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Characterization of growth and arachidonic acid production of *Parietochloris incisa* comb. nov (Trebouxiophyceae, Chlorophyta)

Zhang Cheng-Wu[1,2], Zvi Cohen[1], Inna Khozin-Goldberg[1] and Amos Richmond[1,*]
[1]*Microalgal Biotechnology Laboratory, The Albert Katz Department of Dryland Biotechnologies, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede-Boker Campus, 84990, Israel;* [2]*Department of Biological Engineering and Sciences, Nanjing University of Chemical Technology, Nanjing, 210009, China; *Author for correspondence (e-mail: amosr@bgumail.bgu.ac.il; phone: 972-8-6596797; fax: +972-8-6596802)*

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Abstract

Arachidonic acid (AA) is a precursor of biologically active prostaglandines and leukotrienes. The commercial source for AA at present is a fungus, but the recently discovered coccoid green alga, *Parietochloris incisa* comb. nov., in which over 90% of total AA is deposited in triacylglycerols, makes this species a potential candidate for commercial production of AA. We investigated the effect of the light-regime on cell-AA content and on culture productivity, by manipulating the intensity of the light source, the length of the light-path (LPL), and the population density of cultures grown in flat plate glass reactors under both controlled laboratory conditions (continuously illuminated) as well as outdoors. The effect of nitrogen deprivation on culture content of AA and its productivity was also studied. In all experiments, the longer light-path reactors with the highest areal volumes (L m^{-2}) yielded the highest culture-AA or the highest amount of AA harvested per illuminated reactor surface. Highest culture content of AA was obtained in cultures exposed to strong light and nitrogen-deprivation. In contrast, highest cell-AA content was obtained in cultures receiving the lowest light-dose. Maximum culture content of AA obtained in the laboratory was 2667 mg L^{-1}, reached after a 38-day growth period (of which the final 17 days took place in nitrogen-free medium), under continuous exposure to 2000 μmol photon m^{-2} s^{-1}. Maximal culture content of AA outdoors was significantly lower compared with the maximum obtained in the laboratory.

Introduction

Considerable interest has been directed at unicellular algae as a source of oils and fatty acids. Much of this research was initially conducted at the Solar Energy Research Institute, USA, focusing on utilizing algal oils as bio-fuels. The project did not prove commercially feasible, but nevertheless stimulated research on algal oils containing long-chain polyunsaturated fatty acids (LC-PUFAs) as nutritional supplements, most attention given to the omega-3 LC-PUFAs viz., docosahexaenoic acid (22:6ω3, DHA), eicosapentaenoic acid (20:5ω3, EPA) as well as arachidonic acid (20:4ω6, AA).

AA is an essential fatty acid in human nutrition (WHO/FAO 1977) and a precursor for the biologically active prostaglandins and leukotrienes with important functions in the circulatory (Singh and Chandra 1988), as well as the central nervous systems (Innis 1991). Furthermore, AA serves as a starting material for the biosynthesis of the prostanglandin PGE2 (Ahern et al. 1983). AA is a component of human milk and therefore a potentially valuable ingredient in various formulations of artificial baby food (Koletzko et al. 1989). According to Koletzko et al. (1996), AA is necessary for the visual acuity and better cognitive development of infants after birth. Several algae species containing AA have been investigated, e.g., the red alga *Porphyridium cruentum*

(Cohen 1990); some diatoms (Dunstan et al. 1994); *Euglena gracilis* (Euglenophytes, Hiyashi et al. (1994)); *Ochromonas danica* (Chrysophyceae, Eichenberger and Gribi (1994)) two marine unicellular green algae, *Tetraselmis suecica* (Servel et al. 1994), *Nannochloris sp.* (Petkov et al. 1994), *Sargassum* (Wu et al. 1995), and the brown seaweed *Fucus* (Kim et al. 1996). Presently, however, the commercial AA source is a fungus, *Mortierella alpina*, the AA content of which reaches as high as 60% of its total fatty acids (Shimizu et al. 1987).

