Bioethanol Production from Green Alga *Chlorella Vulgaris* Under Different Concentrations of Nitrogen

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ABSTRACT

Ethanol fuel or ethyl alcohol is an alternative to gasoline; it can be used as additive to gasoline, and also as a feed chemical in the transesterification process for biodiesel. A number of bio feed stocks are currently being experimented for biofuel production; algae have emerged as one of the most promising sources for biofuel production. The locally isolated microalga chlorella vulgaris was used in the current study to test their ability to production bioethanol through stimulated in different nitrogen concentration treatments (0, 4, 8, 12 g/l), and effect of nitrogen concentrations on the content of primary products (carbohydrate and protein), also the yield of bioethanol.

The growth curves of C. vulgaris were different among the treatments. The stationary phase was identified as day 5, 9, 12 and 14 in treatments 0, 4, 8 and 12 g/l nitrogen respectively. The growth rate (K) increased from 0.14 to 0.20 for the treatments 8 g/l and 0 g/l respectively. The shortest doubling time (G) was 1.4 days in treatments 0 g/l while the longest was 3.0 days in 12 g/l. The carbohydrate content for C. vulgaris increased from 17.35% at 8g/l (control) to 24.60% at 4g/l treatment and 32.75% at 0g/l treatment of dry weight. While, the carbohydrate content decreased from 17.35% at control to 14.11% at 12g/l treatment. The protein content was decreased sharply when nitrogen concentrations decrease. It is recorded 51.17% at 8g/l (control), 33.60% at 4g/l treatment and 15.07% at 0g/l treatment. While, it is increased in treatment 12g/l in contrast with control treatment. The highest bioethanol yields of 27.08% and 24.09% were obtained in treatments 0g/l and 4g/l respectively compare with control which gave 17.34% ethanol. While, the lower bioethanol yields was recorded in treatment 12g/l which gave 14.87% in contrast with control.

The highest carbohydrate content and bioethanol yield were obtained under nitrogen starvation, but the protein content recorded the lowest content under nitrogen starvation.

Keywords: Biofuel, ethyl alcohol, bioethanol, biodiesel, *Chlorella vulgaris*, transesterification process

INTRODUCTION

Fossil fuel depletion has become a great concern as the world population is increasing at an alarming rate. Current concerns such as global warming, depletion of fossil fuels and increasing price of petroleum-based fuels have forced the search for alternative and cost effective energy sources with fewer greenhouse gas emissions (Jason et al., 2006; Krishna et al., 2001).

Research into the development of renewable and sustainable fuels has recognized bioethanol as a viable alternative to fossil fuels, owing to its low toxicity, biodegradability, and the ability to effectively combine with gasoline without any engine modifications (Harun et al., 2009).

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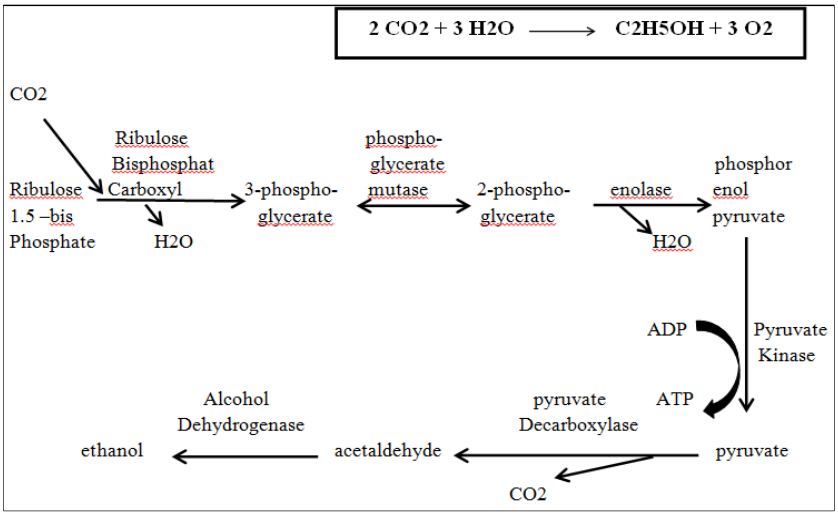
The utilization of crops such as sugar cane, sorghum and corn are considered as traditional approaches for bioethanol production (Jessup,2009).The use of such feedstock for bioethanol production competes in the limited agricultural logistics for food production thus escalating the "food versus fuel debate" (Borjesson and Tufvesson,2011 ; Vries et al., 2010). There has been a considerable interest in the use of microalgal biomass to replace food-based feedstock for renewable transport fuel production (Nigam and Singh, 2010).

