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ORIGINAL ARTICLE

Enhanced lipid production and essential ω-fatty acids synthesis by the hypersaline biodiesel-promising microalga *Tetraselmis elliptica* through growth medium optimization

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Abstract

This study aimed to optimize growth medium composition for enhanced lipid and fatty acid production by the green halophilic chlorophyte *Tetraselmis elliptica*. The impact of different ratios of natural seawater, nitrogen source/concentration, carbon source/concentration, and trace metals on the growth, biomolecules, lipid accumulation, and lipid productivity was studied. In addition, fatty acid profile of the optimized growth medium was compared with that of the typical medium as a control, and biodiesel production as well as essential polyunsaturated fatty acids (PUFAs) were evaluated. Among all studied factors, 0.1 g L^{-1} KNO_3, 0.01 g L^{-1} glycerol, and 7.0 mg L^{-1} $FeSO_4 \cdot 7H_2O$ significantly enhanced the lipid productivity of *T. elliptica*, and were used further to evaluate the impact of their combination. Cultivation of *T. elliptica* in the optimized medium showed enhancement of growth up to 16 days, which resulted in 30.9% and 17% increase in biomass and lipid productivities, respectively. Moreover, optimization of growth medium enhanced PUFAs proportion to 15.05% due to de novo synthesis of arachidonic acid and docosahexaenoic acids which represented 4.17% and 10.88%, respectively, of total fatty acids. Biodiesel characteristics of the optimized medium showed compliance of all studied parameters with the US standards. The present study provides new insights for commercial utilization of marine microalgae cultivated in optimized growth medium for dual purpose of ω-fatty acids and biodiesel production through biorefinery approach.

Keywords Growth medium · Micronutrients · Mixotrophic · Waste recycling · Green energy

1 Introduction

Nowadays, there is exponential increase in the energy demand due to industrialization and fast human activities, which is expected to double by 2050 [1]. In addition, current utilization of non-renewable fossil fuels led to environmental and health problems as a result of greenhouse gas emissions, threatening the life on the earth [2, 3]. It is essential to explore alternative technologies for sustainable and eco-friendly energy production in order to raise the living standards [4]. Bioenergy production has received increasing consideration recently as potential, sustainable, and clean alternative energy source. However, conversion of food crops to biodiesel cannot serve as the right approach due to *food-versus-fuel* dilemma [5]. Alternatively, microalgae are promising source for biofuel due to their relatively higher biomass production and ability of lipid storage comparing to other oil plants [6, 7]. In addition, algae can be cultivated in arid lands using wastewater or seawater [8, 9].

Lipid accumulation is one of the main criteria for selection of a potential microalgal species as a promising biodiesel candidate [10]. However, lipid production is usually inversely correlated to the cellular growth; i.e., microalgal cells accumulate lipids in the form of triacylglycerol at lower growth rates, resulting in low lipid productivity. Recent studies confirmed that certain conditions could enhance both microalgal growth and lipid accumulation. For instance, using 15% lipid-free biomass hydrolysate for microalgal growth stimulated biomass production and lipid

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accumulation of *Scenedesmus obliquus* by 19% and 8%, respectively [11]. Additionally, using 1 mM *p*-coumaric acid stimulated growth and lipid content of *Tetradesmus obliquus* by 34.1% and 145.4%, respectively [12]. Other factors such as plant hormones [13], high salinity adaptation [14], night illumination [15], low-dose cold atmospheric plasma [16], magnetic field [17, 18], and optimization of aeration in municipal wastewater [19] have been also studied which showed different response of microalgae to the applied stress. However, most of these studies were carried out using freshwater microalgae, with very rare studies on marine microalgae.

Marine fish have been used for centuries as the main natural source of polyunsaturated fatty acids (PUFAs), which suffered in recent decades from sever contamination and reduction of fish stocks [20, 21]. In addition, vegetarians prefer to use algal oil than fish oil, which makes marine microalgae an important source of PUFAs [22]. Moreover, halophilic/halotolerant microalgae have been discussed as a potential source for biodiesel due to their potential to cost-effective growth on seawater with combined action of biomass/macromolecules production and water desalination [23]. A wide range of synthetic growth media have been reported for efficient lab-scale microalgae cultivation, such as f/2, artificial seawater, enriched seawater, and many others that can be found in different culture collections [24, 25]. Due to the elevated cost of microalgae cultivation using synthetic media, recent technologies have been suggested through utilization of waste streams for microalgae cultivation [9, 26]. Despite the wide variety of microalgal species in marine ecosystem, only few species have been studied and have limited commercial applications. In a previous screening study on different halophilic microalgal species isolated from the hypersaline Egyptian Bardawil Lagoon, *Tetraselmis elliptica* showed the highest lipid productivity and was recommended as a promising biodiesel feedstock [27]. However, optimization of growth conditions for enhanced lipid productivity and exploring the response of this microalgal species to different stress conditions were not previously evaluated, which is of great importance for further large-scale application. Besides, possible dual production of essential fatty acids and biodiesel production through biorefinery approach could improve the process economic feasibility. Therefore, this study aimed to enhance the biomass yield and lipid production by optimization of growth medium components including ratio of natural seawater, nitrogen source/concentration, carbon source/concentration, and trace metals. In order to evaluate the impact of these conditions, growth, biomolecules, and lipid accumulation were studied. Also, fatty acid profile of the optimized growth medium was compared with that of the recommended typical medium, and biodiesel production as well as essential PUFAs production was evaluated.

2 Materials and methods

2.1 Algal strain and growth conditions

Tetraselmis elliptica was isolated from the hypersaline Bardawil lagoon, North Sinai, Egypt (maintained at Phycology Research Unit, Botany and Microbiology Department, Faculty of Science, Tanta University). It was axenically cultivated in Erlenmeyer flasks containing 700 mL/each of seawater medium (Seewasser/Erdde-kokt/Salze, SWES) using filtered natural seawater [25]. The growth media were inoculated with exponentially grown microalgal cells at initial optical density (OD_{680}) of 0.012 ± 0.002. All inoculated cultures were incubated under continuous illumination of light intensity 3000 lx at 25 °C. In order to provide necessary CO_2 and to avoid cell settling, continuous aeration of 0.2 vvm [19] was provided to the cultures by bubbling filter-sterilized air. Different conditions were modified in order to study the effect of different medium components on growth and lipid production.

The ratio of natural seawater of the medium was modified by replacing seawater with different freshwater ratios (FWR) (0% (control), 25%, 50%, 75%, and 100%). In addition, different nitrogen sources (KNO_3 (control), NH_4Cl, NH_4NO_3, and urea) at 0.2 g L^{-1}, nitrogen concentration of the best source (0.0, 0.1, 0.2 (control), 0.3, 0.4, and 0.6 g L^{-1}), carbon sources including glucose, sodium acetate, $NaHCO_3$ (0.0 (control), 0.1, 0.2, 0.3, and 0.5 g L^{-1}), glycerol (0.0 (control), 0.01, 0.03, 0.05, and 0.10 g L^{-1}), and trace metals using different concentrations of $FeSO_4{\bullet}7H_2O$ at (0.0, 1.75, 3.5 (control), 7.0, and 10.5 mg L^{-1}), $MnSO_4{\bullet}4H_2O$ (0, 5, 10 (control), 20, and 30 μg L^{-1}), and $CuSO_4{\bullet}5H_2O$ (0, 0.013, 0.025 (control), 0.050, and 0.075 μg L^{-1}) were studied. Afterwards, an experiment was performed to evaluate the growth, lipid productivity/composition, and biodiesel characteristics of *T. elliptica* grown in the control and optimized growth media.