Fatty acid content and composition in algal cells may be modulated by conditions of growth (Spoehr and Milner 1949). Cell-growth is retarded in response to growth limitations such as nutrient depletion and light limitation, as well as sub-optimal pH, temperature or salinity, under which conditions, protein synthesis declines and lipid and carbohydrate synthesis may be enhanced (Cohen et al. 1988; Bajpai and Bajpai 1993; Molina Grima et al. (1994, 1995); Cohen et al. 1995). Several studies aimed at achieving maximal cell PUFA content and overall productivity of PUFA in microalgae have applied nutrient stress and nitrogen starvation to enhance fatty acid content in many species of algae (e.g., Shifrin and Chisholm (1981) and Suen et al. (1987), Reitan et al. (1994)). Light-limitation is known to affect fatty acid composition manifested in the proportion of C_{20} PUFA in the fatty acid profile, as well as the ratio of $\omega3$ to $\omega6$ fatty acids (Cohen et al. 1988; Lee and Tan 1988).

In this work, we investigated the effects of factors affecting the light regime as well as nitrogen deficiency on cell-AA content and composition at the stationary phase of growth of *Parietochloris incisa* comb. nov., a coccoid green alga (Trebouxiophyceae, Chlorophyta) recently isolated from the soil of Mt Tateyama, Japan (Watanabe et al. 1996; Bigogno et al. 2002a). This microalga was found to be perhaps the richest plant source of AA, with over 90% of total AA being deposited in triacylglycerols (Bigogno et al. 2002b). Aiming to probe the feasibility for large-scale production, we also conducted preliminary experiments to test the performance of mass cultures of *Parietochloris incisa* comb. nov in flat plate reactors outdoors.

Materials and methods

Organism and culture medium

Parietochloris incisa comb. nov was isolated from Mount Tateyama in Toyoma Prefecture, Japan (Watanabe et al. 1996). It was purified under the microscope with a micropipette and cultivated in modified BG-11 medium of the following composition: $NaNO_3$, 3.0 g L^{-1}; K_2HPO_4, 0.08 g L^{-1}; $MgSO_4.7H_2O$, 0.015 g L^{-1}; $CaCl_2.2H_2O$, 0.072 g L^{-1}; Citric acid, 0.012 g L^{-1}; $FeCl_3.6H_2O$, 6.3 mg L^{-1}; Na_2EDTA, 0.002 g L^{-1}; Na_2CO_3, 0.04 g L^{-1} and A_5 trace elements solution 1 ml L^{-1} (H_3BO_3, 2.86 g; $MnCl_2.4H_2O$, 1.81 g; $ZnSO_4.7H_2O$, 0.222 g; $NaMoO_4.2H_2O$, 0.39 g; $CuSO_4.5H_2O$, 0.079 g and $Co(NO_3)_2$. $6H_2O$, 49.4 mg in 1.0 L distilled water).

Experimental design and culture operations

The influence of light intensity and nitrogen-starvation on culture growth and cell content of arachidonic acid was studied in flat glass photobioreactors (20.0 cm width × 65.0 cm height) with a light path length of either 1.0 or 3.0 cm (Hu et al. 1996). The photobioreactors were placed in a flow-through water tank, regulated to maintain a temperature of 26 ± 1 °C. Low light was supplied by banks of cool-white fluorescent lamps providing 250 μmol photon m^{-2} s^{-1} at the reactor surface. For the high light treatment, one panel of the reactor was illuminated with cool-white fluorescent lamps as above, the opposite panel being illuminated by halogen lamp providing 1750 μmol photon m^{-2} s^{-1}. Light per cell mass was computed as follows; first, the areal cell mass (g m^{-2}) was calculated by multiplying the cell mass concentration (g L^{-1}) by the number of liters irradiated per m^2, e.g. in a 1.0 cm optical path reactor, 2 m^2 (front and back panels, sides and top being covered) correspond to 10 L, i.e. 5.0 L m^{-2} for the front panel and the same for the rear panel (Richmond (in press)). Light per cell mass is than arrived at by dividing the photon flux density (μE m^{-2} s^{-1}) by the areal cell mass, the units of this value being μE s^{-1} g^{-1}. In cultures of high cell density, this quantity is proportional to the average irradiance. Cultures were stirred by applying compressed air containing 2% CO_2 at a flow rate of 0.5 L min^{-1} L^{-1} culture. Upon reaching steady state with respect to cell number, cell-mass was precipitated by closing the air flow and siphoning out the supernatant. The precipitated cells were washed three times with

nitrogen-free growth medium. Finally, nitrogen-free fresh growth medium was added to each reactor, thereby starting the nitrogen-depletion phase. Other conditions were not altered.

