Growth of the green alga *Chlorella vulgaris* as affected by different carbon sources

[1]Battah M. G. [2]El-Sayed, A.B. and [1]El-Sayed, E.W

[1] Botany Department, Faculty of Science, Benha University, Egypt.
[2] Algal Biotechnology Unit, National Research Centre, Dokki- Cairo, Egypt.
bokhair@msn.com

Abstract: Growth of the green alga *Chlorella vulgaris* was studied under BG-II growth medium conditions enriched with different carbon sources. The tested carbon sources were carbon dioxide, sodium acetate, acetic, oxalic and citric-acids. The effect of urea carbon was also eliminated. Dry weight, total chlorophyll and carotenes were daily determined. Urea nitrogen cultures; as a carbon source; exhibited more dry weight comparing with those of nitrate cultures. Concerning carbon source, acetate salt cultures surpassed all of other examined carbon sources. An opposite manner was observed on chlorophyll accumulation, where, nitrate cultures represented the maximum. Maximum carotene content was observed with sodium acetate cultures due to the presence of sodium ions. On the average, biomass growth rate was higher with urea nitrogen and acetate followed by carbon dioxide.
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1. Introduction

Photosynthesis is the key turn making solar energy available in useable forms for all organic life, where photosynthetic microorganisms including algae are able to use such energy to combine water and carbon dioxide to create life biomass. It has long been recognized that a significant portion of the inorganic carbon fixed in actively growing photosynthetic algae is released as dissolved organic carbon **(Bertilsson and Jones, 2002)**. Maximum releasing of total fixed carbon as extracellular dissolved organic carbon (DOC) is closely depending on the nutrients availability that increased by nutrient limitation **(Anderson and Zeutschel, 1970; Mague *et al.*, 1980; Fogg, 1983; Lancelot, 1983; Obernosterer and Herndl, 1995; Malinsky-Rushansky and Legrand, 1996; Moran and Estrada, 2002)**. Most of microalgae are photo-autotrophs, while some microalgae can use organic carbon substances as a source of energy and carbon for cell growth. For some microalgae, photosynthesis and the oxidative phosphorylation of organic carbon substances seem to function independently. The growth rate in mixotrophic conditions is approximately the same as a sum of the growth rate in the photo-autotrophic and heterotrophic cultures, such as *Chlorella regularis* (**Endo *et al.*, 1977**); *Chlorella vulgaris* (**Ogawa and Aiba 1981**); *Spirulina platensis* (**Marquez *et al.*, 1993).**

Organic carbon metabolism may exert an opposing influence on photosynthesis. Glucose can reduce the apparent affinity for CO_2 in CO_2 fixation in some algae species such as *Chlorella sp*. (**Lalucat *et al.*, 1984**), and *Chlorella vulgaris* (**Martinez and Orus, 1991**). Growth of cells under increasing concentrations of acetate culture reduced the photosynthetic CO_2 fixation and net O_2 evolution, without effects on respiration and photosystem II (PS II) efficiency (**Heifetz *et al.*, 2000**). Acetate can also reduce carbonic anhydrase (CA) activity (**Fett and Coleman, 1994**). On the other hand, **kindle (1987)** and **Goldschmidt-Clermont (1986)** showed that acetate can inhibit the light-harvesting chlorophyll a/b binding gene. In the unicellular green alga *Chlorogonium elongatum*, acetate inhibited the synthesis of Calvin cycle enzyme ribulose-bisphosphate carboxylase/oxygenase (RuBPCase) and its mRNAs (**Steinbiß and Zetsche, 1986**). For microalgae that are able to survive hetero-trophically, exogenous carbon sources offer prefabricated chemical energy, which the cells often store as lipid droplets (**Ratledge, 2004**).

Hetero-trophically cultivated alga *Chlorella protothecoides* has been shown to accumulate as much as 55% of its dry weight as oil, compared to only 14% in cells that grown photo-autotrophically (**Wu and Miao, 2006**). Another natural mechanism through which microalgae can alter lipid metabolism is the stress response owing to a lack of bio-available nitrogen (**Tornabene *et al.*, 1983**). Moreover, other factors triggered oil accumulations were also early recognized. Although nitrogen deficiency appears to inhibit the cell cycle and the production of almost all cellular components, the rate of lipid synthesis remains higher, which leads to the accumulation of oil in starved cells (**Sheehan *et al.*, 1998**). Interestingly, nitrogen deprivation also promotes the accumulation of the antioxidant pigment astaxanthin in the green alga *Haematococcus pluvialis* (**Boussiba, 2000**). Both of these adaptive responses help to ensure the cells'

survival during times of stress, while lipids serve as energy stores, astaxanthin seems to play a role in the protection against reactive oxygen species.

