OPTIMIZATION CONDITIONS FOR THE PRODUCTION OF ARACHIDONIC ACID BY THE MICROALGA *PARIETOCHLORIS INCISA* UNDER INDOORS AND OUTDOORS CONDITIONS

BY

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ABSTRACT

The freshwater green microalga *Parietochloris incisa* is the richest known plant source of the polyunsaturated fatty acid (PUFA), arachidonic acid (20:4ω6, AA). Accumulation of AA and docosahexaenoic acid (22:0, DHA) is necessary for brain and retina development in infants. While many microalgae accumulate triacylglycerols (TAG) in the stationary phase or under certain stress conditions, these TAG are generally made of saturated and monounsaturated fatty acids. In contrast, most cellular AA of *P. incisa* resides in TAG. This is of practical value, since TAGs are the preferred chemicals to introduce AA into baby formulae. Studies using different cultures conditions were carried out to assess their effects on AA production.

Under different cell densities, low, medium and high (LD, MD and HD, respectively), the cells of *P. incisa* showed a different pattern of chlorophyll content. At LD, cells followed the process of nitrogen starvation, decrease in chlorophyll content, accumulation of carotenoids and showed signs of bleaching at the end of cultivation period. In the same way, cells grown at MD followed the same pattern though it was not more pronounced compared to the former. However, at HD, cells continued to increase the chlorophyll content, probably by continuing to build the photosynthetic membranes to maximize light harvesting. The AA content increased in all cultures irrespective of the initial cell density. Volumetric biomass accumulation was higher in HD cultures, eventually the volumetric AA yield was also higher.

The growth stage at which cultures were transferred to N-free medium had a pronounced effect on chlorophyll synthesis of *P. incisa*. The rate of chlorophyll synthesis was faster in cultures transferred in EXP (exponential) and TRANS (transition) phases.

Similarly, the biomass accumulation was rapid in cultures transferred in the EXP and TRANS phases. The proportion of AA of total fatty acid increased faster in cultures transferred to N- starvation in the EXP and TRANS phases of growth. The proportion of AA almost doubled from 25.8% to 47.5% and from 25.8% to 49.8% of TFA, respectively at day 14. Cultures transferred in the E.STA (early stationary) and M.STA (middle stationary) phases of growth, had higher initial proportion of AA, amounting to 34.9% and 36.3%, respectively, at day 0. Their proportion of AA had increased slowly throughout the cultivation period, reaching 47.3% and 47.0% at the end of cultivation period.

Cultures growing on complete medium and supplemented with fatty acid precursors were able to incorporate and further modify both incorporated oleic acid and linoleic acid to AA. This was evident only with 3d cultures at the EXP stage. Older cultures did not demonstrate an enhancement in AA production, but rather a reduction. Supplementation of N-starved cultures did not enhance AA production.

Increasing the C/N ratio favors lipid accumulation in microalgae by inducing *de novo* lipid synthesis. Indeed, high CO_2 significantly enhanced the TFA synthesis in *P. incisa* indicating that under enriched CO_2 conditions the rate of *de novo* synthesis of fatty acid is higher. However, this phenomenon was only evident up to 10 days. Cultivation of *P. incisa* on BG11-N with high CO_2 did not favor an increase in the proportion of AA, moreover, the proportion of 18:1 increased at the expense of AA. This might indicate either an inhibition of sequential desaturation or favored synthesis of shorter and less desaturated fatty acids. Increasing the CO_2 level in HD cultures on BG11-N and tap water media did not cause any significant changes in the TFA and AA contents.

In developing a large-scale cultivation for AA, it is necessary to utilize inexpensive medium component to lower the cost. Tap water and brackish water were used to induce starvation in *P. incisa* in HD cultures. Cells of *P. incisa* survived in tap water, which basically contains no nutrients for sustaining growth. More diluted cultures were not able to survive under those conditions. It is therefore clear that growing cells of *P. incisa* under HD can allow the cells to grow under nutrient-devoid medium. Cells of *P. incisa* were able to produce biomass rich in arachidonic acid on tap or brackish water. Not only is this media less expensive but also it saves labor in media preparation, reducing the final cost of the product.

By growing *P. incisa* under outdoors conditions, this study represented an ideal approach to making the realization of commercial production of AA possible. The volumetric productivity of AA and TFA, as well as the daily accumulation of AA were almost the same in outdoors and indoors cultures at day 8. On subsequent days, indoors culture attained higher productivities, probably such differences was due to contaminations that were microscopically observed in outdoors cultures at day 9.

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I dedicate this thesis to Dr. Martha Kandawa Schulz whose generosity, support, humanity and loving example have moved me countless time and also in memory of her daughter, Pandula Schulz, may her soul rest in eternal peace.

DECLARATIONS

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LIST OF ABBREVIATIONS AND SYMBOLS

AA:	Arachidonic acid
BW:	Brackish Water
CHL:	Chlorophyll
CNS:	Central Nervous System
CO_2:	Carbon Dioxide
DHA:	Docosahexaenoic acid
DGTS:	Diacylglyceroltrimethylhomoserine
DW:	Dry weight
EPA:	Eicosapentaenoic acid
EXP:	Exponential
E. STA:	Early Stationary
HD:	High density
IN:	Indoors
LD:	Low density
MGDG:	Monogalactosyldiacylglycerols
MD:	Medium density
M. STA:	Middle Stationary
OUT:	Outdoors
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PUFA:	Polyunsaturated fatty acid
SHAM:	Salicylhydroxamic acid

TAG:	Triacylglycerols
TFA:	Total Fatty acids
TRANS:	Transition
TW:	Tap water
VLC-PUFA:	Very Long Chain Polyunsaturated Fatty acid

1. Introduction

The demand for polyunsaturated fatty acids (PUFAs) in human nutrition, pharmaceutical applications and aquaculture is rapidly growing. The production of PUFA from current sources is expected to be inadequate to supply the expanding market (Gill and Valivety, 1997). Source of PUFAs such as fish oil have several drawbacks including seasonal and climatic variation in the PUFA content, contemporary presence of PUFA with antagonistic properties, pollutants, palatability and odor. The search for alternative sources of high quality PUFAs have been sought with microalgae considered a promising source (Yongmanitchai and Ward 1991). This consideration is attributed to the metabolic flexibility of microalgae as they can be made to overproduce a variety of different products through simple manipulations of the culture environment (Behrens and Kyle, 1996).

Several studies have focused on optimizing microalgae culture conditions to produce biomass rich in PUFAs (Cohen *et al*., 1998; Reitan *et al*., 1994; Otero *et al*., 1997; Khozin-Goldberg *et al*., 2002). Algal groups identified to produce high levels of PUFAs include diatoms, chrysophytes, cryptophytes, dinoflagellates, and others (Cohen *et al.*, 1995). The discovery of *P. incisa* however, to contain higher content of arachidonic acid make this species a potential candidate for commercial production of arachidonic acid (AA). This study focuses on investigating the optimization conditions for the production of AA from *P. incisa* grown under indoor and outdoor conditions. By growing *P. incisa* under outdoors condition, this study represents an ideal approach to making the realization of commercial production of AA possible.

1.1 Arachidonic Acid - a Very Long Chain Polyunsaturated Fatty Acid (VLC-PUFA)

PUFA are long chain fatty acids with two or more methylene interrupted double bonds. The lipids of all higher organisms contain appreciable quantities of polyunsaturated fatty acids (PUFA) with methylene interrupted double bonds, i.e. with two or more double bonds of the *cis*-configuration separated by a single methylene group. PUFAs are divided into different families, according to the distance of the last bonds from terminal methyl group. Arachidonic acid is a very long chain polyunsaturated fatty acid (VLC-PUFA) with 20 carbon atoms and 4 double bonds and it belongs to the family of ω6 (Fig.1).

Arachidonic acid is an important constituent of biological membranes and a precursor of numerous eicosanoids. The eicosanoids occurs and are biologically active in virtually every mammalian tissue (Gill and Valivety, 1997). With respect to infant nutrition, arachidonic acid is essential for infant development as a major PUFA of human milk. Prolonged dietary deficiency of AA impairs the postnatal development of central nervous system (CNS) functions. Studies on premature infants suggested that AA, as well as docosahexaenoic acid (DHA, 22:6ω3), improve infant development, since they are the major acyl components of brain membrane phospholipids (Hansen *et al.*, 1997). DHA and AA are transferred directly from mother to infant, during the last intrauterine trimester and following birth, by human milk feeding (Agostoni *et al.*, 1994). The accumulation of AA and DHA is necessary for brain and retina development; however, the level of the enzymes involved in the synthesis in preterm infants is not sufficient for the demand. Preterm infants therefore, require an external supply of AA and DHA fatty acids, if they are not breast-fed (Carlson *et al*., 1993). The World Health Organization

(FAO/WHO) recommends the addition of AA and DHA to infant formula, in comparable quantities to those in breast milk (Boswell *et al*., 1996).

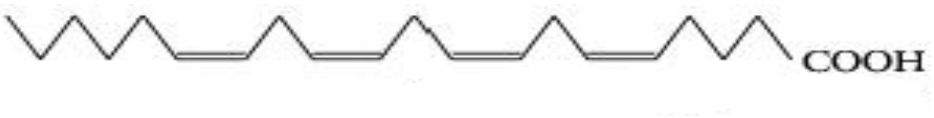

Figure 1. Schematic representation of arachidonic acid (AA; 20:4ω6).

The success of producing arachidonic acid from *P. incisa* would allow the development of an available source of AA that could be added to infant formulae and would improve the nutrition and development of preterm babies in developing countries such as Namibia.

1.2 *Parietochloris incisa*- potential source of arachidonic acid

Parietochloris incisa (Trebouxiophyceae, order Chlorophyte) is a unicellular green freshwater alga, isolated from the slopes of a snow mountain in Japan (Watanabe *et al.*, 1996). The ecological niche is characterized by a wide range of temperatures; from freezing to over 20 ^{o}C, and the intensity of light vary from normal to very high, due to the reflection from the snow. The optimal growth temperature of *P. incisa* is 25^{o}C, but it can withstand temperatures as low as 4 ^{o}C. The alga produces AA, as it is major fatty acid through the whole cell cycle (Bignono *et al.*, 2002a). The polar lipids pattern of *P. incisa* is typical of green algae containing glycolipids, phospholipids and the betaine lipid diacylglycerol trimethylhomoserine (DGTS). However, different from most green algae,

triacylglycerols (TAG) rich in AA, represent the major lipid, reaching up to 80% of total lipids (Bignono et al., 2002a). 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 fatty acids accumulated in TAG, are mostly saturated or monounsaturated. Bigogno *et al.,* (2002a, 2002b) have shown that *P. incisa* is exceptional in its ability to accumulate AA-rich TAG. This makes *P. incisa* outstanding even among other oleaginous algae.

1.3 The biosynthetic pathway of arachidonic acid in *P. incisa*

Several biosynthetic pathways were suggested for AA, EPA and DHA biosynthesis in microalgae (Cohen *et al.,* 1995). The order of elongation and desaturation steps varies in different microorganisms, showing a multiplicity of biosynthetic pathways. In *P. cruentum* (Khozin *et al.,* 1997) 18:1ω9 is stepwise desaturated to 18:2ω6 and 18:3ω6 and then elongated to 20:3ω6 and Δ5 desaturated to AA (Fig. 2, bottom). On the other hand, it was claimed that in *Euglena gracilis*, the elongation of 18:2ω6 precedes any further desaturation (Nichols and Appleby, 1969) as shown in Fig. 2, top.

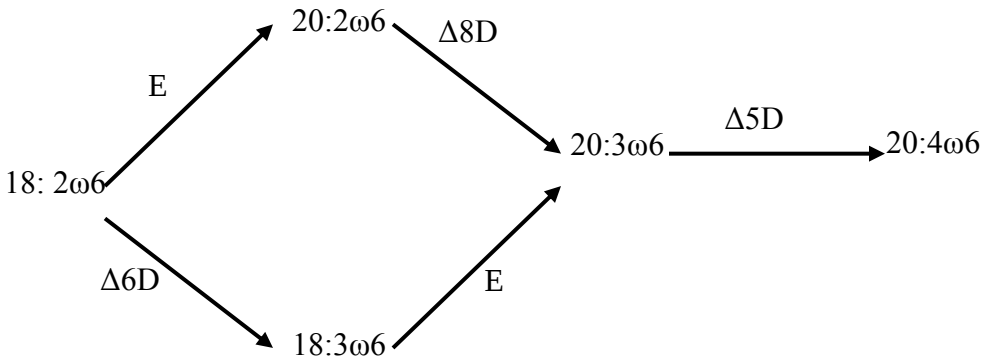

Figure 2. Suggested pathway for the biosynthesis of arachidonic acid in various in microalgae

Bignono *et al*. (2002c) elucidated the biosynthesis pathway of AA in *P. incisa* through radiolabelling experiments with acetate and various fatty acids. Labeling with [1-^{14}C] oleic acid showed that the first steps of the lipid-linked fatty acid desaturation utilize cytoplasmic lipids. PC and DGTS were implicated as the major lipids acting as acyl carriers for the Δ12 and Δ6 desaturation of oleic acid, leading sequentially to linoleic acid (18:2) and γ-linolenic acid (18:3ω6). As elongation of 18:3 occurs at its carboxylic end, the acyl group must be detached from their phospholipids carrier in order to be elongated to 20:3ω6. Since labeled 20:3ω6 was detected first in PE and then in PC, it was then deduced that these lipids (especially PE), are the mostly likely substrates for the Δ5 desaturation of 20:3ω6 to AA. The presence of molecular species common to both PC and PE (Bignono *et al*., 2002) provided further support to this suggestion. Figure 3 shows the outline of the likely biosynthetic pathways of AA in *P. incisa.* This possible biosynthetic pathway of AA was further confirmed by treatments using inhibitors of Δ12 and Δ6 desaturations, salicylhydroxamic acid (SHAM).

In *P. incisa*, AA is synthesized in the phospholipids and is mainly exported to TAG, but also to DGTS and the galactolipids (Bignono *et al.*, 2000b). Once PUFA are produced, they can remain linked to phospholipids and galactolipids or can be accumulated in triacylglycerols. In *Porphyridium cruentum*, AA is transported from PC to TAG (Khozin *et al.*, 1997). Similarly, in *Chroomonas salina*, 18:3ω3 is transferred from DGTS to TAG (Henderson & Mackinlay, 1992).

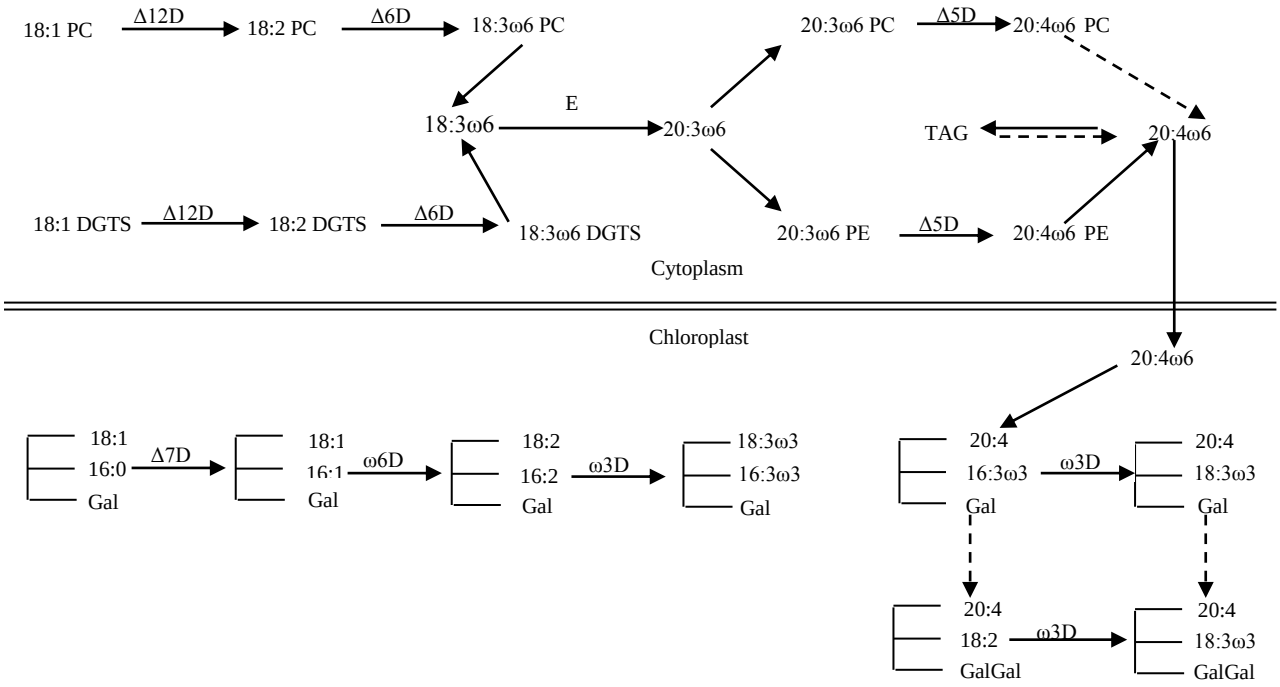

Figure 3. An abbreviated outline of the biosynthesis of AA in *P. incisa* (Adapted from Cohen and Khozin-Goldberg, 2005, unpublished)

1.4 Roles of triacylglycerols in algae

The roles of TAG in algae have not been totally unraveled. TAG is generally considered to be used as storage material. When grown in a light-dark regime, *Nannochloropsis*

forms, during the light period, cytosolic lipid globules, rich in 16:0 and 18:1, which can be reutilized for polar lipid synthesis in the dark (Sukenik and Carmelli, 1990).

Upon a sharp drop in temperature, the cells of *P. incisa* relocate AA, from TAG into polar lipids (Bignono *et al.*, 2002b). A similar pattern has been shown when a stationary phase culture of *P. incisa* was brought back to growth conditions and the production of chloroplastic membranes was enhanced. The translocation of AA to polar lipids, especially to MGDG, appears to occur when *de novo* synthesis of fatty acids is slowed down (Shrestra *et al.*, 2002, unpublished data). In *P. incisa*, under nitrogen starvation, the production of chloroplastic lipids decreased and the accumulation of TAG in oil bodies increased. Following recovery from nitrogen starvation at room temperature (Shrestra *et al.*, 2002, unpublished data), analysis of molecular species and content of MGDG, showed that the content of molecular species of the C18/C18, C20/C18 and C20/C20 series increased. The molecular species of the C18/C16 type were more desaturated but did not increase in content. It was therefore assumed that one role of TAG in *P. incisa* is to serve as a reservoir of AA, that can be rapidly used to construct PUFA-rich chloroplastic membranes, especially at low temperatures, at which *de novo* PUFA synthesis would be very slow, or to enable the organism to quickly acclimate to changes in environmental conditions.

TAG seems to play an important role in reproductive functions in certain species of green algae. In *Chlamydomonas reinhartii*, the accumulation of TAG, induced by nitrogen depletion, promotes the transformation of vegetative cells into male and female gametes forms (Martin and Goodenough, 1975). Appearances of dense lipid bodies, made

up mostly of TAG, were reported to occur in *Chlamydomonas moewusii* during the fusion of the gametes (Brown *et al.,* 1968).

The growth of *Dunaliella salina*, at sub optimal temperature, induces β-carotene synthesis and promotes the formation of lipid-carotene globules. A similar example was found in snow algae in which TAG and secondary carotenoids were accumulated in response to the depletion of nitrogenous nutrients (Bidigare *et al.*, 1993). Unusually large accumulation of astaxanthin esters in extra-chloroplastic lipid globules produces the characteristic red pigmentation typical of some snow algae (*Chlamydomonas nivalis*) (Bidigare *et al*., 1993). Consequently, these compounds greatly reduce the amount of light available to the photosynthetic system, thus limiting photoinhibition and photodamage caused by intense solar radiation. The esterification of astaxanthin with fatty acids represents a possible mechanism by which this chromphore can be concentrated within cytoplasmic globules and can maximize its photoprotective efficiency (Bidigare *et al*., 1993).

1.5 Effects of nutritional and environmental conditions on fatty acid and lipid accumulation in algae

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 some optimal pH, temperature or salinity, under which conditions, synthesis of lipids may be enhanced at the expense of proteins or carbohydrates production (Cohen *et al.,* 1988; Bajpai and Bajpai 1993; Molina Grima *et al* (1995); Cohen *et al.,* 1995).

1.5.1 Nitrogen starvation

Cultivation of algae under nitrogen starvation was shown to result in accumulation of storage lipids such as triacylglycerols (TAG) (Roessler, 1990). The availability of nitrogen during the growth was shown to affect, not only the growth rate, but also the lipid content and composition (Shifrin and Chisholm, 1981). In most oleaginous organisms, the key to lipid accumulation is by allowing nitrogen supplied to the culture to become exhausted. For example, Shifrin and Chisholm (1981) reported a 130-320% increase in oil content under nitrogen deficient conditions in fifteen chlorophyceae strains. The proportion of EPA in *P. cruentum*, under nitrogen starvation, decreased while that of AA increased in neutral and in polar lipids (Cohen, 1992). On the other hand, N-starvation of *Navicula saprophilla* (Kyle *et al*., 1989) resulted in higher lipid content with no difference in the fatty acid composition. Furthermore, Khozin-Goldberg *et al.,* (2002) reported that nitrogen starvation in *P. incisa* has resulted in a fatty acid content of over 35% of dry weight and in a proportion of arachidonic that exceeded 60% of total fatty acids. The experiments in this study will be conducted mainly under AA- inducing conditions, particularly nitrogen starvation.

1.5.2 Feeding with fatty acid precursors

Another strategy that could increase the content of PUFA is by feeding with its precursors. Okumura *et al*., (1986) found that addition of 18:1, 18:2 or 18:3 to the cultivation medium of *Euglena gracilis* enhanced the production of PUFA, especially that of AA and EPA. Similarly, the EPA content of fungi (*Mortierella alpina*) was doubled after supplementation with linseed oil (rich in α-linolenic acid) (Shimizu *et al*., 1998).

Pham Qouc *et al*., (1993) also reported that addition of oleic acid to the growth medium of cyanobacterium (*Spirulina platensis*) caused normally prokaryotic lipids to become partly eukaryotic, producing 18:3ω6/18:3ω6 molecular species of MGDG. In this study, the growth medium of *P. incisa* will be supplemented with oleic acid or linoleic acid, the fatty acid precursor of AA.

1.5.3 Cell density and Light intensity

Cell density and light intensity were shown to have various effects on PUFA production in various algae (Vonshak *et al*., 1982). Generally algal cultures produce more TAG when kept at a low biomass concentration enabling relatively high light per cell (Roessler, 1990). In *P. incisa*, fatty acid production (mostly as TAG) was enhanced under higher biomass concentration in both nitrogen replete and depleted cultures, especially in the latter (Bignono *et al.*, 2002). Increase in cell density has induced a decrease in the EPA proportion of total fatty acids in the red alga *P. cruentum* (Cohen *et al*., 1988), while the EPA content in *Monodus subterraneus* has increased under similar conditions (Cohen, 1994). Vonshak *et al*., (1982) reported that cell density significantly affects the biomass productivity of algal culture.

Equally, light intensity affects not only the growth rate, but also the pigments, the structure and composition of the photosynthetic apparatus. High light intensity was shown to promote the production of PUFA in *P. cruentum* (Cohen *et al.*, 1988), and in *Fucus serratus* (Smith and Harwood, 1984). In the present study, several experiments will be conducted with initial high biomass concentration, since the production of fatty acid in *P. incisa* is enhanced under high biomass concentration.

