Growth of Parietochloris incisa in various organic carbon substrates
Growth of *Parietochloris incisa* in various organic carbon substrates

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**Abstract**

The capacity of *Parietochloris incisa* to grow under mixotrophic and heterotrophic conditions was investigated. Glucose, glycerol, sodium acetate, citric acid and lactic acid at 0.005M concentration were used as organic carbon substrates. Results of this study clearly demonstrate the ability of this microalgae to utilize glucose as carbon source for its growth. Glycerol seemed to support growth under mixotrophic condition but not in heterotrophic growth regime. Growth was not observed with sodium acetate, citric acid and lactic acid. Under specific culture conditions, glucose was shown to increase biomass productivity by 7.8, 15.5 and 6.8 folds higher than photoautotrophic control under mixotrophy without and with supplementation and heterotrophy, respectively. Outdoor mixotrophic cultivation did not yield higher biomass productivity; however, the proportion of arachidonic acid in total fatty acid increased significantly resulting in a higher arachidonic acid volumetric yield. These results open new horizon for the development of *P. incisa* mass cultivation technology for arachidonic acid production employing mixotrophic and heterotrophic cultivation.

**Key words**: arachidonic acid, biomass production, mixotrophic and heterotrophic cultivation, *Parietochloris incisa*, organic carbon

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**Introduction**

Although microalgae are photoautotrophic, some have the ability to assimilate and utilize organic carbon as energy sources for growth either in the presence of light where growth regime involves CO₂ and organic carbon assimilated simultaneously such that respiratory and photosynthetic metabolism operates concurrently (Kaplan et al. 1986; Lee 2004) or in the dark where the fixation of atmospheric CO₂ of autotrophic cultures is replaced by organic substrate dissolved in the culture media as sole carbon and energy sources. For species that can utilize both light energy and organic substrate as source of energy, the mixotrophic cultivation of microalgae can be a superior alternative to phototrophic and heterotrophic growth as both biomass and productivity increases have been reported when microalgae have been grown mixotrophically rather than photoautotrophically or heterotrophically (Ceron García et al. 2000; Chu et al. 1996; Day and Tsavalos 1996; Fang et al. 2004; Wen and Chen 2000). Heterotrophic cultivation is also a sound alternative to photoautotrophic growth since it circumvents the problems posed in photoautotrophic mass cultivation. Although this ability to use organic carbon sources is found from all algal groups (Behrens 2005), heterotrophy is not suitable for most microalgae and more species are obligate autotrophs than facultative heterotrophs (Lee 2001).

Microalgae are potential sources of valuable long chain polyunsaturated fatty acids (LCPUFA). Nutritionally important LCPUFA include eicosapentaenoic acid (20:5 ω 3, EPA), docosahexaenoic acid (22:6 ω 3, DHA) and arachidonic acid (20:4 ω 6, AA). AA and DHA are major components of cell membranes and play important roles in preserving physiological and psychological function. Recent studies demonstrate the benefits of AA and DHA in improving age-related declines in brain and cardiovascular system function (Kiso 2011; Kotani et al. 2006). DHA and AA are found naturally in human milk and are important for the visual acuity and cognitive developments of infants after birth (Koletzko et al. 1996). Several expert groups recommend that infant formulas be supplemented with DHA and AA (Mittmess 2007; Fleith and Clandinin 2005).

