The Effect of Linoleic Acid and Nanoparticle Treatments as Inducers on Biomass and Fatty Acid Content in the Microalga, *Haematococcus lacustris*

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Abstract

Increased biomass density of microalgae is a critical factor in the enhancement of the algal metabolites. In this study, the effects of linoleic acid, TiO_2, and SiO_2 nanoparticles were investigated as elicitors on the production of biomass, and fatty acids in the microalga, *Haematococcus lacustris*. Several treatments of TiO_2 and SiO_2 nanoparticles were analyzed as well as linoleic acid on *Haematococcus lacustris* in two separate designs. Microalgal biomass in nanoparticles was investigated using the Neobar chamber and in linoleic acid using the freeze-dryer methods. Fatty acids compositions were tested by gas chromatography method and five of them named Palmitic, Palmitoleic, Stearic, Oleic, and Linoleic acids (LA) were measured. The results showed that the biomass significantly increased by LA (30 µM) and TiO_2NPs (40 mg/L) treatments, and consequently, these treatments increased the biomass density by 2 and 1.3 times more than the control treatment, respectively. Palmitic and linoleic acids were the most frequent fatty acids produced by 60 and 30µM of LA treatments with 1.4 (53.26 % w/w) and 1.5 (32.51 % w/w) folds, respectively. To conclude, the different concentrations of LA and TiO_2NP boosted the production of algal biomass, and some fatty acids in *Haematococcus lacustris*. Moreover, LA may be used as an effective inducer to increase biomass production in the valuable microalga *Haematococcus lacustris*.

Keywords: *Haematococcus lacustris*, Oxylipin, Salicylic acid, TiO_2NP, SiO_2NP

Introduction

In recent decades, with the increasing population and shortages of food, fuel, and by-products, researchers are making greater efforts to find new alternative sources such as high-potential microalgae for the production of the considerable amounts of proteins, lipids, vitamins, and by-products (Khalili et al., 2019a). To increase the production of this useful substance, it is necessary to increase the production of microalgal cells (biomass). *H. lacustris*, as a high-potential natural producer, is a unicellular microalga living in temperate freshwaters (Khalili et al., 2020).

Scientists have tried to increase biomass production in microalgae via several methods. Using environmental conditions

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and different materials as stresses and inducers are two common strategies, respectively (Hu et al., 2020). Most researchers have shown that some environmental stresses such as high light, salt increase, nitrogen deficiency, and some inducers such as linoleic acid (Khalili et al., 2020), methyl jasmonate (MJ), salicylic acid (SA), and nanoparticles (NPs) affect the production of biomass in microalgae (Hong, 2016). Similar experiments showed that the addition of SA and MJ (500 µM) as inducers in H. lacustris medium culture reduced cell growth (biomass) and carotenoid production (Raman and Ravi, 2011). Linoleic acid is a bio acid (fatty acid), a small molecule in the first stage of oxylipin and arachidonic acid (ARA, C20: 4Δ5), (Khozin-Goldberg et al., 2011) pathways in the plants and microalgae, respectively (Shanab et al., 2018). Two main pathways containing two sidetracks each are derived from Linoleic acid named 13-LOX (lipoxygenase), 9-LOX in plant and macroalgae, as well as ω-6 and ω-3 in microalgae (Shanab et al., 2018).

MJ is produced by some stresses and inducers such as salt, light, wound, LA, and nanoparticles. LA is a precursor of Jasmonic acid (JA) and traumatic acid (TA) phytohormones (Khalili et al., 2019a, Pathak et al., 2018), it is also an allelochemical and a part of its behaviors such as growth inhibition in microalgae is related to this feature. For example, in a resurch which was condected on 32 µM LA increased biomass in *C. sorokiniana* and they displayed that LA disrupted cell membranes and changed cellular functions in *Auxenochlorella pyrenoidosa* (Qian et al., 2018), also inhibited the photosynthetic efficiency in *Chlorella vulgaris* (Qian et al., 2009).

Another inducer of biomass production in microalgae is the nanoparticle. Nanoparticles and other inducers using reactive oxygen species (ROS) stimulate cellular defense mechanisms in the microalgae. ROS in combination with cellular compounds such as DNA, enzymes, and lipids causes algal cell damage or death (Chekanov et al., 2019), thus, the cellular defense mechanism is activated for ROS naturalization.