1.5.4 Enrichment with CO_2

The effects of carbon sources on growth and biochemical composition have been studied for several species of microalgae (Chu *et al*., 1995; Gordillo *et al*., 1998; Wen & Chen, 2000). To optimize the production of the desired chemicals from microalgae, supply of appropriate carbon sources is important. Growth of *Dunaliella viridis* was enhanced when CO_2 (1%, v/v) was included in the aeration (Gordillo *et al.,* 1998). Cultures of *Nitzschia inconspicua* supplemented with glucose (0.1 w/v), acetate (0.1 w/v) or 5% CO_2 attained higher biomasses and their lipid content increased at the expense of proteins when aerated with 5% (v/v) CO_2 and gave the highest yield of eicosapentaenoic acid (EPA) (Chu *et al.,* 1996). On the other hand, cultures aerated with 5% (v/v) CO_2 had a significant increase in carbohydrate content but no in lipids of *Phaeodactylum tricornutum* (Chrismadha & Borowitzka, 1994).

1.5.5 Temperature

Growth temperature is one of the most important factors affecting PUFA productivity. It can influence biomass productivity, lipid and fatty acid content, and the degree of unsaturation of the fatty acids. These factors must be considered at the same time when evaluating the potential of temperature modulation as a means of controlling PUFA production. The effect of temperature on the total fatty acid content of microalgae cannot be generalized, as only a small number of species have been studied and the results are not consistent between species. In most PUFA-producing microalgae studied so far, fatty acid content increases with temperature. The lipid content of *Ochromonas danica* for instance, increased from 39 to 53% as the temperature was raised from 15 ^{o}C to 30 ^{o}C

(Aaronson, 1973) and a similar trend was observed in *Phaeodactylum tricornutum* (Iwamoto and Sato, 1986). On the other hand, in *Nannochloropsis*, the fatty acid content was higher at 18 ^{o}C than at 32 ^{o}C (Sukenik *et al*., 1989). An increase in the degree of fatty acids unsaturation in response to a decrease in growth temperature is an almost universal phenomenon (Williams *et al.*, 1996). At lower temperature, cells of *P. incisa* had an increase in C_{16} PUFA and 18:3ω3, deposited mainly in MGDG and DGDG (Bignono *et al.*, 2002).

An important element of this study is the cultivation of *P. incisa* cultures outdoors. Fluctuation in temperature in outdoor environment is expected to affect fatty acid content, as well as the degree of unsaturation of the fatty acids in the cells of *P. incisa*.

1.6 Aim of this work

The potential value of arachidonic acid produced by *P. incisa* prompted the study of its production under different physiological culture conditions.

To optimize AA production by *P. incisa*, the present work will specifically investigate:

1. Effects of different initial cell density (1, 2, 4 mg/mL) at the same light regime, 170 μmol quanta m^{-2} s^{-1}.
2. Correlation between changes in fatty acid composition, especially the accumulation of AA during nitrogen starvation and the growth stages.
3. Effect of CO_2 enrichment on the fatty acid composition and AA content.
4. Effect of incorporation of oleic acid (18:1ω9) or linoleic acid (18:2ω6) into the growth medium on fatty acid content and composition, more importantly their desaturation to AA.

5. Influence of the growth medium in the oleogenic phase of cultivation (BG11-N, brackish water and tap water) on the production of AA.
6. Outdoor cultivation of *P. incisa* and AA productivity will be assessed.

2. MATERIALS AND METHODS

2.1 Organism

Parietochloris incisa was isolated from Mt. Tateyama in Japan (Watanabe *et al*., 1996). An axenic culture was obtained by means of successive dilution and isolation of individual cell colonies grown on Petri dishes on solidified BG11 medium (1.5% agar) (Stanier *et al.,* 1971).

2.2 Growth conditions

Parietochloris incisa was cultivated on BG11 medium containing the following: $NaNO_3$, 1.5 g L^{-1}; MgSO4.7H2O, 0.075 g L^{-1}; $CaCl_2.2H_2O$, 0.036 g L^{-1}; citric acid, 0.006g L^{-1}; Na_2EDTA, 0.001g L^{-1}; Na_2CO_3, 0.02g L^{-1}; K_2HPO_4, 0.0305 g L^{-1}; ferric ammonium citrate, 0.006 g L^{-1} and A_5 trace elements solution 1 mL L^{-1} (H_3BO_3, 2.86 g; MnCl2.4H2O, 1.86 g; ZnSO4.7H2O, 0.222 g; $Na_2MoO_4.2H_2O$, 0.39 g; CuSo4.5H2O, 0.079 g; Co $(NO_3)_{2.6H2O}$, 0.0494 g in 1L distilled water) at pH 7.6. Cultures were grown in one-liter glass columns placed in a temperature regulated water bath at 25 ^oC. Under nitrogen starvation conditions, $NaNO_3$ was omitted from the medium and ferric ammonium citrate was substituted with ferric citrate.

Cultures were mixed by bubbling with a mixture of 1.5% CO_2 in air (unless otherwise specified), from the bottom part of the column. Illumination was provided by cool white fluorescent lamps external to the water bath at a light intensity of 170 μmol quanta m^{-2} s^{-1}. Light intensity was measured at the center of an empty column with a quantum meter (Lambda L1-185). To study feeding with external fatty acids, cultures were grown on BG11 medium in 150 mL Erlenmeyer flasks under an air: CO_2

(99:1) atmosphere. The flasks were placed in an incubator shaker at 25 ^{o}C and illuminated from above at a light intensity of 115 μmol quanta m^{-2} s^{-1}.

2.3 Measurement of growth parameters

To compensate for the slight loss of water through evaporation, sterile distilled water were added to columns before sampling in order to maintain its original culture volume. Samples were taken to determine growth parameters at specified times.

Since *P. incisa* cells tend to appear in aggregates, making it difficult to count cells, growth was there fore estimated on the basis of dry weight and chlorophyll content. Dry weight was measured by filtering 5-mL samples through pre-weighed glass-fiber filters (GF-52, Schleicher & Schuell). Filtrates were dried at 105 ^{o}C for 2 h.

Chlorophyll concentration (mg/L) was measured by extracting the biomass from 1 mL of culture with 5 mL DMSO at 70 ^{o}C for 10 minutes. If necessary, the extraction was repeated until a white pellet was obtained. The extract was centrifuged and the absorbance of the DMSO extracts was measured at 666, 650 and 480nm for chl a, chl b and total carotenoids, respectively by spectrophotometer (Carry 50, Varian). Chlorophyll content was calculated according to Merzlyak equations (unpublished data):

Chl a (mg/L) = 13.34 *A666-4.85*A650

Chl b (mg/L) = 24.58*A650-6.65*A666

And the total carotenoids: Cx+c= (1000*A480-1.29*Chl a-53.76*Chl b)/220

2.4 Fatty acid analysis

Freeze-dried cells of *P. incisa* were transmethylated with 2% H_2SO_4 in a mixture of dry methanol: toluene (9:1, v/v), under argon atmosphere, at 85 ^oC for 1.5 h. Heptadecanoic acid was added as an internal standard (Sigma Chemical Co.). The transmethylation was terminated by addition of water; fatty acid methyl esters (FAMEs) were extracted with hexane and analyzed using a gas chromatograph HP 6890A equipped with a flame ionization detector. The injector and detector temperature were set at 280 ^oC and 300 ^oC, respectively. Separation was achieved on a Supecowax 10 (Sigma, Inc., Bellefonte, PA) fused silica capillary column (30 m x 0.32 mm) with helium as a carrier gas. One μL of methyl ester solution was injected for each analysis. The oven temperature was programmed as follows: initial temperature of 190^oC was maintained for 10 min, then raised to 210 ^oC at a rate of 10 ^oC min^{-1} for 10 min. Finally, an oven temperature of 230 ^oC was kept for 3 min. Fatty acids methyl esters were identified by co-chromatography with authentic standards (Sigma Chemical Co.), and by comparison of their equivalent chain length (Ackman, 1969). The data shown represent mean values with a range of less than 5% for major peaks (over 10% fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

2.5 Lipid extraction

Biomass was collected in Sorvall tubes and spun down at 35 x 100 rpm. The supernatant was discarded and the residue was frozen at –20 ^oC and lyophilized in freeze dryer (10 Mr-TR, Virtis Co.). Freeze-dried samples of *P. incisa* biomass were extracted with DMSO at 70 ^oC for 10 min, followed by methanol at 4 ^oC for 1 h. The mixture was

centrifuged, supernatant removed, and the pellet was re-extracted with methanol. Peroxide-free diethyl ether containing 0.01% BHT, hexane and water were added to the supernatant so as to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken and centrifuged for 5 min at 35 x 100 rpm and the upper phase collected. The water phase was extracted with a mixture of diethyl ether: hexane (1:1, v/v), acidified with acetic acid and re- extracted. The organic phases were combined and evaporated with a BUCHI Rotavapor R-114.

2.6 CO_2 enrichment

Cultures of *P. incisa* in the exponential phase were cultivated in columns as previously described. Two different levels of CO_2 in the bubbling aeration system were compared, standard CO_2 (1.5%) and high CO_2 (5%). Cultures were cultivated on BG11 and N-free BG11 (BG11-N) medium with initial chlorophyll concentration of 30 mg /L and initial cell density of 1 mg/mL. In another treatment, at aeration of 5% CO_2, cultures were concentrated to initial cell density of 4 mg/mL and Chl 90mg/L and cultivated on BG11-N medium and tap water.

2.7 Induction of nutrient starvation

In the majority of experiments, starvation was induced by transferring the cultures of *P. incisa* in their logarithmic phase to BG11-N medium. In some experiments cells were resuspended and maintained in brackish water (with sodium and chloride ions as major components) and tap water with initial cell density of 4 mg/mL.

2.8 Feeding with oleic and linoleic acids

Cultures of *P. incisa* in the exponential phase of growth were inoculated into flasks and cultivated for 14 days as previously described, in the presence of the following fatty acids, oleic acid (18:1ω9) and linoleic acid (18:2ω6). Initial chlorophyll concentration was adjusted to 30 mg/L in all cultures. Two flasks, each containing 150 mL cultures on BG11 medium were enriched with either 1 mg/mL oleic acid or linoleic acid. Stock solutions of 15 mg fatty acid in 200 μL DMSO were utilized. Same stock solutions were added to two other flasks gradually at 0, 4 and 9 day in sequential amounts, according to the increase in biomass. Cultures in BG11-N medium were supplemented with either 30 mg oleic acid or 30 mg linoleic acid sequentially at 0, 4 and 9 day.

2.9 Cell density

Cells in the logarithmic phase of growth (daily diluted for 3 days) were resuspended and maintained in nitrogen-free medium. Initial cell density was adjusted to either 1 or 2 or 4 mg/mL.

2.10 Growth phases and nitrogen starvation

Cultures of *P. incisa* were transferred to nitrogen-free medium during different growth stages of the alga at initial cell density of 4 mg/mL. For the exponential stage, cultures were diluted daily for 3 days in one-liter columns, on BG11 medium as described above. For the transition stage, cultures were batch cultivated for 3 days. For early stationary stage, cultures were batch cultivated for 7 days. For middle stationary stage, cultures

were batch cultivated for 10 days. In all cultures, cells were washed with DDW and then resuspended in BG11-N medium at initial cell density of 4 mg/mL.

2.11 Outdoors mass cultures

2.11.1 Preparation of inoculums for mass cultures

Cells of *P. incisa* were transferred to BG11 medium and incubated in flasks in a shaker as described above under optimal growth conditions. Cultures were aseptically transferred to columns and daily diluted with sterile BG11 medium for at least 4 days until enough culture was obtained. Cultures were then left undiluted for at least 2 days prior to the onset of the experiment outdoors.

2.11.2 Outdoors cultivation

Cultivation of *P. incisa* was carried out in a 100-L polyethylene sleeves bag from 01/10/2003 to 19/10/2003. Temperature regulation was provided by a chiller, which circulated cooled water inside the bag through a stainless-steel serpentine, when culture temperature exceeded 25 °C. During the night, the culture temperature was allowed to equilibrate to ambient temperature, which was y 22 ^{o}C. The bag was equipped with perforated tube extending along the bottom of the bag, through which a stream of 1.5% CO_2- enriched air passed to affect mixing and supply of CO_2 to the culture. The bag was fitted with a thermometer to measure the temperature, while pH and light intensity were measured using a pH meter and quantum meter, Lambda L1-185, respectively. All measurements were taken during noon (12:00 pm) daily. The top opening of the bag was hold together with a clamp to reduce loss of water as well as air borne particles.

3. RESULTS

P. incisa can be induced to produce AA-rich biomass by cultivation in a two-phase system. The first phase is the cultivation on complete nutrient medium to obtain exponential growth. The second phase is the oleogenic phase in which cultivation is done by depriving medium of nitrogen to attain biomass enriched in AA. It is therefore very important to establish conditions that might have positive influence on AA productivity. Those conditions could be taken into account in subsequent studies aimed at optimization culture conditions for AA production by *P. incisa*. The following presents the findings of different culture conditions such as the effect of cell density, growth phases and nitrogen starvation, nutrient starvation, feeding with oleic and linoleic acid, CO_2 enrichment and outdoors cultivation.

3.1 Cell density

Cells of *P. incisa* were kept in the logarithmic phase of growth by daily dilution from a chl content of 30 down to 15 mg L^{-1}. Cultures were grown in 1 L columns as described in materials and methods. Cultures were resuspended in nitrogen deficient (BG11-N) medium and adjusted to different cell densities. Different cell densities grown under the same light intensity were investigated. Influence of initial cell densities of (1, 2 and 4 mg mL^{-1}) on chl content, fatty acid composition and AA production of *P. incisa* was studied.

3.1.1 Effect of cell density on chlorophyll content of the cultures

As shown in Figure 4, the chlorophyll content of culture grown at high initial cell density continued to increase while cultures grown at lower initial cell density decreased. At

initial cell density of 4 mgmL^{-1} (high density, HD), chlorophyll content has doubled from 90.4 mg L^{-1} to 203.3 mg L^{-1} at day 14, while the chlorophyll content of culture grown at initial cell density of 1 mgmL^{-1} (low density, LD) decreased with time from 22.6 mg L^{-1} to 15.7 mg L^{-1} at day 14. Bleaching of LD culture during nitrogen starvation was observed. In addition, culture grown at initial cell density of 2 mgmL^{-1} (medium density, MD), the chlorophyll content initially increased and started to decrease after 10 d, from 65.4 mg L^{-1} at day 10 to 51.4 mg L^{-1} at day 14. Chlorophyll synthesis appears to continue in cultures grown at HD, despite the nutrient medium being deprived of nitrogen.

In contrast, the dry weights of cultures have continued to increase irrespective of the initial cell density. For example culture grown at HD, MD or LD had increased from 4 to 14.9 mg mL^{-1}, 2 to 8.4 mg mL^{-1} or 1 to 6.1 mg mL^{-1} at day 14, respectively (Fig. 5)

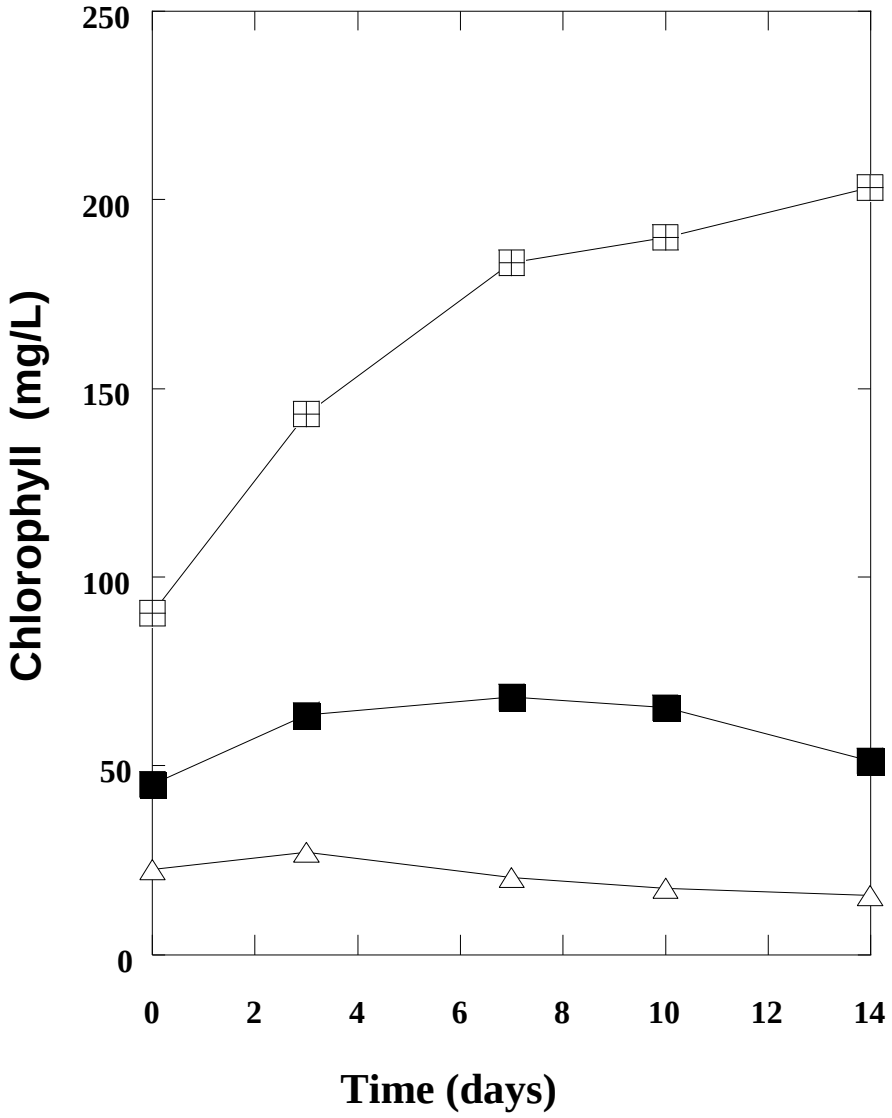

Figure 4. Chlorophyll content of *P. incisa* grown at different cell densities, LD (△), MD (■) and HD (⊞) at 25ºC under light intensity of 170 μmol quanta m^{-2} s^{-1}

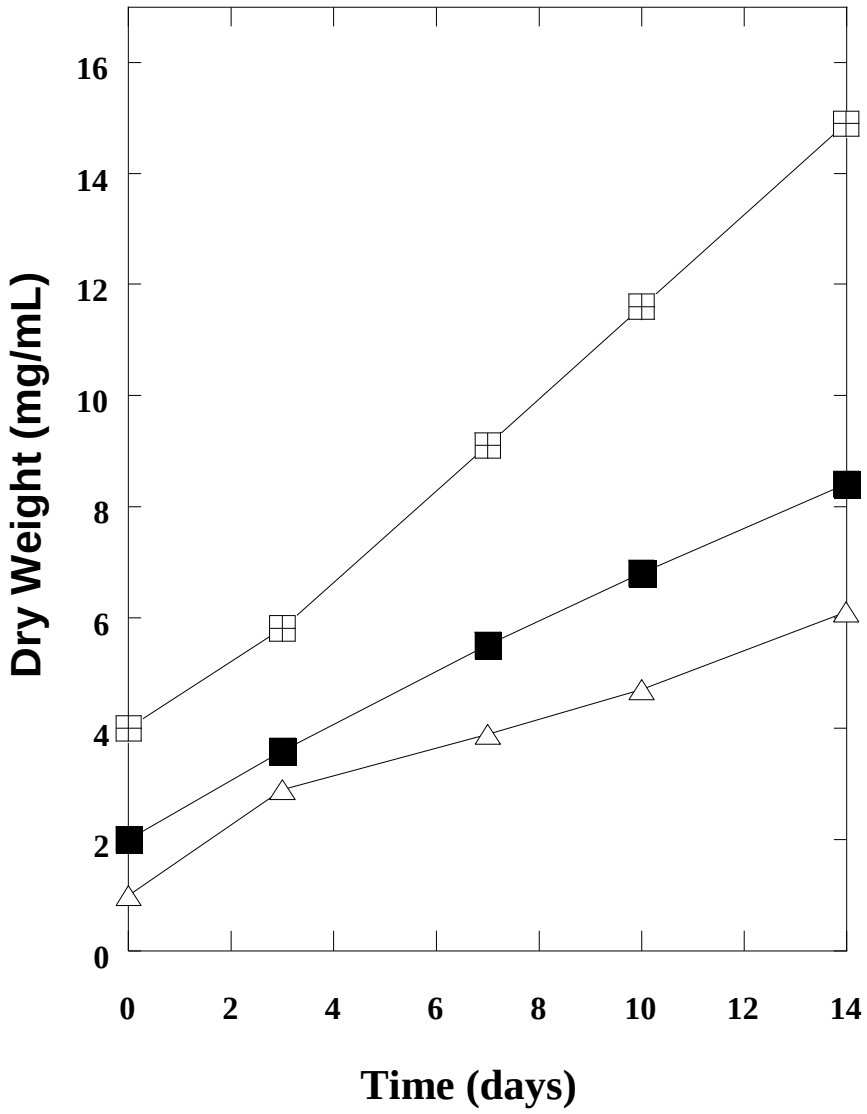

Figure 5. Dry weight content of *P. incisa* grown at different cell densities, LD (∆), MD (■) and HD (⊞) at 25 ºC under light intensity of 170 μmol quanta m^{-2} s^{-1}

3.1.2 Effect of cell density on AA productivity

The effect of cell density on the proportion of AA (% total fatty acids), total fatty acids (TFA, % dry weight) and AA content (% dry weight) of cultures grown under HD, MD or LD is shown in Figure 6. AA accumulation was only slightly affected by the initial cell

density. At LD, the proportion of AA reached 50.2%, while culture grown at MD resulted into higher proportion of AA, 53.9%. Elevation of initial cell density to HD resulted in a proportion of only 51.6% AA in TFA. The AA content increased in all cultures but the culture grown at MD had the highest AA content at day 7 until the end of cultivation period. On day 14, LD, MD and HD cultures attained AA content of 12.2%, 13.0% and 10.0%, respectively. As shown in Table 1, TFA content increased in all cultures, however LD attained the highest TFA content throughout the cultivation period. Furthermore, the proportions of fatty acids were different in all cultures. For example, the HD culture had the highest proportion of 16:1ω11, 16:2ω6 18:2ω6 and 18:3ω3 during the cultivation period, whereas the LD culture had higher proportion of 18:1ω9 amounting to 12.9% on day 14 compared to 9.5% and 7.3% in MD and HD, respectively.

As shown in Figure 6, the initial cell density had an influence on the volumetric content of AA. The highest content occurred in culture grown at HD, reaching 1.48 mg mL^{-1} at day 14. Cultures grown at LD and MD achieved relatively lower volumetric AA content of 0.74 mg mL^{-1} and 1.08 mg mL^{-1} at day 14, respectively. Clearly, the volumetric AA content increases with increasing cell density.

