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Process optimization of fucoxanthin production with *Tisochrysis lutea*

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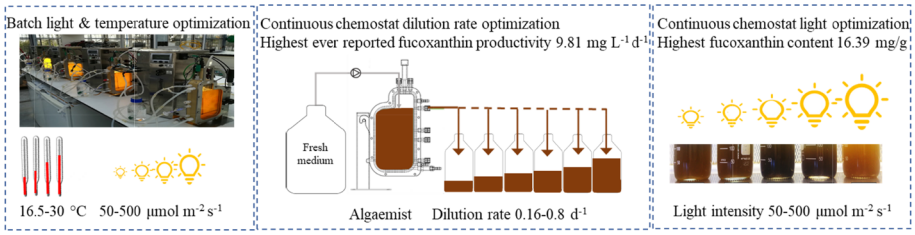
GRAPHICAL ABSTRACT

Process optimization of fucoxanthin production.



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ABSTRACT

To optimize fucoxanthin production in *Tisochrysis lutea*, the effect of different process parameters on fucoxanthin productivity (Pfx) were evaluated using batch and continuous experiments. In batch, the highest Pfx was found at 30 °C and 300 µmol m^{-2} s^{-1}, allowing to design continuous experiments to optimize the dilution rate. The highest ever reported Pfx (9.43–9.81 mg L^{-1} d^{-1}) was achieved at dilution rates of 0.53 and 0.80 d^{-1}. Irradiance was varied (50–500 µmol m^{-2} s^{-1}) to result in a range of absorbed light between 2.23 and 25.80 mol m^{-2} d^{-1} at a fixed dilution rate (0.53 d^{-1}). These experiments validated the hypothesis that light absorbed can be used to predict fucoxanthin content, resulting in 2.23 mol m^{-2} d^{-1} triggering the highest fucoxanthin content (16.39 mg/g). The highest Pfx was found with 18.38 mol m^{-2} d^{-1}. These results can be used to achieve high Pfx or fucoxanthin content during cultivation of *Tisochrysis lutea*.

1. Introduction

Fucoxanthin (Fx) is the main carotenoid in marine brown algae (seaweeds and microalgae) (Miyashita et al., 2011), playing a key role in photosynthesis (Kita et al., 2015; Wang et al., 2019). Industrial production of Fx-rich algal species focuses on niche markets as aquaculture feed due to its biological activity in marine organism reproduction (Kawakami et al., 1998), and in cosmetics because of functions in skin photoaging protection and antiaging (Kang et al., 2020; Urikura et al., 2011). However, its market potential is larger and Fx has gained more attention in the last years due to its biological properties, such as antioxidant, anti-obesity, and antidiabetic (Fung et al., 2013; Maeda et al., 2018). Furthermore, the demand for microalgal functional compounds are becoming increasingly urgent with the fast growing markets of aquaculture and healthy products.

The global Fx production was approximately 500 t in 2016 and was projected to grow at an annual rate of 5.3% between 2016 and 2021 (Joel, 2016). The current Fx production uses edible brown seaweed as feedstock, despite their low concentration in Fx (0.01 to 3.7 mg/g dry weight (DW)) (Terasaki et al., 2012; Verma et al., 2017; Zailanie and Sukoso, 2014). Microalgae have up to 100 times more Fx than seaweeds (Lu et al., 2018). For example, Fx content was reported up to 18.23 mg/g dry weight (DW) in *Isochrysis* aff. *galbana* (Kim et al., 2012). In addition, a novel isolated strain of *Mallomonas* sp. can produce 26.6 mg/g

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DW Fx (Petrushkina et al., 2017), while *Phaeodactylum tricornutum* can reach up to 59.2 mg/g (McClure et al., 2018). Therefore, microalgae can be a more promising commercial source of Fx compared with current feedstocks.

Tisochrysis lutea (also named as *Isochrysis galbana* T-Iso) (Bendif et al., 2013), with cellular Fx contents ranging from 2.24 to 18.23 mg/g DW (Delbrut et al., 2018; Kim et al., 2012; Mohamadnia et al., 2020), is considered a promising microbial cell factory for the production of Fx. The highest Fx content and productivity reported are 23.29 mg/g at 40 μmol m^{-2} s^{-1}, and 2.94 mg L^{-1} d^{-1} at 120 μmol m^{-2} s^{-1}, respectively, measured in *Isochrysis zhangjiangensis* cultivated in 700 mL bubble column photobioreactors under batch mode (Li et al., 2019). Fx productivity in a screened *Isochrysis* strain can reach up to 7.96 mg L^{-1} d^{-1} in semi-continuous mode in bubble column photobioreactors (Sun et al., 2019). The Fx content and productivity in *Tisochrysis lutea* can be improved by process optimization (Gómez-Loredo et al., 2016). Process optimization is a critical factor for the industry to overcome the bottlenecks of low yields and high production cost.

Process parameters, such as light and temperature, are key factors for growth and can affect Fx content and productivity. Increasing light intensity negatively affected Fx content of *Isochrysis galbana* (Gómez-Loredo et al., 2016). Fx content at light intensity of 9.1 μmol m^{-2} s^{-1} was 10.9 times higher than that at 62.0 μmol m^{-2} s^{-1}. In addition to the light intensity, temperature can affect biomass productivity hence affect Fx productivity. The maximum growth rate of *Isochrysis galbana* Parke was reported to be nearly double at 18 °C as at 26 °C (Durmaz et al., 2008). Ippoliti et al. (2016) developed a model for growth prediction of *Isochrysis galbana*, demonstrating that this strain tolerates temperatures up to 35 °C, but it is highly sensitive to irradiances higher than 500 μmol m^{-2} s^{-1}. Therefore, both light and temperature are important parameters for biomass and fucoxanthin production. While many previous studies have reported the production of Fx by *Tisochrysis* (*Isochrysis*), few systematic studies have been done on the influence of process parameters on Fx production. The Fx content in *Tisochrysis lutea* varies widely in literature and no specific strategy has been reported to improve productivity. Process parameters (light and temperature) and cultivation mode (batch and continuous) should be combined to optimize Fx content and productivity.