Microalgae are expensive to produce, and different systems have been designed for the growth and handling of microalgae on a large scale (**Gudin and Chaumont 1980; Richmond 2004; Tredici, 2004; Weissman *et al.*, 1988**). From an industrial standpoint, lipid triggers can be useful for the production of polyunsaturated fatty acids (*e.g.* omega-3) and biodiesel from algal triacylglycerides. Addition of at least 10% of nutrients mainly nitrogen are required to overcome the dry weight accumulation failure (**El-Shafey *et al.*, 1999**). The present work was achieved to determine the effect of different carbon sources; versus industrial carbon dioxide; on growth metabolites mainly dry weight and pigmentation of the green alga *Chlorella vulgaris*.

2. Materials and Methods

Alga and growth conditions

The green alga *Chlorella vulgaris* (NRC); was heterotrophically grown under optimum conditions of BGII nutrient solution (**Stainer *et al.*, 1971**) to obtain the proper inoculums. Continuous light illumination was provided from day light lamps (10x40w) reflexes from one side. Aeration was performed by free oil compressed air from the upper hold throughout 3mm polyethylene tube. Room temperature was recorded to be 27±2^{o}Cduring the whole incubation period. Incubation was employed within fully transparent polyethylene bags (75cm length x5cm diameter and 100µ thickness) containing 2.5 L of the algal broth (**El-Sayed and El-Fouly, 2005**).

Carbon Sources

The original concentration of nitrogen (1.5 g.l^{-1} $NaNO_3$) was substituted by urea at 0.53 g.l^{-1} **(El-Sayed *et al.*, 2001)**. The initial carbon content of such urea concentration was the base of calculation in concerning other carbon sources including acetic acid, sodium acetate, citric acid and oxalic acid. The initial carbon content of 0.53 g.l^{-1} urea was found to be 0.2473 g carbon monoxide (CO) equal to 0.5622 g of acetic acid (the-mono-carboxylic); 0.417 g of oxalic acid (the di-carboxylic) and 0.5943 g citric acid (the tri-carboxylic). Concerning sodium acetate that used as a part of growth media for vegetative growth enhancement it was also used based on its carbon content at 0.804 g.l^{-1}.

Growth measurements

The investigated parameters were dry weight, total chlorophyll and total carotene. For dry weight estimation, 5ml from each replicate were filtered over a pre-weighted Whatman sterile membrane filters (pore size 0.45µm, 0.47 mm in diameter and white grade). After filtration, filters were left to dry for 30 minutes at 105 °C by circulated oven and then kept over anhydrous calcium chloride till room temperature and then re-weighted. The difference between weights monitored the net dry weight of the grown alga within defined sampling time .the dry weight was calculated as g.l^{-1}.

Total chlorophyll was extracted by dimethyl sulfoxide (DMSO) according to **Burnison (1980)**. Five ml of algal suspension was centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded and the residual pellet was re-suspended in 5 ml of 95% DMSO, homogenized and kept for 5 minutes at 70 °C water bath. Such extract contains total chlorophyll and cell carotenoids. The extracted cells were re-centrifuged again at 3500 rpm for 5 minutes. The extracted solution was measured by reading the absorbance (A) of the pigment extract by spectrophotometer at 666m. Total chlorophyll content was calculated (mg.l^{-1}) according to **Seely *et al.* (1972)**. To recover carotenes, saponification of the algal pellet was performed by 5% KOH/30% MeOH and the residual was re-extracted by DMSO after the addition of 5 drops of concentrated acetic acid **(Boussiba *et al.*, 1992)**. Carotenes absorbance was measured at 468nm and concentration was calculated (mg.l^{-1}) according to **Davies (1976)**. Growth rate (µ) was calculated using the methods adopted by **Pirt (1973)**.

