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The influence of culture conditions on biomass and high value product generation by *Nannochloropsis gaditana* in aquaculture

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ABSTRACT

The effect of culture conditions on the productivity of biomass and high value products by microalgae has been studied in order to maximise these parameters and meet the nutritional requirements for aquaculture. To this end, a range of temperatures (15–30 °C), incident photon flux densities (PFD) (250–1600 μmol·s^{-1}·m^{-2}) and dilution rates (0.1–0.5 d^{-1}) have been tested in indoor *Nannochloropsis gaditana* cultures. Predictive models for biomass and high value product productivities are presented. Photoinhibition was observed in cultures at both high incident PFD and temperature. The maximum biomass, EPA, vaucheriaxanthin and β-carotene productivities (0.429 g·L^{-1}·d^{-1} and 17.2, 1.05 and 1.31 mg·L^{-1}·d^{-1}, respectively) were achieved at 25 °C, with an average irradiance of 170 μmol·s^{-1}·m^{-2} and a dilution rate of 0.3 d^{-1}.

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1. Introduction

Microalgae are able to biosynthesise high value chemicals such as proteins, long chain polyunsaturated fatty acids (LC-PUFAs) [1] and pigments [2]. However, microalgal growth and biochemical composition are highly influenced by the culture itself and the environmental conditions to which the culture is subjected [3]. As a result, microalgae find different uses depending on their biochemical profile and the high value products accumulated [4]. Aquaculture is perhaps the most important field in which microalgae are required, with several strains being widely used as direct food for molluscs at all stages of life. In this regard, *Nannochloropsis gaditana* is found in a wide range of temperature and irradiance conditions in natural environments, with high pigment contents, thus suggesting that this microalga is able to regulate its photosynthetic apparatus as a function of culture conditions [2,5]. Carotenoids have antioxidant properties and are therefore used in food and health preservation [6]. *N. gaditana* is also appreciated for its ability to accumulate proteins, lipids [7] and PUFAs [8].

Microalgal growth and the biosynthesis of high value products, such as pigments and PUFAs, are quite sensitive to the temperature and irradiance during culture, and are probably the most critical factors affecting carotenoid content. Sánchez et al. [9] studied the effect of temperature on microalgal growth using the Arrhenius equation model. Similarly, Camacho-Rodríguez et al. [3] studied the combined effect of temperature and irradiance on eicosapentaenoic acid (EPA) synthesis in an outdoor *N. gaditana* culture over a wide range of conditions, thereby establishing the optimal culture conditions for EPA production.

The effect of dilution rate, as a measure of nutrient availability, has been studied by different authors for several strains [10-12],[3] with this parameter being found to have a marked effect on biomass productivity. The biochemical composition is also affected by nutrient availability, as demonstrated by Sukenik et al. [12]. In addition, environmental conditions affect both growth and biochemical composition [13,14] and the synthesis of high value products [3,15,16]. The present study corroborates the effect of temperature and light, and their interaction, on the growth of *N. gaditana* and its biochemical composition. The results obtained at the different culture conditions tested have allowed us to develop models for the prediction of accumulation of high value products that allow us to explain the effect of the studied variables on microalgal growth and the biosynthesis of such products, as well as the interaction with nutrient availability. We have also determined the optimal culture conditions for *N. gaditana* growth under controlled conditions indoors and have compared our findings with studies conducted outdoors on a pilot plant scale while also taking the reactor design into account.

