Differential growth of *Amphora coffeaeformis* (C. Agardh) Kutzing under different treatments of nitrogen sources

Mahmoud A. Marzouk[1], Abo El-Khair B. El-Sayed[2] and Ahmed D. El-Gamal[1]

[1]*Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Nasr City, P. O. Box 11884, Egypt.*

[2] *Algal Biotechnology Unit, National Research Centre, Dokki, Cairo, Egypt.*

Abstract:

The Bacillariophyta *Amphora coffeaeformis* (C.Agardh) Kutzing was grown under indoor laboratory conditions using different nitrogen sources including magnesium nitrate ,nitric acid and urea to evaluate whether the addition of magnesium ions affect the biomass accumulation of the grown alga under this study. Concerning the effect of different nitrogen sources on *Amphora* growth, data revealed that 59.98 $mg.L^{-1}$ of $Mg(NO_3)_2$ gave the best growth by estimating the biomass productivity of the dry weight of the alga, and the best concentration in increasing the growth rate(0.079)followed by urea then nitric acid concentrations. Different concentrations of magnesium nitrate were studied. The results showed that 50% of magnesium nitrate markedly increases the dry weight accumulation of the examined alga and surpasses control and all other treatments. On the other hand, 2 and 3 folds of magnesium concentrations (100 and 150%) resulted in the maximum productivity in which recorded 0.108 and 0.105 of growth rate, respectively. The excess of magnesium nitrate till 2 fold enhanced dry weight accumulation and 1.5 fold of magnesium nitrate resulted in the maximum ($1.38g.l^{-1}$)..Scaling up was established to obtain the *Amphora* biomass based on the laboratory obtained data using 15x14 L photobioreactor with a final capacity of 210 L. The harvested biomass was analyzed for biochemical constituents. Daily growth parameters as dry weight ($g.l^{-1}$), doubling time (g) and percentage increase (Y) were performed. Biochemical analysis of *Amphora coffeaeformis* biomass (%) was composed of 31.02 crude protein, 28.37 of total carbohydrates, 13.2 of oils and 12.91 of moisture under ideal laboratory conditions.

Keywords: *Amphora coffeaeformis*, growth, nitrogen sources, magnesium nitrate, culture biomass, macromolecules, phycochemistry.

Introduction

Amphora coffeaeformis (C. Agardh) Kutzing is one of widely distributed naturally in all water resources ranged from freshwater to brackish to marine habitats (**Bhosleac *et al*., 1993)**.Rare studies have been published concerning the utilizing *A. coffeaeformis* as a rich source of metabolic compounds. Mineral

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

nutrition of algae represented the main costly effective figure. Like higher plants, algae growth is markedly affected by the presence of macro (NPK) and micro-nutrients (Fe, Zn, Mn, Cu, B and Mo). In some cases certain nutrients are much desired including Si for marine and in specific for Bacillariophyta. Other algal groups required vitamins to engage their vegetative growth like *Spirulina* and *Nannochloropsis*. In most cases of algal mass production, some nutrients including Ca and Mg such lack of concentration of these elements in turn lead to the drastic decrease in productivity and biochemical profile. The optimum concentrations of desired nutrients are varied from alga to another even in the same algal group and also based on their natural habitat. In addition, the differences in biodiversity of algae were found to be nutritional and environmental dependence. Salt or salinity margin which monitor the sum of nutrients is the main reason and the interior balance of such nutrients seems to be more effective. Unlike higher plants, algae able to use some nutrients instead of other like K and Na. Magnesium ion (Mg) play an important role in all life cycle including human, animal, plants and microorganism, both as a co-enzyme or as the central part of chlorophyll molecule. In this connection, magnesium (Mg^{2+}) ions are a key component of chlorophyll required as growth factor for green microalgae such as *Chlorella vulgaris* and (**Scheer, 1991; Sarma *et al.*, 2014**) and can be found in stoichiometric ratio to chlorophyll in photosynthetic tissues **(Mehne-Jakobs, 1995; Dinç *et al.*, 2012)**.