2.2 Biomass analysis

Algal growth was monitored every 2 days by measuring the culture optical density at 680 nm (OD_{680}). Cellular dry weight (CDW) was measured at the start and the end of exponential phase, then biomass productivity (BP) was calculated [10] using Eq. 1:

$$BP\left(gL^{-1}d^{-1}\right) = \left(DW_L - DW_L\right) \times \left(t_L - t_E\right)^{-1} \tag{1}$$

where DW_E represents the CDW (as g L^{-1}) at early exponential phase (t_E), while DW_L represents the CDW at the time of late exponential phase (t_L).

2.3 Lipid extraction

Intracellular lipids were extracted according to the modified Folch method [28]. Briefly, about 20 mL of culture was centrifuged at 3000 rpm for 10 min. To the cell pellet, 10 mL of chloroform:methanol (2:1, *v/v*) was added followed by incubation for 2 h on a shaker at 150 rpm. After separation of organic phase and organic solvent evaporation, lipid content was calculated as milligrams per gram of CDW, while lipid productivity (LP) was calculated [10] using Eq. 2:

$$LP\left(\mathrm{mgL}^{-1}\mathrm{d}^{-1}\right) = \left(TL_L - TL_E\right) \times \left(t_L - t_E\right)^{-1} \tag{2}$$

where TL_L represents the total lipids (as mg L^{-1}) at the time of late exponential phase (t_L), while TL_E represents the total lipids at the time of early exponential phase (t_E).

2.4 Fatty acid profile and biodiesel characteristics

After lipid extraction as described above, lipids were converted to fatty acid methyl esters (FAMEs) using the modified method of Christie [29, 30]. The main biodiesel characteristics including unsaturation degree (*Du*), specific gravity (*SG*), kinematic viscosity (*KV*), cloud point (*CP*), higher heating value (*HHV*), iodine value (*IV*), and cetane number (*CN*) were calculated from fatty acid profile according to Hoekman et al. [31] as previously described by Song et al. [32] using Eqs. 3–9. Long-chain saturation factor and cold filter plugging point (*CFPP*) were calculated according to Ramos et al. [33] using Eqs. 10–11:

$$Du = \sum C_{=} \times Mf \tag{3}$$

$$KV(mm^2s^{-1}) = -0.6316DU + 5.2065 \tag{4}$$

$$SG(kg^{-1}) = 0.0055Du + 0.8726 \tag{5}$$

$$CP(^\circ C) = -13.356Du + 19.994 \tag{6}$$

$$CN = -6.6684Du + 62.876 \tag{7}$$

$$IV(gI_2/100goil) = 74.373Du + 12.71 \tag{8}$$

$$HHV(MJkg^{-1}) = 1.7601Du + 38.534 \tag{9}$$

$$LCSF(wt\%) = \left(0.1 \times C_{16}\right) + \left(0.5 \times C_{18}\right) + \left(1.0 \times C_{20}\right) + \left(1.5 \times C_{22}\right) + \left(2.0 \times C_{24}\right) \tag{10}$$

$$CFPP(^\circ C) = (3.1417 \times LCSF) - 16.477 \tag{11}$$

where $C_{=}$ refers to the number of C=C double bonds, while *Mf* represents mass fraction of each individual fatty acid. C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} refer to mass fractions of the saturated fatty acids including C16, C18, C20, C22, and C24, respectively.

2.5 Statistical analysis

Experiments were conducted in three replicates and results are shown as the mean and standard deviation of the three replicates. The statistical analyses were performed by SPSS (IBM, version 22) using one-way analysis of variance followed by least significant difference at $P \leq 0.05$.

3 Results and discussion

3.1 Effect of freshwater supplementation

The effect of freshwater supplementation at different ratios on the growth pattern and biomass productivity of *T. elliptica* is shown in Fig. 1. The obtained results showed that freshwater supplementation at 25%, 50%, and 75% enhanced the algal growth compared to the control, while full replacement of seawater with freshwater resulted in sharp reduction in the growth pattern (Fig. 1A). Therefore, biomass productivity of *T. elliptica* significantly augmented by increasing the FWR, reaching the highest significant recorded value of 0.0816 g L^{-1} day^{-1} at 50% FWR, which represented 20.7% higher than the control (Fig. 1B). Further increase in FWR led to reduction of biomass productivity, which showed the lowest value of 0.0378 g L^{-1} day^{-1} at 100%, representing 44.1% lower than the control. In that context, salt stress was reported to create disturbance of physiological activities of algal cells and significantly affects the growth. Adaptability of microalgae to saline conditions varies according to their salt tolerance level and from a species to another [34]. Alsull and Omar [35] found that *Tetraselmis* sp. and *Nannochloropsis* sp. showed remarkably reduction in the growth and biomass yield under lower salinity conditions. It might be attributed to the hypersalinity of Bardawil lagoon which was reported as high as 39.4‰ [27]. Thus, supplementation of freshwater reduces the salinity of the medium to the optimum values of enhanced microalgal growth. However, extreme reduction of salinity results in growth retardation, which was previously confirmed by Adarme-Vegas et al. [36] who observed significant reduction in biomass yield of *Tetraselmis* sp. in near-freshwater salinity levels.

It can be noted from Table 1 that lipid content significantly decreased by increasing FWR, reaching the lowest value of 179.6 mg g^{-1} at 75% freshwater, which increased to 217.1 mg g^{-1} by further increase in FWR to 100%.

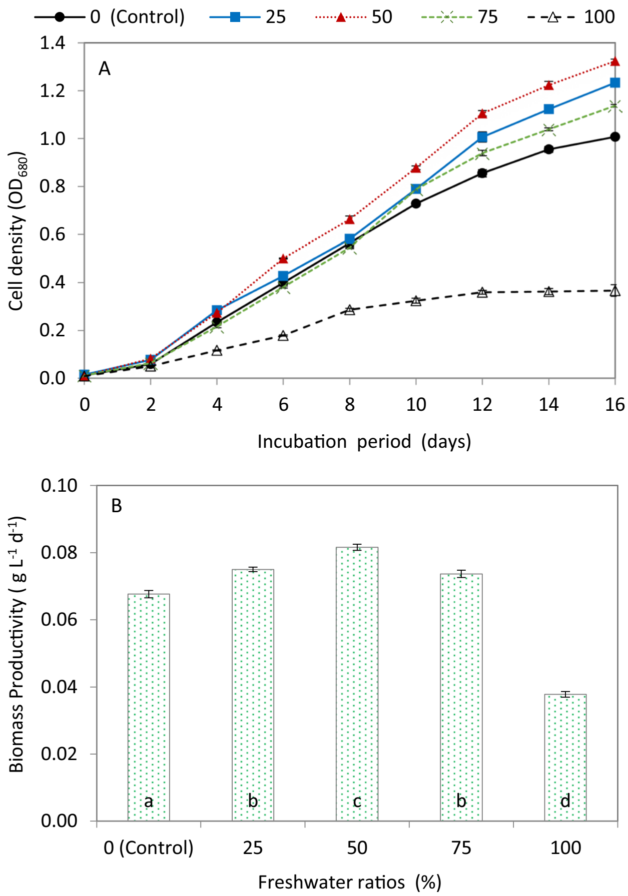

Fig. 1 The growth curve (**A**) and biomass productivity (**B**) of *Tetraselmis elliptica* grown in SWES medium with different ratios of freshwater (0, 25, 50, 75, and 100%). Error bars show the SD for three replicates, columns with the same letter showed insignificant differences (at $P \leq 0.05$)

Reduction of lipid accumulation by freshwater addition could be attributed to reduction of salinity stress. In that context, different stress conditions were reported to enhance lipid accumulation in microalgal cells [9, 37]. Lipid reduction was compensated with enhanced protein synthesis, which might play a significant role in physiological activities of microalgal cells towards growth and biomass accumulation. Due to sharp reduction in lipid content, lipid productivity showed reduction at all studied FWR, reaching the lowest value of 8.20 mg L^{-1} day^{-1} at 100% FWR, which represented 64.3% lower than the control (Table 1). Thus, these results suggested the potential of freshwater supplementation to enhance biomass yield, which is not desired for lipid production.