EPA and DHA are found abundant in microalgae. *Phaeodactylum tricornutum* (Yongmanitchai and Ward 1992), *Monodus subterraneus* (Hu et al. 1997), *Porphyridium cruentum* (Cohen 1999), *Nannochloropsis sp* (Seto et al. 1984) were well studied due to their potential as source of EPA and *Schizochytrium* (Wu et al. 2005), *Cryptothecodinium cohnii* (Jiang et al. 1999) and *Chroomonas salina* (Henderson and Mackinlay 1992) for DHA. Arachidonic acid, however, is notably rare and if found present in marine species, it amounts to a few percent of total fatty acids (Thompson 1996). A marine microalgal species, *P. cruentum* was reported to produce AA in appreciable amount under specific growth conditions (Cohen 1990). In freshwater microalgal
species, AA is almost excluded from the lipid profile. However, the freshwater green microalga, *Parietochloris incisa* (Trebuxiophyceae, Chlorophyta), isolated from the snowy slope of Mt. Tateyama, Japan (Watanabe et al. 1996), is exceptionally capable of accumulating high amounts of arachidonic acid and is recognized as the richest vegetal sources of AA (Bigogno et al. 2002). Under conditions conducive to oil accumulation, *P. incisa* was shown to accumulate AA content exceeding 20% of dry weight, and the proportion of AA reaches 50% of total fatty acids in which over 90% of AA is deposited in triacylglycerols (TAG) (Khozin-Goldberg et al. 2002). This ability of *P. incisa* to deposit AA in triacylglycerols in cytoplasmic oil bodies is of practical economic concern since photoautotrophic cultivation is relatively costly with regards to infrastructures and operations is a huge constraint can be utilized as bioreactor and light is not required thus resulting in significant reduction in cost for most processes (Gladue and Maxey 1999). *P. incisa* is a potential candidate as a commercial source for arachidonic acid.

Commercially mass cultivated algae are generally cultured photoautotrophically. Limitations of photoautotrophic cultures such as ineffective light and carbon dioxide distribution for optimal growth as well as economic concerns since photoautotrophic cultivation is relatively costly with regards to infrastructures and operations is a huge constraint in the industrial mass production of microalgae. A feasible alternative to photoautotrophic cultures is mixotrophic and heterotrophic growth regime. Mixotrophic cultivation was shown to be good strategy to obtain a large biomass and high growth rates (Ogawa and Aiba 1981; Lee and Lee 2002). Heterotrophic growth approach, on the other hand, cancels out two major deficiencies of photoautotrophy *i.e.* any fermentor can be utilized as bioreactor and light is not required thus resulting in significant reduction in cost for most processes (Gladue and Maxey 1994).

Several authors have reported different algal species use organic carbon substrates as source of cellular carbon and energy (Bouarab et al. 2004; Hellebust and Lin 1978; Richardson and Fog 1982; Feuillade and Feuillade 1989) under mixotrophic and/or heterotrophic conditions. Glucose has been demonstrated as organic carbon source for microalgae (Schmidt et al. 2005; Ogawa and Aiba 1981; Wen and Chen 2000). Glycerol was reported to be assimilated as carbon source by some algal species such as *Nostoc* sp., *Navicula pelliculosa*, *Agnmenelium quadruplicatum* and *Goniottichium elegans* in the presence of light and without the external supply of CO₂ (Ingram et al. 1973; Kaplan et al. 1986). Similarly, Ceron Garcia et al. (2000) have shown that growth of *Phaeodactylum tricornutum* was significantly enhanced when glycerol, in the presence of light, was used as substrate for growth. Acetate is also one of the most common carbon sources for many microbial species including microalgae (Droop 1974). *Euglena gracilis* strain L incorporate acetate efficiently in the presence of light (Cook 1967) while *Cryptothecodium* can grow in the dark on acetate (Vazhappally and Chen 1998). Uptake of dissolved carboxylic acids such as acetic, citric, fumaric, glycolic, lactic, pyruvic and succinic under microalgal heterotrophic cultivation has been reported (Bollman and Robinson 1977). The ability of microalgae to assimilate organic carbon substrates for growth, either in the dark or in the presence of light is species dependent.

The present study was undertaken to investigate the possibility of utilizing different growth regimes in enhancing biomass productivity and arachidonic acid accumulation of *P. incisa* by cultivating the microalgae in various organic carbon sources *i.e.* glucose, glycerol, sodium acetate, lactic acid and citric acid under mixotrophic and heterotrophic conditions.

