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Production of eicosapentaenoic acid (EPA) in *Monodus subterraneus* grown in a helical tubular photobioreactor as affected by cell density and light intensity

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Abstract

The effect of cell density (1–4.5 g L^{-1}) and light intensity (44 and 82 μmol m^{-2} s^{-1}) on fatty acid composition and eicosapentaenoic acid (EPA, 20:5 ω3) production was studied in a semi-continuous culture of *Monodus subterraneus* grown in a helical tubular photobioreactor ('Biocoil') under laboratory conditions. Under low light, the highest proportion of EPA (31.5% of total fatty acids) and EPA content (3.5% of dry weight), biomass productivity (1.3 g L^{-1} 24 h^{-1}) and EPA productivity (44 mg L^{-1} 24 h^{-1}) occurred at optimal cell density of about 1.7 g L^{-1}. Cell density had no effect on the total fatty acid (TFA) content and was maintained at *ca*. 11% of dry weight. Under high light, the highest proportion of EPA to fatty acids (31.8%), the total fatty acids content (13.4%) and EPA content (4.3% of dry weight) occurred at cell density of about 3.4g L^{-1}. But the highest biomass productivity (1.7 g L^{-1} 24 h^{-1}) and EPA productivity (56 mg L^{-1} 24 h^{-1}) were obtained at a cell density of 1.6 and 2.6g L^{-1}, respectively. Our results suggest that manipulating the cell density and light intensity can modify the composition of fatty acid and production of eicosapentaenoic acid (EPA) in *M. subterraneus*.

Introduction

Polyunsaturated fatty acids in general and eicosapentaenoic acid (EPA, 20:5 ω3) in particular are gaining increasing importance because of their emerging pharmaceutical use (Simopoulos, 1991; Rambjor et al., 1996). Currently, the main commercial source of EPA is fish oil, but since its purification involves many drawbacks (Cohen & Cohen, 1991; Cohen, 1994), the search for pure EPA from alternative sources has rapidly increased. Microalgae have been recognised as one of the most promising EPA producers (Cohen et al., 1995).† Deceased.

M. subterraneus, a yellow-green alga with relatively high EPA content (Iwamoto & Sato, 1986), has been suggested as a potential natural source for EPA (Cohen et al., 1995). The effects of light intensity, the mode of cultivation, CO_2, and nitrogen starvation on the fatty acid composition and EPA content in this species have been studied (Cohen, 1994). However, the effect of cell density and light intensity on the composition of fatty acids and productivity of EPA remains unclear.

It has been reported that cell density significantly affects the biomass productivity of algal culture (Vonshak et al., 1982), yet less attention has been paid to the effect of cell density on the composition of fatty acids. Although there are some reports concerning the effect of cell density on the fatty acid composition, the results seem to be equivocal. It has been shown that an increase in cell density induced a decrease in

the EPA proportion of total fatty acids in the red alga *Porphyridium cruentum* (Cohen et al., 1988), while the EPA content in *M. subterraneus* increased under similar conditions (Cohen, 1994). On the other hand, Chrismadha & Borowitzka (1994) have studied the effect of cell density and irradiance on eicosapentaenoic acid production of *Phaeodactylum tricornutum* growth in a tubular photobioreactor and observed little effect of cell density on the chemical composition of the diatom *Phaeodactylum tricornutum*. It should be pointed out that all previous studies used relatively low cell density i.e. up to 2 g dry weight of biomass per litre. Moreover, since the cells receive less light with an increase in cell density, the changes in the composition of fatty acids at higher cell density may thus be the result of self-shading. For further understanding how the cell density affects the fatty acid composition, it is necessary to investigate the changes in the fatty acid composition in higher cell density of algal culture grown under different light intensities.

In this study, we have investigated the effect of cell density (1–4.5 g L^{-1}) on the fatty acid composition and EPA production of *M. subterraneus* grown in a helical photobioreactor ('Biocoil') at two different light regimes, viz. 44 and 82 μmol m^{-2} s^{-1}.

Materials and methods

Organism and culture conditions

Monodus subterraneus (Eustigmatophyta) (Hibberd 1991) UTEX 151 was grown under laboratory conditions at 26 ± 2 °C in BG-11 medium as described by Iwamoto & Sato (1986), in which the concentrations of nitrogen and phosphate were doubled to avoid possible nutrient limitation. The culture was illuminated continuously by cool white fluorescence lamps. The pH value was maintained at 7.2 by adjusting the CO_2/air ratio.

