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FATTY ACIDS PROFILE IN A HIGH CELL DENSITY CULTURE OF ARACHIDONIC ACID-RICH *PARIETOCHLORIS INCISA* (TREBOUXIOPHYCEAE, CHLOROPHYTA) EXPOSED TO HIGH PFD*

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Abstract The changes in arachidonic acid (AA) and fatty acids profiles along the growth curve of *Parietochloris incisa*, a coccoid snow green alga, were studied in a 2.8 cm light-path flat photobioreactor, exposed to strong photon flux density [PFD, 2400 μEmol/($m^2 \cdot s$)]. Sixteen fatty acids were identified by gas chromatography showing that AA was the dominant fatty acid (33% - 41%) followed by linoleic acid (17% - 21%). AA content was closely investigated with respect to total fatty acids (TFA), ash free dry weight (AFDW) of cell mass as well as total culture content. These parameters were influenced significantly in a similar manner by culture growth phase, i.e., slightly decreasing in the lag period, gradually increasing in the logarithmic phase, becoming maximal at the early stationary phase, starting to decrease at the late stationary phase, sharply dropping at the decline phase. The increase in AA per culture volume during the logarithmic phase was not only associated with the increase in AFDW but also connected with a corresponding increase in AA/TFA, TFA/AFDW as well as AA/AFDW. The sharp decrease in AA content of the culture during the decline phase was mainly due to the decrease in AA/TFA, TFA/AFDW and AA/AFDW, although AFDW declined only a small extent. Maximal AA concentration, obtained at the early stationary phase, was 900 mg/L culture volume, and the average daily net increase of AA during 9 days logarithmic growth was 1.7 g/($m^2 \cdot$ day). Therefore, harvesting prior to the decline phase in a batch culture, or at steady state in continuous culture mode seems best for high AA production. The latter possibility was also further confirmed by continuous culture with 5 gradients of harvesting rate.

Key words: arachidonic acid, *Parietochloris incisa*, growth, high cell density culture

INTRODUCTION

Some ω3 and ω6 long-chained polyunsaturated fatty acids (PUFAs) have been recognized as an essential components in human nutrients due to their therapeutic effects and roles in maintaining cell membranes structure, fluidity and function. Among these PUFAs, arachidonic acid (AA, 20:4ω6), a precursor of human prostaglandin and leukotrients, may play an important physiological role in humans and so, has attracted much attention recently. This is because AA can be metabolized by two forms of the cyclooxygenase (COX) enzyme into prostaglandin and leukotrients which are highly proinflammatory, bronchospastic and vasodilatory. Any imbalance of the AA cascade may cause many kinds of diseases. Further studies on adults and infants showed that infants, especially those not breast-fed ones, benefit from AA and DHA enriched diet. AA and DHA are major acyl components of brain membrane phospholipids and their concentrations in milk decline during lactation

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(Xiang et al., 1999). Infants have poor ability to transform PUFAs to AA and DHA, therefore, external supplementation to promote growth and neural development is required (Agostoni et al., 1997; Wibert et al., 1997; Crawford et al., 1997; Bougle et al., 1999). AA is found to be present in animals because their bodies can synthesize AA from acetyl-CoA de novo. AA has also been found widely in many aquatic plants, especially in marine and fresh algae because algae carry out chain elongation and further desaturation of linolenic acid to PUFAs. Commercial AA at present comes from pig liver because its concentration in other species in the animal kingdom is quite low and usually less than 0.2% of dry weight. The pharmaceutical and health value of AA coupled with its limited availability prompted further exploration for new AA enriched bio-resources. A newly isolated photoautotrophic snow green alga *Parietochloris incisa* (Watanabe et al., 1996) is regarded as a hopeful alternative AA source. This potential, however, has not been exploited outdoor. Discussed below are some physiological changes of fatty acids profile, with special emphasis on AA, in a high cell density culture of this alga exposed to very high PFD (equal to natural sunshine irradiance).