Accordingly, astaxanthin and other metabolites are produced as the main or bypass products of the defense mechanism for ROS naturalization in microalgae, macroalgae, and plants (Barati et al., 2019; Howe and Schilmiller, 2002). In addition, nanoparticles (NPs) have recently been used by scientists as inducers to produce biomass in microalgae. For instance, the addition of Cadmium (Cd) NP decreased the cell growth rate in *H. lacustris.* Also, Ag, Cd, Fe, and Zn NPs decreased biomass and increased astaxanthin contents by high concentrations in different microalgae. In general, nanoparticles in low concentrations have a positive effect on biomass production (Hong, 2016).

It is important to investigate the type and concentrations of fatty acids in microalgal cells due to their nutritional and fuel value, and find a way to increase them through different treatments. Fatty acids in the endoplasmic reticulum (ER) of microalgae esterify natural astaxanthin (Hempel et al., 2012). It was found by a test that fatty acid accounts for 33% of *H. lacustris*, while its main part was composed of polyunsaturated

fatty acids (PUFAs) that are very helpful for humans, fatty acids have important roles in microalgae cells, for example, the permeability of cell walls and environmental relationships. (Tan et al., 2020) In a study, linoleic, palmitic, and oleic acids were identified as the highest fatty acids among 38 screened-microalga strains in *H. lacustris* respectively (Kabir et al., 2020). Based on the results, oleic, palmitic, and linoleic acids were the most abundant fatty acids that esterify astaxanthin in *Haematococcus pluvialis*. (Zhekisheva et al., 2002). It found that in the microalga, *Bracteacoccus aggregatus* BM5/15 palmitic (C16:0), oleic (C18:1Δ9), and linoleic (C18:2Δ9, 12) acids were the most, respectively (Chekanov et al., 2021).

Materials and methods

The total salts and chemicals required for microalgae cultivation in this experiment were obtained from Merck (Germany) or Sigma (USA) in an analytical grade. Silicon dioxide Nanoparticles (SiO_2NPs) were amorphous and powdery with a size of 10-15 nm and purity of 99.999%, which were purchased from TECNAN Inc (Tecnología Navarra de Nanoproductos S.L., Navarra Spain). Titanium dioxide Nanoparticles (TiO_2NPs) had ~25 nm size, 55-m^2g^{-1} external surface, a combination of anatase and rutile with more ratio of anatase (89.2%), purity of 99.9%, and provided from Degussa Inc (Frankfurt, Germany).

Microalgae culture condition

The unicellular freshwater microalga, *Haematococcus lacostris* CCAP34/7, was obtained from the Microalgae Laboratory of Alzahra University, Tehran, Iran. It was kept at 25 °C until use. In the cultural stage, *H. lacustris* cells with a concentration of 20×10^4 cells ml^{-1} were incubated in 250 ml of Erlenmeyer flasks containing 150 ml Bold's Basal Medium (BBM) culture (Sorokina et al., 2020). Then, the cultures was exposed to 139.35 foot-candle fluorescent light in a growth chamber at 25 ± 2 °C at PH of 6.5 -7 without aeration (shaking), and kept for 12:12 h light-dark cycles. Furthermore, Samples were exposed to 325.16 foot-candle fluorescent light from the seventh until the end of the experiment (19^{th} day). NaOH regulated the pH of the medium culture before autoclaving (Khalili et al., 2019b).

Linoleic acid and Nanoparticles treatments

The treatments were selected at three concentrations of linoleic acid (0, 30, and 60 µM) and two concentrations of TiO_2 and SiO_2 NPs (0 and 40 mg/L) each with three repetitions, which were added to the BBM culture in two separate tests in three days after inoculation. The linoleic acid was added to BBM culture by Tween 20 and NaOH method (Grosch and Schwarz, 1971). TiO_2 and SiO_2NPs stocks were freshly prepared in deionized water, and then autoclaved) It was performed at 120° C for 15 to 20 minutes (and placed in a water bath sonicator at the frequency of 50 Hertz for 15 min. They were kept at 4° C until use (Kahila et al., 2018).

