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Interactive effects of light and temperature on pigments and n-3 LC-PUFA-enriched oil accumulation in batch-cultivated *Pavlova lutheri* using high-bicarbonate supply

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ABSTRACT

We investigated the simultaneous effects of light and temperature on pigments, lipid remodeling, and omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) partitioning into lipid classes of *P. lutheri*. Biomass was produced in batch cultivation using high bicarbonate supply, and hence, any potential accumulation of lipid and triacylglycerols (TAG) containing n-3 LC-PUFA was triggered by nitrogen (N) limitation. The maximum productivities of both eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids were obtained after nitrate-depletion when *P. lutheri* was cultivated at optimal growth conditions; these occurred at an intermediate temperature of 18 °C and low light intensity (40 μmol photons·m^{-2}·s^{-1}) for EPA, but there was no impact of light (40 or 200 μmol photons·m^{-2}·s^{-1}) for DHA production. Under these respective optimum conditions, TAG accounted for 82–84% of TFA on day 15 of cultivation, which contained up to 76–84% of the total cellular EPA and 67–81% of total cellular DHA. Our results clearly demonstrate the process of lipid remodeling in *P. lutheri* and, for the first time, a potential membrane lipid turnover with transfer of n-3 LC-PUFA (EPA and DHA) from membranes (polar lipids, PL) to storage lipids (TAG), highlighting the accumulation of n-3 LC-PUFA-rich oil during N-starvation (i.e., TAG containing: EPA ~12–13% and DHA ~5–6% of TFA). When cultivated under low light conditions, *P. lutheri* additionally accumulated substantial quantities of antioxidant pigments (i.e., fucoxanthin, diadinoxanthin and β-carotene), which add value to extracted bioactive oil for functional food applications. Omega-3 rich oil accumulation and pigment levels in *P. lutheri* appear to be simultaneously regulated by both light and temperature, in addition to N-limitation during batch-cultivation.

1. Introduction

The haptophyte *Pavlova lutheri* is a marine microalga known to be rich in long-chain polyunsaturated fatty acids (LC-PUFA) and able to produce large amounts of omega-3 (n-3) fatty acids such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [1]. Due to this characteristic, *P. lutheri* represents a valuable food source in mariculture hatcheries [2]. Recently, a *P. lutheri* crude lipid extract rich in EPA and DHA as well as pigments, was reportedly effective in the prevention of chronic inflammation-linked metabolic diseases such as cardiovascular disease, and it was suggested that lipid extract can inhibit lipopolysaccharide-induced inflammatory pathways in human macrophages [3].

Carotenoids, as well as other pigments derived from algae, have received much attention due to their potential health benefits (e.g., anti-obesity, anti-diabetic, anti-cancer and anti-oxidant properties) [4,5]. There is also an increasing interest in naturally produced pigments with potential use as natural colorants [6]. Fucoxanthin (Fx), β-carotene (β-car), lutein, astaxanthin and others carotenoids are especially well known for their photoprotective and antioxidant properties [7–10]. The pigment composition of *P. lutheri* may, thus, be of particular interest due to the presence of Fx and β-car, diadinoxanthin (DD) and diatoxanthin (DT) [3,11,12].

Light quality and quantity, as well as temperature, are critical parameters for the regulation of autotrophic growth of microalgae, and specifically the synthesis and accumulation of high-value products. Optimized and cost-effective cultivation strategies need to be developed that enhance the production of several different algal cell components, with the potential to apply the 'biorefinery concept' to serve multiple

Abbreviations: AFDW, ash-free dry weight; β-car, β-carotene; Chl, chlorophyll; DHA, docosahexaenoic acid; DD, diadinoxanthin; DT, diatoxanthin; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; Fx, fucoxanthin; GC-FID, gas chromatography-flame ionization detector; HPLC-DAD/FLD, high performance liquid chromatography-diode array detector/fluorescence detector; n-3 LC-PUFA, omega-3 long-chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; N, nitrogen; PDAT, phospholipid:diacylglycerol acyltransferase; PL, polar lipids; ROS, reactive oxygen species; SFA, saturated fatty acids; SD, standard deviation; TAG, triacylglycerols; TChl, total chlorophylls; TCar, total carotenoids; TFA, total fatty acids; TP, total pigments.