MATERIALS AND METHODS

Organisms and culture conditions

Parietochloris incisa isolated from Mount Oyama in Toyoma Prefecture, Japan, was used in this study. Axenic *P. incisa* in logarithmic growth phase was centrifuged, and the fresh algal residues (about 9.5 g AFDW in weight) were inoculated into new culture medium. The fresh culture media were prepared from autoclaved stock solutions containing double BG-11 nutrients: KNO_3, 2.932 g/L; KH_2PO_4, 61.0 mg/L; Na_2CO_3, 40.0 mg/L; $MgSO_4 \cdot 7H_2O$, 150.0 mg/L; $CaCl_2 \cdot 2H_2O$, 72.0 mg/L; citric acid, 12.0 mg/L; 0.25 M of Na_2EDTA solution, 2.2ml; ferric ammonium citrate, 12.0 mg/L; $CuSO_4 \cdot 5H_2O$, 1.58 mg/L; $ZnSO_4 \cdot 7H_2O$, 4.44 mg/L; $MnCl_2 \cdot 4H_2O$, 37.2 mg/L; $MnCl_2 \cdot 4H_2O$, 37.2 mg/L; $Na_2MoO_4 \cdot 2H_2O$, 7.8 mg/L; HBO_3, 57.2 mg/L and $Co(NO_3)_2 \cdot 6H_2O$, 0.988 mg/L.

A flatglass photobioreactor with 2.8 cm internal light-path was sterilized by 70% ethanol. Very high PFD [ca. 2400 μEmol/($m^2 \cdot s$)], equal to full sunshine irradiance, was used in this experiment on the principle that the stronger the light intensity, the higher is the biomass photoproduction; and that light is always one of the growth-limiting factors in high cell density culture. The continuously strong light on the surface of the algal suspension was provided by halogen lamp at the front panel [2200 μEmol/($m^2 \cdot s$)] and by four parallel fluorescent lamps [200 μEmol/($m^2 \cdot s$)] at the back panel. A stable temperature (25℃) was maintained by a thermally balanied system (cooling by running water coupled with a fan and heating mainly by lights above. The on/off of halogen light, and the topwater flow, were controlled by a sensor immersed in the water bath. Light from a halogen lamp was directed on a big flatglass enclosed vessel (with flowing topwater) set in front of and attached to the bioreactor. Continuously turbulent flow of the culture medium was effected by a pre-filtered (by sterilzed cotton) mixture of air and CO_2 bubbled at flow rate of 0.7 L/(min·L) through a perforated tube extending along the bioreactor. The pH of the algal suspension was regularly determined with computerized pH meter. The pH was maintained stable at a favorable range (7.5 - 8.0) by adjusting CO_2 level. The photobioreactor was covered by plastic membrane on the top to reduce the chance of biotic contamination and dusts pollution from the air.

Sterilized pippett (soaking in 70% ethanol) was used to collect algal samples daily for analysis. Extra double BG-11 nutrients (nutrients stocks only) were added every three days during the culture period so as to avoid nutrient deficiency. The algal culture harvested in continuous cultiva-

tion mode was replaced by the same volume of autoclaved fresh double BG-11 medium.

Measurement of ash free dry weight

Duplicates of certain volumes of algal suspension were vacuum filtered through pre-dried and pre-weighed Whatman glass fiber filters (GF/A). The cells were washed twice with an equal volume of acidified water (pH 4.0) to reduce mineral precipitates in the algal residue and at the same time to prevent cell lysis. The residues were then dried thoroughly in an oven at 105℃ overnight to a constant weight. The filter papers with the dried algal residues were weighed while they were naturally cooled down to room temperature in a desiccator, then combusted in a muffle at 550℃ for 2 hours, and were re-weighed when cooled down to room temperature. Ash free dry weight can be calculated as the dried cell mass weight (DW) minus the ash content of the cell mass. Specific growth rate (μ) was calculated using the equation: $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$ where X_1 and X_2 are the mean ash free dry weight at time t_1 and t_2 respectively.

Fatty acid analysis

Algal suspensions were centrifuged by KS - 5200C KUBOTA at 3500 rev/min (equal to 2270 g) for 5 min. The supernatant medium was discarded. The precipitates were re-suspended in distilled water by vortex super-mixer (LAB-LINE INSTRUMENTS, INC) and re-centrifuged. After repeating the above process three times, the pellets were frozen in refrigerator (- 20℃), then fully dried in vacuum freeze dryer. There were two replicates for each sample. Twenty-five mg of algal powder, 0.25 mg heptadecanoic acid (C_{17}, an internal standard) and a magnetic bar were put into a small bottle with Teflon cap, then 2 ml H_2SO_4 methanol solution (2% H_2SO_4) was added to break the lipid molecules and modify a CH_3^- group. After charging it with argon gas, the liquid sample in the Teflon tube was stirred and heated in 80℃ sandy bath on a hotplate magnetic stirrer for 1 hour so as to free the bounded fatty acids.

One ml distilled water and 1 ml hexane added into the Teflon tube above were well mixed by vortex, then centrifuged at 3500 r/min for 5 min. The supernatant was transferred into another stoppered vial and concentrated by nitrogen, then stored in freezer for gas chromatography analysis.