In this study, batch experiments were conducted in flat panel photobioreactors to obtain the optimum growth conditions (light intensity and temperature) for *Tisochrysis lutea*. Following, continuous experiments were done to determine the optimum dilution rate for Pfx. Using this dilution rate, irradiance was varied to assess the effect of absorbed light on Fx content and productivity.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Tisochrysis lutea (originally isolated from a tropical region of the Pacific) (Bendif et al., 2013) and commercial culture medium stock NutriBloom Plus (Fernandes et al., 2016) were obtained from NECTON, S.A. (Olhão, Portugal). Mineral composition of nutribloom: $NaNO_3$ 2 M, KH_2PO_4 100 mM, $ZnCl_2$ 1 mM, $ZnSO_4$ 1 mM, MnCl 1 mM, Na_2MoO_4 0.1 mM, CoCl 0.1 mM, $CuSO_4$ 0.1 mM, EDTA 26.4 mM, $MgSO_4$ 2 mM, $FeCl_3$ 20 mM, Tiamina 35 mM, Biotina 5 mg/L, vitamin B_{12} 3 mg/L. The cultivation medium was prepared with natural seawater from the North Sea (The Netherlands) enriched with 2 mL/L of the NutriBloom Plus stock with a final pH = 8.0 (containing 20 mM HEPES). The strain was cultured in 250 mL flasks containing 100 mL medium at 25 °C, light intensity 140 μmol m^{-2} s^{-1} and 2% CO_2. In the present study, 18:6 day:night photoperiod was applied for cultivation under phototrophic conditions, which has been reported to lead to higher biomass productivities than photoperiods of 12:12 and 24:0 (Babuskin et al., 2014; Che et al., 2019).

2.2. Batch experiments

2.2.1. Batch experiments with different light intensities

Tisochrysis lutea cultures were inoculated into flat panel photobioreactors (Algaemist, light path 14 mm) (Breuer et al., 2012) containing 400 mL NutriBloom medium, at 25 °C. The aeration rate was 400 L min^{-1} for all experiments performed in the Algaemists. The pH was kept constant at 8.0 by CO_2 injection. The incident light intensities studied were 50, 150, 300, and 500 μmol $m^{-2}s^{-1}$, with an 18:6 day:night cycle. The biomass was harvested by centrifugation (4255 × *g* 5 min) at the end of the batch and washed twice with 0.5 M ammonium formate. The supernatant was removed, and the biomass was flushed with N_2 gas and stored at −20 °C. The microalgal biomass was grinded after freeze drying for 13 h and posteriorly used for Fx analyses.

2.2.2. Batch experiments at different temperatures

Tisochrysis lutea was cultivated in Algaemists at 16.5, 20, 25, and 30 °C. The incident light intensity was 300 μmol m^{-2} s^{-1} with an 18:6 day:night cycle, for all experiments. The biomass was harvested, washed, and freeze-dried according to the methods mentioned above. Biomass concentration and Fx content were analyzed at the end of each run.

2.3. Continuous experiments

2.3.1. Chemostat experiments at different dilution rates

NutriBloom medium was enriched with extra nitrogen ($NaNO_3$) to a final concentration 40 mM for all continuous experiments in present research. *Tisochrysis lutea* was inoculated into five Algaemists at a light intensity of 300 μmol m^{-2} s^{-1} with an 18:6 day:night cycle and at 30 °C. *Tisochrysis lutea* was grown under batch mode for 3 days to reach the desired cell density (optical density at 750 nm (OD_{750}) > 4), then the chemostat started. The cultures were diluted at different specific dilution rates: 0.16, 0.29, 0.35, 0.45, 0.53 d^{-1} (chemostat experiments). The dilution rate of the Algaemist at 0.53 d^{-1} was increased at the end of the experiment to investigate a higher dilution rate (0.8 d^{-1}). The growth at 0.8 d^{-1} was combined with the other groups for comparison. The dilution rates were selected based on the growth rates obtained in batch experiments. Since steady state cannot be reached when dilution rate is higher than the maximum growth rate, dilution rates higher than 0.8 d^{-1} were not tested. The overflow cultures from the Algaemists were harvested every day and washed and freeze-dried based on the methods mentioned above.

2.3.2. Chemostat experiments with different light intensities

Tisochrysis lutea were inoculated into Algaemists with 400 mL medium. For the first four days, the microalgae were grown in batch mode with an incident light intensity of 300 μmol m^{-2} s^{-1} under an 18:6 day:night cycle. When biomass concentrations reached an OD_{750} > 4, the light intensities were changed to 50, 100, 150, 300, and 500 μmol m^{-2} s^{-1}. The chemostat experiments were started with a dilution rate of 0.53 d^{-1} at the same time when the light changing. The biomass was harvested every day for Fx measurement.

