The influence of nutrient manipulation on growth and cultivation constituents of *Anabaena variabilis*

Hanaa Hassanein Morsi [1], Mohamed M. Gharieb [1], Ahmed M. Abd El-Monem [2] and Khalil Mohammed Doman [3*]

1- Botany and Microbiology Department- Faculty of Science - Menoufia University - Shebein El-Koam- Egypt

2- Department of Fresh Water and Lakes, National Institute of Oceanography & Fisheries, Cairo, Egypt.

3- Department of Biology, Faculty of Science, Ibb University, Ibb, Yemen

Abstract:

The cyanobacterium identified as *Anabaena variabilis* is a filamentous cyanobacterium that is capable of differentiating specialized cells, the heterocysts and akinetes, to survive under different stress conditions. The objective of the present study is to investigate the impact of abiotic stresses, namely nitrogen (N), phosphorus (P), and sulphur (S), on the axenic culture of *Anabaena variabilis* and its production of pigments such as chl a and carotenoids, as well as metabolites like proteins, carbohydrates, phenols, and flavonoids. The growth rate was evaluated using optical density (OD)750 nm, and the nutrient supplementation of nitrogen (N), phosphorus (P), and sulphur (S) at 2.5 g/L, 0.06 g/L, and 0,095g/L, respectively, resulted in the highest algal dry weight and maximum production of pigments and biochemical productivity. This demonstrated that the composition of the culture constituents changes as a result of the growth of *A. variabilis* when exposed to different nutritional compositions.

Keywords: *Anabaena variabilis*, chemical constituents, biochemical production, modified BG-11 medium.

Introduction

Microalgae are unicellular, multicellular, filamentous photosynthetic microorganisms that can be classified as eukaryotic or prokaryotic **(Pereira *et al.*, 2020**), With almost 200,000 species, microalgae are also the largest primary producers in the world (**Norton *et al.*, 1996**). Contains unique nutrient contents and bioactive substances that are used in a wide range of commercial applications

Corresponding author: email:* **domankhalil@gmail.com** (ISSN: 1110-8649)

in numerous sectors of the economy, including as nutraceuticals, medicines, biofuels, cosmeceuticals, wastewater treatment, biofertilizers, feed, and proteomics (**Balasubramaniam *et al*., 2021**).

Microalgae are produced by mass cultivation, biomass recovery, and downstream processes to ensure a sustainable output for food, chemicals, feed, biofuels, and other high-value goods. Temperature, salinity, light, and the availability of nutrients are inherent factors that affect the chemical composition of biomass. Among the many compounds that have been utilised for commercial advantage are fatty acids, carotenoids, vitamins, minerals, polysaccharides, and bioactive molecules. (**El-Sheekh *et al*., 2020**). Several sectors depend on the vast production of algal biomass (**Benedetti *et al*., 2018**). Various researchers have created various nutrient media to culture *Anabaena* and *Nostoc*. These are both heterocystous cyanobacteria. The need for natural alternatives to manufactured materials has greatly increased the potential market for bio pigment research. (**Patil and Singh, 2022**).

With the advancement of technology in this area, numerous methods for developing microalgae-based products and downstream processes have been established (**Balasubramaniam *et al.*, 2021**). The primary perceived advantage of microalgae over other organisms is that they are photoautotrophs and thus do not require organic substances for energy, making large-scale cultivation theoretically simpler and cheaper. The basic requirements for algal growth are sunlight, water, CO_2, and inorganic nutrients. Cyanobacteria can grow in mineral medium without any source of organic carbon (C) since they are autotrophic organisms capable of oxygenic photosynthesis.(**Norena-Caro *et al*., 2021**). Nonetheless, new evidence suggests that feasible large-scale cyanobacterial biotechnology should focus not only on biofuel production, but also on the production of biofertilizers, biopolymers, pigments, antioxidants, vitamins, and secondary metabolites (**Norena-Caro and Benton, 2018**). From this standpoint,

the biotechnological significance of *Anabaena* sp. is enhanced by their ability to utilise various nitrogen sources to fuel photosynthetic biosynthesis **(Norena-Caro *et al*., 2021**). All algal cells require the macronutrients nitrogen, phosphorus, and sulphur, therefore shortages in these substances result in a range of metabolic processes in both algae and higher plants**.** Cyanobacteria, on the other hand, are regarded as a great support for examining the effects of nutrient limitation due to their rapid growth, high rate of reproduction, and simple culture system of research **(Doman *et al.*, 2020; El-Fayoumy *et al.*, 2021**).

