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Enhancement of *Chlorella vulgaris* bioethanol production by optimizing phosphorus concentrations

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ABSTRACT

This study investigated the bioethanol production from green algae *Chlorella vulgaris* depending on its carbohydrate-enriched biomass. Four different phosphorous concentrations were employed to stimulate bioethanol production from *Chlorella vulgaris*. The impact of various phosphorous values on *Chlorella vulgaris* growth rate as well as primary product (carbohydrate) were evaluated. High performance liquid chromatography was utilized in this work. The stationary phase was identified as day 14, 12, 10 and 6 in treatments 6, 4, 2 and g/L, respectively. The findings suggest that the treatment without phosphorous addition had the highest record of carbohydrate content (22.64% dry weight) as well as the highest bioethanol yield (20.66% dry weight). It was also found that at 0 g/L treatment, the growth rate was the highest with 0.75 (day^{-1}) while the lowest was recorded at 0.42 with at 6 g/L. Finally, with the treatment of 0 g/L, the shortest doubling time was obtained with 1.35 days, while the highest one was observed with 2.4 days at 6 g/L treatment.

Keywords: bioethanol, doubling time, *Chlorella vulgaris*, carbohydrate, growth curve.

INTRODUCTION

In the past few decades and due to the expected fossil fuel depletion, there has been extensive investment in renewable resources such as biofuel production. Lately, there has been growing interest in the production of bioethanol from algal waste, which is considered cost-effective and requires short time and simple techniques. Another advantage of bioethanol is reducing the pollution when adding it to a conventional fuel which in turn achieves sustainable development goals in terms of energy (Da Costa *et al.,* 2020). Such a nontoxic compound can be produced via multiways such as sugar fermentation, as well as ethylene hydration from petroleum which might have long term advantages compared to fossil fuels (Dave *et al.* 2019; Nanda, 2020).

Usually, corn and soybean are vital sources to produce bioethanol. However, they require an arable land for cultivation, unlike algae that do not require land which make them a favorable option to produce bioethanol (Ramachandra *et al.,* 2013). Algae, which included diverse groups of microorganisms, namely macroalgae, have the ability to live under various ranges of pH and temperature in both fresh and saline water (Yew *et al.,* 2019). Microalgae are able to store carbohydrates in many forms, such as pentoses, hexoses, glycogen as well as starch/cellulose (Obaid *et al.*, 2024). Through the process of fermentation, these forms can be converted to fermentable sugars for bioethanol production (Wayman, 1996). The bioethanol produced from microalgae has many advantages compared with the one produced from lignocellulose materials. First of all, the bioethanol produced from microalgal has high caloric value, as well as low viscosity and density (Deviram *et al.,* 2020). Secondly, they can adapt to various climates, they have inherently high-lipid content, and achieve semi-steady state production (Bastos, 2018). Many researchers have worked on bioethanol generation to improve the output by employing fresh and marine algae (Salman

and Ali, 2014; Mushlihah *et al.*, 2020; Zhou *et al.,* 2020). Recently, studies have utilized microalgae *Chlorella Vulgaris* (*C. vulgaris*) to produce biofuel (Wagih *et al.,* 2017; Varaprasad *et al.,* 2020; Abdulla *et al.,* 2020; Thirugnanasambantham *et al.,* 2020) while others used *Codium tomentosum* (Gengiah *et al.,* 2020), *Padina tetrastromatica* (Ashokkumar *et al.,* 2017) and *polysaccharides* (Lakatos *et al.,* 2017) for the same purpose.

Phosphorus is a vital compound for algal growth (Guo *et al.,* 2018). Carbohydrate in *Chlorella Vulgaris* can be increased by reducing the protein content in the algae caused by phosphorus starvation (Juneja *et al.,* 2013). This work focused mainly on testing *Chlorella vulgaris* growth in the media supplemented with different phosphorous concentration and examined the effect of these conditions on the content of cells carbohydrates and bioethanol yield. It is noteworthy that the used algae cells in this study were isolated from local habitats and purified using reliable techniques. The identification process of the studied *C. vulgaris* was accomplished according to past literature which followed Bellinger and Sigee (Bellinger and Sigee, 2010) and (Prescott, 1982) as represented in Figure 1.

MATERIALS AND METHODS

Isolation and purification of algae

Two methods were applied in this study for the isolation of algae. The first one was the serial dilution method. This was done by utilizing 10 test tubes, each tube has 9 mL of Chu-10 media. Algal culture of about 1 mL was poured into the first tube carefully and mixed by gentle inversion. After that, a one mL was transferred to the second tube and so on to the last tube of the series. Finally, the tubes were incubated at 25 °C for two weeks (Stein *et al.,* 1973). The second method was streaking on an agar plate. Chu- 10 with about 45 to 50 °C was placed into petri-dishes and left a while in order to solidify. The sterile loop was utilized for streaking straight line of algal culture. Afterwards, the plates were incubated in a cooled aluminum incubator with the following conditions: 10–14 days, 16:8 as a light: dark cycle, the temperature of 25±2 °C with a light intensity of 268 µE\m²\s.

Growth media preparation

In order to grow the algae cells, a modified Chu-10 was employed for this purpose (Kassim *et al.,* 1999). Prior to the experiment, the salts mixture solution was prepared based on the recipe reported in (Kassim *et al.,* 1999) as demonstrated in Figure 2. The pH was set at 6.4 using 0.01N of HCl.

