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Influence of Medium Recipe on Biomass and Lipid Profile of an Aquatic Green Microalga *Monoraphidium convolutum* for Biodiesel Production

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ABSTRACT

Changing the growth medium for microalgae causes a remarkable effect on cell metabolism. The growth and lipid content of *Monoraphidium* species, a promising source for biodiesel production, are significantly influenced by various nitrogen sources, phosphorus, and iron concentrations. Three different *Monoraphidium* species were isolated, identified and evaluated for their growth, lipid content and fatty acid profile on the Bold's Basal medium. *Monoraphidium convolutum* exhibited the highest biomass (0.34g L^{-1} ± 0.02) and lipid content (22.6± 1.6%). The effects of BG 11, Bold's Basal and modified *Navicula* media on biomass and lipid production of *M. convolutum* were tested and compared. Different nitrogen sources; calcium nitrate, ammonium sulphate, ammonium acetate, and urea were tested for biomass production and lipid content. *M. convolutum*, which grew on ammonium acetate (12mg L^{-1} N), maintained the highest lipid content (36± 0.6%), with a large amount of saturated fatty acid methyl esters. The effect of different phosphorous and iron concentrations on biomass and lipid accumulation of *M. convolutum* was separately tested. *M. convolutum* grown on phosphorous concentration equal to 0.475mg L^{-1} recorded the highest lipid content (38.4± 1.5 %), with a high amount of monounsaturated, saturated, and polyunsaturated fatty acid methyl esters. On the other side, *M. convolutum* showed the highest lipid content (39.1± 0.14%) when grown on 0.75mg L^{-1} Fe^{+3} with saturated > monounsaturated > polyunsaturated fatty acid methyl esters. Thus, it is advisable to optimize *M. convolutum* growth on a modified *Navicula* medium containing ammonium acetate and low phosphorous and iron concentrations to obtain high lipid content potential as a biodiesel feedstock.

INTRODUCTION

Global energy shortage, rapid industrialization, the destructive effect of fossil supplies, and the increasing standard levels of nitrogen and sulpher oxides in the atmosphere have spurred a significant research era in the field of biofuel **(Bórawski *et al.*, 2019; Curtin *et al.*, 2019; Li *et al.*, 2019; Ganesan *et al.*, 2020)**. In this concern, finding alternative energy sources characterized by renewability and sustainability is a

pressing issue **(Aziz *et al.*, 2020; Kong *et al.*, 2020; Singh *et al.*, 2020)**. Therefore, several efforts are being considered for biofuel advancement, particularly bioethanol and biodiesel, which serve as alternatives to petrodiesel **(Schenk *et al.*, 2008)**.

Biofuels are mainly produced from organic feedstocks **(Mata *et al.*, 2010)** and are categorized into three generations. Edible crops, such as maize, sugarcane, and wheat comprise the first-generation biodiesel. In contrast, non-edible lignocellulosic feedstock, leaves and husks are included in the second-generation biodiesel **(Zhu *et al.*, 2015; Alishah Aratboni *et al.*, 2019)**. The reliance of these generations on the availability of arable lands and their competition with crops raised for human use is considered to be the fundamental hindrance, and they can't practically substitute fossil fuels **(Zhu *et al.*, 2016)**.

Microalgae comprise the third-generation, which is superior to second-generation biofuels since they do not depend on farmlands of food crops, grow quickly, require less water, may be able to obtain nutrients from wastewater, minimize the effects of greenhouse gas emissions, and certain strains possess more than 70% oil **(Ng *et al.*, 2017; Pang *et al.*, 2019; Refaay *et al.*, 2022b; Song *et al.*, 2022)**. The efficiency of biofuel production, particularly biodiesel from microalgae, faces numerous challenges. Therefore, it is critical to distinguish promising strains and develop growth techniques to induce biomass and lipid yield **(Pineda-Camacho *et al.*, 2019)**. The best strains for biodiesel are *Chlorella* spp. and *Monoraphidium* spp. since they were both observed for their maximum diversification, biomass, and lipid yield **(He *et al.*, 2015; Shanmugam *et al.*, 2020; Mathimani *et al.*, 2021)**.

Microalgal lipids improved under surviving in comparatively extreme conditions **(Manzoor *et al.*, 2020; Leong *et al.*, 2022; Mohammad *et al.*, 2023)**. For instance, nutrient starvation or depletion, such as restriction of phosphorous, nitrogen, iron, and salinity, stimulates the biosynthesis of the lipid in microalgae **(Ma *et al.*, 2018; Khamoushi *et al.*, 2020; Su *et al.*, 2021; Yaakob *et al.*, 2021)**. The microalgal synthesis of lipids, carbohydrates, proteins and cellular growth necessitates nitrogen as a vital macronutrient **(Yodsuwan *et al.*, 2017; Zarrinmehr *et al.*, 2020)**; hence, nitrogen content significantly impacts the biochemical constituents and growth of microalgae. For instance, reducing nitrogen concentration in the growth medium causes growth reduction, but on the other hand, lipid content increases **(Van Vooren *et al.*, 2012; Feng *et al.*, 2020)**.

Phosphorus is intensively required for algal growth, lipid production, and critical metabolic processes **(Ota *et al.*, 2016; Yang *et al.*, 2018)**. About 1% of the total algal biomass is phosphorus, which must be included in the growth medium at concentrations between 0.03 and 0.06% to support the biosynthesis of nucleic acids and cellular components **(Procházková *et al.*, 2014; Ota *et al.*, 2016)**. Furthermore, iron is very critical in the redox reactions in nitrogen assimilation and glycerolipid synthesis; as a

result, iron concentration in the growth medium is a limiting factor for these reactions **(Urzica *et al.*, 2013; Che *et al.*, 2015; Marchetti & Maldonado, 2016)**.

This research aimed to determine how lipid-producing microalgae stimulate biomass production and lipid content in response to nitrogen, phosphorous, and iron concentrations for biodiesel production.

MATERIALS AND METHODS

1. Tested isolates

Freshwater samples were collected from the River Nile in Meet Khamees village, Delta region, Egypt (31°02'39.9"N, 31°20'24.2"E) using a device of a plankton net to collect phytoplankton from standing bodies of water. It is divided into three sections. The upper section has bridles and towing line to hold it. The central section is a funnel-shaped consisting of nylon mesh net, successfully captures plankton of different sizes and filters the plankton in the water sample. The lower section has a cod end at the end of a funnel containing a collecting cylinder, opening and shutting valve **(Gutkowska *et al.*, 2012)**. A low-speed boat was utilized to tow the net horizontally in order to collect a plankton sample **(Valdecasas *et al.*, 2010)**. The sampling process involved a 2min duration, after which a plankton sample was collected in a sample bottle by rotating the horizontal valve to open the cod end above it.

The samples were centrifugated at 4,000rpm for 10min, after that the supernatant was discarded. The planktonic pellets were collected into a sterile test tube containing a sterilized Bold's Basal Medium (BBM) **(Nichols & Bold, 1965)**. A possible axenic culture was reported by **Stein (1973)**. A septic technique was employed to make parallel streaks of fresh microalgal biomass on the agar (1.5%) using a flame sterilized wire loop. The plate was covered and incubated at 26^{o}C and 16:8h light: dark duration cycle of 50µmol photons m^{-2} s^{-1}. Furthermore, a stereomicroscope was used to determine *Monoraphidium* colonies that are free of other organisms. Subsequently, *Monoraphidium* colonies were marked and used for further isolation using a fine and sterilized wire needle. Streaking procedure was repeated with algal units from single colony on agar nutrient medium. Finally, *Monoraphidium* cells were transferred to liquid BBM. The purity of the culture was ensured by regular microscopic examination.