Growth and biomass measurement

The number of cells per day in the experimental section of the effect of nanoparticles on the characteristics of microalgae (NPs TiO_2 and SiO_2) was counted using a hemocytometer and Neobar lam from the first to the 19^{th} day due to the

escape of light absorption and weight gain of NPs. According to the specific method (Ahmed et al., 2015) biomass was dried by the Freeze Dryer (model: FDB-5503 Model, company: Operon, Seoul, Korea) and then measured by a digital scale with an accuracy of 10^{-3} g in LA treatment test.

Fatty acids Measurement

To extract and measure fatty acids at ten to twelve days after inoculation, 0.3 g of each algal sample (measured with an accuracy of 0.0001 g) was transferred to 10 ml tubes. 3 ml of methanol and acetyl chloride solution (20 by 1 volume/volume) was added to each. The tubes were placed in Ben Marie at 85° C for 50 minutes. During this time, the tubes were shaken and after cooling, 1 ml of distilled water and 3 ml of hexane were added, the mixture was centrifuged in tubes (4000 rpm for 15 minutes). Then, the upper phase (hexane with fatty acids) was separated. Identification of fatty acids using Gas chromatography was performed with the following conditions. (Nazeri et al., 2017).

The carrier gas was Argon, column RTX-2230 RESTK, Pennsylvania, USA, Inlet: Heater of 240° C, Pressure of 27.939 psi, Septum purge Flow of 2 ml/m, Split of 1:20, Detector: Heater of 240° C, H_2 Flow of 27 ml/ m, Air Flow of 270 ml/m, and N_2 Flow of 27 ml/ m.

Statistics and data analysis

All data were analyzed in a Randomized Complete Design (RCD). Mean± SD of three replications for Nanoparticles and LA treatments, compared with the post-hoc Tukey test. The one-way *analysis* of variance *(*ANOVA*)* was used to determine any significant differences using SPSS software (v16, USA). The statistical probability level less than 5% ($p< 5\%$) was statistically significant (without LA and NPs).

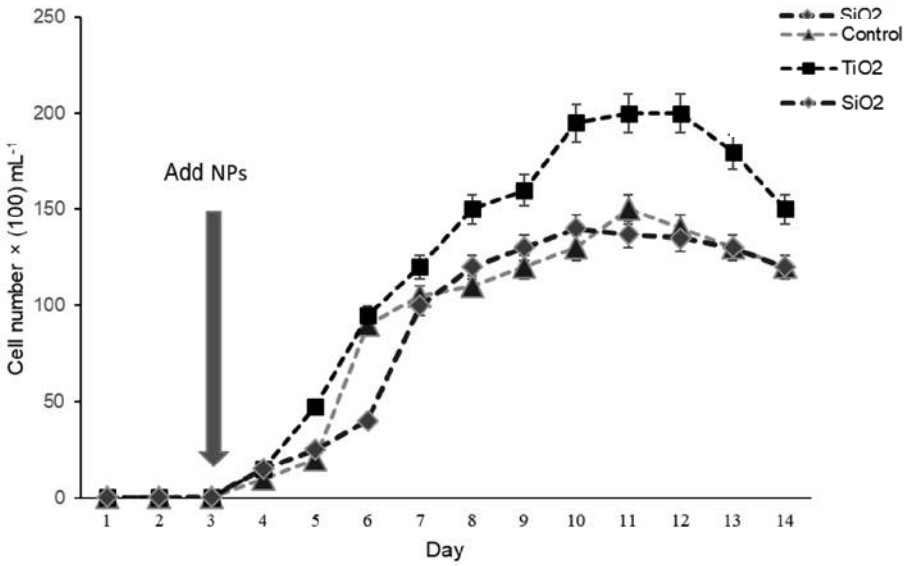

Fig. 1. Effect of nanoparticles on the vegetative growth of *Haematococcus lacustris*. Vertical red arrow: Nanoparticle addition. Average values ± standard deviation are shown (n = 3)

Results

Effect of TiO_2 and SiO_2NPs on biomass accumulation

The results showed that the cellular growth under control and two nanoparticles treatments (TiO_2 and SiO_2) increased rapidly after three days of inoculation, but TiO_2NP (40 mg/L) had a higher effect on the cellular growth on the 11th day and an average of 1.3 (20000 cells/ml) and 1.3 times that of the control, respectively ($p < 0.05$) (Fig. 1).