Helical photobioreactor and operation system

The cultivation system consisted a helical tubular photobioreactor ('Biocoil') constructed from transparent PVC tubing to contain a total volume of 4.5 liter. A schematic diagram of the helical photobioreactor system has been described in detail in our previous study (Watanabe et al., 1995). The photostage consisted of a food-grade, transparent PVC tube (1.6 cm internal diameter with 0.3 cm wall thickness) wound helical on a vertical supporting structure using iron mesh gardening material. Recycling of culture was provided by an air-lift system creating an effective circulation of the culture. The cylindrically shaped photostage was illuminated continuously on the inside and/or outside with an array of 12 cool white fluorescent lamps. In this study, two light levels, 44 (low light) and 82 (high light) μmol m^{-2} s^{-1}, were used by illuminating from the inside or both sides, respectively.

The experiments were carried out in a semi-continuous culture mode to study the effect of cell density on the fatty acid composition and EPA productivity. The cultures were harvested daily by removing appropriate volume of the culture suspension and replacing it with the same volume of fresh medium to maintain the pre-set cell density. The culture was maintained at the indicated cell density for 6–7 consecutive days and thereafter samples were withdrawn for various parameter analyses.

Determination of biomass

The dry weight of *M. subterraneus* biomass was determined on triplicate aliquots of the algal suspension (5–10 mL each) by filtration through pre-weighed Whatman No. 5 filter paper, washed with distilled water and dried for two hours at 105 °C to constant weight. Chlorophyll *a* was extracted by DMSO and assayed spectrophotometrically (Bennet & Bogorad, 1973).

Fatty acid analysis

Freeze-dried cells were transmethylated with methanol-acetyl chloride as described by Cohen & Cohen (1991). Heptadecanoic acid was added as an internal standard. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Co., St. Louis, MO) and by calculation of the equivalent chain length. Fatty acid contents were determined by comparing each peak area with that of the internal standard. Gas-chromatographic analysis was performed on a Supelcowax 10 (Supelco, Bellefonte, PA) fused silica capillary column (30 × 0.32 mm) at 200 °C (FIP, injector and flame ionisation detector temperature 230 °C, split ratio 1:100).

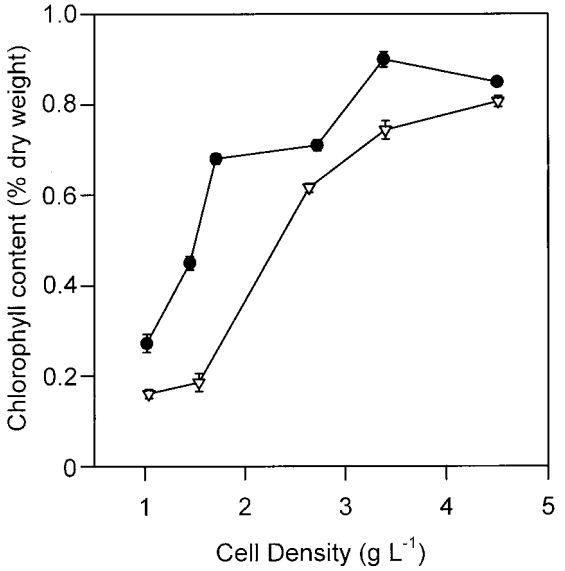

Figure 1. Effect of cell density on chlorophyll content expressed as % of dry weight in *M. subterraneus* cultures grown under 44 (●) and 82 μmol m^{-2} s^{-1} (▽). Data are means ± SE (n = 3–4).

Results

Chlorophyll content

Chlorophyll content increased with the increase of cell density and was higher in the culture grown under low light (44 μmol m^{-2} s^{-1}) than the one grown under high light (82 μmol m^{-2} s^{-1}) (Figure 1). When the cell density increased from 1 to 4.5 g L^{-1}, the chlorophyll content per cell, as a percentage of dry weight, increased from 0.27 to 0.85 under low light and from 0.16 to 0.81 under high light. Figure 1 also shows that the difference in chlorophyll content between the cultures grown under low and high light was more significant at the lower cell density and decreased with the increase of cell density.

