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Differential growth and biochemical composition of photoautotrophic and heterotrophic *Isochrysis maritima*: evaluation for use as aquaculture feed

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Abstract The growth and biochemical composition of photoautotrophic and heterotrophic *Isochrysis maritima* in 50 L of Walne's medium were compared. Heterotrophic *I. maritima* fed with 0.02 M glucose had a 4.6-fold higher maximum cell density ($38.17 \pm 0.23 \times 10^6$ cells mL^{-1}) than photoautotrophic cells ($8.29 \pm 0.70 \times 10^6$ cells mL^{-1}). The carbohydrate content was slightly higher in heterotrophic cells at all growth stages (mid-exponential, 40.8%; early stationary, 48.3%; and late stationary, 47.6%), but there was no significant effect on the protein content under either trophic condition. The total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) were higher under heterotrophic conditions than those under photoautotrophic conditions. However, because omega-3 PUFAs are the most essential element in feed nutrition, low results for eicosapentaenoic acid (EPA) ($0.28 \pm 0.06\%$) and docosahexaenoic acid (DHA) ($3.22 \pm 0.26\%$) in the heterotrophic cells compared to the photoautotrophic cells (EPA: $0.44 \pm 0.11\%$; DHA: $8.58 \pm 0.73\%$) plus a low omega-3/6 PUFAs ratio (heterotrophic: 0.16–0.47; photoautotrophic: 2.60–2.88) and high value of (SFA + MUFA)/PUFA (heterotrophic: 5.50–6.81; photoautotrophic: 2.64–3.60) showed that this species is not suitable for aquaculture feed when cultivated under heterotrophic conditions.

Introduction

The importance of microalgae in aquaculture is not surprising, as microalgae are a natural food source for most aquaculture species. Microalgae are used for either direct consumption, such as for molluscs and penaeid shrimp, or for indirect consumption as food for live prey, such as rotifers and *Artemia* that feed the small larvae of fish (Brown 2002; Patil et al. 2005; Guedes and Malcata 2012). In addition, the cultivation of microalgae, as the primary source of polyunsaturated fatty acids (PUFAs), has been shown to be a profitable area of biotechnology research in the food and feed industries (Pulz and Gross 2004; Mansour et al. 2005). The inability of aquatic animals to synthesise both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) makes microalgae the ideal source of PUFAs for the growth and development of these animals (Meireless et al. 2003). The microalgae species most commonly utilised as feed are members of the genera *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Spolaore et al. 2006; Hemaiswarya et al. 2010).

The marine microalgae *Isochrysis* spp. have received overwhelming research interest due to their ability to provide resources in the form of novel genetic material. These resources contribute to the production of various nutritional and pharmaceutical products, proteins (essential amino acids), energy and other key nutrients, such as vitamins, essential PUFAs and pigments, which are transferred through the food chain (Brown 2002). The most commonly used *Isochrysis* strain is *Isochrysis galbana* (note that the T-Iso strain of *Isochrysis*

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which has sometimes also been referred to as *I. galbana* is now known as *Tisochrysis lutea*—Bendif et al. 2013). *Isochrysis* strains have been widely used as mariculture feed due to their high content of long-chain PUFAs, especially DHA, and their ease of cultivation (Lin et al. 2007; Nalder et al. 2015). These strains are also able to tolerate cultivation at relatively high temperatures, which can be particularly advantageous under unpredictable tropical conditions (Babuskin and Radhakrishnan 2014). Additionally, they have a high growth rate, lack a cell wall and are robust under outdoor culture conditions (Devos et al. 2006; Lin et al. 2007; Liu et al. 2013), making them a popularly utilised microalgal taxa in aquaculture.

Various studies have attempted to enhance the production of both the cell biomass and biochemical composition of algae. Such attempts have included the manipulation of chemical variables such as nitrogen (Arumugam et al. 2013) and phosphorus concentrations (Sun and Wang 2009), physical variables such as temperature (Durmaz et al. 2009) and salinity (Rao et al. 2007) and cultivation modes including photoautotrophic, heterotrophic and mixotrophic modes (Chojnacka and Noworyta 2004). Microalgae are usually cultivated under photoautotrophic conditions (Gouveia and Oliveira 2009), which involve the use of light as an energy source and inorganic carbon as a carbon source. Although metabolite production is relatively high, these cultivation conditions are frequently associated with a low biomass concentration due to light limitations (Wen and Chen 2001; Chen and Chen 2006). The dependence of photoautotrophic cultivation on light energy prevents the cells from reaching a high density due to self-shading or photoinhibition problems. To eliminate the light requirement, microalgae have begun to be heterotrophically cultivated to possibly increase the cell density (Chen 1996; Huang et al. 2010; Pahl et al. 2010). Heterotrophic cultivation involves the utilisation of organic carbon as the energy and carbon sources for growth in dark conditions (Wen and Chen 2003; Chojnacka and Marquez-Rocha 2004; Chen et al. 2011). Although these conditions are advantageous in terms of microalgal growth, not all microalgae can grow in the dark (Perez-Garcia et al. 2011). The cell density was reportedly enhanced with the elimination of light and the utilisation of an organic carbon source. Thus, this culture mode may be a cost-effective alternative for the mass cultivation of microalgae (Chen 1996; Pahl et al. 2010).

In addition to its ability to improve cell biomass, heterotrophic cultivation also received much attention because of its ability to increase the fatty acid content compared to that obtained with photoautotrophic cultivation (Miao and Wu 2006; Xiong et al. 2008; Liu et al. 2011; Wang et al. 2012). A study by Wen and Chen (2000) on the heterotrophic production of EPA by the diatom *Nitzschia laevis* indicated that the production of PUFAs, including DHA and EPA, was higher under dark conditions. A study by Liu et al. (2011) on *Chlorella*

zofingiensis also showed an increase in monounsaturated fatty acids (MUFAs) under heterotrophic cultivation (37.4%) compared to under photoautotrophic cultivation (20.1%). Our preliminary investigation showed that *Isochrysis maritima* isolated from Penang National Park coastal waters was a suitable candidate for aquaculture, meeting all of the general attributes, including being the appropriate size for ingestion and exhibiting rapid growth rates, amenability to mass culture, stability when faced with fluctuation in culture conditions and a good nutrient composition. Thus, the present study aimed to determine the possibility of culturing *I. maritima* photoautotrophically with different nitrogen sources and heterotrophically with different carbon sources. Subsequently, the best nitrogen and carbon sources were then utilised at a higher cultivation volume under both trophic conditions to evaluate the cell biomass and biochemical compositions of this species at different growth stages for use in aquaculture feed.