The objective of this study is to evaluate the impact of several chemically constructed modified BG-11 medium components on the biochemical products of *Anabaena variabilis.*

Materials and Methods

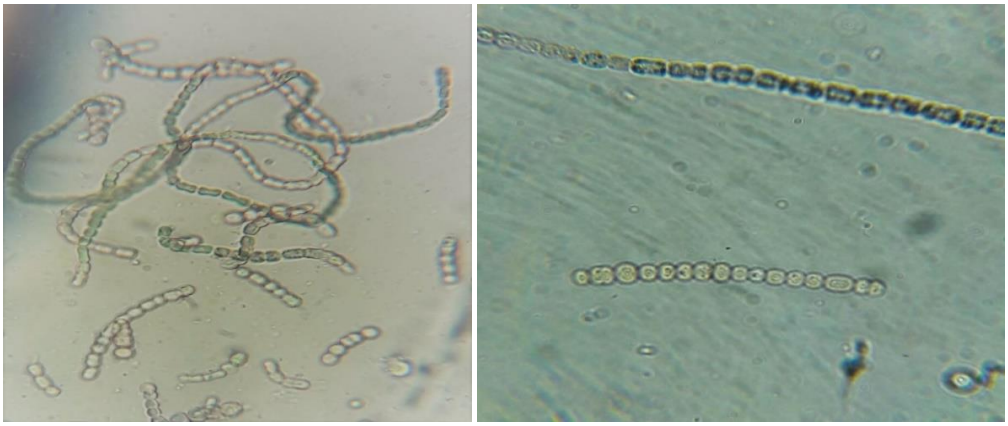
Experimental organism and culture medium

A. variabilis was obtained from Al-Azhar University (ACCAZ), Cairo (Egypt) Plate (1). It was grown axenically utilising modified BG-11 medium in a batch culture (**Ilavarasi *et al*., 2011**). It was grown in Erlenmeyer flasks (500 ml), each flask containing 350 ml of modified BG-11 medium, adjusted at different chemical stresses. The stresses concentrations were:

$NaNO_3$- [1.0, 1.5, 2.0 and 2.5 g/L], $MgSO_{4-}$ [0.055, 0.075, 0.095 and 0.115 g/L], finally KH_2PO_{4-} [0.02, 0.04, 0.06 and 0.08 g/L]. Each flask was inoculated with 35 ml of a pre-culture of the experimental organism. The flasks were placed on shelves illuminated by fluorescent lamps at light intensity of 35 $\mu E/m^2/s$, pH7.5 and 31±1°C. At the end of the experimental period, growth,

production of chlorophyll *a*, carotenoids, carbohydrate, protein, flavonoids, and phenols were determined.

The optical density of the culture was measured at 750 nm (OD_{750}) according to **Abd El-Monem *et al*. (2018)** and the cellular dry weight was determined to monitor cyanobacterial growth (CDW). According to **APHA (2005)** the biomass productivity was calculated.



(A) **(B)**

Plate (1): photomicrographs of *A. variabilis* under lens (**A)**- low power (10x) and (**B)**- High power (40x). Growth assessment.

Estimation of pigment

A known volume (10 ml) of *A. variabilis* culture was centrifuged for 10 minutes at 4000 rpm. After decanting the supernatant, an equal volume of methanol was added to the pellet, then incubated in a water bath at 55°C for 30

minutes before being centrifuged. At 650, 665, and 452 nm by spectrophotometer (MeterteksP-850), the absorbance of the extract (A) was measured against a blank of free methanol. The following equation (**Mackinney, 1941**) was used to calculate chlorophyll and carotenoids as μg ml^{-1} of culture suspension:

Chlorophyll *a* (μg ml^{-1}) = 10.3 E665 - (0.918 E650) and Carotenoids (μg ml^{-1}) = 4.2 E452 – (0.0246 chl.*a*).

Estimation of total soluble proteins:

Microalgae cells were extracted with 1 N NaOH in a boiling water bath for two hours after pigment extraction, according to **Payne and Stewart (1988)** instructions. The method described by **Lowry (1951)** was used to quantitatively determine total soluble proteins. In this procedure, 1 ml of samples (1 N NaOH extract) were added to 3 ml of the alkaline reagent solution in a culture tube, carefully mixed, and let to stand for at least 10 mm at room temperature. A quick addition of 1 ml of the diluted (2:1, v/v) Folin reagent was made to the mixture solution 30 minutes were given for the mixture to stand. At 750 nm by spectrophotometer (MeterteksP-850), the mixture solution was measured against a blank. The amount of protein in the unidentified solution was estimated after preparation of a calibration curve using bovine serum albumin as a standard protein.