Table 1. Fatty acid composition of cultures of *P. incisa* grown at different cell densities under N-starvation

Cell density	Time (Days)	TFA* □□□ (% of dw)	Fatty acid composition (% of fatty acids)												
			16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
LD	3	15.6	9.9	1.2	1.0	0.8	2.3	13.3	3.2	10.9	1.4	1.9	1.0	38.1	0.7
MD	3	14.7	10.2	2.3	1.9	1.0	2.3	8.0	2.7	14.5	1.5	2.6	0.9	36.6	0.8
HD	3	12.1	11.8	3.4	2.5	1.4	1.7	6.3	2.6	16.2	1.1	3.8	0.6	30.0	1.1
LD	7	17.8	9.9	1.0	0.6	0.5	2.7	12.6	3.8	9.7	0.9	1.1	1.0	43.1	0.6
MD	7	17.4	9.6	1.4	1.1	0.5	2.4	8.6	3.4	11.1	1.0	1.2	1.0	45.9	0.6
HD	7	15.5	8.8	2.6	1.5	0.6	2.4	7.5	2.9	13.0	0.9	1.8	0.8	42.7	0.8
LD	10	23.1	9.1	0.7	0.4	0.4	2.7	13.0	3.7	9.2	0.9	0.7	1.1	47.7	0.6
MD	10	22.3	8.6	1.0	0.8	0.4	2.4	9.3	3.2	10.6	0.9	0.9	1.2	50.4	0.5
HD	10	18.1	8.7	1.9	1.3	0.5	2.5	7.1	3.0	13.0	0.5	1.3	0.8	46.7	0.7
LD	14	24.3	8.6	0.5	0.3	0.3	2.5	12.9	4.1	8.4	0.7	0.7	1.1	50.2	0.6
MD	14	24.1	8.0	0.6	0.5	0.3	2.4	9.5	3.3	9.2	0.7	0.7	1.2	53.9	0.4
HD	14	19.3	6.6	1.3	1.0	0.3	2.4	7.3	3.1	11.7	0.8	0.9	1.0	51.6	0.6

LD - Low Density, MD - Medium density, HD - High Density. TFA*- Total Fatty Acids (% of dry weight). 16:1ω9, 16:1ω7, 20:0, 20:1, 20:2ω6 and 22:0 were present at less than 0.5%. The data shown represent mean values with a range of less then 5% for major peaks (over 10% fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

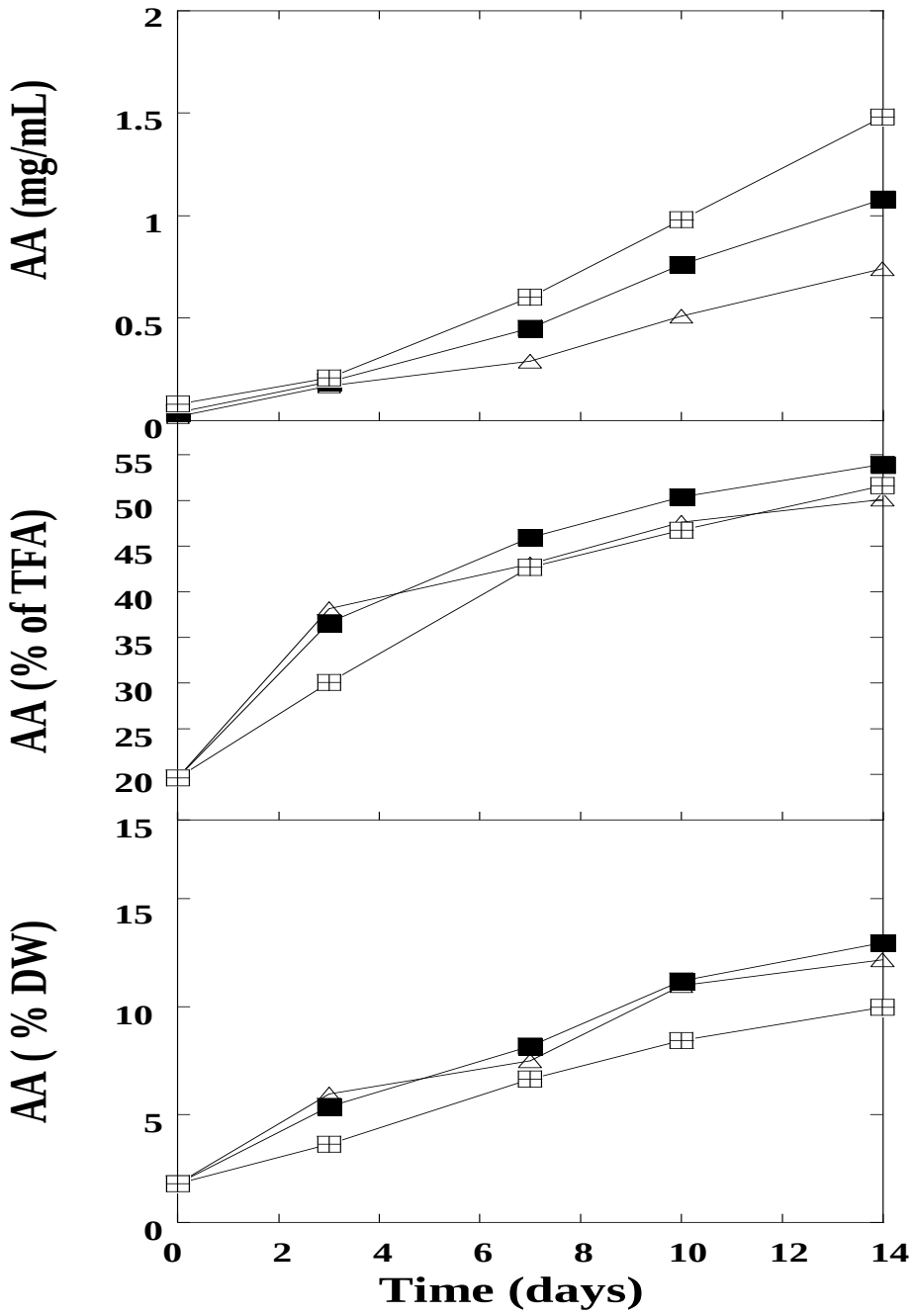

Figure 6. AA content (% of dry weight), AA in total fatty acids and volumetric AA of cultures of *P. incisa* grown at different cell density of LD (Δ), MD (■) and HD (⊞)

3.2 Growth stages and nitrogen starvation:

In order to study in which phase of growth, the cells of *P. incisa* accumulate more AA in a shorter time when transferred to N-free medium, cultures were grown on complete medium to reach different growth phases. Cells were withdrawn at different growth stages, resuspended in N- free medium at a cell concentration of 4 mg mL^{-1} (high-density, HD) for each case. The combined effect of initial culture age and nitrogen starvation on the chlorophyll and biomass content, fatty acid composition and AA production was investigated.

3.2.1 Growth of *P. incisa* on complete BG11 medium

In order to keep the cells of *P. incisa* in exponential (EXP) phase, cultures were daily diluted with fresh BG11 medium to chlorophyll content of 15 mg mL^{-1}. When these cultures were batch cultivated, exponential growth continued for about 3d and reached a transition (TRANS) phase. The cultures continued to be batch cultivated and reached early stationary (E.STA) phase in 7d. When these cultures were batch cultivated for another 3d, middle stationary (M.STA) phase was reached (Fig. 7). Preliminary studies carried out in our laboratory on the growth of *P. incisa* showed that cultures have long stationary phase, which remained for more than 10 days, we thus referred to the growth stage of cultures in stationary phase for 3d as middle stationary phase.

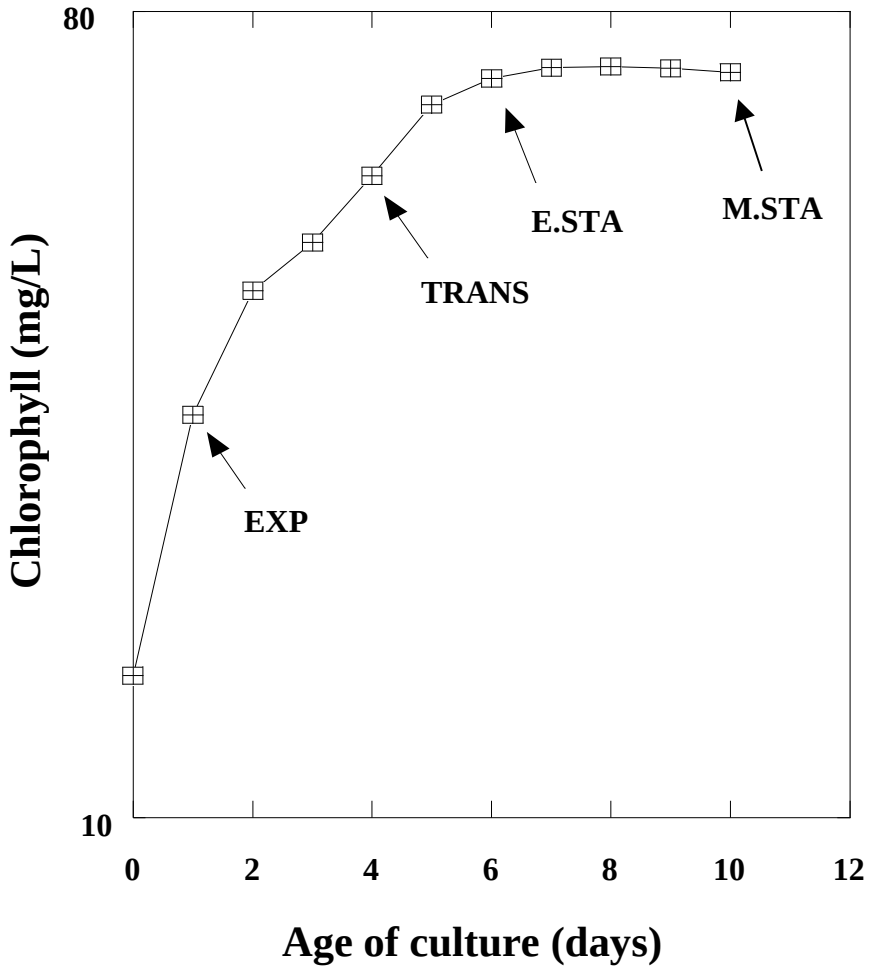

Figure 7. Growth curve of *P. incisa* showing four different growth phases in which samples for the study (growth phases and nitrogen starvation) are withdrawn

3.2.2 Chlorophyll content and biomass accumulation in *P. incisa* cultures transferred to N-starvation at different stages of growth

As shown in Fig. 8, the growth stage at which cultures were transferred to N-free medium had a pronounced effect on chlorophyll synthesis of *P. incisa*. The rate of chlorophyll synthesis was faster in cultures transferred in EXP and TRANS phases. Their chlorophyll

content has doubled from 94.4 to 214.6 mg L^{-1} and from 89.2 to 185.1 mg L^{-1}, respectively at day 14. Cultures transferred to the E. STA phase had a slight increase in chlorophyll content, from 85.1 to 114.3 mg L^{-1}. However, the chlorophyll content of culture transferred in M.STA phase decreased from 84.0 to 58.6 mg L^{-1} at day 14.

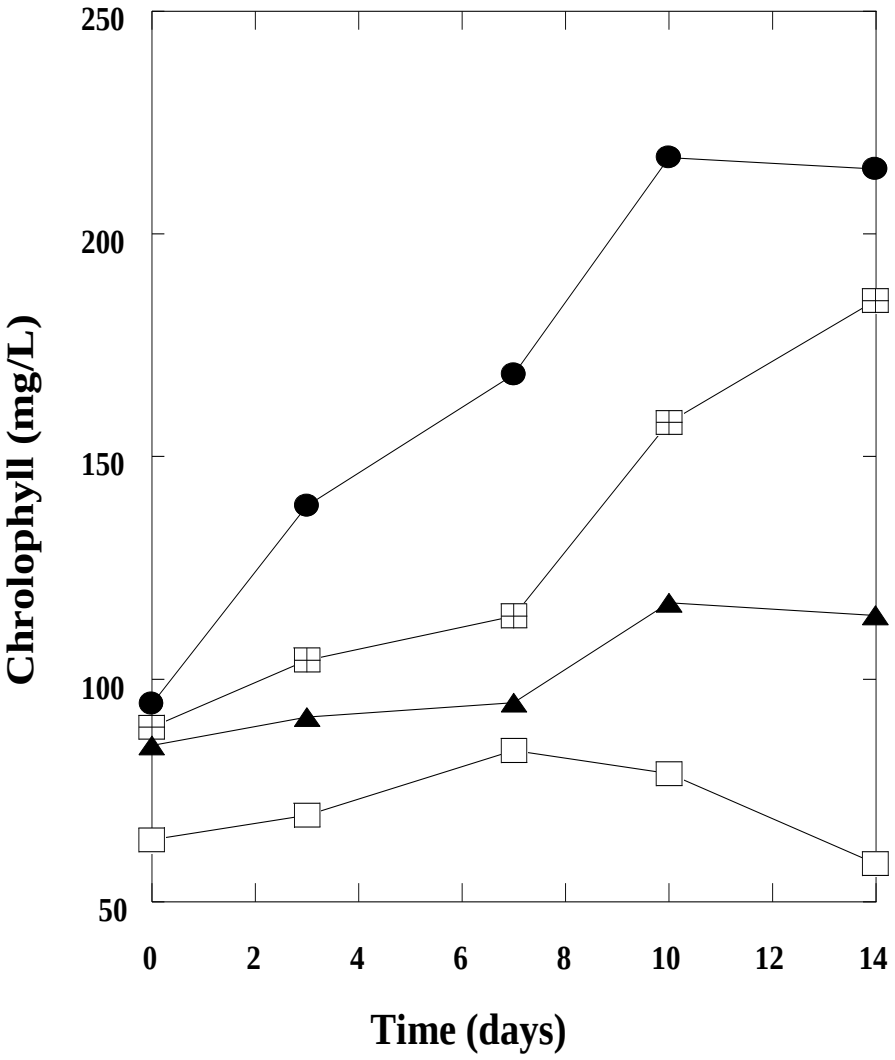

Figure 8. Effect of growth stages [EXP (●), TRANS (⊞), E.STA (▲) and M.STA (□)] on the chlorophyll synthesis of *P. incisa* cultures grown with HD under nitrogen starvation.

Similarly, the biomass accumulation was rapid in cultures transferred in the EXP and TRANS phases attaining biomass content of 16.6 and 16.7mg mL^{-1}, respectively on day 14 compared to 14.3 and only 10.3 mg mL^{-1} attained in cultures of E.STA and M.STA phases, respectively, on the same day (Fig. 9).

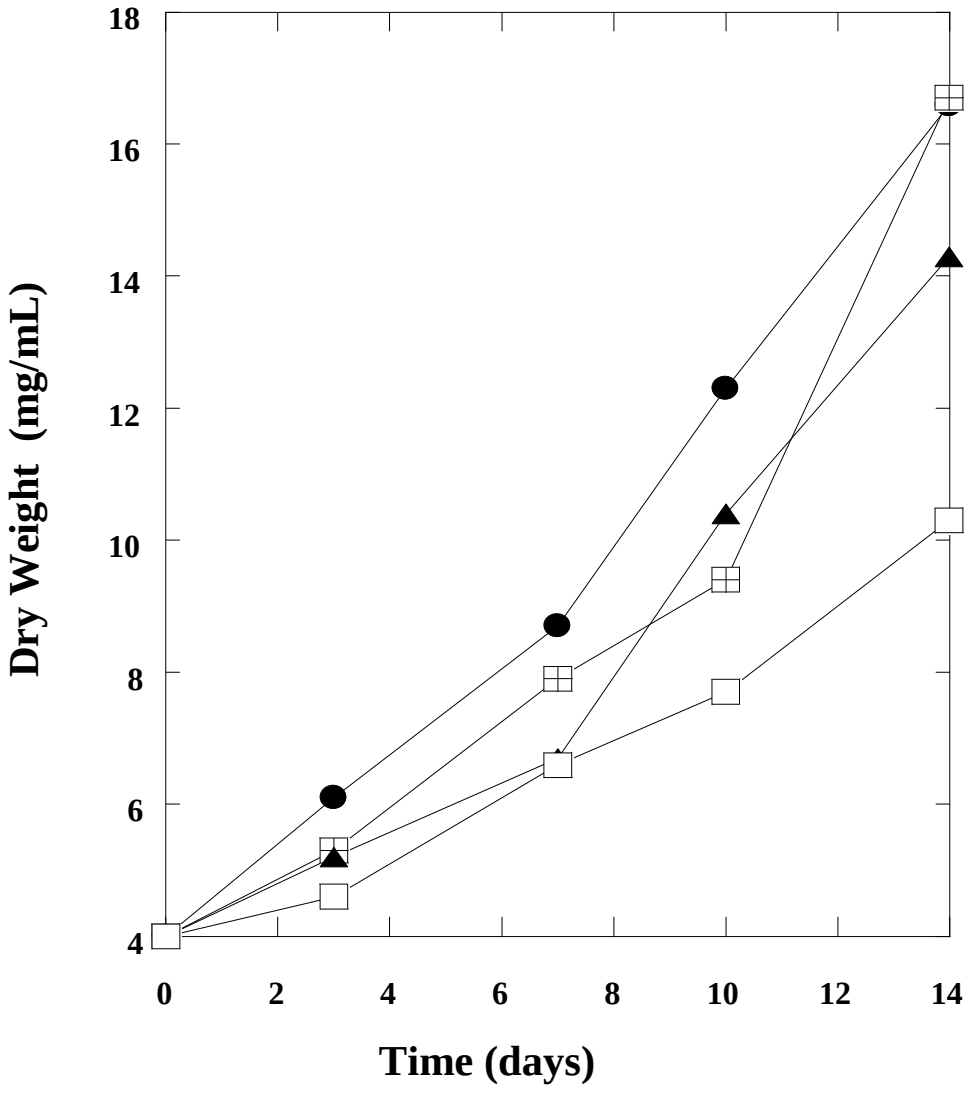

Figure 9. Dry weight content of *P. incisa* cultures transferred to N- starvation at different growth phases [EXP (●), TRANS (⊞), E.STA (▲) and M.STA (□)]

3.2.3 Effect of growth stages on AA accumulation in the cultures of *P. incisa* during nitrogen starvation

The growth stage at which cultures were transferred to N-free medium had a significant influence on the proportion of AA in TFA, as well as on the AA and TFA content of the biomass (Table 2, Fig. 10). The proportion of AA in total fatty acid increased faster in cultures transferred to N- starvation in the EXP and TRANS phases of growth. The proportion of AA almost doubled from 25.8% to 47.5% and 25.8% to 49.8% of TFA, respectively at day 14 (Fig., Table 2). It is evident that even young cells demonstrate a potential towards increasing the AA proportion of total fatty acid under N-starvation (Fig. 10). Cultures transferred in E.STA and M.STA phases of growth, had higher initial proportion of AA, amounting to 34.9% and 36.3%, respectively at day 0. Their proportion of AA had increased slowly throughout the cultivation period, reaching 47.3% and 47.0% at day 14.

The AA content increased in all cultures. The AA content reached to 12.1%, 14.2%, 17.4 % and 18.7% in culture transferred in the EXP, TRANS, E.STA and M.STA phases, respectively (Fig. 10). Total fatty acid content showed the same pattern (Table 2).
As shown in Figure 10, the volumetric AA was higher in cultures of TRANS and E.STA phases reaching 2.38 and 2.49 mg mL^{-1}, respectively at day 14 comparing to 2.01 and 1.92 mg mL^{-1} obtained from cultures of EXP and M.STA, respectively, This shows that average cell age had an impact on volumetric AA content which decreases with the aging of initial culture. It is also worth noting that the M.STA culture had the highest AA content, but lowest volumetric AA content.

Growth stage had an influence on proportions and ratios of C16 and C18 fatty acids as well. The proportions of 16:2ω6, 16:3ω3 and 18:3ω3, which are components of chloroplastic lipids were higher in cultures transferred to N-free medium in the EXP and TRANS phases, whereas that of 18:1ω9 was lower (Table 2). On the other hand, culture transferred in the E.STA phase reached a plateau on day 10, when the proportion of 18:1ω9 started to increase and 16:2ω6, 16:3ω3 and 18:3ω3 decreased. The same pattern was observed in culture transferred during the M.STA phase. This pattern is associated with the decrease of chloroplastic lipids and an increase of 18:1ω9 in triacylglycerols, which accompanies the aging of the cells. The observed trends were significantly lower proportions of 16:0 and 18:1ω9 and higher proportion of 18:2 the biomass of EXP and TRANS compared to biomass of E.STA and M.STA phases (Table 2). Therefore, selection of the initial growth stage of *P. incisa* cultures for transferring to N-starvation, can affect the final fatty acid composition of biomass and accordingly of the accumulated TAG.

Table 2. Effect of growth stages on the fatty acid composition of *P. incisa* cultures under N-starvation

			Fatty acid composition (% of total fatty acids)												
Growth Phase	Time (days)	TFA* (%of dw)	16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
EXP	0	11.5	12.5	2.1	2.1	2.5	1.4	6.5	3.6	15.0	1.5	6.4	0.7	25.8	1.0
TRANS	0	13.3	11.8	3.4	2.2	1.7	2.0	8.3	3.3	17.1	1.5	4.4	1.3	25.8	0.9
E.STA	0	20.7	10.0	0.6	1.8	1.1	1.8	12.2	3.7	16.2	1.3	2.6	1.8	34.9	0.9
M.STA	0	25.9	8.8	1.1	1.2	0.7	2.0	17.4	2.9	15.6	1.0	1.8	1.5	36.3	0.7
EXP	3	13.1	12.1	3.0	0.1	1.6	1.5	4.8	3.7	14.6	1.1	4.1	0.6	33.3	1.4
TRANS	3	17.3	10.6	1.6	1.6	1.0	2.3	8.7	4.1	14.9	1.4	2.6	1.0	36.1	0.9
E.STA	3	21.0	9.3	1.4	1.4	0.8	1.9	10.3	3.6	13.8	1.1	1.9	1.2	41.7	0.9
M.STA	3	25.4	8.3	0.9	0.9	0.6	1.8	13.6	4.0	12.9	1.0	1.6	1.3	43.1	0.8
EXP	7	19.0	10.2	1.7	1.4	0.8	2.1	5.3	4.1	13.0	1.1	2.0	0.9	44.7	1.0
TRANS	7	23.6	9.3	1.0	1.1	0.6	2.2	9.2	3.9	13.5	1.0	1.6	1.0	44.8	0.8
E.STA	7	26.1	8.3	0.7	1.0	0.5	2.2	10.3	4.1	12.6	1.0	1.2	1.2	47.4	0.7
M.STA	7	31.0	7.7	0.6	0.7	0.4	2.0	13.6	4.2	12.4	0.8	1.1	1.2	47.1	0.7
EXP	10	22.7	9.7	0.4	1.3	0.3	2.3	6.1	4.1	13.5	0.9	1.5	0.9	48.1	0.9
TRANS	10	25.2	8.8	0.9	0.9	0.5	2.2	8.7	4.4	12.7	0.9	1.2	0.9	48.2	0.7
E.STA	10	32.4	7.8	0.5	0.7	0.3	2.4	12.2	4.0	13.7	0.7	0.9	1.0	47.3	0.6
M.STA	10	36.4	7.5	0.4	0.5	0.3	2.0	14.9	4.3	12.1	0.8	0.9	1.3	47.5	0.6
EXP	14	25.5	8.8	0.9	1.0	0.4	2.5	7.6	4.0	14.3	0.8	1.1	0.8	47.5	0.7
TRANS	14	28.8	8.2	0.6	0.8	0.4	2.3	8.8	4.4	13.3	0.7	0.9	0.8	49.3	0.6
E.STA	14	36.7	7.5	0.4	0.5	0.3	2.4	13.8	4.2	13.3	0.8	0.8	1.2	47.3	0.5
M.STA	14	39.7	7.5	0.4	0.3	0.3	2.0	16.5	4.5	11.7	0.8	0.9	1.2	47.0	0.5

20:0, 20:1, 20:2ω6, 20:3ω6 and 22:0 were present at less than 0.5%. TFA*- Total Fatty Acids (% of dry weight). EXP- Exponential. TRANS-Transition. E.STA-Early stationary. M.STA-Middle stationary.