Materials and methods

Microalgae strain and growth media

Isochrysis maritima USMAC 19 was isolated from the coastal waters of Teluk Aling, Penang National Park, Malaysia, and identified by Biotech International R & D (BIRD). The strain was purified using mechanical separation to discriminate between the cells and other substances using a vortex mixer (Reax 2000, Heidolph, Germany) at $2000 \times g$ for 1 min and centrifugation at $6000 \times g$ for 10 min. This procedure was followed by antibiotic treatment involving a mixture of penicillin G, dihydrostreptomycin sulphate and gentamicin sulphate in the ratio of 4:1:1 (Guillard 2005) for 18 to 72 h. The sterility of the culture was examined by inoculating the cells onto a culture plate with sterility test medium, including a mixture of 100 mg of yeast extract and 200 mg of tryptone with 100 mL of 2% agar growth medium. The plates with the algae were incubated for 1 week to observe any bacterial growth. When there was no visible bacterial growth, *I. maritima* was transferred to a liquid medium and was photoautotrophically maintained in 100 mL of sterilised seawater enriched with Walne's medium (Walne 1970) at 3‰ salinity. The culture was incubated at a constant temperature of 25 ± 1 °C under a light intensity of 50 μmol photons $m^{-2} s^{-1}$, with a 12:12-h light/dark cycle. Agitation of the culture medium was conducted by shaking the flasks twice daily.

Photoautotrophic cultivation

Isochrysis maritima was cultivated in triplicate in 1 L of Walne's medium with three different nitrogen sources, i.e., nitrate ($NaNO_3$), nitrite ($NaNO_2$) and urea ($(NH_2)_2CO$), at a

concentration of 4 mg atom N L^{-1} (Fidalgo et al. 1998). The Walne's medium was sterilised by autoclaving (HVE-50, Hirayama, Japan) at 121 °C for 20 min. The initial cell density was adjusted to 2.5×10^5 cells mL^{-1} after the cells were introduced into the culture medium. The cultures were grown in an algae culture room (temperature: 25 ± 1 °C) with a 24-h light duration (light intensity: 50 µmol photons m^{-2} s^{-1}) and were continuously bubbled with 0.2-µm filtered air at a rate of 1 L min^{-1}. The number of cells was counted daily until the culture reached the late stationary phase. The nitrogen source that produced the highest yields, in terms of cell density, was used to culture this species in 50 L of culture medium. The 50 L of Walne's medium was sterilised using 50 mL of sodium hypochlorite and was neutralised with 12.5 mL of sodium thiosulphate (250 g L^{-1}) (Kawachi and Noël 2005). The initial density of *I. maritima* was adjusted to 2.5×10^5 cells mL^{-1} (Fidalgo et al. 1998). The cultures were grown at a temperature of 28 ± 2 °C under white fluorescent light (50 µmol photons m^{-2} s^{-1}), with a light duration of 24 h, and they were aerated at 2 L min^{-1}. The number of cells was counted daily until the culture reached the late stationary phase. The microalgae were harvested at three different growth stages (mid-exponential, early stationary and late stationary) by flocculation, and the cell biomass, proteins, carbohydrates and fatty acids were analysed.

Heterotrophic cultivation

Isochrysis maritima was cultivated in triplicate in 1 L of Walne's medium with urea as the nitrogen source and then sterilised by autoclaving at 121 °C for 20 min. All of the culture vessels were fully wrapped with aluminium foil to ensure no light penetration. The carbon sources, including glucose, sucrose, acetate, lactose and fructose, were added at three different concentrations (0.02, 0.05 and 0.1 M). All of the carbon sources were sterilised by filtration through 0.2-µm membranes before injection into the culture medium. The inocula were taken from the same stock culture at the early stationary growth phase, and the initial densities were adjusted to 2.5×10^5 cells mL^{-1}. All of the cultures were incubated in an algae culture room at 25 ± 1 °C. The cultures were continuously bubbled with 0.2-µm filtered air at a rate of 1 min^{-1}. The cell density was recorded daily until the culture reached the late stationary growth phase. The carbon source and concentration that produced the highest cell growth were used in a subsequent experiment, in which *I. maritima* was heterotrophically cultivated in larger volumes. This experiment was conducted in triplicate in 50 L of Walne's medium sterilised using 50 mL of sodium hypochlorite and neutralised with 12.5 mL of sodium thiosulphate (250 g L^{-1}) (Kawachi and Noël 2005) in a batch culture system. The inocula were taken from the same stock culture at the late exponential growth phase, and the initial densities were adjusted to 2.5×10^5

cells mL^{-1}. The cultures were incubated in the closed hatchery laboratory at 28 ± 2 °C and were continuously bubbled with filtered air at a rate of 2 L min^{-1}. *Isochrysis maritima* was harvested at different growth phases (mid-exponential, early stationary and late stationary), and cell biomass, proteins, carbohydrates and fatty acids were analysed.

Analytical methods

The cell density was counted daily using a haemocytometer (Neubauer-improved haemacytometer, Laboroptik, UK), and the cell biomass was determined using the dry weight and the ash content at the end of the experiment (Liu et al. 2013). The specific growth rate was calculated during the exponential phase, according to the following equation:

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)}$$

where X_2 and X_1 are the cell densities (cells mL^{-1}) at times t_2 and t_1 (day), respectively.

Samples were collected by flocculation at the same time for all growth phases by adjusting the pH to 10–11 using 1 M NaOH. The flocculated cells were centrifuged at $3000 \times g$ for 5 min. The pellets were washed with distilled water and centrifuged again at the same speed and for the same amount of time. The cell pellets were freeze dried for total protein, total carbohydrate and fatty acid analyses. The total protein was extracted using a method proposed by Rausch (1981) and was measured using the Lowry assay (Lowry et al. 1951). The carbohydrates were extracted using a method proposed by Chu et al. (1996) and were measured using the phenol–sulphuric assay, according to DuBois et al. (1956).

Fatty acid analysis

Isochrysis maritima was extracted by the direct transesterification method as described by Abel et al. (1963), with some modifications. One hundred milligrams of dried cells was weighed and transferred into screw cap bottles, and 2 mL each of methanolic sulphuric acid (15%) and chloroform were added. The mixture was bubbled with nitrogen gas for 15 s and subsequently vortexed for 2 min. After the mixture became visibly homogenous, it was heated using a heating block (HB-48, WiseTherm, Germany) at 80 °C for 30 min. Then, 1 mL of distilled water was added and two layers were formed in the solution. Subsequently, the lower layer of the mixture was transferred to a 2-mL vial, which was then centrifuged at $10,000 \times g$ for 5 min. Ten milligram of anhydrous sodium sulphate was added to ensure the complete removal of water from the mixture. Finally, 0.75 µL of the lower layer was transferred into a gas chromatography (GC)

vial, which was then sealed and kept at −20 °C until GC analysis.

