MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Stimulation of growth and lipid accumulation of *Monoraphidium braunii* in response to different concentrations of sodium chloride and glucose

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Received:22/10/2023
Accepted: 5/12/2023

Abstract The algal physiological and biochemical constituents including fatty acid synthesis can be improved by salinity stress. Also, organic carbon sources particularly glucose stimulates efficiently the biomass and lipid accumulation in microalgae. This study aimed to investigate the effect of different concentrations of sodium chloride or glucose on growth performance and lipid accumulation of *Monoraphidium braunii*. *M. braunii* was grown on Bold's Basal medium with 0.0, 0.5, 1.0 and 2.0 g L^{-1} NaCl or 0.0, 1.25, 2.5, and 5.0 g L^{-1} glucose. The results revealed an increment in the growth in terms of dry weight (0.5 g L^{-1}) and lipid production (40% DW) at 2.5 g L^{-1} glucose more than those grown at 0.5 g L^{-1} NaCl which they recorded 0.35 g L^{-1} and 22.5% DW, respectively. Therefore, the results specify NaCl at a certain extent or adequate concentration of glucose as an organic carbon source to improve *M. braunii* lipid production.

keywords: *Monoraphidium braunii*; NaCl; Glucose; Lipid; Dry biomass.

1.Introduction

Microalgae are a diverse group of prokaryotic cyanobacteria and eukaryotic photosynthetic microorganisms that are characterized by evolving O_2 and manufacturing numerous bioactive and valuable constituents such as lipids, sterols, vitamins, pigments, proteins, polysaccharides, and phytohormones [1, 2]. The family Selenastraceae including various spescies of *Monoraphidium* spp are free-floating, attached to surfaces in water, or in soils [3]. Also, *Monoraphidium* spp such as *Monoraphidium contortum* and *Monoraphidium braunii* are characterized by their high biomass productivity, and lipid content which can be utilized for multiple future applications [4, 5].

Microalgal cultivation is classified into three types, photoautotrophic, heterotrophic and mixotrophic. Currently, photoautotrophic the most popular mode of microalgal cultivation [6]. According to photoautotrophic cultivation, algae use light energy to absorb and consume CO_2, helping to resolve the world's CO_2 crisis. However, this method of cultivation restricts growth rate and biomass production due to cellular shadowing and low water solubility of carbon dioxide [7-9]. As a result, low biomass productivity will eventually result in relative to the cost of harvesting and producing biomass. This bottleneck can be resolved by growing algae on organic carbons, which eliminates the negative consequences of limiting light penetration as cell concentration rises.

Organic carbon sources have the ability to quickly and considerably boost cell concentration, which lowers the cost of subsequent processes [10-12]. Through mixotrophic cultivation, CO_2 and organic carbon sources were simultaneously uptaken [13] producing more biomass than photoautotrophic or heterotrophic cultivations [13-15].

Glucose, acetate, and glycerol are affordable sources because they are leftovers of other industries and are readily accessible sources of carbon that can be utilized to enhance microalgal growth [16-18]. By establishing a closed system, the risk of contamination in this cultivation may be readily avoided. However, because a significant amount of organic carbon is required to meet the microalgae growth

requirement, mass production will be more expensive. Additionally, throughout the growth, the resulted carbon dioxide from breaking down of organic carbon could trigger the global warming effects [12, 19].

Also, the accumulation and production of lipids in microalgae can be improved by salinity stress. Meanwhile, it has an impact on various biochemical and physiological processes in algae [20]. The microalgal lipids can be enhanced by a propriate rise in Na^+ content in the growth medium [21]. Additionally, it was recognized that a high salt content in the medium could cause oxidative stress in the microalgae cells, which would enhance lipid accumulation [19].

It has been well documented that increasing salinity can stimulate the palmitic acid and oleic acid compositions in microalgae [20, 22]. However, salinity stress in the cultivating medium promotes the induction of neutral lipids which play a significant role in cell membrane rigidity and keeping control of the mineral ions of microalgae cells [23]. Microalgae are being used to produce bioenergy, dietary supplements, and pharmaceutical compounds. The accumulation of beneficial chemicals and biomass productivity in microalgae have been assessed using mixotrophic conditions [24-26]

Therefore, this study attempts to enhance the growth (i.e. dry weight) and lipid accumulation of *M. braunii* through its cultivation under salinity stress or mixotrophic cultivation with an appropriate concentration.

