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Original Article

The growth and biochemical composition of *Nannochloropsis oculata* under influence of different phosphorus sources for aquaculture live food

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ABSTRACT: Phosphorus is one of the most important nutritional factors that regulates cell growth and plays a significant role in most cellular processes. The effect of different phosphorus sources on the growth and biochemical composition of *Nannochloropsis oculata* (*N. oculata*) was investigated in this study. Four treatments: mono potassium phosphate (KH_2PO4), potassium di phosphate (K_2HPO_4), calcium super phosphate ($CaH_6O_9P_2$), and media without any phosphorus sources were all compared to F/2 media, which has sodium di hydrogen phosphate (NaH_2PO_4) as a control at a concentration of 36 µmol -1. The experiment lasted for 15 days under the conditions of 100 µmol photons per m2s of light intensity, with a light-to-dark (L/D) ratio of 24:0, a salinity of 25 g/L, temperature maintained at 23± 1 °C, and a pH value of pH 7.5 ±0.3. The growth of N. oculata based on cell density was inhibited in the medium without any phosphorus sources (5.3×106 cell/ mL) and increased significantly in the medium with NaH_2PO_4 (16.4×106 cell/ mL). All phosphorus sources showed that there were significant differences ($P \leq 0.05$) between all treatment media in chlorophyll content; KH_2PO_4 achieved the highest significant chlorophyll a content (Chl a) with 4.25 mg/l, followed by K_2HPO_4 4.12 mg/l. Cells treated with NaH_2PO_4 produced the highest total protein and lipid content (31.14% and 46.63% by dry weight, respectively). Media without phosphorus had the highest total carbohydrate (22.48%), while the lowest total lipids and total carbohydrate achieved by KH_2PO_4 and $CaH_6O_9P_2$ had the lowest total protein (25.90%). Arachidonic acid (C20:4 ω6) recorded its highest value (0.83%) with K_2HPO_4. While γ-Linolenic acid (C18:3 ω6) recorded its highest value (1.43%) with NaH2PO4. Whereas eicosapentaenoic acid (C20:5 ω3) was achieved the highest level in NaH_2PO_4 (9.53%) and $CaH_6O_9P_2$ (0.37%). In conclusion, this study demonstrated the variation of growth, fatty acid profile, and biochemical composition in *N. oculata*, benefiting the production of microalgae for aquaculture live food.

Key word: Phosphorus sources, Cell Density, Biochemical Composition, Fatty acids Profile.
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1. INTRODUCTION

Aquaculture is the fastest-developing sector of global food production, accounting for more than half of the worldwide fish production (Galappaththi *et al.*, 2020; Chan *et al.*, 2021).

Egypt has the largest aquaculture production in Africa, with a total production of around 1.8 million tons annually, which is concentrated in the production of freshwater fish (Kaleem and Sabi, 2021).

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Recently, Egypt has faced the problem of freshwater scarcity which led to the necessity of mariculture (Shaalan *et al.,* 2018). In this respect, the Egyptian Government has recently established some mega mariculture projects, such as the Gholion project in Kafer ElShiekh Governorate (2800 acres), Fayrouz project in Port Said at Shark El Tafriaa (15000 acres), the Diba triangle in Damietta Governorate (103 acres), the Suez Canal Authority project in the eastern side of the Suez Canal, and Gargob in Matroh Governorate (7000 acres), that is mainly focusing on sea bream, sea bass, and shrimp cultures (Feidi, 2018). These projects necessitate the production of huge amounts of high-quality, healthy fish larvae by marine hatcheries. However, these marine hatcheries are facing a multitude of critical challenges, of which the cost and availability of live food are at the top to secure the required fish larvae feeding. Microalgae are widely used in marine hatcheries as live food for zooplankton, bivalves, crustaceans, and the early stages of fish larvae (Villar-Navarro *et al.,* 2021). Phosphorus is a vital component of algal growth, lipid biosynthesis, fatty acid production, and biological pathways like energy transmission, cell signaling, and photosynthesis (Yang *et al.,* 2018).

Phosphorous is an important nutrient that accounts for less than 1% of total algal biomass and is required in the medium at a concentration of 0.03–0.06% to keep algae functioning (Ota *et al.,* 2016). Phosphorous is crucial for the creation of cellular components such as phospholipids, DNA, RNA, and ATP as well as metabolic processes involving energy transfer and nucleic acid synthesis in microalgal cells (Atiku *et al.,* 2016).

The genus *Nannochloropsis* is considered the leading algae in marine hatcheries with significant importance and also plays a key role in aquaculture development (Kaparapu, 2018). Some of *Nannochloropsis* species are utilized utlized in marine hatcheries for rotifer feeding and as a source of eicosapentaenoic acid (EPA) (Ferreira *et al.,* 2009). This experiment was designed to investigate the impacts of different phosphorus sources on the growth performance of *Nannochloropsis oculata* and determine the biochemical composition and fatty acids profile to enhance the culturing of *N.oculata* and produce highly nutritional algae with high content of enriched fatty acids to use as a live food for marine hatcheries.