The chlorophyll content of the microalgae is known to vary with the growth conditions such as light intensity (**Wright and Jeffrey, 2005; Leonardos and Harris, 2006**) and CO_2 concentration (**Clément-Larosière *et al.*, 2014**). Chemical composition of algae in specific to oil production is very closely related to the source and concentration of magnesium. $MgSO_4$ is the common used source; while some growth medium included $Mg(NO_3)_2$or $MgCl_2$; especially with large scale production. The average of Mg ion concentrations in growth medium ranged from5.0 to 50.0 ppm based on the tested or produced algae. This study aimed to investigate the effects of magnesium nitrate in comparison with urea in addition to nitric acid as a nitrogen source on growth of algal biomass and its metabolic products (crude protein, carbohydrate and lipid contents) of the investigated diatom *Amphora coffeaeformis*. In addition, following up the

optimization of the condition, in particular the Magnesium nitrate concentrations that support the growth of the investigated diatom and its productivity.

Materials and Methods

Alga and growth conditions

The diatom *Amphora coffeaeformis* was obtained from Algal Biotechnology Unit, National Research Centre (NRC); Giza, Egypt. Growth medium (F/2) according to **Guillard and Ryther (1962**).This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms for inoculum preparation and also for subculturing. Indoor growth was performed using fully transparent tubes of 75cm length x 75mm diameter with 100µ in thickness) containing 2 L of algal medium. As for growth conditions, continuous illumination was provided from one side light bank of white fluorescent lamps (5 lamps x 40 watt) adjusted at 120-200 µE m^{-2} s^{-1}.Aeration was performed from free oil compressed air from the lower end of columns throughout 3mm acrylic tubes (air left technique). The experiments were performed at room temperature (23±2^{o}C).

Growth unit specification

Two growth units were employed to achieve the proper growth and scaling up of *Amphora* alga. Both of them were made from fully transparent Plex-Glass. The first has the dimension of 63 mm diameter, 100 cm long and 3mm in thickness containing 2L of algal slurry, while the second was 110 mm diameter, 200 cm long and 5mm in thickness with a final capacity of 14L (Fig.1)

Measurements and analysis

The periodically determination of dry weight ($g.l^{-1}$) was assessed for both growth units in terms of crude protein, total carbohydrates and oils. Dry weight was determined by filtering a defined volume of algal slurry over pre-weighted membrane filter 0.45µm, then drying (50^{o}C) and the weight variation expressed the biomass accumulated. Protein was determined based on Microkjeldahl method

(**Ma and Zauzag, 1942**) and crude protein was estimated by multiplying the nitrogen content by 6.25. Carbohydrates were determined by white phenol method (**Dubois *et al.*, 1956**); while oil was determined by soaking followed Soxhlet technique (**El-Sayed *et al.*, 2020**). Samples were calcinated at 500ºC for ash determination. Moisture content was determined by oven drying till constant weight.

Fig.1: Laboratory growth units a) 110 mm in diameter with 14 L capacity

Effect of different N-sources and Mg-nitrate concentrations on *Amphora* growth

Treatments were done based on the initial concentrations of nitrogen and magnesium of the recommended growth medium (F/2). The experiments were carried out using (F/2) medium to investigate the effect of different nitrogen sources as well as the different concentrations of $Mg(NO_3)_2$ on *Amphora coffeaeformis* growth, tested nitrogen sources were included Urea , HNO_3 and

$Mg(NO_3)_2$. Nitrogen was added to F/2 medium at equal-molecular weight to that located in 75mg.l^{-1} of $NaNO_3$as urea nitrogen. Two liters of medium containing inoculum were placed in each Plexi-Glass column in three replicates. On the other series, different concentrations of Mg as $Mg(NO_3)_2$ were tested at 0.0, 50, 150, 200 % versus to 100 % (control) of F2 growth medium. The experiment was performed under the previous mentioned culture conditions of temperature, light and aeration.