**Materials and methods**

**Organism and culture maintenance**

*Parietochloris incisa* is an isolate from Mt. Tateyama in Japan. Cultures of *P. incisa* were cultivated under 22 ± 0.2°C in 300 ml flasks containing 150 ml Bold Basal Medium (BBM) composed of (per liter) 1.5g NaNO₃, 0.075g K₂HPO₄, 0.175g KH₂PO₄, 0.075g MgSO₄·7H₂O, 0.084g CaCl₂·2H₂O, 0.00498g FeSO₄·7H₂O, 0.05g EDTA.2 Na salt, 0.025g NaCl, 0.031g KÖH, 11.42mg H₃BO₃, 1.44mg MnCl₂·4H₂O, 8.82 μg ZnSO₄·7H₂O, 1.57 μg CuSO₄·5H₂O, 0.049 μg Co(NO₃)₂·6H₂O, 0.71 μg MoO₃, and carbon substrates. Illumination was provided above white cool fluorescent lamps at a light intensity of 80 μmol m⁻² s⁻¹. Flasks were incubated in a shaker at 65 rpm. Cultures are maintained by weekly changing the entire culture medium. To ensure that cells were in exponential phase of growth prior to an experiment, cultures were transferred in fresh medium and diluted daily for at least 4 days before the algal cells were used for experimental purposes.

Inocula for outdoor experiments were cultured in 5 liter Erlenmeyer flasks containing 4 liters medium and mixed by air containing 1% CO₂ (ν/ν) at a flow rate of 0.2 l min⁻¹. Cultures were maintained by changing medium at two weeks interval.

**Laboratory experimental conditions**

Carbon substrates, glucose, glycerol, sodium acetate, citric acid and lactic acid, were added to the culture medium at the rate of 0.005 M. Inorganic medium was autoclaved at 15 psi for 20 minutes and carbon substrates were separately filtered through 0.2 μm membranes. The cultures were incubated for 14 days in 300 ml flasks containing 125 ml culture medium on a shaker at 65 rpm and illuminated at light intensity of 80 μmol m⁻² s⁻¹ under 22 ± 0.2°C. Flasks were moved twice a day to eliminate positional differences in culture condition. For heterotrophic cultivation, the flasks were wrapped in aluminum foil and incubated as described above. For cultures with supplementation, carbon substrates were added at a 3 day interval while base medium was
added to control. The photoautotrophic control (control) was cultivated in BBM base medium without any organic carbon and incubated under same condition as those of mixotrophic cultures.

The effect on different glucose concentrations from 0.0025 to 0.01M was performed in a flat glass culture vessel with conical bottom (20cm width × 50cm height) and a light path of approximately 1.2cm. Photoautotrophic control was grown in BBM without glucose. The cultivation vessels were placed in transparent water bath with temperature regulated at 25 ± 1 °C. Illumination at the reactor surface was supplied at the side at 130μmol m⁻² s⁻¹ provided by cool white fluorescent lamps. Cultures were mixed by air containing 1% CO₂ (v/v) passed through 0.47μm filtration cartridge before being introduced at the bottom of the culture vessel at a flow rate of 0.31 min⁻¹. Air flow was controlled by a flow meter. The cultures were incubated for 20 days.

Outdoor experimental conditions

Vertical tubular photobioreactors (VTPR) with 5cm inner diameter, 2 meters height and a working volume of 4 liters were used for outdoor cultures. Laboratory grown inoculum was transferred to the VTPR at least 4 days prior to the start of the experiment. Mixing and CO₂ was provided by enriched air with 1%CO₂ (v/v) at the rate of 1 l min⁻¹ through perforations at the bottom of the reactor. The rate of gas flow was controlled by a flow meter. The temperature was controlled by water mist and temperature did not exceed 30°C. The cultivation period was 14 days.

Base medium was BBM (photoautotrophic) and glucose was added to BBM to a final concentration of 0.005M for mixotrophy. One gram glucose was added daily to the mixotrophic cultures. Without the supplemental addition of carbon substrate, a significantly higher cell density of 0.71 g l⁻¹ was observed in glucose grown cells. This was three times higher than that of photoautotrophic control (Fig. 1a & b). Glycerol seemed to slightly enhanced growth while growth in other carbon substrates did not differ significantly from photoautotrophic control (Fig. 1a & b). Glycerol seemed to slightly enhanced growth while growth in other carbon substrates did not differ significantly from photoautotrophic control, either they have a negligible effect or do not support growth of P. incisa. Supplementation of glucose to the culture medium further enhanced the algal growth and the cell density attained were twice that of the cell density achieved without supplementation and corresponded to six-fold increase of productivity during inoculation and 2) additional carbon substrates were provided at 3 days interval as supplements. Figure 1 shows the final biomass concentration and productivity of P. incisa after 14 days of cultivation in different organic carbon substrates in the presence of light. Glucose significantly stimulated growth under both conditions with a more pronounced effect in supplemented cultures. Without the supplemental addition of carbon substrate, a significantly higher cell density of 0.71 g l⁻¹ corresponding to productivity of 0.037 g l⁻¹ d⁻¹ was observed in glucose grown cells. This was three times higher than that of photoautotrophic control (Fig. 1a & b). Glycerol seemed to slightly enhanced growth while growth in other carbon substrates did not differ significantly from photoautotrophic control.