Isolates were identified according to **Komárková-Legnerová (1969)** as *Monoraphidium griffithii* (Berkeley) Komárková - Legnerová, *Monoraphidium contortum* (Thuret) Komárková - Legnerová and *Monoraphidium convolutum* (Corda) Komárková – Legnerová.

2. Growth assessment

The tested *Monoraphidium* isolates were cultivated in (three replicates of each isolate) 250ml Erlenmeyer flasks containing 90ml BBM, inoculated by 10% (v/v) with two weeks old culture and incubated for 21 days at 26^{o}C, and 16:8h light: dark duration cycle of 50µmol photons m^{-2} s^{-1}. Direct cell count using a standard haemocytometer technique was utilized to measure the tested algal growth **(Moheimani *et al.*, 2012)**. In addition to the specific growth rate (µ), divisions per day (Dd^{-1}), and division time (Td) were calculated following the subsequent equations according to **Guillard (1973)**.

(1) $\mu = \frac{\mathrm{Ln}(\frac{N}{N_0})}{dt}$

(2) Division per day; $Dd^{-1} = \frac{\mu}{\ln 2}$

(3) Division Time; $Td = \frac{1}{Dd^{-1}}$

Where, N_0 is the initial cell count, and N is the cell count at a given time t.

3. Biomass harvesting

A membrane filter (Nylon Lab Pak mesh opening 1 micron 121n×121N PK/6) was used to harvest the biomass through a filtration process, and distilled water was utilized for washing, then dried at 60°C **(Samori *et al.*, 2010)** to a persistent weight. The gravimetric estimation, expressed as g L^{-1} of dry weight (wt.), was conducted **(Dayananda *et al.*, 2005)**. The calculation of biomass algal productivity was calculated according to the equation outlined by **Sánchez-García *et al.* (2013)**, as follows:

(4) $P_x = (X_f - X_0)/(t_f - t_0)$

Where, P_x is the biomass of maximum production; X_f is the biomass concentration (g L^{-1}) at time t_f (the time of cultivation of maximum concentration), and X_0 is the initial biomass concentration at time t_0=0 days.

4. Determination of the total lipid content

The amount of total algal lipids was estimated by the method of sulfo-phosho-vanillin, and the resultant color was spectrometrically measured at 530nm. Then, the lipid content was calculated throughout an oleic acid standard curve (10- 100µg/ ml) **(Byreddy *et al.*, 2016)**. Accordingly, the lipid productivity was estimated according to the subsequent equation:

(5) $P_L = P_X Y\ [\text{lipids g} \cdot L^{-1} \cdot d^{-1}]$

Where, P_L and P_X are the maximum production of lipids and biomass, and Y is the % of algal lipids at stationary growth phase.

5. Gas chromatography and mass spectroscopy (GC/MS) analyses

Samples were analyzed at the National Institute of Oceanography and Fisheries, Alexandria, Egypt. Briefly, the apparatus of a gas chromatograph mass spectrometer (7890A GC system, USA) was used to analyze the composition of total algal fatty acid (FAs) lipids as methyl esters. Apparatus adjustment conditions were as follows: helium was introduced as a carrier gas, with a flow rate of 1.5ml/ m, the input temperature of a sample was adjusted at 290°C, programming an initial temperature of 90°C for 1min to 300°C at a rate of 8°C, and a HP-5MS capillary column with a diameter of 0.25mm and a length of 30m. The identification of FAs, and mass spectra were employed. The retention times were compared to standards fatty acid methyl esters standard mixture, Sigma-Aldrich, USA.

6. Molecular identification and phylogenetic analysis of *Monoraphidium convolutum*

Given the analyzed data of the previous experiment, the most promising isolate was furtherly identified using total genomic DNA extracted from the isolate via E.Z.N.A.®Water DNA Kit (D5525-00, Omega Bio-TEK, USA) following the manufacturer protocol. The forward primer (5′-AACCTGGTTGATCCTGCCAG-3′) and the reverse primer (5′-CACCAGACTTGCCCTCCA-3′) were utilized to amplify the genomic DNA via 18S rRNA (~560 bp) using GeneAmp Polymerase Chain Reaction (PCR) system cycler (Creacon, Thermo cycler, Holand) **(Adhoni *et al.*, 2016)**. The sequences were analyzed through ABI PRISM® 3100 Genetic Analyzer (Micron-Corp, Korea). The resultant data were analyzed through a gel documentation system (Geldoc-it, UVP, England) and TotalLab analysis software (Ver.1.0.1) **(Haddad *et al.*, 2014)**.

7. Effect of different growth media

Due to *M. convolutum* maintaining maximum growth characteristics, dry weight, lipid content, and fatty acid methyl esters (FAMEs) wt.%., this isolate was selected for further experiments. *M. covolutum* was cultivated on three different nutrient media: BG11 nutrient media **(Stanier *et al.*, 1971)**, and BBM, modified *Navicula* **(Starr & Zeikus, 1993)**. The experiment was carried out in a sterilized 10-L transparent plastic carboy. Each carboy was inoculated separately with 1-L (0.031g L^{-1} of fresh weight (FW)) of the tested isolate and 8-L growth medium. All treatments were incubated for 12 days at 26°C under 16:8h light duration cycle of 50µmol photons m^{-2} s^{-1} and continuous sterilized air bubbling. The oven was employed to dry the harvested biomass at 60°C for 48h and then used for lipid extraction.

8. Effect of different nitrogen sources

It is relevant to mention that the modified *Navicula* medium recorded the most significant dry weight and lipid content for *M. convolutum*. The tested alga was cultivated on four different nitrogen sources; ammonium acetate [$C_2H_7NO_2$], ammonium sulphate

[$(NH_4)_2SO_4$] and urea [$CO(NH_2)_2$], compared with standard modified *Navicula* medium (Calcium nitrate, [$Ca(NO_3)_2.4H_2O$]) by 12mg L^{-1} of nitrogen concentration. Additionally, the experiment was carried out in 10-L transparent plastic carboy. Each carboy was inoculated separately with 1-L (0.031g L^{-1} FW) of the tested isolate and 8-L growth medium then incubated for 12 days at 26°C under 16:8h light duration cycle of 50µmol photons m^{-2} s^{-1} and continuous air bubbling. The harvested biomass was dried in the oven at 60°C for 48h and then used for lipid extraction.

9. Effect of ammonium acetate concentrations

Ammonium acetate, an alternative nitrogen source of the modified *Navicula* medium, maintained the maximum lipid content of *M. convolutum*. Therefore, the experiment used different nitrogen concentrations of ammonium acetate (15, 9, 6 and 3mg L^{-1}) to compare the dry weight and lipid production of such microalga with 12mg L^{-1} N (control culture). As previously mentioned, the experiment was carried out in 10-L transparent plastic carboy. Each carboy was inoculated separately with 1-L (0.031g L^{-1} FW) of the tested isolate and 8-L growth medium then incubated for 12 days at 26°C under 16:8h light duration cycle of 50µmol photons m^{-2} s^{-1} and continuous air bubbling. The harvested biomass was dried in the oven at 60°C for 48h, and then used for lipid extraction.

10. Effect of phosphorous (P) concentrations

M. convolutum was cultivated on different doses of phosphorous (1.425, 0.95, 0.475 and 0.237mg L^{-1}) to test the algal dry weight and lipid content with 19mg L^{-1} P (control culture). Similarly, as mentioned before, the experiment was carried out in 10-L transparent plastic carboy. Each carboy was inoculated separately with 1-L (0.031g L^{-1} FW) of the tested isolate and 8-L growth medium then incubated for 12 days at 26°C under 16:8h light duration cycle of 50µmol photons m^{-2} s^{-1} and continuous air bubbling. The oven was utilized to dry the harvested biomass at 60°C for 48h, and then used for lipid extraction.