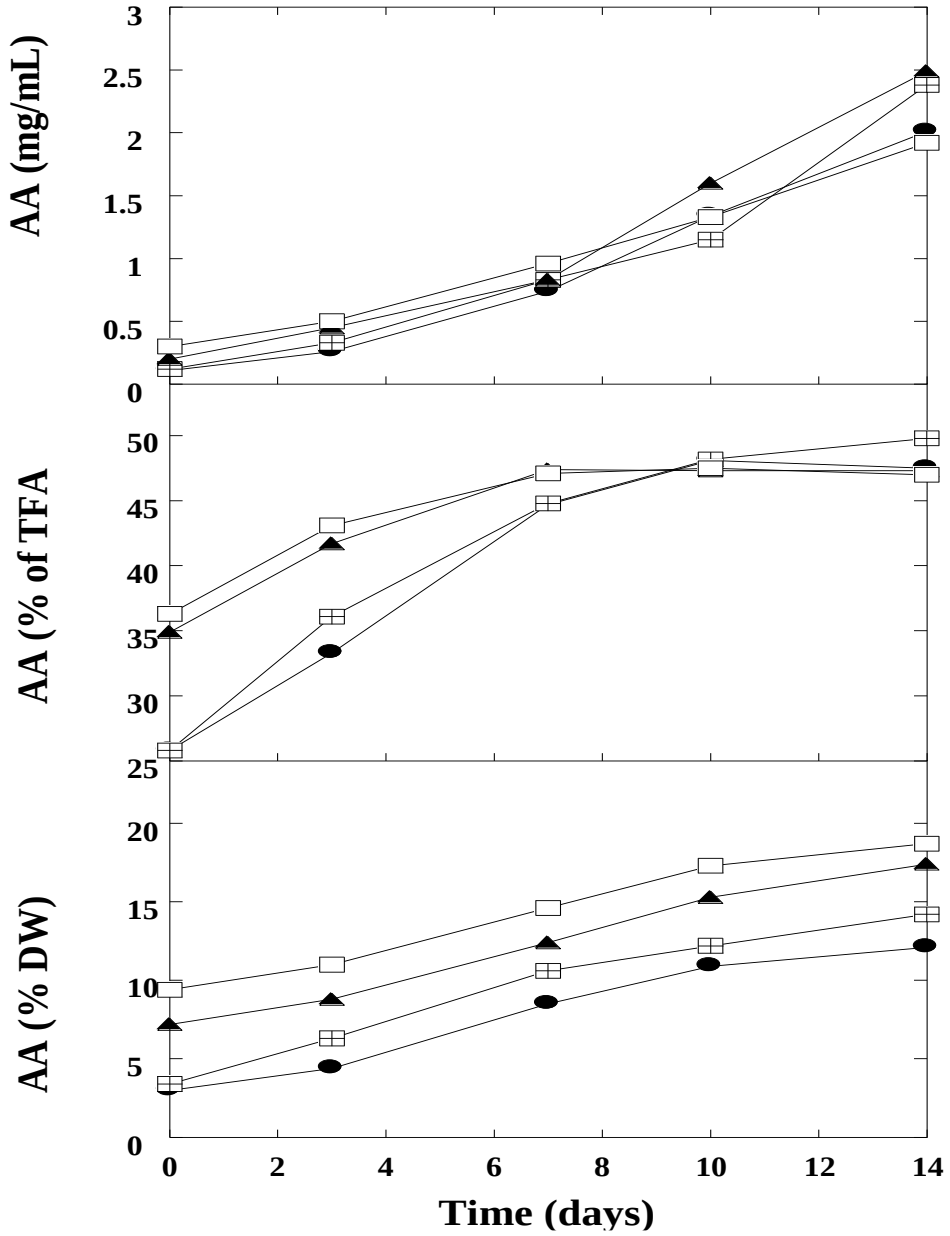

Figure 10. Effect of growth phases [EXP (●), TRANS (⊞), E.STA (▲) and M.STA (□)] on the volumetric AA content, proportion of AA of TFA and AA content (% of dry weight) of *P. incisa* cultures grown with HD under nitrogen starvation

3.3 Nutrient starvation in the oleogenic phase of cultivation

Nutrients deficiency, especially N-starvation, during the oleogenic phase of cultivation can determine the oil productivity of the microalga (Khozin-Goldberg *et al*., 2002). This part of the study focused on the possibility to substitute the starvation medium (BG11-N) with either tap water or brackish water during the starvation phase of cultivation, aimed at decreasing the cost of biomass production and to utilize the cheapest water sources. As shown above, cultures of *P. incisa* maintained in starvation medium (BG11-N) with initial cell density of 4 mg mL^{-1} (HD) are able to increase the chl content, indicating delay of chloroplast degradation and chl bleaching that occurred with LD and MD cultures (Fig. 4). Concurrently, these cultures produced biomass rich in AA and higher volumetric AA productivity under N-starvation. Preliminary studies carried out in our Lab have shown that *P. incisa* grown with initial cell density not less than 4 mg mL^{-1} (HD) is able to increase the chl content and produce biomass rich in AA even when transferred to tap water. Thus, HD cultures of *P. incisa* were maintained in either tap water or brackish water to induce starvation. The effects of using tap water or brackish water on the chl and volumetric biomass contents, fatty acid composition and AA production of *P. incisa* were studied.

3.3.1 Chlorophyll and volumetric biomass contents of *P. incisa* cultures maintained in different starvation media

Cultures of *P. incisa* exhibited an increase in chl content in either of the starvation media. As shown in Figs. 11-12, it was rather surprising, that cells of *P. incisa* can survive in tap water, that basically contain no nutrients for sustaining growth. It is therefore clear that

keeping cells of *P. incisa* under HD can allow the cells to increase biomass and even chl under nutrient-devoid medium. The same pattern of chl content was observed in all cultures, with the chl content increasing up to 7d and further decreasing. The volumetric biomass increased continuously in both cultures. Cultures of *P. incisa* maintained in tap water and brackish water attained the same biomass of 9.1 mg mL^{-1}, respectively at day 14 (Fig. 12).

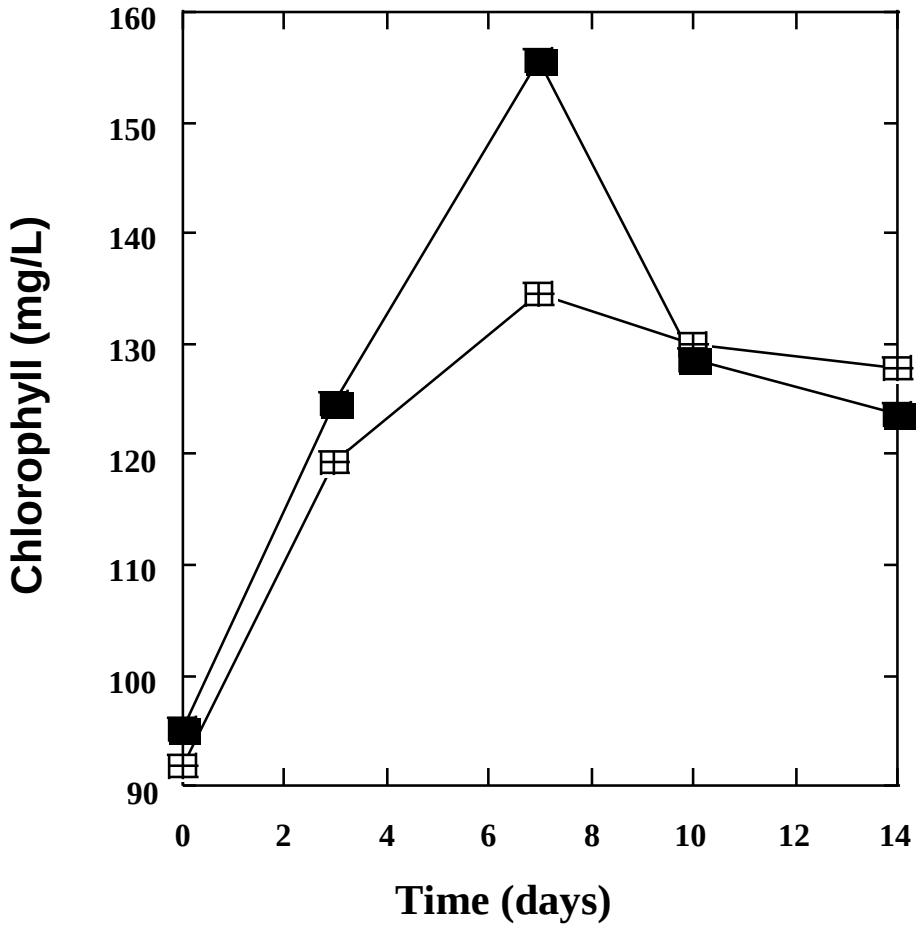

Figure 11. Chlorophyll content of *P. incisa* cultures maintained in tap water (■) and brackish water (⊞) at 25 ^{o}C under 170 μmol quanta m^{-2} s^{-1}.

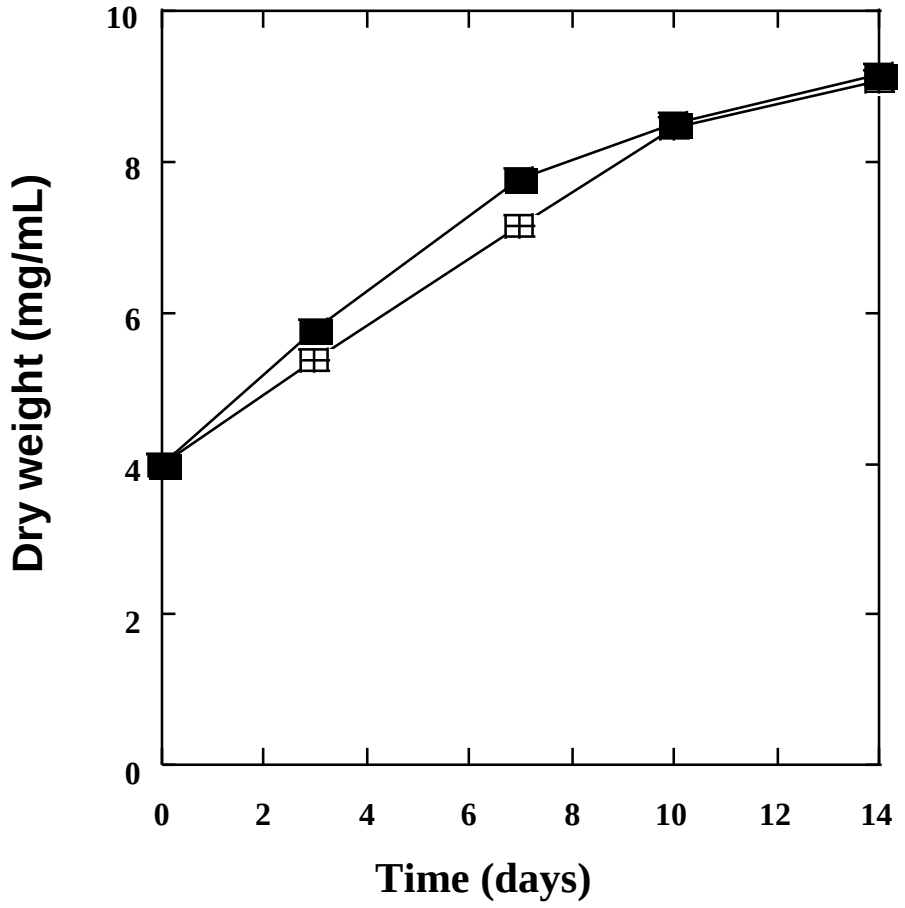

Figure 12. Dry weight content of *P. incisa* cultures maintained in tap water (■) and brackish water (⊞) at 25 ºC under 170 μmol quanta m^{-2} s^{-1}.

3.3.2 Effect of nutrients starvation media on AA production

No significant differences in the fatty acid composition of *P. incisa* biomass were observed as a result of maintaining cultures on TAP or BW. The observed trends were an increase in the proportions 18:1ω9 and 20:4ω6 and a decrease in the proportions of 16:0, 16:1ω11, 16:2ω6, 16:3ω3, 18:3ω3 in biomass of both cultures The proportion of AA of cultures maintained in tap water and brackish water increased from 22.9 to 43.4 % and

22.1 to 44.8 %, respectively at day 14 (Table 3). The AA content continued to increase in both cultures almost at similar rate (Fig. 13). As shown in Fig. 14, the volumetric contents of AA and TFA increased in both cultures and doubled at day 10.

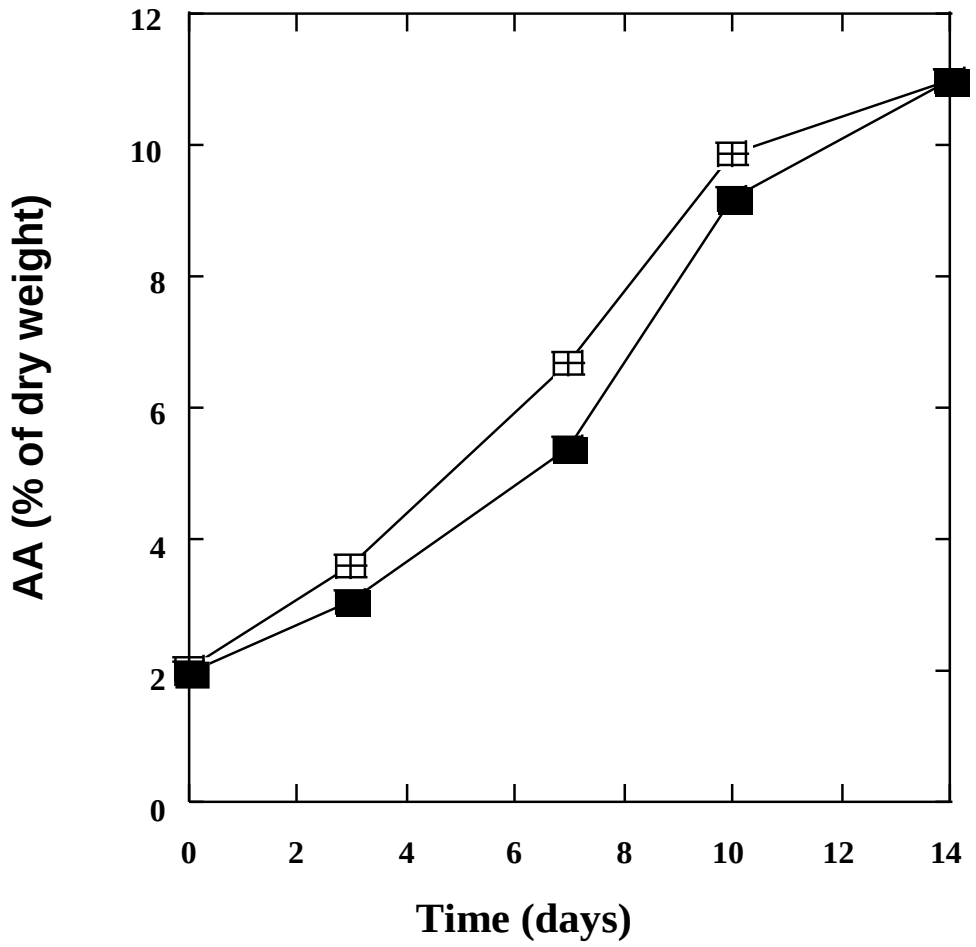

Figure 13. AA content (% of dry weight) of P. incisa cultures maintained in different starvation media [tap water (■) and brackish water (⊞)]

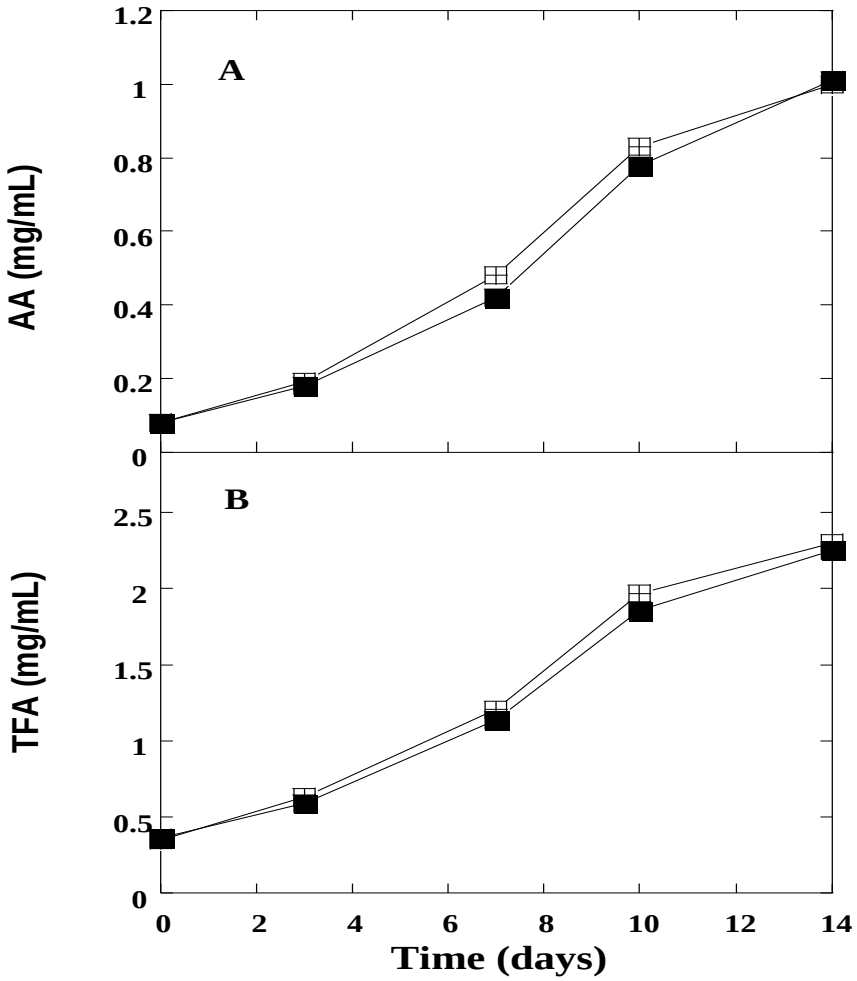

Figure 14. Volumetric AA (**A**) and TFA (**B**) contents of *P. incisa* cultures maintained in different starvation media [tap water (■) and brackish water (⊞)]

Table 3. Fatty acid composition of cultures of *P. incisa* maintained in tap water and brackish water

Starvation medium	Time (days)	TFA* (% of dw)	Fatty acid composition (% of fatty acids)											
			16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:4 ω6	20:5 ω3
TW	0	8.9	17.6	6.6	4.0	3.5	1.1	4.4	5.0	22.7	0.9	8.7	22.9	1.2
BW	0	8.9	18.3	6.9	4.1	4.1	1.3	4.1	4.6	21.6	1.1	9.5	22.0	1.1
TW	3	11.8	16.4	4.4	2.7	1.7	2.3	6.8	5.7	20.9	1.2	4.6	30.7	1.1
BW	3	10.3	17.6	4.9	3.0	2.0	2.0	5.9	5.7	20.1	1.2	5.3	29.9	1.3
TW	7	16.9	12.5	2.4	1.7	0.9	3.3	8.6	5.9	19.9	1.2	2.4	39.5	0.3
BW	7	14.7	14.0	2.7	2.0	1.0	3.2	7.9	6.6	20.5	1.2	2.6	36.7	0.5
TW	10	23.3	10.7	1.3	1.2	0.5	3.7	10.4	5.6	19.4	1.1	1.6	42.3	0.4
BW	10	21.9	11.0	1.0	1.3	0.5	4.1	11.2	4.7	18.9	1.2	1.5	42.0	0.8
TW	14	25.3	10.4	0.9	1.1	0.5	3.6	11.2	5.4	19.2	1.0	1.5	43.4	0.4
BW	14	24.6	10.4	1.0	1.1	0.4	4.1	11.0	4.6	17.5	1.1	1.3	44.8	0.6

TW- Tap water; BW- Brackish water; TFA*- Total Fatty Acids (% of dry weight). The fatty acids: 20:0, 20:1, 20:2ω6, 20:3ω6 and 22:0 were present at less than 1.0%.

3.4 Feeding with oleic and linoleic acid

Oleic acid and linoleic acid are precursors in the biosynthesis of arachidonic acid in *P. incisa* (Bigogno *et al*., 2002). These fatty acids were incorporated into the culture medium of *P. incisa* aiming to increase the production of AA. This approach appeared to be successful with enhancing the production of the PUFAs, AA and eicosapentaenoic acid (EPA) in cultures of *Euglena gracilis* (Okumura *et al.*, 1986). The effects of supplementing cultures on complete BG11 and N-free BG11 media with fatty acid precursors, on the synthesis of AA, were studied. This is important because cultures grown on complete or N-free medium differs in fatty acids composition and content, and reveal different potentials for accumulation of AA-rich TAG. Preliminary experiments were conducted to determine effective concentrations of fatty acid precursors, since extensive supplementation of precursor resulted either in the inhibition of AA production or incorporation into TAG rather than being further desaturated and elongated. Under N-starvation, when TAG synthesis is enhanced, these fatty acid precursors have to be added gradually in order to avoid direct acylation of TAG with precursors. However, cultures grown on complete medium (BG11) were supplemented with fatty acids precursors in two modes: 0.1 mg mL^{-1} of either fatty acid was added at once at time-0 or added gradually as follows from Table 4.

Table 4. Supplementation of cultures of *P. incisa* with fatty acid precursors

	Fatty acid added (µg mL^{-1})	
Days	**BG11**	**BG11-N**
0	16.6	33.0
4	62.5	125.0
9	187.5	375.0

The effect of oleic and linoleic acid on the chlorophyll and biomass volumetric contents, fatty acid composition and AA production were studied.

3.4.1 Influence of oleic and linoleic acid on chl and biomass content of *P. incisa* culture

As shown in Fig. 15, cultures grown on complete BG11 and supplemented with either fatty acid demonstrated lower chlorophyll content, showing growth inhibition. The only exception was the culture supplemented with oleic acid at once that initially had lower chlorophyll content but later recovered and attained higher chlorophyll content than the control on day 14.

The chlorophyll content of cultures cultivated on BG11-N and fed with fatty acids, changed in a pattern similar to that of he control (Fig. 16), however the values were higher. On the other hand, there were only slight differences in the volumetric biomass accumulation of control and supplemented cultures. The control (BG11) culture produced more biomass (9.15 mg mL^{-1} on day 14) compared to supplemented cultures (Fig. 17). As shown in Fig. 18, the gradual addition of fatty acids did not affect biomass accumulation

up to 7 d. However, at day 14 the effect of supplementation was evident, both supplemented cultures produced higher biomass. The culture supplemented with oleic acid had the highest biomass concentration of 6.76 mg mL^{-1} at day 14, while the control (BG11-N) and culture supplemented with linoleic acid attained 5.23 and 6.50 mg mL^{-1}, respectively.