2. MATERIALS AND METHODS

2.1. Inoculation and seawater preparation

The *Nannochloropsis oculata* inoculum originally obtained from SEAFDEC, Iloilo, Philippines, was maintained under controlled conditions at the algae culture lab. and cultured in seawater collected from El Attaka Port, the Red Sea, Suez (29°54'07.6"N 32°28'00.4"E). The physicochemical characteristics of seawater are presented in Table 1. The seawater was filtered twice: first through a layer of three (5 microns) cotton cartridge bags and then by a filtration disc (0.2 microns) to obtain pathogen-free water. Filtered seawater was sterilized by chlorex 5% for 24 hours, and then eliminated chlorine through continuous aeration.

Table 1. The Physicochemical characteristics of seawater.

Parameter	Value
Salinity $g \cdot L^{-1}$	43±1
pH-	7.85±0.2
Dissolved oxygen (Do) $Mg \cdot L^{-1}$	5.06±0.5
Temperature °C	29.5±2
Ammonia (NH_4) μM^{-1}	10.18±1.1
Nitrite (NO_2) μM^{-1}	4.01±0.2
Nitrate (NO_3) μM^{-1}	8.53±0.6

2.2. Culturing procedures of *N.oculata*

Nannochloropsis oculata was cultivated in sterilized seawater supplemented with F/2 culture medium of Guillard (1975) containing (75 g L^{-1} $NaNO_3$, 5 $g \cdot L^{-1}$ $NaH_2PO_4.2H_2O$, 1 mL of stock trace metal solution (per 1 L; 3.15 g $FeCl_3.6H_2O$, 4.36 g $Na_2EDTA.\ 2H_2O$, 1mL; 9.8 $g \cdot L^{-1}$ $CuSO_4.5H_2O$, 1mL; 6.3 $g \cdot L^{-1}$ $Na_2MoO_4.2H_2O$, 1mL; 22.0 $g \cdot L^{-1}$ $ZnSO_4.7H_2O$, 1 mL; 10.0 $g \cdot L^{-1}$ $CoCl_2.6H_2O$ and 1 mL; 180.0 $g \cdot L^{-1}$ $MnCl_2.4H_2O$), vitamin B1 100 $\mu g \cdot L^{-1}$ and B12 1000 $\mu g \cdot L^{-1}$ in the form of (Tri-B ampoules). The culture was loaded in 20 litters Carboys bags (**Fig. 1**) maintained under controlled conditions of continuous illumination with 100 µmol photons m^{-2} s^{-1} of cool-white fluorescent, salinity (25 ± 2 $g \cdot L^{-1}$), and temperature (21±2°C) with continuous aeration.

Fig.1. Algal carboys bags under controlled conditions at the algae culture Lab.

2.3. Experimental design

The experiment was designed to investigate the effect of different phosphorus sources on *N. oculata* cell growth and biochemical composition by replacing sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$ at a concentration of 3.62 × 10^{-5} mmol/L) in F/2 medium with other sources of phosphorus such as potassium mono phosphate (KH_2PO_4), calcium super phosphate ($CaH_6O_9P_2$), potassium diphosphate (K_2HPO_4), and media without any phosphorus sources ($-PO_4$).
In the medium. The cultures were grown for 15 days. All of the experiments were carried out in triplicate.

2.4. The growth pattern of *N. oculata*

2.4.1. Cell density

Algal cell densities (CD) were determined first by hemocytometer, and then confirmed with UV-VIS spectrophotometer (model: T60 UV) by measuring the optical densities at 540 nm (Rocha *et al.*, 2003). The dry weight of the algal samples was determined according to Olguín *et al.* (2001) by filtration 10 ml of the algal sample through a dried pre-weighed Gelman whatman filter (47 mm and nominal pore size 0.45 µm) using Buchner setup connected to a vacuum pump.

2.4.2. Specific growth rate (μ) and doubling time (T_d)

The maximum specific growth rate (μ, division$\cdot day^{-1}$) and doubling time (T_d) were calculated according to the following equations recommended by Durmaz and Erbil (2017).

μ (division/day)$=\ln(X_2/X_1)/(t_2-t_1)$

t_d (doubling/day)$=\ln 2/\mu=(0.693)/\mu$

Where X_1=cell concentration at time t_1, and
X_2= cell concentration at time t_2.

2.4.3. Estimation of pigment content (chlorophyll a,b and carotenoids)

A known volume (5mL) of *N. oculata* culture was centrifuged at 6000 rpm for 10 min. The supernatant was decanted and the same volume of water- free methanol was added to the pellet. The sample was then incubated in a water bath at 55°C for 15min, and subsequently centrifuged. The absorbance of the extract was measured against a blank of free methanol at 650, 665, and 452nm. Chlorophyll a and carotenoids were estimated as µg ml-1 of culture suspension using the following equations (MacKinney, 1941):

The concentration of each pigment fraction (chlorophyll a, chlorophyll b and carotenoids) was determined as μg/ml using the following equations:
Chlorophyll a =10.3 E665 - 0.918 E650
Chlorophyll b =19.7 E650 – 4.87E665
Carotenoids = 4.2 E 452 – (0.0246 chl. a + 0.426 chl. b)

2.5. Biochemical composition analysis

2.5.1. Total protein and carbohydrate

Total protein and carbohydrates were extracted after pigment extraction following the method of Payne and Stewart (1988). Total soluble protein was quantitatively determined using the method described by Bradford (1976) which used bovine serum albumin as a standard protein. Carbohydrate content was estimated by the method of phenol-sulfuric acid as reported by Kochert (1973) using glucose as a standard reference.