11. Estimation of iron (Fe^{3+}) concentrations

The dry weight and lipid content of *M. convolutum* were investigated under different doses of Fe^{3+} of $FeCl_3.6H_2O$ (0.75, 0.50, 0.25 and 0.125mg L^{-1}) compared with 1.0mg L^{-1} Fe^{3+} (control culture). Correspondingly, as previously mentioned, the experiment was carried out in 10-L transparent plastic carboy. Each carboy was inoculated separately with 1-L (0.031g L^{-1} FW) of the tested isolate and 8-L growth medium then incubated for 12 days at 26°C under 16:8h light duration cycle of 50µmol photons m^{-2} s^{-1} and continuous air bubbling. The oven was used to dry the harvested biomass at 60°C for 48h, and then used for lipid extraction.

12. Statistical analysis

All analyses were tested in triplicate, and values were averaged. The standard errors (SE) were computed as well. For all experiment results, the statistical package for the social sciences (SPSS) software was used to apply the analysis of variance (ANOVA) followed by the least significant difference tests (LSD). Probabilities less than 0.05 were considered significant (n= 3).

RESULTS

1. Growth curves of the tested *Monoraphidium* isolates on bold's basal medium

Growth curves of different *Monoraphidium* species were plotted using cell count, as illustrated in Fig. (1)**Error! Reference source not found.** and Table (1). At the end of the 18th day, *M. contortum* (Fig. 2a) maintained the highest growth (1360×10^4 cell mL^{-1}) because of the smallest size of lunate cells (1.2 x 15µm) compared to the straight cells of both *M. convolutum* (Fig. 2b), (2 x12µm), and *M. griffithii* (Fig. 2c), (3 x 30µm), while at the end of the 12th and 15th days, *M. convolutum* and *M. griffithii* exhibited the highest growth (288×10^4 and 272×10^4 cell mL^{-1}), respectively.

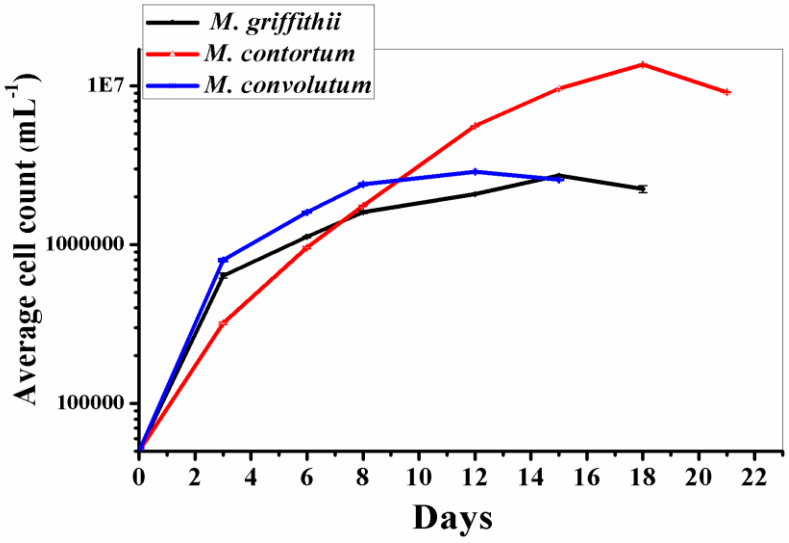

Fig. 1. Growth curves of tested *Monoraphidium* isolates grown on BBM under lab-controlled conditions

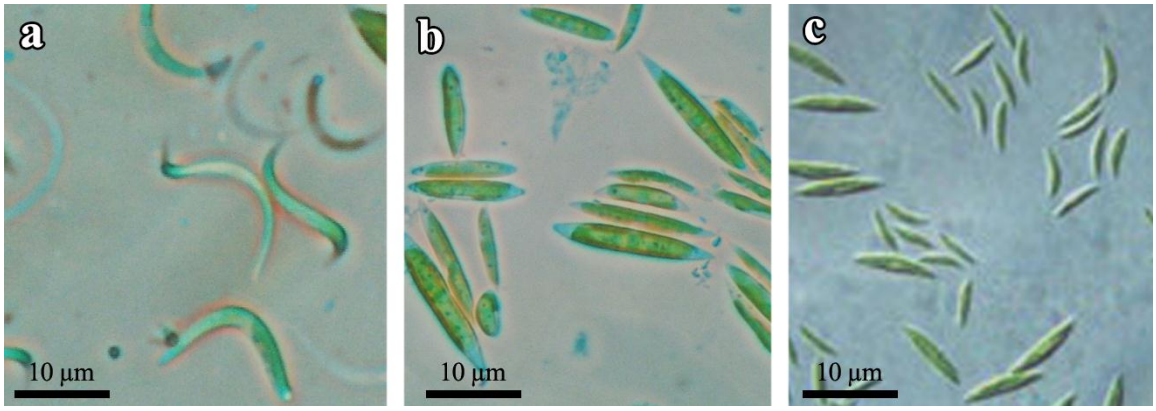

Fig. 2. Photo of isolates showing: **a)** *M. contortum*; **b)** *M. convolutum* , and **c)** *M. griffithii*

1.1. Growth rates of tested Monoraphidium isolates

In order to compare the growth rates of tested *Monoraphidium* isolates on BBM accurately, the specific growth rate (µ), division per day (Dd^{-1}), and division time (Td) were calculated (Table 1). The highest µ, Dd^{-1} and Td were recorded for *M. convolutum* (0.5± 0.1, 0.73± 0.15, and 1.4± 0.3) and *M. contortum* (0.5± 0.07, 0.72± 0.16, and 1.4± 0.32), respectively. The lowest values were recorded for *M. griffithii* as 0.45± 0.09, 0.65± 0.13, and 1.6± 0.13, respectively. According to the growth characteristics (Table 1) and duration of growth cultivation that lasted for 12 days (Fig. 1), *M. convolutum* was selected for the following studied experiments.

Table 1. Specific growth rate (µ), division per day (Dd^{-1}), division time (Td), and cell size of tested *Monoraphidium* isolates

Isolate	µ	Dd^{-1}	Td	Cell size
M. griffithii	0.45 ± 0.09	0.65 ± 0.13	1.6 ± 0.13	3 x 30 µm
M. contortum	0.5 ± 0.07	0.72 ± 0.16	1.4 ± 0.32	1.2 x 15 µm
M. convolutum	0.5 ± 0.1	0.73 ± 0.15	1.4 ± 0.3	2 x 12 µm

2. Dry weight and lipid content of tested *Monoraphidium* isolates

The tested *Monoraphidium* species exhibited different dry weights and lipids content, as shown in Fig. (3), *M. convolutum* exhibited a significant increase in dry wt. and lipid content (0.34± 0.02 g L^{-1}), (22.6± 1.6 %) compared to *M. contortum* (0.14± 0.003 g L^{-1}), (16 ± 0.3 %), respectively. As a result of dry wt. and lipid content, *M. convolutum* was selected for further experiments.