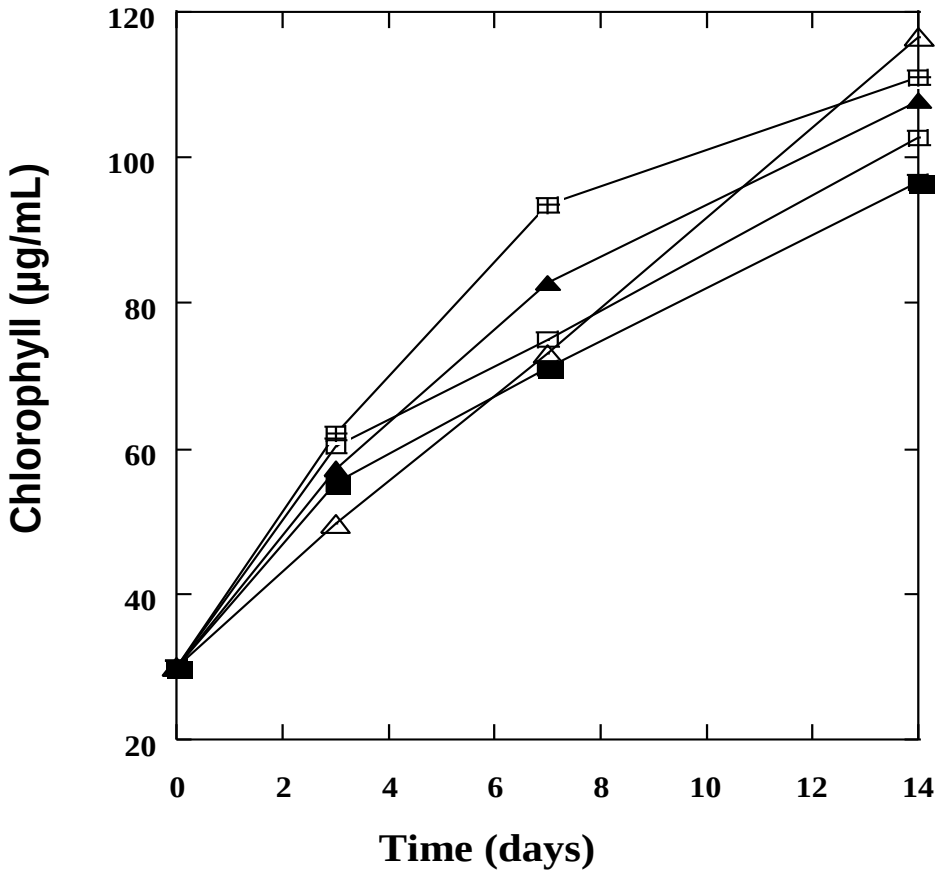

Figure 15. Chlorophyll content of *P. incisa* cultures grown on BG11 medium and supplemented with either of the fatty acids [control (⊞), oleic acid added gradually (■), oleic acid added at once (Δ), linoleic added gradually (□), linoleic acid added at once (▲)]

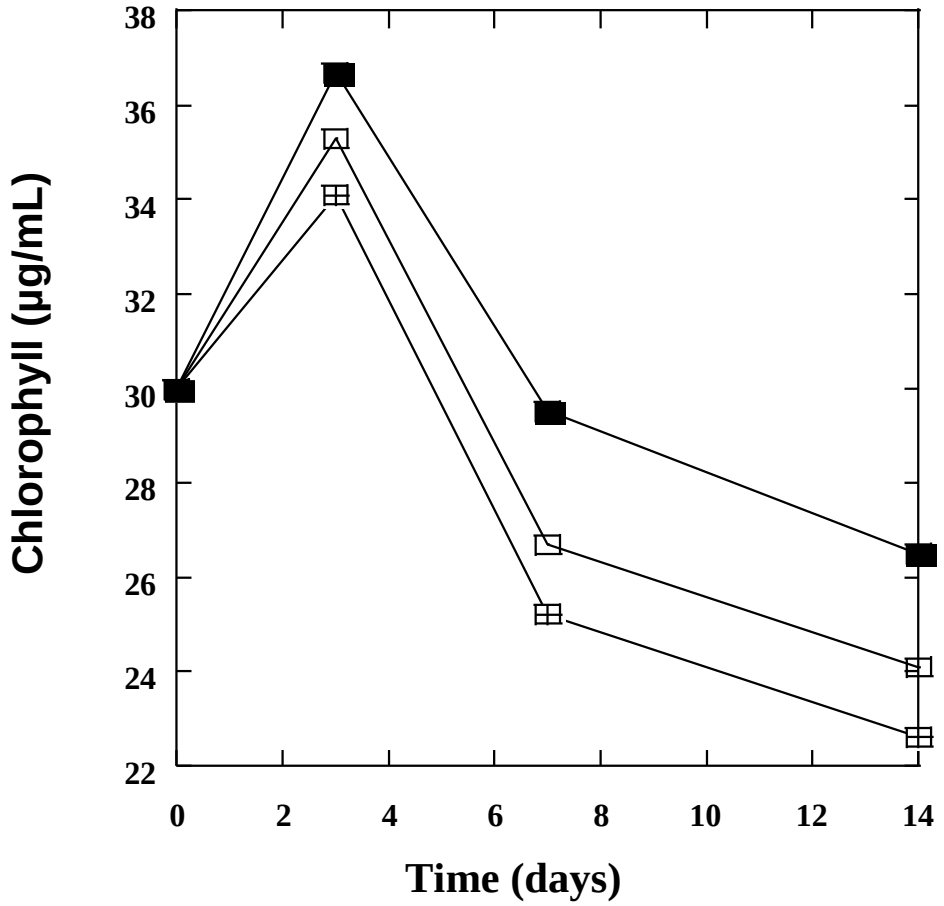

Figure 16. Chlorophyll content of *P. incisa* cultures maintained on N-free medium (BG11-N) and supplemented with either of the fatty acids [control (⊞), oleic acid added gradually (■) and linoleic added gradually (□)]

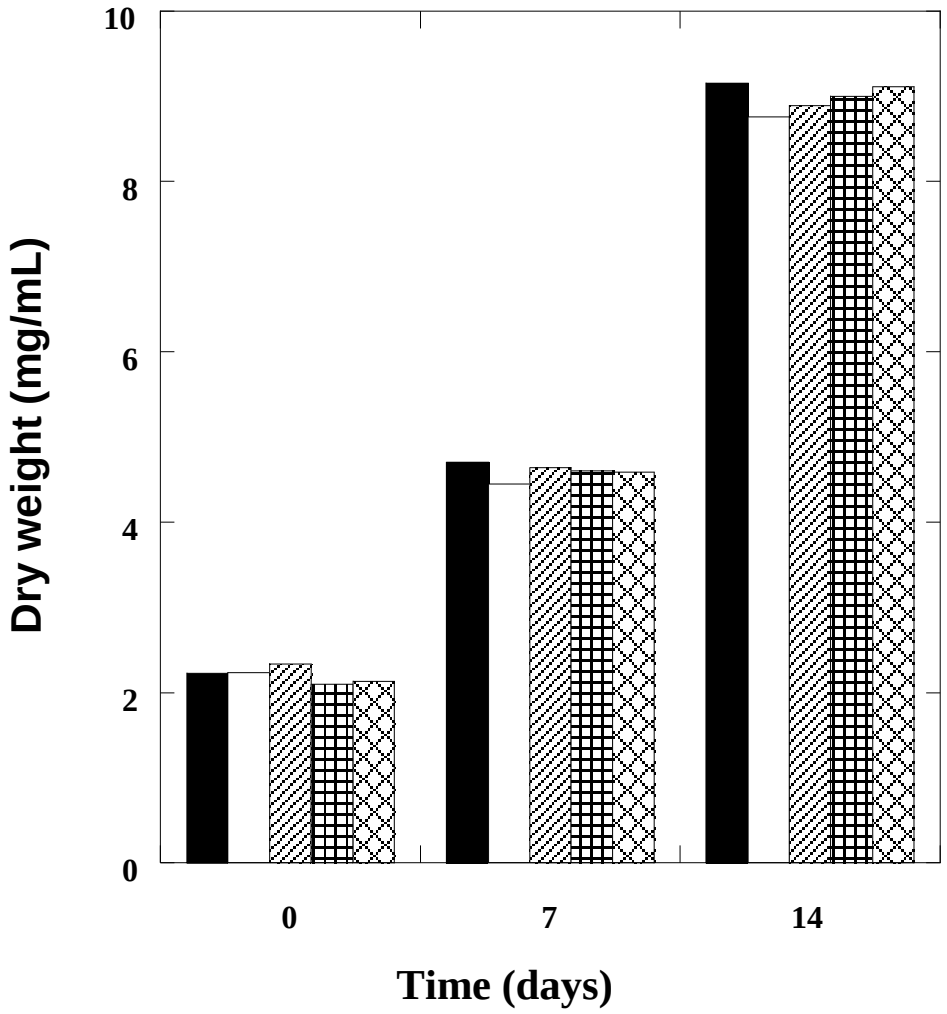

Figure 17. Dry weight content of *P. incisa* cultures supplemented with either of the fatty acids [control (■), oleic acid at once (□), oleic acid gradually (▨), linoleic acid at once (▦) and linoleic acid gradually (▩)] grown on complete nutrient medium (BG11)

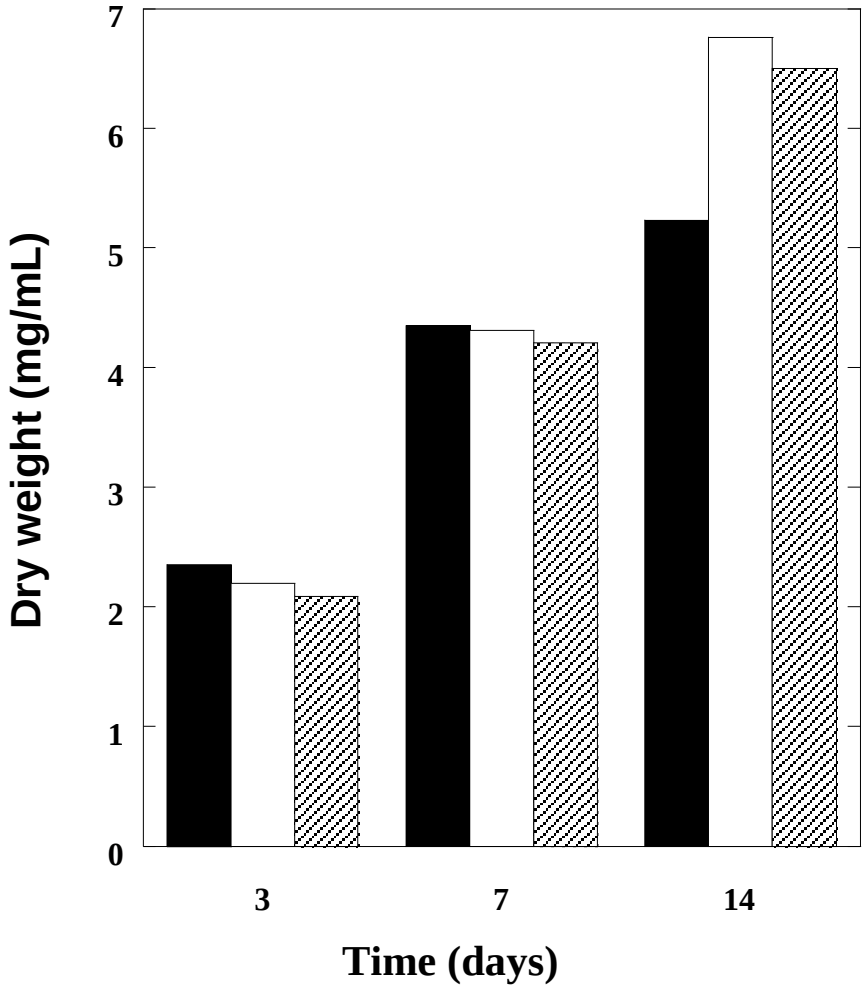

Figure18. Dry weight content of *P. incisa* cultures maintained on N-starvation medium (BG11-N) and supplemented with either of the fatty acids [control (■), oleic acid added gradually (□) and linoleic acid added gradually (▨)]

3.4.2. Effects of feeding with oleic acid on fatty acid composition and content of *P. incisa* cultures

During the time-course, cultures grown on complete BG11 medium and supplemented with oleic acid had a slight decrease in the proportion of C16 PUFA compared to the control, showing the increased contribution of C18 fatty acids (Table 5). On day 3, the culture supplemented with oleic acid added at once (batch cultivation), had a higher total fatty acid content of 10.0% compared to 7.9% and 8.8% in the control and culture supplemented gradually with oleic acid (Table 5). The proportions of 18:1 and 18:2 were also significantly higher when culture cultivated in batch, amounting to 12.6% and 23.8%, respectively, but had only slightly lower proportion of AA amounting to 28.6% in comparison with 31.5% in the control, suggesting that 18:1 was incorporated, to18: 2 and further converted to AA (Fig. 20). Moreover, the proportion of AA in the control and the gradually supplemented culture were 31.5% and 33.4 %, respectively, similarly indicating the sequential conversion of 18:1 to AA. On day 14, however, the culture gradually supplemented had a higher proportion of oleic acid and total fatty acid content amounting to 18.4% and 17.6%, respectively, and a slightly lower proportion of AA (from 34.2% at day 7 to 32.9%). The treated cultures had higher AA biomass content at day 3 compared to control cultures, but attained lower biomass AA content at day 7 and day 14 (Fig. 22A).

On day 3, the volumetric AA content was slightly higher in both gradual supplemented and batch cultivated cultures compared to the control. However, at day 14, the batch culture attained the lowest volumetric AA (Fig. 19A)

The culture maintained on BG11-N and supplemented gradually with oleic acid had a slightly higher proportion of oleic acid (13.1%) compared to the control (10.1%) at day 7 (Fig. 23). On day 14, the total fatty acid and AA contents were higher in the control compared to supplemented culture, and there were no differences in their proportion of AA (Fig. 23, 24 and Table 6). The volumetric AA content was slightly higher in the control at days 3 and 7, except at day 14, when supplemented culture slightly surpassed the control (Fig. 19B).

3.4.3. Effects of feeding with linoleic acid on the fatty acid composition and content in cultures of *P. incisa*

Cultures supplemented with linoleic acid on BG11 medium had a decrease in 16:0 and C16 PUFA and an increase in shares of C18 PUFAs compared to the control on day 3 (Table 5). As shown in Table 5, on day 3 the batch culture had a higher total fatty acid content as well as a substantially higher proportion of linoleic acid (33.9% vs. 18.6% in control) but insignificantly lower proportion of AA (29.6% vs. 31.5% in control), suggesting an incomplete conversion of 18:2 to AA (Fig. 21). On days 3 and 7, gradual supplemented cultures showed an increase in the proportions of linoleic acid, 18:3ω6 and AA, compared to the control and the batch culture (Fig. 21). At day 14, there was a sharp decrease in the proportion of linoleic acid in both supplemented cultures, but the proportion of oleic acid increased sharply in culture gradually supplemented with linoleic acid. The proportion of AA on day 14 remains the same as that of 7 d. When supplemented with linoleic acid added at once, higher increase in the proportion of AA was observed in culture on day 14. The control culture had lower AA biomass content at

day 3 compared to supplemented cultures, but it attained the highest AA content at day 7 and day 14 (Fig. 22B). On day 3, the volumetric AA content was slightly higher in both supplemented cultures in which linoleic acid was either added gradually (66 g mL^{-1}) or added at once 59 g mL^{-1} compared to 56 g mL^{-1} in the control. However, at day 14, control attained slightly higher volumetric AA (Fig. 19A)

Cultures maintained on N-free medium and supplemented with linoleic acid had a higher proportion of linoleic acid compared to the control on 7 d (Fig. 23). On day 14, supplemented culture, had an increase in the proportion of oleic acid and a decrease in the proportion of linoleic acid and AA. In both the control and supplemented culture, there was a decrease in 18:3ω6 with the control decreasing from 1.3% to 0.8%, while in the supplemented culture, the proportion decreased from 1.4% to 1.2% (Fig. 23). Control culture attained highest contents of AA and total fatty acids amounting to 19.1%and 36.2%, respectively at day 14 compared to 10.7% and 23.6% attained in supplemented cultures on the same day (Fig. 24 and Table 6). The volumetric AA content was slightly higher in the control throughout the cultivation period (Fig. 19B).

Table 5. Effect of oleic and linoleic acid feeding on the fatty acid composition, TFA and AA biomass contents of *P. incisa* cultures grown on complete nutrient medium (BG11)

Medium	Time (days)	TFA* (% dw)	AA (% dw)	Fatty acid composition (% of total fatty acids)											
				16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
BG11	3	7.9	2.5	15.8	5.6	2.6	2.5	0.6	3.3	6.8	18.6	0.7	9.1	0.3	31.5	2.1
BG11+ 18:1 batch	3	10.0	2.9	10.6	5.1	1.3	1.7	0.4	12.6	4.9	23.8	0.7	7.4	0.3	28.6	1.7
BG11+ 18:1 gradual	3	8.8	2.9	13.6	4.4	2.0	2.0	0.7	4.9	6.2	19.9	0.7	8.7	0.4	33.4	2.1
BG11+ 18:2 batch	3	9.4	2.8	9.7	3.5	1.1	1.6	0.3	3.5	4.2	33.9	0.9	8.6	0.4	29.6	1.5
BG11+ 18:2 gradual	3	8.9	3.1	13.5	4.6	2.0	2.1	0.5	2.9	6.4	19.9	0.8	8.9	0.4	35.0	2.3
BG11	7	10.2	3.7	14.1	5.0	2.8	1.6	0.8	4.2	6.9	17.7	1.1	5.8	0.7	36.5	2.3
BG11+ 18:1 batch	7	9.6	3.1	11.7	6.1	2.0	1.3	0.5	6.0	5.1	23.4	0.8	6.4	0.4	32.7	1.8
BG11+ 18:1 gradual	7	10.5	3.6	12.3	3.8	2.5	1.3	0.6	8.6	5.5	21.0	1.1	5.7	0.6	34.2	1.9
BG11+ 18:2 batch	7	9.9	3.1	12.3	3.5	2.7	1.1	0.5	5.7	4.3	27.7	0.9	6.6	0.5	31.4	1.7
BG11+ 18:2 gradual	7	10.7	4.0	12.3	4.4	2.4	1.4	0.5	3.4	5.7	22.0	1.4	6.0	0.6	37.1	2.1
BG11	14	13.7	5.7	12.4	1.6	1.5	1.3	1.1	9.1	6.4	17.0	0.8	3.7	1.0	41.7	1.6
BG11+ 18:1 batch	14	12.2	4.5	12.0	3.3	1.7	1.0	0.8	9.7	5.9	20.6	0.7	4.1	0.7	37.3	1.4
BG11+ 18:1 gradual	14	17.6	5.8	11.0	1.8	1.6	0.5	1.0	18.4	5.2	20.5	1.4	2.6	1.3	32.9	0.9
BG11+ 18:2 batch	14	13.7	5.5	10.7	2.6	1.2	1.1	0.7	7.3	5.7	22.7	0.8	4.2	0.7	40.2	1.5
BG11+ 18:2 gradual	14	14.9	5.5	10.6	2.1	1.3	0.8	0.8	15.9	5.1	19.6	0.8	3.1	0.8	37.1	1.1

20:0, 20:1, 20:2ω6, 20:3ω6 and 22:0 were present at less than 1.0%. TFA*- Total Fatty Acids (% of dry weight). 18:1- Oleic acid. 18:2- Linoleic acid

Table 6. Effect of oleic and linoleic acid feeding on the fatty acid composition and TFA biomass content of *P. incisa* cultures maintained on N-starvation medium (BG11-N)

Medium	Time (days)	TFA* (% dw)	AA (% dw)	Fatty acid composition (% of total fatty acids)												
				16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
BG11-N	3	13.4	5.6	13.6	2.1	1.8	1.3	1.2	7.4	6.4	14.2	1.7	4.6	1.2	42.0	2.1
BG11-N + 18:1 gradual	3	12.3	5.0	13.4	3.3	1.9	1.5	0.9	5.8	6.7	15.2	1.5	5.6	0.8	40.5	2.3
BG11-N + 18:2 gradual	3	11.0	4.4	13.7	3.2	1.8	1.6	0.8	4.8	6.9	15.8	1.5	5.9	0.7	40.2	2.2
BG11-N	7	20.5	10.0	12.7	1.4	0.9	0.6	1.5	10.1	5.9	11.5	1.3	2.0	1.4	48.6	1.2
BG11-N + 18:1 gradual	7	19.9	9.2	11.8	1.6	0.9	0.6	1.4	13.1	6.1	11.3	1.4	2.1	1.2	46.2	1.4
BG11-N + 18:2 gradual	7	20.7	9.7	11.7	1.0	0.8	0.6	1.5	10.3	6.1	14.1	1.4	2.2	1.2	46.8	1.4
BG11-N	14	36.2	19.1	12.1	0.0	0.3	0.3	2.1	12.7	5.8	9.3	0.8	0.8	1.2	52.9	0.9
BG11-N + 18:1 gradual	14	28.8	15.0	9.5	0.1	0.3	0.3	1.8	14.9	5.7	10.4	0.8	1.0	1.1	52.2	1.1
BG11-N + 18:2 gradual	14	23.6	10.7	10.9	1.0	0.4	0.4	1.9	16.2	6.5	12.0	1.2	1.2	1.0	45.5	1.0

20:0, 20:1, 20:2ω6, 20:3ω6 and 22:0 were present at less than 1.0%. TFA*- Total Fatty Acids (% of dry weight). 18:1- Oleic acid. 18:2- Linoleic acid