2.5.2. Total lipid and Fatty acid methyl ester

Total lipid was extracted as described in the study of Ren *et al.* (2017). According to the modified method of Zahran and Tawfeuk (2019), fatty acids were determined by converting the oil to fatty acid methyl esters (FAME), adding 1.0 mL of hexane to 15 mg of oil, followed by 1.0 mL of sodium methoxide (0.4 mol). After 30 seconds of vortexing, the mixtures were allowed to settle for 15 minutes. The upper phase containing the FAMEs was collected and analyzed by gas chromatography (GC-FID, Perkin Elmer Auto System XL), equipped with a flame ionization detector (FID). ZB-Wax fused silica capillary column was employed as the packing column (60 m x 0.32 mm i.d). Helium was used as carrier gas at a flow rate of 1 mL.min-1. The injector and detector temperatures were set at 250°C and 250°C, respectively. Ultimately, the fatty acids were recorded as percentages of total fatty acids.

2.6. Statistical analysis

Version 22 of the Statistical Package for Social Science (SPSS) was used to analyze the resulting data (Allen et al., 2014). According to Duncan (1955), ANOVA differences between means were assigned as significant at $P \leq 0.05$ via the least significant difference (LSD) for the multiple ranges of post hoc comparisons to determine the differences among the means of replication. The results were presented as mean ± standard deviation (SD).

3. RESULTS

3.1. Effect of different phosphorus sources on growth parameters of *N.oculata*:

3.1.1. Cell density

The cell density of *N. oculata* was increased by different phosphorus sources after the first cultivation day. The cell density of cultures on the first day was 1×10^6 cell/ml in the different media. The medium supplemented with NaH_2PO_4, KH_2PO_4, K_2HPO_4, $CaH_6O_9P_2$, and -PO_4 showed significant difference ($P \leq 0.05$) on the first day after cultivation achieved 1.37×10^6 cell/ ml, 2.92×10^6 cell/ ml, 2.15×10^6 cell/ ml, 3.90×10^6 ml, and, 3.23×10^6 cell/ml, respectively (**Table 2)**. Cell density with NaH_2PO_4 increased significantly ($P \leq 0.05$) and reached the maximum cell density of 18.75×10^6 cell/ ml after 8 days. On the last day of the experiment K_2HPO_4 showed the highest cell density of 16.43×10^6 cell/ ml, followed by $CaH_6O_9P_2$ 15.32×10^6 cell/ ml and NaH_2PO_4 at 14.75×10^6 cell/ ml which significantly differed ($p \leq 0.05$).

Table 2: Effect of different phosphorus sources on cell density ($\times10^6$) of *N. oculata* for 15 days incubation.

Days	NaH_2PO_4	KH_2PO_44	K_2HPO_4	$CaH_6O_9P_2$	(-)PO_4
0	1.00	1.00	1.00	1.00	1.00
1	1.37±0.06^{e}	2.92±0.10^{c}	2.15±0.13^{d}	3.90±0.13^{a}	3.23±0.08^{b}
3	3.23±0.15^{c}	3.72±0.13^{b}	3.22±0.08^{c}	4.48±0.03^{a}	3.25±0.05^{c}
5	6.61±0.03^{a}	4.42±0.10^{e}	6.28±0.08^{ab}	5.08±0.24^{d}	5.78±0.08^{c}
7	9.90±0.16^{b}	11.65±0.13^{a}	11.43±0.35^{a}	11.22±0.25^{a}	6.52±0.32^{c}
9	18.75±0.53^{a}	13.10±0.05^{b}	13.22±0.13^{b}	12.83±0.30^{c}	8.65±0.05^{d}
11	17.31±0.15^{a}	14.38±0.28^{b}	14.12±0.14^{b}	14.45±0.30^{b}	10.55±0.33^{c}
13	16.43±0.07^{a}	14.64±0.14^{c}	15.31±0.22^{b}	14.99±0.11^{bc}	8.21±0.33^{d}
15	14.75±0.07^{c}	13.52±0.10^{d}	16.43±0.26^{a}	15.32±0.18^{b}	5.30±0.20^{e}

Different superscripts within the same row indicate significant differences among treatments at level of $p \leq 0.05$,

3.1.2. Optical density

The growth curves of *N.oculata* in the growth medium for different phosphorus sources were recorded as optical density (OD) at 540 nm, at 2 days intervals, for 15 days of incubation and illustrated in **Fig. 2.**