2. Materials and methods

Algal material and growth conditions

The algal isolate was obtained from the algal culture collection, Faculty of Science, Mansoura University, Egypt. After centrifuging at 4000 rpm for 10 minutes [27], the algal isolate was purified by streaking technique on solid Bold's Basal medium (BBM), [28, 29]. The algal culture was incubated for one week at 26 ^oC and 16:8 h light: dark cycle of 50 µmol m^{-2} s^{-1}. The unialgal cells were transferred to liquid BBM to identify according to Komárková-Legnerová [30]. The alga was maintained on the BBM medium, and the culture was renewed at regular intervals to maintain the alga in the exponential phase of growth.

Experimental design

Growth assessment

M. braunii growth was measured by direct cell count using standard haemocytometer technique [31]. In addition, the specific growth rate (µ), divisions per day (Dd^{-1}), and doubling time (Td) were calculated according to Andersen [32] using the following equations:

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

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

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

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

Biomass harvesting

A membrane filter was used to harvest the algal biomass. The algal biomass was washed by distilled water, and dried at 60°C [33] to a constant weight. The dry weight of algal biomass was determined gravimetrically and expressed as g L^{-1} [34].

Determination of total lipid content

The harvested biomass of 1.0 g was dried in the oven at 60 °C for 48h and then used for lipid extraction by soxhlet apparatus according to [35] using dichloromethane (250 mL) as the extraction solvent. The extraction process continued for at least 18 hours. At the end of the extraction, the resultant mixture containing the extracted lipids and the extraction solvent was collected. The excess solvent was removed using a vacuum rotary evaporator at 40°C and the crude lipids were collected into a pre-weighed dry clean beaker and left open for 24 hours under mild continuous fan air current until constant weight using a sensitive balance. The lipid fraction was expressed as % (DW) g g^{-1} of algal dry weight [34].

Experimental layout of different NaCl or glucose concentrations on growth and lipid content of *M. braunii*

One liter (0.031g FW L^{-1}) of the starting *M. braunii* culture was inoculated in 10-L transparent plastic bottles containing sterile BBM nutrient medium. The stock BBM contained solution 1 - $NaNO_3$ – 25 g L^{-1},

$MgSO_4.7H_2O$ – 7.5 g L^{-1}, NaCl – 2.5 g L^{-1}, K_2HPO_4 – 7.5 g L^{-1}, KH_2PO_4 – 17.5 g L^{-1}, $CaCl_2.2H_2O$ – 2.5 g L^{-1}, and H_3BO_3 – 11.4 g L^{-1} ; Solution 2 – Trace metal solution consisting of $ZnSO_4.7H_2O$ – 8.82 g L^{-1}, $MnCl_2$. $4H_2O$ – 1.44 g L^{-1}, $Na_2MoO_4.2H_2O$ – 0.71 g L^{-1}, $CuSO_4.5H_2O$ – 1.57 g L^{-1}, and $Co(NO_3)_2.6H_2O$ – 0.49 g L^{-1}; Solution 3 – Alkaline EDTA solution consisting of EDTA – 50 g L^{-1}, and KOH – 31 g L^{-1}; Solution 4 – Acidified iron solution consisting of $FeSO_4.7H_2O$ – 4.98 g L^{-1}, and Conc H_2SO_4 – 1 g L^{-1}. Stress was imposed by either adding NaCl at concentrations of 0.0 (control), 0.5, 1.0 and 2.0 g L^{-1} or glucose at concentrations of 0.0, 1.25, 2.5, and 5.0 g L^{-1} to the medium. The pH was adjusted to 9 ± 1. The experiments were incubated for 12 days at 26 °C under 16:8 h light: dark cycle of 50 µmol m^{-2} s^{-1} and continuous air bubbling.

Statistical analysis

All analyses were tested in triplicate and values were averaged. The standard errors (SE) were computed as well. For the experiments results, the Statistical Package for the Social Sciences (SPSS) programme was used to apply Analysis of Variance (ANOVA) followed by Least Significant Difference tests (LSD). Probabilities less than 0.05 were believed significant (n=3).

