New BIOTECHNOLOGY 66 (2022) 16–24

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Full length Article

Fucoxanthin and docosahexaenoic acid production by cold-adapted *Tisochrysis lutea*

Fengzheng Gao [a,*], Iago Teles Dominguez Cabanelas [a], René H. Wijffels [a,b], Maria J. Barbosa [a]

[a] Wageningen University, Bioprocess Engineering, AlgaePARC, P.O. Box 16, 6700 AA, Wageningen, Netherlands
[b] Faculty Biosciences and Aquaculture, Nord University, N-8049, Bodø, Norway

ARTICLE INFO

Keywords:
Tisochrysis lutea
Fluorescence-activated cell sorting
Fucoxanthin
Fatty acids
Winter strain

ABSTRACT

Tisochrysis lutea is an important microalgal species for fucoxanthin and docosahexaenoic acid (DHA) production with an optimum cultivation temperature of approximately 30 °C. The aim of the present work was to develop a winter strain with high productivity at 15 °C. The response of the original strain to a decrease in temperature from 30 °C to 15 °C was investigated in continuous turbidostat experiments. This was followed by adaptation for >180 days at 15 °C and 2 rounds of sorting for cells with high chlorophyll fluorescence (top 5%) using fluorescence-activated cell sorting (FACS). For the original strain the productivity of biomass, fucoxanthin, and DHA decreased by 92 %, 98 % and 85 % respectively when decreasing the temperature from 30 °C to 15 °C. In the sorted cold-adapted 'winter strain', biomass, fucoxanthin, and DHA productivities were similar to those at 30 °C. In addition, the fucoxanthin concentration increased from 1.11 to 4.24 mg g^{-1} dry weight and the polar lipid fraction in total fatty acids increased from 21 % to 55 %. The winter strain showed a robust and stable phenotype after one year of cultivation, expanding the outdoor fucoxanthin and lipid production seasons for this species.

Introduction

Microalgae are among the most promising feedstocks for sustainable production of food, feed, materials, chemicals, and fuels [1–5]. The commercial application of microalgal biomass relies on large-scale cultivation and ideally strains can function in fluctuating light and temperatures. For outdoor production without temperature control, microalgae will experience temperature fluctuations between 10 °C and 45 °C in temperate regions [6], including temperatures above the thresholds of survival of most commercialized microalgal species [7]. Above 30 °C, most microalgae cannot grow and active cooling is required. Most microalgal species are able to grow between 15 °C and 30 °C, with their optimal being between 20 °C and 25 °C [8]. Thus, the production location and season are limited, even in southern Europe where temperature fall below 20 °C during the winter [9].

The microalga *Tisochrysis lutea* (*T. lutea*) is considered a promising feedstock for production of fucoxanthin (Fx) and fatty acids, especially docosahexaenoic acid (DHA) [10,11] and is able to produce up to 20.3 mg g^{-1} ash-free dry weight (DW) Fx and 320 mg g^{-1} DW total fatty acids (TFA) [12–14]. Fx is receiving attention due to its biological properties, including anti-obesity, antioxidant and antidiabetic effects [15–17]. DHA is associated with numerous health benefits, such as improving cardiovascular condition and promoting fetal development [18]. In addition, both Fx and DHA play key roles in the health and development of aquatic organisms, and *T. lutea* is for this reason a widely used feed in aquaculture [10,19].

T. lutea was originally isolated from a tropical region of the Pacific [20]; its optimal growing temperature is between 25 °C–30 °C [21]. Its production is low at temperatures below 20 °C [21,22]. For instance, in Portugal (the main *T. lutea* production area in Europe), the mean temperature (1941–2006) during winter varied from 4.2 °C to 13.1 °C, which is too low for *T. lutea* production [23]. A heating system can be used, but would impact the economics of the process. Thus, developing winter strains would benefit microalgae production in winter.

Temperature adaptation can be achieved by a process of directed evolution [24]. A long-term continuous experiment makes it possible to enrich a culture with improved phenotypes which can function in the applied conditions, but a mixture of adapted and non-adapted cells will

Abbreviations: DHA, docosahexaenoic acid; Fx, fucoxanthin; TFA, total fatty acids; FACS, fluorescence-active cell sorting; QY, quantum yield; DW, dry weight; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

* Corresponding author.
E-mail addresses: fengzheng.gao@wur.nl (F. Gao), iago.dominguezteles@wur.nl (I.T.D. Cabanelas), rene.wijffels@wur.nl (R.H. Wijffels), maria.barbosa@wur.nl (M.J. Barbosa).

https://doi.org/10.1016/j.nbt.2021.08.005
Received 30 January 2021; Received in revised form 30 August 2021; Accepted 31 August 2021
Available online 6 September 2021

remain in the culture. Single cell isolation is required to obtain an improved strain more quickly from one clone. A promising technique based on fluorescence-active cell sorting (FACS) offers high throughput analysis and selection of microalgal cells [25] and has been applied for selection of microalgal cells with high lipid or pigment content [26–29].

The aim of the present study was to develop new phenotypes of high-performance *T. lutea* under the suboptimum temperature of 15 °C. Experiments were performed at 4 different temperatures to investigate the effect of decreasing temperature on Fx and lipid production, and single-cell fluorescence was assessed periodically by FACS. Thereafter, a continuous low temperature adaptation experiment was performed to obtain improved strains in which cells were sorted for high-chlorophyll fluorescence under low temperatures. The selected strains were compared to the original, showing better performances. The combined strategy of temperature adaptation and FACS selection provided a method for winter strain development.

Materials and methods

Strain and maintenance conditions

The original *T. lutea* and commercial culture medium stock (NutriBloom Plus) were obtained from NECTON, S.A. (Olhão, Portugal). The culture medium (containing 20 mM HEPES) was prepared with natural seawater (from the North Sea, the Netherlands) enriched with 2 mL L^{-1} of the NutriBloom Plus stock and 40 mM $NaNO_3$. The pH of the medium was adjusted to 8.0 using 2 M NaOH. For maintenance, the original *T. lutea* strain was inoculated in Erlenmeyer flasks (250 mL) with 100 mL medium. The flasks were placed in incubators (Infors, Bottmingen-Basel, Switzerland), at 25 °C, light intensity ≈140 µmol m^{-2} s^{-1}, 2.0 % CO_2, 18/6 h day/night cycle, and under constant agitation of 125 rpm. For the maintenance of the winter strains selected by FACS, cultures were inoculated in 100 mL Erlenmeyer flasks (SCHOTT AG, Mainz, Germany) with 50 mL medium, at 15 °C, light intensity ≈60 µmol m^{-2} s^{-1}, 18/6 h day/night cycle, and under constant agitation of 125 rpm.

Reactor set-up and experimental conditions

Flat-panel photobioreactors (Algaemists [30], light path 14 mm, cultivation volume 400 mL, with white LED panel as illumination) were used for all experiments. The temperature was controlled using a heating/cooling water-jacket and the pH was kept constant as 8.0 by automatic CO_2 injection.

Continuous turbidostat experiments at different temperatures

T. lutea cultures were inoculated in Algaemists at optical density OD_{750} of 0.7 in batch mode. During batch cultivation, all Algaemists were operated at 30 °C, light intensity of 300 µmol m^{-2} s^{-1}, 18/6 h day/night cycle, and a constant pH of 8.0. The turbidostat cultivation was active at day 3 when OD_{750} reached ≈3.5 and the temperature was set to 15, 20, 25 or 30 °C, respectively. The outgoing light intensity (≈16.5 µmol m^{-2} s^{-1}) was fixed at the time of starting turbidostat cultivation. The incident and outgoing light were measured per min using light-meters (LI250A; LI−COR, Lincoln NE, USA). A black-sheet cover was used to avoid interference by environment light on the outgoing light measurement. When the outgoing light intensity decreased below the set-point due to increased biomass concentration, the fresh sterile medium (in a 5 L bottle) was pumped into the reactor to dilute the culture back to the biomass concentration at the set-point. Culture overflow was collected in a harvesting vessel for Fx and lipid measurement. These continuous turbidostat experiments lasted for 15 d.