3. Results and Discussion

Dry weight

Growth dry weight of the green alga *Chlorella vulgaris* (Fig.1a) was dramatically varied as they grown under different carbon sources even with non-universal used carbon source like urea. Thus, it could be mentioned that urea surpasses nitrate growth own to the initial carbon generated from urea utilization. This finding is in the harmony with those early obtained by **El-Shafey *et al.* (1999)** who reported that urea at 0.53g.l^{-1} could replace the nitrate content at 1.5g.l^{-1}.

When such cultures were supported by gaseous carbon source (1.5% CO_2); a considerable increase in dry weight accumulation was observed. The variation between the two results might be return to the effect of carbon on alga growth. Data also showed a slight inhibitory effect on dry weight accumulation by such alga as they fed by carbon in the presence of nitrate nitrogen. The inhibitory effect could be ascribed to the liberated sodium as cells stimulate nitrate nitrogen.

Regarding the effect of some organic carbon sources, variable results were obtained. Nitrate nitrogen cultures supported by CO_2 exhibited the greatest positive effect as compared with acetic carbon cultures result. On the contrary, carbon as sodium

acetate represented slightly lower result which might goes back to liberated sodium ion **(El-Shafey *et al.*, 1999)**. In this connection, supplying growth media by super optimal concentrations of urea as N source enhanced vegetative growth of *Scenedesmus* sp. This might be led to increase some nutrient solubility and/or to decrease pH values to near acidic reaction, Here; urea also acts as a complementary source of carbon (**El-Sayed. *et al.*, 2008)**.

The high initial carbon content of urea (46.5% CO) with high solubility rate to form carbonic acid might be enhanced the vegetative growth of algae. Furthermore, the cleavage of urea molecule might allow the fast utilization of ammonia nitrogen part by the used alga. Urea degradation as a nitrogen source involves two specific enzymatic systems (urease and urea amydolayase); which are absent in algal metabolic matrix. The degradation might be achieved by bacteria; or due to the media reaction, mainly acid reaction, light, aeration and/or hydrolysis by extracellular algal excretions. Other organic carbon including citric and oxalic-acids showed the lowest increase on dry weight accumulation. Here, the slight inhibitory effect of oxalic and citric acids on growth could be return to their effect on the acid reaction of growth media.

Also, growth as dry weight was enhanced at first by the addition of citrate wastes even at the higher concentrations. This would be mainly due to the presence of organic carbon that allows the fast carotenoids accumulation on the expense of carbohydrate metabolism and green pigments. Addition of extra citrate amount would increase the salt potential of the growth medium; even though they are serve as utilizable nutrients as a source of different macronutrients including carbon, nitrogen, phosphorous, potassium and some micronutrients **(El-Sayed, 2010 and El-Sayed *et al.*, 2012)**. The dry weight in case of oxalic acid was lower than in case of $NaNO_3$ and this might due to acidity of the solution which negatively affects the biomass accumulation.

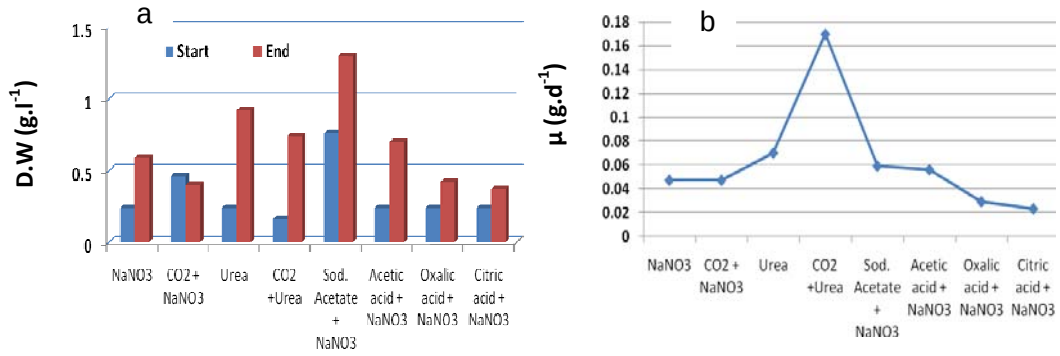

Fig. 1. a) Dry weight (g.l^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

On the average, growth rate was higher with urea nitrogen cultures as compared with nitrate cultures due to the extra nitrogen quantities from urea metabolism (Fig.1b). This finding could be confirmed as both cultures were enriched by carbon dioxide. Among the various organic carbon sources used, acetate carbon represented the maximum. The enhancing effect of acetate might be goes back to their lower molecular weight that making it easy to stimulate via carotene fixation as compared with oxalic or citric acids.