Fatty acid methyl esters (FAMEs) were separated and quantified by a gas chromatograph (GCMS-2010, Shimadzu, Japan) equipped with a flame ionisation detector and a 30×0.22 mm 70% cyanopropyl polysilphenylene–siloxane column (BPX70, SGE). Nitrogen was used as the carrier gas, and the temperature programming was set to increase from 100 to 210 °C at 2 °C min^{-1} and was then maintained at 210 °C for 30 min. The injector and detector temperatures were set at 250 and 260 °C, respectively. Menhaden oil and Supelco 37 Component FAME Mix were used as the standards for fatty acid identification by the comparison of the peak retention times between the samples and standards at a ratio of 3:1. The concentration of the fatty acids was estimated from the peak area on the chromatogram using 99% methyl enanthate (C7:0) (Sigma Aldrich, USA) as an internal standard.

Statistical analysis

The statistical analyses were conducted using the SPSS v20.0 software. A one-way ANOVA was used to test for possible significant differences in the means of the dependent variables for the microalgae grown with different nitrogen sources, and a 5×3 factorial design was used to test for possible significant differences in five carbon sources at three different concentrations. The significant differences between photoautotrophic and heterotrophic cultivations in 50 L of Walne's medium were analysed using independent sample t test. Multiple comparisons were tested by Duncan's post hoc analysis, when the main treatment effect was significant at $P < 0.05$. All of the experiments were conducted in triplicate, and all data are presented as the mean ± standard error.

Results

Effect of different nitrogen sources on photoautotrophic growth

The present study aimed to determine the best nitrogen sources for the maximum growth of the tropical microalga *I. maritima* in photoautotrophic cultivation. Figure 1 shows the growth curves for *I. maritima* cultivated with three different nitrogen sources. After 14 days of cultivation, urea showed a significantly higher maximum cell density ($11.6 \pm 0.27 \times 10^6$ cells mL^{-1}) than the other nitrogen sources tested ($P < 0.05$) (Fig. 1). There was no significant difference in the maximum cell densities between nitrate and nitrite, for which $9.64 \pm 0.25 \times 10^6$ and $9.38 \pm 0.26 \times 10^6$ cells mL^{-1} were recorded, respectively (Fig. 1). The dry weight (DW) analysis and the specific growth rates (SGR) were also significantly greater in urea than in nitrate and nitrite (Table 1).
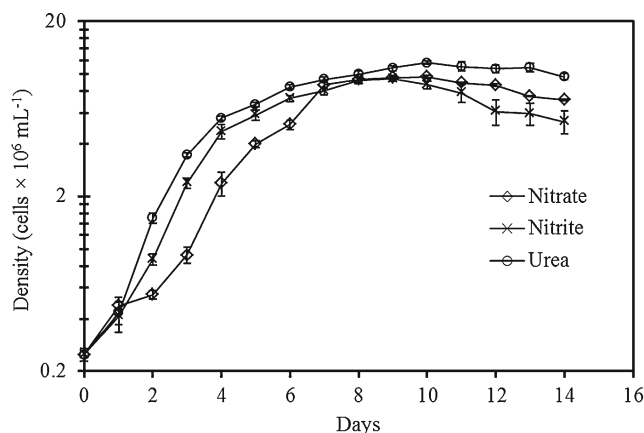

Fig. 1 Growth curves of *Isochrysis maritima* cultured with different nitrogen sources (mean ± SE. n = 3)

However, the ash-free dry weight (AFDW) was not significantly different ($P > 0.05$) across all nitrogen sources (Table 1).

### Effect of different carbon sources on heterotrophic growth

The effects of various carbon sources at different concentrations on the cell density and specific growth rate of *I. maritima* are shown in Figs. 2 and 3. Glucose was the most suitable carbon source for culturing this species under heterotrophic conditions. Glucose, at the concentrations of 0.02 and 0.05 M, achieved significantly higher cell densities than the other carbon sources at all concentrations, with maximum cell densities of $41.83 \pm 0.36 \times 10^6$ and $36.09 \pm 0.27 \times 10^6$ cells mL^{-1}, respectively, and SGR of 1.30 ± 0.16 and 0.97 ± 0.12 day^{-1}, respectively. Other carbon sources, such as fructose, only demonstrated an average maximum cell density of 5.00–6.00×10^6 cells mL^{-1}, which was also obtained at 0.02 and 0.05 M concentrations, with SGR of 0.54 ± 0.16 and 0.54 ± 0.13 day^{-1}, respectively. The effectiveness of lactose on *I. maritima* growth was the lowest among the other carbon sources studied, with a maximum cell density of less than 2.00×10^6 cells mL^{-1} recorded at all concentrations.

Table 1 Cell density, dry weight (DW), ash content and ash-free dry weight (AFDW) of *Isochrysis maritima* grown in different nitrogen sources

Parameters	Nitrate	Nitrite	Urea
Density ($\times 10^6$ cells mL^{-1})	9.64 ± 0.25a	9.38 ± 0.26a	11.6 ± 0.27b
DW (g L^{-1})	3.50 ± 0.12a	3.41 ± 0.13a	4.21 ± 0.11b
SGR (day^{-1})	0.94 ± 0.17a,b	0.76 ± 0.06a	1.24 ± 0.12b
Ash (% DW)	24.5 ± 0.57b	20.8 ± 0.88a	30.4 ± 0.95c
AFDW (g L^{-1})	2.64 ± 0.1a	2.7 ± 0.1a	2.93 ± 0.07a

The values presented are means of three replicates and standard errors. Means were compared using the multiple range test of Duncan ($\alpha = 0.05$); differences were not significant for groups with the same letter

