تأثير درجة حرارة التخمير وتركيز نترات الصوديوم في معدل النمو والكتلة الحيوية للطحلب المحلي *Chlorella vulgaris* المنمى في المفاعل الحيوي الدوار

Influence of Fermentation Temperature and Sodium Nitrate Concentration on Growth Rate and Biomass Production of Local Chlorella Vulgaris Using Stirred Tank Photobioreactor

Hussaam Okkou[1] Adnan Ali Nizam[2] Mohammad Naddaf[3] Mohammad Fawaz Azmeh [4]

(1) Department of Biodiversity, NCBT, Damascus, Syria.
(2) Plant Biology Department, Faculty of Science, Damascus University,Syria.
(3) Department of Food Science, Agriculture faculty, Tishreen University, Latakia, Syria.
(4) protection of Plant Department, Agriculture faculty, Damascus University, Syria

hus77sam@yahoo.com

الملخّص

تُعد الكلوريلا (*Chlorella*) مصدراً غذائياً مدهشاً يستحق الاهتمام، لذا نفذت هذه التجربة في الفترة الممتدة بين عامي 2013 و 2015 في محاولة لأمثلة بعض ظروف نموها لزيادة إنتاجيتها من الكتلة الحيوية. عُزل نوع الطحالب الخضراء المدروس بنجاح من المياه العذبة السورية ، ومن ثم حُضِّن لتنميته في مخابر الهيئة العامة للتقانة الحيوية في دمشق (سورية) باستخدام المفاعل الحيوي، تم دراسة تأثير ثلاث درجات حرارة (15، 25 و 35°س)، وثلاثة تراكيز من نترات الصوديوم (0.1، 0.25 و 0.4 غ/ل)، في الكتلة الحيوية ومعدل النمو للنوع *C. Vulgaris*، حيث سجل وزن الكتلة الحيوية الجافة الأعلى (0.361 غ/ل)، ومعدل النمو الأعلى (0.237/يوم) عند درجة حرارة 25 °س، ولم يلحظ وجود أي فروق معنوية سواء بين متوسطات الكتل الحيوية أو متوسطات معدلات النمو عند درجتي الحرارة 15 و 35°س. من ناحية أخرى، بيّنت النتائج أن تركيز النترات ليس له أي تأثير في إنتاج الكتلة الحيوية، أما فيما يتعلق بمعدل النمو، فقد سجلت أعلى قيمة (0.257/اليوم) عند تركيز 0.4 غ/ل من نترات الصوديوم، في حين لم يكن الفرق معنوياً بين تراكيز النترات الأخرى. يستنتج أن ظروف النمو المتمثلة بدرجة حرارة 25 °س وتركيز نترات الصوديوم 0.1 غ/ل هي الأفضل لأغراض إنتاج كتلة حيوية كبيرة من الكلوريلا.

الكلمات المفتاحية : *Chlorella vulgaris*، المفاعل الحيوي، الكتلة الحيوية، معدل النمو.

Abstract

The aim of this study was to optimize some culture conditions for the highest biomass production and growth rate of a local isolate of the microalgae *C. vulgaris*. The experiments were carried out between 2013 - 2015. Studied green algal strain was successfully isolated from Syrian freshwaters, and then incubated in the laboratory of national commission for biotechnology by bioreactors for the growth. The effect of three temperatures (15, 25, 35°C) and three sodium nitrate concentrations (0.1, 0.25, 0.4 g/L) on biomass production and growth rate of *C. Vulgaris* was determined. The dry biomass weight (0.361 g/L) and growth rate (0.237/ day) were highest at 25°C. No significant difference was observed neither between the averages of dry biomass or the averages of growth rate at 15°C and 35°C. On the other hand, the

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results showed that the nitrate concentration had no effect on biomass production, but not on the growth rate; as the maximum growth rate (0.257 day) was at 0.4g $NaNO_3$/L, with no significant difference with the other nitrate concentrations. So that, the recommended culturing parameters for high biomass production purpose were: 25°C and 0.1 g $NaNO_3$/L.
Keywords: *Chlorella vulgaris*, Photobioreactor, Biomass, Growth rate.

Introduction

Microalgae are a large group of fast growing unicellular or simple multicellular microorganisms (Wang *et al*., 2008) which have several advantages, including higher photosynthetic efficiency, higher growth rates and higher biomass production compared to other energy crops (Goswami and Kalita, 2011).