SiO_2NP (40 mg/L) showed a slightly less cell growth rate than that of the control and, in turn, biomass production for the 11th day and the entire growth cycle with 13700 and ~6400 (cells/ml) were 0.91 and 0.86 times that of the control, respectively. TiO_2NP (40 mg/L) effects on SiO_2NP (40 mg/L) on average and the 11th day (maximum point) were 1.56 and ~1.46 times, respectively.

Biomass production under NPs treatments showed an overall superiority of TiO_2 compared to the control, but SiO_2NPs were less than TiO_2NPs, generally. These results showed that SiO_2NP at a concentration of 40 mg/L could not stimulate the growth of microalgae cells and may damage the cells. Briefly, the order of biomass accumulation was as follows: TiO_2NP (40 mg/L)> control> SiO_2NP (40 mg/L) (Table 1).

Linoleic acid effects on biomass accumulation

Biomass measurement showed that 30 µM LA had the highest effect on the biomass increase on average and twelfth day (maximum biomass) by 2 and 1.8 times that of the control (1.4 and 2.2 mg/ml), respectively. There is a slight difference in the biomass production of 60 µM LA concentration with the control treatment on the 4th and 11th days and the mean, which is 0.2, 1.3 and 0.7 mg/ml biomass production respectively (Figure 2). 30 µM of LA, significantly outperformed the control and 60 µM of LA treatments in biomass production. The effect of 30 µM LA on biomass accumulation was 1.9 times more than that of 60 µM LA concentration, on average (average of 19 days). The order of biomass accumulation was briefly as follows: 30 µM LA>60 µM LA> control (Table 1).

Fatty acid composition

The results of HPLC for astaxanthin measurements agreed well with the spectrophotometry method. Gas chromatography results showed that the contents of palmitic and oleic acids in 60 µM LA and control treatments were the highest and the least fatty acids with 53 and 2.3% w/w, i.e., 1.4 and 0.84 times that of the control, respectively. Also, after palmitic acid, oleic acid in control, oleic acid in SiO_2NPs (40 mg/L), and Palmitoleic acid in TiO_2NPs (40 mg/L) treatments were the highest fatty acids by 1.56, 0.49, and 3.33 times compared to the controls respectively, (Fig. 3). The quantity of palmitic (53.3% w/w)> linoleic (32.5% w/w)> oleic (17.7% w/w)> Palmitoleic (9.7% w/w)> stearic (2.3%w/w) acids had the highest concentrations in one treatment sequentially, also on average, palmitic (44.8% w/w)> linoleic (29.7% w/w)> oleic (15.4%, w/w)> stearic (5.5% w/w)> Palmitoleic (4.7%w/w) were the uppermost fatty acids. The concentration order of palmitic acid in treatments was 60µM LA (53.3% w/w)> 30 µM LA (47.8 % w/w)> SiO_2NP (42.8% w/w)> TiO_2NP

Table 1. The effects of LA and NP treatments on Biomass and Fatty acids in *H. lacustris*

Treatments / Traits	Control	30 µM LA	60 µM LA	TiO_2NPs (40 mg/L)	SiO_2 NPs (40 mg/L)
*Biomass (Cell number×100)/ ml, the average of 19 days	7398.7			9966.4	6383.6
*Biomass mg/ml, the average of 19 days	0.68	1.4	0 .7		
Biomass to the control		2	1.02	1.3	0.86
	Max	Palmitic	Palmitic	Palmitic	Palmitic
	Min	Stearic acid	Stearic acid	Palmitoleic acid	Stearic acid

(35.3% w/w). In addition, for linoleic acid, it was 30 µM LA (32.5 %w/w)> TiO_2NP (30.4 %w/w)> SiO_2NP (28.7 %w/w)> 60µM LA (~27.3%w/w).