Total Chlorophyll

On the contrary with dry weight accumulation data, nitrate nitrogen surpasses ammonical nitrogen regarding chlorophyll accumulation (Fig.2a). The effect of CO_2 supplementation was similar in both nitrogen sources (nitrate and urea). On the other hand, acetate carbon represented wide variations, where acetic carbon enhanced the biosynthesis of chlorophyll as compared with urea cultures, but less in nitrate case. Furthermore, sodium acetate enhanced chlorophyll accumulation of nitrate cultures and inhibited those of urea cultures. This finding was in agreement with **Belcher (1968);** who reported that 10 mM of acetate increased oxygen consumption by *Botrycoccus*

braunii; however **Weetall (1985)** showed that sodium acetate had no effect on the growth of *Botrycoccus braunii*. In addition, **Tenaud *et al.* (1989)** reported that 5 mM acetate did not affect growth, but significantly increased respiration and inhibited photosynthesis. Sodium acetate effect seems to be concentration depending, where its variable concentration used 5-45mM depending on growth stage. Low concentration enhanced vegetative growth, while higher concentration resulted in chlorophyll de-composition especially in the presence of oxygen species agent like ferrous sulfate. As early observed by **Droop (1954)** and **Borowitzka *et al.* (1991)**, acetate at small quantities; appeared to be an important carbon source; enhancing both growth and carotenogensis, however, the effect of acetate was concentration dependent. Higher concentrations inhibiting growth, but markedly increasing astaxanthin content per cell. Acetate addition in excess may generate a relative shortage of nitrogen inducing cyst formation and astaxanthin accumulation triggered by a high C:N ratio **(Kakizono *et al.*, 1992)**. The algal cells seem to reduce their nitrogen uptake and begin to use the cellular nitrogen as in typical N-deficiency conditions, despite presence of nitrogen in the culture medium.

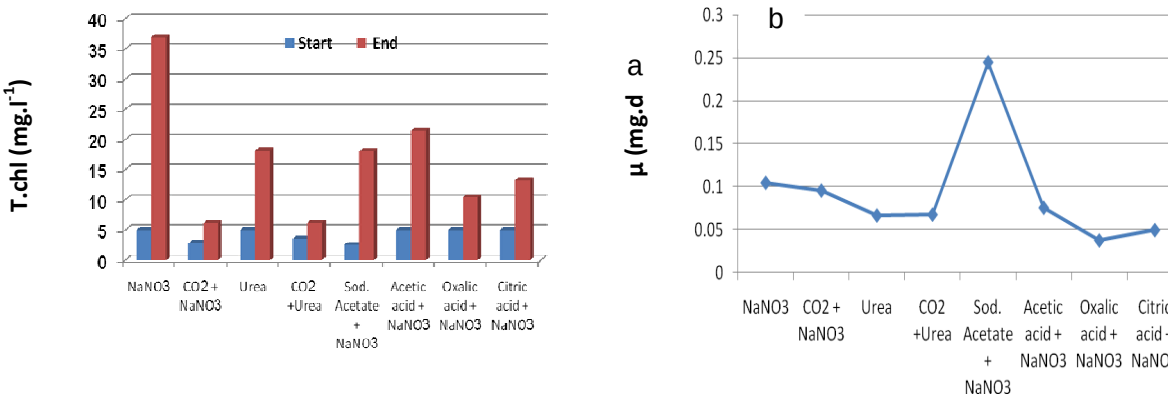

Fig. 2. a) Total chlorophyll (mg.l^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

Addition of more than 10 mM sodium acetate to the cultures increased chlorophyll fluorescence intensity, while it decreased with 20 mM acetate. However, in the presence of glucose under both light and dark culture conditions, the ratios of chlorophyll to dry weight were lower than those under autotrophic culture. The decline in chlorophyll content might be caused by inhibition of chloroplast development by the presence of glucose or by a relative increase in the level of other substances other than chlorophyll **(Tanoi *et al.*, 2011)**. Other organic acids such as oxalic and citric enhanced the chlorophyll accumulation. The last effect could be attributed to the possible assimilation of such organic acid in the absence of chlorophyll function.

