuspended in a N-free medium.

Analytical methods

Algal growth was determined by measuring the following parameters: optical density at 540 nm, ash-free dry weight (AFDW)[11] and total chlorophyll by methanol extraction.[12] Protein was determined according to the Lowry procedure[13] using bovine serum albumin as the standard. Lipids were extracted by a modified Bligh-Dyer[14] procedure as follows: freeze-dried samples were extracted with three volumes of methanol/chloroform/water (10:5:4 v/v) at room temperature and centrifuged. Water and chloroform were added so as to form a methanol/chloroform/water mixture of 10:10:9. After extraction, the chloroform phase was washed with water and then evaporated to dryness.

The photosynthetic efficiency (PE) was calculated by dividing the calorific value of the cell biomass (5·5 kcal = 1 g AFDW) by the measured

solar irradiation (in calories) in the photosynthetically active spectral range (PAR) impinging on the surface of the culture.

RESULTS AND DISCUSSION

A prerequisite for obtaining maximum productivity under outdoor conditions is to ensure that there is no nutritional limitation and that culture-growth is limited by light and temperature alone. Ideally, light should be the sole factor limiting growth, light utilization being optimized by the proper adjustment of pond depth and cell concentration throughout the year.

Optimization of growth under laboratory conditions

The optimum conditions for growth of *Nannochloropsis salina* in the laboratory were determined (Table 1), yielding a $\mu_{max.}$ of $0{\cdot}030.h^{-1}$, which corresponds to a doubling time of 23 h.

Effects of growth conditions on cell lipid content

Under laboratory conditions the lipid content of the algal cells changed with the growth phase. Cells sampled in the early stationary phase (25 mg chlorophyll.l^{-1}) had about twice as much lipid per mg dry weight (Table 2) as cells from cultures in the early logarithmic phase (5 mg chlorophyll.l^{-1}). This could be due to accumulation of lipids as storage products when growth became limited by light and nutrients.

TABLE 1

Determining Growth Conditions and Optima for *Nannochloropsis salina* in the Laboratory

Variable	*Range permitting growth*	*Optimum*[a]
Temperature	17–32°C	28°C
pH	5–10·5	7·5–8·0
NaCl	0·1–1·0M	0·6M
KNO_3	3·0–100 mM	10 mM
$NaHCO_3$	0–5 mM	0

Algae were cultivated in ASW (see 'Materials and methods').

[a] Optimum growth was obtained in glass tube cultures through which 1·5% CO_2 in air was bubbled (see 'Materials and methods'). Under these conditions, the $\mu_{max.}$ obtained was $0{\cdot}031.h^{-1}$ which corresponds to a doubling time of 23 h.

Under outdoor conditions, cultures maintained at several constant cell concentrations differed in their lipid contents. As with the laboratory cultures, the highest lipid content was obtained at the highest cell concentration (Table 2).

A higher level of lipids was found in cells of *Nannochloropsis salina* grown outdoors in the summer (21–28%) than in the winter (13–17%) (Fig. 1). Likewise, increasing the temperature from 15° to 30°C increased the total lipid fraction in *Ochromonas danica*.[15] Although temperature thus appears to considerably affect the lipid content, other environmental factors such as light intensity, cannot be excluded.

Optimization of outdoor cultures for maximizing lipid production

Source of sea water and pH

The effects of ASW or ESW and pH (7·0–7·5 *vs* 8·0–8·5) on the lipid content of the cell and on their total productivity were tested in outdoor cultures which were maintained at steady state (at a cell concentration corresponding to 5–7 mg chlorophyll l^{-1}). The source of sea water and pH had no effect on the lipid content of the cells. Decreased productivity was observed only at the higher pH conditions.

Nitrogen starvation

Thomas *et al*,[8] Tornabene[16] and Shifrin & Chisholm[6] obtained contradicting results when subjecting *Nannochloropsis salina* to nitrogen star-

TABLE 2
The Effect of Cell Concentration on Lipid Content of *Nannochloropsis salina*

Chlorophyll ($mg.l^{-1}$)	*Lipid content (% of AFDW*[a]*)*	
	Laboratory[b]	*Outdoors*[c] *steady-state*
3	—	16·5
5	10·0	16·0
10	—	18·0
15	13·0	—
20	—	21·0
25	20·0	—

[a] AFDW — ash-free dry weight.
[b] Growth conditions as in Table 1.
[c] Monitored in the summer in 2·5 m^2 ponds, ASW medium.