3.2 Effect of nitrogen source/concentration

Figure 2 shows the growth pattern and biomass productivity of *T. elliptica* at different nitrogen sources. Among the studied nitrogen sources, urea showed the highest growth, with biomass productivity of 0.0822 g L^{-1} day^{-1} representing 21.6% over the control (using KNO_3). In that context, urea was reported to enhance biomass yield of *Chlorella vulgaris* under mixotrophic cultivation [38, 39]. It might be attributed to the high solubility rate of urea forming carbonic acid as a complementary carbon fraction to ammonia as nitrogen fraction. However, Arumugam et al. [40] reported that sodium nitrate is more effective to enhance the growth of freshwater microalga *Scenedesmus bijugatus* than urea. Thus, suitable nitrogen source is species-specific and should be justified for individual cases. The present results showed that ammonium nitrate showed 14.9% higher biomass productivity than the control, while 5.5% lower than urea (Fig. 2B). For that, both ammonia and nitrate fractions can be utilized as efficient nitrogen sources, increasing nitrogen assimilation. However, 21.9% reduction in biomass productivity was recorded in the culture provided with ammonium chloride. Different from the growth, the highest lipid content of 382.9 mg g^{-1} dw was recorded using ammonium chloride as nitrogen source, which was 12.6% and 65.5% over control and urea, respectively (Table 2). In case of ammonium chloride, utilization of ammonia as nitrogen source releases chloride ions that forms acid resulting in drop of pH value, which is in consistence with previous studies [41]. However, the highest lipid content recorded in case of ammonium chloride might be attributed to acidity stress conditions which lead to lipid

Table 1 Content of the main macromolecules and lipid productivity of *Tetraselmis elliptica* grown in SWES medium with different ratios of freshwater (0, 25, 50, 75, and 100%) and measured at late exponential phase

Freshwater (%)	Macromolecules (mg g^{-1} dw)			LP (mg L^{-1} day^{-1})[a]
	Carbohydrates	Proteins	Lipid content	
0 (Control)	300.5 ± 4.77	65.99 ± 0.91	340.1 ± 7.16	23.00 ± 0.13
25	296.3 ± 2.64 [ns]	77.33 ± 0.91*	279.8 ± 3.42*	20.99 ± 0.08*
50	268.6 ± 3.44*	68.24 ± 0.80*	184.0 ± 3.79*	15.01 ± 0.15*
75	208.2 ± 4.58*	87.40 ± 0.95*	179.6 ± 3.86*	13.22 ± 0.14*
100	66.4 ± 1.08*	68.40 ± 1.40*	217.1 ± 4.02*	8.20 ± 0.07*

[a] LP refers to lipid productivity

* and [ns] denote significant and insignificant differences, respectively, of each studied treatment compared to the control (at $P \leq 0.05$)

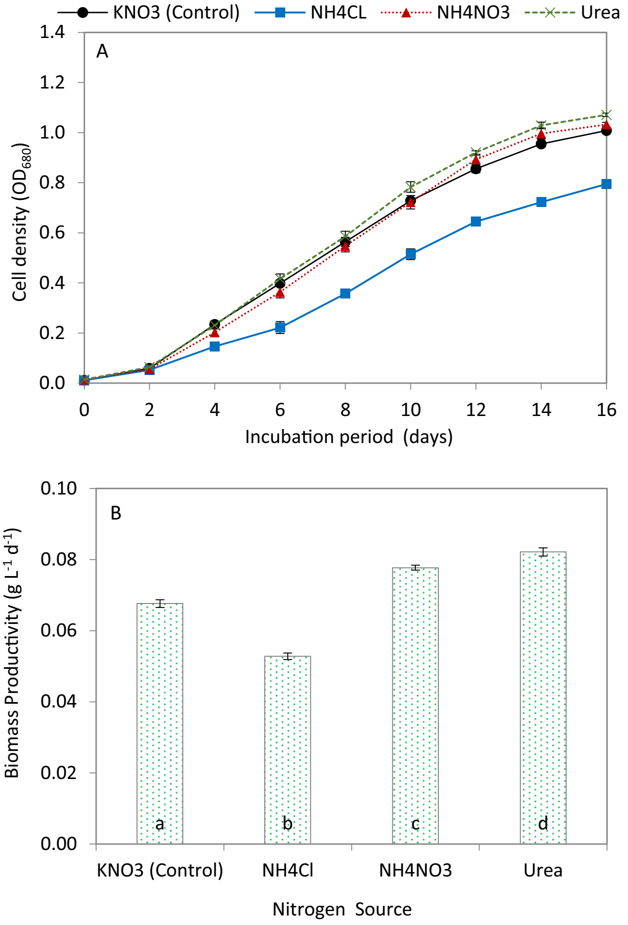

Fig. 2 The growth curve (A) and biomass productivity (B) of *Tetraselmis elliptica* grown in SWES medium with different nitrogen sources. Error bars show the SD for three replicates, columns with the same letter showed insignificant differences (at $P \leq 0.05$)

accumulation [42]. Due to severe reduction in growth and/or lipid content using different nitrogen sources, the highest lipid productivity of 23.0 mg L^{-1} day^{-1} was recorded in the control using potassium nitrate as nitrogen source, which was 13.7%, 18.4%, and 21.0% higher than ammonium chloride, ammonium nitrate, and urea, respectively (Table 2). Based on these results, potassium nitrate was selected as the most favorable nitrogen source for enhanced lipid production in *T. elliptica*, and the effect of different concentrations on growth and lipid production was further evaluated.