2.4. Fucoxanthin extraction and measurement

Small samples of biomass (~1 mg) were extracted with 1 mL of ethanol using bead beater tubes (MP Biomedicals, REF 6914-500) in a beater (Bertin Technologies). The beating procedure was 3 × 60 s with a 2-min pause between each beating. The first 3 beatings were done at 2500 rpm, followed by a round of 2 × 60 s at 2500 rpm, with a 2-min pause. The supernatant was pelleted by centrifugation (10 000 × *g* 2 min) and transferred into an empty glass tube. The bead beater tubes were washed with 1 mL ethanol and the supernatants were transferred into the glass tubes after vortexing for 5 s and centrifugation (10 000 × *g* 2 min). The washing step was repeated three times. The

Table 1
Cell diameter, final cell concentration, and biomass productivity at different light intensities and a temperature of 25 °C.

Light (μmol m^{-2} s^{-1})	50	150	300	500
Growth rate (d^{-1})	0.21	0.61	0.74	0.72
Diameter (μm)	4.67 ± 0.08	4.90 ± 0.05	4.82 ± 0.07	4.88 ± 0.07
Final biomass concentration DW (g/L)	0.62 ± 0.03[d]	1.62 ± 0.08[c]	1.91 ± 0.10[a]	1.80 ± 0.09[b]
Biomass Productivity (g L^{-1} d^{-1})	0.09 ± 0.02 [d]	0.29 ± 0.01[c]	0.35 ± 0.01 [a]	0.32 ± 0.01[b]

Note: values are the means ± SD. Means with different letters are significantly different from each other (comparisons were made between groups for each variable) ($p < 0.05$).

samples were dried by evaporation under nitrogen gas and resuspended with 2 mL of methanol. The samples were filtered through a 0.2 μm filter (SPARTAN™ 13/0.2 RC) into glass vials (2 mL, Agilent Technologies) using 1 mL syringe. To quantify the concentration of Fx, samples were run in a high-performance liquid chromatography (HPLC) (Shimadzu, Nexera UHPLC) according to Grant (2011). The injected sample volume was 20 μL, the stationary phase was a C18 column (Kinetex C18 5 μm 100 Å 150 × 4.6 mm). The standard of Fx was bought from Sigma-Aldrich and were diluted with methanol to different concentration of 0, 2, 4, 6, 8, 10 μg/mL for concentration calibration.

2.5. Growth measurement

The optical density of culture was measured at 750 nm with a 1.0 cm light path cuvette in a HACH LANGE DR600 spectrophotometer. Cell size was measured using a Beckman Coulter Multisizer 3. To measure the dry weight (DW), 2 mL of culture was diluted with 50 mL 0.5 M ammonium formate and filtered through a pre-dried Whatman GF/F filter (0.7 μm pore size) and washed twice with 50 mL 0.5 M ammonium formate. Cells on the filters were dried at 100 °C in the oven until constant weight and were subsequently cooled to room temperature in a desiccator for 2 h before weighting. The DW is the difference value between the filter weight with and without microalgae.

2.6. Calculations

The growth rate (μ; d^{-1}) in batch was calculated according to Eq. (1);

$$\mu(d^{-1}) = \ln(OD_2/OD_1)/(t_2 - t_1) \quad (1)$$

where OD_2 is the OD_{750} value at the end of the exponential growth phase and OD_1 is the OD_{750} value at the start of exponential growth phase. The time of exponential growth is represented by t_2 and t_1.

The volumetric biomass productivity (P_X; mg/L/d) in batch experiments was calculated using Eq. (2);

$$P_X = (DW_2 - DW_1)/(t - t_0) \quad (2)$$

where DW_2 and DW_1were the biomass DW concentrations (g L^{-1}) on days t (end-point of cultivation) and t_0 (start-point of cultivation), respectively.

The volumetric biomass productivity (P_X; mg/L/d) in continuous experiments was calculated using Eq. (3);

$$P_X = Cx \times D \quad (3)$$

where *Cx* was the biomass concentration (g L^{-1}) and D was the dilution rate (d^{-1}) at steady state. The growth rate in continuous experiments during steady state is equal as the dilution rate D (d^{-1}) which was calculated by the ratio between the overflow culture volume and total cultivation volume (400 mL).

In addition, at steady-state, the daily absorbed light intensity (I_{abs}; mol m^{-2} d^{-1}) was calculated as Eq. (4);

$$I_{abs} = \sum_0^t (I_i - B_I) \quad (4)$$

where I_i is the incident light intensity (μmol m^{-2} s^{-1}), B_I is the backlight intensity (μmol m^{-2} s^{-1}) measured by light meter (LI-COR, LI250A). The illumination time (*t*) was 18 h (translated to second in I_{abs} calculation) for all the experiments in the present study.

2.7. Statistical analyses

The results were analyzed based on the data from at least three different cultivation points during steady state. Experimental results were expressed as mean value ± SD. Differences between groups were tested for significance by the least significant difference mean comparison using the IBM® SPSS® Statistics software program (version 25). The relationship between variables was determined by one-way ANOVA at a significance level of 0.05 using a Duncan Post-Hoc test.

3. Results & discussion

3.1. Effect of light and temperature on batch fucoxanthin production

3.1.1. Effect of light on growth and fucoxanthin production

This section shows the effect of different light intensities (50, 150, 300, and 500 μmol m^{-2} s^{-1}) on *Tisochrysis lutea* grown in batch mode for 5 days.