Low temperature adaptation and winter strain selections using FACS

The original *T. lutea* strain was inoculated in an Algaemist and grown in batch mode at 25 °C for 1 d and then at 20 °C for 5 d, after which the temperature was lowered to 15 °C and the culture was grown in turbidostat mode for 26 d. All reactor set-ups, except temperature setting, were as described above.

A Cell Sorter (SH800S, Sony, San Jose CA, USA) with a 100 µm microfluidics sorting chip (Sony, LE-C3210) was used for single-cell fluorescence measurement and strain selection. The sorting chip was calibrated using automatic setup beads (Sony, LE-B3001). A 488 nm wavelength laser was used to excite chlorophyll a and a bandpass filter (720/60 nm) was used to detect the emission signal. To select cells with high fucoxanthin content, single cells with high autofluorescence at 720 nm were sorted according to the previously reported method [31]. According to the previous report, cell size had no effect on either biomass or lipid productivity of microalgae [32]. Therefore, cell size was not a sorting criteria in the present study. A 1.5 mL Eppendorf tube containing the culture (OD_{750} of 0.2) from the low temperature adaptation experiment was placed in the sample chamber. Cells were selected from three different density plots (Fig. 1), according to the method previously reported [33]. The single-cell fluorescence was recorded for 2 min to collect 10^5 events. A selection gate was created to select cells with a high emission signal (top 5%) at 720 nm (Fig. 1d). From the selection gate, single cells per well were selected into a sterile 96-well plate, containing 100 µL sterile NutriBloom medium (nitrogen = 4 mM, pH 8.0). The cells were incubated at 15 °C, light intensity ≈60 µmol m^{-2} s^{-1}, 18/6 h day/night cycle in a climate room. After >40 d incubation, the surviving cells were grown in flasks for 15–30 d and then scaled up in Algaemist for more rounds of continuous turbidostat cultivation. In total, >180 d cultivation in Algaemist was performed at 15 °C for further adaptation. Thereafter, the cells were selected for a second round from the same gate as above. The strains obtained from the first and second rounds of the FACS selection were designated Sorted-1 and Sorted-2, respectively. The procedure of winter strain development is shown in Fig. 2.

Comparison of different strains for growth, fucoxanthin and lipid productivity

Continuous turbidostat experiments (constant biomass concentration ≈1 g L^{-1}) were performed to compare the performance of the three strains (original, Sorted-1, and Sorted-2) in growth, fucoxanthin and lipid productivity. The experiments were run from 8 to 29 d after the culture reached steady state. To compare with the original performance at 15 °C, the original strain was cultivated in turbidostat mode at 30 °C and 15 °C. All other settings and sample collection were as described above.

Daily measurement

During turbidostat experiments, approximately 10 mL of culture was removed daily from the reactor using a Luer-Lock syringe, approximately 1 h before the light was switched off. From this culture, OD_{750}, cell count, quantum yield (QY), and dry weight (DW) were measured according to the methods previously reported [31,34]. In addition, approximately 100 mL of the culture was harvested for cell component analysis. The harvested cells were washed as previously reported [31].

Fucoxanthin and fatty acids measurement

The harvested *T. lutea* cells were freeze dried (Sublimator 2 × 3 × 3–5, Zirbus Technology Benelux B.V., Tiel, The Netherlands) for 41 h before pigment and lipid analysis. Fucoxanthin extraction (using ≈2 mg dried biomass) and measurement were performed according as described previously [33,34]. Fatty acids extraction (using ≈10 mg dried biomass), separation into polar and neutral lipids and quantification were performed as described by [35].

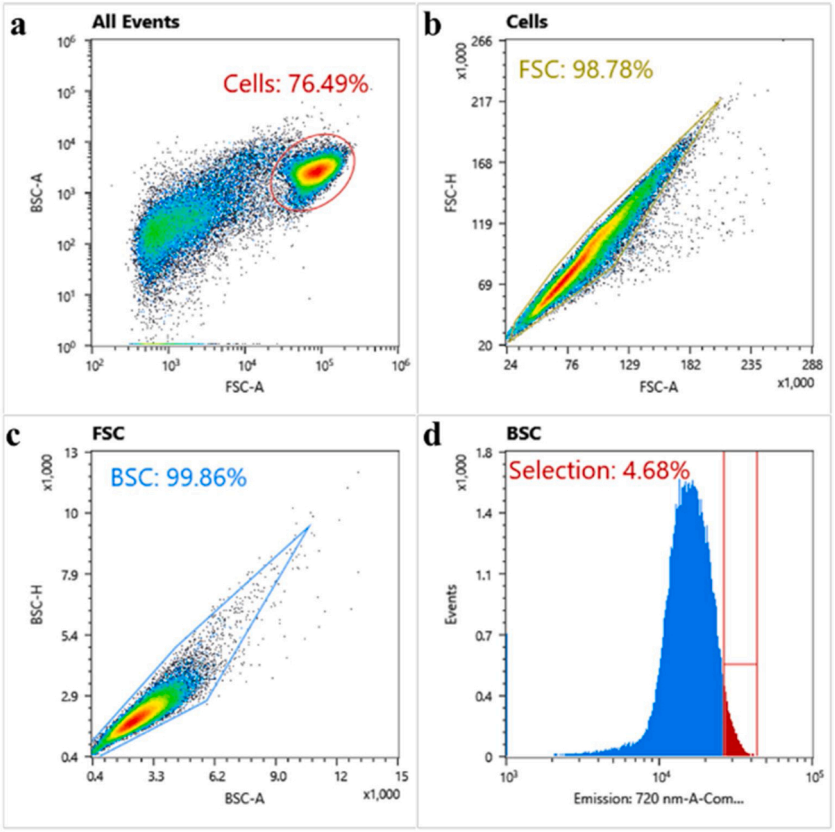

Fig. 1. Density plots in FACS selection. a: Complexity (BSC-A) versus cell size (FSC-A) and gates to distinguish cells from bacteria; b: Cell size height (FSC-H) versus cell size area (FSC-A) with a gate to select singlets; c: Complexity height (BSC-H) versus complexity area (BSC-A) to select all algal cells; d: Single-cell fluorescence at 720/60 nm. A gate was created for high fluorescence cells (top 5%). BSC: backward scatter; FSC: forward scattered; A: area; H: height.

Data calculation and analysis

Calculations

The growth rate (μ; d^{-1}) in continuous turbidostat experiment during steady state was equal to the dilution rate D (d^{-1}); and was calculated according to Eq. 1;

$$\mu = D = V_h/V_R \quad (1)$$

Where V_h (mL) is the volume of the daily harvest culture and V_R (400 mL) is the volume of cultivation volume of the Algaemist.

The volumetric biomass productivity (P_X; g L^{-1} d^{-1}) was calculated using Eq. 2;

$$P_X = Cx \times D \quad (2)$$

Where Cx is the biomass concentration (g L^{-1}) at steady state and D is the dilution rate (d^{-1}); and is expressed as an average productivity.

The volumetric Fx productivity (P_{Fx}; mg L^{-1} d^{-1}) was calculated using Eq. 3;

$$P_{Fx} = C_{Fx} \times P_X \quad (3)$$

Where C_{Fx} is the Fx concentration (mg g^{-1} DW) at steady state and P_X is the biomass productivity (g L^{-1} d^{-1}); and is expressed as an average productivity.