Results showed that increasing of potassium nitrate concentration from 0.2 g L^{-1} in the control to 0.3 and 0.4 g L^{-1} enhanced the growth and biomass productivity of *T. elliptica* (Fig. 3). The highest biomass productivity of 0.0821 g L^{-1} day^{-1} was record at 0.4 g L^{-1}, which was 21.4% higher than the control (Fig. 3B). Further increase or reduction in potassium nitrate concentration resulted in significant reduction in biomass productivity, reaching the lowest recorded value of 0.0048 g L^{-1} day^{-1} in nitrogen-free medium. Stimulation of growth at relatively high nitrogen concentrations is attributed to the importance of nitrogen as an essential element for all functional and structural molecules such as amino acids, nucleic acids, proteins, chlorophylls, and carbohydrates that is required in sufficient quantities [43]. With respect to lipid content, results confirmed that cultures provided with 0.3 and 0.4 g L^{-1} KNO_3 showed significant reduction in lipid content (16.1% and 9.0%, respectively, lower than the control) (Table 3). However, lipid content significantly increased by 53.0% and 33.5%, over the control, by reduction of nitrogen source to 0.1 and 0 g L^{-1} KNO_3, respectively, where the highest lipid content (520.5 mg g^{-1} dw) was recorded at 0.1 g L^{-1} KNO_3. In accordance with the current results, Battah et al. [44] reported that nitrogen deficiency results in reduction of microalgal growth with extraordinary lipid content. Under nitrogen deficiency, microalgal cells shift their metabolism from biomass production towards lipid accumulation as a means of energy storage [45]. These results confirm the inverse relationship between growth rate and lipid content in microalgae, which was previously reported in different freshwater microalgae [10]. Interestingly, using 0.1 g L^{-1} KNO_3 resulted in the highest significant enhancement in lipid productivity to 26.27 mg L^{-1} day^{-1}, representing 14.2% over the control (Table 3).

3.3 Effect of carbon source/concentration

Carbon is an essential element for microalgal growth, photosynthesis, and lipid production. One of the main objectives of this study was to evaluate the growth and lipid production

Table 2 Content of the main macromolecules and lipid productivity of *Tetraselmis elliptica* grown in SWES medium with different nitrogen sources and measured at late exponential phase

N-source	Macromolecules (mg g^{-1} dw)			LP (mg L^{-1} day^{-1})^a
	Carbohydrates	Proteins	Lipid content	
KNO_3 (control)	300.5 ± 4.77	65.99 ± 0.91	340.1 ± 7.16	23.00 ± 0.13
NH_4Cl	401.2 ± 7.13*	23.18 ± 0.31*	382.9 ± 9.56*	20.22 ± 0.15*
NH_4NO_3	289.1 ± 2.54*	62.69 ± 0.55*	250.1 ± 3.98*	19.43 ± 0.16*
Urea	260.6 ± 3.77*	59.06 ± 0.96*	231.3 ± 2.85*	19.01 ± 0.15*

^{a}LP refers to lipid productivity

*Significant differences of each studied treatment compared to the control (at $P \leq 0.05$)

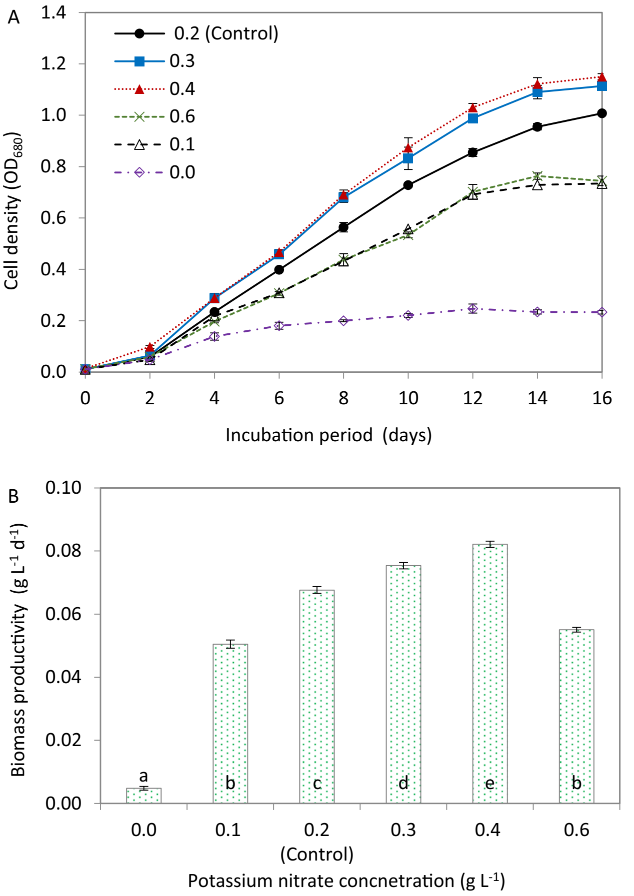

Fig. 3 The growth curve (**A**) and biomass productivity (**B**) of *Tetraselmis elliptica* grown in SWES medium with different concentrations of KNO_3. Error bars show the SD for three replicates, columns with the same letter showed insignificant differences (at $P \leq 0.05$)

of *T. elliptica* under mixotrophic cultivation using different carbon sources at different concentrations. Overall, results revealed that *T. elliptica* can grow under autotrophic and mixotrophic conditions, while maximum biomass and lipid productivities were recorded under mixotrophic cultivation. Figure 4 shows the impact of carbon source on growth of *T. elliptica*. Similar growth trends were recorded using different concentrations of different carbon sources, where increasing of carbon concentration to some extent resulted in enhancement of the algal growth, followed by growth reduction at higher concentrations. Specifically, supplementation of glucose, sodium acetate, and sodium bicarbonate at 0.1 g L^{-1} and supplementation of glycerol at 0.01 g L^{-1} enhanced the algal growth over the control. However, further increase in all carbon sources resulted in pronounced reduction of growth below the control. Initially, biomass productivity significantly increased from 0.0676 g L^{-1} day^{-1} in the control to 0.0816, 0.0749, 0.0774, and 0.0730 g L^{-1} day^{-1} by supplementation of 0.1 g L^{-1} glucose, sodium acetate, sodium bicarbonate, and 0.01 g L^{-1} glycerol, respectively (Fig. 5). Further increase in all carbon sources' concentrations resulted in significant reduction in biomass productivity. Thus, glucose was suggested as the best carbon source to achieve higher biomass production. In that context, glucose was reported as the most simple and preferred organic carbon source for microalgae because it can be assimilated easily to produce complex molecules such as carbohydrates and storage lipids [46]. The lower biomass productivity under photoautotrophic growth can be attributed to self-shading caused by microalgal cells in high-density cultures that limits its light availability for photosynthetic growth. In contrast, mixotrophic growth could overcome light limitations caused under photoautotrophic conditions at late growth stages [47], where microalgal cells can get the energy from both oxidation of supplemented organic carbon and photosynthesis [48]. It can be confirmed by enhancement of lipid content by increased concentrations of all studied carbon sources (Table 4).

The maximum lipid content of 521.7 mg g^{-1} dw was recorded in glycerol-supplemented culture at 0.1 g L^{-1}, which was 53.4% over the control (Table 4). These results suggest that microalgal cells use carbon at low concentrations as energy source for cell division and growth stimulation rather than lipid storage. However, carbon is converted directly to lipids at high concentrations of carbon source, resulting in growth retardation. Specifically, glycerol was reported to be integrated directly in lipid biosynthetic

Table 3 Content of main macromolecules and lipid productivity of *Tetraselmis elliptica* grown in SWES medium with different concentrations of KNO_3 and measured at late exponential phase

Concentrations (g L^{-1})	Macromolecules (mg g^{-1} dw)			LP (mg L^{-1} day^{-1})[a]
	Carbohydrates	Proteins	Lipid content	
0	77.1 ± 1.13*	54.27 ± 4.31*	453.9 ± 30.04*	2.20 ± 0.83*
0.1	362.1 ± 9.29*	59.50 ± 1.46*	520.5 ± 15.31*	26.27 ± 0.36*
0.2 (control)	300.5 ± 4.77	65.99 ± 0.91	340.1 ± 7.16	23.00 ± 0.23
0.3	263.4 ± 3.59*	74.80 ± 0.96*	285.4 ± 4.50*	21.50 ± 0.66*
0.4	246.2 ± 3.09*	72.35 ± 0.94*	309.4 ± 3.10*	25.42 ± 0.88*
0.6	188.4 ± 2.71*	71.36 ± 0.67*	243.1 ± 2.90*	13.37 ± 0.38*