3. Results and Discussion

Growth curve of *M. braunii* on BBM nutrient growth medium

The growth curve of *M. braunii,* in terms of cell number, exhibited a lag period, followed by an exponential phase and eventually maybe a stationary phase. The cell number of *M. braunii* exhibited a progressive increase from the 3rd day to the 12th day, with a peak of 1.6×10^5 cell mL^{-1} at the end of the 12th day (Figure1). The obtained result is in consistent with Pineda-Camacho, de María Guillén-Jiménez, Pérez-Sánchez, Raymundo-Núñez and Mendoza-Trinidad [36], Shrivastav, Mishra, Suh, Farooq, Moon, Kim, Kumar, Choi, Park and Yang [37] who revealed that *Monoraphidium* sp was capable of growing on Bold's Basal medium and producing high biomass through its maximum growth.

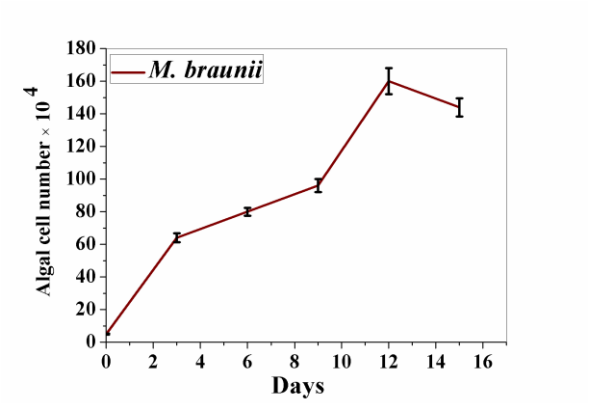

Figure 1. Growth curve of *M. braunii* grown on BBM under lab-controlled conditions

Growth rate of *M. braunii*

The specific growth rate (µ), division per day (Dd^{-1}) and doubling time (Td) were calculated as 0.29 ± 0.06, 0.42 ± 0.08, and 2.4 ± 0.25 respectively.

Biomass production and lipid content of M. braunii

M. braunii was grown on BBM medium to estimate the dry wt. and lipid content. Whereas the dry wt. was 0.3 ± 0.01 g L^{-1}, its lipid content was 16.5 ± 1.12 % DW (Figure 2). Microalgal biomass and lipids are the best precursors exploited for applicable industry. In order to produce huge amounts of biomass and lipids, it is imperative to choose the optimal strains and advance the growth routes technically [36]. The potency of biomasses and lipids of *Monoraphidium* species have great attention by numerous researchers and have concluded that the Selenastraceae family are characterized by their high biomass and lipid productivity [38]. This indicates that *M. braunii* is an ideal candidate to be examined and utilized for different purposes such as biodiesel production.

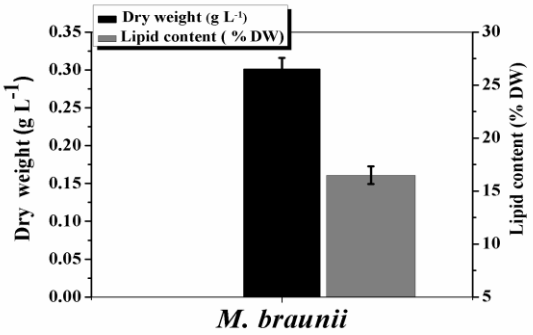

Figure 2. Dry wt. (g L^{-1}) and lipid content (% DW) of *M. braunii* grown on BBM under lab-controlled conditions

Effect of different NaCl concentrations on growth and lipid content in *M. braun*i