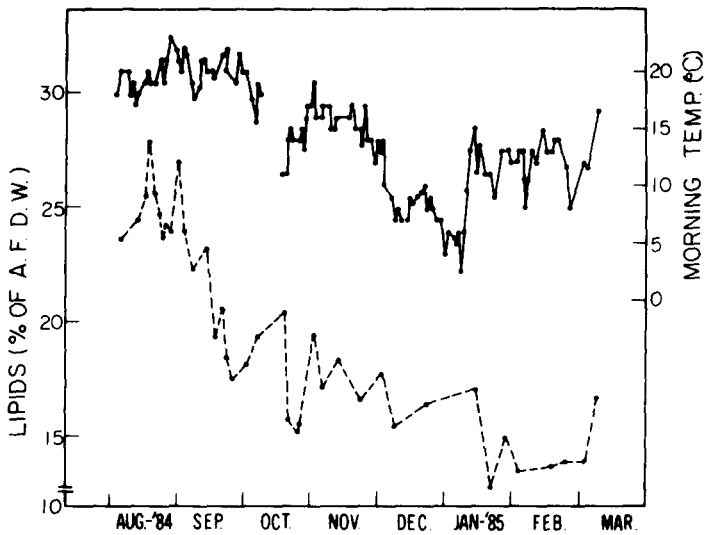

Fig. 1. Changes in the relative lipid content of *Nannochloropsis salina* cultivated outdoors. ●——● temperature; ●– – –● lipid content (% of AFDW).

vation. The first group[8] did not observe any changes in cell lipid content during N starvation, whereas the others[6,16] reported an increase in the cell lipid content in response to the same stress. The reported increase in lipid content caused by N starvation[6,16] could have been affected by a combination of several factors, such as light intensity, pH, age of the culture and CO_2 concentration, which may not have been adequately controlled during N starvation. This interpretation is further supported by the findings of Ben Amotz[17] that an increase in lipid content is observed when cells were grown in high pH, when cells were starved for nitrogen at optimal pH no increase in the lipid content was observed.

When steady-state cultures of *Nannochloropsis salina* were N-starved either outdoors or indoors, there did not appear to be any measurable increase in the lipid content per unit dry weight (Tables 3 and 4). The decrease in the output rate of biomass observed under N starvation (Fig. 2) caused, however, a sharp decrease in the total lipid production rate (Table 4), permitting the establishment of some predator organisms in the culture.

Effect of cell concentration on output rate

The cell concentration in an algal culture affects the amount of light that reaches the individual cells in the culture. Thus, the lower the population density, the higher the specific growth rate to be expected in a light-

TABLE 3
Effect of Nitrogen Starvation on Lipid Content of *Nannochloropsis salina* Cultivated in the Laboratory

Period of N starvation (days)	*Lipid content*[a] *(% of AFDW)*
0	22·0
2	26·0
4	25·8
8	25·0

Growth conditions as in Table 1.
[a] No changes in lipid content were observed in comparable N-sufficient cultures of *Nannochloropsis salina.*

TABLE 4
Effect of Nitrogen Starvation on the Rate of Biomass and Lipid Production in *Nannochloropsis salina* Cultivated Outdoors

Culture	*Output of biomass* $(g.m^{-2}.day^{-1})$	*Lipid content (% of AFDW)*	*Rate of lipid production* $(g.m^{-2}.day^{-1})$
Control	13·5	22	3·0
N-starved[a]	3·5	25	0·9

Algae were cultivated in a 1 m^2 pond, ASW medium (summer).
[a] Cell starved for 25 days.

limited system. The major environmental factor limiting growth and output rate is light in the summer, and temperature in the winter. The effect of the population density on the output rate should therefore be more pronounced in the summer than in the winter.[1,12]

We tested the effect of cell concentration on the overall rates of biomass and lipid production in outdoor cultures throughout the year. Although the ratio of protein to dry weight was constant (0·43), the ratio of chlorophyll to dry weight increased (0·01–0·016) with increasing cell concentration, as expected for a photoautotrophic organism. The results presented in Table 5 indicate the great influence of cell concentration on the biomass productivity, which was more pronounced in the summer. The output rate was clearly lower in December–February than that obtained in June–July, when the temperature was close to the optimum