[a] LP refers to lipid productivity

[*] Significant differences of each studied treatment compared to the control (at $P \leq 0.05$)

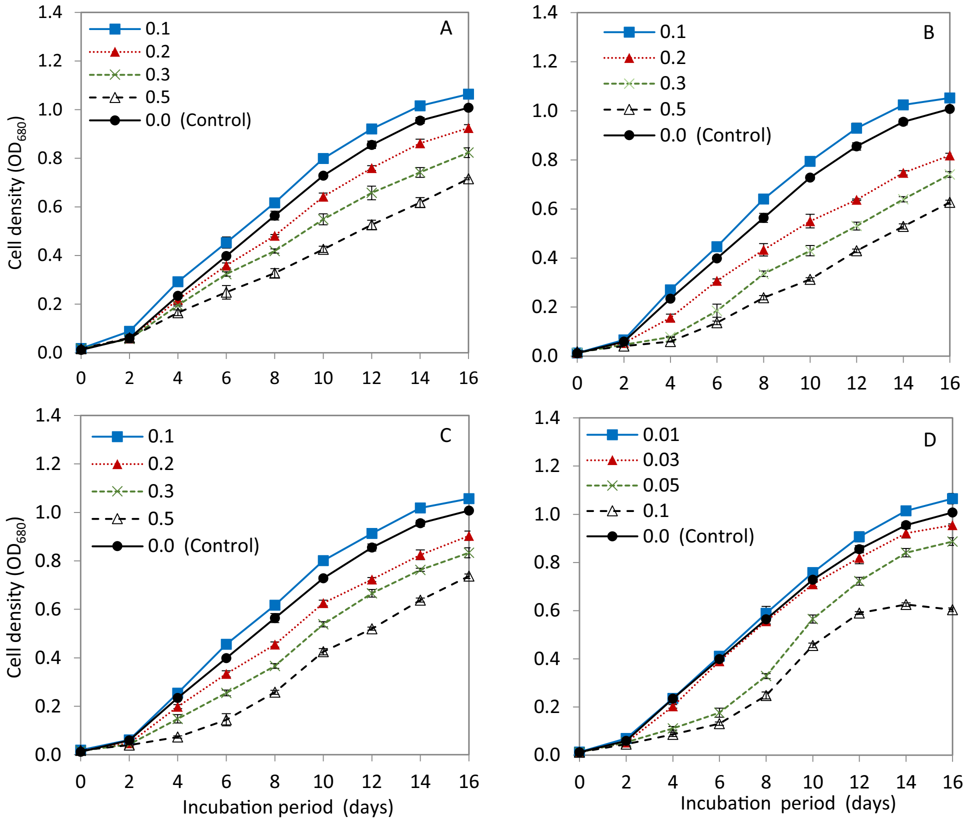

Fig. 4 The growth curve of *Tetraselmis elliptica* grown in SWES medium with different concentrations of glucose (**A**), sodium acetate (**B**), sodium bicarbonate (**C**), and glycerol (**D**)

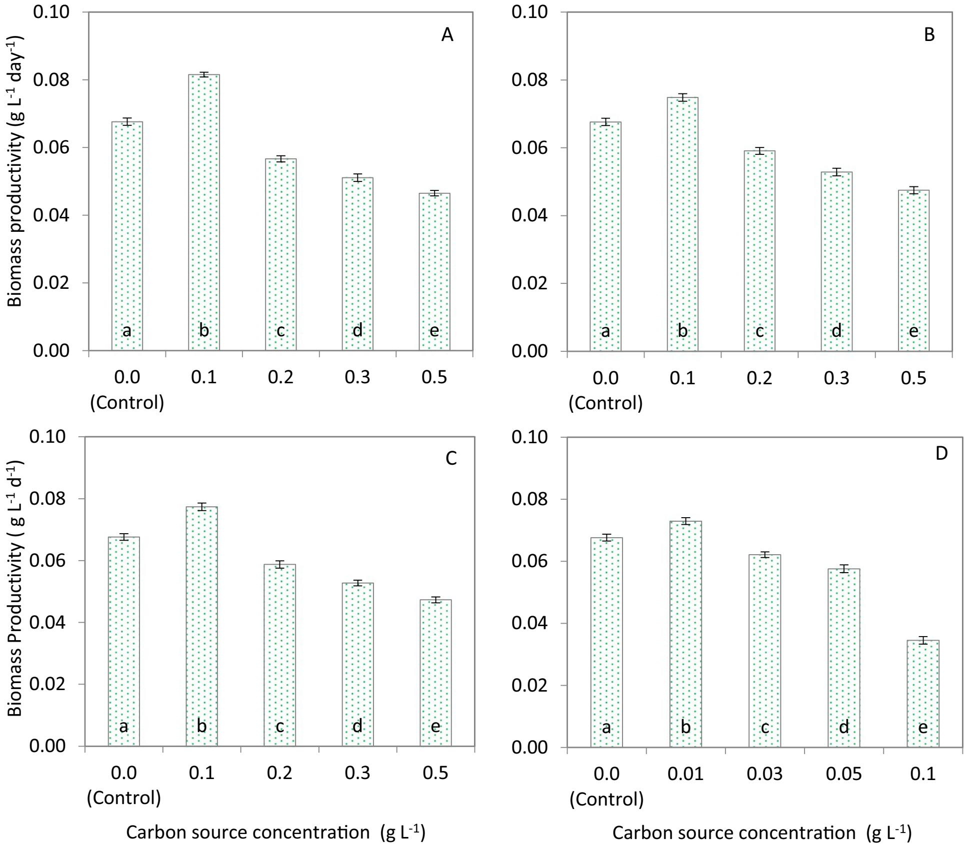

Fig. 5 Biomass productivity of *Tetraselmis elliptica* grown in SWES medium with different concentrations of glucose (**A**), sodium acetate (**B**), sodium bicarbonate (**C**), and glycerol (**D**). Error bars show the SD for three replicates, columns with the same letter showed insignificant differences (at $P \leq 0.05$)

pathway leading to enhancement of lipid accumulation in microbial cells [11, 49]. Kong et al. [50] reported that glycerol supplementation as a carbon source improved both growth and lipid accumulation of *Chlorella vulgaris*, leading to high lipid productivity. Among all treatments, the highest lipid productivity of 25.92 mg L^{-1} day^{-1} was also recorded in glycerol-supplemented culture at 0.01 g L^{-1}, which was 12.7% higher than that of the control (Table 4). Thus, glycerol supplementation plays a significant role as a carbon source for enhancement of both growth and lipid accumulation in microalgal cells, resulting in higher lipid productivity.