On the contrary with dry weight results, growth rate; on the average; was found to be the maximum with nitrate cultures free or enriched by carbon dioxide suggesting the trigger effect of nitrate on chlorophyll accumulation rather than urea (Fig.2b). Acetate urea culture also represented the lowest. In general, Nitrate acetate cultures surpass all of the examined cultures.

Carotenes

Carotene's pigment in the green alga *Chlorella vulgaris* was affected by different carbon sources (Fig. 3a). A slight difference was observed on carotene content between cultures of nitrate or urea nitrogen due to the same nitrogen content regardless the liberated sodium ions. When nitrate cultures were enriched by carbon dioxide, a slight reduction was observed as compared with both nitrate or urea nitrogen free carbon. This might be goes back to the formation of carbonate compounds mainly sodium carbonate. Also, Carotene bio-accumulation is not conditioned by the presence of carbon dioxide, but di-carbon fragment is very necessary for fatty acid accumulation via carotene metabolism. This hypothesis could be confirmed by the obtained result of acetic acid supplementation of nitrate cultures that

reduces pH values and also inhibit carotene formation comparing with carbon cultures.

Oxalic and citric acid cultures exhibited extra inhibitory response on carotene formation. The most effective enhancing action was observed with cultures that fed with sodium acetate plus nitrate nitrogen. Influence of adding an organic carbon source such as acetate increases the growth rate and carotene level this agree with **Borowitzka *et al*. (1991); Kobayashi *et al*. (1993); Meyer and Du Preez (1993); Calo *et al*. (1995) and Orosa *et al*. (2005)** who, stated that acetate and malonate appear to be an interesting carbon sources, enhancing both growth and carotenogenesis. Moderate carotene production was observed when acetic acid and citric acid were used, but depression was occurred when oxalic acid was used due to the high acidic effect which lacking carotenogensis. Concerning growth evaluation (Fig.3b); sodium acetate represented the maximum due to the direct effect of sodium acetate on carotenogensis and librated sodium ion as a result of acetate consumption.

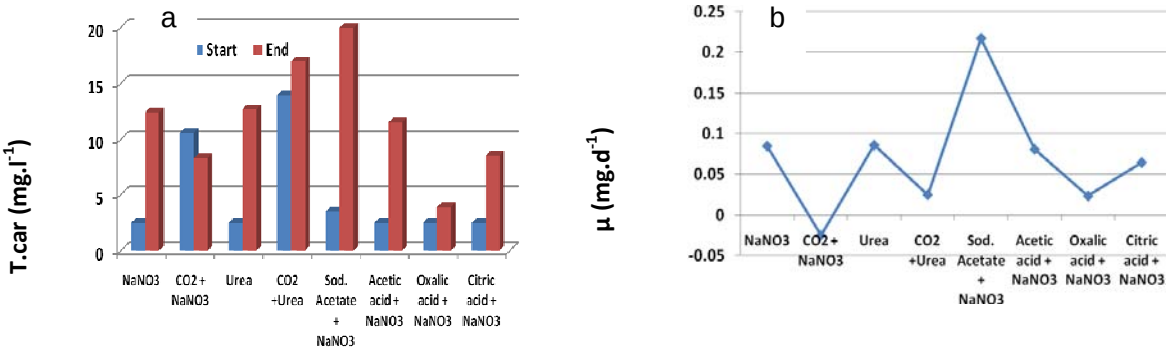

Fig. 3. a) Carotenes (mg.l^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

4. Conclusion

Nitrate was early recognized as the prefer nitrogen source for many algal species. Here, urea seems to be the best as providing algal growth media by extra carbon amount. *Chlorella vulgaris* able to grow and survive under the entire examined carbon source, but acetate carbon (acetic or sodium acetate) was the more efficient. This finding opens the way to use organic carbon wastes (starch effluent) for algae biomass production which in turn reduces the production costs.

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Corresponding author:
El-Sayed, A. B.
bokhair@msn.com

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