

Growth, pigments production and phytochemicals variation in *Tetraselmis chui* under different cultivation parameters

Norhayati Yusuf  . Nurul Shafiq Yusof . Malinna Jusoh . Hazlina Ahmad Zakeri . Nurfarha Muhamad Zalan

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Abstract This study investigates the impact of different cultivation conditions on *Tetraselmis chui*, a microalgae species with vast potential in renewable energy, pharmaceuticals, and nutraceuticals. Various parameters, including media strength (F, F/2, F/4, and F/8), sucrose concentration (0-50 g/L), medium pH (5-9), and light intensity (0-600 lux), were examined. The study focused on growth, pigment content, and phytochemical composition, which is crucial for its commercial value. After eight days of cultivation in F2 media, *T. chui* exhibited typical growth phases, with full-strength media being the most effective in promoting cell growth. The addition of sucrose positively affected cell growth, especially at 40 g/L. The pH of the medium did not significantly impact growth, except at pH 5, where growth was affected negatively. Higher light intensities gradually increased cells' fresh and dry weights. Furthermore, chlorophylls a, b and carotenoids accumulated more in full-strength media supplemented with higher sucrose concentrations and under higher light intensities. Lower pH levels inhibited the synthesis of these pigments. Additionally, total phenolic compounds and flavonoid production were higher in a full-strength medium supplemented with higher sucrose concentrations, light intensities, and pH levels. These findings have significant implications for the development of sustainable microalgae cultivation strategies. By optimising cultivation parameters, including media composition, sucrose concentration, light intensity, and pH levels, the production of desired compounds and biomass can be enhanced, contributing to the advancement and sustainability of various sectors.

Keywords *Tetraselmis chui* . Growth . Pigments . Phytochemicals . Cultivation parameters

Introduction

Microalgae have gained considerable attention as versatile organisms with the potential to produce various bioactive compounds such as lipids, protein, carbohydrates, pigments, vitamins, and phenolics compounds, thus making them valuable sources of renewable energy, pharmaceuticals, and nutraceuticals (Eze et al. 2023). Although a lot of research has been done on the health benefits of these bioactive compounds, there seems to be an agreement in the literature that microalgae research is still in its infancy and that further investigation is required to identify the mechanisms of action that underlie the effectiveness of microalgal compounds.

Norhayati Yusuf . (✉) Malinna Jusoh . Hazlina Ahmad Zakeri . Nurfarha Muhamad Zalan
Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
e-mail: yatiyusuf@umt.edu.my

Norhayati Yusuf . Hazlina Ahmad Zakeri
Biological Security and Sustainability Research Interest Group (BIOSES), Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

Nurul Shafiq Yusof
SATREPS-COSMOS Laboratory, Centre of Research and Field Service, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

Nurul Shafiq Yusof
Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

Several microalgae species, *Chlorella* sp. (Chlorophyta), *Nannochloropsis* sp. (Ochrophyta, Eustigmatophyceae), *Arthrospira/Spirulina* sp. (Cyanobacteria), *Dunaliella* sp. (Chlorophyta), *Synechococcus* sp. (Cyanobacteria), *Phaeodactylum* sp. (Bacillariophyta), *Rhodomonas salina* (Cryptista, Cryptophyceae), *Limnospira maxima* (Cyanobacteria), and *Tetraselmis chui* (Chlorophyta) are among the highlighted species that are rich in bioactive compounds including pigments, various phytochemical as well as natural antioxidants with free radical scavenging abilities (Olasehinde et al. 2017). Among these species, *T. chui* is of great interest in this study owing to its higher growth rate, robustness, and remarkable phytochemical composition.

Tetraselmis chui benefits various industries due to its diversified phytochemical composition and high chlorophyll concentration (Moser et al. 2022). One distinguishing feature is the presence of lipids, notably triacylglycerols (TAGs), which are important for generating biodiesel and renewable energy (Hu et al. 2021a). The food, cosmetics, and textile sectors may use other bioactive substances, including pigments like carotenoids and phycobiliproteins, as natural colourants (Li et al. 2021). Aquaculture and animal feed can both benefit from proteins generated from *T. chui* (Yan et al. 2020). This microalga's carbohydrates can make biofuels, bioplastics, and other products with added value. Additionally, *T. chui* is recognised for producing several antioxidants, such as tocopherols and phenolic compounds (Paterson et al. 2023), which can be utilised to create functional meals and nutraceuticals and provide considerable health advantages.

The efficient photosynthesis of *T. chui* is one of its distinguishing characteristics. Each cell has a single flagellum, a pyrenoid, and a cup-shaped chloroplast that allow maximum light absorption and efficient solar energy conversion into organic compounds. Its high chlorophyll content, which includes chlorophyll a and b, are crucial primary pigments for absorbing light energy and starting photosynthesis, reflecting this photosynthetic activity. Hsu et al. (2013) explored the mechanisms where both chlorophylls a and b can decrease the oxidation of other molecules, particularly DNA and lipids, by inhibiting the formation of reactive oxygen species, thus confirming that both pigments are significant health-promoting dietary components which can shield the body through a variety of chemical mechanisms. Carotenoids are another valuable accessory pigment that can be obtained from microalgae. They are regarded as powerful functional constituents with intriguing uses in various industries, including agro-food, aquaculture, biotechnology, medicine, and pharmacy (Jalal et al. 2013). Microalgae contain a class of bioactive molecules called phytochemicals that have a variety of biological properties, such as antioxidant, anti-inflammatory, and antibacterial properties. The most prevalent phytochemicals in microalgae are polyphenols, which are made up of phenolic acids, flavonoids, isoflavonoids, stilbenes, lignans, and phenolic polymers. These compounds exhibit a range of biological functions, such as anti-inflammatory, anti-cancer, anti-allergic, anti-diabetes, anti-ageing, and antibacterial capabilities (Galasso et al. 2019).