Fig. 2 Growth profiles (mean ± SE. n = 3) of *Isochrysis maritima* cultured at different glucose (**a**), sucrose (**b**), acetate (**c**), fructose (**d**) and lactose (**e**) concentrations
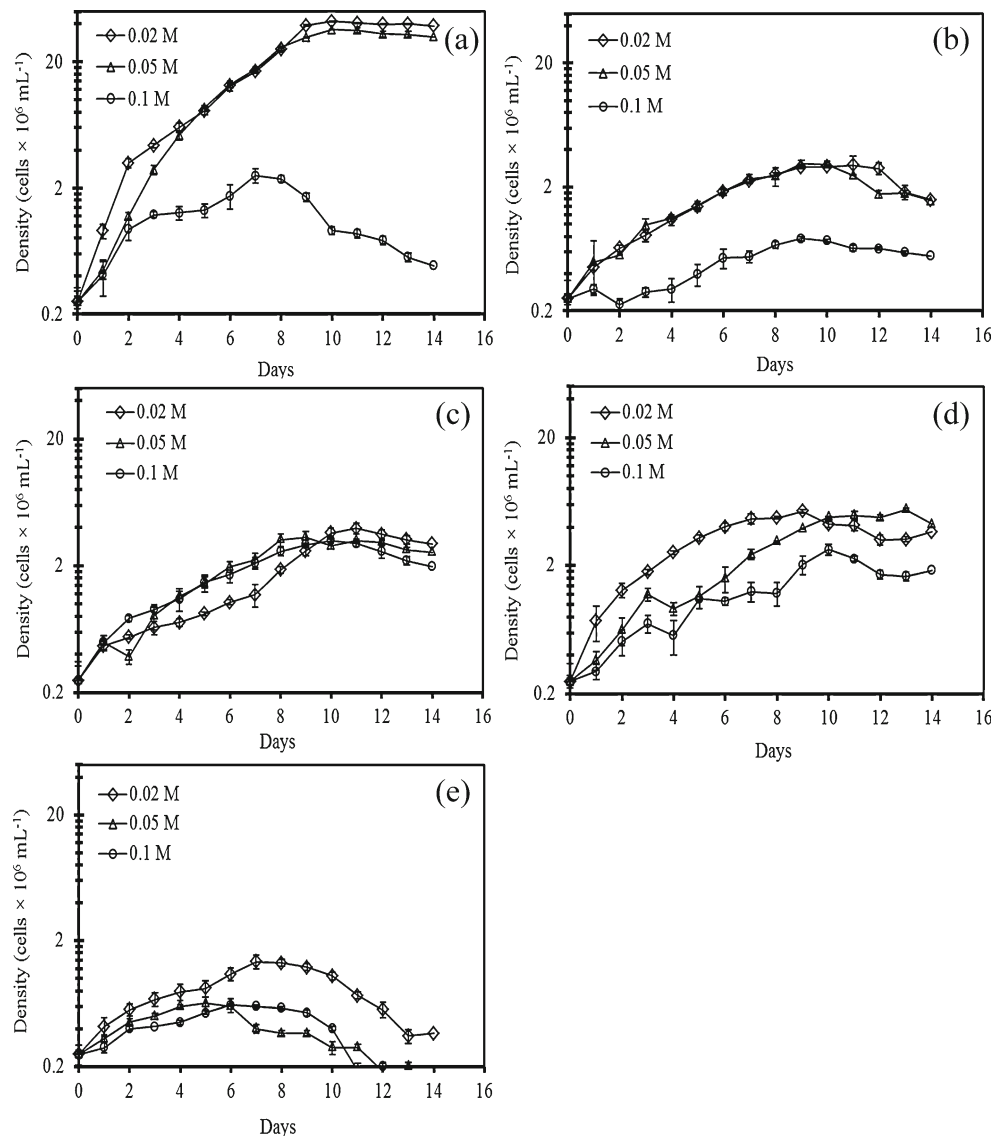
Isochrysis maritima grown in sucrose and acetate also demonstrated low maximum cell densities in the range of 0.70–3.10 × 10^6 and 3.10–4.00 × 10^6 cells mL^{-1}, respectively, at all of the tested concentrations.

Growth, biochemistry and fatty acid composition under photoautotrophic and heterotrophic conditions

Isochrysis maritima photoautotrophically cultured in 50 L of Walne's medium had a significantly lower maximum cell density (8.29 ± 0.70 × 10^6 cells mL^{-1}) (Fig. 4) than *I. maritima* cultured in 8 L of culture medium (11.6 ± 0.27 × 10^6 cells mL^{-1}, Fig. 1) under the same culture conditions ($P < 0.05$). Nevertheless, the SGRs were not significantly different between 50 L of Walne's medium and 8 L of Walne's medium, (0.98 ± 0.09 and 1.24 ± 0.12 day^{-1}, respectively; $P > 0.05$). Different results were attained in 50 L of

Walne's medium heterotrophically. The maximum cell density was not significantly different between 50 L of Walne's medium (38.17 ± 0.23 × 10^6 cells mL^{-1}, Fig. 4) and 1 L of Walne's medium (41.83 ± 0.36 × 10^6 cells mL^{-1}, Fig. 2) ($P > 0.05$). A similar result was recorded in the SGR for both culture volumes, which were 1.31 ± 0.07 day^{-1} in 50 L of Walne's medium and 1.30 ± 0.16 day^{-1} in 1 L of Walne's medium from the previous study with the same culture conditions ($P > 0.05$, Fig. 3).