The volumetric TFA productivity (P_{TFA}; mg L^{-1} d^{-1}) was calculated using Eq. 4;

$$P_{TFA} = C_{TFA} \times P_X \quad (4)$$

Where C_{TFA} is the TFA content (mg g^{-1} DW) at steady state and P_X is the biomass productivity (g L^{-1} d^{-1}); and is expressed as an average productivity.

The volumetric DHA productivity (P_{DHA}; mg L^{-1} d^{-1}) was calculated using Eq. 5;

$$P_{DHA} = C_{DHA} \times P_X \quad (5)$$

Where C_{DHA} is the DHA content (mg g^{-1} DW) at steady state and P_X is the biomass productivity (g L^{-1} d^{-1}); and is expressed as an average productivity.

Statistical analyses

The results of continuous turbidostat experiments, expressed as mean value ± standard deviation (SD), were analyzed based on the data from at least 3 different cultivation points during steady state. Differences between groups were tested using the IBM® SPSS® Statistics software program (version 25). The relationship between variables was determined by one-way ANOVA using a Duncan Post-Hoc test (significance level of 0.05).

Results and discussion

Effect of decreasing temperature on Tisochrysis lutea

The original *T. lutea* was grown at different temperatures to study the effect of decreasing temperature. The growth rate of *T. lutea* fell by 95 % from 0.57 to 0.03 d^{-1} with decreasing temperatures from 30 °C to 15 °C

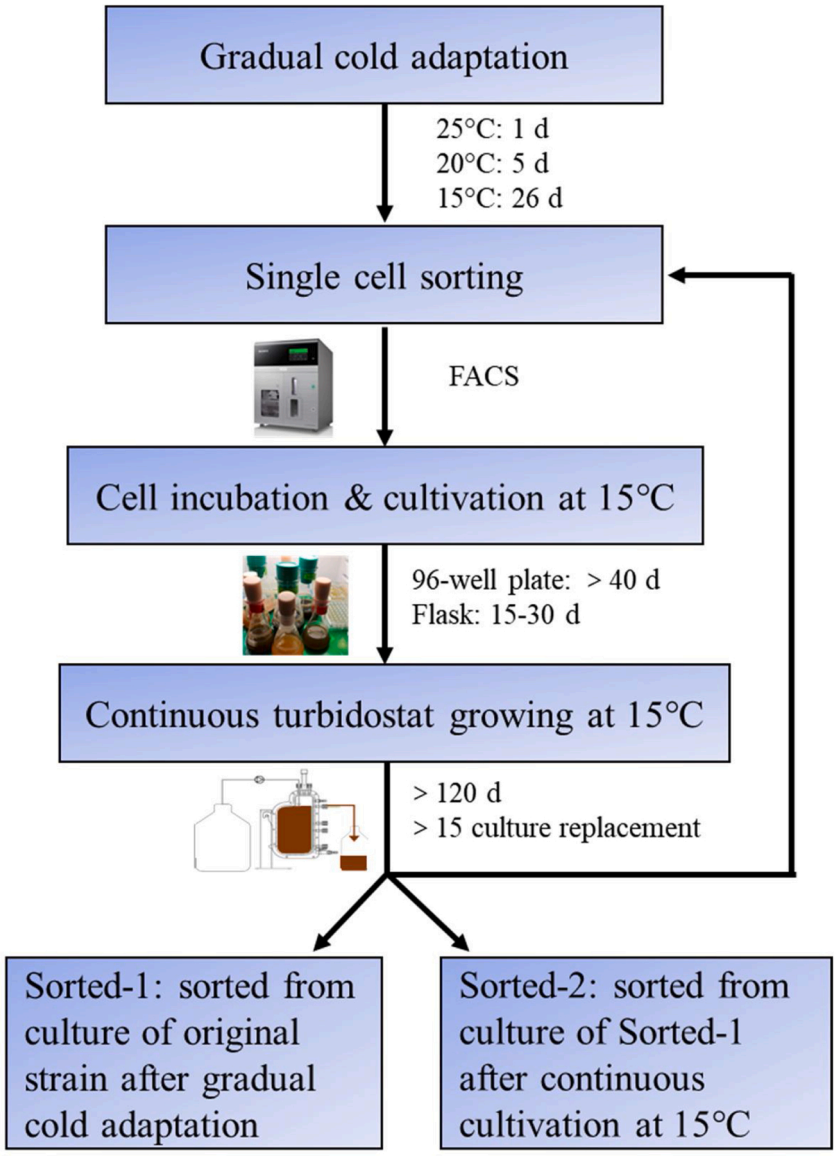

Fig. 2. Cold adaptation and selection procedure of winter strain.

Table 1
Growth and cell parameters obtained in turbidostat experiments at different temperatures.

Temperature (°C)	15	20	25	30
μ (d^{-1})	0.03 ± 0.02^{d}	0.20 ± 0.01^{c}	0.51 ± 0.06^{b}	0.57 ± 0.09^{a}
Diameter (μm)	5.91 ± 0.09^{a}	5.23 ± 0.06^{c}	5.14 ± 0.09^{d}	5.53 ± 0.13^{b}
Biomass Concentration (g L^{-1} DW)	3.17 ± 0.28^{a}	2.64 ± 0.15^{b}	1.81 ± 0.20^{c}	1.50 ± 0.24^{d}
Biomass Productivity (g L^{-1} d^{-1})	0.07 ± 0.05^{c}	0.53 ± 0.05^{b}	0.94 ± 0.11^{a}	0.85 ± 0.22^{a}
QY (Fv/Fm)	0.62 ± 0.02^{d}	0.72 ± 0.01^{c}	0.74 ± 0.01^{a}	0.73 ± 0.01^{b}
Cell number ($\times 10^{6}$ mL^{-1})	59.44 ± 3.34^{c}	85.68 ± 8.95^{a}	66.74 ± 11.85^{b}	50.80 ± 8.03^{d}
Cellular Fx content (pg $cell^{-1}$)	0.20 ± 0.04^{c}	0.16 ± 0.01^{c}	0.28 ± 0.04^{b}	0.47 ± 0.04^{a}

Note: values are the means ± SD. Means with different letters are significantly different ($p < 0.05$). DW: dry weight; QY: quantum yield.

($p < 0.05$; Table 1). The biomass, Fx, total fatty acids (TFA) and DHA productivities followed the same trends, and decreased by 92 %, 98 %, 82 % and 85 %, respectively (Table 1 and Fig. 3). In addition, the Fx content decreased from 16.05 to 3.67 mg g^{-1} DW, and the polar lipids fraction in TFA from 63.53 % to 19.56 % (Fig. 3). In colour, the cells of the original strain grown at 30 °C (Fig. 4a) were slightly brown, but yellow at 15 °C (Fig. 4b). Although the initial biomass concentration (≈2 g L^{-1}) in each photobioreactor was the same when turbidostat mode started at day 2 (Fig. 2a), the biomass concentration at steady state decreased to 1.50 g L^{-1} at 30 °C, whereas it increased to 3.17 g L^{-1} at 15 °C (Table 1), implying that a higher biomass concentration was needed to maintain the same absorbed light at lower temperatures. This may be explained by the fall of 57 % in cellular Fx content at 15 °C from 0.47 to 0.20 pg $cell^{-1}$ (Table 1).