EPA productivity

The effect of cell density on the proportion of EPA (% total fatty acids), total fatty acids (TFA, % dry weight) and EPA content (% dry weight) in the culture grown under low and high light is shown in Figure 2. Under low light, the proportion of EPA as a percentage of total fatty acids reached the maximum of 31.5% at 1.7 g L^{-1} of cell density. Under high light, the proportion of EPA as a percentage of total fatty acids increased with increasing cell density and was highest (31.5%) at 3.4 g L^{-1} (Figure 2A). Under low light

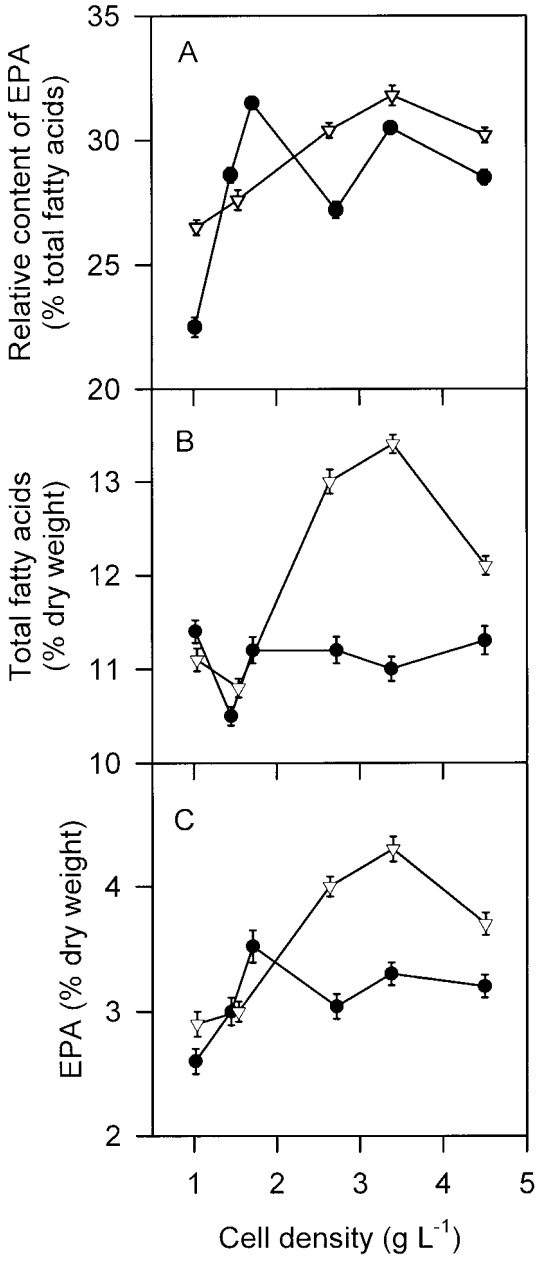

Figure 2. Effect of cell density on (A) the proportion of EPA, (B) the total fatty acid content and (C) EPA content (% of dry weight) in *M. subterraneus* cultures grown under 44 (●) and 82 μmol m^{-2} s^{-1} (▽). Data are means ± SE (n = 3–4).