Fatty acid analysis

Fifty ml of culture aliquot were centrifuged at 2,000g for 5 minutes and the algal pellets were immediately frozen and freeze dried. Total fatty acid content of the dry biomass was determined following the method of Bligh and Dyer (1959). Freeze-dried cells were transesterified with methanol-acetyl chloride according to Cohen et al. (1993), heptadecanoic acid (C17) was added as an internal standard. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Supelco, Bellefonte, PA) and by calculation of the equivalent chain length. The methyl esters were analyzed with a Shimadzu GC-8A using Supelco 2560 capillary column (100 m × 0.25 mm) at 150°C (FID, injector and flame ionization detector temperature 250°C, split ratio 1: 100). Rate of temperature increase was 3 degrees per minute. Fatty acid content was determined by comparing each peak area with that of the internal standard and corrected accordingly.

Results

P. incisa was cultivated mixotrophically in various carbon substrates under two different conditions: 1) one time addition of carbon substrate during inoculation and 2) additional carbon substrates were provided at 3 days interval as supplements. Figure 1 shows the final biomass concentration and productivity of P. incisa after 14 days of cultivation in different organic carbon substrates in the presence of light. Glucose significantly stimulated growth under both conditions with a more pronounced effect in supplemented cultures. Without the supplemental addition of carbon substrate, a significantly higher cell density of 0.71 g l⁻¹ corresponding to productivity of 0.037 g l⁻¹ d⁻¹ was observed in glucose grown cells. This was three times higher than that of photoautotrophic control (Fig. 1a & b). Glycerol seemed to slightly enhanced growth while growth in other carbon substrates did not differ significantly from photoautotrophic control.

Growth measurements

Dry biomass

Ten ml of culture aliquot was filtered on tarred pre-dried GF/C Whatman filters. Filtered biomass was washed with 10 ml of acidified distilled water (pH 4) to remove inorganic salts and dried at 105°C for 3 hours. Dried samples are allowed to cool to room temperature in a desiccator over silica gel before being weighed.

Chlorophyll determination

Two ml of culture aliquots were centrifuged at 2,000g for 5 minutes. Pigments were extracted from algal pellets with DMSO in 70°C water bath for 20 minutes. The extracts were mixed, centrifuge at 2,000g for 5 minutes and measure spectrophotometrically based on Wellburn (1994).
Fig. 1. Mixotrophic growth of *Parietochloris incisa* in different organic carbon substrates. (a & b) without carbon substrate supplementation; (b & c) with carbon substrate supplementation; bars represent the standard deviation (DMRT, p=0.05, n=3).

**Carbon substrate**

Fig. 2. Changes in the biomass of *Parietochloris incisa* grown mixotrophically in different organic carbon substrates. (a) without carbon substrate supplementation; (b) with carbon substrate supplementation; Bars represent the standard deviation (DMRT, p=0.05, n=3).
In dark incubation, under heterotrophic cultivation, glucose was demonstrated to enhance the growth of *P. incisa* (Fig. 3). Glycerol, sodium acetate, citric acid and lactic acid did not exhibit any growth. It was noted that the cell density attained by glucose cultures after 14 days in dark incubation was 0.58 g l⁻¹ corresponding to a productivity of 0.028 g l⁻¹ d⁻¹, about half the values attained in the mixotrophic supplemented cultures.