Estimation of total soluble carbohydrates:

The microalgae cells were extracted with 1 N NaOH in a boiling water bath for 2 hours after pigment extraction, as described by **Payne and Stewart**

(1988). Total carbohydrates were determined quantitatively using **Kochert (1978)**, Phenol-Sulphuric acid method. In a culture tube, one ml of 5% phenol was added to 0.5 ml of sample (1 N NaOH extract), followed by a 5 ml cone. Sulphuric acid was directly applied to the surface of the culture tube. The sample was left at room temperature for 30 minutes. At 490 nm by spectrophotometer (MeterteksP-850), absorbance was measured in comparison to a blank. After preparing a calibration curve with glucose as the standard carbohydrate, the carbohydrate concentration of the unknown solution was estimated.

Preparation of microalgae extract:

About 0.1 gm of algal was soaked in 70% ethanol (5 ml) for 24h and centrifugation. The residues were repeated soaked in 70% ethanol (5ml) for 24h and centrifugation. The filtrate was taken and concentrated in vacuum until drying. Extracts were stored in airtight glass bottles in refrigerator until use. For estimation of total phenols and flavonoids the extracts were mixed with 5 ml of the same solvent extract.

Estimation of total phenolic compounds

Contents of the total phenolic of microalgae were estimated quantitatively according to **Jindal and Singh (1975)**. 1 ml combined extract was mixed with 1 ml folin reagent and 1 ml sodium carbonate solution (20% w/v) was added, then the mixture was completed up to a known volume with distilled water. The tubes were incubated in dark for color development. After 30 minutes absorbance was

measured at 650 nm by spectrophotometer (MetertekssP-850). A standard curve was prepared using different concentrations of pyrogallol.

Estimation of total flavonoids compound

Aluminum chloride colorimetric method, as modified by **Chang *et al*. (2002)** was used to estimate flavonoid content of the cultivated microalgae. A definite volume of alga extract (0.5 ml) was mixed with 1.5 ml of 95% ethyl alcohol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. Different concentrations of quercetin equivalents (mg/g of dry weight) were used to make the calibration curve.

Statistical analyses

Results are presented as mean ± SD (standard deviation) for three replicates. All the data were subjected to one – way analysis of variance (ANOVA) using SPSS software, version 21. Test of significance was carried out using TUKY test at the significance level $P \leq 0.05$.

Results

Effect of sodium nitrate concentration of modified BG-11 medium on growth parameters and biochemical composition of *A. variabilis*:

A. variabilis was cultivated in modified BG-11 with various $NaNO_3$ (1, 1.5, 2.0, and 2.5 g/L) concentrations. The best conditions were used to incubate each experimental flask. The biochemical composition and growth parameters were measured on day 21 of incubation. The effect of different nitrogen concentrations (in the form of sodium nitrate) on growth of *A. variabilis* was recorded as OD_{750} at 3 days intervals for 30 days of incubation as shown in Fig. 1.

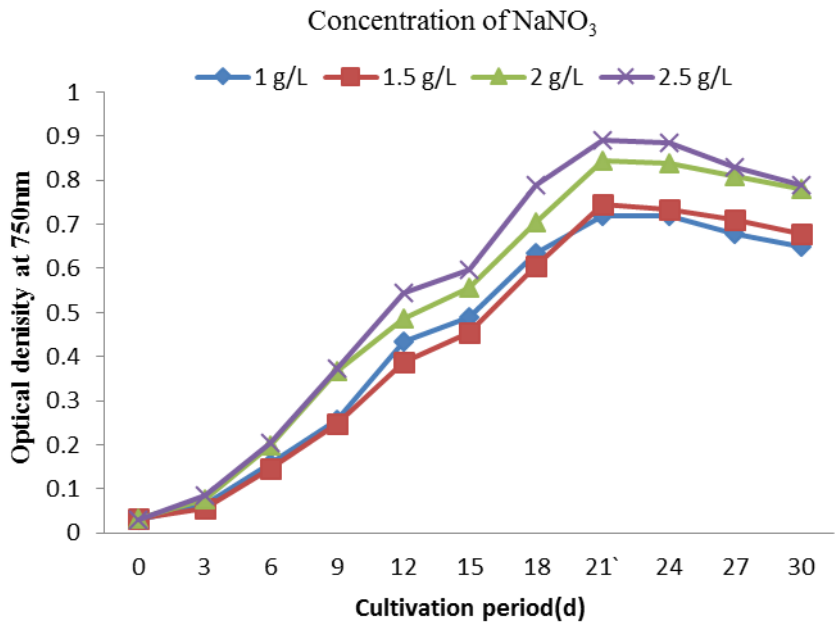


Fig. 1. Growth curve of *A.variabilis* grown in modified BG-11 medium with different concentration $NaNO_3$ over 30 days incubation.