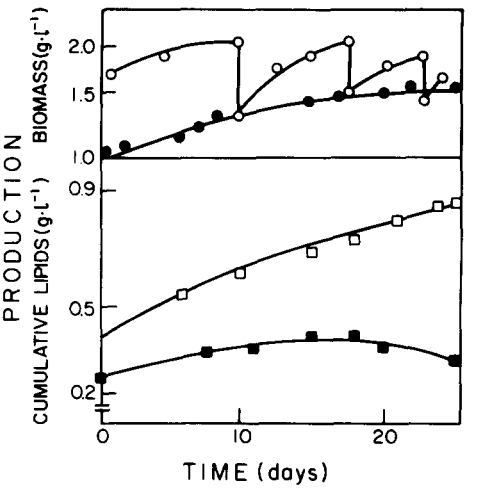

Fig. 2. Effect of nitrogen starvation on output of biomass and accumulation of lipids in *Nannochloropsis salina* cultivated in a 1 m^2 pond (June 1984). o——o control — biomass ($g.l^{-1}$); ●——● nitrogen starved — biomass ($g.l^{-1}$); □——□ control — lipid production; ■——■ nitrogen starved — lipid production.

for this organism (25°–28°C) (Table 5). Lipid production, however, was affected to a lesser extent in response to changes in cell concentration, due to the inverse relation between cell concentration and cell-lipid. The maximum rates of biomass and lipid production under our experimental conditions were 24·5 and 4·0 $g.m^{-2}.day^{-1}$, respectively (Table 5).

Maintenance of monoalgal cultures

Maintenance of a monoalgal culture is frequently hindered by contamination with other algae and zooplankton. Outdoor cultures of *Nannochloropsis salina* suffered mainly from invasion by diatoms and some predators. Under steady-state conditions, *Nannochloropsis* was the dominant alga in the pond (> 95% of the biomass). In some cases, however, other microorganisms developed and, if not treated, became deleterious to the growing cultures. Contamination with other algae (mainly diatoms) and predators (ciliates) was mainly observed in the nitrogen-starvation experiments and when the cell concentration was kept at a low of 350 $mg.l^{-1}$.

It was concluded that a monoalgal culture could be maintained best at the high cell concentration (500–1300 $mg.l^{-1}$). At the lower concentration (350 $mg.l^{-1}$), contaminative organisms occasionally accumulated to *ca.* 10–15% of the total biomass.

TABLE 5
The Effect of Cell Concentration on Output of Biomass, Photosynthetic Efficiency and Lipid Production in *Nannochloropsis salina*

	Cell concentration ($mg.l^{-1}$)	*Biomass* ($g.m^{-2}\,day^{-1}$)	*Photosynthetic efficiency* (%)	*Lipids* (% of AFDW)	*Rate of lipid production* ($g.m^{-2}\,day^{-1}$)
Winter[a]	500	7·1	3·1	15	1·1
	750	5·3	2·4	17	0·9
	1300	5·1	2·1	19	0·9
Summer[b]	350	24·5	4·3	16	4·0
	500	23·0	4·0	16	3·7
	750	16·7	3·5	18	3·0
	1300	14·0	3·3	21	2·9

Cultivated in 2·5 m^2 ponds, ASW medium, in winter (Dec–Feb) and in summer (Jun–Aug).
[a] Maximum diurnal temperature 30°–35°C, minimum 17°–23°C.
[b] Maximum diurnal temperature 15°–20°C, minimum 5°–12°C.

Proliferation of diatoms was apparently prevented by adding urea as a nitrogen source (0·12 $g.l^{-1}$) instead of KNO_3 (1 $g.l^{-1}$). In preliminary experiments it was observed that diatoms have disappeared within 1 to 2 days when urea was used as a nitrogen source. For the elimination of predators, NH_4^+ was used in a concentration range of 2–5 mM, toxic to the predators.[18]

CONCLUSIONS

To maximize biomass production by *Nannochloropsis salina* it is imperative to maintain steady-state conditions in the growing culture. This should also prevent proliferation of some contaminant species.

Except for the temperature, cell concentration had the greatest effect on biomass output under outdoor conditions, as was also observed for *Spirulina*.[19]

To prevent contamination with undesired microorganisms, we recommend maintaining outdoor cultures of *Nannochloropsis salina* at a cell density of 500 $mg.l^{-1}$, even if it may be slightly above the optimal and may result in some decrease in productivity. The ability to maintain this organism outdoors as a monoalgal culture suggests its potential as a model organism for studying lipid production in mass algal cultures.