Scaling up, harvesting and biomass drying

Serial dilution of the 2 L Plexi-glass grown alga to obtain the proper inoculum was achieved to reach 15 columns x 14L per each (210 L). Growth conditions and performance was employed as mentioned above. When growth of alga reached the maximum in term of dry weight, aeration was overnight braked-down to allow algal sedimentation or floatation. The clear part of growth slurry was eliminated and the remainder was dewatered by laboratory centrifuge at 3000-4000 rpm. The obtained paste was then hot air dried at 45^{o}C and fine powdered for the further analysis.

Results and Discussion

Effect of different nitrogen sources on dry weight of *Amphora coffeaeformis*

Three different sources of nitrogen were examined to evaluate their effect on dry weight accumulation of the Bacillariophyta alga *Amphora coffeaeformis*. They include urea, magnesium nitrate and nitric acid. The applied concentrations were adjusted based on the initial nitrogen concentration of the original growth medium (F/2). However, the net biomass seems to be the maximum value with urea nitrogen (1.3g.l^{-1}); compared with those obtained by magnesium nitrate (1.13g.l^{-1}) or with nitric acid (0.92 g.l^{-1}), respectively as shown in Table 1 and Figure 2.

Table 1. Dry weight growth parameters of *Amphora coffeaeformis* under different nitrogen sources

Treatment	X_o	X	μ	G	N	Y
Urea	0.61	1.30	0.06	11.1	1.07	211.2
Mg Nitrate	0.43	1.13	0.07	8.72	1.37	259.2
Nitric acid	0.65	0.92	0.02	24.3	0.49	140.7

X_o= biomass at zero time (g.l^{-1}); X= biomass at the end of experiment (g.l^{-1}); μ= growth rate (g.d^{-1}); G= doubling time; n= Degree of value multiplication and Y= percentage increase

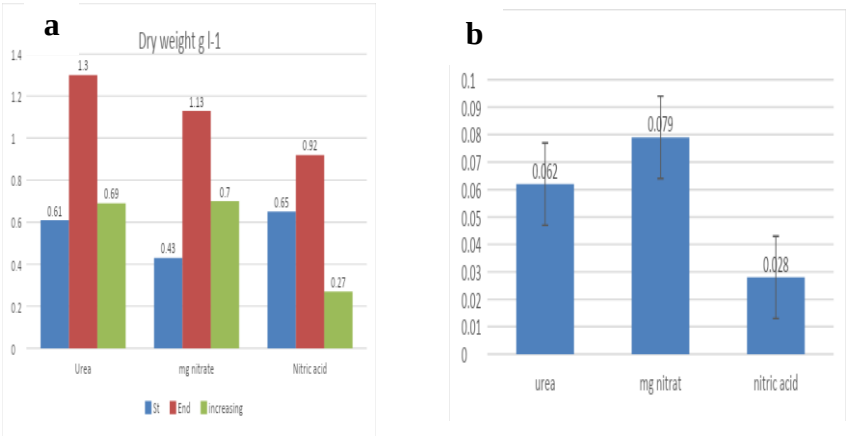

Fig. 2: a) Dry weight, dry weight productivity (mg.L^{-1}) and b) growth rate of *Amphora coffeaeformis* under different nitrogen sources

On the other hand, magnesium nitrate seems to be equal to those of urea nitrogen when data were subjected to the net biomass gain referring to the initial biomass at zero time. Thus, the net differences were calculated as 0.69, 0.7 and 0.27 g.l^{-1}. The inhibitory effect of cultures grown under nitrate nitrogen might be attributed to the acidic effect of nitric acid. In addition, urea supported the growth medium by extra amount of carbon dioxide, while magnesium nitrate was rich in

magnesium. Modifications in culture medium such as nitrogen, phosphorus and silicate concentrations affect the growth rate of microalgae, cellular composition, fatty acid profile of the lipid fraction, as well as the final yield of the *Isochrysis galbana* (**Sanchez et al., 2000)**.These results are in complete agreement with the results of the current study.