Chlorophyll, fatty acid and arachidonic acid content were determined at the end of each culture. In all mixotrophic cultures, glucose exhibited significantly higher chlorophyll concentrations over the photoautotrophic control. However, under heterotrophic condition, glucose attained the lowest chlorophyll content (Fig. 4). In mixotrophy, non-supplemented cultures, a significantly higher TFA content was obtained in glycerol and glucose attained same level as that in photoautotrophic culture. The proportion of AA in TFA and AA content did not show significant differences between treatments (Table 1). On the other hand, with carbon substrate supplementation, glucose attained the lowest TFA and AA content in biomass. In dark cultivated cultures, the TFA content did not vary significantly among all carbon substrates tested and photoautotrophic control attained highest value. Glucose grown cell obtained a higher AA content relative to all the other carbon substrates tested. It was noted that under condition of mixotrophy with supplement, ARA content of glucose culture was lower than in heterotrophic and mixotrophic without supplementation.

Different concentrations of glucose from 0.0025 to 0.01 M were added to growth medium and incubated for 20 days to determine optimal concentration for biomass production. In all glucose concentrations tested, mixotrophic cultures significantly exhibited higher biomass productivities relative to photoautotrophic. A gradual decline in productivity was observed from 0.0075 M to 0.01 M glucose concentrations (Fig. 5a). TFA content did not exhibit any significant differences between treatments whereas the proportion of AA in TFA was significantly low-
Table 1: Total fatty acid and arachidonic acid content of *Parietochloris incisa* cultivated with various carbon substrates under different culture conditions.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>AA(%) in TFA</th>
<th>TFA(%) in biomass in biomass</th>
<th>AA(%) in biomass</th>
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<tbody>
<tr>
<td>Carbon substrate</td>
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<tr>
<td>Mixotrophic:</td>
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<tr>
<td>without supplement</td>
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<td></td>
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<tr>
<td>Control</td>
<td>21.47 a</td>
<td>8.27 b</td>
<td>1.77 c</td>
</tr>
<tr>
<td>Glucose</td>
<td>26.49 a</td>
<td>8.65 b</td>
<td>2.33 c</td>
</tr>
<tr>
<td>Glycerol</td>
<td>19.01 a</td>
<td>10.81 b</td>
<td>2.04 c</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>30.45 a</td>
<td>6.83 b</td>
<td>2.12 c</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>19.09 a</td>
<td>5.46 b</td>
<td>1.03 c</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>24.38 a</td>
<td>5.97 b</td>
<td>1.34 c</td>
</tr>
<tr>
<td>Mixotrophic:</td>
<td></td>
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<tr>
<td>with supplement</td>
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<tr>
<td>Control</td>
<td>40.12 ab</td>
<td>21.43 b</td>
<td>8.67 b</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.49 f</td>
<td>7.43 f</td>
<td>0.80 f</td>
</tr>
<tr>
<td>Glycerol</td>
<td>26.64 a</td>
<td>8.39 f</td>
<td>2.14 f</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>37.00 b</td>
<td>14.82 c</td>
<td>5.35 f</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>46.54 a</td>
<td>27.96 c</td>
<td>13.02 f</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>42.29 a</td>
<td>18.67 b</td>
<td>7.88 f</td>
</tr>
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<td>Heterotrophic:</td>
<td></td>
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<td>with supplement</td>
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<td>Glucose</td>
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<td>10.74 b</td>
<td>1.90 b</td>
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<td>Glycerol</td>
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<td>4.35 d</td>
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</tr>
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<td>1.41 b</td>
</tr>
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<td>Citric Acid</td>
<td>13.45 b</td>
<td>5.77 d</td>
<td>0.91 e</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>17.05 b</td>
<td>7.74 d</td>
<td>1.30 e</td>
</tr>
</tbody>
</table>

*Carbon substrate was added at 0.005M in all treatments.
-14 days of incubation.
*Carbon substrate was added at 3 days interval,
-means followed the same letter are not significantly different at 5% level using DMRT.