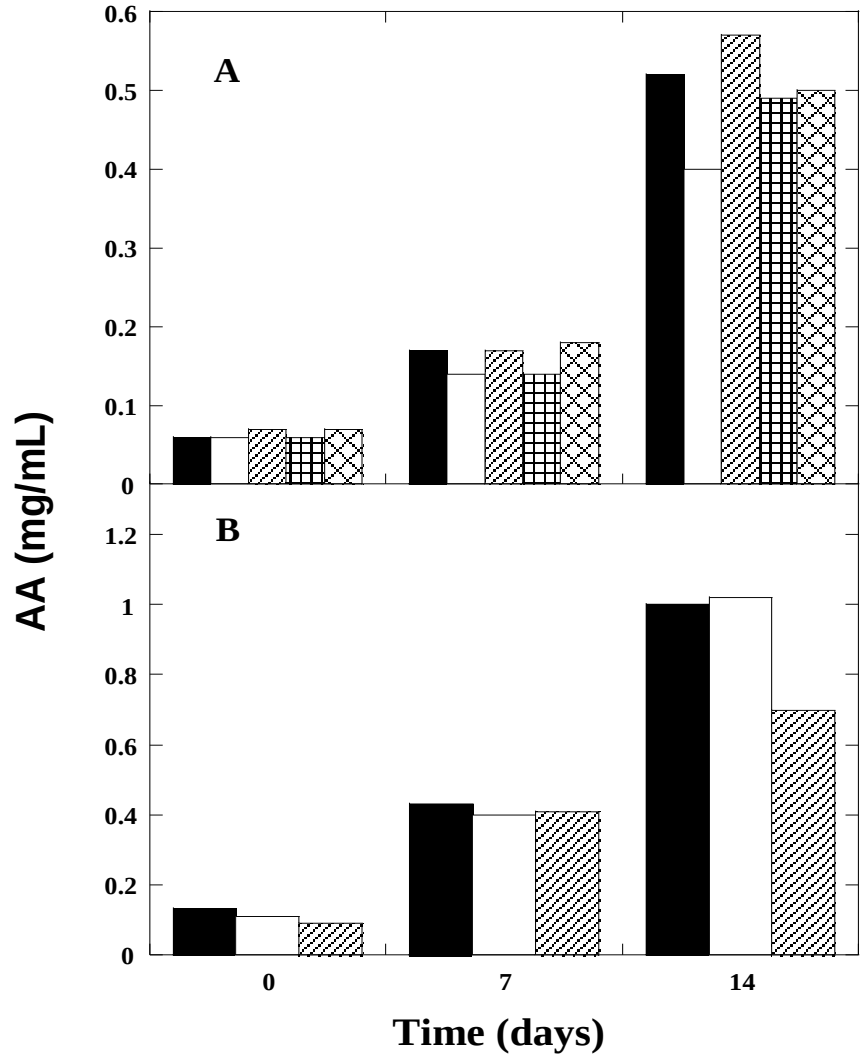

Figure 19. Volumetric content of AA in cultures of *P. incisa* supplemented with fatty acids. **A** [control-BG11 (■), oleic acid at once (□), oleic acid gradually (▨), linoleic acid at once (▦) and linoleic acid gradually (▩)] and **B** [control-BG11-N (■), oleic acid (□) and linoleic acid (▨)

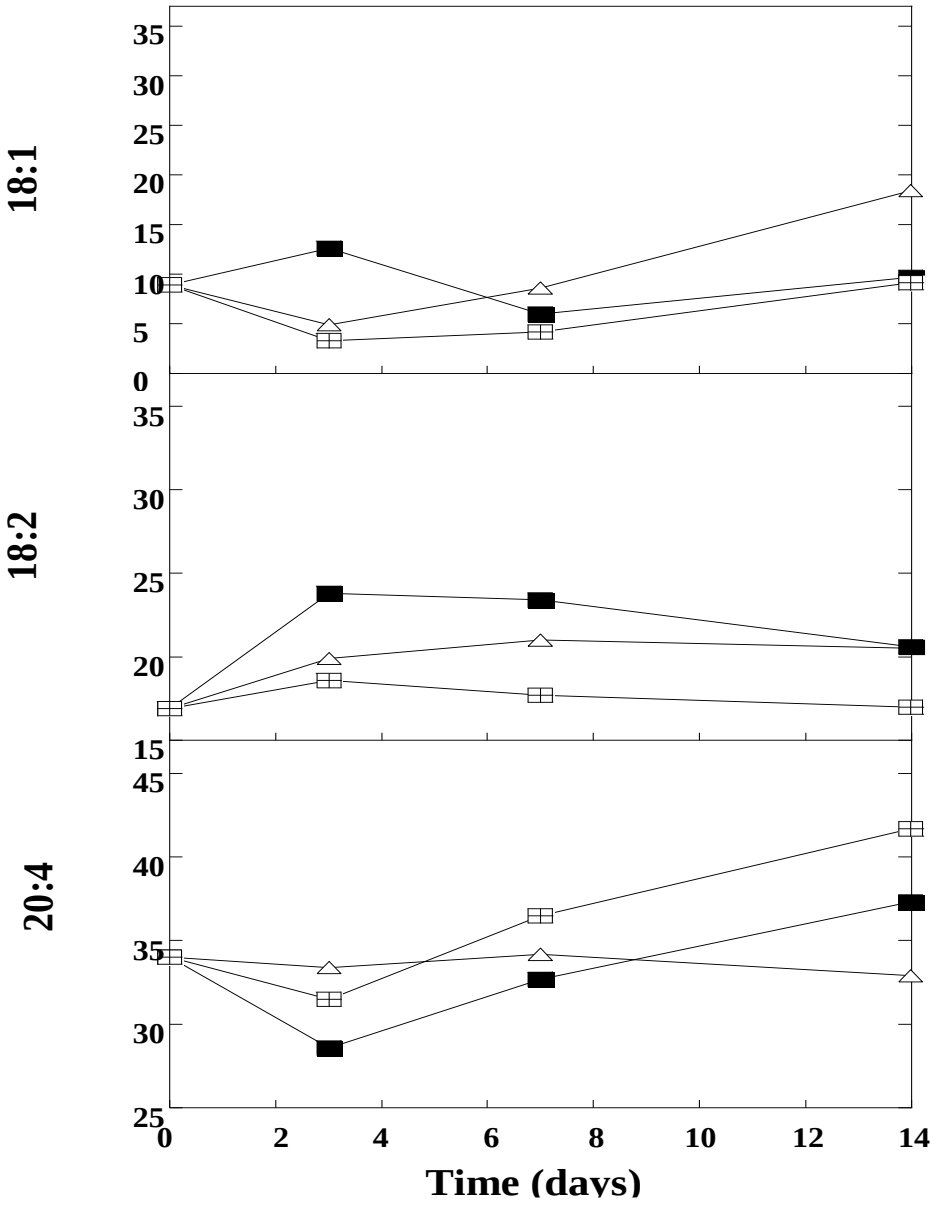

Figure 20. Proportions of three major fatty acids [18:1, 18:2 and 20:4] in cultures of *P. incisa* supplemented with oleic acid added batch (■) or gradual (Δ) and control (⊞) on complete medium (BG11)

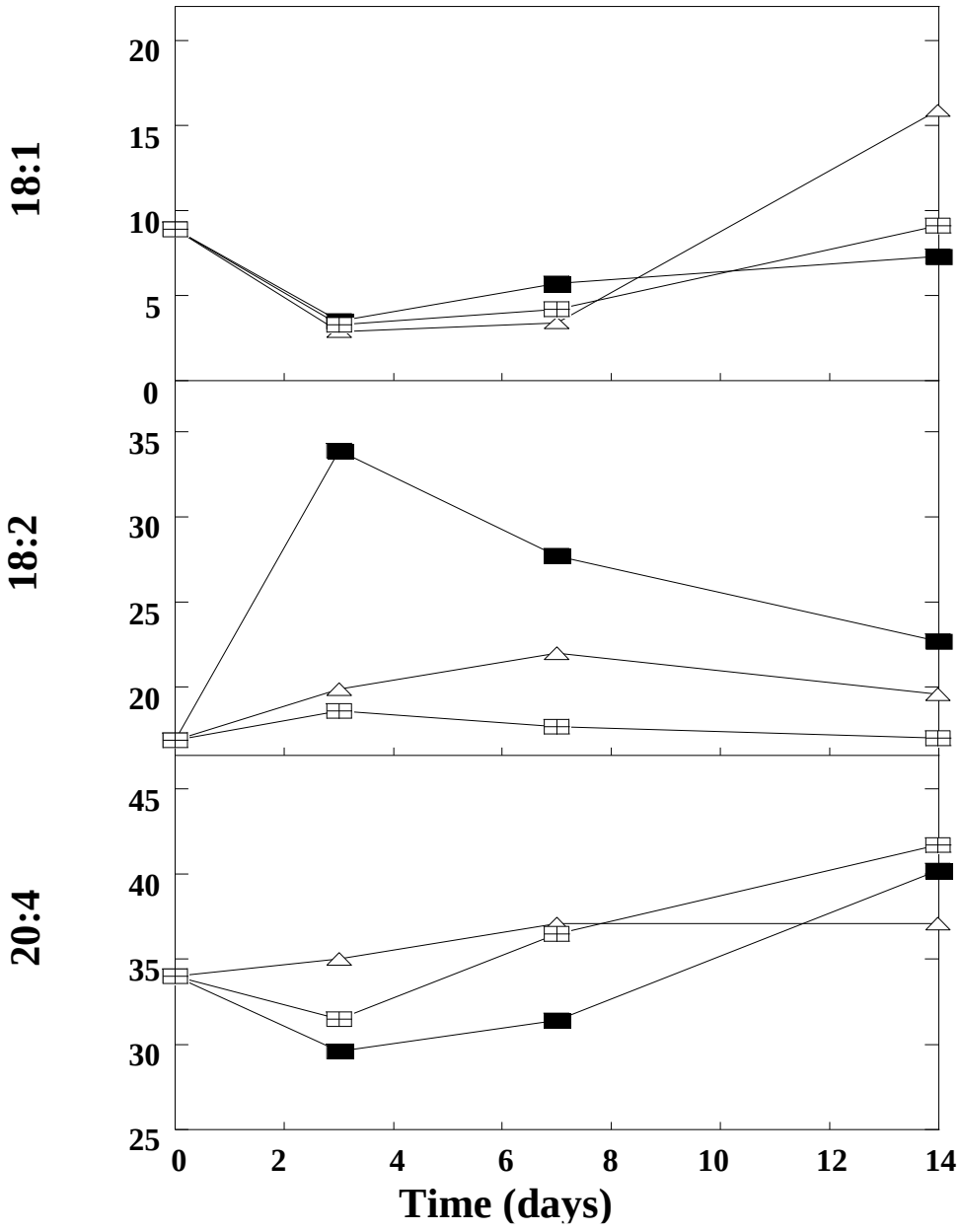

Figure 21. Proportions of three major fatty acids [18:1, 18:2 and 20:4] in cultures of *P. incisa* supplemented with linoleic acid added batch (■) or gradual (Δ) and control (⊞) on complete medium (BG11)

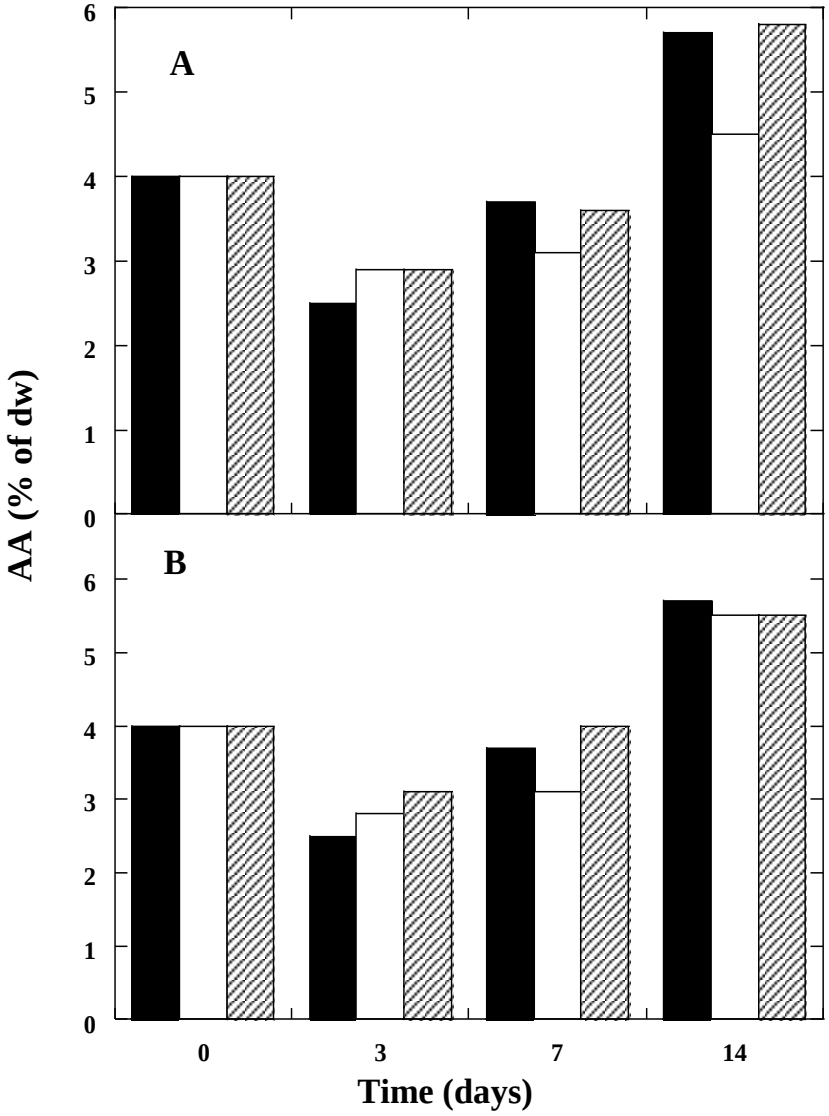

Figure 22. AA content of *P. incisa* cultures grown on BG11 and supplemented with either of the fatty acids **A** [control (■), oleic acid added gradually (□), oleic acid added at once (▨)] and **B** [control (■), linoleic added gradually (□), linoleic acid added at once (▨)]

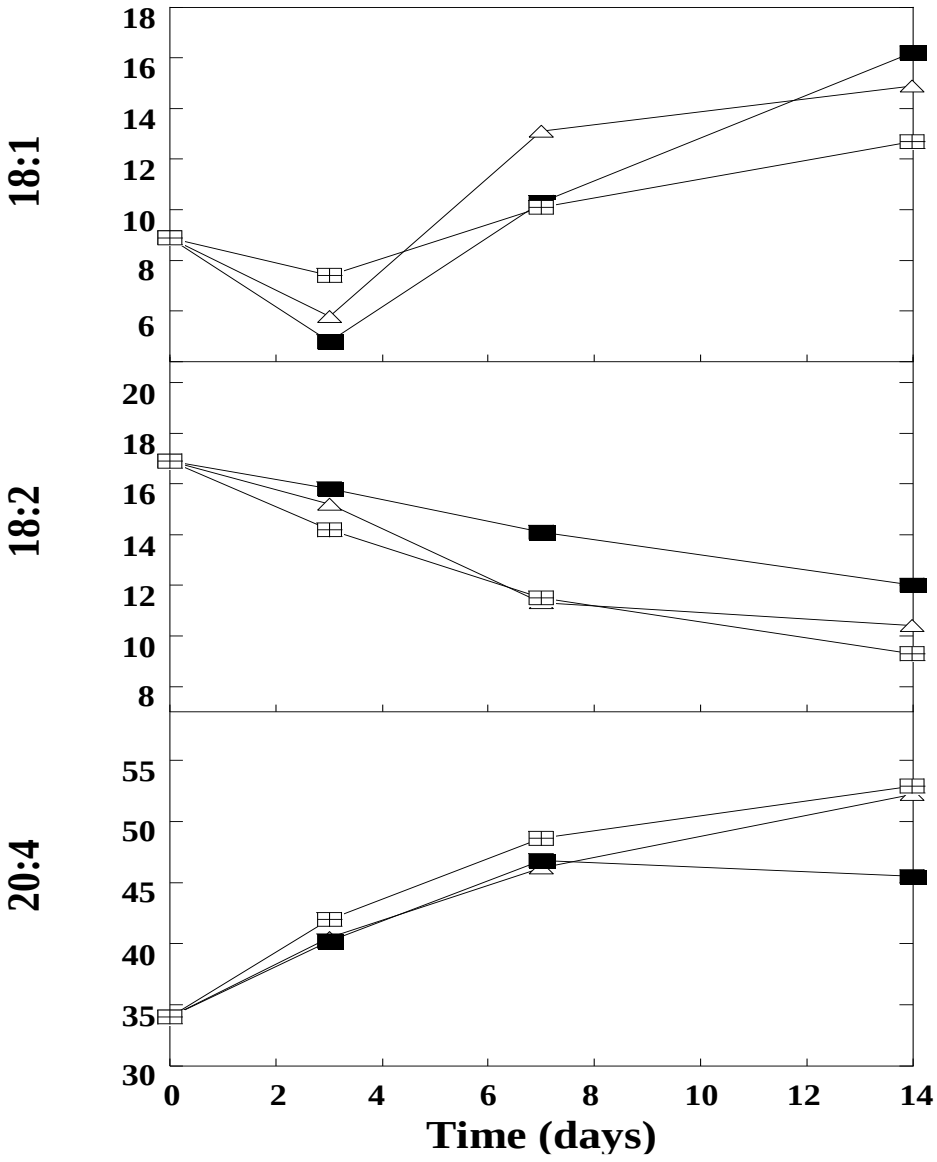

Figure 23. Proportions of three major fatty acids [18:1, 18:2 and 20:4] in cultures of *P. incisa* supplemented with oleic acid added gradual (Δ), linoleic acid added gradual (■) and control (⊞) N-starvation medium (BG11-N) and supplemented with either oleic or linoleic acid

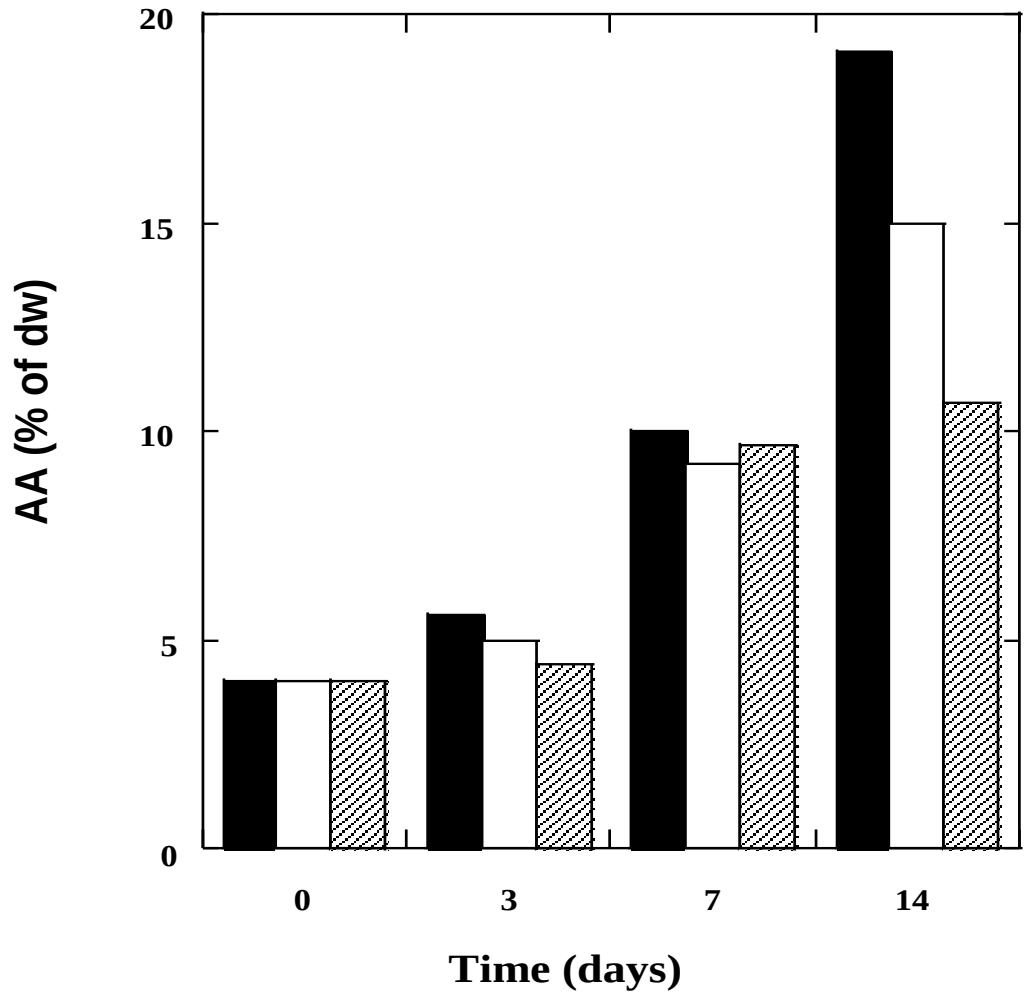

Figure 24. AA content of *P. incisa* biomass maintained on N-free medium (BG11-N) and supplemented with either of the fatty acids [control (■), oleic acid added gradually (□) and linoleic added gradually (▨)]

3.5 CO_2 enrichment

To optimize the production of AA from *P. incisa*, appropriate supply of carbon is important. In this part of the study we analyzed the effects of increasing CO_2 concentration with cultures grown on complete nutrient medium (BG11) and N-free medium (BG11-N) on the chlorophyll and biomass content, fatty acid composition and AA production in the chlorophyte *P. incisa*. Exponential cultures of *P. incisa* were adjusted to initial cell concentration of 1 mg mL^{-1}, grown on complete and nitrogen deprived medium and aerated with air enriched with either 1.5 or 5% CO_2. In another treatment, high dense cultures (initial cell density of 4 mg mL^{-1}) were maintained on BG11-N and tap water media and aerated only with 5% CO_2.

3.5.1 Effect of CO_2 enrichment on chl content and biomass accumulation in *P. incisa*

Increasing CO_2 from the standard 1.5% to 5% did not have a significant effect on the chlorophyll content of *P. incisa* cultures grown on BG11. Cultures supplied with either standard or high CO_2 had nearly the same chlorophyll content throughout the cultivation period. As shown in Fig. 25, the chlorophyll content of cultures on complete medium and aerated with either standard CO_2 or high CO_2 after reaching the maximum at day 10, decreased from 153.9 mg L^{-1} to 115.9 mg L^{-1} and from 146.8 mg L^{-1} to 111.2 mg L^{-1}, respectively at day 14. Cultures on N-free medium and aerated with high CO_2 had slightly higher chlorophyll content compared to cultures aerated with the standard CO_2 and maintained on the same medium at day 10 and 14.

Our results clearly show that cultures aerated with high CO_2 either on complete medium or N-free medium had a higher volumetric biomass up to 7 d. However,

volumetric biomass accumulation began to increase more in cultures aerated with standard CO_2 from day 10. They attained a volumetric biomass of 6.74 and 6.16 mg mL^{-1}, respectively at day 14, compared to 6.67 and 5.57 mg mL^{-1} attained in cultures aerated with high CO_2 (Fig. 26).

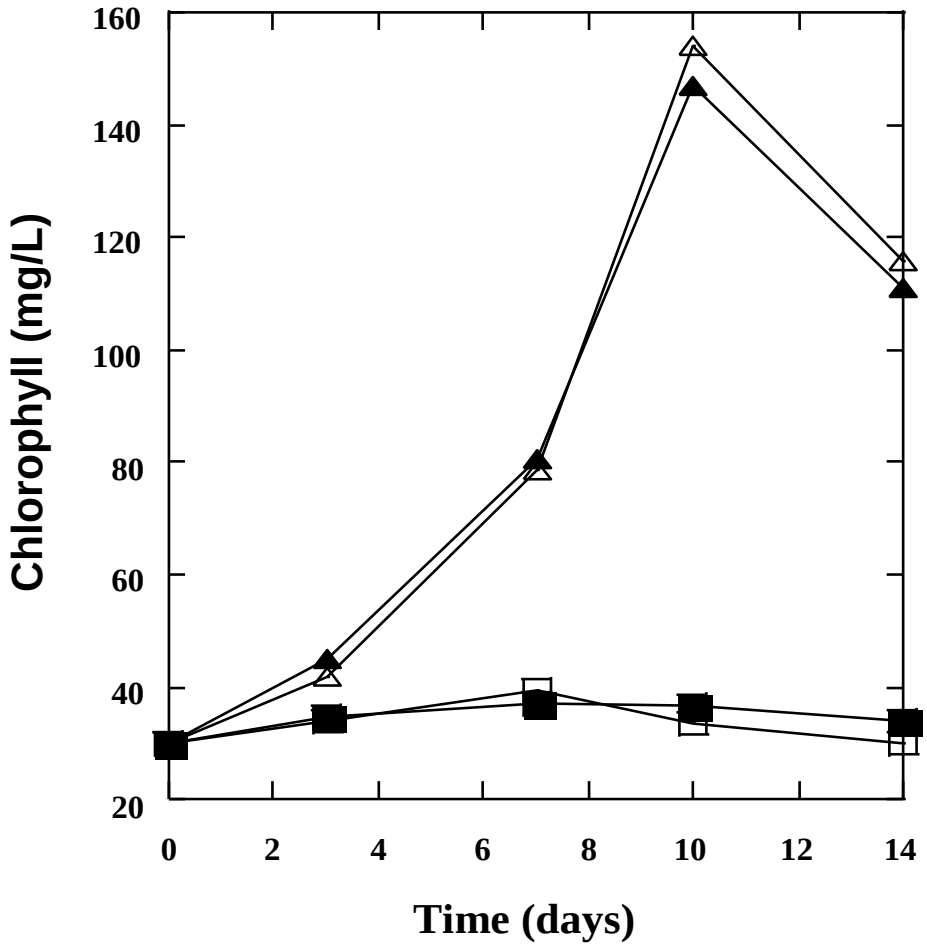

Figure 25. Chlorophyll content of *P. incisa* cultures grown on complete nutrient medium (Δ, ▲) and N-free medium (□, ■) aerated with 1.5% CO_2 (Δ, □) or 5% CO_2 (▲, ■) and maintained at a temperature of 25 ^oC and light intensity of 170 μmol quanta m^{-2} s^{-1}.