The cell density, DW, ash percentage and AFDW of *I. maritima* were affected by different trophic conditions, as shown in Table 2. *Isochrysis maritima* cultivated heterotrophically reached a significantly higher (4.6-fold) maximum cell density than that cultivated in photoautotrophically condition ($P < 0.05$). The DW, ash percentage and AFDW were also significantly higher in the heterotrophic culture (6.12 ± 0.24 g L^{-1}, 41.0 ± 0.49% and 3.61 ± 0.21 g L^{-1},
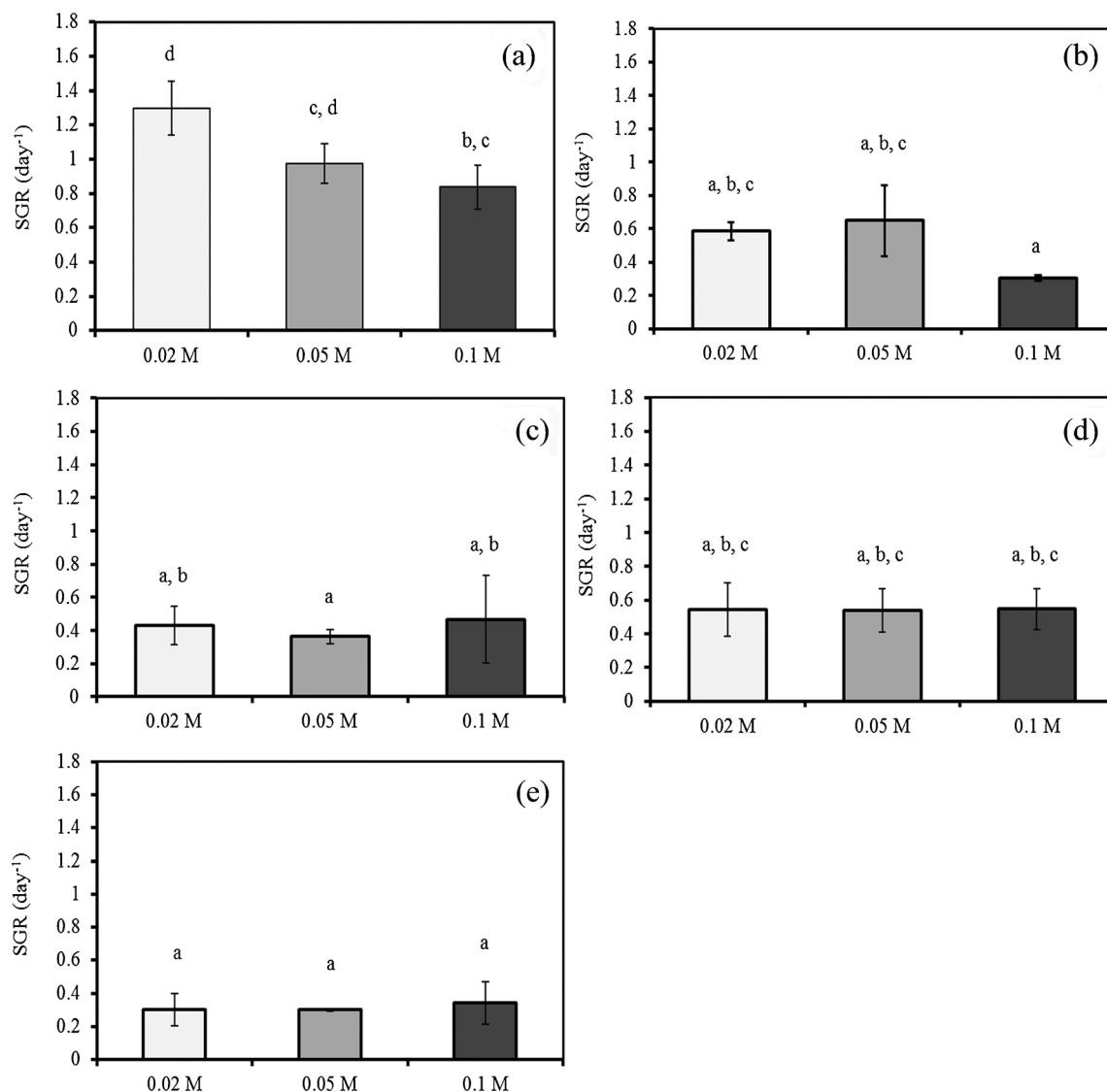
Fig. 3 Specific growth rate (mean ± SE. n = 3) of *Isochrysis maritima* cultured at different glucose (**a**), sucrose (**b**), acetate (**c**), fructose (**d**) and lactose (**e**) concentrations

respectively) than in the photoautotrophic culture (3.01 ± 0.25 g L^{-1}, 21.9 ± 0.66% and 2.35 ± 0.23 g L^{-1},
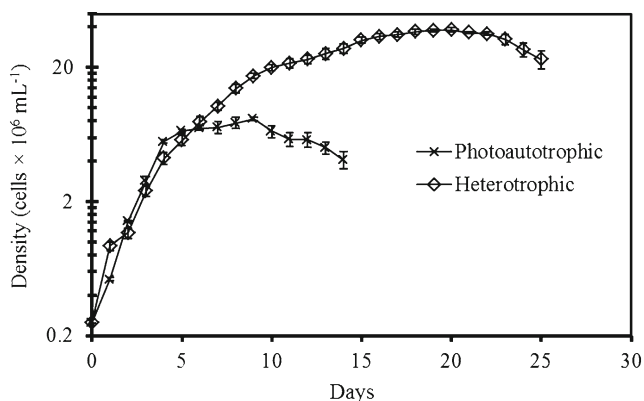

Fig. 4 Growth curves (mean ± SE) of *Isochrysis maritima* cultured in 50 L Walne's media under photoautotrophic and heterotrophic conditions

respectively). In addition, other than different trophic conditions, the cell density, DW, ash percentage and AFDW were affected by different growth phases (Table 2). The cell density for the photoautotrophic and heterotrophic cultivations increased from 5.62 ± 0.19 × 10^6 and 20.04 ± 0.19 × 10^6 cells mL^{-1} at the mid-exponential phase, respectively, to approximately 8.29 ± 0.70 × 10^6 and 36.90 ± 0.25 × 10^6 cells mL^{-1} at the early stationary phase, respectively. Cellular DW was also increased from 2.14 ± 0.09 and 3.32 ± 0.17 to 2.63 ± 0.07 and 6.12 ± 0.24 g L^{-1}, respectively. Similar results were obtained for the AFDW content, which significantly increased from 1.59 ± 0.07 to 1.91 ± 0.12 g L^{-1} in the photoautotrophic culture and from 2.1 ± 0.12 to 3.61 ± 0.21 g L^{-1} in the heterotrophic culture.

The total protein was more affected by the growth phase than the trophic condition (Table 2). The protein content was higher for both trophic conditions at the exponential phase

(23.01 ± 3.4 and 26.2 ± 2.10%, respectively) and decreased at the late stationary phase (10.64 ± 3.62 and 18.3 ± 0.60%, respectively). There was a slight increase in the carbohydrate content from the mid-exponential phase to the late stationary phase for both trophic conditions. Meanwhile, there was a significant difference between the photoautotrophic and heterotrophic cultures at all growth phases ($P < 0.05$). The carbohydrate content was 40.8 ± 3.6% in the heterotrophic culture and 18.8 ± 1.5% in the photoautotrophic culture at the mid-exponential phase, 48.3 ± 1.52 and 21.8 ± 2.4%, respectively, at the early stationary phase and 47.6 ± 2.5 and 26.9 ± 3.4%, respectively, at the late stationary phase.