On the contrary, nitric acid affected the media reaction toward acidic range in which inhibits the saline water grown algae. For instance, Growth of *Tisochrysis lutea* under alkaline conditions increases the lipid content, as cells accumulate oils as a defense mechanism against stress conditions (**Almutairi *et al*., 2020**). Otherwise, Specific pH-dependent processes which are of prime importance in microalgal cultivation are speciation and differential availability of inorganic carbon species (CO_2/HCO^{1-}/$CO3^{2-}$) in the culture medium (**Moss, 1973; Azov, 1982**); co-precipitation of phosphate with calcium, magnesium and carbonate at high carbonate levels; low solubility and availability of trace metals at high pH (**Sunda *et al*., 2005**), intracellular pH regulation, and changes in the uptake of essential nutrients such as nitrate and phosphate (**Meseck and Smith, 2004; Smith and Meseck, 2004; Meseck *et al*., 2007**).

Growth analysis (Table 1) by calculating dry weight revealed the high inhibitory effect of nitrogen delivered as nitric acid and resulting in 0.02 $g^{-1}.l.d^{-1}$ of growth rate, with doubling time of 24.3 hours, 0.49 degree of multiplication and only 140.7 of percentage increase. Comparing with those of nitrogen provided in the form of magnesium nitrate, the results markedly increased to be 0.07 of growth rate, 8.72 hours of generation time, 1.37 of multiplication degree and 259.2 of percentage increase. These current results may be promising preliminary results for the use of effective and cheap alternatives from nitrogen sources instead of the high-priced sodium nitrate, which is used in almost all algal cultures. This in turn opens the way to reduce the cost of algal cultivation.

In addition, nitrogen as urea form resulted in the moderate values between magnesium nitrate and nitric acid. Urea is a nitrogen source, and algae needs nitrogen to grow. However, the large amounts of urea have oversaturated the water with nitrogen. This could have caused the algae to have too much nitrogen and lead to a decrease in cell growth (**Fiebiger *et al*., 2018).**

In spite of the enhancing effect of magnesium ion in algal growth, the liberated nitrate ions affecting the rise of media reaction (pH) due to algal growth which ten to alkaline side. Microalgal growth is accompanied by processes affecting the pH, such as excretion of certain cell metabolites and absorption of acidic compounds (*e.g*., amino acids and nitrogenous compounds. Thus, the growth medium tends to become more alkaline over time. Thus, pH is not simply a static factor that affects growth, but rather is also an indicator for healthy algal growth. Thus, the natural decline in acid reaction of the algal growth medium gives rise to a decline in algal growth, indicating a high death rate (**Almutairi *et al*., 2020**).

Effect of different Mg concentrations on *Amphora coffeaeformis* growth dry weight

Among the different concentrations used, enriching of growth medium with 50% of magnesium nitrate markedly increases the dry weight accumulation of the examined alga and surpasses control and all other treatments ($MgNO_3$) and concentrations. This effect might attribute to the accompanied nitrate ions released from magnesium nitrate. Apart from having multiple roles in algal physiology, Mg is the central atom of chlorophyll. On the other hand, 2 and 3 fold of magnesium concentrations (100 and 150%) resulted in the maximum productivity in which recorded 0.108 and 0.105 of growth rate, respectively (Fig.3, Table 2) with the lowest generation time comparing with those of control and other magnesium concentration grown cultures.