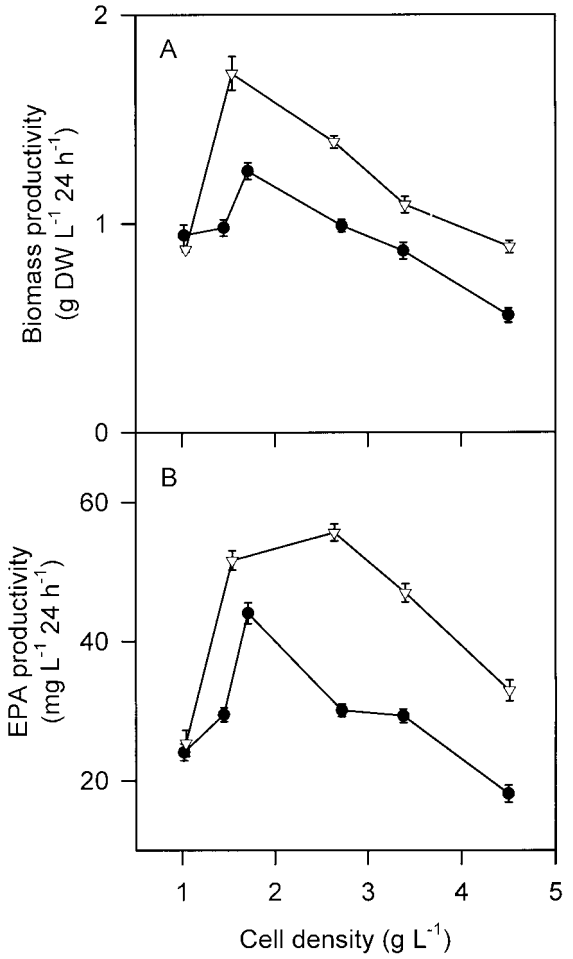

Figure 3. Effect of cell density on (A) biomass productivity and (B) EPA productivity in *M. subterraneus* cultures grown under 44 (●) and 82 μmol m^{-2} s^{-1} (▽). Data are means ± SE (n = 3–4).

TFA was largely constant at around 11%. However, in cells grown at high light, TFA increased with increasing cell density and reached its maximum value at a cell density of 3.4 g L^{-1} (Figure 2B). Similarly, the EPA content as a percentage of dry weight also reached the maximum at 1.7 and 3.4g L^{-1} of cell density grown under low and high light, respectively (Figure 2C).

The effect of cell density and light intensity on biomass productivity and EPA productivity are shown in Figure 3. Under low light, with increasing cell density, biomass and EPA productivity increased when biomass concentration was low (1–1.7 g L^{-1}) and decreased at biomass concentrations higher than 1.7 g L^{-1}. The highest biomass productivity and EPA productivity in cells grown under low light, were 1.3 g L^{-1} 24 h^{-1} and 44 mg L^{-1} 24 h^{-1}, respectively, and occurred at a biomass concentration of 1.7 g L^{-1}. In contrast, under high light both biomass productivity (Figure 3A) and EPA productivity (Figure 3B) increased at low cell density and decreased at cell densities higher than 1.6 and 2.6 g L^{-1}, respectively. The maximum biomass and EPA productivity were 1.7 g L^{-1} 24 h^{-1} and 56 mg L^{-1} 24 h^{-1} at cell densities of 1.6 and 2.6g L^{-1}, respectively. It is worth noting that under high light the EPA productivity (56 mg L^{-1} 24 h^{-1}) was not only higher than the low light grown cultures (44 mg L^{-1} 24 h^{-1}), but was also achieved at a higher cell density (2.6 g L^{-1}) as compared to (1.6 g L^{-1}) for the latter.

Discussion

Any attempt to develop the biotechnology for production of microalgal biomass in order to extract a high value product will require a better understanding of the parameters that are governing its accumulation. The fatty acid content of many microalgae is affected by nutritional as well as environmental factors (Cohen, 1999). Light availability plays a major role on the lipid content of cells grown outdoors, mainly due to its effect on the energy supply to many of the biosynthetic pathways, as well as its effect on the ultrastructure of the cell organelles where lipids are an important component of their membrane composition. Light availability to the outdoor grown cells can be modified not only by a direct change in the light intensity but also via changing pond depth, tube diameter or cell density. Thus development of a carefully designed maintenance protocol to adjust the cell density in order that light availability will be maintained to ensure maximal productivity of the desired component is of utmost importance for the commercial development of this biotechnology. Our results show that changing the cell density can be used to modify light availability to outdoor grown cells. This is clearly depicted in Figure 1, which demonstrates the changes in the chlorophyll content of the biomass as a function of cell density. A typical response is observed in which an increase in cell density, which is also correlated to a decrease in light availability, results in an increase in chlorophyll content. This phenomenon which is observed in higher plants as well is known as shade adaptation. It is worth noting that at any given cell density the chlorophyll content is higher in the low

light grown cells as compared to the high light grown cells. The fact that this pattern is not maintained when following the fatty acid and EPA content may indicate that the massive accumulation of lipids in *M. subterraneus* is not taking place in the chlorophyll bound membrane structure of the cells. In cells grown under low light conditions, the changes in the EPA and TFA content were relatively low and took place only at the relatively low cell density. This may be due to the fact that under higher cell density the light limitation was too severe and the energy supply was highly limited. In cells grown at an higher light intensity it was clearly demonstrated that fatty acid composition in *M. subterraneus* cells can be modified by cell density of the culture. The optimal cell density at which the highest proportion of EPA and the EPA content is achieved was raised to 3.4 g L^{-1}. Unlike the changes in the proportion of EPA and the total fatty acid observed under low light intensity, the proportion of EPA and the total fatty acid content increased with increasing cell density and reached the highest value at 3.4g L^{-1} (Figures 2A and 2B). Thus, the changes in EPA content under high light resulted from the changes in both the proportion of EPA and the total fatty acid content (Figure 2C). Our results suggest that the optima for the proportion of EPA and EPA content changed with the cell density as well as growth light intensity.