For outdoor cultures, inoculum (grown in the laboratory), at mid to late logarithmic growth phase was transferred to the flat plate glass-photobioreactors (of 5 or 10 cm light path, 40 × 65 cm), to which 5 or 10 L of growth medium were added, respectively. Mixing and CO_2 were provided by continuous supply of air enriched with 2% CO_2, injected through the perforated plastic tube placed in the base of the reactor (Hu et al. 1996; Richmond and Zhang 2001). Turbulence was induced by adjusting the rate of air passing through a gas flow meter at 0.7 L min^{-1} L^{-1} culture. The outdoor experiments were carried out in winter (December to February). Maximum daily temperature did not exceed 26 ± 1 °C, this was achieved by spraying water to affect evaporative cooling (Hu et al. 1996; Richmond and Zhang 2001). The minimum night temperature was 10 °C. Upon reaching steady state cell-mass was precipitated by shutting off aeration and removing the supernatant by siphon. Nitrogen starvation was induced as described above. The range of global horizontal irradiance in winter, at Sede-Boker (33 Latitude) is 3 to 4 kwh m^{-2} day^{-1}. On vertical surfaces in a South-North orientation, irradiance in winter ranges between 4 to 4.5 kwh m^{-2} day^{-1}, this value includes ca. 0.5 kwh reflected light, assuming an albedo of 0.3. Irradiance coming from the north side in winter is ca. 1.0 kwh m^{-2} day^{-1} (Faiman D., personal communication).

All experiments were carried out at least twice, yielding similar results. Data shown are from one representative experiment.

Measurements of growth and cell contents

Algal biomass was determined by filtering 10 mL of the culture onto tarred GF/C Whatman filters, precombusted in a Eurotherm combustion furnace at 560 °C. The filtered sample was then washed with 10 mL of pH 4.0 distilled water (acidified with drops of 0.1 N HCl) to remove adhering inorganic salts, dried at 105 °C overnight and finally dried in a desicator over silica gel for at least 2 h, before being weighted.

Fifty mL of algal suspension were centrifuged (2000 × g, 5 min), the pellets frozen immediately (−80 °C), and freeze-dried. Freeze-dried cell-mass was transmethylated with methanol-acetylchloride according to Cohen et al. (1993), heptadecanoic acid being added as an internal standard. Gas chromatographic analysis was performed on a supecowax 10 (Supelco, Bellefonte, PA) fused silica capillary column (30 m × 0.32 mm) at 200 °C (FID, injector and flame ionization detector temperature was 230 °C, split ratio 1:100). Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma, St Louis, MO) and by calculation of the equivalent chain length. Fatty acid content was determined by comparing each peak area in the chromatogram with that of the internal standard and correcting accordingly.

Results

Cell-mass concentration maxima were strongly affected by the rate of radiation and were higher in the 1 cm than in the 3 cm light path (LP) reactor (Figure 1). The length of the LP exerted a strong effect on cell growth in low light, enhancing cell concentration in the 1 cm – by some 40% over the 3 cm LP reactor. In contrast, a much smaller effect (ca. 10%) of the LP on cell growth was observed in high light, indicating an overly high exposure to light in the 1 cm LP reactor resulting in inefficient use of strong light. The areal cell mass in the 3 cm LP exposed to low light was twice as high as in the 1 cm LP reactor, this difference being somewhat higher in high light (Figure 1).

The length of the LP, a basic factor related to the light regime to which cells in a reactor are exposed (Richmond 2000), unexpectedly exerted a rather small effect on cell AA content. Highest AA content in cell-mass was obtained with the 3 cm LP reactor at the low light dose, following 21 days of nitrogen deprivation (Table 1).

At high light, there was a maximal increase of 18% in cell-AA in the 3 cm- over the 1-cm LP reactor, the quantity of light per cell mass being some 2.5 times lower in the former. Replenishment of nitrogen resulted in an immediate decline in the AA percent of TFA as well as in percent total fatty acids (TFA) of cell mass (Figure 2). Significant differences in mg AA per gram cell mass between ″low″ and ″high″ light were clearly observed: In 1 cm LPL at low light, 164 mg AA was obtained, compared with 127 mg in high light. Likewise, 192 mg in 3 cm LPL at low light, compared with 141 mg in high light. Nevertheless, the percent increase of cell-AA after 21 days of N-