3.4 Effect of trace metals

Trace metals have potential impact on microalgal growth, photosynthesis, and lipid accumulation, especially under high heavy metal stress [18, 51, 52]. Increasing of $FeSO_4•7H_2O$ in the media to certain levels was reported to improve the microalgal growth [53, 54]. It is attributed to the importance of iron in photosynthetic pigments composition, and it is involved in many crucial enzymatic reactions as well as formation of photosynthetic electron transport proteins such as cytochrome and ferredoxin [55, 56]. Manganese also is an essential metal for microalgal growth, as it has catalytic activity during photosynthesis and works as enzyme activator in tricarboxylic acid cycle and glycolysis [57]. In addition, copper is an essential micronutrient for some physiological activities and metabolic processes of microalgae, as it plays vital roles to form the active sites of many algal enzymes [58, 59]. Therefore, this study evaluated the impact of different concentrations of heavy metals used in SWES medium including $FeSO_4•7H_2O$, $MnSO_4•4H_2O$, and $CuSO_4•5H_2O$.

It can be noted that *T. elliptica* growth increased by increasing of $FeSO_4•7H_2O$ or $MnSO_4•4H_2O$ and reduction of $CuSO_4•5H_2O$ than those recommended in SWES medium (Fig. 6). Using typical SWES (3.5 mg L^{-1} $FeSO_4•7H_2O$, 10 µg L^{-1} $MnSO_4•4H_2O$, and 0.025 µg L^{-1} $CuSO_4•5H_2O$) showed average biomass productivity of 0.06 g L^{-1} day^{-1} (Fig. 7). However, the highest biomass productivities for $FeSO_4•7H_2O$, $MnSO_4•4H_2O$, and $CuSO_4•5H_2O$ treatments were 0.0889, 0.0748, and 0.0700 g L^{-1} day^{-1} at 7.0 mg L^{-1}, 30 µg L^{-1}, and 0.013 µg L^{-1}, respectively. The control culture showed 340.1 mg g^{-1} dw lipid content, which was the highest among all treatments except for Mn-devoid culture, representing 6.9% higher lipid content than the control (Table 5). Among all studied trace metal treatments, the highest lipid productivity of 25.9 mg L^{-1} day^{-1} was recorded at 7.0 mg L^{-1} $FeSO_4•7H_2O$, which was 17% higher than the control. Interestingly, this concentration

Table 4 Content of main macromolecules and lipid productivity of *Tetraselmis elliptica* grown in SWES medium with different carbon sources and measured at late exponential phase

C-source	Conc. (g L^{-1})	Macromolecules (mg g^{-1} dw)			LP (mg L^{-1} day^{-1})[a]
		Carbohydrates	Proteins	Lipid content	
0.0 (control)		300.5 ± 4.77	65.99 ± 0.91	340.1 ± 7.16	23.00 ± 0.13
Glucose	0.1	281.8 ± 2.56*	59.35 ± 0.38*	291.2 ± 2.80*	23.76 ± 0.29*
	0.2	327.9 ± 7.39*	67.4 ± 2.00*	316.3 ± 6.98*	17.92 ± 0.14*
	0.3	363.5 ± 9.56*	70.2 ± 0.18*	419.2 ± 6.75*	21.41 ± 0.13*
	0.5	415.4 ± 3.82*	73.3 ± 3.55*	424.3 ± 7.38*	21.14 ± 0.12*
Na-acetate	0.1	326.4 ± 4.90*	79.27 ± 1.26*	218.0 ± 3.17*	16.32 ± 0.13*
	0.2	347.4 ± 3.59*	81.1 ± 0.54*	382.4 ± 6.50*	22.60 ± 0.27 ^{ns}
	0.3	363.9 ± 1.61*	84.6 ± 0.44*	366.3 ± 6.46*	19.36 ± 0.08*
	0.5	422.9 ± 7.57*	88.6 ± 0.48*	402.0 ± 11.26*	19.09 ± 0.13*
$NaHCO_3$	0.1	349.0 ± 5.54*	77.09 ± 1.30*	257.3 ± 5.62*	19.90 ± 0.13*
	0.2	362.7 ± 1.44*	79.14 ± 1.64*	357.9 ± 8.50*	21.01 ± 0.28*
	0.3	390.1 ± 8.93*	87.52 ± 1.45*	345.6 ± 7.41 ^{ns}	18.22 ± 0.08*
	0.5	416.5 ± 2.20*	89.7 ± 0.19*	427.7 ± 4.16*	20.22 ± 0.21*
Glycerol	0.01	315.7 ± 5.16 ^{ns}	59.39 ± 1.17*	355.3 ± 6.01*	25.92 ± 0.18*
	0.03	369.8 ± 5.42*	61.08 ± 0.81*	403.3 ± 6.61*	25.05 ± 0.16*
	0.05	365.0 ± 8.03*	59.15 ± 1.46*	425.4 ± 11.65*	24.49 ± 0.46 ^{ns}
	0.10	319.2 ± 10.83*	67.66 ± 2.60 ^{ns}	521.7 ± 7.53*	21.42 ± 0.13*

[a] LP refers to lipid productivity

* and ^{ns} denote to significant and insignificant differences, respectively, of each studied treatment compared to the control (at $P \leq 0.05$)

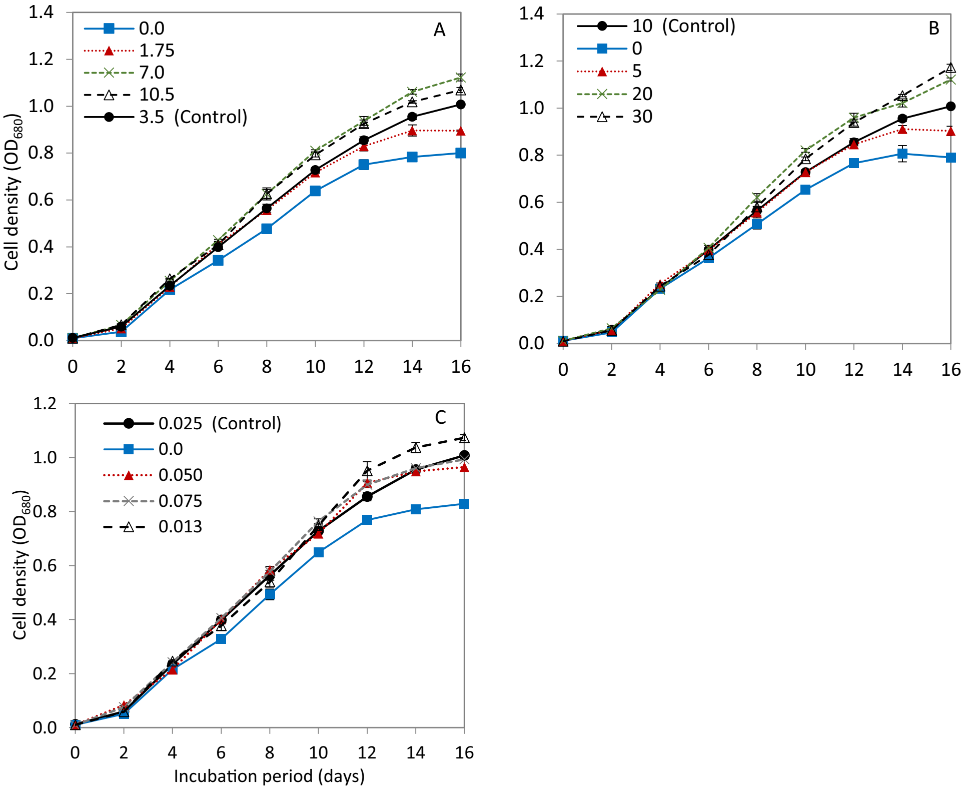

Fig. 6 Growth curve of *Tetraselmis elliptica* grown in SWES medium with different concentrations of trace metals including $FeSO_4•7H_2O$ (**A**), $MnSO_4•4H_2O$ (**B**), and $CuSO_4•5H_2O$ (**C**)

resulted in 11.1% reduction in lipid content compared to the control. Thus, the recorded higher lipid productivity is attributed mainly to enhancement of growth at this concentration. Putting altogether, the present study showed that 0.1 g L^{-1} KNO_3, 0.01 g L^{-1} glycerol, and 7.0 mg L^{-1} $FeSO_4•7H_2O$ significantly enhanced the lipid productivity of *T. elliptica*, and were used further to evaluate the impact of combination of these factors.