The fatty acid compositions of *I. maritima* photoautotrophically and heterotrophically grown at different growth phases are listed in Table 3. The main fatty acids (as % of total fatty acids) were C14: 0 (8.79–14.66%), C16: 0 (11.12–15.7%) and C22: 6 (n-3) (5.68–9.31%) in the photoautotrophic culture and C16: 0 (21.34–36.19%), C18: 0 (7.08–17.38%) and C18: 1 (n-9) (10.32–19.48%) in the heterotrophic culture. The major saturated fatty acid (SFA) for both photoautotrophic and heterotrophic cultures was palmitic acid (C16: 0) (10.7–15.70 and 21.34–36.19%, respectively) (Table 3). Meanwhile, the major monounsaturated fatty acid (MUFAs) for both trophic conditions was oleic acid (C18: 1 (n-9)), which were 3.23–3.67% photoautotrophically and 10.32–19.48% heterotrophically. Docosahexaenoic acid (DHA; C22: 6 (n-3)) was the major polyunsaturated fatty acid (PUFA) in photoautotrophic cells, while linoleic acid (LA; C18: 2 (n-6) was the major PUFA in heterotrophic cells.

The PUFA content significantly increased from the mid-exponential phase to the late stationary phase ($P < 0.05$). The SFAs increased from the mid-exponential phase towards the late stationary phase for both trophic conditions. There was no difference in the MUFA content in photoautotrophic cells but was slightly decreased from the mid-exponential phase to the late stationary phase in heterotrophic cells. The composition of PUFAs, especially omega-3 and omega-6 fatty acids in the dietary microalgae, determines the suitability of these microalgae as aquaculture feed. In the present study, the highest content of α-linolenic acid (ALA) C18: 3 (n-3), arachidonic acid (AA), C20: 4 (n-6) eicosapentaenoic acid (EPA) C20: 5 (n-3) and docosahexaenoic acid (DHA) C22: 6 (n-3) were all obtained at the late stationary phase in photoautotrophic cells, which were 1.04 ± 0.12, 0.38 ± 0.08, 0.44 ± 0.11 and 8.58 ± 0.73%, respectively. However, the highest content of linoleic acid (LA) C18:2 (n-6) was obtained at the early stationary phase in heterotrophic cells, which was 9.04 ± 0.36%. There were no significant differences in the total omega-3 and total omega-6 PUFAs for all three growth stages in both trophic conditions ($P > 0.05$). The total (n-3)/(n-6) index represents the quotient between the fatty acids corresponding to the groups (n-3) and (n-6) and also a parameter to estimate the nutritional value of microalgal cells. In this study, the ratio of total (n-3) and total (n-6) PUFAs showed optimal values in photoautotrophic cells (2.60–2.88) compared to heterotrophic cells (0.16–0.47) (Table 3). The ratio of (SFA + MUFA)/PUFA was higher in heterotrophic cells (5.50–6.81) than in photoautotrophic cells (2.64–3.60).

Discussion

Nitrogen is the most commonly reported nutrient-limiting factor in the growth and lipid accumulation of microalgae (Xu et al. 2001; Li et al. 2008; Lin and Lin 2011). Hence, the first experiment aimed to choose the best nitrogen sources for the maximum growth of the tropical microalga *I. maritima* in photoautotrophic cultivation. The results showed that *I. maritima* could be cultivated in nitrate, nitrite and urea because the SGR showed no significant difference between them. However, different nitrogen sources did affect the total biomass of the cells. Urea recorded the highest cell density, DW, ash and AFDW compared to the others. Changes in the cell biomass of microalgae due to different nitrogen sources were reported by other researchers, including Fidalgo et al. (1998) on *I. galbana*, Feng et al. (2011) on *Isochrysis zhangjiangensis*, González-Garcinuño et al. (2014) on *Scenedesmus abundans* and *Chlorella ellipsoidea*, Lin and Lin (2011) on *Scenedesmus rubescens*, Arumugam et al. (2013) on *Scenedesmus bijugatus* and Campos et al. (2014) on *Nannochloropsis salina*. In fact, studies on other haptophytes by Fidalgo et al. (1998) and Feng et al. (2011) also reported that *I. galbana* and *I. zhangjiangensis* grown with urea recorded the highest cell biomass compared to other tested nitrogen sources. Most microalgae take up nitrate–nitrogen only if ammonium nitrogen is depleted (Xu et al. 2001), because nitrate–nitrogen must be transformed into ammonium nitrogen before being utilised by algae (Flores et al. 1980). In the present study, this species inclination towards urea might be related to the statement by Sakamoto and Bryant (1998), who reported that urea assimilation to ammonium required less energy than nitrate, because it existed in reduced form. Carvalho et al. (2004) also suggested that the simultaneous actions of urease, which is an enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia (Carvajal et al. 1980), and alkaline conditions (Danesi et al. 2002) could have promoted urea hydrolysis to ammonia at a rate consistent with its assimilation by the cell, thereby minimising ammonia inhibition that might occur during the growth phase. On the other hand, compared to other nitrogen sources, urea can easily pass through the plasma membrane (Rashid et al. 2014). Urea as a nitrogen source can provide a slight advantage for commercialization, because urea was among the cheapest nitrogen sources compared to others (Hulatt et al. 2012), and urea may also act as a readily available source of CO_2 carbon for photosynthetic organisms (Berman and Chava 1999).

Table 2 Cell density, dry weight (DW), ash content, ash-free dry weight (AFDW), protein and carbohydrate content of *Isochrysis maritima* grown in 50 L Walne's medium and different phases of growth cycles

Parameters	Phase	Photoautotrophic	Heterotrophic
Density (× 10^6 cells mL^{-1})	ME	5.62 ± 0.19a	20.04 ± 0.19b
	ES	8.29 ± 0.70a	38.17 ± 0.23b
	LS	6.68 ± 0.68a	35.72 ± 0.09b
DW (g L^{-1})	ME	2.04 ± 0.06a	3.32 ± 0.17b
	ES	3.01 ± 0.25a	6.12 ± 0.24b
	LS	2.42 ± 0.25a	5.92 ± 0.19b
Ash (% DW)	ME	25.5 ± 1.96a	36.7 ± 1.50b
	ES	21.9 ± 0.66a	41.0 ± 0.49b
	LS	19.4 ± 0.41a	40.2 ± 0.84b
AFDW (g L^{-1})	ME	1.52 ± 0.02a	2.1 ± 0.12b
	ES	2.35 ± 0.23a	3.61 ± 0.21b
	LS	1.95 ± 0.24a	3.54 ± 0.14b
Protein (% AFDW)	ME	23.0 ± 3.4a	26.2 ± 2.1a
	ES	15.4 ± 4.1a	20.1 ± 2.5a
	LS	10.6 ± 3.6a	18.3 ± 0.6a
Carbohydrate (% AFDW)	ME	18.8 ± 1.5a	40.8 ± 3.6b
	ES	21.8 ± 2.4a	48.3 ± 1.52b
	LS	26.9 ± 3.4a	47.6 ± 2.5b