Microalgae are photosynthetic organisms which have the ability to fix CO2 while capturing solar energy with efficiency of 10-15 times greater than that of terrestrial plants, and produced biomass for biofuels production (Khan et al., 2009). They have been suggested as very good candidates for fuel production because of their advantages of higher photosynthetic efficiency, higher biomass production and faster growth compared to other energy crops and they can grow partially anywhere also use far less water than traditional oilseed crops so there will be no competition with food crops also they are only feedstock that have the potential to replace transportation fuels (Maio and Wu, 2006). Certain species of microalgae have the ability of producing high levels of carbohydrates instead of lipids as reserve polymers. Ethanol is produced from the incomplete metabolism of glucose in plants, called alcoholic fermentation. Fermentation is the decomposition of organic compound in to simpler compound with the help of microorganism that produce energy (Hogg, 2005). Most commercial-scale ethanol fermentation is by yeast, one of *Saccharomyces cerevisiae* that produce ethanol and the bacterium *Zymomonas mobilis* (Hutkins, 2006). The production of ethanol through alcoholic fermentation is shown in scheme 1 (Emma and Rosalam, 2012).

The microalgae *Chlorella vulgaris*, particularly, has been considered as a promising feedstock for bioethanol production because it can accumulate up to 37% (dry weight) of starch.However, higher starch contents can also be obtained for optimized culture conditions (Hirone and Hirayama,1997). During photosynthesis, using only light and nutrients, algae produce lipids, proteins, and carbohydrates. The relative amounts of these metabolic products are tightly linked to environmental and nutrient conditions including: the amount and intensity of sunlight; CO2 levels; pH; temperature; available nutrients; and, the presence (or absence) of other organisms (Apt and Behrens, 1999). Cultivated *Chlorella vulgaris* and *Scenedesmus obliquus*, respectively, under nitrogen starvation conditions, which resulted to accumulation of carbohydrates. The carbohydrate-enriched biomass was used for bioethanol production (Ho et al., 2013; Miranda et al., 2012).



Scheme 1. The production of ethanol through alcoholic fermentation

The present study aims are isolation and purification of *Chlorella vulgaris* from local habitats, examining the ability of it to produce ethanol and catalyze ethanol production from it in different nitrogen concentrations.

MATERIALS AND METHODS

Isolation and Purification

C. vulgaris was isolated from ponds and artificial canal of University of Babylon. Modified Chu-10 was used for the algal growth shown in Table 1 (Kassim et al., 1999].

Table 1. The components concentration of modified Chu-10 medium and the concentration of each component

No of Stock Solution	*Chemical Formula of Each Salt*	*Concentration g/l*
1	$MgSo_4.7H_2O$	10
2	K_2HPO_4	4
3	$NaNo_3$	8
	$CaCl_2$	16
4	$FeCl_3$	0.32
5	EDTA-Na	4
6	NaCl	30
7	Na_2Co_3	8
8	$MnCl_2.4H_2O$	0.02
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.028
	$ZnSo_4.7H_2O$	0.224
	$CuSo_4.5H_2O$	0.08
	$CoCl_2.6H_2O$	0.004
	H_3BO_3	0.288
9	Na_2Sio_3	5.7

Serial dilution method and streaking on plate agar techniques were used for algae isolation and purification in this study. For algae cultivation, 10 ml of isolated culture was added to a flask containing 100 ml of Chu-10 media and incubated for 14 days, then transported to 1000 ml of media and incubated for 14 days; finally the growth was transported to glass pools 5 L for mass culture. The growth curve was determined for the studied algae. Cell growth was measured by determining the optical density (OD) daily. Optical density was measured by using spectrophotometer UV-VIS at 540 nm. All measurements of the study were triplicates. The growth rate (K) and doubling time (G) were obtained according to the following equations:

$$K = \frac{(\log OD_t - \log OD_0)}{t} * 3.322 \quad \text{(Huang et al., 2002a)}$$

$$G = \frac{0.301}{K} \quad \text{(Huang et al., 2002b)}$$

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t: time (day)