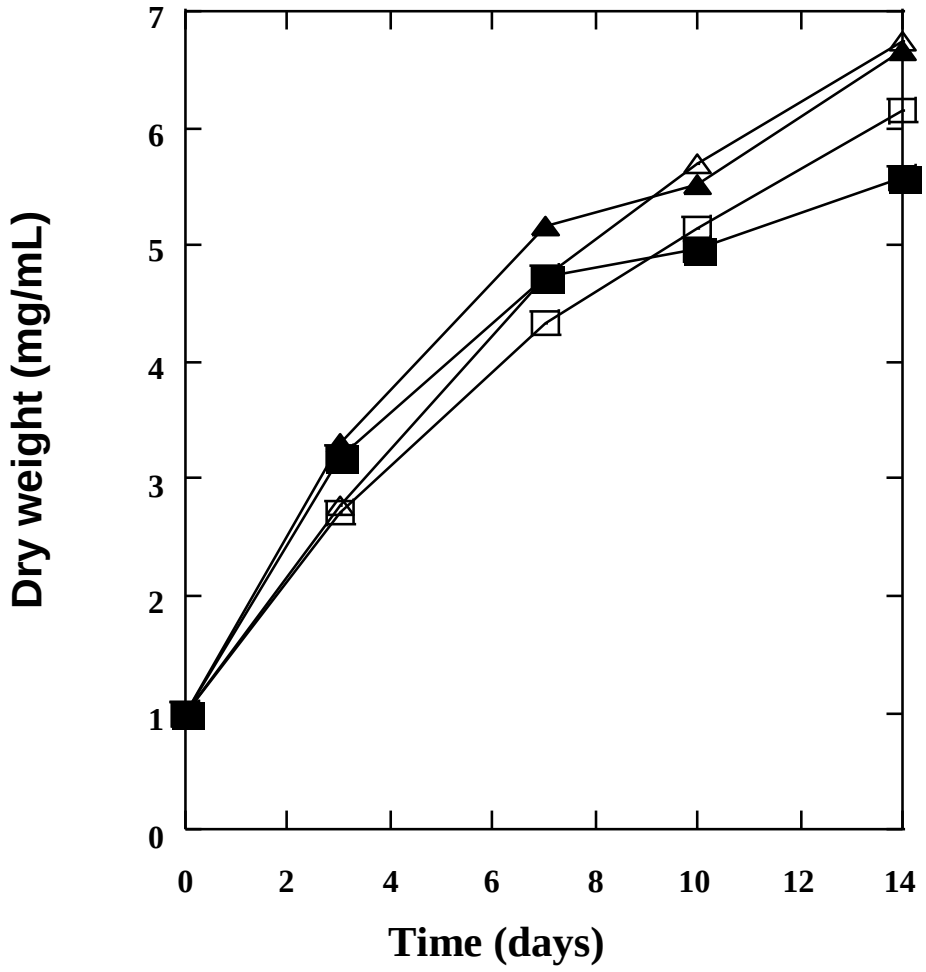

Figure 26. Volumetric biomass content of *P. incisa* cultures grown on complete nutrient medium (Δ, ▲) and N-free medium (□, ■) aerated with 1.5% CO_2 (Δ, □) or 5% CO_2 (▲, ■) and maintained at a temperature of 25 ^oC and light intensity of 170 μmol quanta m^{-2} s^{-1}.

3.5.2 Influence of CO_2 enrichment on fatty acid composition and AA production

The important finding is that the total fatty acid contents were significantly higher in cultures aerated with high CO_2 in both BG11 and BG11-N media up to day 10. As shown

in Table 7, at day 7, the TFA content increased from 14.2% to 21.8% (53.5% increase) in BG11 culture and from 26.7% to 32.3% (21% increase) in BG11-N culture, when the CO_2 in aeration was raised to 5% CO_2 compared to cultures aerated with standard CO_2. This shows that under enriched CO_2 supply, the fatty acid synthesis was enhanced. Similarly, the AA biomass content increased from 5.1 to 8.2% (37.8% increase) in BG11 culture and from 13.8 to 15.7% (12.1% increase) in BG11-N as CO_2 concentration was raised from 1.5 to 5% (Fig. 27). In addition, the volumetric AA content was higher in cultures aerated with high CO_2 at day 7 and 10. However, at day 14, the volumetric AA content was almost the same in cultures on BG11 irrespective of CO_2 concentration. Conversely, the volumetric AA content was slightly higher in culture aerated with standard CO_2 on BG11-N compared to cultures aerated with high CO_2 on the same medium (Fig. 28).

As shown in Table 7, the proportion of AA of fatty acids was slightly higher in cultures aerated with standard CO_2 on BG11-N medium. Thus, high CO_2 did not favor AA biosynthesis. The major change was the increase in proportion of 18:1 in cultures aerated with high CO_2, which increased at the expense of AA. Lower proportion of AA, might indicate an inhibition of sequential desaturation or inability to desaturate the excess 18:1. The culture on BG11 had slightly higher proportion of 16:0 under standard CO_2 than the high CO_2 condition. Similarly, cultures aerated with high CO_2 on BG11 had higher proportions of 18:1 as of day 7, amounting to 16.6% compared to 8.1% in cultures aerated with standard CO_2 on BG11. There were no significant differences in the proportion of AA in both cultures throughout the cultivation period, although, relatively the proportion of AA was slightly higher in culture under high CO_2 considering the

higher proportions of 18:1 + 18:2, the proportion of 18:1 was almost similar on day 14 in both cultures indicating a start in aging of the cells irrespective of the CO_2 concentration supplied.

Table 7. Fatty acid composition of *P.incisa* cultures grown on BG11 and BG11-N media aerated with 1.5% CO_2 or 5% CO_2

			Fatty acid composition (% of total fatty acids)												
CO_2 %	Time (days)	TFA* (% of dw)	16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
BG11															
1.5	3	9.9	14.9	6.5	3.6	2.0	1.4	6.8	3.6	22.6	1.1	6.4	0.5	28.5	1.3
5	3	11.1	15.8	1.7	3.4	2.4	1.9	7.0	6.4	21.2	1.4	6.2	0.5	30.4	0.9
1.5	7	14.2	13.8	1.5	3.5	1.0	2.2	8.1	3.7	22.6	1.4	3.0	0.8	36.2	1.0
5	7	21.8	10.7	0.6	1.8	0.8	3.6	16.6	3.1	18.4	1.2	2.5	1.1	37.4	0.7
1.5	10	15.6	12.7	2.4	3.0	0.8	2.6	9.4	3.8	21.2	1.2	2.4	1.0	37.2	0.8
5	10	22.6	10.5	0.4	1.5	0.8	3.6	16.5	3.4	18.1	1.0	2.3	1.0	38.7	0.7
1.5	14	25.5	10.1	1.1	1.8	0.5	2.7	15.1	3.6	18.7	1.0	1.6	1.2	40.9	0.6
5	14	26.5	9.6	0.9	1.3	0.7	3.0	16.2	3.5	16.5	0.9	1.8	1.0	42.7	0.7
BG11-N															
1.5	3	16.5	12.6	0.4	1.4	1.1	2.3	13.0	4.4	15.1	1.8	3.0	1.2	41.7	1.0
5	3	18.7	13.2	1.4	0.9	1.4	2.0	12.1	10.9	13.0	1.8	2.8	0.7	38.8	0.4
1.5	7	26.7	10.5	0.2	0.5	0.4	3.2	12.5	4.6	11.2	1.0	1.1	1.1	51.9	0.8
5	7	32.3	10.6	0.1	0.3	0.5	2.3	18.1	4.7	9.8	1.1	1.3	1.1	48.6	0.8
1.5	10	28.1	10.2	0.4	0.5	0.3	3.3	12.1	4.8	10.5	0.9	1.1	1.2	53.1	0.8
5	10	33.1	9.9	0.1	0.3	0.4	2.3	16.7	4.9	9.3	1.0	1.1	1.1	51.2	0.8
1.5	14	32.6	9.3	0.4	0.3	0.2	3.1	11.8	4.7	9.8	0.9	0.8	1.2	55.9	0.8
5	14	35.6	9.2	0.4	0.2	0.3	2.3	16.8	5.0	9.4	0.8	1.0	1.1	52.1	0.7

The fatty acids, 20:0, 20:1, 20:2ω6, 20:3ω6 and 22:0 were present at less than 1.0%. TFA*- Total Fatty Acids (% of dry weight).

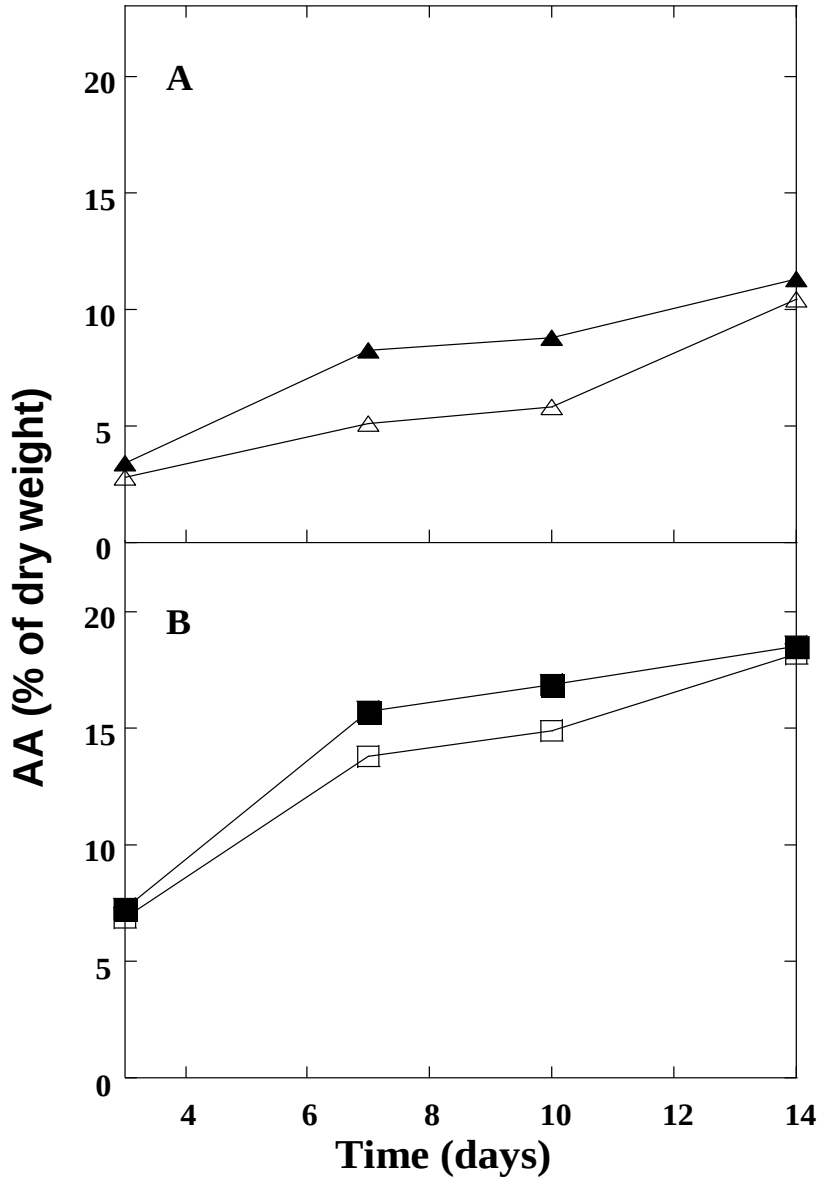

Figure 27. Biomass content of AA in *P. incisa* cultures grown on complete nutrient medium (**A**) (Δ, ▲) and N-free medium (**B**) (□, ■) aerated with 1.5% CO_2 (Δ, □) or 5% CO_2 (▲, ■) and maintained at a temperature of 25 ºC and light intensity of 170 µmol quanta m^{-2} s^{-1}.

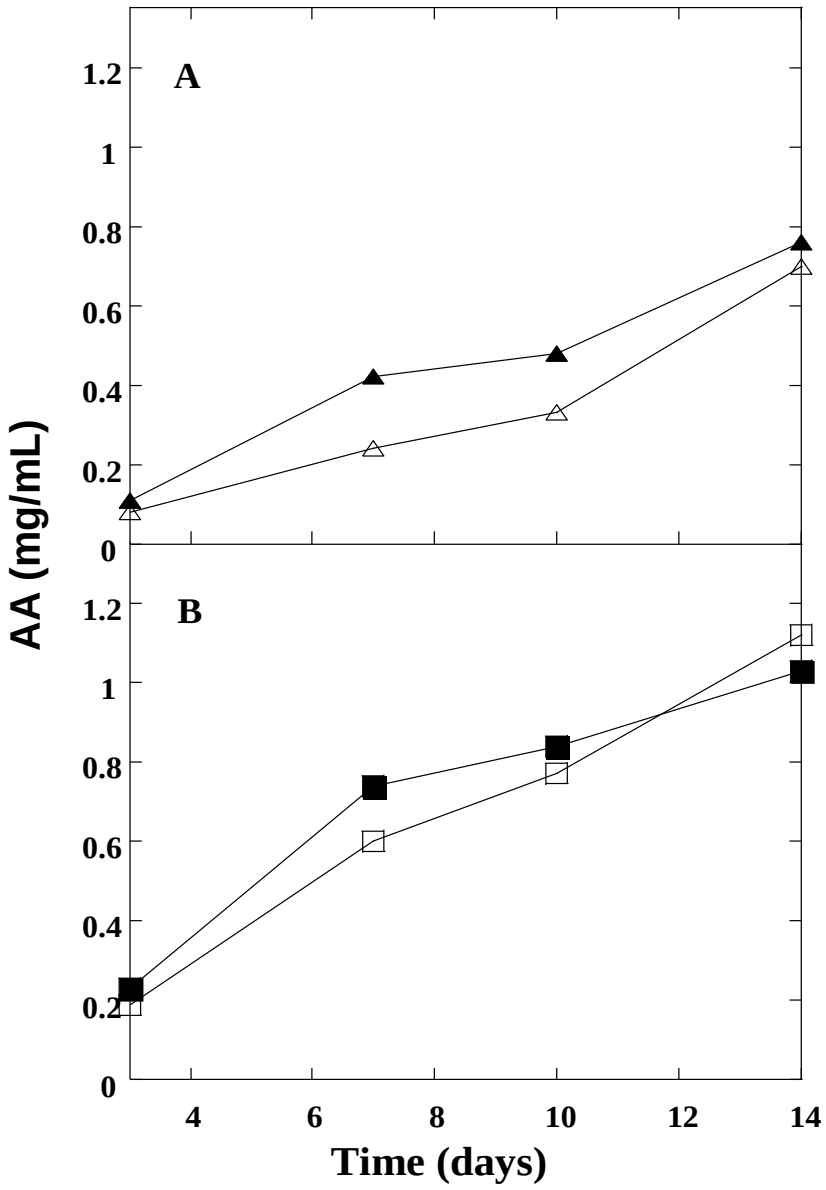

Figure 28. Volumetric content of AA in *P. incisa* cultures grown on complete nutrient medium **A** (Δ, ▲) and N-free medium **B** (□, ■) aerated with 1.5% CO_2 (Δ, □) or 5% CO_2 (▲, ■) and maintained at a temperature of 25 ºC and light intensity of 170 µmol quanta m^{-2} s^{-1}

In another series of experiments we cultivated HD cultures under 5% CO_2. We suggested that additional carbon supply would enhance AA production especially in dense culture. In addition, according to the objectives of our study, tap water was used as a possible cheap starvation medium instead of BG11-N. As mentioned previously, only HD cultures are able to withstand moderate light intensities utilized in the study when kept on tap water in starvation stage. At day 3, the proportion of AA was slightly higher in HD on tap water (37.8%) compared to HD on BG11-N (35.2%). On day 14, both HD cultures had almost the same proportion of 18:1, concurrently culture on tap water had higher proportion of 18:2 and lower proportion of AA compared to culture on BG11-N (Table 7). The fatty acid content of culture on tap water medium was almost similar to culture on BG11 aerated with 5% CO_2 throughout the cultivation period.

The volumetric AA content was almost similar in both HD cultures at day 3 and 7. However, HD culture on BG11-N medium increased continuously from day 7 until day 14. The two cultures attained volumetric AA content of 1.7 and 1.1 mg mL^{-1}, on BG11-N and tap water, respectively at day 14 (Fig. 29A). The volumetric TFA content doubled at day 7 in both cultures (Fig. 29B).

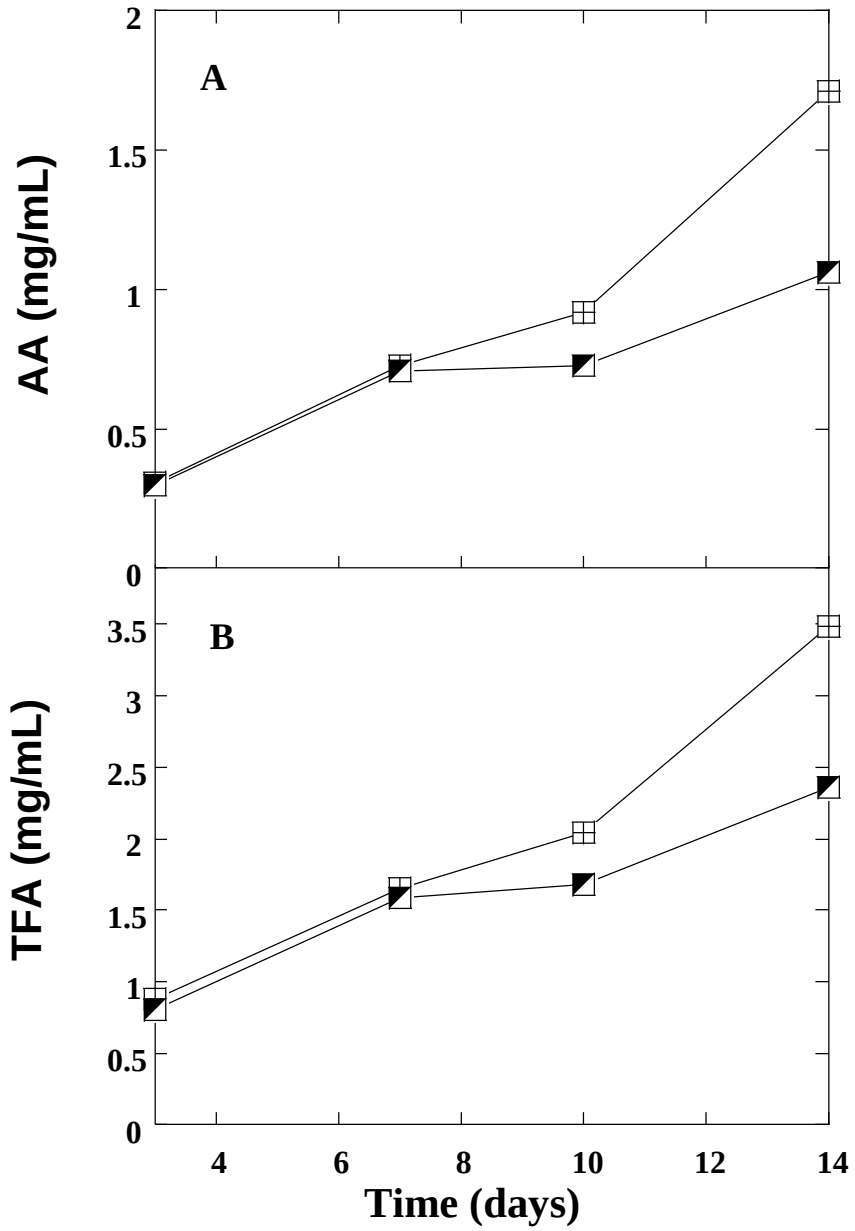

Figure 29. (**A**) volumetric AA and (**B**) volumetric TFA contents of HD cultures of *P. incisa* on BG11-N medium (⊞) and Tap water (◩) aerated with 5% CO_2 and maintained at a temperature of 25 ^{o}C and light intensity of 170 µmol quanta m^{-2} s^{-1}

Table 8. Fatty acid composition of higher dense (HD) cultures of *P. incisa* on BG11-N and tap water aerated with 5% CO_2

			Fatty acid composition (% of total fatty acids)													
Nutrient starvation	Time (days)	TFA* (% of dw)	16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
BG11-N	3	15.2	12.9	2.3	2.2	1.5	2.2	10.7	5.8	19.4	1.4	4.0	0.2	0.6	35.2	0.9
Tap water	3	15.4	12.9	1.9	2.1	1.5	2.0	7.4	7.6	19.5	1.3	3.9	0.1	0.8	37.8	0.8
BG11-N	7	20.9	9.3	1.3	1.5	0.6	2.8	13.4	3.7	16.9	1.2	1.9	0.4	0.9	44.5	0.7
Tap water	7	22.1	9.6	1.4	1.4	0.6	2.5	12.9	3.9	17.2	1.0	2.0	0.5	0.8	44.5	0.8
BG11-N	10	22.9	9.1	1.4	1.3	0.6	2.4	14.1	3.9	16.1	1.0	1.7	0.6	0.9	45.3	0.7
Tap water	10	20.4	9.7	1.1	1.5	0.6	2.7	13.0	4.1	17.5	1.0	1.9	1.0	0.8	43.6	0.8
BG11-N	14	29.6	7.9	0.7	0.9	0.4	2.4	15.2	4.1	14.7	0.8	1.2	0.5	0.9	49.1	0.6
Tap water	14	24.9	8.5	1.2	1.2	0.5	2.3	14.8	4.3	17.0	0.8	1.6	0.6	0.8	45.1	0.6

The fatty acids, 20:0, 20:1 and 22:0 were present at less than 0.5%. TFA*- Total Fatty Acids (% of dry weight).