Among the microalgae, Chlorella species have the most desirable features for efficient and economic combination of CO_2 fixation, and wastewater treatment (Blersch *et al*., 2013). *Chlorella*, considered as a potential source of a wide spectrum of nutrients (e.g. carotenoids, vitamins, minerals), is used widely in the healthy food market, as well as for animal feed and aquaculture (Gouveia *et al*., 2008). *Chlorella* can be a health promoting factor on many kinds of disorders, such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes infant malnutrition and neurosis (Yamaguchi, 1997).

The most important species of *Chlorella* genus is *C. vulgaris*, due to its good growth ability and good tolerance of different environmental conditions (Yaakob *et a*l., 2014). *C. vulgaris* is highly valued for its protein and minerals content (Sankar and Ramasubramanian, 2012).

Recently, many studies reported various cultivation technologies for the production of microalgae (Olaizola, 2003). Open ponds are one of the primary and effective methods for the large-scale production of microalgae (Chisti, 2007). Photobioreactors are alternative methods (Lebeau and Robert, 2003; Sato *et al*., 2006) which provide a better opportunity to meet specific demands, and to optimize the control of cell growth parameters. In the design of Photobioreactors, many things need to be considered depending on what the end goal is (Sacasa-Castellanos, 2013). The most important and common design is Stirred-Tank Reactor (STR), being operationally and structurally simple, so it is an ideal device for cultivating different types of cells, including microalgae (Yang and Wang, 1992). STR can be beneficial in preliminary studies for optimizing the growth conditions, since it is easy to modeling and control the main experimental parameters (Sacasa-Castellanos, 2013).

Several strategies have been applied to improve microalgae growth, biomass production and lipid content. These include optimization of the medium composition (e.g., type of carbon source, nitrogen, phosphorus, vitamins and salts) (Mata *et al*., 2010), and physical parameters (e.g., pH, temperature and light intensity) (Rawat *et al*., 2013).

Temperature is an important element for growing algae. It strongly influences the growth rates for every species of algae. It is known that the growth rate will increase with the increase in temperature up to its optimum and then decrease drastically by increasing temperature (Cassidy, 2011). For *Chlorella vulgaris*, the optimum temperature ranges from 25 to 30°C (Cassidy, 2011), while Chinnasamy *et al*. (2009) reported an increase in biomass content at optimum temperature (30°C). *Chlorella vulgaris* can also grow in high temperatures up to 35°C (Converti *et al*., 2009).

Nitrogen was quantitatively the most important nutrient affecting the biomass growth and lipid productivity

of various microalgae (Griffiths and Harrison, 2009). Therefore, it is important to utilize the appropriate nitrogen source at a suitable concentration, (Yeh and Chang, 2012). Many studies investigated the effect of nitrogen sources and concentration on biomass growth. The growth of *Chlorella vulgaris* is proportionally correlated to increasing concentrations of sodium nitrate more than 3mM $NaNO_3$ concentration in Bisschoff and Bold medium (BBM) (Battah *et al*., 2014).

The aim of this study was to evaluate the effect of temperature and nitrogen at various levels on *C. vulgaris* growth using a modified Photobioreactor, and to identify optimal conditions for the cell growth and biomass production.

Material and Methods

Microalgae and culture conditions

This study was carried out at the National Commission for Biotechnology (NCBT) during 2012. *C. vulgaris* was isolated from fresh water ponds located in Quneitra Province (South of Syria). The species was identified in laboratory of Plant Biology, Faculty of Science, Damascus University In collaboration with prof. Mostafa EL-Sheekh-Tanta University, Faculty of Science, Botany Department, Egypt. The way of identification depending on Characteristics and Morphological feature of the isolate have demonstrated its close similarity with genus chlorella vulgaris. The individual cells of the colonies were in the range of 10µm. Cells are green color, unicellular, spherical in shape.

The solid algae broth medium (sigma) was used to isolate and purify the studied algae by Petri-Dishes. The samples were incubated in illumined incubator at 25◦C, 4000lux for 8 days. Then the inoculate were prepared by transferring the cells from Petri dishes, and incubated aseptically in 250 ml Erlenmeyer flasks containing 100ml liquid algae broth medium (Sigma Company). Erlenmeyer flasks were incubated in the same illumined incubator which has orbital shaker set at 150 rpm, temperature 25◦C and illumination 4000lux for 8 days, then the volume was increased up to 300 ml in 1000 ml Erlenmeyer flasks to form a stock culture to inoculate the experimental media after 8 days as Modified methods of (Wang *et al*., 2010).