OD_t: Optical density after (t) day

OD_0: Optical density at beginning of the experiment zero time

Experimental Design

Different concentrations of nitrogen were used in the current study, to stimulate the isolated algae for production bioethanol. Nitrate was used as a source of nitrogen in media (NaNO3) by 8g/l and considered as control treatment in the study; also other three concentrations of nitrate (6, 4, 0 g/l) were used as treatments.

Determination of Protein and Carbohydrate

Algae samples were centrifuged by cooling centrifuge for 5000 r/min for 30 min, 4C° in the stationary phase. The supernatant was collected and the protein determined according to Bradford (1976) and the carbohydrate according to phenol sulphuric acid method (Dubios et al., 1956).

Hydrolysis Process

C. vulgaris as much as 50 ml put in Erlenmeyer and heated on a hot plate. Heating process lasts for ± 2 hours with a heating temperature of ±100°C and then cooled until the temperature reaches ±45°C (Zhang and Feng, 2010), and α-amylase enzyme is added with concentration 0.06 grams/50 ml substrate and incubated for 80 minutes (Sulfahari et al., 2011). Once hydrolyzed, hydrolysates filtered using filter paper to be taken supernatant. Supernatant was then centrifuged at 9000 rpm for 15 minutes. Centrifugation the supernatant was sterilized, and will be used for the substrate of fermentation.

Fermentation Process

Saccharomyces cerevisiae was added to a concentration of 10% (OD600nm = 0.5) into the 100 ml bottle fermented containing 50 ml of substrate C. vulgaris, incubated for 120 hour at room temperature (±30°C). If still experiencing increased levels of ethanol, the fermentation was continued. The fermentation process was stopped if ethanol levels have been reduced (Zhang and Feng, 2010).

Ethanol Analysis

Samples analyzed by HPLC system (Hong et al., 2014), model Knauer. HPLC consist from Aceternitril (100%) as a mobile phase, while the stationary phase is C18 column, dimension (25cm× 4.6mm ×5µm), injection flow is 1.2 ml/min, the absorption at UV 210, and ethanol standards used in the experiment were analytical grade and were purchased from Sigma-Aldrich. The samples were filtered by Millipore filter 0.45 µm, and the time taken for a sample to pass through the system is recorded as its retention time and is one of the characteristics used to identify the compound.

Statistical Analysis

General Treatment Structure was used as an experimental design. Data were analyzed by using gene stat discovery (2012) to study the effect of different nitrogen concentrations on the carbohydrate, protein and ethanol production. Least significant difference (LSD) was used to compare the significant difference between means at $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolated Algae and Effect of Nitrate on the Growth Culture

Chlorella vulgaris was obtained successfully, and it was identified according to Bellinger and Sigee (2010) and Prescott (1982).

Class: Chlorophyceae

Order: Chlorococcales

Family: Chlorococcaceae

Genus: *Chlorella vulgaris* Bejerinck

Different growth was observed for isolated algae in the treatments, figure 1 illustrates the effect of different nitrate concentration on *C. vulgaris* biomass growth. The biomass growth of *C. vulgaris* entered a stationary phase in different days among treatments. The stationary phase was identified as day 4, 8, 12 and 14 in treatments 0, 4, 8 and 12 g/l nitrogen respectively.

The growth rate (K) increased from 0.14 to 0.20 for the treatments 8 g/l and 0 g/l respectively. A significant difference were recorded in K value ($p \leq 0.05$) between control and all treatments except treatment 4g/l. Figure 2 shows the K value of *C. vulgaris* at different nitrate concentrations. The shortest doubling time (G) was 1.4 days in treatments 0 g/l while the longest was 3.0 days in 12 g/l. A significant difference were recorded in G value ($p \leq 0.05$) between control and all treatments except treatment 0g/l. Figure 3 shows the doubling time of *C. vulgaris* at different nitrate concentrations.