Mixotrophic cultivation in outdoor conditions was investigated. Growth and arachidonic acid content were measured. Photoautotrophic cultures exhibited a significantly higher biomass concentration and productivities over mixotrophic cultures up to 11 days of cultivation and the values converged at day 14 (Fig. 6a & b). Chlorophyll content was observed to be significantly lower in mixotrophy (Fig. 6c). TFA in biomass did not vary significantly between photoautotrophic and mixotrophic cultures, however mixotrophy significantly increase the proportion of AA in TFA, AA content in dry biomass (Fig. 7a) resulting in a higher volumetric arachidonic acid yield (Fig. 7b).

**Discussion**

Currently, there are no available report on the mixotrophic and heterotrophic growth of *P. incisa*. This may be the first attempt to grow this alga using organic carbon sources as substrate for growth.

The obtained result of this study clearly demonstrates that *P. incisa* has the capability to utilize organic carbon substrates for growth in the light (mixotrophic) and in the dark (heterotrophic). Glucose, glycerol, sodium acetate, lactic acid and citric acid were added to the medium at a single dose. Only glucose resulted in substantial stimulation of growth, the other carbon substrates tested did not appreciably enhance growth though glycerol seemed to slightly stimulate growth. Glucose is the most commonly used organic carbon source and was reported to increase microalgal productivities by mixotrophic growth. Higher rates in 0.0025M and AA content of dried biomass was significantly lower in concentrations 0.0025 (Fig. 5b).

![Graph](image)

**Fig. 5.** Response of *Parietochloris incisa* to different glucose concentrations. (a) biomass productivity; (b) total fatty acid and arachidonic acid content; Growth conditions: light intensity of 130μmol m⁻²s⁻¹; temperature of 25±1°C; Mixing and CO₂ was supplied by 1% CO₂-enriched air (v/v) bubbled from the bottom of the culture vessel. Bars represent the standard deviation (DMRT, p=0.05, n=3).
of growth and respiration are obtained with glucose than any other substrate because glucose contains more energy per mole compared to other substrate (Griffiths et al. 1960). A 2.4 times higher productivity than that obtained by photoautotrophic for *Spinifer platensis* cultures was reported by Cheng and Zhang (1997).

It was hypothesized that supplementation of carbon sources in the growth medium throughout the duration of the cultivation time would likely sustain higher cell concentration and biomass productivities than a single dose of the organic carbon source. Indeed, supplementation of glucose increased the biomass productivity from 0.037 g l⁻¹ d⁻¹ attained for single dose to 0.07 g l⁻¹ d⁻¹ for supplemented cultures. This indicated that glucose was actively utilized and was translated to biomass. Depletion of supply in the medium could have resulted in the reduction in the potential productivity in the single dose regime. This was supported by the observed surge in growth of the supplemented glucose culture after the third addition of the organic carbon substrate. Supplementation of glycerol, sodium acetate, citric acid and lactic acid resulted in the inhibition of growth. Since these carbon sources were not utilized for growth as the result suggested in the single dose batch cultures, their accumulation in the medium due to supplementation could have resulted to inhibitory levels. Organic carbon sources above the critical level suppress algal growth. *Galdieria sulphuraria* grown in high concentrations of glucose or fructose was observed to thrive up to 0.9 M concentration but growth was inhibited at higher concentration (Schmidt et al. 2005). Glycerol noticeably supported *P. incisa* growth in the presence of light; however the alga did not grow in glycerol when incubated in the dark. This indicates that this algal species requires light for glycerol assimilation. Algal species such as *Nostoc* sp., *N. pelliculosa*, *A. quadruplicatum* and *G. elegans* were demonstrated to assimilate glycerol only in the presence of light (Ingram et al. 1973; Kaplan et al. 1986). Ceron-Garcia et al. (2006) reported that glycerol was stimulatory to growth of *P. tricornutum* under mixotrophy, however the microalga was incapable of heterotrophic growth in glycerol.