The microalgae cultural conditions have a relative influence on their biomasses and active growth stages. The culture parameters, including the media's nutritional composition, pH level, and light intensity, are crucial in controlling the physiological reactions and metabolic activities of *T. chui*. Kim et al. (2020) and Johnson and Wen (2010) suggested that changing the photoperiod and light intensity impacts microalgae's growth rate and chlorophyll content. Proteins, carbohydrates, lipids, pigments and antioxidants are all included in the phytochemical makeup and may be impacted (Chen et al. 2019; Hu et al. 2021b). The availability of essential nutrients such as nitrogen, phosphorus and other micronutrients affects the accumulation of these bioactive chemicals (Yen et al. 2019; Patel et al. 2021).

Optimising the cultivation conditions is crucial for enhancing productivity and desired phytochemical compounds. Thus, this study aims to investigate and analyse the impact of manipulating the cultivation conditions on the growth, pigment accumulation, and phytochemicals produced in *T. chui*. The outcomes of this study can potentially contribute to the development of sustainable microalgae cultivation strategies and the efficient production of desired bioactive compounds. This optimisation can significantly advance pharmaceuticals, cosmetics, functional foods, aquaculture, and the renewable energy sector.

Materials and methods

Microalgae culture

Tetraselmis chui was provided by The Institute of Marine Biotechnology, UMT. The cells were grown in an F2 medium (Guillard 1975; Guillard and Ryther 1962) at 30 ppt salinity and pH 8.0 ± 0.2 . The cultures were



incubated at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under continuous illumination using a 6,500 K daylight white light-emitting diode lamp. The aeration Hailea HAP-120 pump (Hailea Group Co., China) was supplied with an airflow rate of around 120 L/min of air under the pressure of 0.018 MPa, filtered through a 0.22 μm Minisart® Sartorius syringe filter to avoid contamination. Before use in experiments, the stock culture was grown in a 500 ml liquid medium and harvested at a 1×10^6 cells/ml density.

Manipulation of culture growth conditions

For this part, algae were treated in similar culture conditions as above, except that there were changes in:

- 1) Media strength: Cultures were treated with full strength, F, F/2, F/4 and F/8 of F2 medium.
- 2) Effect of sucrose concentrations: Cultures were treated with different sucrose concentrations, i.e. 0, 10, 20, 30, 40 and 50 g/L. 30 g/L sucrose was used as a control.
- 3) Effect of different pH values: The effect of initial pH values was examined in the range of 5 to 9, with 8 as the control pH. The pH of the media was adjusted using 0.1 N NaOH or 0.1 HCl and was determined using a pH meter (Corning, USA).
- 4) Effect of light intensities: Cells were cultured in different light intensities, i.e. 0, 0.42, 1.37, 6.51, 18.85, 31.03 and 103.41 $\mu\text{mol}/\text{m}^2/\text{s}$ under cool, white fluorescent lamps with constant illumination.

Growth assessment

Cell density was determined daily for 14 days. Approximately 200 μL of cultures were diluted with 800 μL of Lugol's solution (White et al. 2014). Ten μl of diluted solution were transferred into a Neubauer haemocytometer, and the cells were counted using a compound microscope (Leica CME, Leica Microsystems GmbH, Germany). Cell densities were calculated according to the method described by Sahastrabudhe (2016) using the following formula:

$$\text{Cell density (cells/ml)} = \frac{\text{Total number of cells} \times \text{dilution factor}}{\text{Total number of squares} \times \text{Volume of the square at 0.01 mm depth}}$$

Based on our previous preliminary experiments, *T. chui* was harvested on day 8 (at the end of the exponential and early stationary phase) for fresh and dry biomass determination. Microalgae culture was centrifuged for 10 min at $1,000 \times g$. The supernatant was discarded, and fresh microalgae paste was washed twice with distilled water. Fresh microalgae paste was rinsed twice with distilled water after discarding the supernatant. For the dry weight, the fresh paste was dried in the oven at $70^{\circ}\text{C} \pm 2^{\circ}\text{C}$ until a constant weight was obtained (Kong et al. 2011).

Chlorophylls a, b and total carotenoid contents measurement

The method of Lichtenthaler (1987) was used to extract the chlorophylls a and b and total carotenoids. The cells were homogenised for 10 min with acetone using an ultrasonic bath. The homogenate was centrifuged at $9,168 \times g$ for 10 min at 4°C . The supernatant obtained was transferred to a 96-well plate, with acetone used as control. The plate was shaken in a microplate reader for 10 sec. The absorbance of the supernatant was measured at 470, 653, and 666 nm. The amounts of Chlorophyll a (Ch a), Chlorophyll b (Ch b) and Total carotenoids (Car) were calculated according to the following formulae:

$$\begin{aligned} \text{Chl a (mg/g FW)} &= 15.65 A_{666} - 7.340 A_{653} \\ \text{Chl b (mg/g FW)} &= 27.05 A_{653} - 11.21 A_{666} \\ \text{Car (mg/g FW)} &= (1000 A_{470} - 2.860 \text{ Chl a} - 129.2 \text{ Chl b})/245 \end{aligned}$$

Phytochemicals determination

The folin-Ciocalteu method was used to measure the total phenolic content (Tiong et al. 2020). Approximately 20 μL of extract was added into 100 μL of Folin-Ciocalteu's phenol reagent. The mixture was allowed to stand for 5 min at room temperature. Then, 80 μL of 7.5 % sodium carbonate (Na_2CO_3) solution



was added. The mixture was incubated in the dark for 120 min at room temperature. Then, the absorbance was measured at 750 nm. The total phenolic contents of the samples were expressed as mg gallic acid equivalent per gram of microalgae extract (mg GAE/g).