The highest growth rate (0.74 d^{-1}) was observed at 300 μmol m^{-2} s^{-1} while the lowest was obtained at 50 μmol m^{-2} s^{-1} (0.41 d^{-1}) (Table 1). Similar to growth rate, the highest biomass productivity (0.35 g L^{-1} d^{-1}) was observed at 300 μmol m^{-2} s^{-1}, and the lowest (0.09 g L^{-1} d^{-1}) at 50 μmol m^{-2} s^{-1} (Table 1). Microalgae growth is closely related to the light intensity experienced by the cells inside a reactor. An increase (from ~0.4 to ~1.1 d^{-1}) of growth rate in *Isochrysis galbana* was reported with increasing light intensity from 30 to 300 μmol m^{-2} s^{-1}, and a slight decrease at 500 μmol m^{-2} s^{-1}, possibly due to photoinhibition, in a continuous turbidostat experiment (Sukenik et al., 1993). Similar results were reported where the specific growth rate decreased with increasing light intensity from 300 to 600 μmol m^{-2} s^{-1} at 30 °C (Kurpan Nogueira et al., 2015). However, no difference was found in growth rate between 300 and 600 μmol m^{-2} s^{-1} at 20 °C (Kurpan Nogueira et al., 2015), indicating combined effects of light and temperature.

Regarding Fx content, batch experiments showed a 3.0-fold increase ($p < 0.05$) with decreasing light from 500 to 50 μmol m^{-2} s^{-1} (Fig. 1a), from 1.74 mg/g to 5.24 mg/g. To optimize Pfx, both biomass production and Fx content need to be considered. The highest Pfx was found at 150 μmol m^{-2} s^{-1} and the lowest at 500 μmol m^{-2} s^{-1} (Fig. 1a). Similar to our results, Li et al. (2019) reported that the Fx content in *Isochrysis zhangjiangensis* cultivated in 700 mL bubble column photobioreactors increased as the light decreased from 300 to 40 μmol m^{-2} s^{-1}.

These experiments show the optimum conditions for biomass and Pfx, i.e. the balance between high pigment and biomass production.

3.1.2. Effect of temperature on growth and fucoxanthin production

Results from the different batches done under different temperatures (16.5, 20, 25, and 30 °C) and a constant light intensity of 300 μmol m^{-2} s^{-1} show that *Tisochrysis lutea* grew faster (0.78 d^{-1}) at 30 °C than at lower temperatures (Table 2). The biomass productivity increased from 0.06 to 0.42 g L^{-1} d^{-1} with increasing temperature

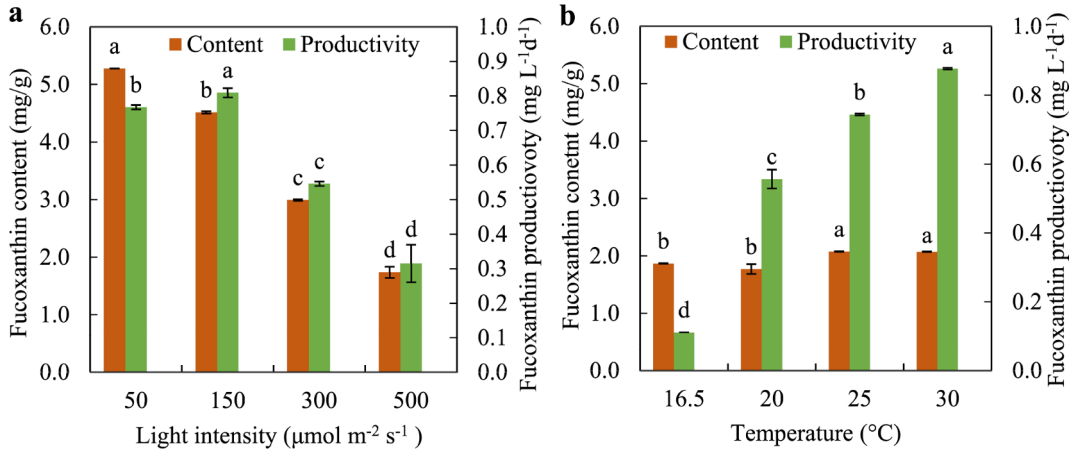

Fig. 1. Fucoxanthin contents and productivities of *Tisochrysis lutea* at different light intensities (a) and different temperatures (b). Note: values are the means ± SD; Bars with different letters are significantly different from each other (comparisons were made between bars with the same color only) ($p < 0.05$).

from 16.5 to 30 °C (Table 2). The final biomass at 30 °C was 5.2-fold higher than at 16.5 °C. In literature, the cultivation temperature of *Isochrysis* ranges from 5 to 35 °C (Araie et al., 2018; Ippoliti et al., 2016; Li et al., 2016), with the most commonly used temperatures between 25 and 30 °C (Abu-Rezq et al., 1999; All et al., 2012; Renaud et al., 2002). The optimum temperature varies for different strains of *Isochrysis* species; for example, Kaplan et al. (1986) reported a reduced biomass yield in *Isochrysis galbana* at temperatures higher than 32 °C or lower than 19 °C. Likewise, the cell density of *Isochrysis galbana* Parke was reported to be 5 times higher at 20 °C than at 35 °C (Su et al., 2017). These differences are strain-specific and are usually associated with the origin of the strains. *Tisochrysis lutea* strain was originally isolated from a tropical region of the Pacific (Bendif et al., 2013), which explains its adaptation to higher temperatures. In addition, the growth rate of *Isochrysis* sp. decreased from 0.89 to 0.04 d^{-1} with increasing temperature from 30 to 35 °C (Renaud et al., 2002). Hence, temperatures higher than 30 °C were not studied in the present study.