The alga recorded a significant increase ($P \leq 0.05$) of growth (3.2×10^6 cell mL^{-1}) when grown on 0.5 g L^{-1} NaCl compared to control culture (0.0 g L^{-1} NaCl), (2.24×10^6 cell mL^{-1}), (Figure 3A) . Also, the concentration of 0.5 g L^{-1} NaCl exhibited a significant increase of dry wt. (0.35 ± 0.01 g L^{-1}) and lipid content (22.5 ± 0.5 %) compared to control culture (0.31 ± 0.04 g L^{-1} NaCl), (16.5 ± 0.5 %) respectively. This means that such a concentration of NaCl was more suitable for algal growth. The microalgal growth and lipid induction are regulated and induced in response to various factors, such as salinity, and mineral stress [39-43]. The obtained data revealed that, 0.5 g L^{-1} of NaCl maintained significant increment in dry weight and lipid content (Figure 3B). The results discussed by Affenzeller [44] and [20] are in accordance with our findings (Figure 3A & B). Normal developmental cell growth will undergo programmed cell death (PCD) in case of a high limit of salt content. Ionic, osmotic, and oxidative stresses are the main reasons for salinity stress influencing where the unbalancing of ionic homeostasis of Na^+ and K^+ causes suppression of the enzymatic binding site of K^+ according to the Na^+ effect [45].

The salt concentration inside the living cell containing cytosol rises because of decreasing the water amount rapidly and hence osmotic efficiency decreases. Additionally, the contraction of intracellular space occurs and is accompanied by the inactivation of photosynthetic electron transport. After, the algal growth and biomass productivity decline. However, Del Campo [46] who illustrated that, *Chlamydomonas reinhardtii* and *Scenedesmus* sp have the ability to develop the cellular biomass in response to a certain extent of salt because Na^+/H^+ antiporters were produced and balance the photosynthetic machinery [47-49]. The obtained data (Figure 3B) are in great harmony with Gour [50] and [41] who illustrated that, the addition of NaCl to the cultivating nutrient medium enhances the lipid accumulation of either *Chlorella vulgaris* and *Scenedesmus* sp. CCNM 1077. It was reported [19, 22, 23, 43, 51, 52] that, microalgal lipid content including saturated fatty acids increased significantly in comparison to polyunsaturated fatty acids due to enhancing the oxidative stress under high salinity stress. Additionally, it was noted that, microalgae tend to synthesize more neutral lipids under salt stress. This is due to the fact that neutral lipids rigidify cell membranes, which act as a maintenance control of mineral ions in microalgae cells [53].

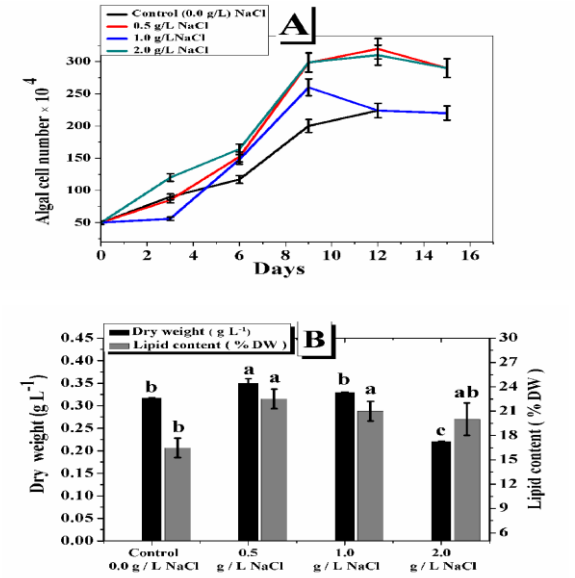

Figure 3. 3A) Average cell count (cell mL^{-1}), and 3B) dry wt. (g L^{-1}), and (% DW) lipid content of *M. braunii* grown on different NaCl concentrations under lab controlled-conditions