T. lutea showed very little growth at 15 °C. As reported previously, the growth rate of *T. lutea* decreased markedly below 19 °C and above 32 °C [36] and was 2.05-fold higher at 30 °C than at 16.5 °C in batch cultivation [34]. Temperature is one of the most important factors

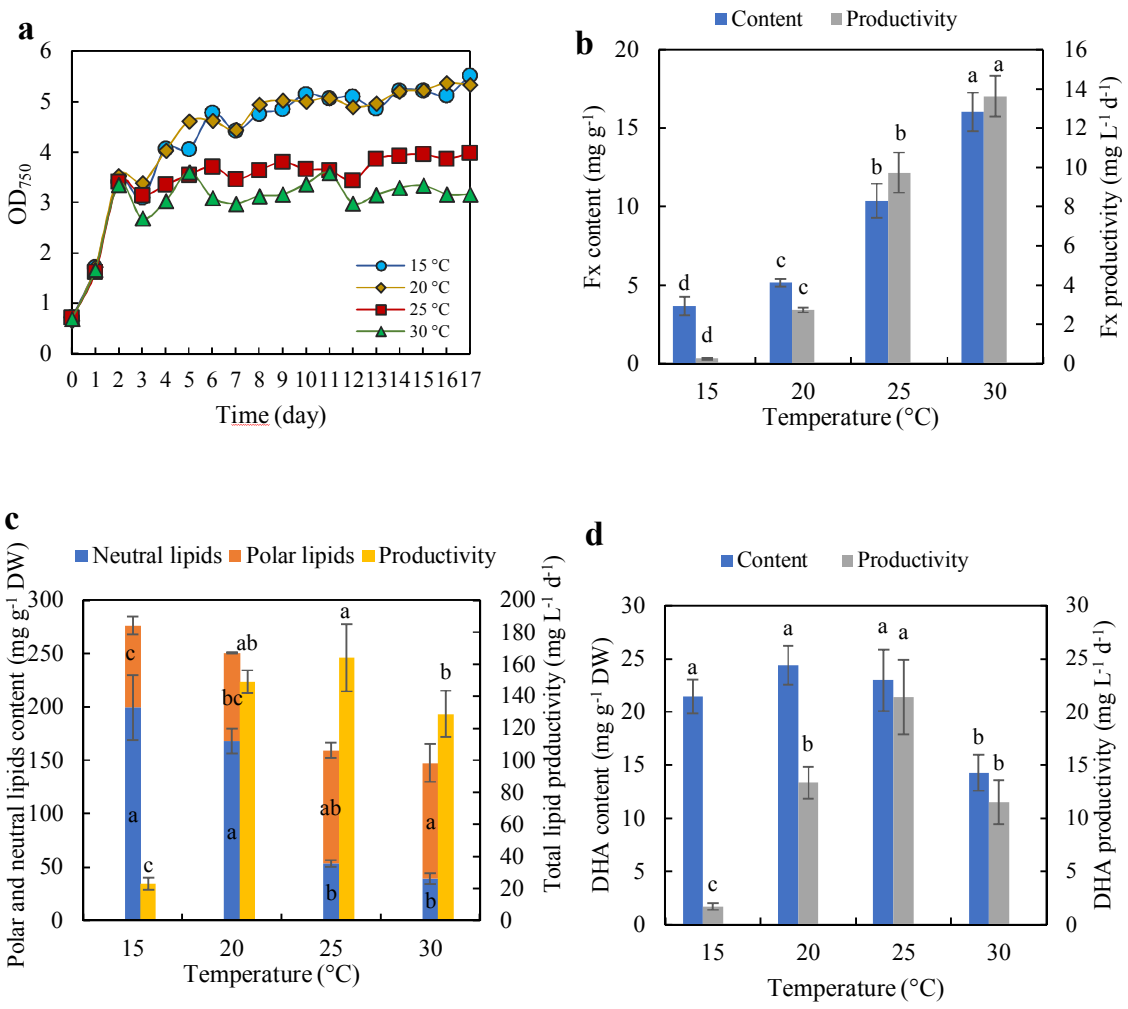

Fig. 3. Effect of decreasing temperature on growth and cellular composition of original *Tisochrysis lutea*. (a) Continuous growth, (b) fucoxanthin content and productivity, (c) polar and neutral lipids content and TFA productivity, (d) DHA content and productivity at different temperatures in turbidostat experiments. 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) ($p < 0.05$).

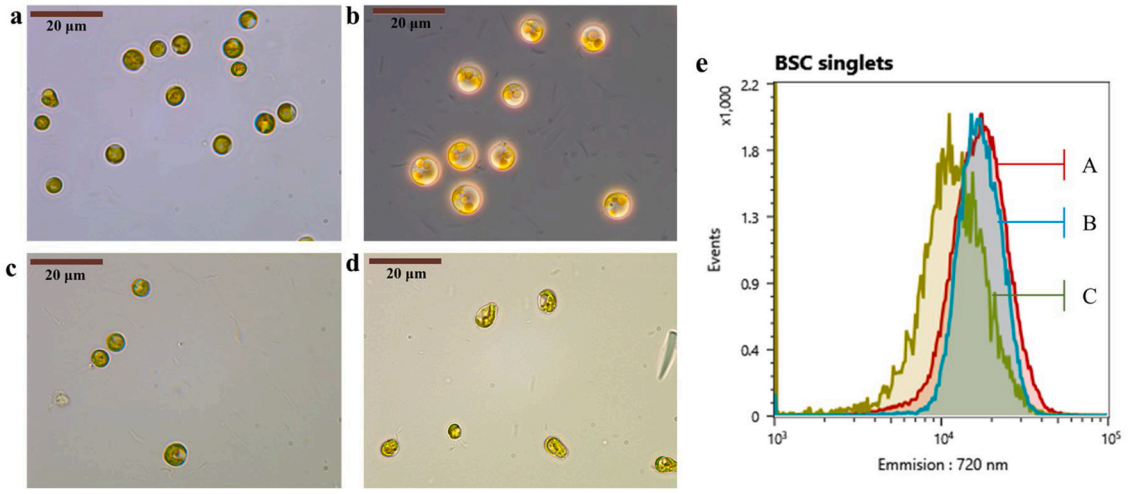

Fig. 4. Microscope images of microalgal cells (a-d) and single-cell fluorescence signals at 720 nm (e). a: Original *T. lutea* cells grown at 30 °C; b: Original *T. lutea* cells grown at 15 °C; c: Sorted-1 cells grown at 15 °C; d: Sorted-2 cells grown at 15 °C; e: Single-cell fluorescence of original and cold-adapted strains: A, the original *T. lutea* grown at 30 °C; B, the Sorted-2 strain grown at 15 °C; C, the original *T. lutea* grown at 15 °C.

affecting the accumulation of carotenoids and lipids in microalgal cells [37,38]. Higher temperatures resulted in increased accumulation of carotenoids due to greater photo-oxidative stress [39]. Low temperatures led to increased neutral lipids accumulations in *Isochrysis* cells [40]. The cells were stressed due to the perturbed equilibrium between energy input and energy consumption at 15 °C [41], which was also indicated by the lowest QY (0.62) and greater cell size (5.91 μm) (Table 1).

Adaptation, and cell sorting and monitoring using FACS

Adaptation and cell sorting

The Sorted-1 strain was obtained by FACS after a 26 day adaptation at 15 °C, and 3.6 times replacement of the culture in continuous turbidostat cultivation. A long term cold-adaptation cultivation was operated again with Sorted-1 for >120 d with more than 15 times replacement of the reactor volume (Fig. 2). Thereafter, another sorted strain (Sorted-2) was obtained from another round of sorting performed with the same selection parameters. The whole procedure of two selection rounds took ≈180 d since the incubation and growth periods of the sorted cells were long at 15 °C: >40 days for incubation in 96-well plate and 15–30 days for inoculation preparation in flask. Unlike the original *T. lutea* cells grown at 15 °C, no visible lipid bodies were observed in Sorted-1 (Fig. 4c) and Sorted-2 (Fig. 4d), indicating that the sorted strains were not stressed like the original strain at low temperature. Moreover, microscope observation showed that the shape of part Sorted-2 cells changed to elliptical instead of round.