A distinction is usually made between primary and secondary carotenoids. Primary carotenoids are those synthesized under normal, favourable growth conditions and are associated with chlorophylls, β-carotene, violaxanthin and vaucheriaxanthin in *Eustigmatophyceae* strains [17]. So-called secondary carotenoids are produced under nitrogen-depleted stress conditions. They are synthesized from primary carotenoids and accumulate after the cell growth phase [18]. Secondary

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carotenoids are located outside the chloroplast. Mendoza et al. [19] studied the effect of incident photon flux density (PFD) irradiance on β-carotene accumulation and found that the higher the incident PFD, the higher the β-carotene content, with a maximum value of 10 pg·cell^{-1} at 1500 μmol·m^{-2}·s^{-1}. This fact is explained by Müller et al. [20] as being due to the photoprotective role of carotenoids in the case of long-term exposure to high light intensities. The significant effect of temperature on carotenoid content in *N. gaditana* was also established by Lubián et al. [2].

Some xanthophyll pigments can interconvert under certain conditions. For example, in the presence of an excess of light, violaxanthin is interconverted into zeaxanthin by removal of the epoxides via the mono-epoxy carotenoid antheraxanthin [20]. In contrast, zeaxanthin is epoxidised under low light conditions [21].

In the present paper, the influence of irradiance and temperature on biomass productivity, chlorophyll fluorescence and EPA and carotenoid biosynthesis by the strain *N. gaditana* is analysed. The objective of this work is to model the influence of irradiance and temperature on growth rate and product biosynthesis rate in order to obtain overall models that will allow us to determine the optimal conditions for maximum biomass and product generation. The results reported here will allow the design and development of improved systems for the efficient production of EPA, β-carotene and vaucheriaxanthin-rich cells of *N. gaditana* on an industrial scale.

2. Materials and methods

2.1. Microorganism and culture conditions

N. gaditana strain B-3 was obtained from the Marine Culture Collection of the Institute of Marine Sciences of Andalucía (CSIC, Cádiz, Spain). Stock cultures were maintained photoautotrophically in 1 L Erlenmeyer flasks containing 700 mL of culture. The inoculum was grown aseptically in a thermostated room at 25 °C, under a constant incident PFD of 100 μmol·m^{-2}·s^{-1} and continuously bubbled with air at 0.5 v·v^{-1}·min^{-1}. The culture medium used was commercial Algal medium (Bionova, Santiago de Compostela, Spain), with the following composition: 1.13 g·L^{-1} of KNO_3, 0.0759 g·L^{-1} of $NaH_2PO_4·H_2O$, 0.0147 g·L^{-1} of $C_6H_5FeO_7·H_2O$, 0.00162 g·L^{-1} of $Na_2MoO_4·2H_2O$, 0.00119 g·L^{-1} of $MnCl_2·4H_2O$, 0.0008 g·L^{-1} of $ZnCl_2$, 0.0001 g·L^{-1} of $CoCl_2·6H_2O$, 0.00021 g·L^{-1} of $CuSO_4·5H_2O$, 0.016 g·L^{-1} of EDTA, 0.00294 g·L^{-1} of thiamine, 0.000038 g·L^{-1} of biotin and 0.000027 g·L^{-1} of cyanocobalamin.

2.2. Optimisation of temperature, average irradiance and dilution rate in continuous operation

Experiments were performed indoors in continuous mode in glass bubble column photobioreactors with a volume of 1.8 L, a diameter of 0.07 m and a height of 0.50 m. The reactors were continuously bubbled with air from a compressor at a rate of 0.5 v·v^{-1}·min^{-1} (Atlas Copco Airpower B.V. — GA7FF, Atlas Copco S.A.E., Madrid, Spain) via an inlet at the bottom of the column. The top of each bioreactor was fitted with ports for the addition of fresh medium, a gas outlet and a pH probe, as well as a harvest valve. The pH was kept in a range from 7.8 to 8.0 by injection of carbon dioxide on-demand. The culture medium was sterilized as indicated in the previous section. Experiments were initiated at 0.17 $g_{biomass}·L^{-1}$.