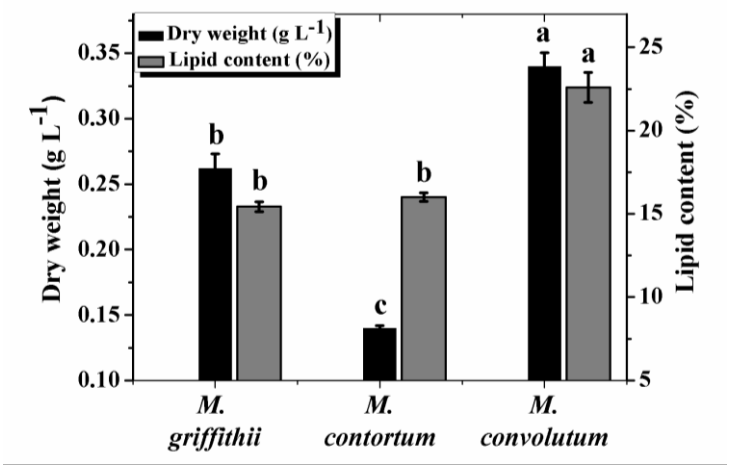

Fig. 3. Dry weight (g L^{-1}) and lipid content (%) of tested *Monoraphidium* isolates grown on BBM under lab-controlled conditions

3. GC/MS analyses of the extracted lipids of tested isolates

Fatty acid methyl esters (FAMEs) of tested *Monoraphidium* species were investigated by GC/MS analysis. Variations in FAMEs of tested *Monoraphidium* species are illustrated in Table 2. Compared to other tested isolates, *M. convolutum* maintained relatively higher wt.% of FAMEs, including methyl alpha-linolenate ($C_{19}H_{32}O_2$), (31.30 %), methyl 9- hexadecenoate ($C_{17}H_{32}O_2$), (14.38%), methyl hexadecanoate ($C_{17}H_{34}O_2$), (12.46%), methyl cis-9-octadecenoate ($C_{19}H_{36}O_2$), (12.51%), methyl 9,12-octadecadienoate ($C_{19}H_{34}O_2$), (6.06%), methyl heptadecanoate ($C_{18}H_{36}O_2$), (3.94%), methyl octadecanoate ($C_{19}H_{38}O_2$), (3.40%) and methyl pentadecanoate ($C_{16}H_{32}O_2$), (3.36%).

Table 2. Composition of FAMEs of tested *Monoraphidium* species

FAME	**Chemical formula**	***M. griffithii***	***M. contortum***	***M. convolutum***
Methyl pentadecanoate	$C_{16}H_{32}O_2$	ND*	ND	3.36
Methyl 9-hexadecenoate	$C_{17}H_{32}O_2$	ND	ND	14.38
Methyl hexadecanoate	$C_{17}H_{34}O_2$	11	ND	12.46
Methyl heptadecanoate	$C_{18}H_{36}O_2$	ND	ND	3.94
Methyl alpha-linolenate	$C_{19}H_{32}O_2$	ND	ND	31.30
Methyl 9,12-octadecadienoate	$C_{19}H_{34}O_2$	ND	6.0	6.06
Methyl cis-9-octadecenoate	$C_{19}H_{36}O_2$	0.5	ND	12.51

Methyl octadecanoate	$C_{19}H_{38}O_2$	ND	ND	3.40
Methyl 11-eicosenoate	$C_{21}H_{40}O_2$	2.0	ND	ND
Methyl 6,9,12,15-docosatetraenoate	$C_{23}H_{38}O_2$	ND	9.0	ND
Sum (wt.%)		**13.5**	**15**	**87.41**

ND*: Not Detected

4. Dry weight and lipid content of *M. convolutum* grown on different nutrient growth media

Fig. (4) shows that a significant increase in the dry wt. and lipid content of *M. convolutum* was maintained when grown on modified *Navicula* medium (0.32± 0.001 g L^{-1}), (26± 0.82 %) compared to BBM (control medium), (0.28± 0.002 g L^{-1}), (22.6± 1.55%), respectively. Accordingly, the modified *Navicula* medium was selected as a control medium for subsequent experiments.

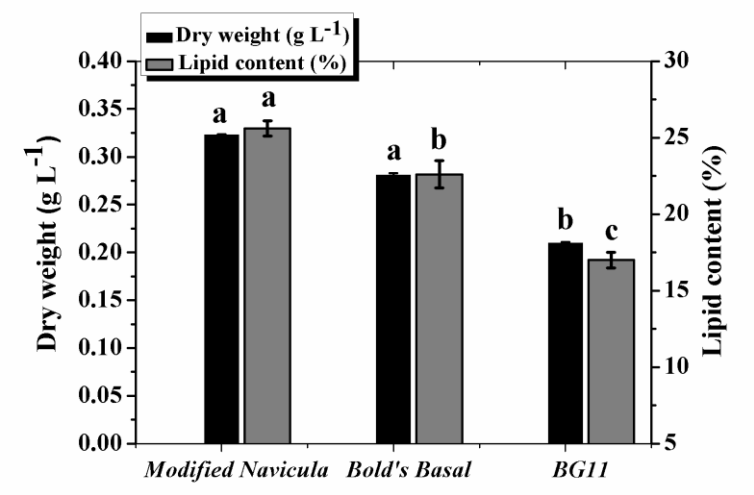

Fig. 4. Dry weight (g L^{-1}) and lipid content (%) of *M. convolutum* grown on different nutrient media under lab-controlled conditions

5. Dry weight and lipid content of *M. convolutum* grown on different nitrogen sources

The results illustrated in Fig. (5) display the dry wt. and lipid content of *M. convolutum* cultivated on different nitrogen sources. The tested alga recorded a significant increase in dry wt. when grown on ammonium sulphate (0.33± 0.001g L^{-1}) compared to standard medium-N source ($Ca(NO_3)_2.4H_2O$), (0.31± 0.001g L^{-1}). However, ammonium acetate exhibited a significant increase in lipid content (36± 0.6%) compared to ($Ca(NO_3)_2.4H_2O$), (26± 0.37%). The lipid productivity was estimated for ammonium

sulphate (6.4± 0.05mg L^{-1} d^{-1}), and ammonium acetate (8± 0.14mg L^{-1} d^{-1}). Therefore, ammonium acetate-N source was replaced by calcium nitrate for further experiments.

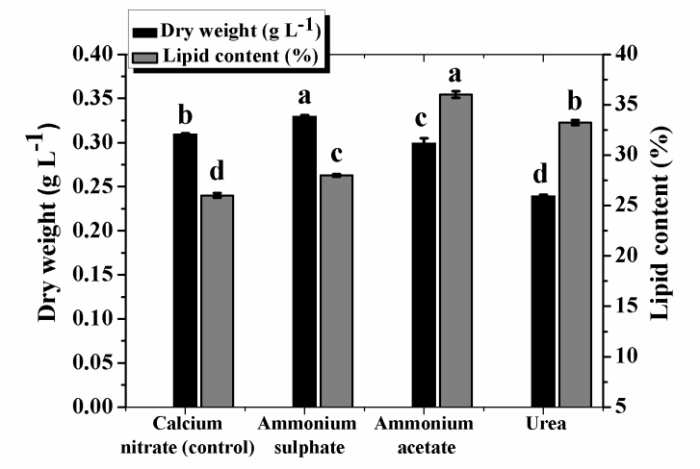

Fig. 5. Dry weight (g L^{-1}) and lipid content (%) of *M. convolutum* cultivated on different nitrogen sources of modified *Navicula* medium under lab-controlled conditions

6. Dry weight and lipid content of *M. convolutum* grown on different nitrogen (N) concentrations of ammonium acetate

The results of dry wt. and lipid content of *M. convolutum* grown on different nitrogen concentrations of ammonium acetate are displayed in Fig. (6A). It was obvious that 15 and 12mg L^{-1} of nitrogen ammonium acetate recorded a significant increase in dry wt., with values of 0.3± 0.003 and 0.3± 0.004g L^{-1}, respectively, while the concentration of 3mg L^{-1} N maintained a significant increase in lipid content (50± 0.68%) compared to 12mg L^{-1} N (36± 0.88%). By comparing lipid productivity (Fig. 6B) of either 15mg L^{-1} N (7± 0.66mg L^{-1} d^{-1}), 12mg L^{-1} N (8± 0.45mg L^{-1} d^{-1}), and 3mg L^{-1} N (3± 0.23mg L^{-1} d^{-1}), the concentration of 12mg L^{-1} ammonium acetate maintained a greatest lipid productivity value, therfore it was decided to select such a concentration for further experiments.