Long-term selection under specific temperatures is a strategy to produce adapted strains. Others have used a long term (7 month) selection approach with *T. lutea* (daily variations of temperature, 10 cycles with low temperatures from 12 °C to 28 °C and high temperatures from 28 °C to 36 °C), which resulted in a thermal niche (temperature range for which growth is possible) increase of 3 °C (+16.5 %), with a 9% enhancement of the maximal growth rate [21]. Two new *T. lutea* strains with increase in thermal niches (+ 11 % and + 22 % respectively) were obtained after 41 generations in fed-batch and approximately 157 generations in turbidostat. In the present study, this process was accelerated by using targeted selection and isolation of single cells with high pigment content under the desired temperature, by using FACS. This approach significantly reduced the number of generations needed (≈20 generations) to obtain adapted strains.

Single-cell fluorescence changes

As shown above, Fx and polar lipids content decreased at low temperatures. Under this condition, the cells with the highest Fx and polar lipids are assumed to be robust and adapted to low temperatures. The mean single-cell autofluorescence at 720 nm in original *T. lutea* cells at 15 °C of 12,325 RFU was significantly lower by 31 % than that at 30 °C, 17,949 RFU (Fig. 4e), indicating a pigment decrease at low temperature. The mean single-cell autofluorescence of the Sorted-2 cells at 15 °C of 17,859 RFU was similar to that in original *T. lutea* cells at 30 °C (Fig. 4e). The single cell fluorescence was in line with cellular Fx content (Table 2). In addition, Sorted-2 cells had the lowest coefficient of variation (29 %) in fluorescence distribution compared with the original *T. lutea* at 30 °C (40 %) and 15 °C (50 %), indicating a more homogeneous population of the Sorted-2 strain. Multi-rounds of FACS selection have been shown to be an efficient strategy for microalgal strain improvement [26,28]. Here, two selection rounds resulted in the improved winter strain with a single-cell autofluorescence comparable to the original strain.

Comparison between winter strains and original strain

Sorted-1, Sorted-2 and original *T. lutea* strains were cultivated (continuously) at 1 g L^{-1} to compare their performances in growth, Fx, and fatty acids content and productivity.

Table 2
Growth and cell parameters in turbidostat experiments with original and winter *Tisochrysis lutea*.

Strain	Sorted-1	Sorted-2	Original-30	Original-15
Temperature (°C)	15	15	30	15
Experiment period (day)	29	8	7	10
μ (d^{-1})	0.30 ± 0.02^b	0.37 ± 0.01^a	0.41 ± 0.06^a	0.01 ± 0.01^c
Diameter (μm)	5.62 ± 0.03^b	5.79 ± 0.09^a	5.78 ± 0.25^a	5.56 ± 0.06^b
Biomass Concentration (g L^{-1} DW)	1.03 ± 0.09^a	1.06 ± 0.14^a	0.92 ± 0.08^a	1.07 ± 0.12^a
Biomass Productivity (g L^{-1} d^{-1})	0.31 ± 0.00^b	0.39 ± 0.00^a	0.37 ± 0.00^a	0.01 ± 0.00^c
QY (Fv/Fm)	0.65 ± 0.05^b	0.63 ± 0.02^b	0.73 ± 0.00^a	0.54 ± 0.04^c
Cell number (×10^6 mL^{-1})	30.73 ± 0.85^a	30.56 ± 0.40^a	28.91 ± 2.88^a	28.23 ± 2.97^a
Cellular Fx content (pg $cell^{-1}$)	0.10 ± 0.01^b	0.15 ± 0.01^a	0.14 ± 0.02^a	0.04 ± 0.00^c

Note: values are the means ± SD. Means with different letters are significantly different ($p < 0.05$). DW: dry weight; QY: quantum yield.

Growth of original and winter strains

The growth rate of the original strain at 15 °C was 40 times lower than that at 30 °C, indicating 15 °C to be an extreme growth condition. The growth rate of Sorted-2 grown at 15 °C was similar to the original strain growing at 30 °C, and higher than that of Sorted-1 (Table 2). A permanent cold environment results in temperature adaptation or acclimation of photosynthetic microorganisms [42]. In the current work, long-term cold adaptation improved the growth of *T. lutea* significantly. The biomass productivity followed the same trends as the growth rate. Although the QYs of winter strains grown at 15 °C was still lower than that of the original strain at 30 °C, they were significantly higher than that of the original strain grown at 15 °C ($p < 0.05$; Table 2). These results demonstrated Sorted-2 to be a robust strain with biomass productivities at 15 °C similar to the original *T. lutea* grown at 30 °C.

Fucoxanthin production with original and winter strains

Chlorophyll autofluorescence is linearly correlated to Fx content and can be used to estimate the photosynthetic activity of microalgal cells [31,43]. Sorted-1 and Sorted-2 were selected from the gate of the top 5% chlorophyll autofluorescence at 720 nm, expecting to have high pigment content. During steady state, the Fx content in Sorted-2 was 1.6-fold higher than that of Sorted-1 (Fig. 5a). Fx content decreased by 76 % from 4.53 mg g^{-1} to 1.11 mg g^{-1} with decreasing temperature from 30 °C to 15 °C in the original *T. lutea*. However, Sorted-2 had a similar Fx content at 15 °C to that of the original *T. lutea* grown at 30 °C (Fig. 5b). The cellular Fx content (pg $cell^{-1}$) followed the same trends as Fx content (mg g^{-1} DW) (Table 2).

The Fx productivity of Sorted-2 was significantly higher than that of Sorted-1 ($p < 0.05$). The highest Fx productivity (1.65 mg L^{-1} d^{-1}) was found with Sorted-2 grown at 15 °C, which was similar to that of original *T. lutea* (1.68 mg L^{-1} d^{-1}) grown at 30 °C (Fig. 5a). The Fx productivity was the lowest (0.01 mg L^{-1} d^{-1}) in original *T. lutea* grown at 15 °C (Fig. 5a).

Fatty acids production with original and winter strains

TFA content, 280.05 mg g^{-1} DW, was the highest in original *T. lutea* grown at 15 °C ($p > 0.05$; Fig. 5b). It decreased significantly in Sorted-1 and Sorted-2, with the lowest, 131.76 mg g^{-1} DW, being found in Sorted-2 grown at 15 °C, which was 54 % of the value found in the original *T. lutea* grown at 15 °C and identical to that of the original strain at 30 °C (142.89 mg g^{-1} DW) (Fig. 5b). The relative amount of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in TFA increased by 22.11 % and 36.10 % while polyunsaturated fatty acids (PUFA) decreased by 46.29 % as temperature were reduced from 30 °C