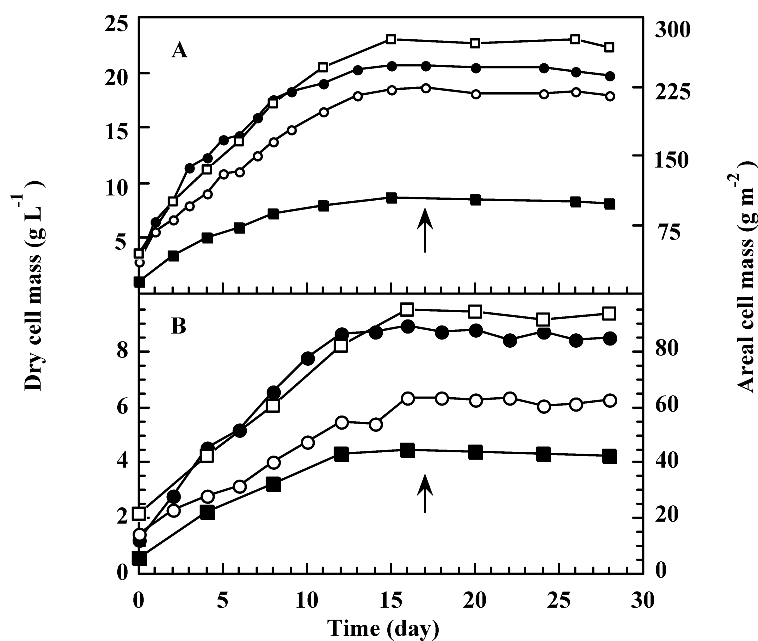

Figure 1. Dry cell mass concentration and areal cell mass of *P. incisa* growth in 1 and 3 cm flat plate glass reactors exposed to A) ″high″ light; 2000 μmol photon m^{-2} s^{-1} and B) ″low″-light; 250 μmol photon m^{-2} s^{-1}; -●- 1 cm and -○- 3 cm (concentration of cell mass); -■- 1 cm and -□;- 3 cm (areal cell mass). Arrow-nitrogen deprivation initiated.

deprivation in low light was very similar to that found for high light, irrespective of LPL (Table 1).

Light path length in outdoor cultures affected both the overall yield of dry cell mass (DCM) as well as the percent of AA in DCM (Table 1). Similar to results in the laboratory, DCM concentration outdoors was higher in the shorter (5 cm) LP reactor. Deprivation of nitrogen, which was induced when net increase in cell number ceased, did not affect the DCM concentration but approximately doubled the percent AA of DCM. AA reached 9.6% of DCM in the 10 cm LP reactor, the highest obtained outdoors and 36 percent higher over that obtained in the 5-cm LP reactor.

Only a small effect of the LP on the AA proportion in TFA and on cell TFA content was obtained in the 1- and 3-cm LP reactors in the laboratory (Figure 2), but in contrast, a clear effect of the LP was observed outdoors. A much higher cell-TFA content was obtained in the 10-cm reactor than in the 5-cm LP reactor (21.6 vs. 17.2%, respectively). The effect of the LPL on the AA proportion of TFA was, however, rather small. Radiation greatly affected the culture volume content of AA (i.e., mg AA per liter culture), seemingly due to increased productivity of cell mass in high light. The cumulative culture content of AA after 38 culture days in high light was ca. twice as high compared with the low light treatment. Highest AA-proportions of TFA obtained in the laboratory were 58 and 56 percent, under low and high light, respectively. AA proportions of TFA attained in outdoor cultures were always lower, reaching up to 46%.

Adding nitrogen to nitrogen-deprived cultures after 38 culture days, when culture volume content of AA reached a maximum, resulted in an immediate decline in both TFA as well as AA (Figure 2). Maximal areal dry cell mass (DCM m^{-2} of illuminated reactor surface) as well as maximal average areal yield of AA (1030 mg AA m^{-2} day^{-1}) was obtained in the 3-cm LP cultures exposed to strong light. The areal yield of AA in the 1 cm LP reactor was less than one half as high.