ACKNOWLEDGEMENTS

This work was supported by Grant No. XK-4-0410-1 from SERI.

We wish to thank Ms S. Didi and Ms R. Milkis for skilful technical assistance, and Dr Marjorie A. Tiefert for editing the manuscript.

REFERENCES

1. Richmond, A. (1983). Photosynthetic microalgae. In: *Biotechnology,* Vol. 3 (Rehm, J. & Redd, G. Eds). Weinheim, Verlag Chemie, 109–43.
2. Soeder, J. (1980). Massive cultivation of microalgae: results and prospective. *Hydrobiologia,* **72**, 197–204.
3. Benemann, J. R., Weissman, J., Koopman, B. L. & Oswald, W. J. (1977). Energy production by microbial photosynthesis. *Nature,* **268**, 19–23.
4. Aaronson, S., Berner, T. & Dubinsky, Z. (1980). Microalgae as source of chemicals and natural products. In: *Algae biomass* (Shelef, G. & Soeder, C. J., Eds). Amsterdam, Elsevier/North Holland Biomedical Press, 575–601.
5. Shifrin, M. S. & Chisholm, S. W. (1980). Phytoplankton lipids: environmental influences on production and possible commercial applications. In: *Algae biomass* (Shelef, G. & Soeder, C. J., Eds). Amsterdam, Elsevier/ North Holland Biomedical Press, 625–45.
6. Shifrin, N. S. & Chisholm, S. W. (1981). Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. *J. Phycol.,* **17**, 374–84.
7. Ben-Amotz, A. & Tornabene, T. G. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. *J. Phycol.,* **21**, 72–81.
8. Thomas, W. H., Seibert, D. L. R., Alden, M., Neori, A. & Eldridge, P. (1984). Yields, photosynthetic efficiencies and proximate composition of dense marine microalgal cultures. III. *Isochrysis* sp. and *Monollantus salina* experiments and comparative conclusions. *Biomass,* **5**, 299–316.
9. Pohl, P. & Zurheide, F. (1979). Fatty acids and lipids in marine algae and the control of their biosynthesis by environmental factors. In: *Marine algae in pharmaceutical sciences* (Hope, H. A., Levring, T. & Tanaka, Y., Eds) Berlin, Walter de Gruyter, 433–523.
10. Wolf, F. R. (1983). *Botryococcus braunii*: an unusual hydrocarbon producing alga. *Appl. Biochem. Biotech.,* **8**, 249–60.
11. Vonshak, A., Boussiba, S., Abeliovich, A. & Richmond, A. (1983). Production of *Spirulina* biomass: maintenance of monoalgal culture outdoors. *Biotechnol. & Bioeng.,* **25**, 341–9.
12. Vonshak, A., Abeliovich, A., Boussiba, S., Arad, S. & Richmond, A. (1982). Production of *Spirulina* biomass: effects of environmental factors and population density. *Biomass,* **2**, 175–85.
13. Lowry, O.H., Rosebrough, N.H., Farr, A. L. & Randall, R. J. (1951). Protein measurements with folin-phenol reagent. *J. Biol. Chem.,* **193**, 265–75.
14. Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.,* **37**, 911–17.

15. Aaronson, S. (1973). Effect of incubation temperature on the macromolecular and lipid content of the phyto-flagellate *Ochromonas danica. J. Phycol.*, **9**, 111–13.
16. Tornabene, T. G. (1984). Chemical profile of microalgae with emphasis on lipids. In: *Aquatic species program review.* Procs, April 1984, SERI publication SERI/CP 231-2341, 64–79.
17. Ben Amotz A. (1985). Development of out door raceway capable of yielding oil reach halotolerant microalgae. In: *Aquatic species program review.* Procs, March 1985, SERI publication SERI/CP 231-2700, 230–43.
18. Lincoln, E. P., Hall, T. W. & Koopman, B. (1983). Zooplankton control in mass algal culture. *Aquaculture,* **32**, 331–7.
19. Richmond, A. & Vonshak, A. (1978). *Spirulina* culture in Israel. *Arch. Hydrobiol. Beih.*, **11**, 274–80.