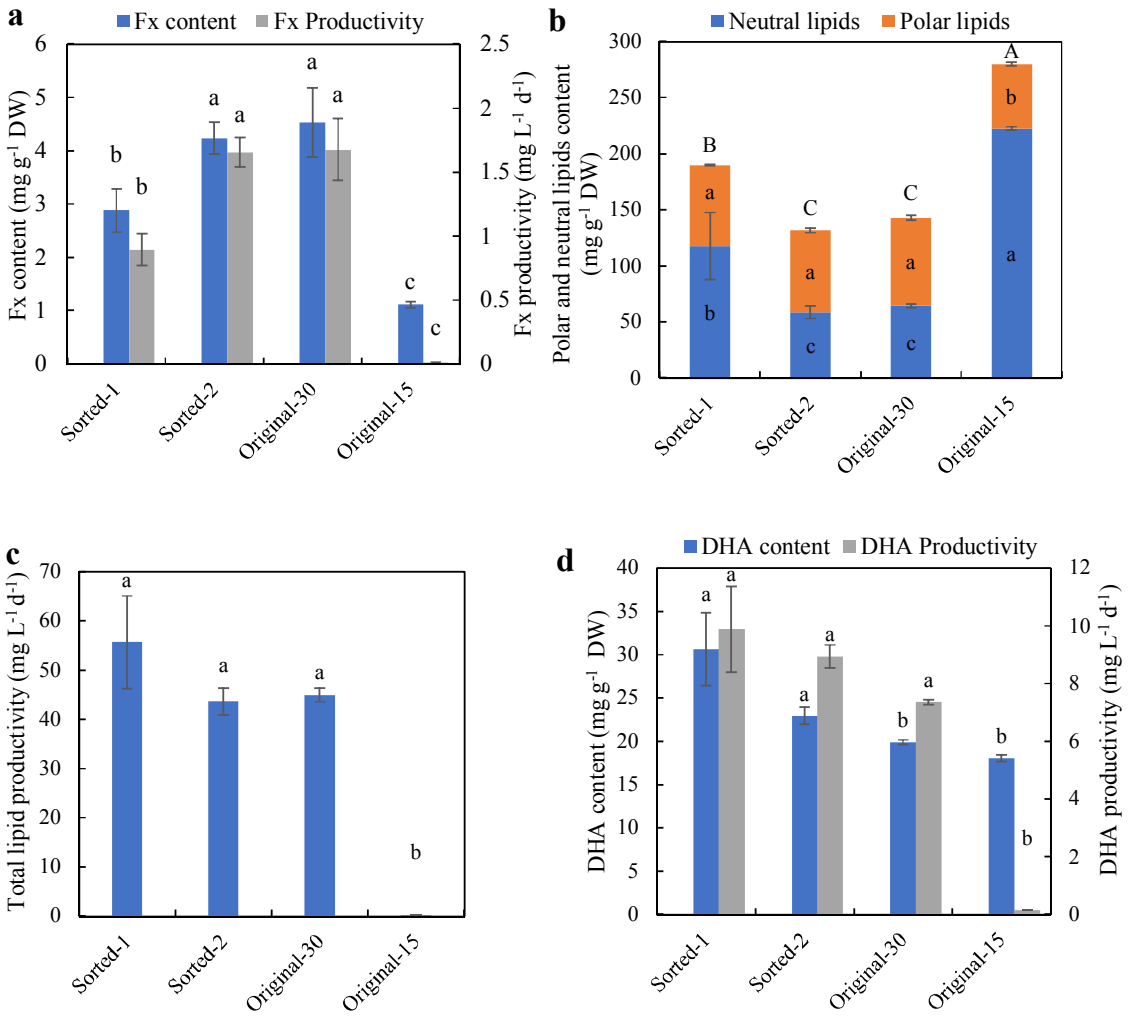

Fig. 5. (a) Fucoxanthin content and productivity, (b) polar and neutral lipids content, (c) total fatty acids productivity, (d) DHA content and productivity with original and winter *Tisochrysis lutea* in turbidostat experiments. Values are the means ± SD; Bars with different letters are significantly different from each other (comparisons were made between bars with the same color only) ($p < 0.05$). Original-30: Original *Tisochrysis lutea* grown at 30 °C; Original-15: Original *Tisochrysis lutea* grown at 15 °C.

to 15 °C with the original *T. lutea* strain (Table 3). However, the PUFA in winter *T. lutea* was higher than that of original *T. lutea* grown at 30 °C, which was twice that of original *T. lutea* grown at 15 °C ($p < 0.05$; Table 3). Hence, the winter strain is a promising bioresource of PUFA.

The TFA productivities in Sorted-1 and Sorted-2 grown at 15 °C, (43.61–55.64 mg L^{-1} d^{-1}), were similar to those from original *T. lutea* grown at 30 °C (Fig. 5c). The lowest was in the original *T. lutea* grown at 15 °C, (0.22 mg L^{-1} d^{-1}), (Fig. 5c). The lower TFA resulted from the decreased neutral lipids fraction. Neutral lipids content (58.65 mg g^{-1} DW) was lowest in Sorted-2, corresponding to 44.51 % TFA (Fig. 5b), and highest (222.42 mg g^{-1} DW) in the original strain grown at 15 °C, corresponding to 79.42 % of the TFA (Fig. 5b). Neutral lipids are commonly accepted as energy storage and are therefore characterized by low metabolic activity [44]. In contrast, the polar lipids fraction increased from 20.58 % to 55.49 % in *T. lutea* at 15 °C from the original to the winter strain. Polar lipids play special roles in the optimal maintenance of membrane fluidity for a variety of metabolic and biosynthetic processes [45]. A major adaptation of metabolic functions influencing photosynthesis and microalgal growth at low temperatures is in membrane fluidity maintenance [46]. Thus, favourable changes, such as increased polar lipids fraction and Fx content, were in line with the improved growth rate of the winter strain.

The DHA content, 22.95–30.64 mg g^{-1} DW, was similar in Sorted-1 and Sorted-2 ($p > 0.05$; Fig. 5d), and 1.9–2.6 fold higher than the 11.90 mg g^{-1} DW reported at a light intensity of 150 µmol m^{-2} s^{-1} and batch cultivation [47]. The DHA content was lower in the original strain at both 30 °C (19.89 mg g^{-1} DW) and 15 °C (18.01 mg g^{-1} DW) (Fig. 5d). DHA productivities in Sorted-1 and Sorted-2 grown at 15 °C were identical to original *T. lutea* grown at 30 °C, 60–66 fold higher than original *T. lutea* grown at 15 °C (0.15 mg L^{-1} d^{-1}) (Fig. 5d).

Seasonal variation in microalgal biomass production is a well-known challenge. Here, cultivation at low temperature combined with FACS was found to be a fast approach to obtaining a winter strain. Strains obtained from this method are non-GMO (genetically modified organisms) that can be used in food or feed industries [48].

Table 3
Fatty acids profile of original and winter *Tisochrysis lutea*.

Strain	Sorted-1	Sorted-2	Original-30	Original-15
C14:0	19.05 ± 0.22^{a}	19.39 ± 0.37^{a}	15.98 ± 0.81^{b}	18.32 ± 0.09^{a}
C16:0	12.33 ± 0.0.28^{c}	10.97 ± 0.27^{d}	15.64 ± 0.13^{b}	20.33 ± 0.20^{a}
C18:0	1.15 ± 0.07^{ab}	0.87 ± 0.05^{c}	1.07 ± 0.05^{b}	1.27 ± 0.05^{a}
∑SFA	**32.52 ± 0.56^{b}**	**31.23 ± 0.69^{b}**	**32.69 ± 0.93^{b}**	**39.92 ± 0.17^{a}**
C14:1 cis-9	0.05 ± 0.07^{a}	0.13 ± 0.01^{a}	0.14 ± 0.02^{a}	0.04 ± 0.03^{a}
C16:1	1.58 ± 0.18^{c}	1.98 ± 0.18^{b}	2.72 ± 0.03^{a}	1.58 ± 0.02^{c}
C18:1	23.88 ± 0.78^{b}	21.01 ± 0.10^{c}	23.51 ± 0.87^{b}	36.98 ± 0.09^{a}
C20:1	4.08 ± 0.53^{a}	4.43 ± 0.03^{a}	2.69 ± 0.14^{b}	0.95 ± 0.02^{c}
∑MUFA	**29.59 ± 0.16^{b}**	**27.55 ± 0.31^{c}**	**29.06 ± 0.71^{b}**	**39.55 ± 0.09^{a}**
C16:2	0.55 ± 0.0^{4b}	0.81 ± 0.08^{a}	0.81 ± 0.04^{a}	0.40 ± 0.04^{c}
C16:3	0.23 ± 0.03^{bc}	0.53 ± 0.06^{a}	0.26 ± 0.05^{b}	0.13 ± 0.02^{c}
C18:2	6.78 ± 0.23^{a}	6.73 ± 0.07^{a}	6.41 ± 0.48^{a}	6.06 ± 0.04^{a}
C18:3	4.01 ± 0.19^{c}	4.46 ± 0.02^{b}	5.70 ± 0.06^{a}	2.75 ± 0.04^{d}
C18:4	10.05 ± 0.21^{b}	11.71 ± 0.20^{a}	10.01 ± 0.13^{b}	4.54 ± 0.06^{c}
C20:5-n3	0.08 ± 0.12^{b}	0.27 ± 0.27^{b}	0.71 ± 0.16^{a}	0.22 ± 0.00^{b}
C22:6	16.05 ± 0.55^{a}	16.73 ± 0.99^{a}	14.34 ± 0.63^{b}	6.43 ± 0.16^{c}
∑PUFA	**37.89 ± 0.68^{b}**	**41.23 ± 1.00^{a}**	**38.24 ± 0.68^{b}**	**20.54 ± 0.25^{c}**

Note: values (% TFA) represent mean ± SD. Means with different letters are significantly different from each other ($p < 0.05$). TFA: total fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Sorted 1 and Sorted 2 were cultivated at 15 °C. The original strain was cultivated at both 30 °C (Original-30) and 15 °C (Original-15).