Maximal culture content of AA obtained in the laboratory with continuous exposure to 2000 μmol m^{-2} s^{-1} was 2667 mg L^{-1}, reached after 38 days of culture (first 17 days in full growth medium followed by 21 days of nitrogen deprivation). Culture content of AA outdoors, in contrast, was 6 times lower (Table 1). Nitrogen deprivation both in low and high light increased the AA content of DCM by over 100%. Arachidonic acid was the most prevalent fatty acid in *P. incisa*, total fatty acid (9.5 to 22.6%) and AA content (3.2 to 12.7%) as well as percent of AA in TFA (29.5 to 57.9%) increasing steadily in response to nitrogen deprivation and culture age.

Table 1. AA productivity in *Parietochloris incisa* grown in 1 and 3 cm light-path flat plate glass reactors exposed to 250 or 2000 μmol photon m^{-2} s^{-1} in the laboratory and in 5 and 10 cm light-path plate reactors outdoors

Light source (μE m^{-2} s^{-1})	Reactor light-path length (cm)	Cell mass conc.[a] (g L^{-1})	Areal dry cell mass[a] (g m^{-2})	Light per cell mass (μmol m^{-2} s^{-1}) g^{-1}	AA content (in full growth medium)[b] (mg g^{-1})	AA content (following N-deprivation)[c] (mg g^{-1})	% increase of AA content due to N deprivation	Cumulative culture content of AA[c] (mg L^{-1})	Total culture period (days)	Average[a] areal AA yield[d] (mg m^{-2} d^{-1})[e]
250	1	8.8 ± 0.3	44 ± 2	5.7	73 ± 5	164 ± 7	124	1443 ± 13	38	190 ± 5
250	3	6.3 ± 0.2	95 ± 3	2.5	90 ± 3	192 ± 8	112	1210 ± 11	38	478 ± 7
2000	1	21.0 ± 0.3	103 ± 5	19.4	58 ± 2	127 ± 5	119	2667 ± 13	38	351 ± 9
2000	3	18.5 ± 0.4	275 ± 6	7.3	65 ± 4	141 ± 7	114	2609 ± 9	38	1030 ± 11
outdoors	5	5.3 ± 0.3	138 ± 7		45 ± 3	72 ± 6	59	382 ± 7	32	298 ± 8
outdoors	10	4.2 ± 0.1	213 ± 5		57 ± 6	96 ± 9	69	403 ± 8	32	630 ± 7

[a]After a total 38 days (17 days in full growth medium and 21 days in nitrogen-free growth medium) in laboratory or 32 days (15 days in full growth medium and 17 days in nitrogen-free growth medium), outdoors.
[b]At stationary stage, 17 days (laboratory) or 15 days (outdoors) in full growth medium.
[c]Following 21 days (in laboratory) or 17 days (outdoors), in nitrogen free medium.
[d]Based on the total illuminated area, *i.e.* both the front and back panels.
[e]d = day; in laboratory: 24 h of continuous illumination. Outdoors: 12 h day^{-1} average light period.

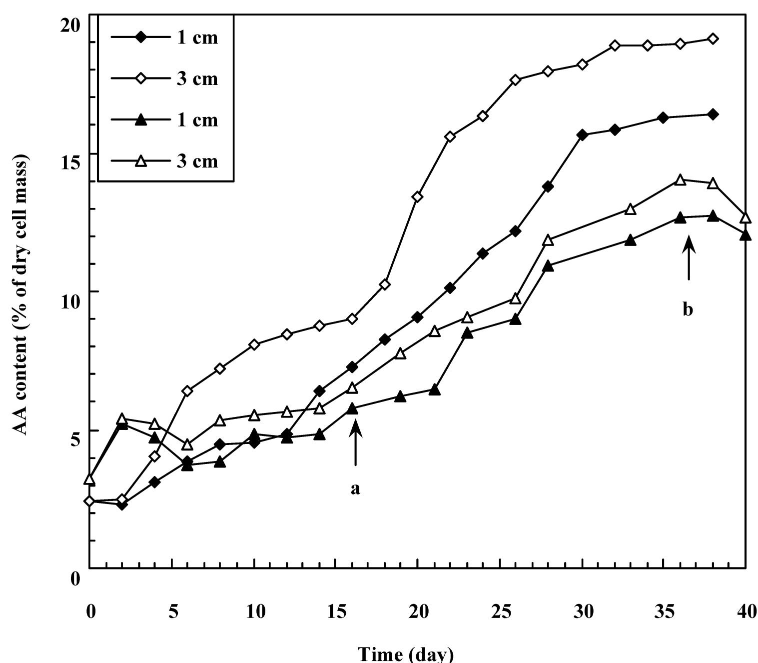

Figure 2. Production of arachidonic acid (AA) and total fatty acid (TFA) content of *P. incisa* as affected by light path length. -▲- 1 cm and -△- 3 cm (AA); -◆- 1 cm and -◇- 3 cm (TFA); a – nitrogen deprived, b – nitrogen added. Light −250 μmol photon m^{-2} s^{-1}.