The total flavonoid content was assayed based on the method of Tiong et al. (2020). Approximately 100 μL distilled water, 10 μL of 50 g/L NaNO_2 and 25 μL of the extract were loaded into 96 wells plate. Then, 50 μL of 100 g/L AlCl_3 was added to the mixture. The plate was incubated for 5 min. Following this, 50 μL of 1 mol/L NaOH and distilled water were added, and the plate was incubated on a shaker for 50 min at room temperature. The absorbance was measured at 510 nm. The total flavonoid content was expressed as quercetin equivalent per gram of microalgae extract (mg QE/g).

Statistical analysis

The experiment was conducted in five replicates ($n=5$), and data were presented as mean \pm standard deviation. The significant differences were determined using a one-way analysis of variance (ANOVA) and tested by Duncan's Multiple Range Test (DMRT) to compare the differences between treatments at 0.05% significance level.

Results and discussion

Tetraselmis chui has successfully grown in an F2 medium at 30 ppt salinity and pH 8.0 ± 0.2 . They were incubated at $24^\circ\text{C} \pm 2^\circ\text{C}$ under constant illumination using a 6,500 K daylight white light-emitting diode lamp, aerated with an airflow rate of around 120 L/min of air under the pressure of 0.018 MPa. Cell density (growth curve), fresh and dry weights, pigments (chlorophylls a and b, carotenoids) and total phenolic and flavonoid compounds at different media strengths, sucrose concentration, pH and light intensities were determined.

Cell density

Media strength plays a crucial role in the growth and productivity of *T. chui*. The media provide essential nutrients for the microalgae to carry out photosynthesis, cellular division, and metabolic processes, thus affecting their growth rate and overall biomass production. Altering the media strength can significantly impact microalgae growth due to the availability of nutrients for their physiological processes. In this current study, full-strength media are the best media in inducing the cells' growth, followed by F/2, F/4 and F/8 (Fig. 1A). Adequate nutrient availability in the full-strength media can promote faster growth rates and increased biomass production in microalgae. The macronutrients and micronutrients in full-strength media facilitate enhanced photosynthesis, leading to higher carbon fixation and energy production. This, in turn, promotes cell division and overall growth (Safi et al. 2014). On the other hand, lower media strength with insufficient macronutrients, such as nitrogen and phosphorus, can limit photosynthesis and cellular division, decreasing cell density and biomass accumulation (Hu et al. 2019). Similarly, deficiencies in micronutrients can impair various enzymatic reactions, affecting metabolic pathways and hindering optimal growth (Hu et al. 2021a; Udayan et al. 2023).

T. chui can also grow well in media supplemented with different sucrose concentrations. Adding sucrose to the growth media can serve as an additional carbon source for microalgae, affecting their metabolic processes and biomass accumulation. Increasing the sucrose concentration slowly increased the cell growth for 8 days, especially at 40 g/L. However, excessively high sucrose concentrations (at 50 g/L) decreased cell density due to the osmotic stress imposed on the microalgae (Fig. 1B). Andreeva et al. (2021) also reported the same response in *Chlorella vulgaris* (Chlorophyta) but with lower sucrose concentrations ranging from 2.0 to 5.0 g/L. It is important to note that the optimal sucrose concentration may vary depending on the microalgae species and strain. Each species has specific metabolic requirements and preferences for carbon sources (Bashir et al. 2019). Therefore, determining the ideal sucrose concentration for a particular microalgae species requires experimentation and optimisation.

The influence of media pH on microalgae growth can be attributed to its impact on nutrient availability and cellular processes. pH affects the solubility and availability of nutrients in the media, such as carbon dioxide (CO_2) for photosynthesis and various macronutrients and micronutrients (Andreeva et al. 2021). Deviations from the optimal pH range can affect nutrient uptake, reducing growth rates and cell density.