Fx contents were similar at 30 °C and 25 °C ($p > 0.05$) and significantly higher than at 20 °C and 16.5 °C ($p < 0.05$; Fig. 1b). Pfx was higher ($p < 0.05$) with increasing temperatures due to higher biomass productivities. The Pfx at 30 °C was 7.9-, 1.6-, and 1.2-fold higher than at 16.5, 20, 25 °C, respectively. The optimal temperature was 30 °C for both biomass and Pfx. The same optimum temperature was obtained from a prediction model in a previous study (Marchetti et al., 2013).

In our results, Pfx improved at higher temperatures due to higher biomass productivity. Moreover, the cellular diameter was larger at 16.5 °C ($p < 0.05$) (Table 2), which is a known response to low temperatures due to intracellular lipid accumulation (Skau et al., 2017). Lipid bodies can be seen under microscope at 16.5 °C. This alteration in cell diameter, associated with lower growth, indicates that lower temperatures are not lethal but lead to cellular stress in *Tisochrysis lutea*. A similar phenomenon was reported before where negative growth rates (~0.1 d^{-1}) and increased lipid contents in *Isochrysis galbana* were observed at 10 °C (Roleda et al., 2013).

Overall, the batch experiments provided the optimum parameters, 300 μmol m^{-2} s^{-1} and 30 °C, for growth and biomass production, and supplied evidence for the effect of light on Fx production, which were used when designing the continuous experiments, described in the following sections.

3.2. Continuous fucoxanthin production

Continuous experiments were performed to determine the dilution rate resulting in the highest biomass productivity; and therefore, the highest Pfx. Following, the dilution rate was fixed, and light was varied, leading to a range of absorbed light values. These second experiments were used to investigate the influence of absorbed light on Fx content. Together, these data were used to indicate the conditions to increase Fx content (achievable at lab scale), and productivities (applicable to pilot/outdoor conditions).

3.2.1. Chemostat experiments with different dilution rates

Steady state was kept for at least 7 consecutive days (Fig. 2a). The biomass concentration decreased from 3.04 to 1.23 g/L with increasing the dilution rate from 0.16 to 0.80 d^{-1} (Table 3) while biomass productivity increased from 0.49 to 1.01 g L^{-1} d^{-1} (Table 3), showing a linear relation between both variables (Fig. 2b; R^2 0.96) at an incident light of 300 μmol m^{-2} s^{-1}. A different relation has been found for *Dunaliella tertiolecta* at an incident light intensity of approximately 1000 μmol m^{-2} s^{-1} (Barbosa et al., 2005); a plateau of volumetric biomass productivity was reported over a range of dilution rates (0.03 to 0.05 h^{-1}). In the present work, 94.5–99.8% of the incident light (300 μmol m^{-2} s^{-1}) was absorbed by the culture, meaning that light limitation may have taken place, specially at low dilution rates (i.e. high biomass concentrations). In addition, the relation between absorbed light and biomass productivity may differ per strain. Experiments with varying light intensity were designed in the next section to further study the effect of light on fucoxanthin productivity.

The lowest biomass productivity (0.49 g L^{-1} d^{-1}) in continuous chemostat experiments was higher than the highest biomass productivity in batch experiments (0.42 g L^{-1} d^{-1}). The biomass productivity of *Isochrysis galbana* was reported up to 0.51 g L^{-1} d^{-1} at

Table 2
Cell diameters and biomass productions at different temperatures and an incident light intensity of 300 μmol m^{-2} s^{-1}.

Temperature (°C)	16.5	20	25	30
Growth rate (d^{-1})	0.38	0.74	0.76	0.78
Diameter (μm)	6.05 ± 0.31^{a}	4.89 ± 0.16^{b}	4.30 ± 0.22^{c}	4.57 ± 0.22^{bc}
Final Biomass Concentration DW (g/L)	0.35 ± 0.02^{d}	1.36 ± 0.07^{c}	1.54 ± 0.08^{b}	1.81 ± 0.09^{a}
Biomass Productivity (g L^{-1} d^{-1})	0.06 ± 0.01 ^{d}	0.32 ± 0.02^{c}	0.36 ± 0.02^{b}	0.42 ± 0.02 ^{a}

Note: values are the means ± SD. Means with different letters are significantly different from each other (comparisons were made between groups for each variable) ($p < 0.05$).