The same organic carbon substrates for mixotrophic cultivation were used to explore the capacity of *P. incisa* for heterotrophic growth. Similar to those incubated in the presence of light, glucose supported the
growth of the alga in the dark attaining a biomass productivity of 6.8 fold higher than photoautotrophic control. Some studies suggested that mixotrophic growth is the sum of heterotrophic as well as phototrophic growth (Lee 2001). Wen and Chen (2000) reported that mixotrophic productivity was slightly lower than the sum of phototrophic and heterotrophic cultures of Nitzchia laevis and attributed the low productivity to light limitation in the mixotrophic cultures as caused by mutual shading as an effect of rapid growth which was not attained in the photoautotrophic culture. In this study it was be noted that the productivity of glucose mixotrophic cultures with supplementation was 2.3 times higher than that of heterotrophic cultures which was also supplemented. This may probably be explained by the stimulatory effect of light on metabolism of sugar. In the presence of light, algae are reported to utilize glucose more efficiently (Ogawa and Aiba 1981; Marquez Sasaki et al. 1995; Lalucat et al. 1984). The presence of weak light (8 μEm⁻²s⁻¹) induces a growth rate relatively higher than total darkness (Bouarab et al. 2004). The role of weak light was interpreted by Neilson and Lewin (1974) as a result of a stimulation of the synthesis of the necessary cytochromes for the respiratory chain and these cytochromes could be destroyed by high light in some algal species. Since the cultures were not supplemented with CO₂ and that it is known that presence of sugar as carbon substrate enhance respiration, the CO₂ evolved in respiration could have been utilized for photosynthesis thus contributing to photosynthetic growth. Furthermore, Pearce and Carr (1978) demonstrated that in the presence of light, restricting the availability of CO₂ to the level found in air induces Anabaena variabilis to use organic compounds as major source of cellular carbon. The lower productivity obtained in the non-supplemented mixotrophic cultures may probably be attributed to the depletion of glucose in the culture medium due to rapid assimilation.

The higher chlorophyll content of cells grown mixotrophically with glucose in relation to control is in line with the other reports that mixotrophic cultures have higher growth rates and chlorophyll content compared to photoautotrophic controls (Ellis et al. 1975; Ogawa and Aiba 1981; Marquez Sasaki et al. 1995). However, in heterotrophically incubated cultures, the glucose cultures exhibited the lowest chlorophyll. This observation is supported by others that one major disadvantage of heterotrophic growth is the reduction in levels of light induced products such as pigments, chlorophyll and carotenoids (Chintzi et al. 2000; Day and Tsavalos 1996, Ogbonna et al. 1997). Glucose modifies the pigmentary system (Lalucat et al. 1984) and was reported to induce physiological changes in Chlorella vulgaris, which strongly affects the metabolic pathways of carbon assimilation, size of cells, and volume densities of storage materials, such as starch and lipids grains (Martinez et al. 1991) and protein, chlorophyll and vitamin contents (Endo et al. 1974).

Glucose grown cells exhibited higher TFA content in heterotrophic condition relative to mixotrophy. The higher total lipids in heterotrophically grown cells are hypothesized to be due to the accumulation of more storage lipids arising from the increased availability of carbon in the substrate (Roesller 1990) or the competition in phototrophic cultures of the lipid and photosynthetic enzyme for the available CO₂ (Radwan and Mangold 1980). Results obtained in this study were not conclusive but demonstrate that the effect of organic carbon on TFA and AA content of P. incisa seemed to be affected by the specific organic substrate, substrate concentration and life cycle of the alga as well as other growth conditions and needs further detailed investigations.

It is important to mention that one drawback that was noted and must be addressed before mixotrophy and heterotrophy can be used for biomass production of P. incisa is the formation of zoospores. Massive zoospore formation was observed in glucose cultures with carbon substrate supplementation and to a limited extent in heterotrophic cultures. This phenomenon resulted in young small unicellular cells and basically explains the very low TFA and ARA content obtained in mixotrophic glucose supplemented cultures. Since the only variable factor between the mixotrophic with supplementation and heterotrophy was light, it can be postulated that glucose at a certain concentration can induce zoospore formation and this effect was further enhanced by the presence of light. Previous experiences in outdoor cultivation of P. incisa have indicated low temperature as another factor that triggers massive zoospore formation in this microalgal species. Currently, literature on zoospore formation of P. incisa is nil.