Computer controlled gas chromatographic (hp Hewlett 5890, Packard series II) analysis was performed on a fused silica capillary column (30 m × 0.32 mm, Supelco, Bellefonte, PA) at 200℃ (FID, injector and flame ionization detector temperature 230℃, split ratio 1:100). Fatty acid methyl esters were identified by chromatography with authentic standards (Sigma, St Louis, MO). Fatty acids were calculated by comparing each peak area, with that of standard C_{17} amount and its peak area. Fatty acids were expressed on the bases of total fatty acids and ash free dry weight (Hu et al., 1997; Adlerstein et al., 1997).

RESULTS AND DISCUSSION

1. Growth of *P. incisa* culture under conditions of high cell mass density and high PFD

The increase in cell mass of *P. incisa* exposed to strong light irradiance is shown in Fig.1 indicating clearly the growth phase of *P. incisa* in high cell mass density culture was in the form of an untypical "S". A visible increase in ash free dry weight (AFDW) in the first day of culture was observed. This result showed that the generally long lag period in cell cultivation can be avoided by culturing at high cell density. The following linear increase in AFDW indicated that the culture went into the logarithmic growth phase. The maximal AFDW content was obtained after 10 days cultivation when the algal suspension reached the stationary phase. The average daily net increase in AFDW (or

photoproduction of this alga in this bioreactor) was extremely high, about 28 g/(m^2 · d) during the whole logarithmic growth phase. The final sudden decrease in AFDW means the culture went into the decline phase, which also showed the high cell density culture system was relatively unstable.

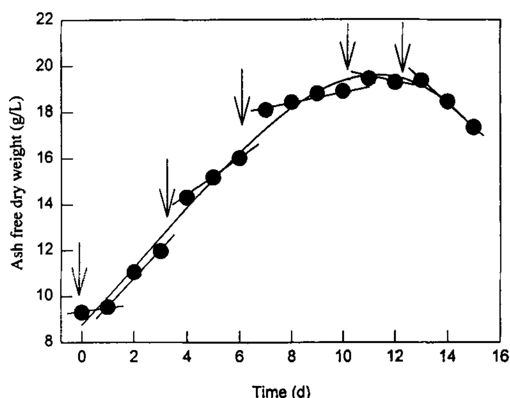

Fig.1 Changes in biomass of *P. incisa* along growth phase. Arrows indicate the points in time when double nutrients of BG-11 were added

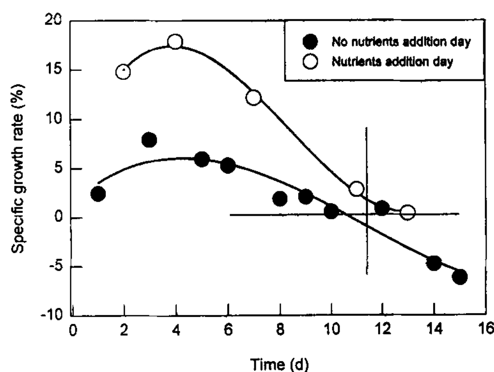

Fig.2 Nutrients addition effect on specific growth rate of *P. incisa* along growth phase

Close study of Fig.1 shows that AFDW always visibly jumped following the addition of nutrients. Fig.2 on the specific growth rate (SGR) on days when nutrients were added or not added shows that SGR on nutrients added days was much higher than that in no nutrients added days. Furthermore, the SGR increased more significantly when nutrients were added at the early logarithmic growth phase than when nutrients were added at the late logarithmic growth phase and the stationary phase, and increased only slightly when nutrients were added at the decline phase. The above increase of SGR on nutrient addition days indicated nutrients deficiency problem still existed in this culture system although a large amount of nutrients (double BG-11) was added every three days. These results also showed that nutrients were consumed rapidly when the cells grew fast, so that much more nutrients (> 1.0 g nitrate per day) were required to maintain fast algal growth.

As mentioned above, the relatively unstable high cell mass density culture at the late stationary or decline phase was not mainly caused by nutrients deficiency, because the double nutrients were still added and had less and less effect (Fig.2). This SGR decrease was largely due to light limitation as a result of mutual shading, so that light available to individual cells was the lowest at maximal algal cell mass. In fact, the light penetration distance was quite limited although the illumination was as strong as full sunshine in this experiment. We found that only the cells within an about 3 mm narrow zone near the front surface panel had light for photosynthesis. Fortunately, all individual cells in this flat photobioreactor moved back and forth between the light zone and dark zone due to the vigorous stirring of the culture, and so, could receive irradiance intermittently. Transient light:dark cycle is a crucial parameter in high (especially ultra high) cell density cultivation.