Initially, in order to study the effect of temperature, the cultures were thermostated in cooling jackets, using water as cooling agent and a refrigerated bath chiller circulator (Thermo Scientific — Neslab RTE 7, Thermo Scientific, Newington (N.H.), United States), at different culture temperatures (15, 20, 25 and 30 °C). At each temperature, the incident PFD at different maximum values of 250, 500, 1000 and 1600 μmol·m^{-2}·s^{-1} on the reactor surface was controlled by a computer software that simulates a 12 h light/12 h dark circadian cycle, with the equations proposed by Duffie and Beckman [22]. The lamps used were white-light Philips PL-32 W/840/4p. The combined effect of temperature and incident PFD on *N. gaditana* growth and biochemical composition was studied. Experiments were started in batch mode with an initial biomass and nitrogen concentration of 0.17 g·L^{-1} and 11.3 mM, respectively, until a biomass concentration of more than 1 g·L^{-1} was reached. Cultures were then shifted to continuous mode at a dilution rate of 0.3 d^{-1}, which was found to be optimal for biomass production in a previous study (data not shown). A volume of fresh medium corresponding to the dilution rate of 0.3 d^{-1} was supplied daily in a single addition carried out over a period of 15 min at the beginning of the light period. The overflow collected during the fresh medium addition was disposed of. A steady state was considered to have been attained when the biomass concentration fluctuated by less than 5% over five consecutive days. Samples were then harvested for biomass composition analysis by centrifugation at 7500 rpm for 5 min (SIGMA 4K15 Sartorius, Goettingen, Germany), washing with a 0.5 M aqueous ammonium bicarbonate solution to prevent mineral salts from precipitating [23] and freeze-drying (TELSTAR Cryonos — 50, Madrid, Spain). The dry biomass was analysed immediately or stored at −20 °C for a maximum of 10 days. The effect of different dilution rates on *N. gaditana* growth and biochemical composition was also studied. Thus, after optimizing the temperature and incident PFD, different dilution rates ranging from 0.1 to 0.5 d^{-1} were tested under these temperature and incident PFD conditions. Each test was carried out in triplicate, with three samples in each replication for the last 3 days of each steady state. As such, the average values correspond to nine experimental measurements.

2.3. Analytical procedures

Absorbance measurements (Helios Omega UV–vis spectrophotometer, Thermo Scientific, Horsham, England) were conducted in order to estimate the biomass concentration in the culture [8], although this was also checked periodically by dry-weight determination.

Chlorophyll fluorescence was determined as an indicator of the physiological status of the cells through the Fv/Fm ratio, Fm being the maximum value of fluorescence attributed to chlorophyll *a* and Fv the variable fluorescence value (the maximum minus the minimum fluorescence value), using a fluorimeter (AquaPen-C AP-C 100, Photon Systems Instruments, Czech Republic). In addition, cell viability was checked by flow cytometry (Cell Lab Quanta SC, Beckman Coulter). The biochemical composition was determined following established methods: total lipids as described by Kochert [24], ashes using the method described by Brown et al. [25] and protein content according to González-López et al. [26]. The carbohydrate content (%) was obtained as the difference between 100 and the sum of the percentages of the other fractions (ashes, proteins and total lipids). The fatty acid content was determined by gas chromatography as described by Rodríguez-Ruíz et al. [27], and the carotenoid content according to Cerón-García et al. [6]. Neoxanthin, violaxanthin, antheraxanthin and β-carotene standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vaucheriaxanthin and zeaxanthin standards were purchased from DHI (Hørsholm, Denmark).

2.4. Kinetic parameters

Eq. (1), established by Molina-Grima et al. [28], was used to calculate the average irradiance, I_{av}, to which cells were exposed in the culture:

$$I_{av} = \frac{I_0}{K_a \cdot r \cdot C_b}(1 - \exp(-2 \cdot K_a \cdot r \cdot C_b)) \quad (1)$$

where I_0 (μmol·m^{-2}·s^{-1}) is the maximum incident PFD on the reactor surface, r (m) the bubble column photobioreactor radius (relative term to the optical light path length), C_b (kg·m^{-3}) the biomass

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