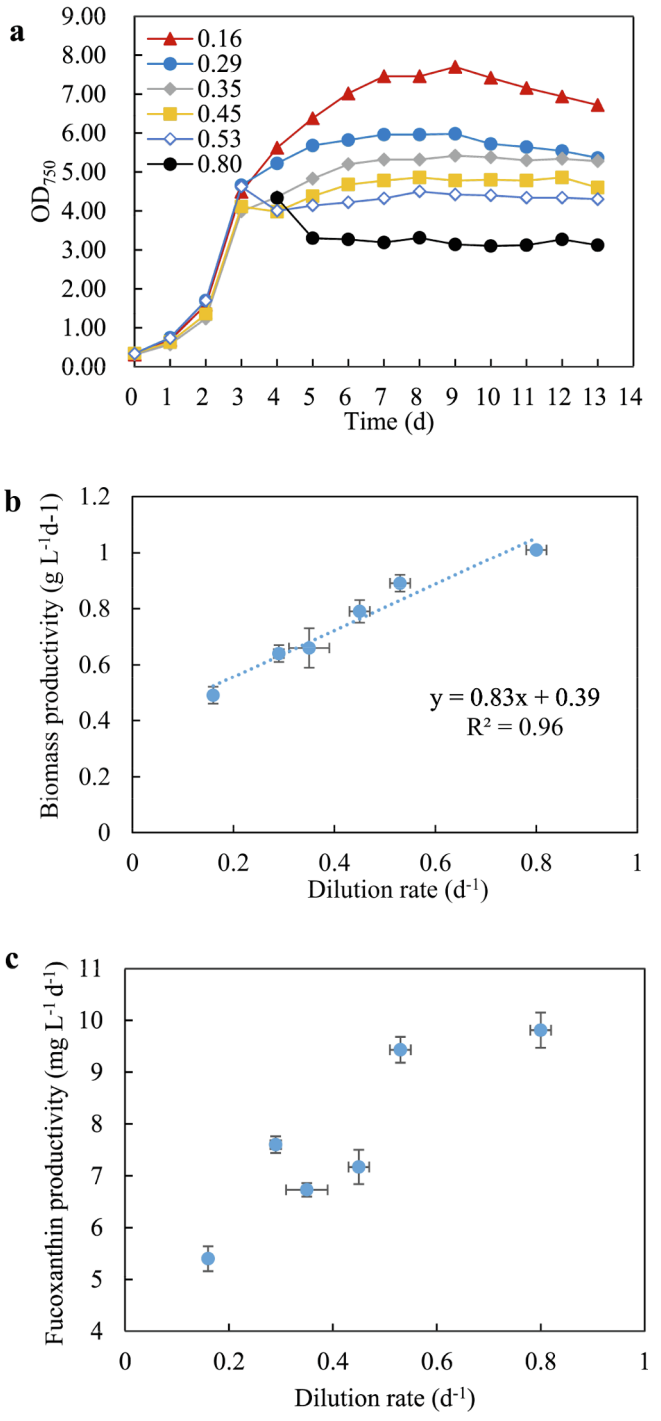

Fig. 2. Continuous growth (a), correlation between biomass productivity and dilution rate (b) and correlation between fucoxanthin productivity and dilution rate (c) of *Tisochrysis lutea* at different dilution rates and an incident light intensity of 300 μmol m^{-2} s^{-1}, 30 °C. Note: values are the means ± SD; Bars with different letters are significantly different from each other (comparisons were made between bars with the same color only) ($p < 0.05$).

semi-continuous mode, which was higher than in batch and intermittent fed batch modes (Picardo et al., 2013). In the present study, the biomass productivity obtained with continuous chemostats was the double of the productivity obtained with semi-continuous operation. Chemostat cultivation showed the highest biomass production among different cultivation modes.

The Fx content ranged from 9.16 to 12.02 mg/g without obvious trends (Table 3). The cells were observed with similar dark brownish colors under microscope, indicating high pigments contents. In literature, the carotenoid content per cell in *Isochrysis* increased with increasing growth rate from 0.33 to 1.0 d^{-1} in continuous chemostat mode (Saoudi-Helis et al., 1994). High growth rate leads to more intracellular Fx. However, we didn't find a higher Fx content at higher growth rate in the present study. This could be explained by the small variation of the total daily light absorbed among the different experiments (18.38–19.40 mol m^{-2} d^{-1}), leading to a similar Fx content in all groups.

The Pfx increased from 5.40 to 9.81 mg L^{-1} d^{-1} as the dilution rate increased from 0.16 to 0.80 d^{-1}, which was mainly affected by biomass productivity (Fig. 2c). The Pfx at dilution rate of 0.53 d^{-1} was similar to that at 0.80 d^{-1} without significant differences ($p > 0.05$). To our knowledge, 9.81 mg L^{-1} d^{-1} is the highest Pfx in *Tisochrysis/Isochrysis* in literature (2.94–7.96 mg L^{-1} d^{-1}) (Li et al., 2019; Sun et al., 2019). Li et al. (2019) reported a higher Fx content (23.29 mg/g) in *Isochrysis zhangjiangensis* at 40 μmol m^{-2} s^{-1} in batch; however a lower Pfx (2.94 mg L^{-1} d^{-1}) was obtained in their studies. Moreover, 7.96 and 6.43 mg L^{-1} d^{-1} Pfx were obtained at day 3–5 and day 5–7, respectively, in a screened *Isochrysis* strain at semi-continuous cultivation mode with a light intensity of 60 μmol m^{-2} s^{-1} (Sun et al., 2019). No significant differences were found between the dilutions of 0.53 and 0.80 d^{-1} in Pfx. Hence, 0.53 d^{-1} was selected as the fixed dilution rate to investitive the effect of light on Fx production in next section.

3.2.2. Chemostat experiments with different light intensities

To investigate the effect of absorbed light on Fx production, different light intensities (50, 100, 150, 300, 500 μmol m^{-2} s^{-1}) were tested under the dilution rate of 0.53 d^{-1} to investigate the effect of absorbed light on Fx production in *Tisochrysis lutea*.

The chemostat experiments started after day 4 of batch (Fig. 3a). Biomass concentration increased from 0.31 to 1.50 g/L with increasing light intensity from 50 to 300 μmol m^{-2} s^{-1} (Table 4). At 500 μmol m^{-2} s^{-1} steady state could not be reached, the biomass concentration decreased during the entire chemostat period, which indicated photoinhibition. The culture was yellowish at 500 μmol m^{-2} s^{-1} whereas brownish at low light intensities. Moreover, visible (under the microscope) lipid bodies can only be observed in cells exposed to 500 μmol m^{-2} s^{-1}. The cell dimeter at 500 μmol m^{-2} s^{-1} was significantly larger ($p < 0.05$) than other groups (Table 4). Biomass productivity also increased as light intensity increased from 50 to 300 μmol m^{-2} s^{-1} and decreased at 500 μmol m^{-2} s^{-1}. These results were in line with the results from the batch experiments at different light intensities.