Another reason for the rapid decrease in growth in the late stationary or decline phase may partly be due to strong inhibition caused by auto-inhibitor (to be discussed in another paper) in old culture.

In general, even with doubled nutrients added every 3 days and very strong PFD (equal to sunshine) provided, nutrient deficiency and insufficiency of light irradiance, instead of photoinhibition, are still the two main factors limiting growth in high cell mass culture.

The reason for expressing the cell mass growth in term of AFDW, not DW, is due to the evident ash content, which in *P. incisa*, was less than 4% during the first several days, but could reach 10% or more, at the late culture stages during high cell density culture. So AFDW is a better

parameter for expressing the cell mass growth.

2. Fatty acids composition and their changes along the growth curve

Gas chromatography showed that at least 16 fatty acids, mostly unsaturated, existed in this algal lipid, although their proportions varied at different growth phases (Table 1). Most prevalent of those fatty acids were palmitic acid (16:0), stearic acid (18:0), three kinds of palmitoloeic acids (16:1ω11, 16:1ω9 and 16:1ω7), oleic acid (18:1ω9), linoleic acid (18:2), γ-linolenic acid (18:3ω3), γ-linolenic acid (18:3ω6), dihomo-γ-linolenic acid (20:3ω6), arachidonic acid (20:4ω6), and ecosapentaenoic acid (20:5ω3). AA was the dominant fatty acid (ca. 33% - 41% of TFA or 4% - 6% of AFDW) followed by linoleic acid (ca. 15% - 21% of TFA). Table 1 shows that the unsaturated fatty acids accounted for 80% of TFA in *P. incisa*. Why this snow green alga accumulates so much unsaturated fatty acids is unclear. It is presumably related to an adaptive mechanism to keep membrane fluidity at low temperature.

Table 1 The proportion of each fatty acid to TFA in high cell mass density *P. incisa* cultured in 2.8 cm light-path flat bioreactor exposed to strong PFD

Fatty acid	Minimum(%)	Maximum(%)	Fatty acid	Minimum(%)	Maximum(%)
14:0	0.1	0.4	18:1ω7	0.0	5.2
16:0	11.2	14.8	18:2	15.0	21.2
16:1ω11	1.1	2.7	18:3ω6	0.4	1.1
16:1ω9	0.3	0.3	18:3ω3	3.1	6.5
16:1ω7	1.7	3.2	20:2ω6	0.3	1.1
16:2	1.3	1.7	20:3ω6	0.5	0.7
18:0	1.4	1.8	20:4ω6	33.1	41.8
18:1ω9	5.1	9.1	20:5ω3	1.7	2.4

Many aquatic plants, especially algae, carry out chain elongation and further desaturation of linolenic acid to PUFAs. Up to date, AA has been only found at low level in the following marine algae: 1. Brown seaweed *Fucus* (Kim et al., 1996), *Sargassum* (Wu et al., 1995) and *Laminaria* (Rorrer, 1995). 2. Red algae *Porphyridium* (Cohen, 1990; Adlerstein et al., 1997; Akimoto, et al., 1998; Fabregas et al., 1998; Robles et al., 1999), *Laurentia obtusa* (Mabrouk et al., 1999) and *Gracilaria* (Sajiki, 1997a,1997b). 3. Diatom *Chaetoceros* (Napolitano et al., 1990). 4. Green algae *Spirogyra crassa* (Stefanov et al., 1996), *Nannochloris* (Petkov et al., 1994), Ulva (Floreto et al., 1994). 5. Cyanobacteria *Spirulina* (Dubacq and Pham, 1993). 6. Eustigophyceae *Euglena* (Hayashi et al., 1994). 7. *Tetraselmis suecica* (Servel et al., 1994; D'-Souza and Loneragan, 1999). Some fresh algae like *Scenedesmus*, *Anabaena* and *Aphanizomenon* also have AA, but in too low level (commonly 0.06 to 0.2 mg/g DW both for batch and continuous culture, Gunnel Ahlgren, 1999, private communication). Due to its unique ability to accumulate large amount of AA, *P. incisa* is a hopeful alternative AA bio-resource.

AA/TFA, TFA/AFDW in high cell mass culture of *P. incisa* exposed to high PFD were influenced significantly by the culture growth phase too (Fig.3). Both AA/TFA and TFA/AFDW parameters slightly decreased in the lag period, gradually increased in the logarithmic phase, maximized at the early stationary phase, then started to decrease at the late stationary phase and sharply dropped at the decline phase. The dynamic changes in AA/AFDW and AA per liter of culture in high cell mass culture of *P. incisa* exposed to high PFD are shown in Fig.4. Along the growth curve, the variation pattern of AA/AFDW and AA per culture volume were similar to AA/TFA and TFA/AFDW but differed in extent.