Concentration of $NaNO_3$ present in the basal medium 2.5 g/L, was proven to be the optimal for the growth and production of pigmented biomass. Table 1 revealed that the culture of *A. variabilis* recorded the maximum dry weight 0.074 g/15 ml, protein content 490 mg/g, carbohydrates 240

mg/g, Chl. *a* 7.6 µg/ml and carotenoids 5.49 µg/ml at concentration 2.5 g/L of $NaNO_3$.

Table 1. Effect of $NaNO_3$ concentrations on growth, proteins, carbohydrates, chlorophyll *a* and carotenoids contents of *A. variabilis*

$NaNO_3$ g/L	D. Wt. g/15ml	Proteins mg/g d.wt	Carbohydrates mg/g d.wt	Chlorophyll *a* µg/ml	Carotenoids µg/ml
1.0	0.031 ± 0.004^{d}	311 ± 6.0^{d}	110 ± 1.7^{d}	3.3 ± 0.031^{d}	2.42 ± 0.009^{d}
1.5	0.053 ± 0.003^{c}	$366\pm7.0^{c.}$	150 ± 0.9^{c}	4.7 ± 0.022^{c}	3.09 ± 0.012^{c}
2.0	0.064 ± 0.0002^{b}	441 ± 4.0^{b}	200 ± 0.7^{b}	5.1 ± 0.024^{b}	4.58 ± 0.019^{b}
2.5	0.074 ± 0.0002^{a}	490 ± 5.0^{a}	240 ± 2.0^{a}	7.6 ± 0.022^{a}	5.49 ± 0.015^{a}

Each value is the mean of three readings ± standard deviation. Values with the same small letter in the same column showed insignificant difference (at $p\leq0.05$).

Effect of the different concentration of $NaNO_3$ on the total flavonoid and phenolic compounds of *A. variabilis* are presented in Fig. 2. The highest phenolic and flavonoids contents were found 9.67 mg/g and 2.98 mg/g respectively, at 2.5 g/L $NaNO_3$.

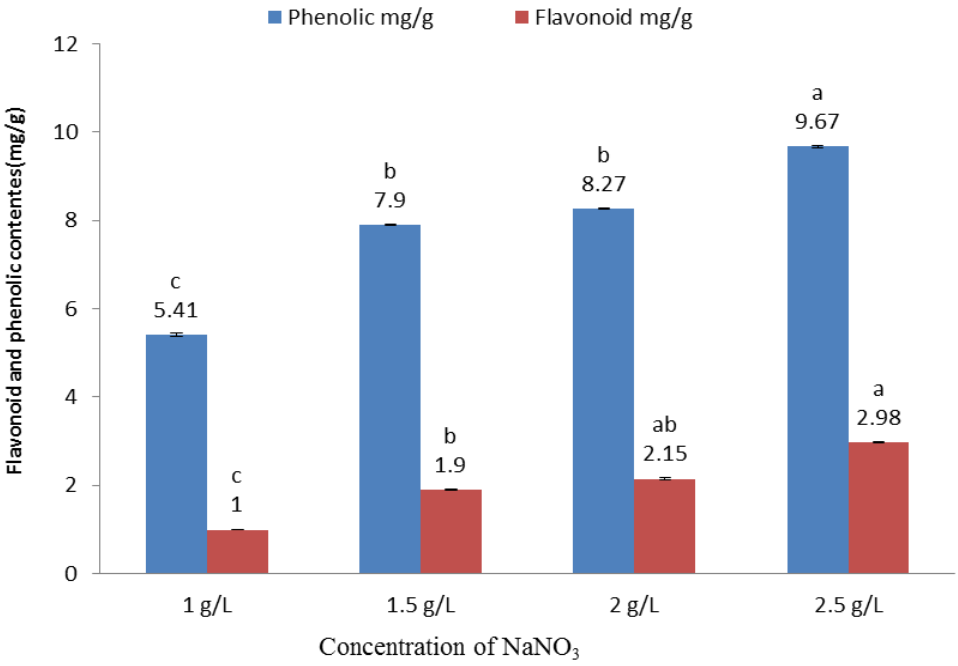


Fig. 2. Effect of $NaNO_3$ concentrations on phenolic and flavonoid contents of *A. variabilis*.