The Fx content increased from 3.68 to 16.39 mg/g as the light intensity decreased from 500 to 50 μmol m^{-2} s^{-1} (Table 4). The highest Fx content at 50 μmol m^{-2} s^{-1} was 4.5-fold higher than at 500 μmol m^{-2} s^{-1}. Light intensity affected Fx content in *Tisochrysis lutea*. Previous studies also showed that Fx content was decreased by high light intensities, and increased with low light intensities in *Isochrysis* (Gómez-Loredo et al., 2016; Li et al., 2019). In the present study, the Fx content ranged from 1.73 to 16.39 mg/g in batch and continuous experiments. The maximum Fx content obtained at 50 μmol m^{-2} s^{-1} is comparable to the results from Kim et al. (2012), who reported a 18.23 mg/g Fx content by optimization of extraction solvent; this is the highest content in *Tisochrysis* found in literature. However, they did not report the cultivation conditions of the biomass they used in their study.

The Pfx at 150 and 300 μmol m^{-2} s^{-1} were significantly higher ($p < 0.05$; Fig. 3b) than those at 50, 100, and 500 μmol m^{-2} s^{-1}; Pfx at 50, 150, 300, and 500 μmol m^{-2} s^{-1} was 3.1-fold, 6.0-fold, 8.9-fold, and 7.8-fold higher than those in batch experiments at the same light intensity (respectively). Continuous cultivation mode showed clear advantages in Fx production. The light intensity of 300 μmol m^{-2} s^{-1} was considered optimum for both highest biomass and Fx productivities.

The continuous chemostat at different light intensities were

Table 3
Growth details of continuous experiments at different dilution rates.

Dilution rate (d^{-1})	0.16	0.29	0.35	0.45	0.53	0.80
μ (d^{-1})	0.16 ± 0.0^{f}	0.29 ± 0.01^{e}	0.35 ± 0.04^{d}	0.45 ± 0.02^{c}	0.53 ± 0.02^{b}	0.80 ± 0.02^{a}
Diameter (μm)	5.40 ± 0.05^{b}	5.28 ± 0.02^{c}	5.31 ± 0.02^{c}	5.25 ± 0.02^{c}	5.55 ± 0.02^{a}	5.59 ± 0.03^{a}
Biomass Concentration DW (g/L)	3.04 ± 0.01^{a}	2.18 ± 0.01^{b}	1.86 ± 0.01^{c}	1.74 ± 0.03 ^{cd}	1.67 ± 0.01^{d}	1.23 ± 0.09^{e}
Biomass Productivity (g L^{-1} d^{-1})	0.49 ± 0.03^{e}	0.64 ± 0.03^{d}	0.66 ± 0.07^{d}	0.79 ± 0.04^{c}	0.89 ± 0.03^{b}	1.01 ± 0.01^{a}
Absorbed light (mol m^{-2} d^{-1})	19.40 ± 0.01^{a}	19.32 ± 0.02^{ab}	19.25 ± 0.02^{b}	18.97 ± 0.09^{c}	19.02 ± 0.05^{c}	18.38 ± 0.20^{d}
Fx content (mg/g)	11.10 ± 0.49^{ab}	12.02 ± 0.26^{a}	10.34 ± 0.20^{bc}	9.16 ± 0.42^{b}	10.69 ± 0.28^{a}	10.01 ± 0.35^{a}
Pfx (mg L^{-1} d^{-1})	5.40 ± 0.24^{d}	7.60 ± 0.16^{b}	6.73 ± 0.13^{c}	7.17 ± 0.33^{b}	9.43 ± 0.25^{a}	9.81 ± 0.34^{a}

Note: values are the means ± SD. Means with different letters are significantly different from each other (comparisons were made between groups for each variable) ($p < 0.05$).

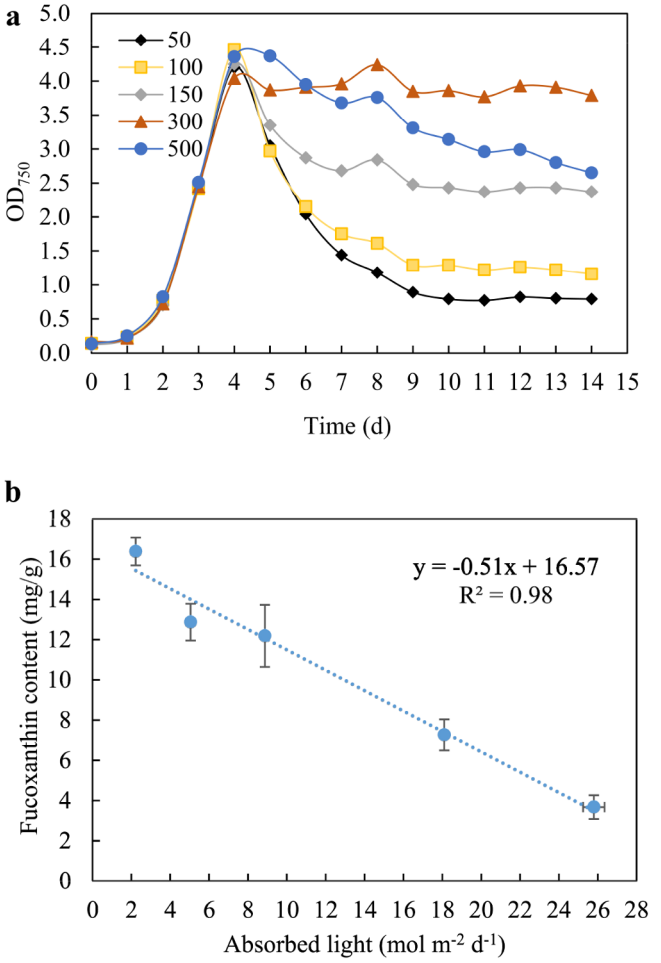

Fig. 3. Biomass concentration (a) and correlation between absorbed light and Fx content (c) of *Tisochrysis lutea* grown under chemostat operation at different light intensities with dilution rate of 0.46–0.54 d^{-1}. Note: values are the means ± SD; Bars with different letters are significantly different from each other (comparisons were made between bars with the same color only) ($p < 0.05$).