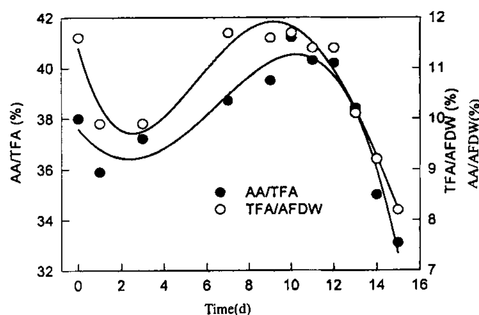

Fig.3 Changes in AA/TFA and TFA/AFDW of *P. incisa* along the growth curve

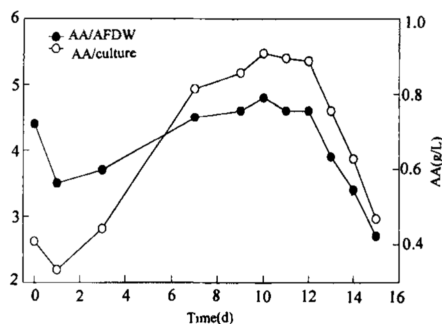

Fig.4 Changes in AA/AFDW and AA per culture volume of P. incisa cultured in 2.8 cm light-path flat photobioreactor and exposed to high PFD

The increase in AA per culture volume (Fig.4) during logarithmic phase was not only associated with the increase in AFDW (Fig.1) but was also connected with the corresponding increase in AA/TFA and TFA/AFDW (Fig.3) as well as AA/AFDW (Fig.4). The sharp decrease in AA content of the culture during the decline phase was mainly due to the decrease in AA/TFA, TFA/AFDW and AA/AFDW (Fig.4), although AFDW (Fig.1) declined to only a small extent. In batch culture, maximal AA concentration was obtained at the early stationary phase (reached 900 mg/L culture volume) when AA/TFA, TFA/AFDW, AA/AFDW and AFDW also reached to the maximum. The average net AA daily increase during the 9 days logarithmic growth phase of P. incisa was about 1.7 g/(m^2·day), which may be the highest obtained AA production from autophototrophic organism so far as we know.

Harvesting at the late logarithmic phase or early stationary phase in a batch culture mode (prior to the decline phase), seems best for maximal AA production by *P. incisa*. Any method like semi-continuous, continuous and chemostat culture to avoid culture reaching to the decline phase should be taken for maximal AA production. This hypothesis was studied in detail by continuous culture of *P. incisa* in 2.8 cm light path photobioreactor with 5 gradients of harvesting rate (from 7% – 20%, Table 2).

Table 2 Daily fatty acids and AA production in each square meter surface from continuous culture of *P. incisa* in 2.8 cm light path photobioreactor and exposed to high PFD

Daily harvesting rate (%)	7.00	10.00	13.50	17.00	20.00
Nitrate input [NI, g/(m^2·d)]	5.74	8.20	11.08	13.95	16.41
AFDW concentration (g/L)	9.0	9.8	8.7	6.9	6.3
AFDW production [g/(m^2·d)]	17.64	27.4	32.88	32.84	35.25
Ratio of AFDW production to NI	3.07	3.34	2.97	2.35	2.15
TFA production [g/(m^2·d)]	4.52	5.43	7.00	4.91	3.58
Ratio of TFA production to NI	0.79	0.66	0.63	0.35	0.22
AA production [g/(m^2·d)]	1.53	2.32	2.58	1.64	0.97
Ratio of AA production to NI	0.27	0.28	0.23	0.12	0.06

Very high daily biomass, TFA and AA production were achieved when the culture of *P. incisa* reached steady state. The maximal biomass concentration (9.8 g/L) and biomass production per

photosurface [35.25 g/(m^2·d)] were obtained with 10% and 20% harvesting rate, respectively. 10% harvesting rate seemed the most economic mode for biomass production with highest AFDW to NI ratio (3.34). The maximal TFA production [7.0 g/(m^2·d)] and AA production [2.58 g/(m^2·d)], the maximal AA production in batch culture mode > 1.7 g/(m^2·d) could be achieved in continuous culture mode with daily harvest of 13.5% of the culture volume. Therefore, daily replacement of 13.5% of the whole culture with new medium is suggested in practice, although the biomass production was not the highest and the nitrate inputs related to AFDW, TFA and AA profiles were seemingly not the most economic.

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