The values presented are means of three replicates and standard errors. Means were compared using the *t* test (α = 0.05); differences were not significant for groups with the same letter

ME mid-exponential phase, *ES* early stationary phase, *LS* late stationary phase

The ability of microalgae to grow in media with organic supplementation depends on the algal species and the sources of organic carbon (Chen and Chen 2006; Alkhamis and Qin 2013). Therefore, the second experiment aimed to choose the best carbon sources at different concentrations for the maximum growth of *I. maritima* in heterotrophic cultivation. Based on the obtained results, this species only grew well on glucose at 0.02 and 0.05 M concentrations. This finding was not surprising, as glucose is the most commonly used carbon source for the heterotrophic culture of microalgae (Park et al. 2002; Liang et al. 2009; Li et al. 2013; Liu et al. 2014; Gim et al. 2016). A study by Gim et al. (2016) showed that the supplementation of glucose led to a significant improvement in the growth of *I. galbana*, *Nannochloropsis oculata* and *Dunaliella salina* compared to other studied carbon sources. Moreover, *C. zofingiensis* also demonstrated the highest growth rate, cell biomass, lipid content and lipid yield after being cultivated in glucose as a carbon source (Liu et al. 2010). When glucose was available, a membrane-bound hexose/H$^+$ symport system, which is triggered by the hexose uptake protein gene (*hup1*) (Sauer and Tanner 1989), was most likely activated. This activated system transports glucose and H$^+$ with a stoichiometry of 1:1 (Komor et al. 1973) into the cytosol and invests one molecule of ATP per molecule of sugar transported (Tanner 2000). The oxidative assimilation of glucose begins with the phosphorylation of hexose to form glucose-6-phosphate, which is an important intermediate product for storage, cell synthesis and respiration (Perez-Garcia et al. 2011; Liu et al. 2014), yielding the reducing equivalents (FADH$_2$ and NADPH) from the ATP synthesis. Hence, all of the produced ATP is used for cell growth and lipid accumulation (Gim et al. 2016). On the contrary, Perez-Garcia et al. (2011) also stated that glucose might be considered a "preferred substrate" for the heterotrophic cultivation of microalgae because the cells grown on other substrates may require a lag period (an acclimation period) to develop the specific transport systems necessary for the uptake of other substrates. Nevertheless, this lag phase may not always occur because of other factors, such as the strain used, the bioreactor configuration and the environmental conditions.

The low maximum cell density at 0.1 M (19.8 g L^{-1}) recorded for all carbon sources was most likely due to an excess of organic substrate, which inhibited the growth of *I. maritima* (Perez-Garcia et al. 2011; Liu et al. 2014). Ogawa and Aiba (1981) suggested that the initial concentration of glucose should be limited to 10 and 1 g L^{-1} to promote the cellular growth of *Chlorella vulgaris* and *Scenedesmus acutus*, respectively. Meanwhile, Tan and Johns (1991) stated that inhibition occurred for *Chlorella saccharophila* and *Chlorella sorokiniana* at glucose concentrations of more than 25 and more than 5 g L^{-1}, respectively. *Nitzschia laevis* yields also decreased as the substrate concentration increased from 1 to 40 g L^{-1} (Wen and Chen 2000). However, different species experience carbon inhibition at different concentrations. For example, *Chlorella protothecoides* was cultivated at carbon concentrations as high as 80 g L^{-1} to obtain an optimal yield of biomass (Shi et al. 1999). *Galdieria sulphuraria* was reported to grow under high concentrations of glucose or fructose of up to 166 g L^{-1}, but higher glucose concentrations inhibited its growth (Schmidt et al. 2005).

Based on the results from the first and second experiments, urea was chosen as the nitrogen source for photoautotrophic growth and 0.02 M glucose as the carbon source for the heterotrophic growth to explore the growth and biochemical composition response of *I. maritima* in both trophic conditions when cultivated in 50 L Walne's medium. Growth was affected by cultivation in a larger culture volume. The maximum cell density, the DW, the ash content and the AFDW decreased as the culture medium increased from 8 L from the first experiment to 50 L with the same culture conditions. A study by Renaud et al. (1991) on the effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *N. oculata* for tropical aquaculture also recorded a decrease in microalgal growth as the size of the culture medium increased from 4 to 8000 L. This result was presumably related to the limitation of the physical

Table 3 Fatty acid composition (% of total fatty acids) of *Isochrysis maritima* grown in different glucose concentrations and growth phases