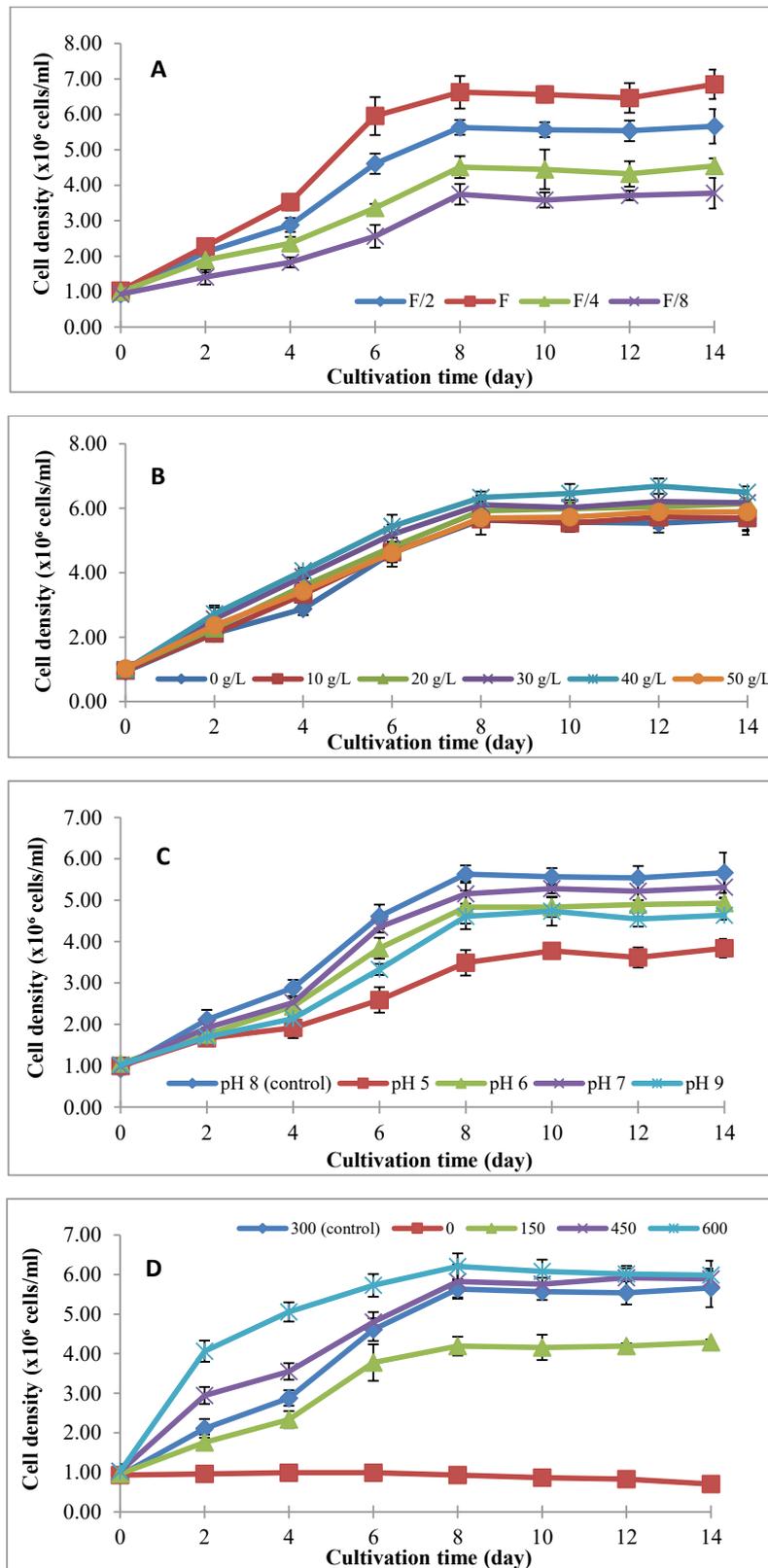


Fig. 1 Growth curve of *Tetraselmis chui* in different A) media strength, B) sucrose concentrations, C) pH of the media and D) light intensities. Data are mean \pm SD, n=5.

Different microalgae species have specific pH preferences, and their growth rates may vary depending on the pH conditions provided in the media (Moheimani 2012). In this study, *T. chui* can grow well in the pH media ranging from pH 6 to pH 9. The lowest cell density was observed at pH 5.0 (Fig. 1C). Adam et al.



(2022) investigated the effect of media pH on the growth of *C. vulgaris*. The study revealed that pH values within the range of 7 to 8 were optimal for promoting higher microalgae cell density. At pH levels outside this range, microalgae growth was significantly reduced. Similarly, a study by Bhattacharya et al. (2022) focused on the impact of media pH on *Scenedesmus quadricauda* (Chlorophyta) growth and found that a pH range of 6.5 to 7.5 supported the highest cell density.

Another factor that contributes to the variation in microalgae cell density is the light intensity. The influence of light intensity on microalgae growth can be attributed to its impact on photosynthetic efficiency and energy balance. Light intensity affects the rate of photon absorption and energy conversion through photosystems, influencing the production of ATP and NADPH, which are essential for photosynthesis (Shi et al. 2022). *T. chui* favoured higher light intensity (300 to 600 lux) to grow. Cell density under 600 lux was 1.1-fold higher than the control (300 lux) at 8 days of culture (Fig. 1D). Insufficient light intensity at 150 lux might limit energy supply, leading to slower growth rates and lower cell density. Research by Gu et al. (2022) focused on the effect of light intensity on the growth of *C. vulgaris*. The study revealed that moderate light intensity (around 100 $\mu\text{mol photons/m}^2/\text{s}$) promoted the highest cell density, while low light intensity hindered growth. Excessively high light intensities above the optimal range caused photoinhibition, reducing growth rates and cell density (Walters 2005). Similarly, a study by Li et al. (2021) investigated the impact of light intensity on the growth of *Arthrospira platensis* (formerly *Spirulina platensis*) (Cyanobacteria) and found that a light intensity of around 150 $\mu\text{mol photons/m}^2/\text{s}$ supported optimal cell density.

Fresh and dry weights

Full-strength media produced the highest fresh and dry weights of *T. chui*, followed by F/2 and F/4, and the least growth was observed in F/8 media (Fig. 2A). The current results demonstrated that the growth of *T. chui* is highly sensitive to some cultured conditions and that it was possible to markedly improve the growth performance by modifying the nutrient concentrations. For instance, cells cultured in full-strength media significantly increased the specific growth rates (3.5-fold higher compared to F/8 media) by increasing the concentrations of some medium components such as nitrogen and phosphorus (Fig. 2A). Nitrogen and phosphorus are important nutrients in microalgal growth, and their concentrations need to be optimised for each species and condition. Nucleic acids, proteins, and ATP synthesis and enzymes involved in cell proliferation require nitrogen (Tongprawhan et al. 2014), whereas many metabolic processes, including energy generation, photosynthesis, and signalling pathways, need phosphorus. Thus, both nutrient concentrations facilitate optimal photosynthetic activity, leading to higher biomass accumulation, as observed in *T. chui* cells grown in full-strength media. Insufficient nutrient concentrations can limit growth and biomass production, while excessive nutrient levels may result in nutrient imbalances or toxicity, affecting cellular processes and biomass yield (Singh et al. 2016). Research studies have demonstrated the correlation between media strength and microalgae biomass production. Adjusting the concentration of nutrients in the growth media makes it possible to optimise the growth conditions for maximum biomass yield. Priyadarshani and Rath (2012) investigated the effect of media strength on the growth of *C. vulgaris*. The researchers varied the nitrogen and phosphorus concentrations in the growth media and observed that higher nutrient concentrations resulted in increased fresh and dry weight of microalgae biomass. Similarly, a study by Hu et al. (2019) explored the influence of different nutrient concentrations on the growth of *S. quadricauda* and found that higher nutrient levels promoted higher biomass production.