Effect of K_2HPO_4 concentration of modified BG-11 medium on growth parameters and biochemical composition of *A. variabilis*:

A. variabilis grown in modified BG-11 medium containing different concentrations of K_2HPO_4 (0.02, 0.04, 0.06 and 0.08 g/L). All the experimental flasks were incubated under the optimal conditions. The biochemical composition

and growth parameters were measured on day 21 of incubation. The effect of different concentrations of K_2HPO_4 on growth of *A. variabilis* wear recorded as OD_{750} at 3 days intervals for 30 days of incubation as shown in Fig. 3.

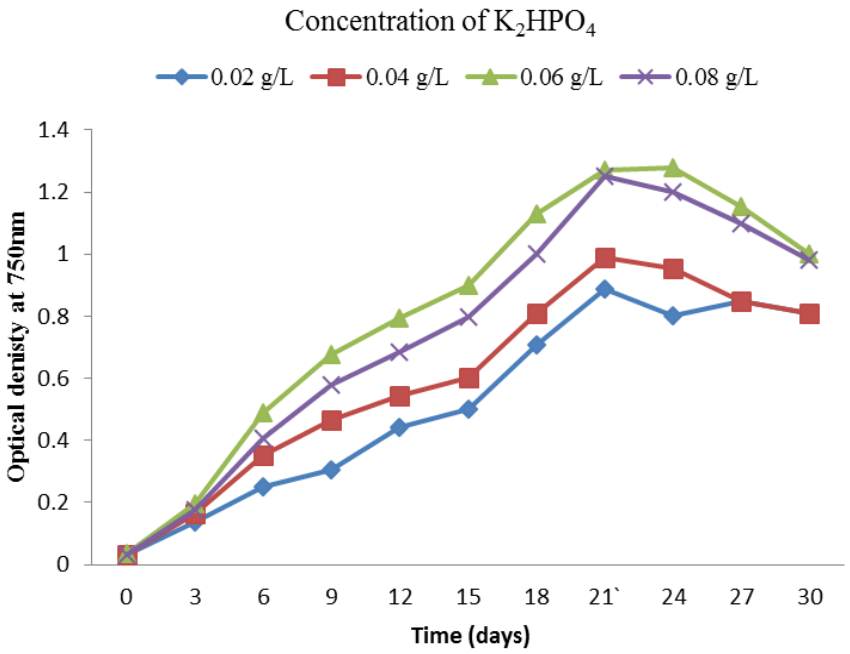


Fig. 3. Growth curve of *A. variabilis* grown in modified BG-11 with different concentration K_2HPO_4 over 30 days incubation.

The optimum concentration of K_2HPO_4 for the most production of pigmented biomass was found to be 0.06 g/L, as shown in Table 2. The culture of *A. variabilis* recorded maximum biomass growth of 0.076 g/15 ml, carbohydrate content of 234 mg/g and protein of 490 mg/g dry weights. In presence of the mentioned level of phosphorus source, pigmentation of *A. variabilis* was increased; carotenoids 7.76 µg/ml and Chl.*a* content 11.5 µg/ml, are shown in Table 2.

The effects of different concentration of phosphorus on total flavonoids and phenolic of *A. variabilis* are shown in Fig. 4. The highest phenolic and flavonoid contents were 7.07 mg/g and 1.99 mg/g, respectively, at 0.06 g/L K_2HPO_4.

Table 2. Effect of K_2HPO_4 concentration on growth, proteins, carbohydrates, chlorophyll a and carotenoids contents of *A. variabilis*

K_2HPO_4 g/L	D. Wt. g/15ml	Proteins mg/g d.wt	Carbohydrates mg/g d.wt	Chlorophyll *a* µg/ml	Carotenoids µg/ml
0.02	0.023 ± 0.0007^{c}	304 ± 7.0^{c}	120 ± 1.5^{d}	6.3 ± 0.137^{c}	2.22 ± 0.045^{c}
0.04	0.037 ± 0.0006^{b}	383 ± 4.0^{b}	168 ± 0.9^{c}	9.9 ± 0.217^{b}	3.29 ± 0.081^{c}
0.06	0.076 ± 0.0019^{a}	490 ± 3.0^{a}	234 ± 0.5^{a}	11.5 ± 0.254^{a}	7.76 ± 0.119^{a}
0.08	0.062 ± 0.0024^{a}	461 ± 6.0^{a}	208 ± 3.0^{b}	10.1 ± 0.142^{b}	5.88 ± 0.255^{b}

Each value is the mean of three readings ± standard deviation. Values with the same small letter in the same column showed insignificant difference (at $p\leq0.05$).