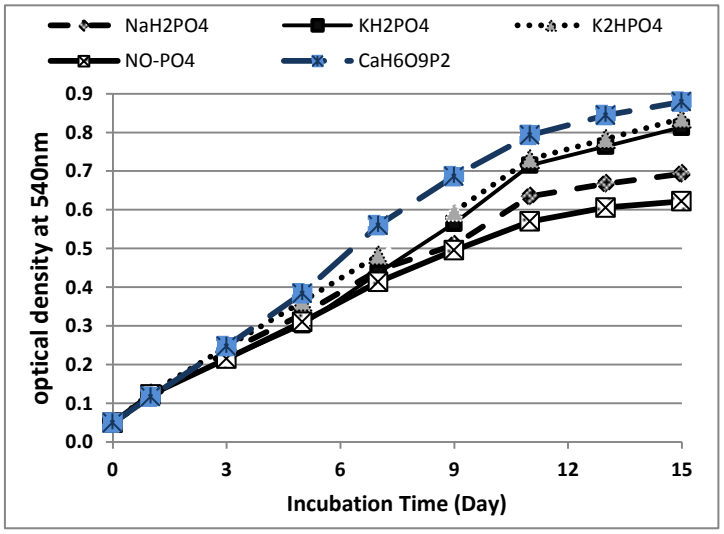


Fig. 2. Effect of different phosphorus sources on optical density at 540 nm of *N. oculata* during the incubation period

3.1.3. Specific growth rate (μ) and doubling time (TD)

Fig. 3 is showed that NaH_2PO_4 achieved the highest significant growth rate ($p \leq 0.05$), with the lowest doubling time of 0.383 and 0.405 doubling/ day, respectively.

While cultures with -PO_4 recorded the lowest specific growth rate (1.28 division/ day) with 0.543 doubling/ day).

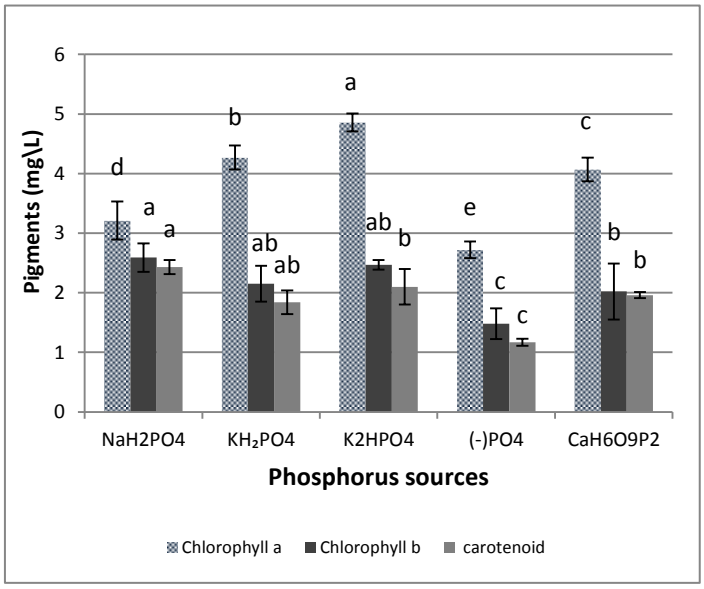
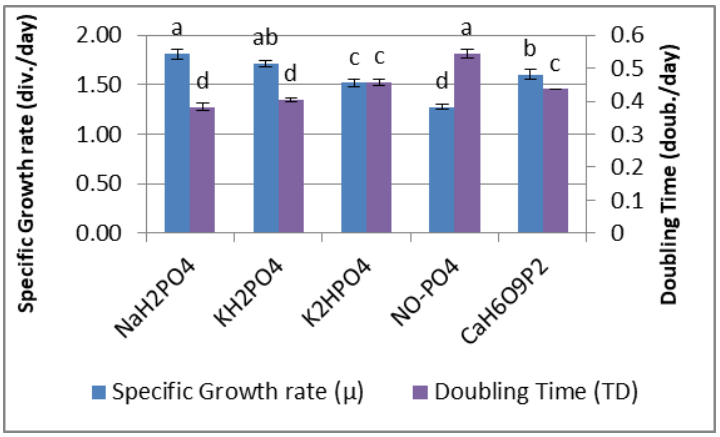


Fig. 3. Effect of different phosphorus sources on the specific growth rate (*u*) and doubling time (Td) at the maximum cell density.

3.1.4. Effect of different phosphorus sources on pigments

The minimum and maximum total Chl a content was achieved in (- PO_4) and K_2HPO_4, as 3.08 and 4.86 µg/L, respectively which showed in **Fig.4**. Decreasing of Chl a content in (- PO_4) had reflected in Chl b and carotenoid content which showed a similar pattern and decreased significantly ($P < 0.05$) than other phosphorus sources**,** while the highest Chl b and carotenoid content 2.59 and 2.43 µg/L, respectively was obtained in the media containing NaH_2PO_4 as phosphorus source.

Fig. 4. Effect of different phosphorus sources on



pigments as µg/L at the maximum cultivation day

3.2. Effect of different phosphorus sources on the biochemical composition of *N. oculata*

Results of total protein, carbohydrate, and lipid as a percentage of dry weight (% DW) are summarized in **Table 3**. The highest dry matter content was found in K_2HPO_4 (0.95 g/L) and $CaH_6O_9P_2$ (0.88 g/L) followed by KH_2PO_4, - PO_4 and NaH_2PO_4 with 0.77 g/L, 0.71 g/L, and 0.69 g/L. The total protein content is increased by 31.14 % and 29.11% in cultures with K_2HPO_4 and KH_2PO_4, respectively. While the culture containing $CaH_6O_9P_2$ had the lowest protein content (25.90%).

