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Combined effects of irradiance level and carbon source on fatty acid and lipid class composition in the microalga *Pavlova lutheri* commonly used in mariculture

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ABSTRACT

Pavlova lutheri, a marine Pavlovophyceae, has been well documented as it is commonly used as a food source in mariculture. In this study, we investigated the combined effects of carbon sources and irradiance levels on the growth, lipid classes and fatty acid profiles of this microalga. The microalga was cultured at 15 °C with a 14 h photoperiod in artificial seawater containing bicarbonate or acetate as carbon source.
The growth and lipid composition of *P. lutheri* were more sensitive to variations in light intensity than in carbon source. However, *P. lutheri* seems to be able to use acetate to growth cell and lipid metabolism. With the both carbon source, the lowest cellular lipid contents were obtained under low light intensity. The proportions of PUFAs, especially EPA, were significantly higher under low light, and saturating fatty acids and DHA levels were significantly higher under high light. In *P. lutheri*, galactolipids, a major component of chloroplast lipid membranes, made up approximately 54-66% of total lipids. The highest PUFA levels, such as those of EPA, were predominantly found in the galactolipid fraction when the cells were grown at low light, regardless of the carbon source. The consequent accumulation of n-3 fatty acids in the galactolipids could facilitate thylakoid membrane fluidity, and therefore the velocity of electron flow involved in photosynthesis during light acclimatization. These results could be used to optimize the culture conditions and the nutritional value of this microalga, which is used to feed marine invertebrates and fish larvae in mariculture hatcheries, and to produce n-3 fatty acids for human health care and nutrition.

1. Introduction

Microalgae are currently used as food sources for larval and juvenile molluscs, crustaceans and fish species in mariculture hatcheries (Brown et al., 1997). Their nutritional value is often correlated to their biochemical composition; especially their lipid content and fatty acid composition (Thompson et al., 1996). Several microalgal species are known to synthesize and accumulate large amounts of polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) (Moreno et al., 1979; Volkman et al., 1989). Long chain n-3 fatty acids are also recognized as having a number of important neutraceutical and pharmaceutical applications (Apt and Behrens, 1999; Ward and Singh, 2005). They are known to have health benefits for the human organism, and to play a major role in preventing medical disorders in three areas: heart and circulation (Dyerberg and Bang, 1979; Din et al., 2004), inflammatory conditions (Babcock et al., 2000), and cancer (Bourre, 2007). As a result, a number of studies have focused on microalgal culture conditions with the aim of improving the nutritional value of algal foods (aquaculture) and/or to boost PUFA production (pharmaceutical industry).

It is well known that environmental factors (temperature, salinity, light, nutrients, etc.) and culture time affect the growth and biochemical composition of microalgae (Reitan et al., 1994; De Castro Araújo and Tavano Garcia, 2005; Liang et al., 2006; Ranga Rao et al., 2007). Specifically, significant variations in lipid contents and fatty acid profiles are observed in response to differing growth conditions (Yongmanitchai and Ward, 1991; Carvalho et al., 2006; Petkov and Garcia, 2007). Much of the published data have reported the influence of temperature on the fatty acid composition of microalgae (Nagashima et al., 1995; Rousch et al., 2003). It is generally recognized that an increase in PUFAs might be one of the ways marine algae acclimatize to low-temperature conditions and maintain membrane fluidity (Tatsuzawa and Takizawa, 1995; Jiang and Gao, 2004). The fatty acid composition is also known to be affected by the level of irradiance (Blanchemain and Grizeau, 1996; Guihéneuf et al., 2008). However, the light conditions that produce the highest proportion of the essential fatty acids are species specific (Thompson et al., 1990). In most species, the highest proportions of EPA were obtained at low levels of irradiance, whereas DHA levels were highest under high light intensities (Thompson et al., 1990; Brown et al., 1993). High PUFA

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levels were observed at low light, suggesting that the biochemical composition of chloroplasts was adapting to low irradiance intensities by increasing PUFA synthesis (Sukenik et al., 1989; Khotimchenko and Yakovleva, 2005).