All experiments were carried out in Stirred-Tank Reactor (STR), designed and realized in NCBT by the senior author. STR was equipped with PLC (programmable logic controller) that controls and monitors all growth parameters (temperature, light, pH, gas flow and mixing speed). Maximum capacity of the bioreactor was 14 L with a maximum working volume of 10 L.

Growth experiments were done at three different temperatures (15, 25 and 35◦C), and three different nitrate concentrations (0.1, 0.25 and 0.4g/L) in 10 L of BBM, and the initial cell concentration was set to be its optical density at 680nm (OD_{680}) 0.150. Each batch cultivation was carried out three times for 15 days at fixed parameters (continuous illumination 6000Lux, mixing speed 250 rpm, pH= 7, air mixed with CO_2 500ppm with flow rate 2L/min, (temperature 25◦C when the nitrate concentration was the variable, and nitrate concentration 0.25g/L when the temperature was the variable). The central values of temperature, 25◦C were chosen according to (Hernandez *et al*., 2009; Brown *et al*., 1998). Then, the additional experiments were done with increasing and reducing the growth temperature by 5◦C. the central concentrations of nitrogen in medium 0.25g/L were selected depending on (Guillard, 1975), and the additional cultivations were run at 0.1 and 0.4g/L.

-Estimation of Algal Growth rate /day (GR)(day)

Microalgae growth was monitored by measuring the optical density at 680 nm. Samples of the culture media were taken every day, for OD_{680} measurement using a spectrophotometer at wave length 680nm (HITACHI U-2900) as the algal density indicator. The growth rate was calculated by fitting OD_{680} in the following formula (Wang *et al*., 2010):

$$GR = (\ln OD_t - \ln OD_0)/t$$

OD_0: the optical density at inoculation day.

OD_t: the optical density measured on day t.

Each recorded OD_t was corrected by taking away that of the corresponding blank sample.

-Determination of Biomass Dry Cell Weight (DCW)

Dry Biomass content was determined according to the modified method of Yadavalli *et al*. (2012) by measuring OD_{680} using a spectrophotometer at wave length 680nm. The conversion factor was established by plotting OD_{680} versus DCW of a series of samples with different biomass concentrations. Samples were diluted by appropriate ratios to ensure that the measured OD_{680} values were within the range of 0.09–2.4. DCW of the sample was determined gravimetrically after drying, and collecting the algal cells by centrifugation (5,000 rpm, 10 min), and washing with water. The linear regression Equation was obtained for *C. vulgaris* species as described in Figure 1.