Discussion

Preliminary experiments indicated that cell-AA reached its maximum approximately when the cultures reached steady state in cell number, i.e. net increase of cells barred because of severe light-limitation resulting from extreme mutual-shading of cells. In response to nitrogen deprivation induced at this stage, AA content as percent of DCM increased significantly.

The longer light paths, associated with lower light per cell (Table 1), affected a larger accumulation of AA, cell-AA and fatty acids for a given LPL at low light being higher than at high light (Figure 2). It is suggested, therefore, that the accumulation of triacylglycerols (TAG) and the biosynthesis of AA in *P. incisa* are inversely related to the quantity of light per cell (Table 1). Accumulation of AA and TAG outdoors was lower than in the laboratory, although the light path of the reactors used outdoors was longer. Laboratory cultures, however, were irradiated continuously at optimal temperature, whereas outdoor cultures were naturally exposed to fluctuating conditions of light and temperature. It was clear that growth conditions affecting high biomass production and accumulation are different from those affecting highest cell-AA. High light flux per cell is required to achieve the highest concentration of cell mass, but it was the areal cell mass (g m^{-2}), which was much higher with 3- or 10-cm LP compared with 1- or 5-cm reactors, respectively, that exerted the dominant effect on the total areal AA yield. Light path length exerted a much greater effect on the population density (g L^{-1}) at ″low″ compared with ″high″ light, cell density being 40% higher in the 1-cm LP at low light and only 13% higher at high light over the 3-cm reactors. This is interpreted as a clear indication that high light, which was 8 times stronger than low light, was relatively much less effective in supporting light-limited growth of *Parietochloris*, particularly so in the 1-cm reactor.

Areal cell mass at the end of 38 days growth, in contrast, was close to 3 times higher in the 3-cm than the 1-cm LP reactor (Table 1).

Maximal culture content of AA in either the laboratory or outdoors was essentially not affected by the LP, which exerted however a substantial effect on the areal output, i.e., the average daily productivity of AA per square meter obtained by dividing culture areal content of AA by the overall culture period (Table 1). In all our experiments, the longer light-path reactors with the higher areal volumes resulted in the highest AA yield, i.e. the highest amount of AA harvestable per illuminated reactor surface.

The increase in the AA-proportion of TFA was accompanied by a concomitant decrease in the proportion of the ω3 fatty acids, *i.e.*, 16:3 and 18:3. These fatty acids were reported to have a role in the protection of photosystem I (Klyachko-Gurvich et al. 1999), and are almost entirely deposited in chloroplast lipids, predominantly monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). The proportions of the ω3 fatty acids to TFA were directly related to light intensity (Cohen et al. 1988), and were much reduced in nitrogen-deprived *P. incisa*.

Numerous studies have shown that in many species of microalgae, imposing nitrogen starvation would result in accumulation of large quantities of TAG (Ben-Amotz et al. 1985; Cohen 1999). The accumulated TAG, however, are generally made of short chained, saturated and monounsaturated fatty acids, PUFAs being generally excluded. Recently, Bigogno et al. (2002a, 2002b) have shown that *P. incisa* is outstanding in its ability to accumulate AA-rich TAG. The very high cell content of AA in *P. incisa* is thus unique among algae. The rate of production in *M. alpina* is higher (Kyle 1995); however, algal oils are likely to be preferred over microbial and fungal oils. Moreover, the results presented here are far from being optimal.

We conclude that the following conditions should be met for maximal AA yield in *P. incisa*: 1) the length of the reactor light-path should be optimized for production of high areal cell mass; 2) the reactor should be exposed to strong light to affect rapid growth and high areal density of cell mass; 3) effective induction of the oleaginous process in mass cultures of *P. incisa* requires cell mass should be resuspended in nitrogen-free medium when net increase of cell mass in the culture ceased; 4) For highest areal yield of AA outdoors, the nitrogen-deprived oleaginous phase should be carried out in reactors of a rather long LP, i.e. at the range of 10 to 20 cm.

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