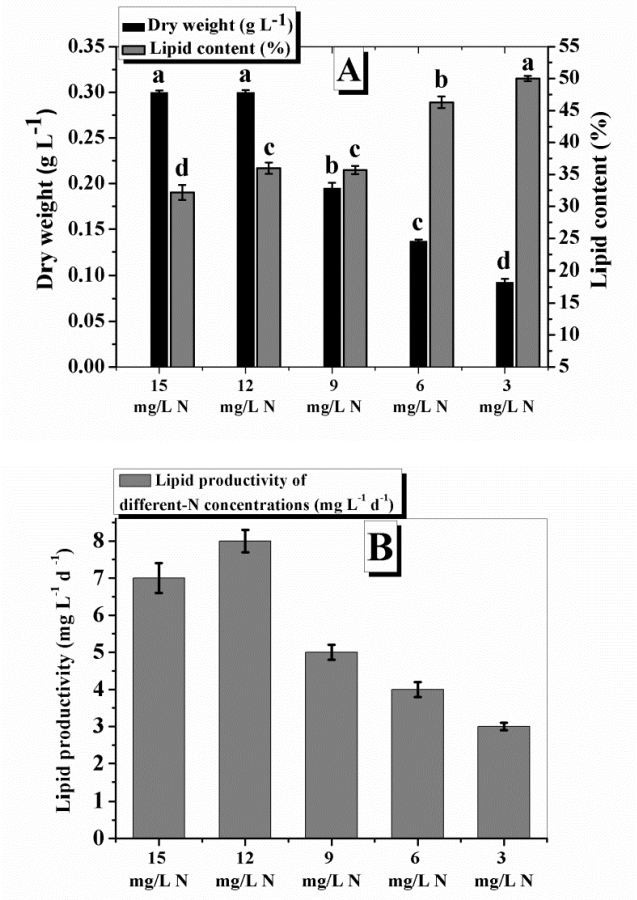

Fig. 6. A) Dry weight (g L^{-1}) and lipid content (%) of *M. convolutum*, and **B)** Lipid productivity of *M. convolutum* cultures grown on different nitrogen concentrations of ammonium acetate of modified *Navicula* medium under lab-controlled conditions

6.1. GC/MS analyses of FAMEs of M. convolutum cultures grown on both 12mg L^{-1} calcium nitrate and ammonium acetate of modified Navicula medium

Table 3) illustrates the FAMEs composition of *M. convolutum* cultures grown on both 12mg L^{-1} calcium nitrate (control culture) and ammonium acetate (alternative nitrogen source) of modified *Navicula* medium. The algal culture grown on calcium nitrate had the following distribution; monounsaturated fatty acid methyl esters (MUFAMEs)> polyunsaturated fatty acid methyl esters (PUFAMEs)> saturated fatty acid methyl esters (SFAMEs)> Diunsaturated fatty acid methyl esters (DUFAMEs). MUFAMEs were mainly represented by C17:1 (methyl 9, hexadecenoate), (14.38%), C18:1 (methyl 10, heptadecenoate), (12.57%), C19:1 (methyl 13, octadecenoate), (12.51%). PUFAMEs were mainly as C19:3 (methyl alpha linolenate), (31.3%). DUFAMEs were mainly categorized by C19:2 (methyl 9,12 octadecadienoate), (6.06%), while the SFAMEs were mainly C17:0 (methyl hexadecanoate), (12.46%), C18:0 (methyl

heptadecanoate), (3.94%), C19:0 (methyl octadecanoate), (3.4%), and C16:0 (methyl pentadecanoate), (3.36%). Whereas the algal culture grown on ammonium acetate displayed the following distribution: SFAMEs> MUFAMEs> PUFAMEs> DUFAMEs. It was observed that the highest values refered to SFAMEs, mainly C24:0 (methyl tricosanoate), (29.6%) and C19:0 (methyl octadecanoate), (13.78%). In relation to MUFAMEs, it was observed to be mainly represented by C17:1 (methyl 9, hexadecenoate), (16.39%), C19:1 (methyl 13, octadecenoate), (10.62%), and C23:1 (methyl 11, docosenoate), (6.94%). PUFAMEs were mainly represented by C21:5 (methyl 5,8,11,14,17 eicosapentaenoate), (0.53%), while DUFAMES were mainly symbolized by C19:2 (methyl 9,12 octadecadienoate), (0.22%).

7. Dry weight and lipid content of *M. convolutum* cultivated on low different phosphorous concentrations

It is worthy to mention that, 12mg L^{-1} of ammonium acetate was used as alternative nitrogen source of modified *Navicula* medium to be tested with low different P concentrations. The dry wt. and lipid content of *M. convolutum* cultivated on low different P concentrations are illustrated in Fig. (7). It was indicated that, the concentration of 19mg L^{-1} P (control culture) exhibited a significant increase (0.28± 0.004g L^{-1}) in dry wt., while the concentration of 0.475mg L^{-1} P induced a significant increase in lipid content (38.4± 1.5%) compared to 19mg L^{-1} P (35.23± 1.1%). Therefore, the concentration of 0.475mg L^{-1} P seemed to be an optimum concentration for algal cultivation since it maintained the highest lipid content and reduced the high phosphorous concentration in the modified *Navicula* medium.

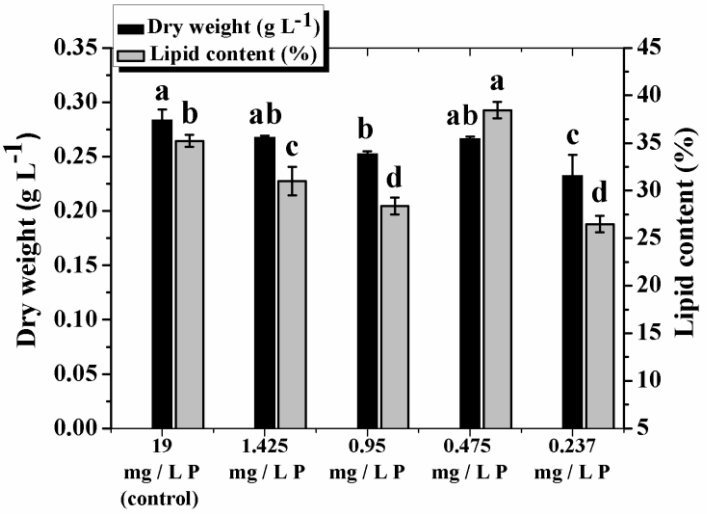

Fig. 7. Dry weight (g L^{-1}) and lipid content (%) of *M. convolutum* grown on low different phosphorous concentrations of modified *Navicula* medium under lab-controlled conditions

Table 3. Composition of FAMEs resulted from cultivation of *M. convolutum* on both 12mg L^{-1} calcium nitrate and ammonium acetate of modified *Navicula* medium