Carbohydrate content was significantly high in (-PO_4) followed by K_2HPO_4 with no significant difference ($p > 0.05$) between each other. The lowest carbohydrate content (12.46 % and 12.36%) was achieved in $CaH_6O_9P_2$ and KH_2PO_4, respectively.

The highest total lipid content was increased in K_2HPO_4 at 43.03%, followed by $CaH_6O_9P_2$ at 42.98%, however KH_2PO_4 showed the lowest level (33.58 %) compared to other treatments.

Table 3. The biochemical composition (% of dry weight) of *N. oculata* at different Phosphorus sources harvested after 9 days of cultivation.

Media	Cell Dry Weight (CDW, g L^{-1})	Protein (%CDW)	Carbohydrate (%CDW)	Lipid (%CDW)
NaH_2PO_4	0.69±0.121^{b}	28.83±1.751^{b}	19.09±1.455^{b}	38.26±2.314^{b}
KH_2PO_4	0.77±0.017^{ab}	29.11±0.491^{a}	12.36±0.221^{c}	33.58±3.014^{d}
K_2HPO_4	0.95±0.020^{a}	31.14±0.614^{a}	20.00±1.274^{ab}	43.03±3.956^{a}
$CaH_6O_9P_2$	0.88±0.010^{a}	25.90±1.627^{c}	12.46±0.191^{c}	42.98±1.771^{a}
(-) PO_4	0.71±0.010^{b}	26.70±1.080^{bc}	22.48±1.406^{a}	35.84±1.286^{c}

Different superscripts within the same row indicate significant differences among treatments at level of $p \leq 0.05$,

3.3. Effect of different phosphorus sources on fatty acids profiles of *N. oculata*

In the comparison of F/2 control media to the other mediums, palmitic acid (C16:0) was the most abundant saturated fatty acid, with a maximum value of 20.31% in $CaH_6O_9P_2$. Following the palmitic acid, was the stearic acid (C18:0), which recorded its highest value (7.01%) with - PO_4 media in addition, palmitoleic acid (C16:1) recorded the highest value (9.21%) with K_2HPO_4 followed by - PO_4 (6.96%), while KH_2PO_4 had the lowest percentage value (6.57%). Linoleic acid (C18:2) was the most abundant polyunsaturated fatty acid (PUFA) throughout all treatments, where the data showed the highest values of this fatty acid (21.11 % and 20.46%) were recorded with $CaH_6O_9P_2$ and - PO_4, respectively. Eicosetrienoic acid (C20:3 ω6) was the second polyunsaturated fatty acid, where its maximum percentage value (2.17%) was recorded with $CaH_6O_9P_2$ medium and arachidonic acid (C20:4 ω6), which recorded its highest value (0.83 %) with K_2HPO_4. While γ- Linolenic acid (C18:3 ω6) recorded its highest value (1.43 %) with NaH_2PO_4. whereas eicosapentaenoic acid (C20:5 ω3) achieved the highest in NaH_2PO_4 (9.53%) and $CaH_6O_9P_2$ (0.37%) (**Table, 4)**.

4. DISCUSSION

4.1. Growth parameters of *Nannochloropsis oculata* under influence of different phosphorus sources:

Our data reported that K_2HPO_4 and NaH_2PO_4 (as sources of phosphorus**)** resulted in significantly higher growth in regard to cell density in *N.oculata*. Our suggestions were in agreement with Roopnarain *et al.* (2014) and yu *et al.* (2019); who demonstrated that Phosphour concentration of 0.25 mg/L, 0.28 mg/L, respectively as NaH_2PO_4 had achieved better growth for *I. galbana* also, Rizal *et al.* (2020) who showed that K_2HPO_4 had

Table 4: Fatty acids profile (% of total fatty acids) of *N. oculata* cultured under different phosphorus sources after 9 days of cultivation.