In addition to nutrients, the carbon source concentration, particularly sucrose in the growth media, also impacts microalgae biomass production. The addition of sucrose slowly increased the fresh and dry weights of *T. chui* to maximum values at 40 g/L and significantly decreased at high sucrose concentration (50 g/L). However, the 0–40 g/L sucrose concentrations do not significantly affect the growth (Fig. 2B). Gradual increases in the fresh and dry weights at 0–40 g/L sucrose are associated with the primary function of sucrose as a source of energy during photosynthesis. Sucrose supplementation in the growth media enhances carbon assimilation and provides microalgae with readily available energy, enabling them to allocate more resources towards growth and biomass production (Hu et al. 2021b). Moderate sucrose concentrations often enhance biomass production by providing an additional carbon source for metabolism and biomass synthesis. However, excessively high sucrose concentrations may inhibit growth observed in cells at 50 g/L sucrose. It is important to note that the optimal sucrose concentration for microalgae biomass production



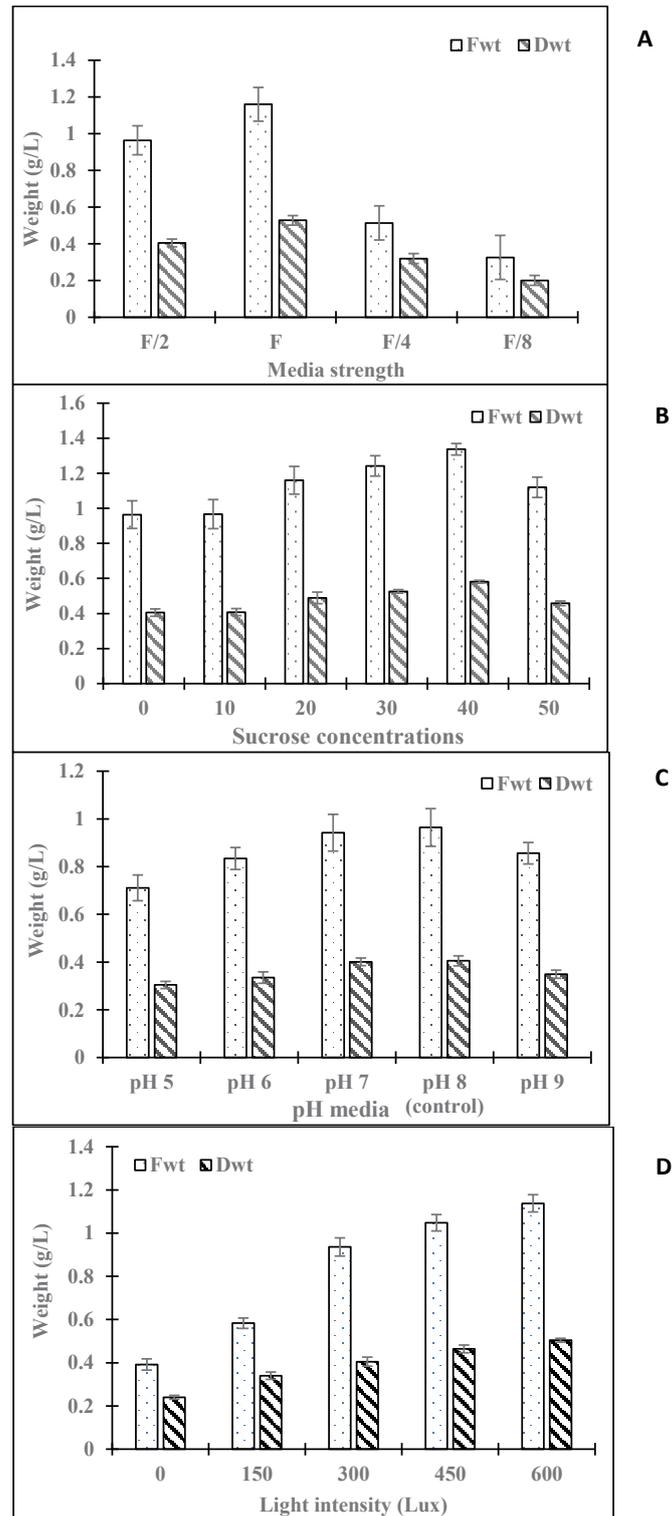


Fig. 2 Fresh and dry weights of *Tetraselmis chui* in different A) media strength, B) sucrose concentrations, C) pH of the media and D) light intensities. Data are mean \pm SD, n=5.

may depend on various factors, including the microalgae species, strain, and growth conditions (Bashir et al. 2019). Different species may have varying preferences and capacities for sucrose utilisation. Additionally, the microalgae culture's specific growth phase and physiological state may influence the response to sucrose supplementation. Other authors found that adding moderate sucrose concentrations (around 10–20 g/L) significantly increased microalgae biomass's fresh and dry weights compared to no sucrose supplementation in *Auxenochlorella pyrenoidosa* (formerly *Chlorella pyrenoidosa*) (Chlorophyta) (Hu et al.



2021b). However, excessively high sucrose concentrations led to reduced biomass yields, possibly due to osmotic stress or substrate inhibition. Similarly, Ren et al. (2013) found that a glucose concentration of 10 g/L resulted in the highest biomass production, while lower or higher concentrations led to reduced growth and biomass yields of *Scenedesmus* sp. strain R-16.

Nutrient uptake and assimilation by microalgae are highly dependent on the appropriate pH range. pH imbalance can hinder nutrient absorption, leading to nutrient deficiencies or toxicities, impacting microalgal growth and biomass production. pH regulation in the growth media helps maintain the optimal pH conditions for enzyme activity, photosynthesis, and cellular metabolism, thereby supporting efficient metabolic processes and biomass production (Qiu et al. 2017). However, different species required different optimal pH to support their growth. In this current study, pH ranging from 5–9 did not significantly affect the growth of *T. chui*, where no significant difference was observed in cell growth of *T. chui* at the selected pH (Fig. 2C). In general, marine microalgae like *T. chui* tend to thrive within a slightly alkaline pH range of around 7.5 to 8.5 is often considered suitable for the growth of *Tetraselmis* strains (Patrinou et al. 2022), while reduced growth rates were recorded with pH values of 5.5 and 9.5 (Khatoun et al. 2014). On the other hand, Wu et al. (2021) and Latsos et al. (2021) found that pH 8.0 and pH 6.5–8.5 supported the highest growth rate and biomass production of *C. vulgaris* and *Rhodomonas salina* (Cryptista, Cryptophyceae), respectively. Deviations from the optimal pH range reduced growth rates and biomass yields.