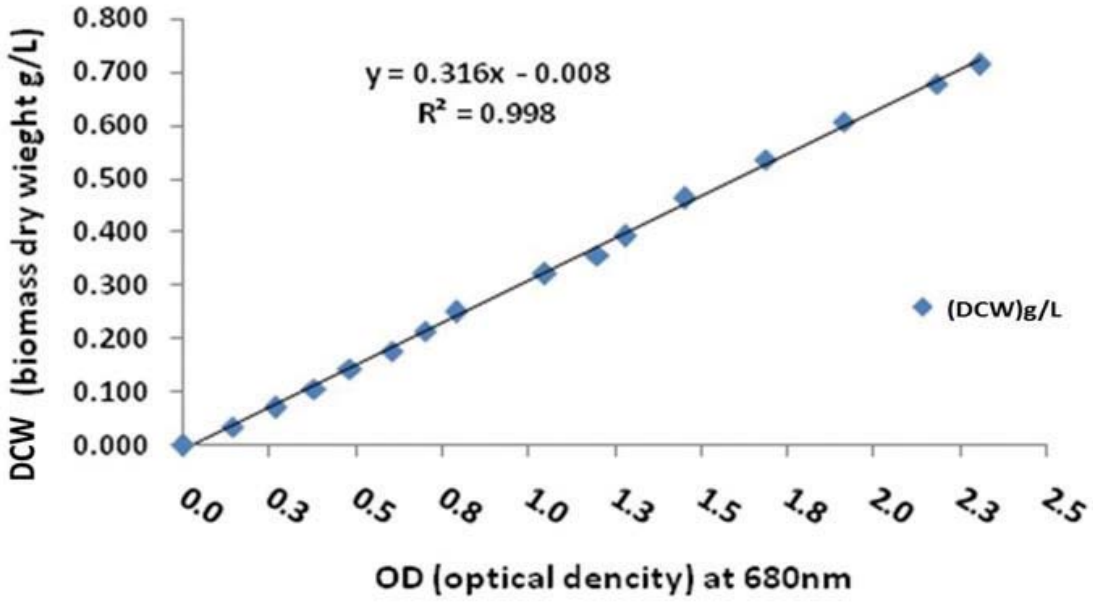

Figure 1. Linear regression equation of relationship between OD and dry biomass. (y): the DCW of algal biomass, and (x): the optical density at 680 nm.

Statistical analysis

The analyses of dry biomass and growth rate variance were performed for the cultures grown in the different culture conditions (Temperatures: 15◦C, 25◦C and 35◦C. Nitrate concentrations: 0.1g/L, 0.25g/L and 0.4g/L. pH: 6, 7and 8). The effect of the culture conditions on the dry biomass and growth rate were analyzed statistically. Significantly different mean values were established by means of one-way ANOVA followed by Tukey Test ($P < 0.01$). Statistical analyses were carried out using Statistical program (SPSS, 2010).

Results and Discussion

-Effect of temperature

Biomass production

The effect of temperature on the daily increase of dry biomass is expressed in Figure 2, which shows that the actual increase began after the second day of inoculation at temperatures 25°C and 35°C, while the increase at 15°C began after the third day.