Increasing Mg concentration in the culture medium showed positive effect on biomass yield. High doses of Mg induced high biomass production, as chlorophyll, the main molecules responsible for trapping the light energy, requires one magnesium ion for synthesis of each chlorophyll molecule. Rise in chlorophyll synthesis with increasing Mg concentration was also observed and MgSO4 nanoparticles were found to improve the lipid production by 118.23 ± 5.67% (**Sarma *et al*., 2014**).

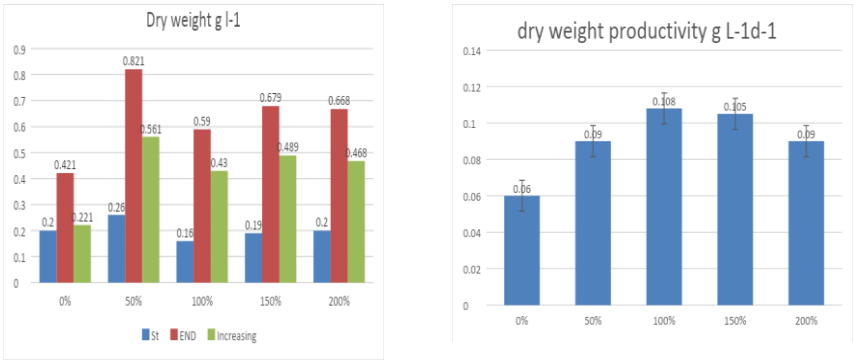

Fig. 3: a) Dry weight and b) growth rate of *Amphora coffeaeformis* under different Mg Nitrate concentrations.

Table 2. Dry weight growth parameters of *Amphora coffeaeformis* under different Mg Nitrate concentrations

Treatment	X_o	X	μ	G	N	Y
F2 (Control)	0.21	0.421	0.06	11.1	1.07	210
50% (2.6 ppm Mg)	0.26	0.821	0.09	7.3	1.65	315
100% (5.2 ppm Mg)	0.16	0.59	0.108	6.3	1.88	368
150% (7.8 ppm Mg)	0.19	0.679	0.105	6.5	1.81	352
200% (10.4 ppm Mg)	0.22	0.668	0.09	6.9	1.7	360

X_o= biomass at zero time ($g.l^{-1}$); X= biomass at the end of experiment ($g.l^{-1}$); μ= growth rate ($g.d^{-1}$); G= doubling time; n= Degree of value multiplication and Y= percentage increase

A distinction was made between the Mg^{2+} ions adsorbed onto the cells and the absorbed Mg^{2+} ions which were more strongly associated with the biomass. The cells became saturated with absorbed ions at approximately 46.1 mg $Mg^{2+}.g^{-1}$ of dried biomass, but the amount of adsorbed Mg^{2+} ions associated with

the biomass increased in proportion with the initial concentration of these ions in the medium.

The original concentration of Mg in N11 medium was 50 $mg.l^{-1}$. Increasing the concentration showed positive effects on biomass yield, and at 3 folds concentration (150 $mg.l^{-1}$) biomass yield was raised up to 1.6 $g.l^{-1}$ (33% rise) for *Chlorella vulgaris*, and 1.5 $g.l^{-1}$ (36% rise) for *Scenedesmus obliquus* on the 18^{th} day of incubation (**Gorain *et al*., 2013**). Absence of Mg is expected to hinder cell division, cessation of chlorophyll synthesis and, hence, the growth yields (**Finkle and Appleman, 1953**). Also, there was no inhibition of *Chlorella vulgaris* growth at the highest Mg^{2+} ion concentration. This supports the results reported by **Sarma *et al*. (2014).**