It has been shown that for most EPA-containing microalage, EPA is concentrated mainly in the galactolipids, suggesting that EPA is largely located in photosynthetic membranes (Sukenik et al., 1989). Our results show that the chlorophyll content of cells increased with the increase of cell density either at low or high light density, which indicates that shade adaptation took place by increasing the cell content of the photosynthetic membranes. Thus, the increasing photosynthetic membranes should be accompanied by an increase in EPA content. However, the changes in EPA content were not followed by the same pattern with the changes in the chlorophyll contents. The cell density for maximal EPA content was 1.7 g L^{-1} when light intensity was 44 μmol m^{-2} s^{-1} and was shifted to 3.4 g L^{-1} when light intensity was increased to 82 μmol m^{-2} s^{-1}. These results suggest that the increase in EPA content in response to the increase in cell density may reflect a light adaptation process and be the result of an increase in photosynthetic membranes. The changes in EPA content below the optimal cell density should be associated with light availability. On the other hand, the decrease in EPA content when cell density was above the optimum may be associated with the fact that under higher cell density the light limitation became very extreme and the energy supply was highly limited. It seems that the changes in fatty acids composition and EPA content affected by cell density may be not due to the difference between growth rates since it has been demonstrated that the changes in growth rate do not necessarily affect the fatty acids composition in outdoor cultures of *M. subterraneus* cells (Hu et al., 1997). How growth rate affects the fatty acids composition and EPA content in *M. subterraneus* cells remains to be further investigated.

The effect of light intensity on the fatty acid composition of algae has been studied previously. In *P. cruentum*, increasing light intensity modified the fatty acid composition by increasing the proportion of EPA in the total fatty acid fraction (Cohen et al., 1988; Cohen, 1991), while in the eustigmatophyte *Nannochloropsis* a decrease in the proportion of EPA was observed in response to higher light intensity (Sukenik et al., 1989). There are also several reports showing that the fatty acid composition is not affected by light intensity (Sevilla et al., 1998).

In batch culture of *M. subterraneus* grown at different light intensities, increasing light intensity resulted in a decrease in the proportion of EPA (Cohen, 1994). In the present study, we used semi-continuous culture and doubled the concentration of nitrogen and phosphate in the medium to avoid possible nutrient stress since nitrogen starvation decreases the proportion of EPA (Cohen, 1994). Furthermore, we compared the effect of direct light intensity on the fatty acid composition at the same cell density. Our results show that the effect of light on the proportion of EPA is complex. No clear pattern was observed on the effects of light intensity on the proportion of EPA. It seems that generally light intensity had only negligible effects on the proportion of EPA in *M. subterraneus* either at low or high cell density. Understandably, the inconsistent results on the effect of light intensity on fatty acid composition may be associated with the differences in species, cultivation modes, and environmental conditions. Also, it is possible that in the previous studies the effect of light intensity on the fatty acid composition was not compared at the same cell density.

Our data also show that cell density had significant effect on the biomass productivity and EPA productivity. Under both light regimes, the maximum biomass productivity reached at a cell density of about 1.7 g L^{-1}. Biomass productivity was always higher under

high light than under low light when cell density exceeded 1 g L^{-1}. In terms of EPA productivity, the maximum values were 44 mg L^{-1} 24 h^{-1} at a cell density of 1.7 g L^{-1} under low light and 56 mg L^{-1} 24 h^{-1} under high light, which occurred at a cell density of 2.6 g L^{-1}. EPA productivity was always higher under high light than under low light when cell density was higher than 1 g L^{-1}. Our data suggest that when cell density was lower than *ca.* 1.7 g L^{-1}, the higher EPA productivity under high light was mainly the result of higher biomass productivity. However, when cell density was higher than *ca.* 1.7 g L^{-1}, the higher productivity of EPA under high light was associated with higher total fatty acid content and higher biomass productivity.

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