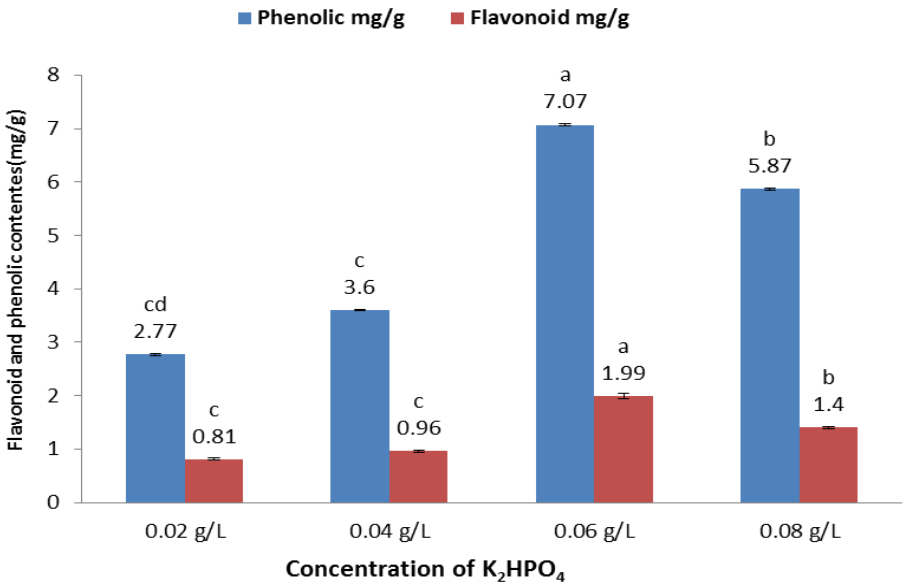


Fig. 4. Effect of K_2HPO_4 concentrations on the phenolic and flavonoid contents of *A. variabilis.*

Effect of $MgSO_4$ concentration of modified BG-11 medium on growth parameters and biochemical composition of *A. variabilis:*

Fig. 5 shows the growth of *A. variabilis* on the modified BG-11 medium under different sulphur of concentrations**.** Therefore, various concentrations of $MgSO_4$ were tested for the maximum growth and formation of pigments. On day 21, the growth and biochemical composition were determined.

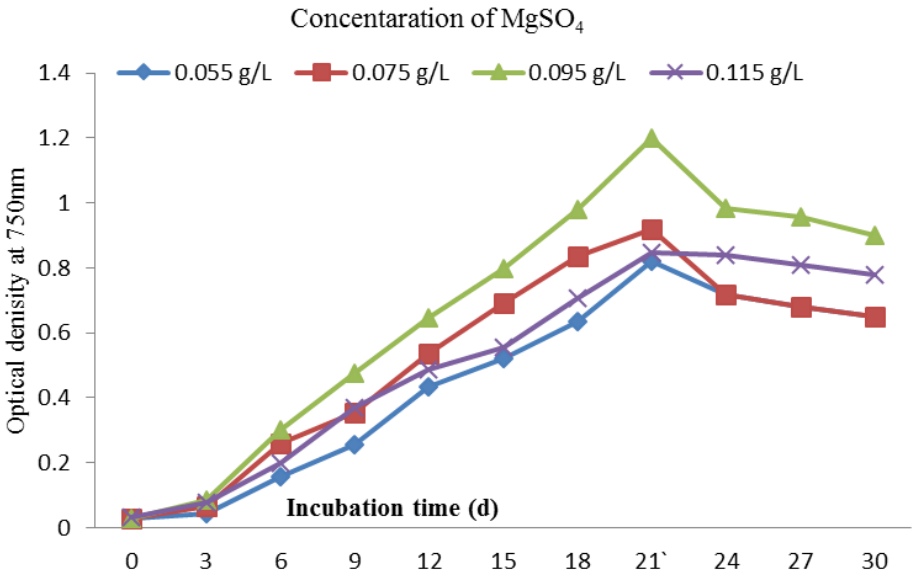


Fig. 5. Effect of $MgSO_4$ concentration on growth of A. variabilis over 30 days

Optimum production of pigmented biomass was found to be at 0.095 g/L concentration of $MgSO_4$, as shown in Table 3. The cultured *A. variabilis* recorded maximum growth of 0.072 g/15 ml, carbohydrate content of 210 mg/g and protein of 401 mg/g dry weights.