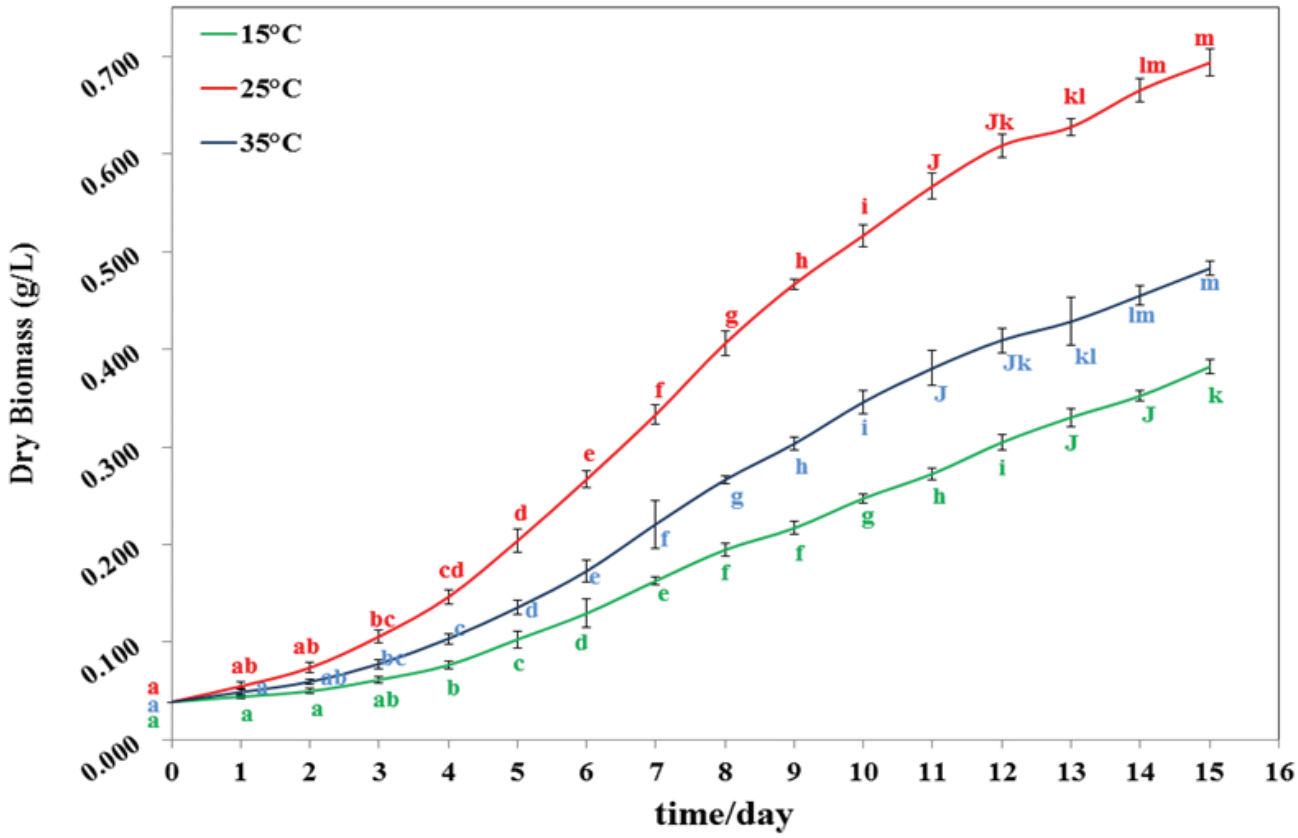

Figure 2. The Dry biomass curve in different temperatures. Different letters above the lines indicate to significant differences (p<0.01) among means within each temperature.

This is due to the Lag phase that algae need, to adapt with the growth medium according to (Spencer, 1954). Therefore, it has been noticed that the lower temperatures led to a longer lag phase. The next phase is the Exponential one, when the dry biomass significantly (P<0.01) increased by time at all temperatures. This result is consistent with (Becker, 1994) who explained the systematic duplication of algae cells during this phase. The highest values of dry biomass were obtained at 25°C, where the average value of the dry biomass at 25°C was 0.361 g/L (Table 1), this result converges with the result of Chinnasamy *et al*., (2009), which showed that the biomass increased significantly (P<0.01) near a temperature of 25°C in *C. vulgaris*.

The insignificance of differences (P<0.01) between biomass formed at 35°C and 15 C° suggested that, 15°C and 35°C had an similar effect on the algae biomass compared to the optimal temperature 25°C.

Growth rate

The highest average of the daily growth was at temperature 25°C (0.237/day) as shown in (table 1), this is consistent with Cassidy (2011) who obtained the maximum growth of *C. vulgaris* between 25°C and 30°C.

Also, no significant increase (P<0.01) in the average growth rate at 35°C (0.183/day) compared with that at 15°C (0.142/day) has been noticed. This corresponds to Converti *et al*., (2009) who indicated that *C. vulgaris* can grow well at relatively high temperature up to 35°C.

Table1. Means ±SD of dry biomasses and growth rate during the entire growth period at three temperatures and three concentrations of sodium nitrate.

		dry biomass ±SD (g/L)	Growth rate ±SD (Day)
°Temperatur	15°C	0.186 ± 0.116[a]	0.142 ± 0.047[a]
	25°C	0.361 ± 0.232[b]	0.237 ± 0.075[b]
	35°C	0.246 ± 0.155[a]	0.183 ± 0.056[a]
$NaNO_3$(g/L)	0.1	0.271 ± 0.182[a]	0.192 ± 0.058[a]
	0.25	0.361 ± 0.232[a]	0.237 ± 0.075[a]
	0.4	0.396 ± 0.246[a]	0.257 ± 0.087[b]

Different letter in each Column for each variable parameter indicates to a significant difference (p < 0.01) between means.

-Effect of nitrate concentrations

Biomass production

The effect of the concentration of sodium nitrate on the daily increase of dry biomass is shown in Figure (3). The daily increase during most days was significant (P<0.01) at all concentrations, and associated with the Exponential phase, which began in the second day of inoculation at concentration 0.4 g/L, but in the third one at both concentrations 0.1g/L and 0.25 g/L, since the high concentration of nitrate led to a rapid end of the Lag phase. Moreover, the highest values of dry biomass through all days was at concentration 0.4 g/L, followed by concentration 0.25 g/L then 0.1 g/L, Figure (3). However, these differences in dry biomass were insignificant (P<0.01), which may indicate to that dry biomass can be produced in the three concentrations. In other words, nitrate concentration does not have any significant effect on the biomass over the range of $NaNO_3$ 0.1 g/L to 0.4 g/L, this was confirmed by the results shown in the (table 1) which refers to the average of dry biomass (0.273, 0.361 and 0.396 g/L) during the total period of growth at three concentrations of sodium nitrate; 0.1, 0.25 and 0.4 g /L, respectively.