Previous studies have also reported that the carbon source affects growth and fatty acid composition in numerous species (Wood et al., 1999; Wen and Chen, 2003). Under natural conditions, the carbon source usually used for photosynthesis by algae is sodium bicarbonate ($NaHCO_3$) and/or carbon dioxide (CO_2). Furthermore, many microalgae are able to grow rapidly and heterotrophically in the dark using organic carbon sources such as sugars or organic acids (Wen and Chen, 2003; Cerón García et al., 2005). When the cells are exposed to light, some microalgae can utilize both inorganic and organic carbon simultaneously, and develop a mixotrophic metabolism (Poerschmann et al., 2004). Wood et al. (1999) have previously reported that acetate is a good carbon source for photoheterotrophic growth, yielding reasonably high PUFA levels in freshwater algae. However, acetate has a negative effect on the growth of some marine microalgae (Wood et al., 1999), such as *Phaeodactylum tricornutum* (Cerón García et al., 2005). Consequently, strategies to improve algal biomass and fatty acid production under indoor conditions whilst keeping production costs low (dependent on the need for light) involve mixotrophic, photoheterotrophic or heterotrophic algal growth systems (De Swaaf et al., 1999; Wood et al., 1999; Cerón García et al., 2005).

Pavlova lutheri (Droop) Green, the organism investigated here, is a common Pavlovophyceae used in microalgal laboratories, known to produce high levels of PUFAs, especially EPA and DHA (Tatsuzawa and Takizawa, 1995; Carvalho et al., 2006), and which is commonly used in the aquaculture industry to feed marine organisms (Thompson et al., 1996; Leonardos and Lucas, 2000; Ponis et al., 2006a,b). Some growth conditions, such as temperature and nutrients, have already been described for this microalga (Tatsuzawa and Takizawa, 1995; Carvalho and Malcata, 2000; Carvalho et al., 2006). However, no studies have so far reported the combined effects of light intensity and carbon source on the lipid composition of *P. lutheri*. We set out to investigate the simultaneous impacts of irradiance level and carbon source on growth, lipid class and fatty acid composition of *P. lutheri*. To do this, cells were cultured at different irradiance levels after changing the carbon source in the culture medium by replacing sodium bicarbonate with sodium acetate.

2. Material and methods

2.1. Microalgal cultures

Pavlova lutheri cells were obtained from the microalgal culture collection of the "Centre de Ressources Biologiques" (Université de Nantes, France). After three successive cultures with 1% antibiotic/antimycotic (A5955, Sigma-Aldrich, St. Quentin Fallavier, France; formulated to contain 10000 units ml^{-1} penicillin G, 10 mg ml^{-1} streptomycin sulfate, 25 µg ml^{-1} amphotericin B), an axenic culture was obtained. Culture "axenicity" was tested on two medium, A (bacto-pectone, 3 g l^{-1}; yeast extract, 1 g l^{-1}; ammonium sulfate, 1 g l^{-1}; sodium glycerophosphate, 25 mg l^{-1}; iron (Fe^{3+})-EDTA, 6 mg l^{-1}; ASW 50%, qsp 1000 ml) and B (bacto-pectone, 4 g l^{-1}; yeast extract, 500 mg l^{-1}; sodium glycerophosphate, 25 mg l^{-1}; iron (Fe^{3+})-EDTA, 6 mg l^{-1}; ASW 75%, qsp 1000 ml), respectively conductive to the growth of fungi and bacteria. The microalgae were then grown under batch conditions in 500 ml Erlenmeyer flasks sealed with cotton plugs that allow exchanges with atmosphere (carbon dioxide and oxygen released during microalgae respiration and photosynthesis, respectively), using a working volume of 300 ml. The culture medium was artificial seawater (Harrison et al., 1980) complemented following De Brouwer et al. (2002). The medium enrichment was composed as previously described by Perkins et al. (2006): Fe-NH_4-citrate (1.37 µM final concentration), $CuSO_4$ $5H_20$ (0.04 µM f.c.), folic acid (0.18 nM f.c.), nicotinic acid (0.0325 µM f.c.), thymine (0.95 µM f.c.), Ca-*d*-pantothenate (8.39 nM f.c.) and inositol (1.11 µM f.c.). The culture medium contains 0.174 g l^{-1} sodium bicarbonate or 0.169 g l^{-1} sodium acetate as the carbon source. These two concentrations correspond to 2.07 mM which is the usual concentration of bicarbonate in artificial seawater. The medium was prepared with deionized water, and had been autoclaved at 121 °C for 20 min. Inocula were carried out using exponentially growing cells, after two generations without antibiotic/antimycotic, and with an initial density of 10^5 cell ml^{-1}. For each carbon source, the cultures were maintained at 15±1 °C under three irradiance levels: low light intensity (LL-20) with 20 µmol photons m^{-2} s^{-1}; medium light intensity (ML-100) with 100 µmol photons m^{-2} s^{-1}; and high light intensity (HL-340) with 340 µmol photons m^{-2} s^{-1}; provided by cool-white fluorescent lamps (PHILIPS 18 W), attenuated by distance and/or neutral density screening, and using 14/10 h light/dark cycle. Light intensity was measured as PAR (Photosynthetic Active Radiation) using a 4π US-SQS/L Quantum Sensor (Walz Instruments, Effeltrich, Germany), coupled to a LI-189 Data Logger (LI-COR Biosciences, ScienceTec, Les Ulis, France). Three replicate cultures were grown under each irradiance level and with each carbon source.