Fatty acids	Photoautotrophic			Heterotrophic		
	ME	ES	LS	ME	ES	LS
Saturated						
C14: 0	11.53 ± 1.65	10.32 ± 1.53	13.21 ± 1.45	1.53 ± 0.05	1.74 ± 0.14	1.89 ± 0.05
C15: 0	0.82 ± 0.32	1.23 ± 0.16	1.27 ± 0.35	0.48 ± 0.04	0.60 ± 0.04	0.72 ± 0.06
C16: 0	13.22 ± 0.47	11.38 ± 0.26	13.20 ± 2.50	24.20 ± 2.86	30.28 ± 3.04	32.45 ± 3.74
C17: 0	0.20 ± 0.04	0.12 ± 0.02	0.28 ± 0.03	0.38 ± 0.10	0.37 ± 0.07	0.52 ± 0.13
C18: 0	1.83 ± 0.06	0.98 ± 0.18	2.18 ± 0.06	9.93 ± 2.85	17.20 ± 0.18	16.45 ± 0.85
C20: 0	0.29 ± 0.05	0.31 ± 0.14	0.38 ± 0.08	0.50 ± 0.04	0.54 ± 0.04	0.57 ± 0.09
C22: 0	0.15 ± 0.02	0.09 ± 0.02	0.23 ± 0.03	–	–	0.22 ± 0.05
Monounsaturated						
C16: 1	0.80 ± 0.08	0.63 ± 0.11	1.08 ± 0.04	5.39 ± 0.48	4.11 ± 0.16	3.63 ± 0.22
C17: 1	0.17 ± 0.02	–	0.11 ± 0.02	0.18 ± 0.08	-	0.32 ± 0.10
C18: 1 (n-9)	3.39 ± 0.10	3.58 ± 0.04	3.45 ± 0.22	16.84 ± 2.64	13.50 ± 3.18	15.80 ± 2.16
C18: 1 (n-7)	1.06 ± 0.10	0.88 ± 0.07	0.84 ± 0.18	0.84 ± 0.04	0.52 ± 0.05	0.64 ± 0.03
C20: 1	–	–	–	–	–	0.06 ± 0.01
Polyunsaturated						
C18: 2 (n-6)	2.28 ± 0.12	1.83 ± 0.18	3.14 ± 0.16	6.94 ± 0.22	9.04 ± 0.36	8.69 ± 0.18
C18: 3 (n-6)	0.12 ± 0.03	0.84 ± 0.07	0.13 ± 0.04	–	–	–
C18: 3 (n-3)	0.76 ± 0.09	0.81 ± 0.14	1.04 ± 0.12	0.82 ± 0.04	0.82 ± 0.10	0.78 ± 0.06
C20: 3 (n-6)	–	–	–	–	–	0.02 ± 0.00
C20: 4 (n-6)	0.18 ± 0.06	0.03 ± 0.00	0.38 ± 0.08	0.08 ± 0.02	0.20 ± 0.10	0.33 ± 0.15
C20: 5 (n-3)	0.11 ± 0.03	0.08 ± 0.01	0.44 ± 0.11	0.18 ± 0.04	–	0.28 ± 0.06
C22: 6 (n-3)	5.84 ± 0.16	6.88 ± 0.35	8.58 ± 0.73	0.83 ± 0.18	0.62 ± 0.26	3.22 ± 0.26
SFA	28.04 ± 2.18a	24.43 + 1.91a	30.75 ± 2.29a	37.02 ± 3.91b	50.73 ± 5.12b	52.82 ± 4.71b
MUFA	5.42 ± 0.70a	5.09 ± 0.94a	5.48 ± 0.72a	23.25 ± 3.85b	18.13 ± 3.87b	20.45 ± 3.00b
PUFA	9.29 ± 0.92 a	10.47 ± 1.06a	13.71 ± 1.34a	8.85 ± 1.30a	10.68 ± 2.13a	13.32 ± 1.38b
(n-3)	6.71 ± 1.81a	7.77 ± 2.16a	10.06 ± 2.62a	1.83 ± 0.22a	1.44 ± 0.1a	4.28 ± 0.91a
(n-6)	2.58 ± 0.71a	2.70 ± 0.52a	3.65 ± 0.96a	7.02 ± 3.43a	9.24 ± 4.42a	9.04 ± 2.84a
(SFA+ MUFA)/ PUFA	3.60	2.82	2.64	6.81	6.45	5.50
(n-3)/(n-6)	2.60	2.88	2.76	0.26	0.16	0.47

The values presented are means of three replicates. Means were compared using the *t* test ($\alpha = 0.05$); differences were not significant for groups with the same letter

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *ME* mid-exponential phase, *ES* early stationary phase, *LS* late stationary phase

– none detected

requirements for microalgae, especially light. This problem occurred when the cell density increased, which led to self-shading phenomena or poor light availability inside the culture (Pruvost et al. 2002; Chen and Chen 2006), even with the supply of aeration. This result was confirmed as *I. maritima* cultivated heterotrophically in 50 L of Walne's medium, with urea as the nitrogen source and glucose as the carbon source, showed no significant difference in the cell density compared to that cultivated in 1 L in the second experiment. The elimination of the light requirement in the heterotrophic cultivation gave an advantage to this species to maximise its growth without the light limitation. As a result, photoautotrophic cells recorded lower cell densities than the microalgae grown under heterotrophic conditions, which were 345% higher than those of the photoautotrophic cells.

The cell density, the cell DW, the ash content and the AFDW were also affected by the growth phase for both trophic conditions. The cell density, the cell DW and the AFDW increased as the culture aged. This result was expected because most of the biochemical compounds, except for protein, increased when the culture aged, especially carbohydrate and lipid (Fidalgo et al. 1998; Lin et al. 2007; Chiu et al. 2009). In the present study, the carbohydrate content slightly increased from the mid-exponential phase to the late stationary phase for

both trophic conditions. The lipid content was not tested in this study. The protein content showed different results, with the values being higher under both trophic conditions at the mid-exponential phase and decreasing at the late stationary phase. The biochemical composition of photosynthetic microalgae is closely related with their cell metabolism, and protein becomes the main component of the cells harvested during the exponential phase of growth and, consequently, replaced by storage products, such as carbohydrate and lipids, which increase along with the nutrient deficiency (Zhu et al. 1997; Phatarpekar et al. 2000).

As previously described, the objective of this study was to compare the growth and biochemical compositions of *I. maritima* cultivated in photoautotrophic and heterotrophic conditions for use in aquaculture feed. The obtained results showed that *I. maritima* grew well heterotrophically compared to photoautotrophic growth. There was no significant difference in the protein content in both trophic conditions. In contrast, the carbohydrate content was significantly higher in the heterotrophic cultures than in the photoautotrophic cultures. However, the content of omega-3, especially EPA and DHA, was low in heterotrophic cells. Because the omega-3 PUFAs are essential in the diets of many commercially important marine fish and shellfish, it is important for an aquaculturist to maximise the content of the omega-3 PUFAs in dietary algae. Therefore, *I. maritima* cultured heterotrophically was not suitable for use as aquaculture feed. This statement was supported by the low amount of omega-3/6 PUFA ratio obtained in all growth phases (0.16–0.47) and the high value of (SFA + MUFA)/PUFA (5.50–6.81) recorded in heterotrophic cells. The microalgal cells produced in the culture had an acceptable nutritional quality in bivalve larvae production when the omega-3/6 PUFAs quotient was within a range value of 2–5 (Sánchez et al. 2000) and with a lower value of (SFA + MUFA)/PUFA (Lin et al. 2007). In contrast, photoautotrophic cells showed optimal values of omega-3/6 PUFA ratio (2.60–2.88) and a low value of (SFA + MUFA)/PUFA (2.64–3.60). In addition, harvesting the cells at the late stationary growth phase may allow a better yield of its composition because the protein, EPA and DHA contents were highest at this phase. The composition of cells grown mixotrophically also should be examined (Alkhamis and Qin 2015).

In conclusion, *I. maritima* cells cultivated under photoautotrophic and heterotrophic conditions displayed different growth characteristics and biochemical compositions, especially in terms of PUFAs. Although the heterotrophic algal cells had much higher yields of cell density and total SFAs, MUFAs and PUFAs than the photoautotrophic cells, the high omega-3/6 PUFAs ratio of heterotrophic *I. maritima* compared to photoautotrophic cells as well as the high value of (SFA + MUFA)/PUFA demonstrated that this species is unsuitable for use as aquaculture feed in the heterotrophic condition.

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