Industrial production with the winter strain

The Sorted-2 strain obtained is robust with a stable phenotype after a year's cultivation. Besides the good performance at 15 °C, it also showed a higher (≈20 %) biomass productivity at 30 °C than the original strain, with an improved performance in both winter and summer. Moreover, in a temperature switch experiment (from 15 °C to 30 °C and then back to 15 °C, data not shown), the growth rates at 15 °C before and after temperature switch were identical, indicating a stable phenotype.

The winter strain was transferred to the industrial company NECTON, S.A. (Olhão, Portugal), who used it for one year for production in flat-panel or tubular photobioreactors with a cultivation volume up to 19,000 L.

Conclusions

In the present study, long-term low temperature adaptation combined with FACS selection was used to develop a *T. lutea* strain that can be grown at low temperatures. The Sorted-2 strain, obtained from 2 rounds of FACS selection, had improved Fx content and polar lipids fraction in TFA from 1.11 to 4.24 mg g^{-1} and 20.58%–55.49%. High Fx and lipid productivities were achieved in this strain at 15 °C, which were similar to the original strain grown at 30 °C. This work has provided a successful experimental strategy to obtain robust strains suitable for outdoor production in winter.

Author contribution

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

Funding

This research belongs to MAGNIFICENT project, funded by the Bio-Based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation program (grant No. 745754).

Declaration of Competing Interest

The authors report no declarations of interest.

References

[1] Draaisma RB, Wijffels RH, Slegers PM, Brentner LB, Roy A, Barbosa MJ. Food commodities from microalgae. Curr Opin Biotechnol 2013;24:169–77. https://doi.org/10.1016/j.copbio.2012.09.012.
[2] Wijffels RH, Barbosa MJ. An outlook on microalgal biofuels. Science 2010;329:796–9. https://doi.org/10.1126/science.1189003.
[3] Fernández FGA, Reis A, Wijffels RH, Barbosa M, Verdelho V, Llamas B. The role of microalgae in the bioeconomy. N Biotechnol 2021;61:99–107. https://doi.org/10.1016/j.nbt.2020.11.011.
[4] Russo GL, Langellotti AL, Oliviero M, Sacchi R, Masi P. Sustainable production of food grade omega-3 oil using aquatic protists : reliability and future horizons. N Biotechnol 2021;62:32–9. https://doi.org/10.1016/j.nbt.2021.01.006.
[5] Li J, Liu Y, Cheng JJ, Mos M, Daroch M. Biological potential of microalgae in China for biorefinery-based production of biofuels and high value compounds. N Biotechnol 2015;32:588–96. https://doi.org/10.1016/j.nbt.2015.02.001.
[6] Béchet Q, Shilton A, Fringer OB, Munoz R, Guieysse B. Mechanistic modeling of broth temperature in outdoor photobioreactors. Environ Sci Technol 2010;44:2197–203. https://doi.org/10.1021/es903214u.
[7] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: a review. Renewable Sustainable Energy Rev 2010;14:217–32. https://doi.org/10.1016/j.rser.2009.07.020.
[8] Li WKW. Temperature adaptation in Phytoplankton: cellular and photosynthetic characteristics. Prim. Product. Sea. Boston, MA: Springer; 1980. p. 259–79. https://doi.org/10.1007/978-1-4684-3890-1_15.
[9] Ras M, Steyer JP, Bernard O. Temperature effect on microalgae: a crucial factor for outdoor production. Rev Environ Sci Biotechnol 2013;12:153–64. https://doi.org/10.1007/s11157-013-9310-6.
[10] Mohamadnia S, Tavakoli O, Faramarzi MA, Shamsollahi Z. Production of fucoxanthin by the microalga *Tisochrysis lutea*: A review of recent developments. Aquaculture 2020;516:734637. https://doi.org/10.1016/j.aquaculture.2019.734637.
[11] Hu H, Li J-Y, Pan X-R, Zhang F, Ma L-L, Wang H-J, et al. Different DHA or EPA production responses to nutrient stress in the marine microalga *Tisochrysis lutea* and the freshwater microalga *Monodus subterraneus*. Sci Total Environ 2019;656:140–9. https://doi.org/10.1016/J.SCITOTENV.2018.11.346.
[12] Balduyck L, Bijttebier S, Bruneel C, Jacobs G, Voorspoels S, Van Durme J, et al. Lipolysis in *T-Isochrysis lutea* during wet storage at different temperatures. Algal Res 2016;18:281–7. https://doi.org/10.1016/j.algal.2016.07.003.
[13] Kim SM, Kang SW, Kwon ON, Chung D, Pan CH. Fucoxanthin as a major carotenoid in *Isochrysis* aff. *galbana*: characterization of extraction for commercial application. J Korean Soc Appl Biol Chem 2012;55:477–83. https://doi.org/10.1007/s13765-012-2108-3.
[14] Gao F, Sá M, Cabanelas IT, Wijffels RH, Barbosa MJ. Production and monitoring of biomass and fucoxanthin with brown microalgae under outdoor conditions. Biotechnol Bioeng 2021;118:1355–65. https://doi.org/10.1002/bit.27657.
[15] Maeda H, Fukuda S, Izumi H, Saga N. Anti-oxidant and fucoxanthin contents of brown alga ishimozuku (*Sphaerotrichia divaricata*) from the west coast of aomori. Japan Mar Drugs 2018;16:1–10. https://doi.org/10.3390/md16080255.
[16] Guo B, Liu B, Wei H, Cheng KW, Chen F. Extract of the microalga *Nitzschia laevis* prevents high-fat-diet-induced obesity in mice by modulating the composition of gut microbiota. Mol Nutr Food Res 2019;63:1800808. https://doi.org/10.1002/mnfr.201800808.
[17] Guo B, Yang B, Pang X, Chen T, Chen F, Cheng KW. Fucoxanthin modulates cecal and fecal microbiota differently based on diet. Food Funct 2019;10:5644–55. https://doi.org/10.1039/c9fo01018a.
[18] Swanson D, Block R, Mousa SA. Omega-3 fatty acids EPA and DHA : health. J Adv Nutr 2012;3:1–7. https://doi.org/10.3945/an.111.000893.Omega-3.
[19] Vizcaíno AJ, Saéz MI, López G, Arizcun M, Abellán E, Martínez TF, et al. *Tetraselmis suecia* and *Tisochrysis lutea* meal as dietary ingredients for gilthead sea bream (*Sparus aurata* L.) fry. J Appl Phycol 2016;28:2843–55. https://doi.org/10.1007/s10811-016-0845-0.
[20] Bendif EM, Probert I, Schroeder DC, de Vargas C. On the description of *Tisochrysis lutea* gen. nov. sp. nov. and*Isochrysis nuda* sp. nov. in the Isochrysidales, and the transfer of Dicrateria to the Prymnesiales (Haptophyta). J Appl Phycol 2013;6:1763–76. https://doi.org/10.1007/s10811-013-0037-0.
[21] Bonnefond H, Grimaud G, Rumin J, Bougaran G, Talec A, Gachelin M, et al. Continuous selection pressure to improve temperature acclimation of *Tisochrysis lutea*. PLoS One 2017;12:1–19. https://doi.org/10.1371/journal.pone.0183547.
[22] Ippoliti D, Gómez C, del Mar Morales-Amaral M, Pistocchi R, Fernández-Sevilla JM, Acién FG. Modeling of photosynthesis and respiration rate for *Isochrysis galbana* (T-