These results revealed that the maximum production of two main fatty acids, palmitic and linoleic acids, occurred at two LA treatments (60 and 30µM) while the ultimate production of three other fatty acids, namely Oleic, Stearic, and Palmitoleic was achieved

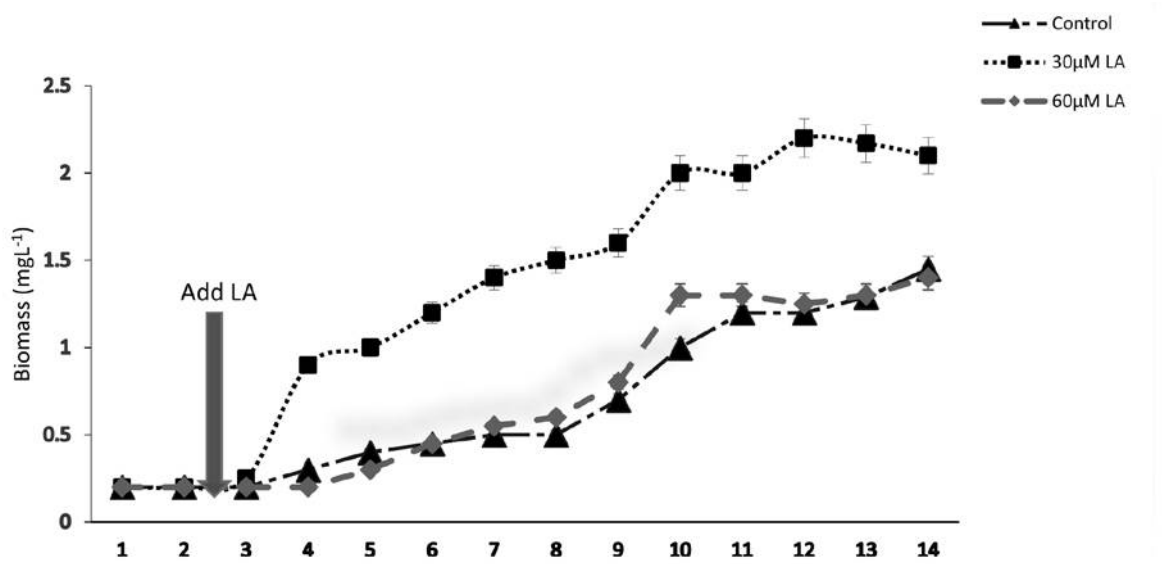

Fig. 2. Effects of linoleic acid on vegetative growth of *Haematococcus lacustris*. Vertical red arrow: Linoleic acid addition, Average values ± standard deviation are shown (n = 3)

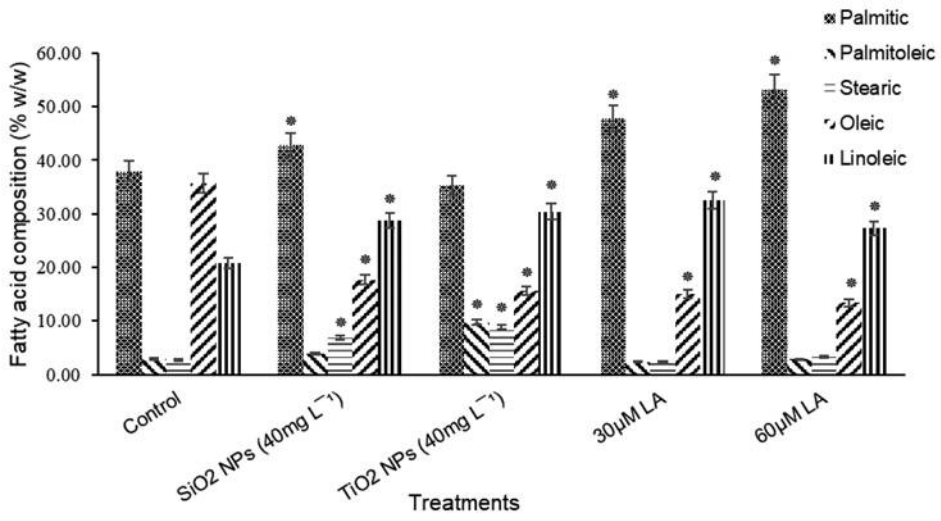

Fig. 3. The results of fatty acid analysis by gas chromatography (*) on the error bars shows the significant difference with control ($p< 0.05$, Duncan's test). Median values± standard deviation (SD) are shown (n = 3).

in NPs (TiO_2 and SiO_2) treatments. In addition, there was a direct relationship between linoleic acid (fatty acid) production, and biomass, as all two were maximized by LA treatment of 30 µM (Table 1).