In the presence of the mentioned level of $MgSO_4$, the pigmentation of *A. variabilis* was increased; carotenoids 6.88 µg/ml and Chl.*a* content 11.1 µg/ml, as shown in Table 3.

The effects of different concentration of sulfur on total flavonoids and phenolic of *A. variabilis* are shown in Fig. 6. The highest flavonoid content (2.0 mg/g) and phenolic was (8.67 mg/g) at 0.095 g/L, $MgSO_4$.

Table 3. Effect of $MgSO_4$ concentration on growth, proteins, carbohydrates, chlorophyll a and carotenoids contents of *A. variabilis*

$MgSO_4$ g/L	D. Wt. g/15ml	Proteins mg/g d.wt	Carbohydrates mg/g d.wt	Chlorophyll *a* µg/ml
0.055	0.033 ± 0.0007^{b}	255 ± 8^{c}	111 ± 2^{c}	6.0 ± 0.157^{c}
0.075	0.040 ± 0.0008^{b}	319 ± 6^{b}	150 ± 7^{b}	8.9 ± 0.257^{b}
0.095	0.072 ± 0.0009^{a}	401 ± 5^{a}	210 ± 6^{a}	11.1 ± 0.194^{a}
0.115	0.061 ± 0.0006^{a}	330 ± 4^{b}	194 ± 3^{a}	9.5 ± 0.132^{b}

Each value is the mean of three readings ± standard deviation. Values with the same small letter in the same column showed insignificant difference (at $p\leq0.05$).

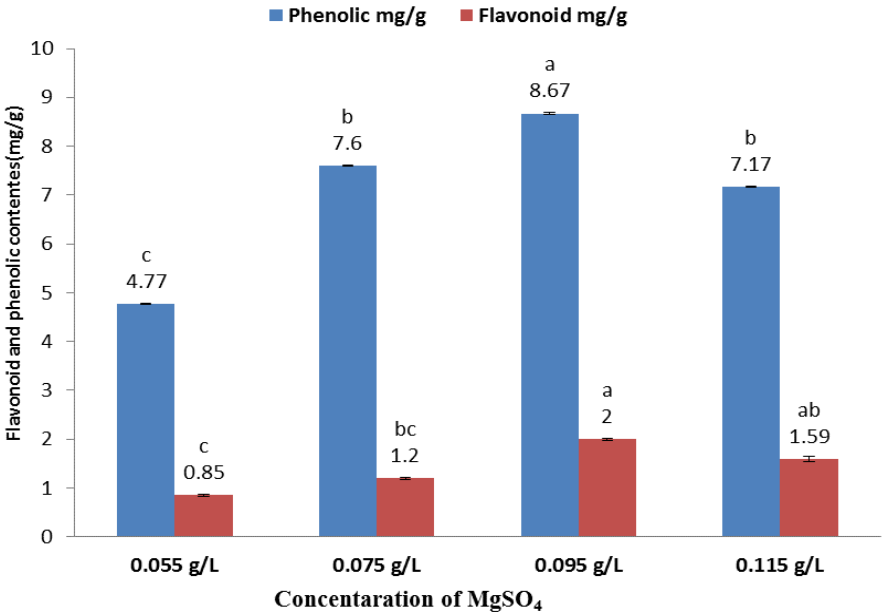


Fig. 6. Effect of $MgSO_4$ concentrations on the phenolic and flavonoid contents of *A.variabilis*.

Discussion

In comparison to previous studies, most of them measured biomass after 14 and 21 days, and none of them used extra time to track biomass depletion **(El-Monem *et al.*, 2021; El-Sheekh *et al.*, 2021**). In the current study, *A. variabilis*

growth began to slow after 21 days of incubation. This agree with the results reported by **Ahamad *et al*. (2021)** reported that the optimum culture conditions were even observed slow after 21 days of batch. **Norena-Caro *et al.* (2021)** discovered that optimal culture conditions could be observed after 14 days of batching.