operated at similar dilution rates, hence light is the main factor that affected Fx production. The absorbed light increased from 2.23 to 25.80 mol m^{-2} d^{-1} with increasing light from 50 to 500 μmol m^{-2} s^{-1} (Table 4). A lower absorbed light resulted in a higher Fx content, but not necessarily higher Fx productivity (Table 4). These results tested the hypothesis that absorbed light could predict Fx content (R^2 0.98), resulting in an absorbed light of 2.23 (mol m^{-2} d^{-1}) causing the highest Fx content (16.39 mg/g) (Fig. 3c). The correlation between absorbed light and Fx content obtained in the present study provided an idea to estimate the Fx content in pilot/outdoor conditions by measuring absorbed light. Many factors such as light, growth, and nutrient can affect Fx content. Based on our findings in the present study, absorbed light is the most direct parameter that affects Fx content, which should be considered as the main assessment criteria in industrial Fx production.

This study showed how the process affects Fx content and productivities in *Tisochrysis lutea*. First, batch experiments were used to obtain the optimum temperature (30 °C) and light intensity (300 μmol m^{-2} s^{-1}) for biomass production. Second, continuous chemostat experiments were designed to investigate the effect of dilution rate (0.16–0.80 d^{-1}) on Fx content and productivity, obtaining the highest ever reported Pfx (9.81 mg L^{-1} d^{-1}). Finally, continuous chemostat at a fixed dilution rate (0.53 d^{-1}) at different light intensities were conducted to analyze the effect of absorbed light (2.23–25.80 mol m^{-2} s^{-1}) on Fx production. Fx content and Pfx were improved by using different cultivation parameters. An increasing dilution rate increased Pfx and an increasing absorbed light decreased Fx content. Dilution rate and absorbed light should be considered in combination to achieve the optimum Pfx in *Tisochrysis lutea*.

4. Conclusions

This study optimized the process parameters for Fx production in *Tisochrysis lutea* at batch and continuous modes. The maximum Fx content (16.39 mg/g) was found at 50 μmol m^{-2} s^{-1}, dilution rate 0.47 d^{-1}, 30 °C; and the maximum Pfx (9.81 mg L^{-1} d^{-1}) was found at 300 μmol m^{-2} s^{-1}, dilution rate 0.80 d^{-1}, 30 °C. Pfx was positively affected by dilution rate and Fx content was negatively corrected to light absorbed (R^2 0.98). Therefore, one should combine absorbed light and dilution to achieve optimal Pfx. Process parameters optimization improved Fx production, which could be translated to industrial applications.

Table 4
Growth details of continuous experiment at different light intensities.

Light (μmol m^{-2} s^{-1})	50	100	150	300	500
μ (d^{-1})	0.47 ± 0.04^{bc}	0.54 ± 0.02^{a}	0.50 ± 0.03^{b}	0.46 ± 0.06^{c}	0.54 ± 0.03^{a}
Diameter (μm)	5.43 ± 0.04^{b}	5.21 ± 0.01^{c}	5.42 ± 0.01^{b}	5.24 ± 0.03^{c}	5.65 ± 0.01^{a}
Biomass Concentration DW (g/L)	0.31 ± 0.04^{e}	0.44 ± 0.05^{d}	0.82 ± 0.02^{c}	1.50 ± 0.03^{a}	1.29 ± 0.05^{b}
Biomass Productivity (g L^{-1} d^{-1})	0.15 ± 0.02^{d}	0.24 ± 0.02^{c}	0.41 ± 0.01^{b}	0.69 ± 0.01^{a}	0.69 ± 0.03^{a}
Absorbed light (mol m^{-2} d^{-1})	2.23 ± 0.07^{e}	5.05 ± 0.11^{d}	8.87 ± 0.12^{c}	18.10 ± 0.15^{b}	25.80 ± 0.55^{a}
Fx content (mg/g)	16.39 ± 0.69^{a}	12.87 ± 0.91^{b}	12.19 ± 1.55^{b}	7.27 ± 4.99^{c}	3.68 ± 0.59^{d}
Pfx (mg L^{-1} d^{-1})	2.39 ± 0.32^{b}	3.05 ± 0.32^{b}	4.99 ± 0.12^{a}	4.99 ± 0.11^{a}	2.55 ± 0.11^{b}

Note: values are the means ± SD. Means with different letters are significantly different from each other (comparisons were made between groups for each variable) ($p < 0.05$).

CRediT authorship contribution statement

Fengzheng Gao: Investigation, Methodology, Formal analysis, Data curation, Software, Visualization, Writing - original draft, Writing - review & editing. **Iago Teles (Cabanelas, ITD):** Project administration, Formal analysis, Methodology, Supervision, Writing - review & editing. **René H Wijffels:** Project administration, Supervision, Writing - review & editing. **Maria J Barbosa:** Project administration, Formal analysis, Funding acquisition, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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