Effect of Nitrate on the Carbohydrate and Protein Content and Amount of Ethanol Production

Carbohydrate content of algae is an important parameter that determines the economy of bioethanol production from algae (Harun et al., 2010). The carbohydrate content for *C. vulgaris* increased from 17.35% at 8g/l (control) to 24.60% at 4g/l treatment and 32.75% at 0g/l treatment of dry weight. While, the carbohydrate content decreased from 17.35% at control to 14.11% at 12g/l treatment. Statistically there are significant differences among treatments.

The protein content of *C. vulgaris* was decreased sharply when nitrogen concentration decrease. It is recorded 51.17% at 8g/l (control), 33.60% at 4g/l treatment and 15.07% at 0g/l treatment. While, it is increased in treatment 12g/l in contrast with control treatment. Statistically there are significant differences among treatments. The present study revealed that concentration of nitrate is significantly affecting carbohydrate content of *C. vulgaris* especially at zero concentration which achieved higher carbohydrate content than control nitrate media and other treatments. Algae carbohydrate content usually increases at nitrogen starvation, because of all the carbon structure produced during metabolic process might be directed towards carbohydrate and lipid and that increased the carbohydrate content of algal cell while in the presence of nitrogen most of the carbon structure was incorporated in nitrogenous compounds like amino acids, proteins and nucleic acids (Giuliano et al., 2011; Afify et al., 2010; Widjaja, 2009).

Kilham et al. (1997) agree with this study, their study showed an increasing in carbohydrate content of the green algae *Ankistrodesmus falactus* 0.3 to 0.5 µg/µm under nitrogen starvation. Another study (Hassan et al., 2013) showed an increasing in carbohydrate content of the *C. vulgaris* from16.5% at 8g/l $NaNO_3$to 25% at 0g/l$NaNO_3$ and decreasing in protein content from 50% to 15% under nitrogen limitation. This change in growth parameters was

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also noticed in the present study that may be the limitation of nitrogen concentration in media growth limited protein biosynthesis thus increasing carbohydrate was recorded (Heraud et al., 2005; Lynn et al., 2000; Huang et al., 2002a).