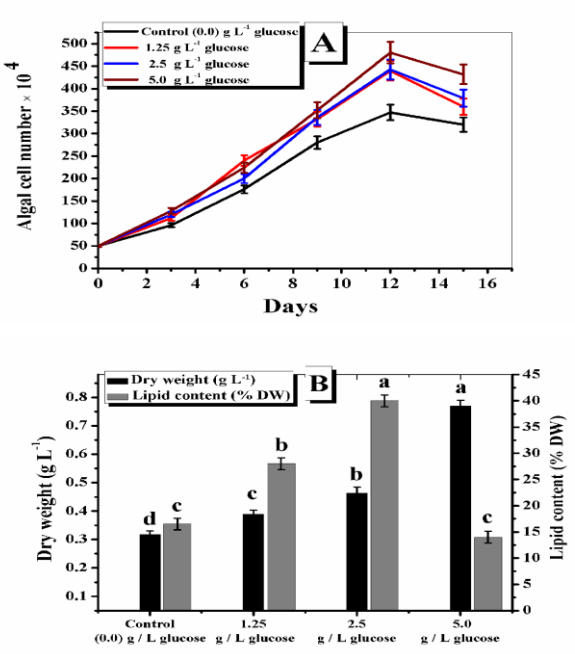

Figure 4. 4A) Average cell count (cell mL^{-1}), and 4B) dry wt. (g L^{-1}), and lipid content (% DW) of *M. braunii* grown on different glucose concentrations under lab controlled-conditions

Effect of different glucose concentrations on growth and lipid content in *M. brauni*

Figures 4 A&B illustrated the cell counts of different glucose concentrations, dry wt. and lipid content of *M. braunii*. The alga exhibited a significant increase ($P \leq 0.05$) of growth (4.8×10^6 cell mL^{-1}) and dry wt. (0.8 ± 0.02 g L^{-1}) when grown on 5.0 g L^{-1} glucose compared to control culture (0.0 g L^{-1} glucose), (3.5×10^6 cell mL^{-1}), and (0.31 ± 0.002 g L^{-1}), respectively. The significant increase in cell number and dry weight of *M. braunii* at 5.0 g L^{-1} glucose was attributed to Ren [54] who stated the utilization of high glucose concentrations increased the dry weight of *Scenedesmus* sp and decreased the lipid content because of the conversion rate of glucose to oil ($Y_{oil/glu}$) was higher at low glucose concentration than higher glucose concentration. However, 2.5 g L^{-1} glucose (Figure 4B) exhibited a significant increase in lipid content (40 ± 1.5 %) compared to control culture (16.5 ± 0.5 %), respectively. According to (% DW) lipid content, 2.5 g L^{-1} glucose seemed to be an optimum concentration for algal cultivation.

Our findings are greatly consistent with those discussed by Shen [55], [56], and [57] who stated that either dry weight or lipid content of different strains of green alga *Chlorella* sp were improved under mixotrophic cultivation. Microalgae have the ability to photosynthesize through the utilization of inorganic carbon source but hinder biomass productivity. In the autotrophic cultivation, microalgae utilize CO_2 as the sole source of energy to be emitted [58]. Accordingly, the released CO_2 cannot be exploited in photosynthesis as a source of carbon. Consequently, pH decreases the culture, influencing the microalgal growth rate and biomass productivity [59, 60]. On the other side and for the purpose of biomass productivity enhancement; glucose was applied to the culture cultivation because it improves the difficulties of autotrophic cultivation as the availability of energy and carbon source. The photosynthesis process was substituted by glucose as a source of energy stimulating the microalgal growth efficiency, and cell density [61-63]. It is well recognized [1, 64] that the availability of acetyl-CoA and NADPH is necessary for stimulating microalgal lipid synthesis. As mixotrophic cultures include both organic and inorganic carbon, therefore, the interaction of autotrophic and heterotrophic metabolism proceeds resulting in the formation and fixation of CO_2. Consequently, there is an increase in the flow of electrons between PSI and PSII, which results in the production of additional energy and NADPH. Besides, Chandra [65] and [56] who revealed that, mixotrophic cultivation stimulated the formation of saturated fatty acids (SFAs) relative to unsaturated fatty acids (UFAs). As a result, the saturated fatty acids with an increment of carbon chain are more valuable and significant for lipid induction. Consequently, more efficient in large-scale industerial application.

Conclusion

The results concluded that, the possibility of *M. braunii* to survive under a certain extent of salinity stress. *M. braunii* was capable of growing on NaCl at a concentration equal to 0.5 g L^{-1} efficiently triggering the algal lipid production. Also, *M. braunii* can grow mixotrophically on 2.5 g L^{-1} glucose to greatly improve lipid content of such alga. It was concluded that, mixotrophic cultivation of *M. braunii* is more convenient and effective for lipid synthesis stimulation.

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