	Carbon no.	% of total algal lipid cultivated on 12mg L^{-1} N		
		FAME	Calcium nitrate	Ammonium acetate
SFAME	C15	Methyl tetradecanoate	ND*	3.81
	C16	Methyl pentadecanoate	3.36	1.02
	C17	Methyl hexadecanoate	12.46	0.05
	C18	Methyl heptadecanoate	3.94	0.05
	C19	Methyl octadecanoate	3.40	13.78
	C21	Methyl eicosanoate	ND	0.57
	C22	Methyl heneicosanoate	ND	0.63
	C23	Methyl docosanoate	ND	2.41
	C24	Methyl tricosanoate	ND	29.6
	C25	Methyl lignocerate	ND	3.57
Sum			**23.16**	**55.49**
MUFAME	C15:1n-5	Methyl 9, tetradecenoate	ND	0.24
	C17:1n-7	Methyl 9, hexadecenoate	14.38	16.39
	C18:1n-7	Methyl 10, heptadecenoate	12.57	1.48
	C19:1n-5	Methyl 13, octadecenoate	12.51	10.62
	C21:1n-11	Methyl 9, eicosenoate	ND	0.09
	C23:1n-11	Methyl 11, docosenoate	ND	6.94
	C25:1n-9	Methyl 15, nervonate	ND	2.53
Sum			**39.46**	**38.29**
DUFAME	C19:2n-6	Methyl 9,12 octadecadienoate	6.06	0.22
	C23:2n-6	Methyl 13,16 docosadienoate	ND	0.13
Sum			**6.06**	**0.35**
PUFAME	C18:3n-3	Methyl 8,11,14 heptadecatrienoate	ND	0.11
	C19:3n-3	Methyl alpha linolenate	31.30	0.04
	C21:5n-3	Methyl 5,8,11,14,17 eicosapentaenoate	ND	0.53
	C23:6n-3	Methyl 4,7,10,13,16,19 docosahexaenoate	ND	0.19
Sum			**31.30**	**0.87**

ND*: Not detected.

7.1. GC/MS analyses of M. convolutum FAMEs grown on the concentrations of 19 and 0.475mg L^{-1} of phosphorous and 12mg L^{-1} ammonium acetate of modified Navicula medium

The *M. convolutum* cultures grown on both concentrations of 19 (control culture) and 0.475mg L^{-1} of P had variations of FAMEs with C15-C25 chain lengths, as shown in Table (4). In general FAMEs of algal culture grown on 19mg L^{-1} P were dominated by SFAMEs> MUFAMEs> PUFAMEs> DUFAMEs. SFAMEs were principally represented by C24:0 (methyl tricosanoate), (29.6%) and C19:0 (methyl octadecanoate), (13.78%). In relation to MUFAMEs, mainly symbolized by C17:1 (methyl 9, hexadecenoate) (16.39%), C19:1 (methyl 13, octadecenoate), (10.66%) and C23:1 (methyl 11, docosenoate), (6.94%). PUFAMEs were principally dominated by C21:5 (methyl 5, 8, 11, 14, 17 eicosapentaenoate), (0.529%). while DUFAMEs were mainly exemplified by C19:2 (methyl 9,12 octadecadienoate), (0.22%).

The dominant FAMEs of algal culture grown on 0.475mg L^{-1} P were: MUFAMEs> SFAMEs> PUFAMEs> DUFAMEs. The highest values referred to MUFAMEs, mainly dominated by C17:1 (methyl 9, hexadecenoate), (22.41%), C19:1 (methyl 13, octadecenoate), (24.28%) and C23:1 (methyl 11, docosenoate), (3.28%). SFAMEs were principally represented by C24:0 (methyl tricosanoate), (19.58%) and C15:0 (methyl tetradecanoate), (1.77%). PUFAMEs were mainly symbolized by C23:6 (methyl 4, 7, 10, 13, 16, 19 docosahexaenoate), (15.89%) and C21:5 (methyl 5,8,11,14,17 eicosapentaenoate), (2.93%), while DUFAMEs were mainly dominated by C19:2 (methyl 9,12 octadecadienoate), (0.91%).

Table 4. Composition of FAMEs resulted from cultivation of *M. convolutum* on 19 and 0.475mg L^{-1} of phosphorous of modified *Navicula* medium

Carbon no.		FAME	% of total algal lipid cultivated on 19mg L^{-1} P	% of total algal lipid cultivated on 0.475mg L^{-1} P
SFAME	C15	Methyl tetradecanoate	3.81	1.77
	C16	Methyl pentadecanoate	1.02	0.14
	C17	Methyl hexadecanoate	0.05	0.04
	C18	Methyl heptadecanoate	0.05	0.03
	C19	Methyl octadecanoate	13.78	0.06
	C21	Methyl eicosanoate	0.57	0.37
	C22	Methyl heneicosanoate	0.63	0.14
	C23	Methyl docosanoate	2.41	0.93
	C24	Methyl tricosanoate	29.6	19.58
	C25	Methyl lignocerate	3.57	1.08
Sum			**55.49**	**24.14**
MUFAME	C15:1n-5	Methyl 9, tetradecenoate	0.24	0.18
	C17:1n-7	Methyl 9, hexadecenoate	16.39	22.41
	C18:1n-7	Methyl 10, heptadecenoate	1.48	1.22
	C19:1n-5	Methyl 13, octadecenoate	10.62	24.28
	C21:1n-11	Methyl 9, eicosenoate	0.09	0.06
	C23:1n-11	Methyl 11, docosenoate	6.94	3.28
	C25:1n-9	Methyl 15, nervonate	2.53	0.97
Sum			**38.29**	**52.4**
DUFAME	C19:2n-6	Methyl 9,12 octadecadienoate	0.22	0.91
	C23:2n-6	Methyl 13,16 docosadienoate	0.13	0.1
Sum			**0.35**	**1.01**
PUFAME	C18:3n-3	Methyl 8,11,14 heptadecatrienoate	0.11	0.08
	C19:3n-3	Methyl alpha linolenate	0.04	0.21
	C21:5n-3	Methyl 5,8,11,14,17 eicosapentaenoate	0.53	2.93
	C23:6n-3	Methyl 4,7,10,13,16,19 docosahexaenoate	0.19	15.89
Sum			**0.87**	**19.11**

8. Dry weight and lipid content of *M. convolutum* cultivated on low different iron concentrations

It is relevant to mention that 12mg L^{-1} of ammonium acetate was used as an alternative nitrogen source with the standard phosphorous concentration of modified *Navicula* medium to be tested with low different Fe^{3+} concentrations. The illustrated results in Fig. (8) indicate that the culture of *M. convolutum* cultivated on 0.75mg L^{-1} Fe^{3+} exhibited a significant increase in lipid content (39.1± 0.14 %) compared to 1.0mg L^{-1} Fe^{3+} (control culture), (35.8± 0.14 %), respectively. Consequently, the concentration of 0.75mg L^{-1} Fe^{3+} was recommended to be the optimum concentration for algal cultivation.

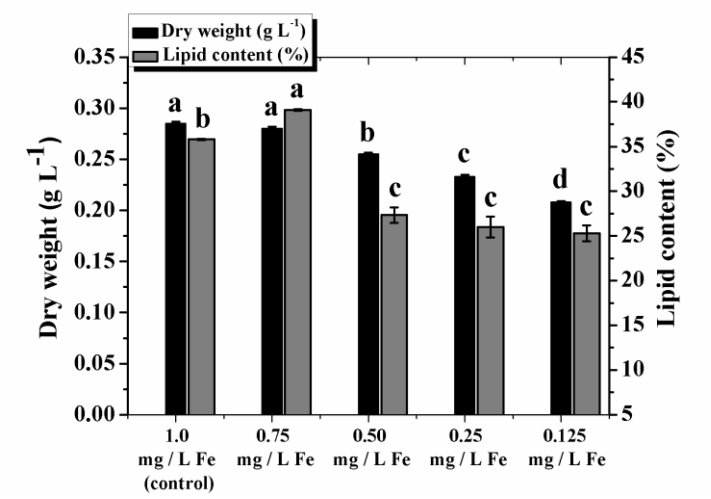

Fig. 8. Dry weight (g L^{-1}) and lipid content (%) of *M. convolutum* cultivated on low different iron concentrations of modified *Navicula* medium under lab-controlled condition

8.1. GC/MS analyses of M. convolutum FAMEs grown on both concentrations of 1.0 and 0.75mg L^{-1} of iron of modified Navicula medium

The FAMEs resulted from cultures of *M. convolutum* cultivated on 1.0 and 0.75mg L^{-1} Fe^{3+} displayed the following distribution, SFAMEs> MUFAMEs> PUFAMEs> DUFAMEs, as illustrated in Table (5). According to the culture grown on 1.0mg L^{-1} Fe^{3+}, SFAMEs were mainly categorized as C24:0 (methyl tricosanoate), (29.6%), C19:0 (methyl octadecanoate), (13.78%) and C15:0 (methyl tetradecanoate), (3.81%). MUFAMEs were represented by C17:1 (methyl 9, hexadecenoate), (16.39%), C19:1 (methyl 13, octadecenoate), (10.62%) and C23:1 (methyl 11, docosenoate), (6.94%). PUFAMEs were mainly dominated by C21:5 (methyl 5, 8, 11, 14, 17 eicosapentaenoate), (0.53%), while DUFAMEs were principally C19:2 (methyl 9, 12 octadecadienoate), (0.22%).