Light intensity is considered one of the most important parameters for microalgal growth and biomass production. Apart from growth aspects, sufficient light intensity is required for photosynthesis to generate ATP and NADPH to produce metabolites necessary for growth (Niccolai et al. 2019). This study shows that an increase in the microalgae fresh and dry weights was observed when irradiance increased up to 600 lux (Fig. 2D). Sufficient light intensity can lead to strong utilisation of CO₂ by microalgae and boost energy production, leading to overwhelmed cell growth. On the other hand, when the light intensity exceeds the saturation limit, photoinhibition can occur. This means the photosystem becomes overloaded, pigments become bleached, and photosystems are destroyed, thus lowering cell growth (Montoya-Vallejo et al. 2023).

Chlorophyll and carotenoid content

T. chui is a microalga known for producing high levels of chlorophylls and carotenoids, essential pigments in photosynthesis. The biosynthesis of these pigments can be influenced by various factors, including media strength, sucrose concentrations, pH medium, and light intensities (Table 1).

Table 1 Chlorophyll a, chlorophyll b and carotenoids content of *Tetraselmis chui* in different A) media strength, B) sucrose concentrations, C) pH of the media and D) light intensities.

Treatments		Pigments		
		Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Carotenoids (mg/g FW)
Media strength	F/2	0.202 ± 0.026 ^b	0.154 ± 0.019 ^b	0.138 ± 0.017 ^b
	F	0.819 ± 0.050 ^a	0.677 ± 0.049 ^a	0.297 ± 0.014 ^a
	F/4	0.081 ± 0.006 ^c	0.067 ± 0.015 ^c	0.042 ± 0.009 ^c
	F/8	0.036 ± 0.004 ^d	0.030 ± 0.003 ^c	0.022 ± 0.002 ^d
Sucrose concentration (g/L)	0	0.202 ± 0.026 ^d	0.154 ± 0.019 ^c	0.138 ± 0.017 ^d
	10	0.333 ± 0.061 ^c	0.200 ± 0.035 ^b	0.155 ± 0.030 ^d
	20	0.488 ± 0.054 ^b	0.294 ± 0.031 ^a	0.246 ± 0.028 ^b
	30	0.505 ± 0.043 ^{a,b}	0.295 ± 0.025 ^a	0.262 ± 0.022 ^{a,b}
	40	0.561 ± 0.078 ^a	0.328 ± 0.045 ^a	0.288 ± 0.038 ^a
	50	0.388 ± 0.020 ^c	0.234 ± 0.013 ^b	0.199 ± 0.012 ^c
pH	5	0.110 ± 0.005 ^b	0.071 ± 0.004 ^{b,c}	0.061 ± 0.002 ^d
	6	0.163 ± 0.027 ^b	0.080 ± 0.002 ^{b,c}	0.132 ± 0.016 ^{b,c}
	7	0.198 ± 0.003 ^b	0.084 ± 0.003 ^b	0.150 ± 0.002 ^a
	8	0.202 ± 0.026 ^a	0.154 ± 0.019 ^a	0.138 ± 0.017 ^{a,b,c}
	9	0.148 ± 0.009 ^c	0.068 ± 0.005 ^c	0.120 ± 0.005 ^c
Light intensity (Lux)	0	0.059 ± 0.001 ^d	0.040 ± 0.000 ^c	0.024 ± 0.001 ^d
	150	0.404 ± 0.028 ^a	0.256 ± 0.017 ^a	0.125 ± 0.009 ^c
	300	0.202 ± 0.026 ^c	0.154 ± 0.019 ^b	0.138 ± 0.017 ^c
	450	0.277 ± 0.024 ^b	0.239 ± 0.020 ^a	0.169 ± 0.021 ^b
	600	0.397 ± 0.020 ^a	0.253 ± 0.009 ^a	0.238 ± 0.015 ^a

Data are mean ± SD with different superscript letters in a column are significantly different (P < 0.05).



The media strength, which includes the nutrients and trace elements concentration, plays a crucial role in the growth and pigmentation of microalgae. The nitrogen content in the medium is necessary for the microalgae to synthesise chlorophylls a and b and produce amino acids. In addition, phosphorus significantly impacts chemicals involved in metabolism, photosynthesis, and energy production. Altering nutrient availability through media strength variations can influence the synthesis and accumulation of valuable bioactive compounds, such as lipids, pigments, and proteins (Zhang et al. 2022). The chlorophylls a, b and carotenoids were highest in the full strength media of 0.819 ± 0.050 , 0.677 ± 0.049 and 0.297 ± 0.014 mg/g FW, respectively. The lowest pigment production was observed in cells grown in F/8 media, which was almost 22-fold lower for chlorophylls a and b and 13.5-fold for carotenoids. The light-harvesting complexes of photosystems I and II are chlorophylls a and b. So, the more substance they contain, the better they can absorb solar radiation. Therefore, the biomass in the full-strength medium was greater than that in other media. As photosynthesis efficiency increases with an increase in chlorophyll a and b contents, alternatively, it is predicted that a metabolism pathway other than the carbon metabolism of biomass will be responsible for the accumulation of carotenoids. Although a prior study revealed that this changes depending on the carotenoid type, it is generally accepted that nutritional deficit drives the synthesis of carotenoids (Cezare-Gomes et al. 2019). Higher pigment biosynthesis in higher media strengths in *T. suecica* was also demonstrated by Montes-González et al. (2021) due to the adequate availability of essential nutrients, such as nitrogen, phosphorus, and trace metals, which act as co-factors in the biosynthetic pathways of these pigments.