Discussion

Due to the importance and value of microalgae biomass, extensive research is being done to increase its production from inexpensive materials and methods. The effect of linoleic acid on Biomass production is related to its precursor of two Phytohormones (Khalili et al., 2019a). The starter of oxylipin and arachidonic acid pathways (Shanab et al., 2018), the acidic nature of this substance (Wu et al., 2006), as well as allelochemical properties (Qian et al., 2018), and the effects of Nanoparticles to the microalga are related to their ability to create ROS. TiO_2NPs are more motivating than SiO_2NPs in the biomass production of microalgae (Manzo et al., 2015).

Effect of TiO_2 and SiO_2NPs on biomass accumulation

The produced biomass under the influence of TiO_2 NP (40 mg/L) in the stationary phase was higher than the other two treatments (SiO_2NP and control). Usually, different NPs limit the growth of microalgae and have toxic effects on cells, but some of them have various effects on cell growth at low concentrations (Adams et al., 2006). In general, TiO_2NP has a greater effect than SiO_2NP on the growth and division of microalgal cells. In this regard, some researchers reported that TiO_2NP neutralizes the toxic effect of Cadmium (Cd) on microalga *Chlamydomonas reinhardtii* at lower concentrations than SiO_2Np (A quarter of the concentration) (Yu et al., 2018). The effect of TiO_2NP on *Dunaliella tertiolecta* microalga is much greater and faster than that of SiO_2NP due to the higher accumulation of TiO_2NP in algal medium culture (Manzo et al., 2015).

that of 60µM LA and their difference was significant. When linoleic acid was added to the medium culture from the third day onwards, a significant increase in biomass production was observed in 30µM LA treatment compared to the other two treatments (Fig. 2). It shows the positive effect of the level of this hormone on the growth of microalgae cells, which is consistent with the results of some researchers (Khalili et al., 2019a). For example, 32 µM LA increased biomass in *C. sorokiniana* the reduction of biomass at LA concentrations higher than 30µM is under debate, where some researchers believe that LA is an allelochemical substance with an allelopathy effect on microalgae. According to some other reports, the cellular growth of *C. sorokiniana* is decreased at high concentrations of LA (100 µM) (Qian et al. 2018) . Some materials and phytohormones, such as IAA, IBA, NAA, and PAA increased and decreased cell growth rate in *Chlorella vulgaris* at low and high concentrations, respectively (Piotrowska-Niczyporuk and Bajguz, 2014).

In a study, the effect of 60µM LA on biomass accumulation was approximately equal to that of the control and their difference was insignificant (Khalili et al., 2019). According to previous study by Khalili et al. (2019), the effect of LA on biomass production was higher than this concentration (> 60 µM) that was gradually decreased or become negative. However, this concentration is different for each species in microalgae (specific species). Interestingly, LA is the precursor to MJ and traumatic acid (TA), both of which act similarly to LA (de Los Reyes

There are three hypotheses for this result. Maybe TiO_2NP kills more bacteria and other prokaryotes, so that microalga cells can grow well in the medium culture without any nutritional competition (poor possibility). TiO_2NP is activated by the UV spectrum so that it produces ROS and consequently, the ROS breaks down medium culture components. TiO_2NP covers the whole surface of the microalgae cell and increases optical absorption which enhances chlorophyll content and biomass (Kulacki and Cardinale, 2012; Vargas-Estrada et al., 2020).

Indeed, the accumulation of TiO_2NPs in the medium culture is higher than SiO_2NPs So TiO_2NPs can penetrate cellular organ and genomes more and faster than SiO_2Nps (Manzo et al., 2015).

In addition, SiO_2NP (40 mg/L) had a slightly greater (non-significant) inhibitory effect on cell growth (biomass) compared to the control group (Pikula et al., 2020. Therefore, SiO_2NP in comparison with TiO_2NP and control treatments at this concentration (40 mg/L) could not stimulate cell growth but reduced it, but why cannot SiO_2NPs stimulate biomass production? It seems that aggregation of SiO_2NPs (40 mg l^{-1}) in medium culture is not enough to penetrate (Manzo et al., 2015) in the genome and affect it but can only damage cellular walls and kill or weak microalgal cell so biomass production becomes less than control treatment in this concentration.