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applications (e.g., human and animal nutrition, pharmaceuticals and biofuels).

Previous studies have demonstrated that irradiance and temperature play a major role in the determination of growth and proximate biochemical composition of *P. lutheri* [1,13,14]. In addition, nutrients, medium pH, and carbon supplies are essential components impacting on growth and lipid metabolism of microalgae [15,16]. In photoautotrophic microalgae, LC-PUFA are mainly accumulated in complex polar lipids (i.e., glycolipids and phospholipids) constituting the membranes, while triacylglycerols (TAG) are predominantly constructed of saturated (SFA) and monounsaturated (MUFA) fatty acids [17–19]. LC-PUFA partitioning into TAG has been reported to occur only within a few microalgal species (e.g., *Parietochloris incisa*, *Nannochloropsis oculata*, *Thalassiosira pseudonana*, and *Phaeodactylum tricornutum*) [18,20,21]. However, we recently demonstrated that accumulation of lipids and TAG containing n-3 LC-PUFA was also inducible in *P. lutheri* [22].

Despite the renewed interest in bioactive pigments, their concentrations, especially under optimal growth conditions, are often too low to make microalgae-based pigment production economically feasible. In some green microalgae (e.g., *Dunaliella salina* and *Haematococcus pluvialis)*, specific stress conditions such as over-saturating light, nutrient-deficiency or high salt concentration induce the overproduction of secondary carotenoids, β-car and astaxanthin [23–25]. Secondary pigments, including astaxanthin, β-car and lutein, are known to play a scavenging role and prevent cell damage by reacting with reactive oxygen species (ROS) [26]. By contrast, the synthesis of primary pigments requires carbon dioxide and nutrients, such as inorganic nitrogen and phosphate in addition to light [27]. Microalgal pigment production requires detailed knowledge of their respective *in vivo* functions, the factors controlling their concentration and composition, as well as the metabolic pathways involved in their synthesis. Although pigment synthesis and production in green microalgae has been investigated intensively, fewer studies have focused on pigment metabolism and accumulation in other taxonomic groups such haptophytes. Zapata et al. [28] descriptively investigated the pigment composition of 37 species (65 strains) of cultured haptophytes using improved high performance liquid chromatography (HPLC) methods. Subsequently, Seoane et al. [29] reported the effect of light intensity on the growth rates and pigment patterns of 11 haptophytes isolated from estuarine waters, showing that changes were highly species-specific. However, the effects of environmental factors or stressors on pigments in haptophytes are even less well documented [11,12,30]. Although *P. lutheri* may be a potential candidate for pigment production, no studies have been performed so far on the effect of abiotic factors on its specific pigment composition.

After demonstrating that adequate bicarbonate addition enhanced growth, lipid and TAG accumulation, as well as n-3 LC-PUFA partitioning into TAG [1,22], this study aimed to optimize further the cultivation process. Therefore, we probed the interactive effects of two light intensities, a non-saturating low light (LL-40, 40 μmol photons$\cdot m^{-2}\cdot s^{-1}$), and a saturating high light (LL-200, 200 μmol photons$\cdot m^{-2}\cdot s^{-1}$), and a large range of temperatures (8–28 °C) on pigments, lipid-remodeling, and n-3 LC-PUFA partitioning into lipid classes of *P. lutheri* in batch cultivation using a pre-determined high bicarbonate supply. This approach allowed the assessment of the effects of both factors, conjointly to nutrient-limitation occurring over time, thus aiding the development of culture strategies to produce lipid extract from *P. lutheri*, rich in n-3 LC-PUFA and pigments, as a potential functional food ingredient.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Axenic *Pavlova lutheri* (Droop) J. C. Green CCAP 931/6, formerly named *Diacronema lutheri* [31], was purchased from the Culture Collection of Algae and Protozoa at the Scottish Marine Institute (SAMS Research Services Ltd., Oban, UK). F/2-RSE, as described by Guihéneuf and Stengel [22], was used as culture medium in the experiment. The medium was sterilized by autoclaving for 20 min at 120 °C.