A study by Xu et al. (2017) examined the effect of different sucrose concentrations on pigment production in *T. chui*. They observed that higher sucrose concentrations increased chlorophyll a and carotenoid content, while chlorophyll b levels remained relatively stable. In *T. chui*, higher sucrose concentrations have been shown to stimulate chlorophylls a, b and carotenoids and maximum production was observed at 40 g/L sucrose and reduced at 50 g/L. Sucrose as a carbon source likely influenced the allocation of metabolic resources towards pigment synthesis, particularly for chlorophyll a and carotenoids. It affects pigment synthesis by providing a carbon skeleton for producing precursors required for pigment biosynthesis (Patel et al. 2022).

Media pH also influences the pigment accumulation in *T. chui*. Notably, chlorophylls a and b were optimal under pH 8 and carotenoids were abundant in media at pH 7 in the current study. In contrast, Du et al. (2020) reported that moderately acidic pH conditions (pH 6.5–7.5) promoted higher chlorophyll a, chlorophyll b, and carotenoid compared to more alkaline pH values in the same species. The optimal pH range likely facilitated enzyme activities and metabolic processes involved in pigment synthesis.

A key factor limiting the formation of carotenoids and biomass is light intensity. The optimal light intensity provided sufficient energy for photosynthesis while minimising the potential for photoinhibition, leading to increased pigment synthesis. The results showed a reversed trend in the production of the studied pigments. Chlorophyll a and b were accumulated at lower light intensities (150 lux), whereas carotenoids were higher at higher light intensities (600 lux). The overexpression of phytoene synthase (PSY), a rate-limiting enzyme in carotenoid production, is thought to be the mechanism by which light causes the increase in total carotenoid concentration (Cichoński and Chrzanowski 2022). It is hypothesised that carotenoids, especially lutein and zeaxanthin, are synthesised to shield the photosynthetic machinery from excessive light energy and are synthesised in large quantities at high light intensities (Kim et al. 2019). Algae may also require less chlorophyll for photosynthesis due to slower metabolic processes caused by decreased light intensities. As a result, they produce more carotenoids and less chlorophyll (Fabrowska et al. 2018). A different investigation by Ferreira et al. (2016) on *Tetrademus dimorphus* (formerly *Scenedesmus dimorphus*) (Chlorophyta) revealed that cells produced under low light intensity ($16.9 \mu\text{mol photon/m}^2/\text{s}$) had higher chlorophyll contents than those grown under high light intensity ($123 \mu\text{mol photon/m}^2/\text{s}$).

Phytochemicals content

Green algae are fast-growing, have high biomass production, fix large amounts of CO_2 , and require little water, making them natural sources of bioactive compounds, including total phenolics and flavonoids (Cezare-Gomes et al. 2019). Several types of flavonoids and phenolic acids, such as simple phenols, flavonoids, flavanones, isoflavone, flavonols, dihydro flavonols, flavones, flavan-3-ols, dihydrochalcones, and



proanthocyanidins, have been identified in different species of marine algae (Goiris et al. 2015). A few factors influence the concentration of algal polyphenols within algal cells, including season, habitat, UV irradiation, light, nutrient availability, and salinity (Freile-Pelegrín and Robledo 2013). Full-strength media increased the production of total phenolics and flavonoids (4.3-fold higher) in *T. chui* compared to those grown in an F/8 medium (Figs. 3A and 4A). These results are in agreement with the findings from Cichoński and Chrzanowski (2022), who demonstrated that higher media strengths significantly increased the levels of total phenolics and flavonoids in *C. vulgaris*. The availability of essential nutrients, such as nitrogen, phosphorus, and trace metals, in higher media strengths likely facilitated the synthesis of precursors and co-factors required for the biosynthesis of these bioactive compounds.

As a carbon source, sucrose can influence microalgae biosynthesis of total phenolics and flavonoids. The total phenolic content slowly increased from 0 g/L to 50 g/L sucrose. However, no significant differences were observed in the total phenolic content at 0–30 g/L and 40–50 g/L sucrose (Fig. 3B). In contrast, 20–30 g/L sucrose induces higher total flavonoids than other treatments (Fig. 4B). This might be due to the availability of sucrose as a carbon source redirecting metabolic resources towards secondary metabolite synthesis, including phenolics and flavonoids, contributing to their enhanced production. Tang et al. (2018) also reported that higher sucrose concentrations increased total phenolics and flavonoid levels in *Tetradesmus obliquus* (formerly *Scenedesmus obliquus*) (Chlorophyta).

The total phenolic and flavonoids can also be found abundantly in cells grown at pH 9.0 (Figs. 3C and 4C). This pH facilitates enzyme activities and metabolic processes involved in the biosynthesis of these compounds, thus significantly increasing both phytochemicals studied. In addition, algae can respond to pH fluctuations by perceiving them as stressors, and they can also upregulate their production of phenolic compounds and flavonoids to protect themselves against oxidative stress and other pH-related stress conditions (Ismail et al. 2016). A different trend was observed in *Arthrospira platensis* where mildly acidic to neutral pH conditions (pH 6.5–7.5) resulted in higher levels of total phenolics and flavonoids than more alkaline or acidic pH values (Shi et al. 2016).