3.6 Outdoors cultivation

This part of the work concerns an attempt to develop large-scale cultivation of *P. incisa* outdoors. Extensive experience indicates that there are main two problems with respect to outdoors cultivation: the difficulty to maintain clean cultures and the low productivities. In this study, cultivation was carried out in a 100 L polyethylene sleeve bag as described in the materials and methods. There are many advantages to using the sleeve bag such as high light availability to the cells, rapid heating and cooling, relatively clean cultures, avoidance of salinity fluctuation (since there is no evaporation), relatively low maintenances and relatively low cost (since the bags can be used continuously over long a period but are also disposable).

Cultures of *P. incisa* were cultivated outdoors on BG11-N medium during October 2003. During the day, temperature was regulated by a chiller when culture temperature exceeded 25 °C as mentioned in materials and methods. However, at night, the culture temperature was allowed to equilibrate to ambient temperature, which was 22 ^{o}C. After 2 d of cultivation outdoors, one liter of culture was transferred to 1L column and cultivated indoors under controlled growth conditions. This was done to compare the growth parameters and fatty acid production of cultures cultivated indoors under constant light and temperature conditions with the one cultivated outdoors. The impact on chl content and production of AA was studied in outdoors cultures.

3.6.1 The chlorophyll and biomass volumetric contents of *P. incisa* cultures cultivated outdoors and indoors on BG11-N medium

As shown in Fig. 30A, the chlorophyll content of *P. incisa* cultures cultivated on N-free medium either outdoors or indoors decreased continuously from day 6. More pronounced decrease was observed in the culture cultivated indoors. The chlorophyll content of the decreased from 38.0 mg L^{-1} at day 0 to 43.1 mg L^{-1} and 36.1 mg L^{-1} in outdoors and indoors, respectively at day 14.

The culture cultivated indoors had higher volumetric biomass content. This culture attained biomass content of 8.1 mg mL^{-1} at day 14. On the other hand, the outdoors culture had only slight increase in biomass content from day 8 and attained biomass content of only 4.6 mg mL^{-1} at day 14, about half as much as that of the indoors culture (Fig. 30B). Thus, we conclude that cultivation outdoors was affected by temperature and light fluctuations at day and night differently of the indoor culture which was kept under continuous illumination and constant temperature.

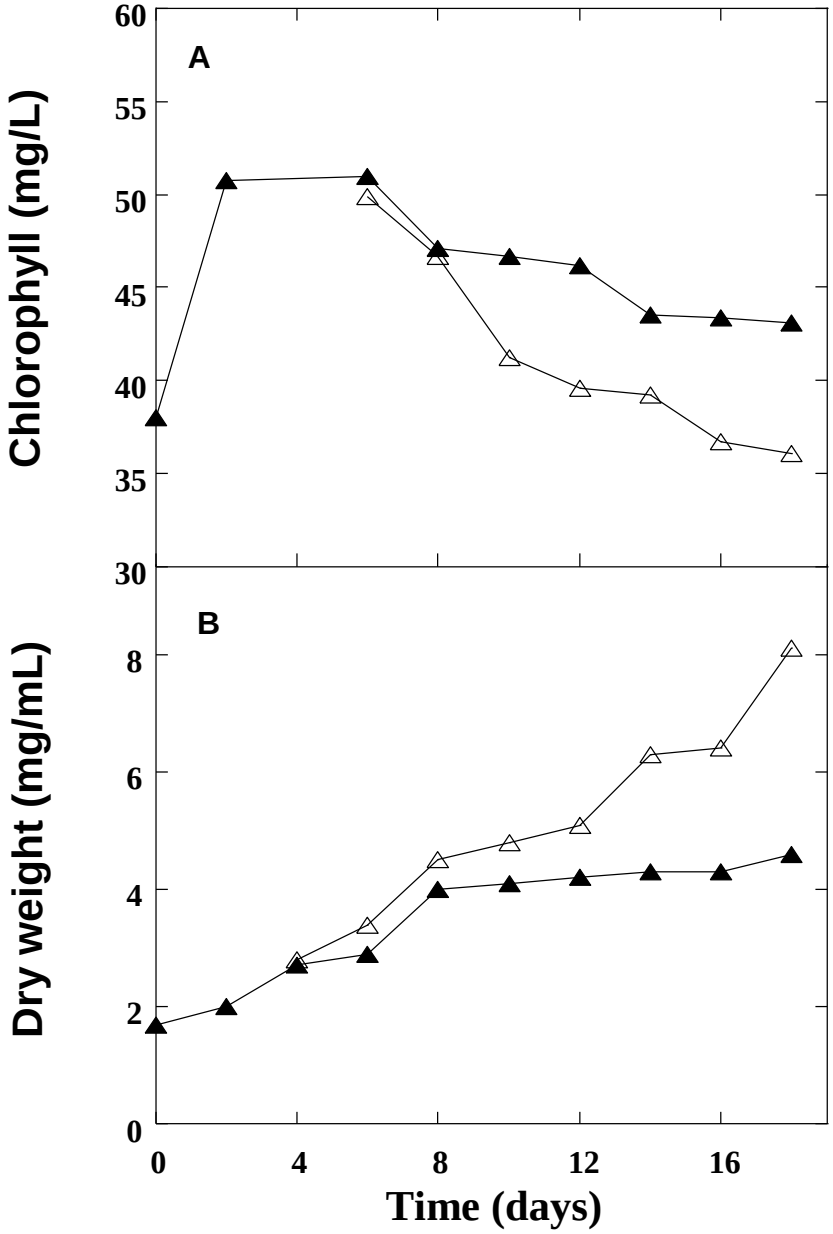

Figure 30. Chlorophyll (**A**) and volumetric biomass (**B**) contents of cultures of *P. incisa* cultivated outdoors (▲) and indoors (Δ) on N-free medium (October 2003). For indoors cultivation, chlorophyll content was taken as of day 4.

3.6.2 Fatty acid composition and AA production in outdoors and indoors cultures of *P. incisa*

The AA and total fatty acid biomass contents increased in both cultures. The culture cultivated indoors, attained 12.9% (of dw) AA and 23.7% TFA, respectively, at day 14 while the outdoors culture only 9.5% AA and 19.3% TFA (Fig. 31A and Table 9). As shown in Figure 31B and Table 10, the volumetric contents of AA and TFA increased in the indoor culture continuously during the time-course of the experiment. At the same time, in the outdoors culture there were little changes the volumetric content of AA and TFA after day 12 (Table 10). It is worth enlightening that, on day 9 contaminations were observed microscopically in outdoors culture. This can be the reason for steady productivities during the subsequent days. However, because of fluctuation in temperatures and light, such productivities are also anticipated. In addition, the daily accumulation of AA reached a peak at day 8 in both cultures.

The proportion of AA increased in both cultures, however the indoor culture had the highest value (Table 9). In the outdoors culture there was no increase in the proportion between 8 d and 10 d, which remained around 41.9%. This might be due to a contamination problem as previously mentioned. Nevertheless, at day 18, the proportions of AA reached 49.1% and 54.4% in the outdoors and indoors cultures, respectively. On the other hand, the outdoor culture had higher proportions of 16:2ω6, 16:3ω3 and 18:3ω3 compared to indoor culture in line with its higher chl contents, suggesting slower chloroplast degradation under light-dark cultivation.

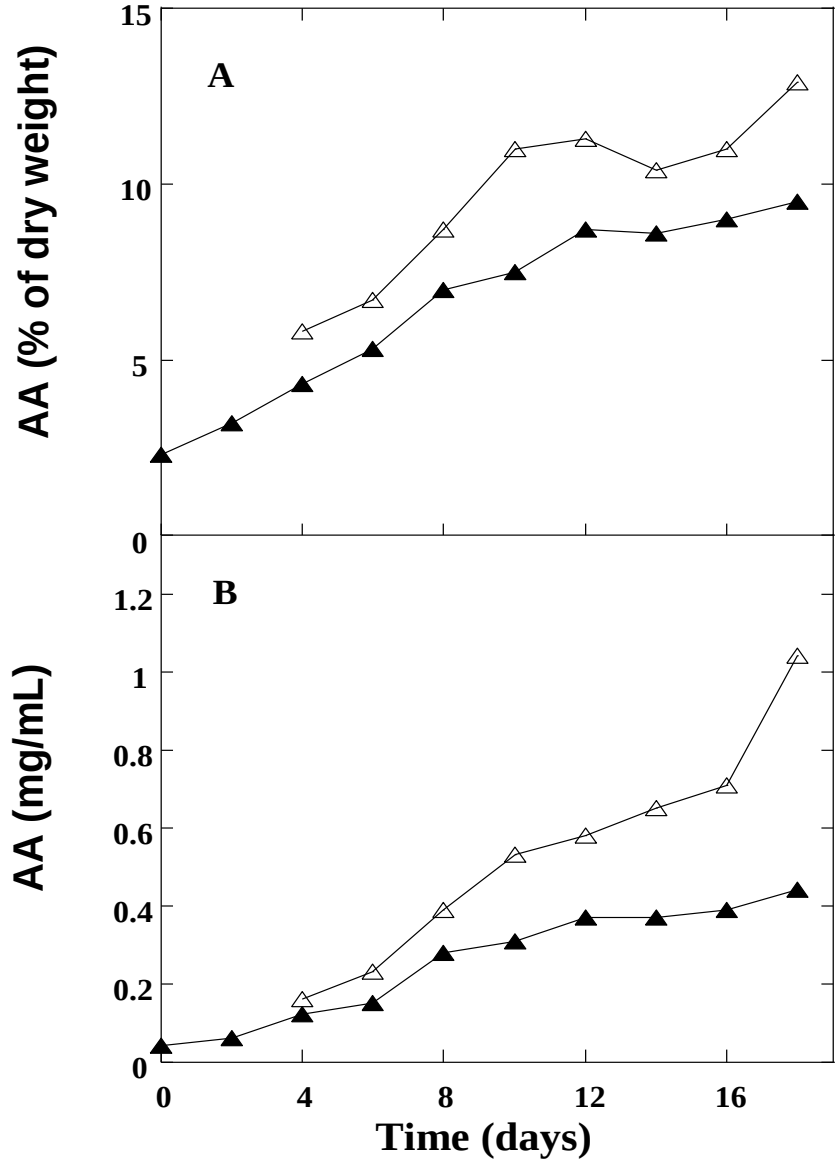

Figure 31. AA (% of dry weight) (**A**) and volumetric AA content (**B**) of cultures of *P. incisa* cultivated outdoors (▲) and indoors (Δ) on N-free medium (October 2003). For indoor cultivation, AA content was taken as of day 4.

Table 9. Fatty acid composition of *P. incisa* cultures cultivated outdoors (Out) and indoors (In) on N-free medium

Environment	Time (days)	TFA* (% of dw)	Fatty acid composition (% of total fatty acids)												
			16:0	16:1 ω11	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
Out	0	10.2	12.4	2.9	2.9	3.2	1.3	4.9	3.5	14.7	1.1	7.7	0.7	22.1	1.6
Out	2	11.4	11.6	2.2	2.3	3.1	1.2	5.3	3.6	12.2	1.2	7.4	0.6	27.9	2.3
Out	4	13.0	11.0	1.9	1.8	2.4	1.2	6.8	4.1	11.4	1.3	5.5	1.0	33.0	2.1
In	4	14.6	10.2	1.7	1.4	1.7	1.7	5.9	4.1	10.9	1.0	4.0	0.9	39.8	1.9
Out	6	14.8	10.3	1.4	1.5	2.0	1.2	6.7	4.3	10.6	0.9	4.5	0.8	38.9	2.2
In	6	15.0	10.9	1.3	1.2	1.3	1.7	7.4	4.6	5.6	1.3	2.9	1.0	44.7	1.7
Out	8	16.7	10.2	1.7	1.4	1.8	1.2	6.4	4.6	10.4	0.8	3.9	0.8	41.6	2.1
In	8	17.9	9.3	0.5	0.9	0.9	1.6	7.0	4.4	9.4	1.0	1.8	1.1	48.5	1.4
Out	10	17.9	10.1	1.4	1.3	1.5	1.2	8.1	4.7	10.6	1.0	3.2	0.9	41.9	1.8
In	10	21.9	9.5	0.9	0.7	0.7	1.7	7.4	4.3	9.5	0.9	1.5	0.9	50.5	1.2
Out	12	19.4	9.1	1.1	1.1	1.3	1.2	8.6	4.6	10.3	0.8	2.8	1.0	44.9	1.8
In	12	21.9	9.4	0.4	0.6	0.6	1.8	7.7	4.4	9.3	0.8	1.2	1.0	51.6	1.1
Out	14	18.4	8.3	0.7	0.8	0.5	1.2	9.3	4.7	9.9	0.8	2.4	1.1	46.7	1.6
In	14	20.8	9.2	0.3	0.5	0.5	1.9	8.7	4.3	10.2	0.7	1.0	1.0	50.1	1.0
Out	16	18.8	8.4	0.8	0.8	1.0	1.1	8.3	4.8	9.3	0.7	2.3	1.0	48.0	1.7
In	16	21.1	9.1	0.4	0.4	0.5	1.4	8.2	4.4	9.5	0.7	1.0	1.0	52.1	1.0
Out	18	19.3	8.4	0.9	0.8	1.0	1.2	7.8	4.9	9.1	0.6	2.3	0.9	49.1	1.7
In	18	23.7	8.2	0.4	0.4	0.4	1.9	8.1	4.3	9.0	0.7	0.9	1.0	54.4	1.0

The fatty acids: 20:0, 20:1, 20:2ω6, and 22:0 were present at less than 1.0%. TFA*- Total fatty Acids (% of dry weight). Out- Outdoors. In- Indoors. For indoors cultivation, fatty acid composition was taken as of day 4.

Table 10. Volumetric productivities of TFA and AA and accumulation in *P. incisa* cultures cultivated outdoors and indoors

Environment	Time (days)	TFA (mg/mL)	AA (mg/mL)	AA accumulation rate (mg/mL/d)
Out	0	0.20	0.04	-
Out	2	0.20	0.06	0.01
Out	4	0.40	0.12	0.03
In	4	0.40	0.16	0.05
Out	6	0.40	0.15	0.02
In	6	0.50	0.23	0.04
Out	8	0.70	0.28	0.07
In	8	0.80	0.39	0.08
Out	10	0.70	0.31	0.02
In	10	1.10	0.53	0.07
Out	12	0.80	0.37	0.03
In	12	1.10	0.58	0.03
Out	14	0.80	0.37	0.00
In	14	1.30	0.65	0.04
Out	16	0.80	0.39	0.01
In	16	1.40	0.71	0.03
Out	18	0.90	0.44	0.03
In	18	1.90	1.04	0.17

For indoors cultivation, data were taken as of day 4.

4. DISCUSSION AND CONCLUSIONS

Any attempt to develop the biotechnology for production of microalgal biomass that is rich in PUFAs of interest, will require a better understanding of the culture conditions that are governing its accumulation. The fatty acid content of many microalgae is affected by nutritional as well as environmental factors (Cohen, 1999). This work aimed at studying the production of AA from the green alga, *P. incisa* under different culture conditions.

4.1 Cell density

Various studies aiming to achieve maximal cell contents and overall productivities of PUFAs have focused primarily on nutrient stress, e.g., Suen *et al*., (1987), Reitan *et al*., (1994). The present study demonstrated that increasing the initial cell density upon transferring cell to N-deprived medium could also enhance cell content and AA productivity in *P. incisa*. In our study, we modified light availability to *P. incisa* cells by changing the initial cell density of the cultures to LD, MD or HD on N-free medium. Light availability plays a major role on the lipid content of cells, 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 the lipids are the important components (Lu *et al*., 2001).

Our results showed that during N-starvation, the chlorophyll content of *P. incisa* cells increased with the increase of initial cell density, which indicates that shade adaptation took place by increasing the cell content of the photosynthetic membranes. Similarly the chlorophyll content of *M. subterraneus* biomass increased as the cell

density increased when cultivated on nutrient sufficient medium (Lu *et al*., 2001). This typical response is correlated to a decrease in light availability, resulting in an increase in cellular chlorophyll content in order to maximize the light harvesting. In contrast, the chlorophyll content in the MD culture increased only initially and decreased thereafter. Moreover, the LD culture bleached by the end of the cultivation period. This may indicate excess light and perhaps photoinhibition.

Cultures of *P. incisa* maintained under N-free medium at either HD or LD or MD, finally had almost similar proportion of AA (of total fatty acids) as well as AA content of biomass. However, the TFA content was slightly higher in the LD and MD cultures, which indicates that under high light per cell, there is an increase in the fatty acid synthesis. Moreover, the results shows that increasing the initial cell density can enhance significantly the biomass and thus, volumetric AA productivity. This is clearly depicted in Figure 6, which demonstrated the changes in volumetric AA of the biomass as a function of cell density.

4.2 Growth stages and nitrogen starvation

The relative amount of each lipid class and fatty acid composition in microalgal cells can change considerably with variations in culture conditions (Yongmanitchai & Ward, 1989; Roessler, 1990). Increase in lipid content is usually associated with culture aging e.g., stationary phase (Piorecck and Pohl, 1984; Hodgson *et al*., 1991). Nitrogen starvation has also been positively associated with an increase in lipid abundance, although the response was species-specific (Shifrin and Chilsom, 1980). Indeed, nitrogen starvation enhanced the accumulation of TAG and AA in *P. incisa* (Khozin- Goldberg *et al*., 2002).

The growth stage at which cultures were transferred to N-free medium had a pronounced effect on the chlorophyll content of *P. incisa* cultures (Fig. 8). The rate of chlorophyll synthesis was faster in culture transferred in EXP and TRANS phases. The cells of these cultures had higher proportions of C16 and C18 PUFAs, major acyl components of chloroplastic lipids in this microalga (Bigogno *et al*., 2002a). These data suggest that in HD cultures, younger cells are still able to build up their photosynthetic apparatus despite the medium being deprived of nitrogen. On the other hand, the chlorophyll content of HD cultures transferred in the E. STA and M. STA phases had only a slight increase initially but decreased with time. These cultures were already ageing because of nutrient depletion. Under these conditions, many algae continue to synthesize fatty acids that are diverted to TAG rather than into construction of new membranes (Shifrin and Chisholm, 1981). Similarly, the biomass accumulation was higher in cultures transferred in the EXP and TRANS phases; probably they are still constructing membranes delaying chloroplast and protein degradation. On the other hand, cultures transferred in the M.STA stage had the lowest biomass accumulation.

The AA content increased in all cultures irrespective of the growth stage when they were transferred to N-free medium. However, culture transferred in M. STA had the highest AA content at the end of cultivation period. This culture was transferred in the stationary phase when AA-rich TAG is already accumulated (Bignono *et al*., 2002b). However, the highest productivity (mg AA mL^{-1} d^{-1}) was obtained in the EXP, TRANS and E. EXP cultures.

4.3 Nutrient starvation

In developing a large-scale cultivation for AA, it is necessary to utilize inexpensive medium component to lower the cost. Tap water and brackish water were used to induce starvation in *P. incisa* in HD cultures. Cells of *P. incisa* survived in tap water, which basically contains no nutrients for sustaining growth. More diluted cultures were not able to survive under those conditions. It is therefore clear that growing cells of *P. incisa* under HD can allow the cells to grow under nutrient-devoid medium. This is also an advantage for outdoors cultivation, especially during summer period, when midday light intensity is too high. HD dense cultures could withstand such light intensity, since the effect of photoinhibition is minimized. At the same time, HD cultures are able to produce biomass rich in AA. Similarly, brackish water demonstrated a similar potential as a possible nutrient starvation medium for *P. incisa* cells to attain biomass enriched with AA. At day 14, cultures on tap water and brackish water attained volumetric AA productivity of 1.00 and 1.01 mg mL^{-1}, respectively. Not only is this media less expensive but also it saves labor in media preparation, reducing the final cost of the product.

4.4 Feeding with oleic and linoleic acid

Cultures growing on complete medium and supplemented with fatty acid precursors were able to incorporate and further modify both incorporated oleic acid and linoleic acid to AA. Increase in AA and fatty acid intermediates were evident only with 3d cultures at the EXP stage. Older cultures did not demonstrate an enhancement in AA production, but rather a reduction. Media containing free fatty acids have been reported to suppress

biosynthesis of other fatty acids of microorganisms (Lees *et al*., 1966). However, addition of oleic, linoleic acids to cultures of *E. gracilis* enhanced the production of PUFAs, especially, AA and EPA (Okumura *et al*., 1986). On the other hand, supplementation of N-starved cultures did not enhance AA production, however, it delayed chlorophyll degradation and enhanced biomass accumulation.

4.5 CO_2 enrichment

The effects of carbon sources on growth and biochemical composition have been studied in several species of microalgae (Chu *et al*., 1995; Gordillo *et al*., 1998). LD cultures of *P. incisa* grown either under standard or high CO_2 did not show significant differences in their growth. The only difference observed was in the LD culture on N-free medium aerated with high CO_2, which slightly increased chlorophyll content during the last days of the cultivation period.

The fate of carbon sources incorporated in microalgal cells varies with species and is related to many factors, such as nitrogen levels (Hu *et al*., 2003). Increasing C/N ratio favors lipid accumulation in microalgae by inducing de novo lipid synthesis. Indeed, high CO_2 significantly enhanced the TFA synthesis in *P. incisa* indicating that under enriched CO_2 conditions the rate of *de novo* synthesis of fatty acid is higher. However, this phenomenon was only evident up to 10 days. Similar findings were observed in *Nannochloropsis* sp. when elevated CO_2 enhanced the rate of fatty acid synthesis (Hu *et al*., 2003).

Cultivation of *P. incisa* on BG11-N with high CO_2 did not favor an increase in the proportion of AA, moreover, the proportion of 18:1 increased at the expense of AA. This

might indicate either inhibition of sequential desaturation or favored synthesis of shorter and less desaturated fatty acids.

Increasing the CO_2 level in HD cultures on BG11-N and tap water media did not cause any significant changes in the TFA and AA contents. Probably, in HD cultures, there is low light per cell; light availability plays a major role on the lipid content of cells, mainly due to its effect on the energy supply. It could be that, cells under HD cultures are not able to metabolize excess CO_2 due to insufficient energy.

4.6 Outdoors cultivation

Until recently, open systems were the most important design principle for microalgal production (Richmond, 1990). There are several shortcomes experienced in the cultivation of microalgae cultures outdoors especially in open systems. The main problems are the difficulty of maintaining clean cultures and the low productivities. The contaminants include other algae, bacteria and predator microorganisms. The low productivities seem to be caused by three factors: the limited availability of light to cells, widely fluctuation day/night temperatures with especially low morning temperatures and salinity fluctuation. In fact, various types of closed systems have been tested. Closed system have fundamental benefits such as reduced contamination risk, reproducible cultivation conditions, controllable temperature and flexible technical design (Pulz, 1992).

In our study, we cultivated the cultures of *P. incisa* in transparent polyethylene sleeves, which is also another type of closed system. During October, the temperature reached at midday is often high and can compromise productivity. In fact, the ideal

temperature level was maintained in this bag throughout the day and allowed to equilibrate with ambient temperature at night, which was 22 ^{o}C during this period. Not so faraway from the optimal growth temperature of *P. incisa*. As depicted in Table 9, the volumetric productivity of AA and TFA, as well as the daily accumulation of AA was almost the same in outdoors and indoors cultures at day 8. Probably, there wouldn't be such significant differences in the productivities of outdoors and indoors on subsequent days, if it were not for contaminations observed in outdoors cultures at day 9. Relatively moderate productivity was obtained in outdoors cultures.

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