P. lutheri was pre-cultivated under batch condition with dilution each 3–4 days, to allow nutrient sufficiency before starting the experiment, in growth chambers with adjustable light cassettes (Binder GmbH, Germany) at 20 °C and continuous light (100 μmol photons$\cdot m^{-2}\cdot s^{-1}$) provided by Lumilux cool daylight fluorescent lamps (OSRAM L18W/865, Germany). Continuous light was used to avoid any effects of changes in time of daily harvesting on the content and composition of the various components analysed in this study.

The combined light and temperature experiment was then performed using identical medium supplemented with a pre-determined high bicarbonate supply in order to promote n-3 LC-PUFA-enriched oil production [22]. After autoclaving and before inoculation, 1.5 $g\cdot L^{-1}$ of sodium bicarbonate ($NaHCO_3$) were added and pH adjusted to ~8.0 by adding 1 M HCl under sterile conditions. *P. lutheri* pre-cultivated was inoculated in 2 L Erlenmeyer flasks containing 1.6 L of fresh medium, reaching an initial cell density of 1×10^6 cell$\cdot mL^{-1}$. Cultures were then placed to grow under batch conditions in different growth chambers at temperatures 8, 12, 18 and 28 °C, under two light intensities and over a total period of 18 days. The two light intensities were selected based on previous studies [1,13,32], as a non-saturating low light (LL-40, 40 μmol photons$\cdot m^{-2}\cdot s^{-1}$) and a saturating high light (HL-200, 200 μmol photons$\cdot m^{-2}\cdot s^{-1}$). The experiment was carried out with three independent replications for each treatment ($n = 3$). Samples were collected on days 2, 4, 7, 10, 15 and 18 to determine growth parameters, nitrate uptake, and pigment, lipid and fatty acid composition, as described following.

2.2. Growth parameters, nitrate uptake, biomass harvesting and storage

Culture growth was estimated on the basis of cell density determined using a Neubauer hemocytometer after immobilizing the cells with Lugol 5% and appropriate dilution. Maximum growth rates (μ_{max}, day^{-1}) were calculated from the increase in cell density during the exponential growth phase (i.e., between days 7–10 at 8 °C, and between days 2–4 at 12, 18 and 28 °C), while average growth rates ($\mu_{average}$, day^{-1}) determined between day 0 and days 15–18, according to the equation $\mu = \text{Ln}(N2/N1) / (t2\text{-}t1)$, where N is the cell density and t is the time. Due to salt precipitation occurring during growth using high initial $NaHCO_3$ concentrations, the biomass and all parameters were estimated and correlated to ash-free dry weight (AFDW) instead of dry weight. Briefly, 10 mL of culture were filtered and further washed with two times 20 mL of 0.5 M ammonium formate using glass microfiber filters (Fisherbrand MF300, 47 mm, nominal pore size 0.7 μm) precombusted at 450 °C for 2 h. The filters were then oven-dried overnight at 95 °C and dry weight determined. Upon drying, the filters were burnt in an oven at 450 °C for 4.5 h and weighed to determine the AFDW.

Nitrate concentration in the medium was determined according to method reported by Collos et al. [33] and modified as described by Guihéneuf and Stengel [22].

Algal biomass was harvested by gently centrifuging (×1200 *g* for 10 min) using a Hettich Rotina 38R centrifuge (Andreas Hettich GmbH, Germany). The cell pellets obtained were then frozen, and stored at −20 °C prior to analysis.

2.3. Total lipid extraction and lipid class separation

All chemicals used were of analytical grade, and were purchased from Fischer Scientific (Leicestershire, LE11 5RG, UK). Total lipid extraction was performed according to a modified version of Bligh and Dyer [34] using a chloroform/methanol/water (2/2/1, v/v/v) system coupled to ultrasounds (twice, 15 and 30 min, respectively). The chloroform fraction containing the total lipids was evaporated under high vacuum using a rotary evaporator (Büchi, Switzerland) and all samples were dissolved in 4 times 1 mL of chloroform, and then concentrated under a