Nitrogen is an essential nutrient for the microalgal biomass **(El-Sheekh *et al*., 2020**) in this work, sodium nitrate present in the basal medium (2,5 g/L), was proven to be the optimal concentration for growth and production of pigments. This agree with the results reported by **Das and Sarma (2015)** who reported that highest values of biomass of *Anabaena variabilis*, grown in BG-11 medium, were attained at 2.5 g/L $NaNO_3$. **El Shafay *et al*. (2021)** reported that the highest values of biomass of *A. variabilis*, grown in Allen's medium, were attained at +50% N-NO_3. **Patil and Singh (2022)** reported that the highest values of biomass of *Anabaena PCC550*, grown in algal culture medium, were attained at 1g/L sodium nitrate. **El-Monem *et al*. (2021)** who reported that highest values of maximum growth parameters, and pigments of *Spirulina platensis*, grown in Zarrouk's medium, were attained at 2.5 g/L $NaNO_3$.

Phosphorus is a macronutrient that is required for plant and algae growth and the production of energy molecules, **El-Monem *et al*. (2021)**. The optimal concentration of K_2HPO_4 to produce the most pigmented biomass was found to be 0.06 g/L in the current studies. These results were in accordance with **El Shafay *et al*. (2021)**. Phosphorus supplementation increases biomass production in *A. circinalis*, according to **Sarkar *et al*. (2021)**. These results are consistent with **Deb *et al*. (2019)**, who found that nitrate and phosphate depletion prompted the total cellular carbohydrate yield of both *Anabaena variabilis* and *Microcystis aeruginosa*. These findings may be attributed to the importance of nitrogen and phosphorus as two main nutrients, without which the cell's metabolic route is diverted, resulting in the accumulation of energy storage molecules. The highest

values of biomass of *Anabaena PCC550*, grown in algal culture medium, were attained at 0.25 g/L P-PO_3 **Patil and Singh (2022)**.

Some vitamins and amino acids contain sulphur as part of their structural makeup. **Møller and Evans (1976)** studied the nutrition and sulphur use by algae. The present study showed optimum concentration of $MgSO_4$ for production of most pigmented biomass was found to be 0.95 g/L. That reduction of sulphur in the growth medium showed insignificant decrease in *A. variabilis* growth and biomass productivity. These results are consistent with **Das and Sarma (2015)** reported that the highest values of biomass of *A. variabilis*, grown in BG11 Modified 5 culture medium, were attained at +50% S-SO_4.

Conclusion

The present investigation emphasizes the effect of nutrient manipulation of NO_3, PO_4 and SO_4 on growth and biochemical constituents using *A. variabilis*. The overall the large biomass of *A. variabilis* was due to the high nitrogen level. Because algae have a limited supply of nutrients, nutrient addition will increase their biomass. According to the results of this research, the nutrients N, S, and P alone, rather than in combination, have different effects on algal biomass.

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تأثير التغيير فى المغذيات على نمو وزراعة طحلب ***Anabaena variabilis***

هناء مرسى[١]، محمد غريب[١]، أحمد عبد المنعم[٢]، خليل دومان[٣]

١ - قسم النبات والميكروبيولوجى، كلية العلوم ، جامعة المنوفية ، شبين الكوم ، مصر

٢ - قسم البحيرات والمياه العذبة، المعهد القومي لعلوم البحار والمصايد ، القاهرة ، مصر

٣ - قسم الأحياء ، كلية العلوم ، جامعة إب ، اليمن

تعتبر *Anabaena variabilis* من السيانوبكتيريا الخيطية المحتوية على بعض الخلايا المتخصصة مثل الهتيروسيست و الاكينات والتى تساعد الكائن فى البقاء على قيد الحياة في ظل ظروف الإجهاد المختلفة. الهدف من هذه الدراسة هو دراسة تأثير الضغوط اللاأحيائية ، مثل النيتروجين (N) ، والفوسفور (P) ، والكبريت (S) ، على نمو طحلب *Anabaena variabilis* وإنتاجه للأصباغ مثل الكلورفيل والكاروتينات، وكذلك البروتينات والكربوهيدرات والفينولات والفلافونويد. تم تقييم معدل النمو باستخدام الكثافة الضوئية (OD)750 نانومتر، و أظهرت النتائج أن المكملات الغذائية من النيتروجين (N)والفوسفور (P) والكبريت (S) عند 2.5 جم / لتر ، 0.06 جم / لتر ، و 0.095 جم / لتر على التوالي ، أدى إلى الوصول لأعلى وزن جاف للطحلب وأقصى إنتاج للأصباغ والكتلة الحيوية. كما وجد حدوث تغير فى مكونات وسط النمو كنتيجة لنمو *A. variabilis* عند التعرض لتركيبات غذائية مختلفة.