Iso) and its influence on the production of this strain. Bioresour Technol 2016;203:71–9. https://doi.org/10.1016/j.biortech.2015.12.050.
[23] Espírito Santo F, De Lima MIP, Ramos AM, Trigo RM. Trends in seasonal surface air temperature in mainland Portugal, since 1941. Int J Climatol 2014;34:1814–37. https://doi.org/10.1002/joc.3803.
[24] Hopes A, Mock T. Evolution of microalgae and their adaptations in different marine ecosystems. ELS 2015:1–9. https://doi.org/10.1002/9780470015902.a0023744.
[25] Gao F, Sá M, Teles I, Cabanelas D, René H, Barbosa MJ. Improved fucoxanthin and docosahexaenoic acid productivities of a sorted self-settling *Tisochrysis lutea* phenotype at pilot scale. Bioresour Technol 2021;325:124725. https://doi.org/10.1016/j.biortech.2021.124725.
[26] Cabanelas ITD, Van Der Zwart M, Kleinegris DMM, Wijffels RH, Barbosa MJ. Sorting cells of the microalga *Chlorococcum littorale* with increased triacylglycerol productivity. Biotechnol Biofuels 2016;9:1–12. https://doi.org/10.1186/s13068-016-0595-x.
[27] Mendoza H, de la Jara A, Freijanes K, Carmona L, Ramos AA, de Sousa Duarte V, et al. Characterization of *Dunaliella salina* strains by flow cytometry: a new approach to select carotenoid hyperproducing strains. Electron J Biotechnol 2008;11:5–6. https://doi.org/10.2225/vol11-issue4-fulltext-2.
[28] Cabanelas ITD, van der Zwart M, Kleinegris DMM, Barbosa MJ, Wijffels RH. Rapid method to screen and sort lipid accumulating microalgae. Bioresour Technol 2015;184:47–52. https://doi.org/10.1016/j.biortech.2014.10.057.
[29] Park S, Nguyen THT, Jin ES. Improving lipid production by strain development in microalgae: strategies, challenges and perspectives. Bioresour Technol 2019;292:121953. https://doi.org/10.1016/j.biortech.2019.121953.
[30] Hulatt CJ, Wijffels RH, Bolla S, Kiron V. Production of fatty acids and protein by *Nannochloropsis* in flat-plate photobioreactors. PLoS One 2017;12:1–17. https://doi.org/10.1371/journal.pone.0170440.
[31] Gao F, Teles (Cabanelas, ITD) I, Ferrer-Ledo N, Wijffels RH, Barbosa MJ. Production and high throughput quantification of fucoxanthin and lipids in *Tisochrysis lutea* using single-cell fluorescence. Bioresour Technol 2020;318:124104. https://doi.org/10.1016/j.biortech.2020.124104.
[32] Cabanelas ITD, Fernandes C, Kleinegris DMM, Wijffels RH, Barbosa MJ. Cell diameter doesn't affect lipid productivity of *Chlorococcum littorale*. Algal Res 2016;19:333–41. https://doi.org/10.1016/j.algal.2016.02.002.
[33] Gao F, Woolschot S, Teles I, Cabanelas D, Barbosa MJ. Light spectra as triggers for sorting improved strains of *Tisochrysis lutea*. Bioresour Technol 2020;321:124434. https://doi.org/10.1016/j.biortech.2020.124434.
[34] Gao F, (Cabanelas Teles, ITD) I, Wijffels RH, Barbosa MJ. Process optimization of fucoxanthin production with *Tisochrysis lutea*. Bioresour Technol 2020;315:123894. https://doi.org/10.1016/j.biortech.2020.123894.
[35] Remmers IM, Martens DE, Wijffels RH, Lamers PP. Dynamics of triacylglycerol and EPA production in *Phaeodactylum tricornutum* under nitrogen starvation at different light intensities. PLoS One 2017;12:1–13. https://doi.org/10.1371/journal.pone.0175630.
[36] Kaplan D, Cohen Z, Abeliovich A. Optimal growth conditions for *Isochrysis galbana*. Biomass 1986;9:37–48. https://doi.org/10.1016/0144-4565(86)90011-9.
[37] Minhas AK, Hodgson P, Barrow CJ, Adholeya A. A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. Front Microbiol 2016;7:1–19. https://doi.org/10.3389/fmicb.2016.00546.
[38] Zienkiewicz K, Du ZY Ma W, Vollheyde K, Benning C. Stress-induced neutral lipid biosynthesis in microalgae — molecular, cellular and physiological insights. Biochim Biophys Acta - Mol Cell Biol Lipids 2016;1861:1269–81. https://doi.org/10.1016/j.bbalip.2016.02.008.
[39] Tripathi U, Sarada R, Ravishankar GA. Effect of culture conditions on growth of green alga - *Haematococcus pluvialis* and astaxanthin production. Acta Physiol Plant 2002;24:323–9. https://doi.org/10.1007/s11738-002-0058-9.
[40] Zhu CJ, Lee YK, Chao TM. Effects of temperature and growth phase on lipid and biochemical composition of *Isochrysis galbana* TK1. J Appl Phycol 1997;9:451–7. https://doi.org/10.1023/A:1007973319348.
[41] Maxwell DP, Falk S, Trick CG, Huner NPA. Growth at low temperature mimics high-light acclimation in *Chlorella vulgaris*. Plant Physiol 1994;105:535–43. https://doi.org/10.1104/pp.105.2.535.
[42] Morgan-Kiss RM, Priscu JC, Pocock T, Gudynaite-Savitch L, Huner NPA. Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. Microbiol Mol Biol Rev 2006;70:222–52. https://doi.org/10.1128/mmbr.70.1.222-252.2006.
[43] Wang J, Sun J, Song Y, Xu Y, Pan X, Sun Y, et al. A label-free microfluidic biosensor for activity detection of single microalgae cells based on Chlorophyll fluorescence. Sensors (Switzerland) 2013;13:16075–89. https://doi.org/10.3390/s131216075.
[44] Solovchenko AE. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russ J Plant Physiol 2012;59:167–76. https://doi.org/10.1134/S1021443712020161.
[45] Lu S, Wang J, Ma Q, Yang J, Li X, Yuan YJ. Phospholipid metabolism in an industry microalga *Chlorella sorokiniana*: the impact of inoculum sizes. PLoS One 2013;8. https://doi.org/10.1371/journal.pone.0070827.
[46] Los DA, Murata N. Membrane fluidity and its roles in the perception of environmental signals. Biochim Biophys Acta Biomembr 2004;1666:142–57. https://doi.org/10.1016/j.bbamem.2004.08.002.
[47] Premaratne M, Liyanaarachchi VC, Nimarshana PHV, Ariyadasa TU, Malik A, Attalage RA. Co-production of fucoxanthin, docosahexaenoic acid (DHA) and bioethanol from the marine microalga *Tisochrysis lutea*. Biochem Eng J 2021;176:108160. https://doi.org/10.1016/j.bej.2021.108160.
[48] Eriksson D, Kershen D, Nepomuceno A, Pogson BJ, Prieto H, Purnhagen K, et al. A comparison of the EU regulatory approach to directed mutagenesis with that of other jurisdictions, consequences for international trade and potential steps forward. New Phytol 2019;222:1673–84. https://doi.org/10.1111/nph.15627.