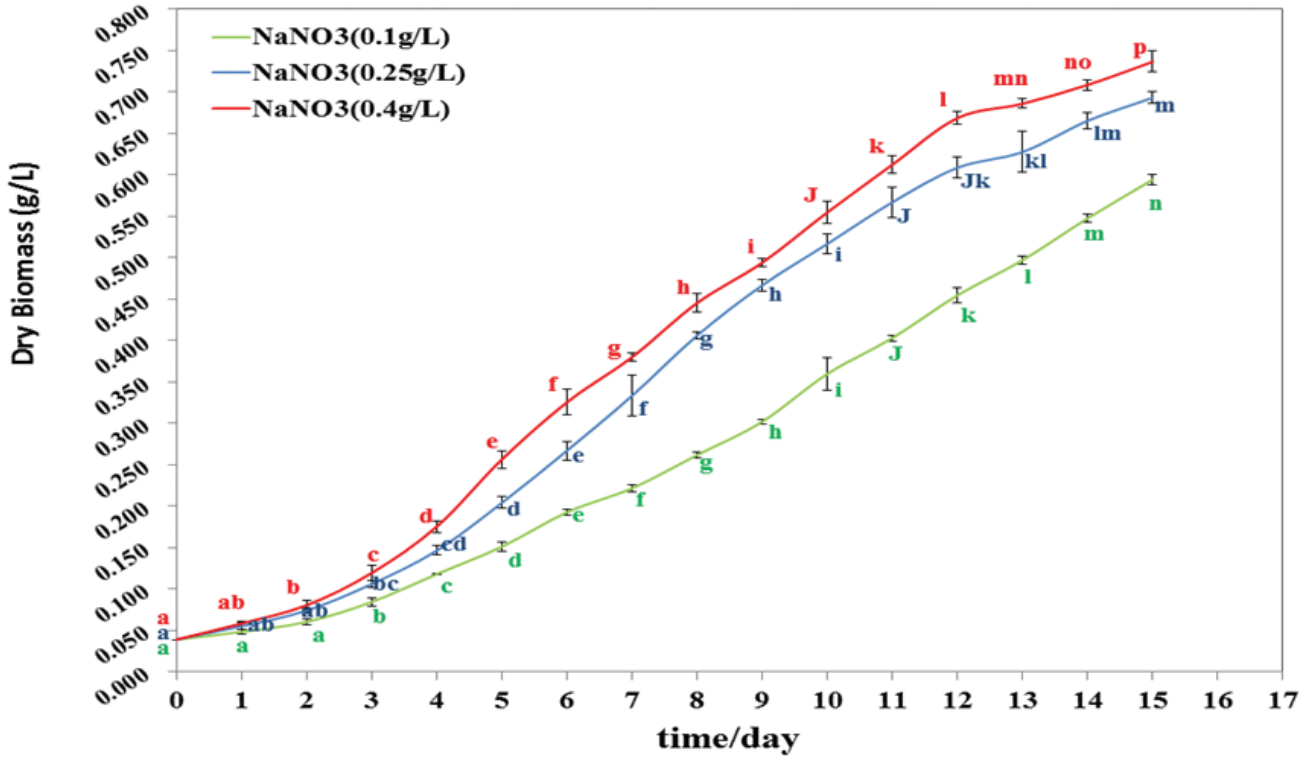

Figure 3. The dry biomass curve grown in the different concentrations of sodium nitrate. Different letters above the lines indicate to significant differences ($p<0.01$) among means within each concentration.

-Growth rate

Table 1 explained that the highest growth rate 0.257/day was at the highest sodium nitrate concentration (0.4 g/L), while no significant differences were recorded between the others, this reveals that there is no mutual effect between growth rate and sodium nitrate concentration within the range (0.1 g/L, 0.25 g/L), and this was incompatible with Battah *et al*., (2014), who suggested that increasing the concentration of sodium nitrate in growth medium above 3mM (0.25 g/L) commensurate with the increasing in the growth rate.

Conclusion

Fermentation at 25°C showed the highest value for biomass and growth rate. The highest growth rate of *C. vulgaris* was at the nitrate concentration 0.4g/L. By contrast, Biomass was not significantly influenced by nitrate concentration, so any increase in nitrate concentration was useless and not economic.

Therefore, we recommend culturing *C. vulgaris*, for its high biomass production purpose, at 25°C and 0.1 g/L $NaNO_3$.

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