Test on optimal glucose concentration showed that glucose from 0.0025 to 0.01M significantly enhanced biomass productivity relative to photoautotrophic control with a decline observed from 0.0075M to 0.01M. It was also noted that the effect of glucose on cell density and biomass productivity in the presence of CO₂ supplementation was not as pronounced as when CO₂ was not supplemented in the cultures as in the first experiment. This seems to suggest P. incisa has a higher affinity for CO₂ over glucose assimilation in the presence of sufficient CO₂ to support its growth. The growth stimulation by glucose observed in the presence of 1% CO₂ is taken as evidence that the cells were still using glucose as an additional carbon source under the experimental conditions. Another explanation that can be forwarded is the effect of light intensity on glucose uptake. Kamiya and Kowallik (1987a) reported that glucose uptake is light sensitive. The illumination provided in this trial was 130μmol m⁻²s⁻¹ which was more than twice the light intensity provided in the first experiment. In the presence of light, Scenedesmus acutus and Spirulina platensis were able to utilize glucose more efficiently (Ogawa and Aiba 1981; Marquez Sasaki et al. 1995; Chen et al. 1996) than in heterotrophic, however another report on C. vulgaris, showed light suppressed glucose uptake by inhibiting expression.
of hexose/H⁺ symport system particularly the blue end of the visible spectrum and the red light is only slightly effective (Kamiya and Kowallik 1987b). This result indicates that under low light, the uptake of glucose may not be inhibited and the presence of light facilitated efficient utilization of glucose. At higher light intensity, the uptake of glucose may have been inhibited. Furthermore, Hellebust and Gresley (1987) reported that at optimal light intensity, the growth rate already reached maximum and thus the effect of organic substrate is minimum.

The presence of CO₂ as well as higher light intensity in this experiment could have reduced the effect of glucose on growth of *P. incisa*.

Though there were no significant differences in the fatty acid contents between treatments, it seemed that glucose concentration form 0.005 to 0.01M partially decreased the AA content of *P. incisa*. Glucose at high concentrations can impose inhibition to algal growth and the inhibitory concentration is species dependent. In all glucose concentrations, zoospores were not observed. This result establishes the possibility of using mixotrophic cultivation for biomass production in the laboratory, though a more detailed growth optimization is essential before this cultivation regime can be utilized.

Under laboratory conditions, it was shown that mixotrophy using glucose as carbon substrate could increase biomass productivity and the possibility of employing mixotrophy in outdoor conditions was explored. Photoautotrophy attained higher biomass productivity over mixotrophy up to day 11 of cultivation. This result does not conform to other reports where mixotrophy were shown to increase productivity over photoautotrophy (Wen and Chen 2000; Behrens 2005; Ceron Garcia et al. 2000; Chu et al. 1996; Day and Travales 1996). Since the cultures are grown outdoors in full sunlight, this may be due to the inhibitory effect of light on glucose uptake as reported by Kamiya and Kowallik (1987b).

TFA content seemed to be unaffected by the presence of glucose in mixotrophy. However, under outdoor conditions, the proportion of AA in TFA, AA content in dry biomass and AA volumetric yield were significantly higher in mixotrophic over photoautotrophic cultures. The high AA content was attributed more on the increase of AA proportion in TFA rather than to the increase of TFA content in dry biomass. This resulted to a significantly higher volumetric yield of AA in mixotrophic culture. The decrease in chlorophyll content and the higher AA proportion in TFA could possibly be attributed to an increase in the cellular C:N ratio which could have shifted the culture to N-limited condition. Nitrogen deprivation is reported to significantly increase AA content in dry biomass of *P. incisa* (Cheng-Wu et al. 2002).

The most significant result of this study was the clear demonstration that *P. incisa* can utilize organic substrate, glucose in particular, for laboratory biomass production under specific culture conditions. Productivity of 7.8, 15.5 and 6.8 fold higher than the photoautotrophic control was attained under mixotrophy without supplementation, mixotrophy with supplementation and heterotrophy with supplementation, respectively. These results unfold novel potential for the development of *P. incisa* mass cultivation for commercial production of AA. More detailed investigations to elucidate the effect of different cultural and environmental conditions and the combination of these factors to effect optimal biomass productivity and AA accumulation in *P. incisa*.

Acknowledgements

This research was supported by T. Akiyama and Co. and Naigai Chemical Products Co., Ltd.

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(Received 6 June, 2011; Accepted 15 July, 2011)