Similarly, the FAMEs of algal culture grown on 0.75mg L^{-1} Fe^{3+} were SFAMEs> MUFAMEs> PUFAMEs> DUFAMEs. SFAMEs were mainly dominated by C24:0 (methyl tricosanoate), (34.25%), C19:0 (methyl octadecanoate), (14.06%) and C15:0 (methyl tetradecanoate), (3.36%). MUFAMEs were represented by C17:1 (methyl 9, hexadecenoate) (16.71%), C21:1 (methyl 9, eicosenoate), (5.73%) and C23:1 (methyl 11, docosenoate), (3.76%). PUFAMEs were principally represented by C21:5 (methyl 5, 8, 11, 14, 17 eicosapentaenoate), (2.56%), while DUFAMEs were mainly, C23:2 (methyl 13, 16 docosadienoate), (0.15%).

9. Phylogenetic analysis and placement of *M. convolutum*

The local pairwise orientation (http://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html) and Smith-Waterman algorithm with a 100% bootstrap value of two sequences construct the phylogenetic tree. The Egyptian isolate *M. convolutum* belongs to a clade with *Monoraphidium* sp HDMA-11 (accession number MH340049.1) (Fig. 9). The tested results indicated that the two sequences are similar (100%). The submitted isolate known as *M. convolutum* was entered into the GenBank database under accession number OQ420741.1.

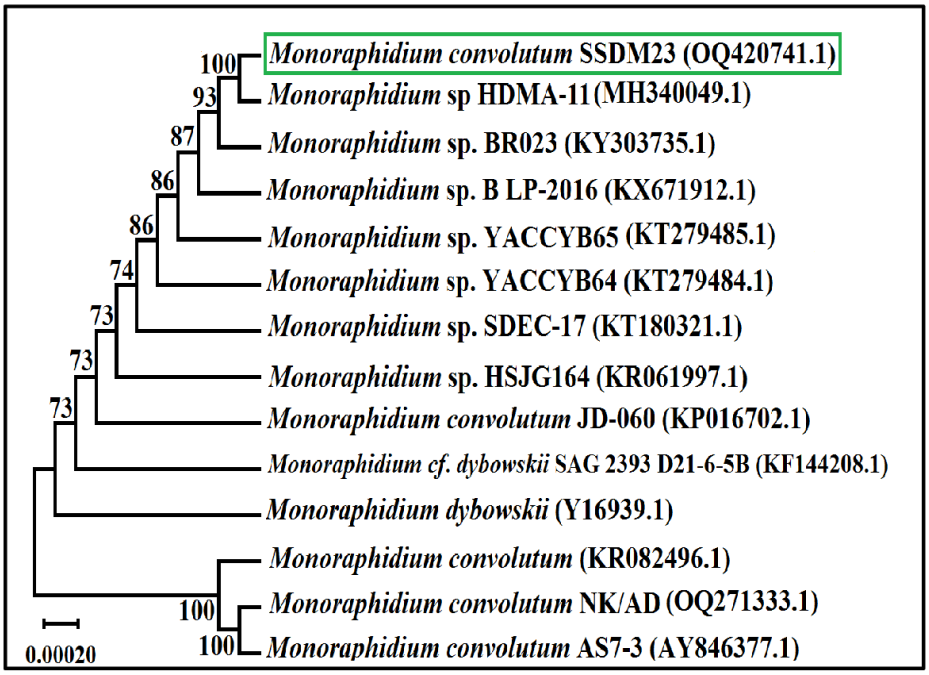

Fig. 9. The construction of 18S rRNA of *M. convolutum* phylogenetic tree using maximum-likelihood (ML) technique

Table 5. Composition of FAMEs resulted from cultivation of *M. convolutum* on 1.0 and 0.75mg L^{-1} of iron of modified *Navicula* medium

	Carbon no.	FAME	% of total algal lipid cultivated on	
			1.0mg L^{-1} Fe^{3+}	**0.75mg L^{-1} Fe^{3+}**
SFAME	C15	Methyl tetradecanoate	3.81	3.36
	C16	Methyl pentadecanoate	1.02	1.46
	C17	Methyl hexadecanoate	0.05	0.06
	C18	Methyl heptadecanoate	0.05	1.6
	C19	Methyl octadecanoate	13.78	14.06
	C21	Methyl eicosanoate	0.57	0.31
	C22	Methyl heneicosanoate	0.63	0.22
	C23	Methyl docosanoate	2.41	1.2
	C24	Methyl tricosanoate	29.6	34.25
	C25	Methyl lignocerate	3.57	1.38
Sum			**55.49**	**57.9**
MUFAME	C15:1n-5	Methyl 9, tetradecenoate	0.24	0.45
	C17:1n-7	Methyl 9, hexadecenoate	16.39	16.71
	C18:1n-7	Methyl 10, heptadecenoate	1.48	1.2
	C19:1n-5	Methyl 13, octadecenoate	10.62	0.1
	C21:1n-11	Methyl 9, eicosenoate	0.09	5.73
	C23:1n-11	Methyl 11, docosenoate	6.94	3.76
	C25:1n-9	Methyl 15, nervonate	2.53	1.53
Sum			**38.29**	**29.48**
DUFAME	C19:2n-6	Methyl 9,12 octadecadienoate	0.22	0.04
	C23:2n-6	Methyl 13,16 docosadienoate	0.13	0.15
Sum			**0.35**	**0.19**
PUFAMEs	C18:3n-3	Methyl 8,11,14 heptadecatrienoate	0.11	0.14
	C19:3n-3	Methyl alpha linolenate	0.04	0.48
	C21:5n-3	Methyl 5,8,11,14,17 eicosapentaenoate	0.53	2.56
	C23:6n-3	Methyl 4,7,10,13,16,19 docosahexaenoate	0.19	0.21
Sum			**0.87**	**3.39**

DISCUSSION

The necessity for the production of biofuels from renewable resources has increased due to the depletion of oil and petroleum reserves. Microalgal lipids are the best precursors exploited for biodiesel production. In order to produce huge amounts of biomass and lipids, it is imperative to choose the optimal strains and advance the growth routes technically **(Pineda-Camacho *et al.*, 2019)**.

M. convolutum maintained relatively higher dry weight, lipid content, growth characteristics (μ, Dd^{-1}, and Td), and wt.% of FAMEs, as shown in Fig. (3) and Tables (1, 2). The potency of biomass and lipid of *Monoraphidium* species have gained great attention of numerous researchers and have concluded that the Selenastraceae family have a viable possibility for the production of biodiesel **(Pineda-Camacho *et al.*, 2019; Lakatos *et al.*, 2023)**. The GC/MS analyses results (Table 2) are in excellent accordance with many similar reported studies **(Knothe, 2009; Engin *et al.*, 2018)**. Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids are most generally acceptable for biodiesel. Accordingly, *M. convolutum* appeared promising for biodiesel of adequate quality since it possessed a majority of saturated and unsaturated fatty acid methyl esters.