2.2. Growth, chlorophyll *a* and lipid contents

After taking a sample of 2 ml in a laminar flow hood, growth was monitored daily by spectrophotometric measurements of the optical density of cell suspensions at 750 nm ($Y=10^{-7}X$, with Y the optical density at 750 nm and X the cell density), by using a 2.5 ml plastic cuve. The cell number was determined with a Malassez improved bright-line hemocytometer, after immobilizing the cells with Lugol 5%. No special precautions have been taken to prevent carbon dioxide re-equilibration during daily sub-sampling. Specific growth rates (µ, d^{-1}) were calculated from the increase in cell density during the exponential growth phase, between days 12 and 19, according to the equation $\mu=\ln(N_2/N_1)/(t_2-t_1)$, where N is cell density and t is the time. Pigments were extracted in dimethylformamide, and total chlorophyll *a* (Chl *a*) was determined by spectrophotometry (Speziale et al., 1984). Cells were gently harvested by centrifuging at low-speed (1200 *g*, 10 min) using a Sigma 4K15 centrifuge (Bioblock Scientific, Illkirch, France). The pellets obtained were then frozen, and stored at -70 °C prior to analysis. Chl *a* and lipid contents were estimated during the mid-exponential growth phase.

2.3. Extraction of total lipids

All chemicals used in the experiments were of analytical grade, and were purchased from Carlo Erba (Val de Reuil, France). Total lipids were extracted with methanol/chloroform (2/1, v/v) after adding 200 µL of 2.8 g l^{-1} NaCl, a modified version of Bligh and Dyer's (1959) method, using manual crushing (Dounce cells grinders) coupled with ultrasonication (twice, for respectively 15 and 30 min). Chloroform (1 ml) was added between the two ultrasonication steps in order to allow phase separation. The chloroform layer, containing the lipids, was collected and second extraction was carried out by adding 2 ml of chloroform to the remaining methanol/water phase. The solvents were removed by evaporating under vacuum, and all samples were dissolved in a known volume of chloroform. The lipid extracts were stored at -20 °C under nitrogen gas (N_2) to limit oxidization until analysis.

2.4. Lipid class separation

Total lipid extracts were fractionated on reversed phase silica gel columns (Sep-Pak Plus silica cartridges, Waters, St. Quentin en Yvelines, France) after an activation step with 20 ml of methanol followed by 20 ml of chloroform. Neutral lipids were eluted using 20 ml of chloroform, polar lipids such as galactolipids were eluted with 40 ml of chloroform/methanol (5/1, v/v), and the phospholipids were recovered in 30 ml of methanol (Sukenik et al., 1989). The fractions were reduced in volume by evaporating under a stream of N_2.