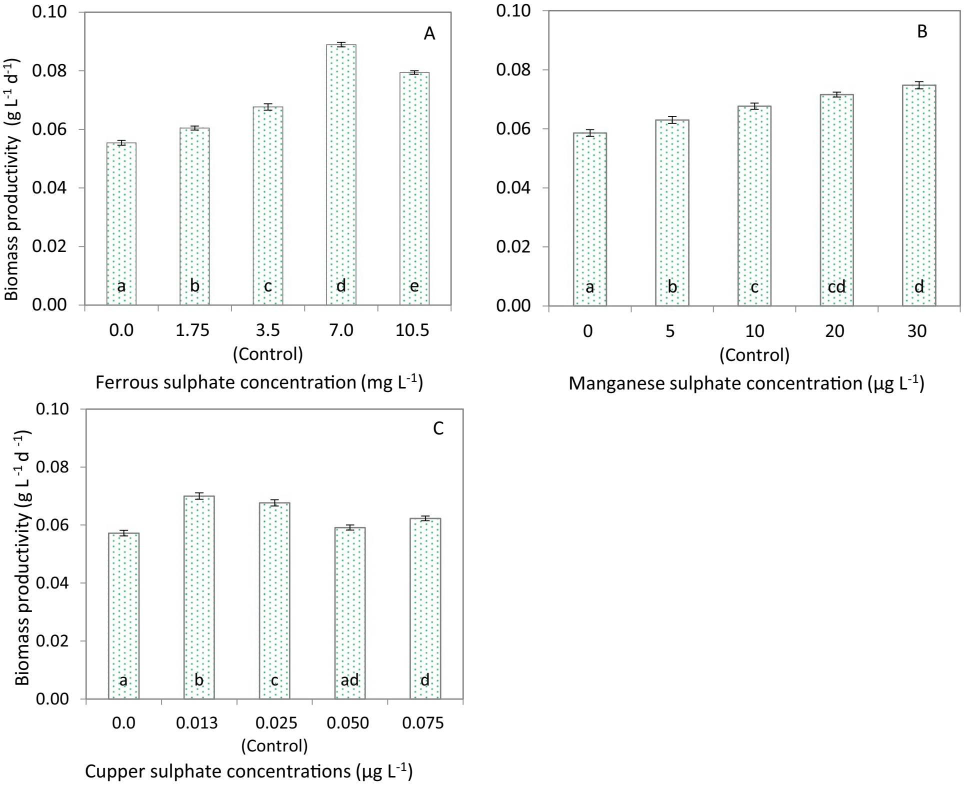

Fig. 7 Biomass productivity of *Tetraselmis elliptica* grown in SWES medium with different concentrations of trace metals including $FeSO_4•7H_2O$ (**A**), $MnSO_4•4H_2O$ (**B**), and $CuSO_4•5H_2O$ (**C**)

Table 5 Content of main macromolecules and lipid productivity of *Tetraselmis elliptica* grown in SWES medium with different concentrations of trace metals and measured at late exponential phase

Trace metals	Concentration	Macromolecules (mg g^{-1} dw)			LP (mg L^{-1} day^{-1})[b]
		Carbohydrates	Proteins	Lipid content	
Control[a]		300.5 ± 4.77	65.99 ± 0.91	340.1 ± 7.16	23.00 ± 0.13
$FeSO_4$•$7H_2O$ (mg L^{-1})	0.00	354.0 ± 5.61*	26.66 ± 0.66*	332.5 ± 3.88 ^{ns}	18.42 ± 0.18*
	1.75	310.4 ± 3.54 ^{ns}	38.63 ± 0.53*	342.9 ± 2.22 ^{ns}	20.72 ± 0.13*
	7.00	258.8 ± 2.36*	36.34 ± 0.36*	302.4 ± 3.37*	25.90 ± 0.21*
	10.5	276.5 ± 2.26*	32.56 ± 0.44*	325.3 ± 3.31*	25.03 ± 0.08*
$MnSO_4$•$4H_2O$ (μg L^{-1})	0	261.9 ± 5.14*	36.82 ± 0.67*	363.4 ± 5.73*	21.28 ± 0.17*
	5	318.8 ± 6.13*	44.25 ± 0.75*	339.6 ± 4.45 ^{ns}	21.39 ± 0.13*
	20	272.3 ± 3.12*	20.71 ± 0.15*	325.2 ± 2.21*	23.28 ± 0.14 ^{ns}
	30	287.5 ± 5.01*	44.01 ± 0.63*	317.3 ± 4.14*	23.72 ± 0.28 ^{ns}
$CuSO_4$•$5H_2O$ (μg L^{-1})	0.00	274.8 ± 4.89*	27.78 ± 0.60*	318.0 ± 6.74*	18.19 ± 0.08*
	0.013	309.8 ± 5.08 ^{ns}	36.05 ± 0.04*	321.6 ± 6.96*	22.50 ± 0.15 ^{ns}
	0.050	289.5 ± 4.25*	24.67 ± 0.39*	314.0 ± 5.95*	18.57 ± 0.07*
	0.075	290.4 ± 4.24*	36.79 ± 0.48*	350.6 ± 5.30 ^{ns}	21.84 ± 0.07*

[a] Control medium contains 3.50 mg L^{-1} $FeSO_4$•$7H_2O$, 10 μg L^{-1} $MnSO_4$•$4H_2O$, and 0.025 μg L^{-1} $CuSO_4$•$5H_2O$

[b] LP refers to lipid productivity

* and ^{ns} denote to significant and insignificant differences, respectively, of each studied treatment compared to the control (at $P \le 0.05$)

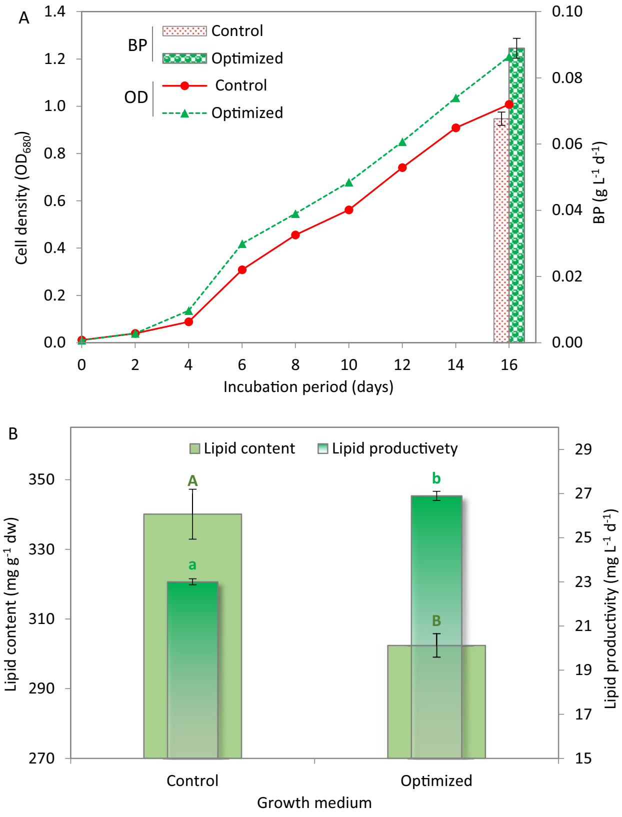

Fig. 8 Growth curve, biomass productivity (BP) and lipid production of *Tetraselmis elliptica* grown in typical SWES medium (control) and the optimized medium