Furthermore, increasing Mg concentration in the culture medium showed positive effect on biomass yield (**Ayed *et al*., 2015**). High doses of Mg induced high biomass production, as chlorophyll, the main molecule responsible for trapping the light energy, requires one magnesium ion for synthesis of each chlorophyll molecule. Rise in chlorophyll synthesis with increasing Mg concentration was also observed by earlier workers. (**Finkle and Appleman, 1953**). Microalgal suspensions are generally stabilized by a negative surface charge on the cell which is generated by hydroxyl, carboxyl, phosphate and/or sulphate groups (**Vandamme *et al*., 2012**) and these could bind positively charged ions such as Mg^{2+} ions (**Sukenik and Shelef, 1984**).On the other hand, Under Mg starvation, growth of both the microalgae was severely affected, whereas a marginal rise in cellular lipid content (% dcw) over control was recorded. Absence of Mg is expected to hinder cell division, cessation of chlorophyll synthesis and, hence, the growth yields. Furthermore, increasing Mg concentration in the culture medium showed positive effect on biomass yield. High doses of Mg induced high biomass production, as chlorophyll, the main molecule responsible for trapping the light energy, requires one magnesium ion for synthesis of each chlorophyll molecule. Rise in chlorophyll synthesis with increasing Mg concentration was also observed by earlier workers. (**Finkle and Appleman, 1953**).

Phyco-chemical compositions of *A. coffeaeformis*

Most of the factors affecting biochemical composition and lipid content were analyzed, such as nutrient starvation, temperature, irradiance, salinity, oxidative stress, metals, CO_2 flux, pH and metabolic engineering (**Morales *et al*., 2021**). The biochemical components of algae like protein, carbohydrates, and lipids have been used in different industries of food, cosmetics, and medicines. The dynamics of microalgae growth, as well as inorganic carbon and nutrients uptake, were extensively studied during the pond start-up and semi-continuous feeding conditions **(Kumar *et al*., 2017**).

Application of preliminarily obtained data on algal biomass scale revealed the enhancing effect of outdoor growth which might return to the technical effect of light and temperature. In addition, the used growth unit (14L) has advanced specification including turbulence and high light exposed area. Chemical analysis of the produced alga (Table 3) showed the increase of lipid content on the expense of other cell metabolites revealing that the rise in lipid content and biomass accumulation as well.

Table 3. Some biochemical composition of *Amphora coffeaeformis* grown at high Mg ions concentration

DW	Crude Protein	Carbohydrates	Oil	Mois.	Ash
$g.l^{-1}$	%				
1.38	31.02	28.37	13.2	10.74	12.91

Data were found in some contrast with these obtained by **El-Sayed *et al*. (2018)** who found that carbohydrates of *Amphora coffeaeformis* reached 33.60% on dry weight basis associated with 15.74% of crude protein, 30.43% of ash and 10.5% of moisture. Such differences could be attributed to the effect of both environmental conditions in concern growth unit and nutritional factor as Mg ions concentration was raised to 3 fold of those received from original growth medium. Beside, Diatoms produce oil drops that are as a reserve material during the vegetative period of growth, with percentages that vary from 23% to 45% of dry cell weight. Physiological and genetic manipulations have also shown the

possibility of increasing the amount of lipids in the cellular mass (**Hildebrand *et al.*, 2012**); while **Rajaram *et al.* (2018)** mentioned that oil content of *Amphora coffeaeformis* ranged from 18.68 to 36.16 based on the examined strain.

It was early mentioned that unfavorable nutritional conditions both deficiency or excess markedly limit the growth and profile of the grown alga. Mostly, such conditions are associated with the decrease in protein and chlorophyll content with a rise in carbohydrates and oils (**El-Sayed *et al.*, 2010**). Otherwise, cellular lipid content showed a significant rise under increasing Mg supplementation. The role of Mg ions in activating the enzyme acetyl-CoA carboxylase, catalyzing the first step of fatty acid biosynthesis, is well established (**Nelson and Cox, 2008**).Moreover, the chloroplast pyruvate dehydrogenase complex that provides acetyl-CoA and NADH for fatty acid synthesis has a very high requirement for Mg ions (**Camp and Randall, 1985**). The overall lipid production ($mg.l^{-1}$) was found to be decreased significantly which could be attributed to the decrease in biomass yield under Mg starvation (**Gorain *et al.*, 2013)**.