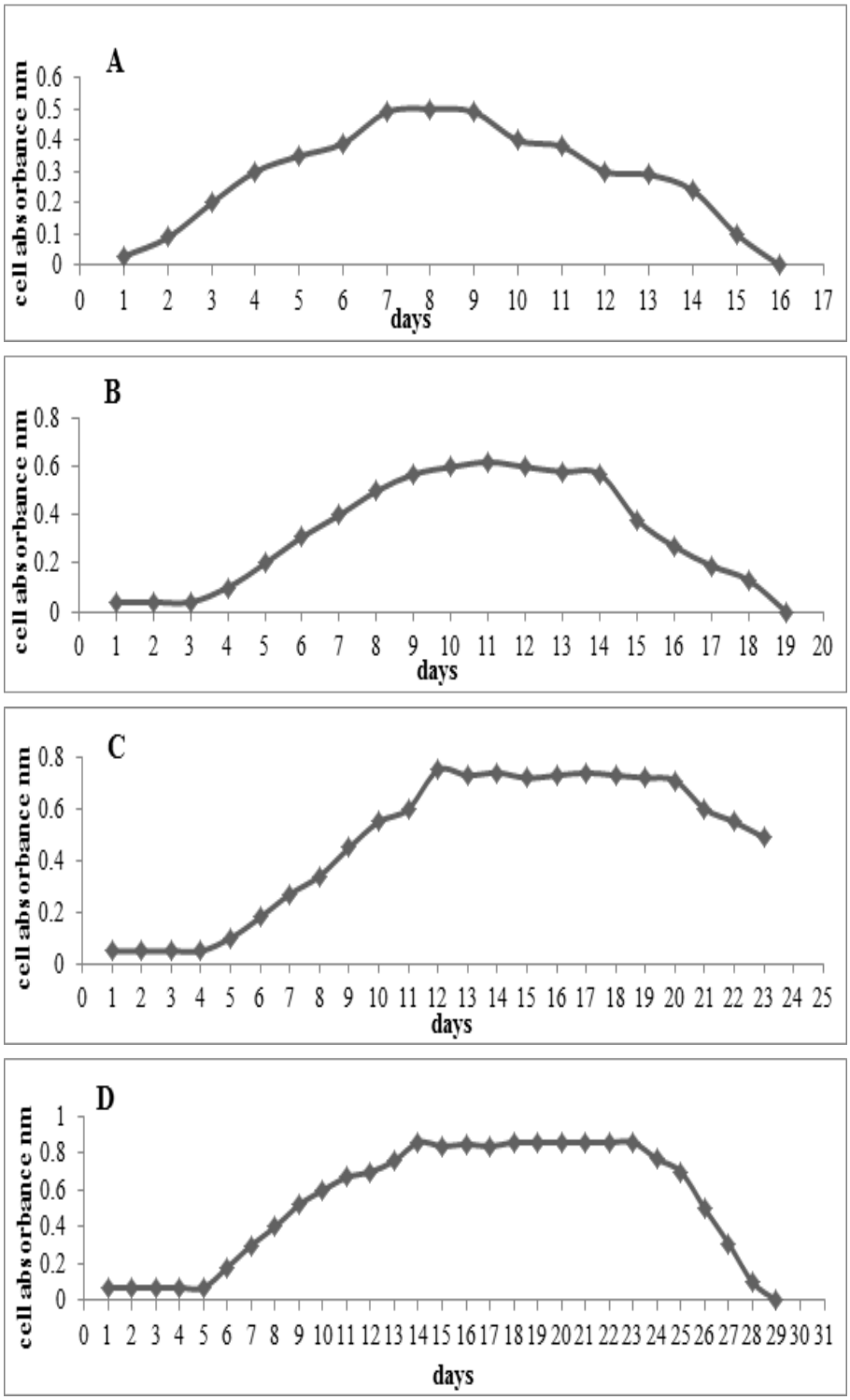

Figure 1. Growth curve of *C. vulgaris* at different nitrate concentrations g/l. (A)= 0. (B)= 4 (C)= 8(control) (D)= 12. Optical density (OD) measurements at 540 nm by UV-Vis spectrophotometer.

Cultivation conditions: light intensity 268 µE/m²/sec, temperature 25± 2 Cº and 16:8 light: dark period.

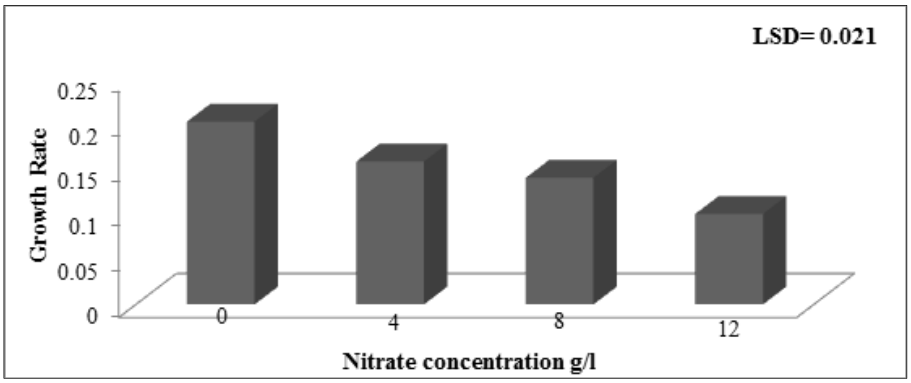

Figure 2. Growth rate (K) of *C. vulgaris* at different nitrate concentrations (g/l). Cultivation condition: light intensity 268 µE/m²/sec, temperature 25± 2 Cº and 16:8 light: dark period. LSD, least significant differences of means.