3.5 Optimized medium and biodiesel characteristics

Cultivation of *T. elliptica* in the optimized medium showed enhancement of growth up to 16 days, which resulted in 30.9% increase in biomass productivity (Fig. 8A). Although lipid content of *T. elliptica* showed 11.1% reduction in the optimized medium, lipid productivity increased by 17% over the control due to enhancement of microalgal growth (Fig. 8B). As shown in Table 6, lipids of *T. elliptica* grown in the control medium showed 86.65% of saturated fatty acids (SFAs), with very low proportion of PUFAs (1.84%). Optimization of growth medium enhanced PUFAs proportion to 15.05%, with simultaneous reduction in SFAs to 59.44%. Increase in PUFAs is attributed mainly to de novo synthesis of arachidonic acid (ARA) and docosahexaenoic acid (DHA) which represented 4.17% and 10.88%, respectively. ARA and DHA are ω-6 and ω-3 fatty acids, respectively, with high nutritional values [60, 61]. Therefore, the present study provides a new insight for enhancement of value-added fatty acids from marine microalgae through growth medium optimization.

It is well known that biodiesel properties depend mainly on fatty acid profile of the used feedstock [62, 63]. Biodiesel characteristics showed compliance of all studied parameters with the US standards [64]. However, kinematic viscosity of biodiesel produced from the control medium did not comply with the European standards [65], as it showed high viscosity due to the recorded high saturation degree. Therefore, it showed higher cetane number,

Table 6 Fatty acid profile of *Tetraselmis elliptica* grown in typical SWES medium (control) and the optimized medium at late exponential phase

Fatty acids	Control	Optimized
Caproic (C6:0)	5.65	0.07
Caprylic (C8:0)	0.66	*nd*
Capric (C10:0)	1.86	1.04
Undecylic (C11:0)	5.03	0.35
Lauric (C12:0)	17.75	1.82
Tridecylic (C13:0)	15.56	7.58
Myristic (C14:0)	13.12	10.92
Pentadecylic (C15:0)	7.59	6.07
Palmitic (C16:0)	14.03	28.45
Margaric (C17:0)	0.74	*nd*
Stearic (C18:0)	4.66	2.43
Arachidic (C20:0)	*nd*	0.71
Myristoleic (C14:1)	6.48	10.48
Pentadecenoic (C15:1)	*nd*	7.33
Palmitoleic (C16:1)	0.73	3.51
Heptadecenoic (C17:1)	0.69	*nd*
Oleic (C18:1)	3.61	3.38
Paullinic (C20:1)	*nd*	0.81
Linoleic (C18:2)	1.84	*nd*
Arachidonic (C20:4)	*nd*	4.17
Docosahexaenoic (C22:6)	*nd*	10.88
SFAs	86.65	59.44
MUFAs	11.51	25.51
PUFAs	1.84	15.05

nd not detectable

as longer fatty acid carbon chains and higher saturation result in higher cetane number [66, 67]. It is one of the main factors influencing biodiesel quality, as it relates to fuel ignition delay time [68]. There is a wide variation in the acceptable cetane number based on the engine speed; where the lower the speed, the lower acceptable cetane number, which can reach as low as 20 [69]. In addition, a high positive correlation was reported between low NOx emissions and high cetane number, which is a desirable factor for clean fuel production [68]. Table 7 shows values of cetane number for the control and optimized algae (61.86 and 55.71, respectively) falling within the range acceptable by international standards (≥ 47). Considering cetane number of 49.6 for fossil diesel [70], the present study confirms the advantage of biodiesel produced from *T. elliptica* under both cultivation conditions for better ignition and lower pollutants. Optimization of growth conditions resulted in slight increase in CFPP from −4.75 °C to −1.49 °C. It is noteworthy to mention that acceptable CFPP value should be generally lower than the ambient temperatures; i.e., it is not a critical biodiesel quality parameter in the tropics as in Egypt. Energy density (i.e., HHV) of the biodiesel is an important property to replace petroleum diesel, which indicates the amount of heat released from combustion of a certain amount of fuel. Interestingly, optimization of growth medium enhanced the HHV by 4.2% over the control (Table 7). Overall, the present results confirmed the suitability of FAMEs produced from both cultures as biofuel, with the advantage of higher energy density and lower kinematic viscosity in case of the optimized culture.

4 Conclusions

The present study confirmed the potential of the hyperhalophilic microalga *T. elliptica* for ω-fatty acid accumulation by optimization of growth medium. In addition, the recorded high lipid productivity with improved fatty acid composition under optimized conditions supports the microalga as biodiesel feedstock. Results confirmed that reduction of KNO_3 to 0.1 g L^{-1}, supplementation of 0.01 g L^{-1} glycerol, and increasing of $FeSO_4$•$7H_2O$ to 7.0 mg L^{-1} in SWES medium significantly enhanced the lipid productivity of *T. elliptica* by 17% compared to the standard medium. Although the

Table 7 The main characteristics of biodiesel produced from *Tetraselmis elliptica* grown in typical SWES medium (control) and the optimized medium in comparison with the international standards

Characteristics	Control	Optimized	Standards	
			US [64]	Europe [65]
Unsaturation degree	0.15	1.08	–	–
Kinematic viscosity (mm^2 s^{-1})	5.11	4.53	1.9–6.0	3.5–5.0
Specific gravity (kg^{-1})	0.87	0.88	0.85–0.9	–
Cloud point (°C)	17.96	5.64	–	–
Cetane number	61.86	55.71	Min. 47	Min. 51
Iodine value (g I_2/100 g oil)	24.01	92.66	–	Max. 120
HHV (MJ kg^{-1})	38.80	40.43	–	–
Long-chain saturated factor (LCSF)	3.733	4.770	–	–
Cold filter plugging point (CFPP, °C)	−4.75	−1.49	Location and season dependent	

present study focused on lipids as a potential feedstock for biodiesel, the relatively high carbohydrate content in the residual biomass could be used for bioethanol production through sequential route of energy recovery. Hence, this research provides new insights for commercial utilization of halophilic oleaginous microalgae for triple purpose of seawater desalination, value-added compound production, and enhanced energy recovery through a biorefinery approach.

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Author contribution R. S. Marey: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft. A. M. Abo-Shady: conceptualization, methodology, writing—review and editing, funding acquisition. H. M. Khairy: formal analysis, writing – review and editing. A. M. Abd El-Moneim: formal analysis, methodology. Abdelfatah Abomohra: conceptualization, data validation, writing—review and editing, funding acquisition.

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Declarations

Ethical approval Not applicable.

Conflict of interest The authors declare no competing interests.

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