Conclusion

Magnesium is the most important element required for proper algal growth and its efficiency could be maximized through the use of nitrate form which more cheap, available and containing a sufficient amount of nitrogen instead of the nitrate from the original or urea from the modified form.

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النمو المتباين لطحلب امفورا كوفيفورميس تحت معاملات مختلفة لمصادر نتروجينية

محمودعلي مرزوق[١]، أبوالخيربدوي السيد[٢]، وأحمددرويش الجمل[١]

١ - قسم النبات والميكروبيولوجى ،كليةالعلوم (بنين) ،جامعة الأزهر،القاهرة،مصر.

٢ - وحدةالطحالب الحيوية،المركزالقومي للبحوث،الدقي،القاهرة،مصر.

تم تنمية طحلب *Amphora coffeaeformis* من الطحالب العصوية في ظروف معملية باستخدام مصادرمختلفة من النيتروجين بمافي ذلك نترات المغنيسيوم بدلاًمن نترات الصوديوم أواليوريا لتقييم ما إذا كانت إضافة أيونات المغنيسيوم تؤثر على تراكم الكتلة الحيوية للطحالب المزروعة قيد الدراسة. فيما يتعلق بتأثير مصادر النيتروجين المختلفة على نمو*Amphora* ، أوضحت النتائج أن 59.98 مجم / لترمن$Mg(NO_3)_2$ أعطت أفضل نتائج في النمو من خلال تقدير إنتاجية الكتلة الحيوية للوزن الجاف للطحلب،وأفضل تركيز في زيادة الوزن الجاف ومعدل نمو (0.079) الطحلب يليه تركيزاليوريا ثم حمض النيتريك. كما تم دراسة التركيزات المختلفة من نترات المغنيسيوم وأظهرت النتائج أن 50٪ من تركيز نترات المغنيسيوم أدى إلى زيادة بشكل ملحوظ في تراكم الوزن الجاف للطحلب تحت الدراسة مقارنة بالكنترول وجميع المعاملات الأخرى. من ناحية أخرى، نتج عن استخدام 2 و 3 أضعاف من تركيزالمغنيسيوم (100 و 150٪) أقصى نشاط من معدل النمو (0.108 و 0.105)على التوالي. أوضحت النتائج أن زيادة تركيز نترات المغنيسيوم أدى إلى مضاعفة الوزن الجاف كما ان تركيز 1.5 ضعف من نترات المغنيسيوم أدى إلى حد أقصى للنمو عند (1.38 جم.ل$^{-1}$). تم تنمية الطحلب على نطاق أكبر خارج المعمل للحصول على الكتلة الحيوية للأمفورا بناءً على البيانات المعملية التي تم الحصول عليها باستخدام مفاعل حيوي ضوئي ابعاده 14 × 15 لتربسعة نهائية قدرها 210لتر. تم قياس معايير النمواليومية مثل الوزن الجاف .($g.l^{-1}$) كما تم تحليل النمو من خلال حساب معدل النمو (μ) ؛مضاعفة الوقت (جم) والنسبة المئوية للزيادة (Y). أوضح التحليل البيوكيميائي لطحلب *Amphora* (٪) باحتوائه على 31.02 من البروتين الخام، 28.37 من الكربوهيدرات الكلية، 13.2 من الدهون و 12.91 من الرطوبة تحت ظروف المعملية المقاسة. بينت الدراسة الحالية امكانية استخدام بدائل رخيصة من المركبات المحتوية على النتروجين مثل نترات الماغنسيوم كبديل عن نترات الصوديوم المركب الاساسي والرئيسي للعديد من المنابت الطحلبية المستخدمة كما كان لها تأثير ملحوظ في النمو والتركيب البيوكيميائي لطحلب امفورا مقارنة بالكنترول والعديد من المركبات النتروجينية.