Fatty acids	NaH_2PO_4	KH_2PO_4	K_2HPO_4	$CaH_6O_9P_2$	PO_4
Saturated fatty acids (SFAs)					
Caprylic acid (C8:0)	nd*	20.39±1.02^{a}	15.66±2.02^{b}	7.96±2.99^{c}	7.43±1.01^{c}
Capric acid (C10:0)	nd*	16.94±0.05^{a}	12.68±1.04^{b}	6.36±2.06^{c}	6.12±0.05^{c}
Umdecanoic acid (C11:0)	2.26±1.02^{a}	0.75±0.30^{bc}	0.92±0.03^{b}	nd*	nd*
Laueic acid (C12:0)	0.86±0.30^{c}	2.95±1.05^{b}	3.94±0.06^{a}	0.23±0.08^{c}	0.63±0.13^{c}
Myristic acid (C14:0)	1.13±0.50^{a}	0.54±0.24^{b}	1.30±0.30^{a}	1.67±0.20^{a}	0.25±0.05^{b}
Pentadecanoic acid (C15:0)	nd*	0.16±0.02^{b}	nd*	0.20±0.05^{b}	nd*
Palmitic acid (C16:0)	19.89±0.50^{a}	6.34±0.34^{c}	7.35±1.00^{c}	20.31±0.31^{a}	15.51±0.50^{b}
Heptadecanoic acid (C17:0)	3.33±0.50^{a}	nd*	nd*	0.13±0.02^{b}	nd
Stearic acid (C18:0)	4.06±1.02^{c}	4.40±0.60^{c}	5.86±0.82^{ab}	4.60±1.20^{c}	7.01±0.50^{a}
Arachidic acid (C20:0)	nd*	7.54±0.54^{c}	8.76±0.60^{b}	8.19±0.70^{bc}	13.00±0.40^{a}
Heneicosanoic acid (C21:0)	nd*	0.44±0.22^{a}	nd*	0.14±0.06^{b}	nd*
Behenic acid (C22:0)	nd*	0.42±0.32^{ab}	1.05±0.52^{a}	0.51±0.23^{ab}	0.49±0.33^{ab}
Lignoceric acid (C24:0)	nd*	4.14± 2.50^{a}	2.27±1.19^{ab}	1.23±0.56^{b}	1.83±0.75^{ab}
∑SFA	31.53	65.01	59.79	51.53	52.27
Monounsaturated fatty acids (MUFAs)					
Myristoleic acid (14:1)	3.30±0.63^{a}	nd*	nd*	0.37±0.25^{b}	nd*
Palmitoleic acid (C16:1ω_7)	1.64±0.62^{ab}	0.76±0.40^{bc}	Nd	1.45±0.56^{b}	2.84±1.20^{a}
Palmitoleic acid (C16:1ω_9)	nd*	6.57±1.07^{b}	9.21±0.36^{a}	6.71±1.05^{b}	6.96±0.69^{b}
Heptadecanoic acid (C17:1)	5.57±0.79^{a}	nd*	nd*	0.15±0.09^{b}	nd*
Oleic acid (C18:1ω_9)	7.95±1.85^{a}	1.42±0.56^{c}	nd*	4.50±2.01^{b}	4.08±0.69^{b}
Erusic acid (C22:1ω_9)	nd*	0.26±0.15^{bc}	0.79±0.23^{a}	0.16±0.07^{bc}	0.35±0.19^{b}
Elaidic acid(18:1ω_9)	nd*	0.48±0.10^{c}	nd*	2.76±0.23^{a}	0.76±0.20^{b}
∑MUFA	18.46	9.49	10.00	16.10	14.99
Polyunsaturated fatty acids (PUFAs)					
Linoleic acid (C18:2ω_6)	3.00±1.50^{c}	15.77±2.80^{b}	17.93±1.60^{ab}	20.46±1.20^{a}	21.11±2.80^{a}
Linolelaidic acid (C18:2ω_6)	nd*	0.72±0.22^{ab}	nd*	1.26±0.23^{a}	1.24±0.96^{a}
Eicosadienoic acid (C20:2)	nd*	0.15±0.05^{ab}	nd*	0.25±0.16^{a}	0.16±0.08^{ab}
Docosadienoic acid (C22:2)	nd*	0.54±0.19^{a}	0.33±0.21^{ab}	0.19±0.09^{bc}	nd*
γ- Linolenic acid (C18:3ω_6)	nd*	3.29±1.02^{c}	6.19±1.45^{a}	3.54±0.45^{bc}	4.91±0.34^{ab}
Linolenic acid (C18:3ω_3)	1.43±0.32^{a}	nd*	nd*	1.19± 0.50^{b}	0.48±0.16^{b}
eicosetrienoic acid (C20:3ω_6)	3.20±0.26^{a}	0.81±0.22^{c}	1.55±0.34^{b}	0.50±0.31^{c}	0.88±0.50^{c}
Eicosetrienoic acid (C20:3ω_3)	nd*	1.19±0.25^{b}	1.99±0.40^{b}	2.17±1.02^{a}	1.58±0.63^{b}
Arachidonic acid (C20:4ω_6)	nd*	0.79±0.35^{a}	0.83±0.35^{a}	0.32±0.09^{bc}	0.46±0.18^{ab}
Eicosapentaenoic acid (C20:5ω_3) EPA	9.00±0.30^{a}	nd*	nd*	0.37±0.20^{b}	nd*
∑PUFA	16.31	23.26	28.82	30.25	30.82

Different superscripts within the same row indicate significant differences among treatments at level of $p \leq 0.05$,

recorded the highest cell density reaching 17.8 × 10^6 cells/ml for *N. oculata* in addition, *Desmodesmus communis* had achieved the highest cell concentration in response to K_2HPO_4 (Akgül *et al.*, 2021). The most possible reason is that dissolved phosphorus (DP) can be directly accumulated in phytoplankton, with dissolved inorganic orthophosphate (PO_4^{-3}, Ortho-P) being the most bioavailable form to support primary production (Giles *et al.,* 2015).