Linoleic acid effects on biomass accumulation

The general effect of 30µM LA on biomass production was approximately two times

et al., 2014). For example, it was shown that 1 µM methyl jasmonate increased the cell number in *Chlorella vulgaris*, while further concentrations diminished it, this result was obtained for TA as well (Pietryczuk et al., 2014), and our results are consistent with mentioned results. Finally, LA is an organic acid and in high concentrations inhibits the cellular growth of microorganisms such as microalgae, but this prevention is different for each microorganism (Wu et al., 2006).

Fatty acid composition

In general, Palmitic> Linoleic> Oleic> Stearic> Palmitoleic were the most abundant fatty acids in microalgae, respectively. The astaxanthin molecule in the microalgal cell is covered with different fatty acids, due to its greater stability, the percentage of which varies from one microalga to another and even at different stages of cell growth. The results of some experiments indicate that fatty acids esterify 90% of astaxanthin molecules in *H. lacustris*, whereas the highest fatty acid that covers and attaches to the astaxanthin molecules is oleic acid (Holtin et al. 2009), which does not confirm our results. In some investigations linoleic and oleic acids (Doan et al., 2019) while in others palmitic acid were the predominant fatty acids in the *H. lacustris* (Boonnoun et al., 2014), which almost confirm our results. Here the question arises, why does the type of fatty acids change? Why are palmitic and linoleic acids the major primary fatty acids in microalgae cells? It can be concluded that linoleic acid is the major fatty acid in both pathways GƆ-3 and GƆ-6 (defense pathways of microalgae), but it is the least fatty acid in the cell walls of microalgae. In contrast, palmitic (structural and saturated fatty acid) is the major fatty acid in the cell walls or cell organelle walls (Sharathchandra and Rajashekhar, 2011; Tan et al., 2020). LA is not produced when cells live in the green motile stage (the first stage of the life cycle of *H. lacustris*), while palmitic acid is the first fatty acid in this stage. However, when the microalga enters later stages of growth, such as the late red stage and aplanospore (haematocyst), linoleic acid and its derivatives (α-linolenic, oleic) are produced and become the first fatty acid in them (Butler et al., 2018), which confirms our findings. In our study, palmitic acid was the predominant fatty acid in all treatments due to the GC assay time (10 to 12 days after inoculation), which was at the end of the logarithmic phase which time that the cells were in the transition from green to red. Most likely, if sampling were done in the last days of the experiment, for example, on the seventeenth to nineteenth days, linoleic acid and its derivatives would be more than palmitic acid. Interestingly, linoleic acid in 30µM LA and TiO_2NP (40 mg/L) treatments was the most produced fatty acid after palmitic acid, while these treatments produced the highest amount of biomass, which means 30 µM LA and TiO_2NP (40 mg/L) were directly related to the accumulation of LA (fatty acid), and biomass in *H. lacustris*.

Briefly, the effects of linoleic acid and nanoparticles (TiO_2 and SiO_2NP_s) on the biomass content and fatty acid composition in *H.lacustris* were investigated. To our knowledge, this is the first time that the effects of LA and NPs (TiO_2 and SiO_2) have been investigated simultaneously

on biomass, and fatty acid in *H. lacustris*. In biomass production, the treatments of 30 μM LA and TiO_2NP (40 mg/L) had the highest production, respectively. GC study showed that palmitic and stearic in 60 and 30 μM LA was maximum and minimum produced fatty acids, respectively. There was a direct relationship between 30 μM LA and biomass production.

It was shown that LA could stimulate biomass production in *H. lacustris*. Since this type of microalgae, *H. lacustris*, which is closely similar to *Haematococcus pluvialis*, has received less attention from researchers around the world. This study was a step towards demonstrating the importance and economic potential of *H. lacustris* in the production of fatty acids. We feel the importance of this work would substantially increase with the performance of additional experiments, such as identifying the mechanism on pathways that nanoparticles induced in *Haematococcus lacustris*. Because of microalgae biomass and fatty acid importance, we hope that in the future, more research about the effects of elicitors and stressors on biomass and fatty acid production take place to produce easier and faster than now in different microalgae.

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