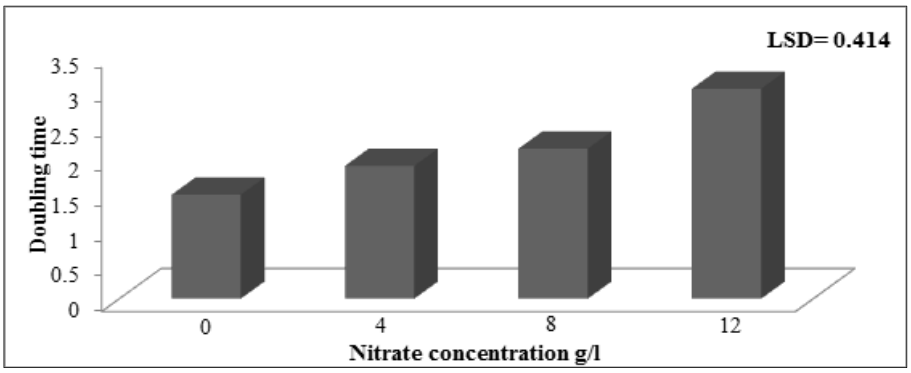

Figure 3. Doubling time (G) of *C. vulgaris* at different nitrate concentrations (g/l). Cultivation condition: light intensity 268 µE/m²/sec, temperature 25± 2 Cº and 16:8 light: dark period. LSD, least significant differences of means.

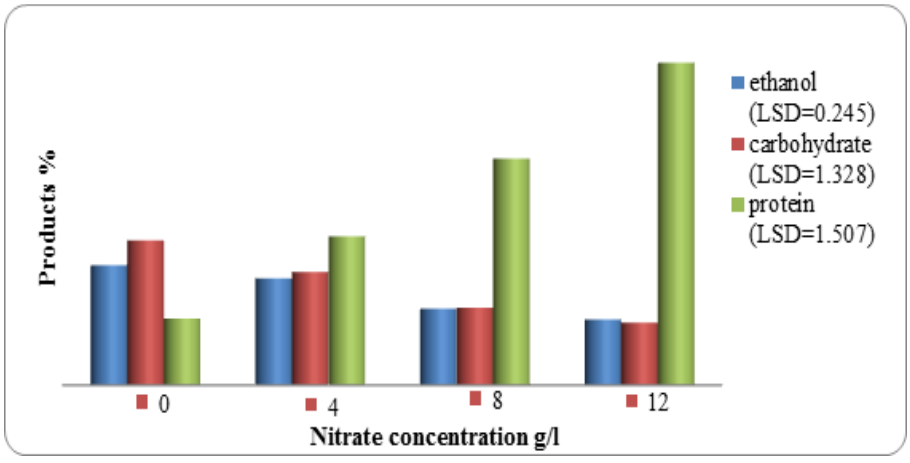

Figure 4. Ethanol, carbohydrate and protein content of *C. vulgaris* at different nitrate concentrations. Cultivation conditions: light intensity 268 µE/m²/sec, temperature 25± 2 Cº and 16:8 light: dark

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period. Fermentation conditions: incubated for 120 hour at room temperature (±30°C). LSD, least significant differences of means.

In the present study the potential of bioethanol production using carbohydrate-enriched biomass of the green algae *C. vulgaris* was studied. The bioethanol yield (%) was significantly affected by the nitrate concentrations. The highest bioethanol yieldsof27.08% and 24.09% were obtained in treatments 0g/l and 4g/l respectively compare with control which gave 17.34% ethanol. While, the lower bioethanol yields was recorded in treatment 12g/l which gave 14.87% in contrast with control. Significant differences were noticed among treatments. Figure 4 illustrates ethanol, carbohydrate and protein of *C. vulgaris* at different nitrate concentrations. These results showed when the nitrate concentrations decrease the carbohydrates content increase so the bioethanol yields increase too, because the carbohydrates consider as a substrate for bioethanol production by fermentation (Ho et al.,2013; Miranda et al., 2012 ;Choi et al.,2010; Nguyen et al.,2009 ; Huang et al., 2002b).

CONCLUSION

Microalgae biomass is a potential feedstock for biofuel production. In particular carbohydrate-rich microalgae can be used for bioethanol production. The different concentrations of nitrogen influenced the carbohydrate and protein content, and affecting on the bioethanol productivity of *C. vulgaris*. The highest carbohydrate content and bioethanol yield were obtained when the nitrate concentration is 0g/l. While, the highest protein content was obtain when the nitrate concentration is 12g/l.

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