Microalgae can store extra phosphates in the form of polyphosphate granules within their cells and then use them for growth during periods of phosphate limitation (Powell *et al.*, 2008). The media of $CaH_6O_9P_2$, K_2HPO_4 then NaH_2PO_4 appeared to have the same pattern of cell density and achieved the highest optical density, these results are compatible with Witono *et al.* (2020) who demonstrated that the optical density of *Haematococcus pluvialis* cultivated at K_2HPO_4 achieved 2.5 fold where it was better than NaH_2PO_4 which achieved 2 fold. The results revealed that the media without phosphate had shown a significant reduction ($P \leq 0.05$) in cell density which was in a parallel line with the results of Yang *et al.* (2018), Hu *et al.* (2019) and Nayak *et al.* (2019)who found that phosphorus deficiency inhibits cell division and suppress of photosynthesis which influences the growth of microalgae.

The inhibition of cell density in the media without phosphate may be due to the photosynthetic activity reduction and metabolic demand transfer (Matsui *et al.*, 2020). The effect of phosphorus restriction includes a reduction in the production and regeneration of substrates in the Calvin-Benson cycle and subsequently, a decrease in the use of light energy for carbon fixation, resulting in low biomass production and decreasing growth rates (Dyhrman, 2016).

It has been demonstrated by (Salam, 2019) that low phosphate concentrations will upset the process of ATP synthesis so that cell growth is limited. Also, Belotti et al. (2013) detected that phosphorus restriction in the medium triggered tiggered the repression of photosynthesis and this situation influences the growth of microalgae (Roopnarain *et al.*, 2014).

Phosphorus is an important component of nucleic acid, protein, and phospholipid in algae cells, as well as a necessary nutrient for chlorophyll synthesis and algae growth (Agrawal, 2012). Parallel to our observations (Lovio-Fragoso *et al.*, 2019) stated the highest Chlorophyll content was found in culture with NaH_2PO_4 at a concentration of 72 µM; while the treatments with P deficiency showed a

lower quantity of Chlorophyll a. As mentioned before, the results illustrated a decrease in the Chlorophyll content of *N. oculata* with P deficiency although chlorophyll does not contain P hence this decline cannot be attributed to the breakdown of chlorophyll for P sequestration. When P becomes limiting, the majority of the photosynthetic is directed toward the synthesis of storage products (lipids) and the biosynthetic pathways (leading to chlorophyll synthesis) become somewhat neglected (Faé Neto *et al.,* 2018).

Also, Matsui *et al.* (2020) confirmed the decline of chlorophyll content, photosynthetic activity, and growth rate due to severe P starvation. This decline might be due to the delay of electron transfer modulated by the rate of CO_2 fixation, which might be strongly reduced due to the depletion of phosphorylated intermediates. Also, Haung *et al.* (2019) demonstrated that the decrease in pigment content might be due to the lack of ATP and NADPH needed for chlorophyll synthesis. It is concluded that chlorophyll production is more negatively influenced by P and by N depletion (Ahmad *et al.,* 2016; Savvidou *et al.,* 2020).

4.2. Biochemical composition of *N oculata* under influence of different phosphorus sources:

The present study revealed that K_2HPO_4 and KH_2PO_4 treatments achieved the highest total protein content; on the other hand, the lowest protein content was achieved by the media without phosphate and CaH6O9P2 with 26.7% DW and 25.9% DW, respectively. Our protein results are compatible with Hamouda and Abou-El-Souod, (2018) detected that, different phosphorus concentrations on BG11 media have effects on growth, primary and secondary metabolites of *Scenedesmus obliquus*. Also, Akgül *et al.* (2021) showed higher protein content in the media containing K_2HPO_4 with 49.59% DW. For our results of the media (-PO_4), these findings agree with Huang *et al.* (2019) who observed that P starvation did not induce protein degradation.

As we have seen, this inhibition might be related to an overall global constraint on RNA and ATP synthesis (Dyhrman, 2016; Mühlroth *et al.,* 2017). In parallel with our study Lovio-Fragoso *et al.* (2019 found a high protein content in the treatment of NaH_2PO_4 with a level of 144 µM compared to treatments with 18 and 72 µM P levels, which would suggest that a greater quantity of phosphorus presence, ATP formation would increase and thus the production of a higher protein content. The most possible reason was explained by Salam (2019) who stated that turbidity can inhibit light penetration and interfere with the photosynthesis process carried out by microalgae so that the efficiency of nutrient absorption is less than optimal for growth. The total carbohydrate content was increased significantly in the media without phosphate followed by K_2HPO_4 ($P \leq 0.05$), followed by NaH_2PO_4, whereas the media containing KH_2PO_4 and $CaH_6O_9P_2$ reduced significantly and achieved the lowest total carbohydrate content.

These results are in consistent with Markou *et al.* (2012) who emphasize that phosphorus limitation causes the accumulation of carbohydrates and lipids in microalgae.

Huang *et al.* (2019) found that carbohydrate accumulation occurred during the late exponential phase and then steady with P, limitation this may be due to P limitation did not hinder the photosynthetic capacity; higher efficiency of carbon fixation fueled the allocation of carbon fluxes to the reserves of carbohydrates and neutral lipids.