It has been well recognized that macronutrients and micronutrients vary from one growth medium to another, which is crucial for algal constituents and the production of particular metabolites **(Hong & Lee, 2008; Refaay *et al.*, 2022a)**. The experimental findings (Fig. 4) exhibit that the modified *Navicula* medium maintained the greatest growth and lipid content among all tested nutrient growth media. The presence of Na_2SiO_3, as a source of silicon element (Si), which is necessary for many organisms from unicellular algae to vascular plants, is responsible for the significant increase in dry weight and lipid content of all tested *Monoraphidium* species **(Sharma *et al.*, 2011; Knight *et al.*, 2016)**. Moreover, **Idenyi *et al.* (2016)** stated that the alkaline buffering of the medium caused by Na_2SiO_3 resulted in the enhancement of algal growth. Consequently, a modified *Navicula* medium was established for further growth experiments.

It has been well documented **(Arumugam *et al.*, 2013; Goncalves *et al.*, 2016; Shin *et al.*, 2018; Zhuang *et al.*, 2018)** that algal cells can accumulate oil and influence directly by a critical nitrogen element. Ammonium acetate was the optimum nitrogen source and external organic carbon for lipid production of *M. convolutum*, as shown from Fig. (5). It has been proposed that acetate can detoxify ammonium when used as a substrate because of the transformation to acetyl- CoA throughout acetyl-CoA synthase. Subsequently, the formation of ATP and NAD(P)H occurred after the entrance to the tricarboxylic acid cycle **(Collos & Harrison, 2014; Gutierrez *et al.*, 2016; Lauersen *et al.*, 2016; Lin *et al.*, 2017)**.

Our findings are in harmony with those attained by **Dittamart *et al.* (2014)**, **Chandra *et al.* (2016)** and **Lin *et al.* (2017)**, who employed ammonium acetate in

mixotrophic cultivation of *Nanochloropsis oculata*, *Scenedesmus*, and *Chlorella* strains for lipid production. The various sources of nitrogen concentrations influence the biochemical constituents and microalgal growth **(Zhuang *et al.*, 2018)**. Therefore, different concentrations of ammonium acetate were studied (Fig. 6A). The experimental data revealed that the concentration of 12mg L^{-1} N maintained the highest lipid productivity (Fig. 6B). It has been well documented that microalgal lipids can increase through nutrient-deficit strategies, however the lipid concentration and productivity are unaffected. Hence, low biomass reduces the lipid productivity in microalgal cells. It appears challenging to grow microalgae with huge amounts of biomass and lipids simultaneously **(Liu *et al.*, 2020; Poh *et al.*, 2020; Gomez-De la Torre *et al.*, 2023; Touliabah & Refaay, 2023)**.

Earlier studies revealed that microalgae possess C14:0, C16:0, C18:1, C18:2 and C18:3 fatty acids **(Tejeda-Benitez *et al.*, 2015)**. As illustrated from the experimental data (Table 3), SFAMEs and MUFAMEs enhanced noticeably in the existence of ammonium acetate as an alternate source of nitrogen. **Liu *et al.* (2018)** assumed that PUFAMEs were transformed more proportionately into SFAMEs and MUFAMEs. In the meantime, the NH^{+4} improved the absorption of acetate, causing more PUFAMEs to shift to SFAMEs and MUFAMEs. Although SFAMEs lower the flow characteristics at low temperatures, they increase biodiesel's burning ability **(Jeong *et al.*, 2008)**. The fluidity of biodiesel is increased by high PUFAMEs concentrations; however, the antioxidant activity is decreased **(D'Alessandro & Antoniosi Filho, 2016)**. MUFAMEs, as C18:1, can significantly minimize the oxidation characteristics of PUFAMEs and improve the flow performance of biodiesel. As a result, ammonium acetate in culture media may affect the FAMEs composition and enhance the quality of the biodiesel.

One of the most crucial nutrients, phosphorus, is essential for cellular development and metabolic functions, particularly energy transmission, signal transduction, macromolecule production, and photosynthesis **(Yang *et al.*, 2018)**. The experimental findings (Fig. 7) reveal that *M. convolutum* maintained a high lipid content at low 0.475mg L^{-1} of P concentration. In this respect, **Yang *et al.* (2018)** observed that decreased phosphorus concentrations trigger the lipid content of *Scenedesmus* sp. Limiting phosphorus availability is a strong environmental pressure that indices lipid buildup. It was hypothesized that phosphorus stress could stop the synthesis of starch or proteins, increasing the carbon flux towards lipids. The signalling mechanisms, however, by which phosphorus stress causes lipid accumulation. Furthermore, it appears that the relationship between biomass productivity and lipid content is contradictory **(Griffiths *et al.*, 2014)**. According to this study, MUFAMEs of C17:1 (methyl 9, hexadecenoate) and C19:1 (methyl 13, octadecenoate) significantly increased in the nutrient deficit-P compared to SFAMEs (Table 4). Similarly, **Sakarika & Kornaros (2017)** reported that the microalga *Chlorella vulgaris* had an increase in MUFAMEs due to the limited availability of $PO4^{3+}$. The quality of lipids such as SFAMEs or MUFAMEs could affect

the properties of biodiesel **(Arias *et al.*, 2013)** that primarily reduce hazardous emissions while enhancing the oxidative stability and cetane number of biofuel without influencing its lubricity, viscosity, or flow qualities **(Arias *et al.*, 2013)**.

The absorption of nitrogen, photosynthetic activity, and cellular respiration process require the trace metal iron **(Che *et al.*, 2015; Marchetti & Maldonado, 2016)**. The concentration of 0.75mg L^{-1} Fe^{3+} exhibited the utmost lipid content, as illustrated in Fig. (8). Researchers have concentrated on how iron concentrations in the culture medium cause lipid buildup in microalgae **(Rajabi Islami & Assareh, 2019)**. Our findings are consistent with those of **Sun *et al.* (2014)** and **Che *et al.* (2015)** who stated that *Neochloris oleoabundans* produces the maximum levels of triacylglyceride at low Fe^{3+} concentrations. However, subsequent supplementation had little effect on lipid content, which is typically unrelated to the maximum biomass. As discussed in Table (5), the amounts of SFAMEs and PUFAMEs in the treated culture of *M. convolutum* increased, compared with the control culture.

Moreover, many scientists **(Ramos *et al.*, 2009; Urzica *et al.*, 2013; Sun *et al.*, 2014; Pádrová *et al.*, 2015)** informed that, iron deficiency had a substantial impact on lipid accumulation as well as the percentage of SFAMEs and PUFAMEs. According to **Ramos *et al.* (2009)**, the significant accumulation of PUFAMEs decreases the oxidative stability of biodiesel but enhances cold-flow properties of biodiesel. In contrast, while oxidative stability and cetane number are highly improved when biodiesel is produced by saturated fatty acids (SFAs), it tends to have relatively poor low-temperature characteristics **(Hu *et al.*, 2008)**.

CONCLUSION

This study revealed that the cultivation of *M.convolutum* on a modified *Navicula* medium with ammonium acetate as an alternative nitrogen source and a concentration of 0.475mg L^{-1} of phosphorous or 0.75mg L^{-1} of iron significantly induced high accumulation of MUFAMEs and SFAMEs which were recommended for large-scale applications of biodiesel. Consequently, *M.convolutum* is mainly nominated as a robust applicant for biodiesel production.

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