Light intensity plays a pivotal role in photosynthesis and the production of bioactive compounds in microalgae. Different light intensities can modulate the synthesis of both compounds. Light intensities at 150 and 450 Lux bring about higher total phenolics followed by 600, 300 Lux and cells grown in the dark (Fig. 3D). Total flavonoids were also built up in *T. chui* grown under 450 lux (Fig. 4D). The results demonstrated that moderate light intensities likely provided the optimal balance between photosynthetic efficiency and

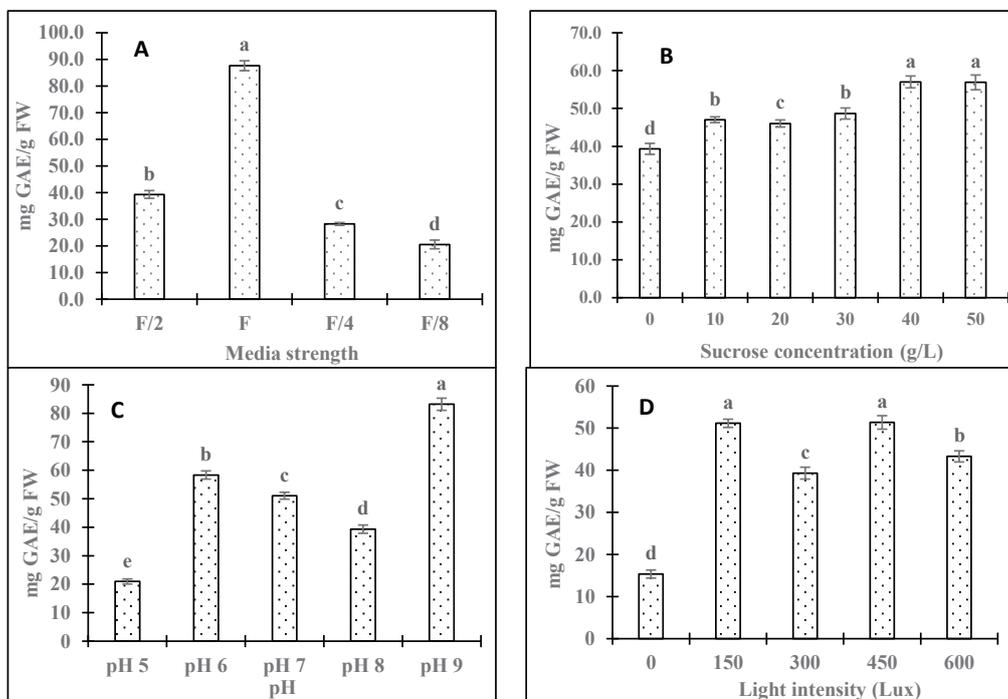


Fig. 3 Total phenolic content of *Tetraselmis chui* in different A) media strength, B) sucrose concentrations, C) pH of the media and D) light intensities. Data are mean \pm SD, n=5.



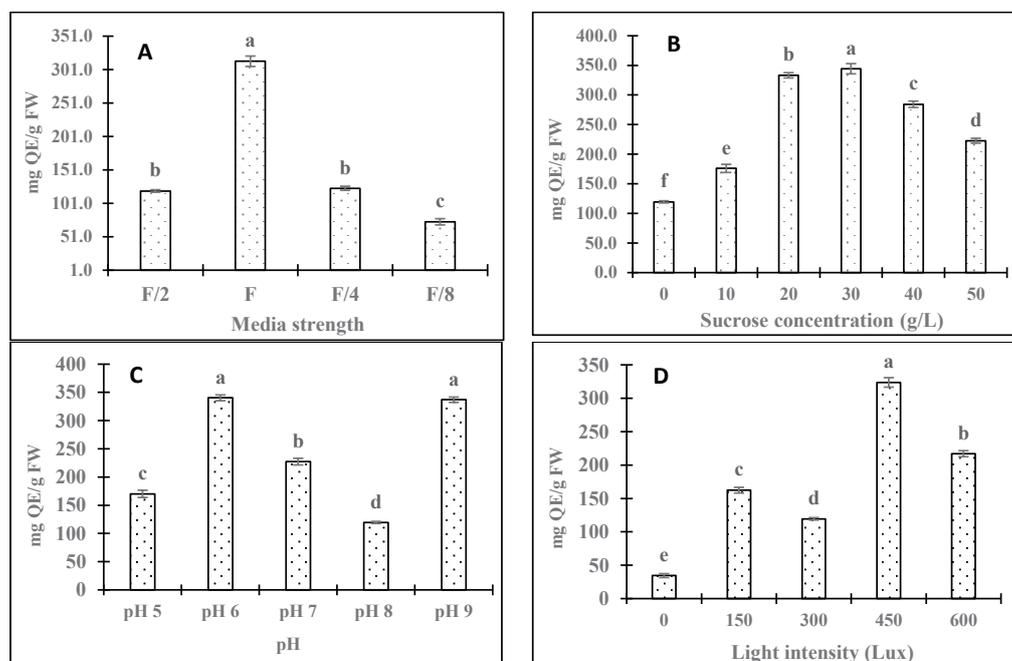


Fig. 4 Total flavonoid content of *Tetraselmis chui* in different A) media strength, B) sucrose concentrations, C) pH of the media and D) light intensities. Data are mean \pm SD, n=5.

the activation of metabolic pathways leading to increased bioactive compound production, hence promoting higher total phenolics and flavonoids compared to lower or higher light intensities.

Conclusion

The cell growth, biosynthesis of pigments, and total phenolics and flavonoids in *T. chui* are influenced by media strength, sucrose concentrations, pH medium, and light intensities. Full media strength, optimal sucrose concentrations, slightly alkaline pH conditions, and moderate to high light intensities have been shown to enhance the growth required for synthesising pigments and selected bioactive compounds in *T. chui*. Understanding the interplay between these factors can assist in developing cultivation strategies to maximise biomass yields, including valuable pigments, phenolics and flavonoid compounds in microalgae, thus enhancing their potential applications in various industries.

Competing interests The authors declare that they have no competing interests.

Authors' contributions NY have made a substantial contribution to the concept and design of all the experiments as well as the acquisition, analysis and interpretation of data for the article and finalised the manuscript. NSY did all the experiments. NMZ analysed the data. MJ and HAZ refined the writing style and language and helped finalise the manuscript. All authors read and approved the final manuscript.

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