These latter accumulated without massive degradation of essential cellular compounds. In addition, carbohydrate and lipid syntheses could compete for the use of precursors (Wang *et al.,* 2014). The total lipid content was increased significantly with the addition of K_2HPO_4 followed by $CaH_6O_9O_2$ ($P \leq 0.05$), followed by NaH_2PO_4, whereas the media without phosphate achieved moderate lipid content. Our findings were consistent with a study carried out by Akgül *et al.,* (2021) who

found that K_2HPO_4 achieved higher lipid than the media without phosphate.

Also, Lovio-Fragoso *et al.* (2019) reported that the culture containing NaH_2PO_4 with the lowest concentration of P (7 µM) achieved the highest total lipid and it was observed that the total lipid content decreased as the P concentration increased.

Although the values reported by Lin *et al.* (2018) are higher than those reported in our study, it is worth to mention that the laboratory's conditions vary among them, as well as the experimental design, the culture media, and the strains; these factors could influence in the final results. Similarly, yang *et al.* (2018) compared phosphate excess (200% phosphate) and phosphate-deplete (6.25–12.5% phosphate) medium, they found a two-fold increase in oil content in response to phosphate limitation in *Scenedesmus* sp.

Further, it has been suggested that lipid amounts in microalgae species could be possible to improve by modifying the culture conditions and nutrient availability. In that sense, it is well documented that under deficient nutrient conditions, neutral lipids synthesis is favored and can be used as an alternative source of biofuels (Zulu *et al.*, 2018). Under low phosphorus cell division rates decrease, although photosynthetic rates are much less reduced.

This leads to an accumulation of carbon, which might be stored in the form of TAG that is rich in saturated fatty acid (Spijkerman and Wacker, 2011), However, nutrient deficiency does not always elevate overall lipid production due to the reduced algal biomass production (Chu *et al.*, 2013). Although nutrient-limiting strategies can enhance the lipid content in microalgal cells, the lipid productivity and lipid concentration of microalgal cells do not improve. For example, the lipid productivity and lipid concentrations of *Chlorella* sp. GN1 under nitrogen or phosphorus restriction conditions was significantly lower than those obtained under nitrogen and phosphorus-sufficient conditions (Feng *et al.*, 2020).

The most possible reason was that algal cell growth was limited when nitrogen or phosphorus was absent compared to when nutrients were sufficient. This phenomenon leads to low biomass production, resulting in low lipid productivity in microalgal cells (Chu *et al.*, 2019; Hu *et al.*, 2019 and Nayak *et al.*, 2019). The lipid content of *Isochrysis galbana*, *Pavlova lutheri*, *Phaeodactylum tricornotum*, *Dunaliella salina* and *Chaetoceros* increased with phosphorus limitation (Gao *et al.*, 2013) but was reduced in *Nannochloris atomis*, *Tetraselmis* and *Chlorella* (Ahmad *et al.*, 2016) suggesting that not all microalgae accumulate lipids during phosphate limitation.In agreement with previous studies, under environmental microalgal cell division is reduced and the synthesis of CO_2 is switched to lipid as storage of energy, so the lipid content per microalgal dry matter basis is increased (Haoujar *et al.*, 2020).

Our results revealed that the media containing NaH_2PO_4, $CaH_6O_9P_2$ and the media without phosphate had a higher content of mono saturated fatty acids (MUFA), these results are compatible with Ahmad *et al.* (2016) who found that monosaturated fatty acids (MUFA) was higher at the treatment of NaH_2PO_4 (36 µM and 72 µM) and in the media with phosphate limitation (9 µM and µM 18) in the culture of *Chlorella* sp. Our results revealed that the media without phosphate and the media containing $CaH_6O_9P_2$ achieved the highest content of polyunsaturated fatty acids also the media containing K_2HPO_4, these results are in parallel with the results of Ahmad *et al.* (2016) who found that the media with the lowest phosphate concentration (9µM) followed by the media with the highest phosphate concentration were achieved the highest polyunsaturated fatty acids among other treatments.

These findings clearly show that fatty acid accumulation is affected by phosphate

availability. Our experiment demonstrated that phosphate deficiency had a significant impact on the production of unsaturated Fatty acids. Supporting our findings, Almutairi (2020) demonstrated that the reductions in nitrogen and phosphorus concentrations lead to an increase in lipid content, including an increase in long chain fatty acids and unsaturated fatty acids, and a decrease in biomass, the most possible reason may be due to the stress induces the production of free radicals, which can injure these cells.

Cells tend to protect themselves against free radicals by accumulating di carbon fragments to form fatty acids and by synthesizing unsaturated fatty acids, which act as free radical scavengers (Valledor *et al.*, 2014). For the same reason, Nouri *et al.* (2019) demonstrated that nutrient limitations do not directly affect fuel properties; rather they affect FAME profiles by increasing both long chain and highly saturated-fatty acids, while having a negative effect on the net gain of dry biomass. As an example of this demonstration the study by Yu *et al.* (2019) found that P restriction increased FA content in N adequate medium.

CONCLUSION

It can be concluded that FA productivity was higher in N adequate and P limitation medium, making this the best condition for biodiesel synthesis from *I. zhangjiangens* is sodium nitrate and sodium dihydrogen phosphate.

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