Algae cultivation

About 100 mL of isolated algae culture prepared in 2.1 was transferred to one liter of Chu-10 media that was kept in the incubator for two weeks prior to transferring the algal culture to it. Afterwards, the growth transferred to glass pools of five liters for mass culture (Kawaguchi, 1980). The effect of phosphorous on algal bioethanol production was examined by supplementing the growth media with phosphorous at four different concentrations of 0, 2, 4 and 6 g/L using K_2HPO_4.

Growth curve determination

In order to determine the concentration of microalga, optical density (OD) measurements were

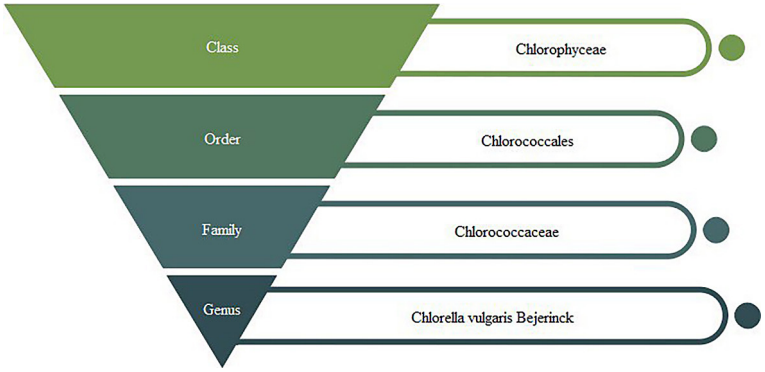

Figure 1. Identification of *C. vulgaris*

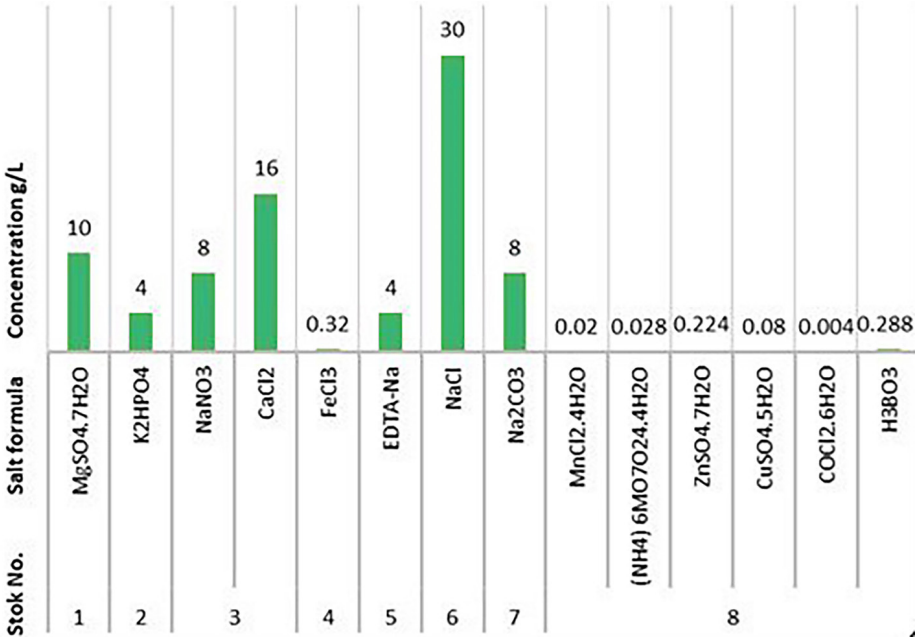

Figure 2. Compenants of algal culture media (Chu-10) used to growth of *C.vulgaris* in the current study

employed in triplicate. The following equations (Fogg, 1975; Jia *et al.,* 2015) were utilized to determine both growth rate (K) (day^{-1}) and doubling time (G) (day)

$$K = ((log\ OD_t - log\ OD0)\ t^{-1}) \times 3.322 \quad (1)$$

$$G = (0.301K^{-1}) \quad (2)$$

where: OD_0 represents the initial optical density at time zero while OD_t is the optical density after time (t) expressed in days.

Protein and carbohydrate calculation

Protein and the carbohydrate contents were determined using Bradford and phenol sulfuric acid methods, respectively (Dubois *et al.,* 1956; Bradford, 1976). Algae cells were extracted from the growth media after reaching the stationary phase by centrifugation at 5 × 10^3 rpm at for 30 min at 4 °C.

Hydrolysis process

A 200 mL of *C. vulgaris* slurry was placed in Erlenmeyer flask and heated to 100 °C for two hours using a hot plate. After that, the slurry was cooled down to 45 °C (Zhang and Feng, 2010). Then, 0.24 g/200 mL substrate of α-amylase enzyme was added to the sample and incubated for 80 min (Sulfahri *et al.,* 2010). The resultant solution was then filtered followed by centrifugation at 9 × 10^3 rpm for 15 min. Finally, the supernatant was sterilized for later use in the fermentation process.

Fermentation process

Saccharomyces cerevisiae was utilized for performing the fermentation process. Saccharomyces cerevisiae was supplied from Microbiology Department at the University of Babylon. About 2.5 mL of 10 percent Saccharomyces cerevisiae solution (OD600nm = 0.5) was added to 200 mL of substrate *Chlorella vulgaris* in a 500 mL bottle fermenter. The mixture was incubated at room temperature for about 120 h (Zhang and Feng, 2010).

Ethanol analysis

High performance liquid chromatography (HPLC) was utilized for ethanol analysis. The mobile phase was Aceternitril with a column (C18) dimension of 25 × 4.6 × 5 µm and injection flowrate of 1.2 mL.min^{-1} (Chen *et al.,* 2014). The samples were passed through a Whatman (0.45 µm) filter paper prior to HPLC analysis.

Statistical analysis

Gene stat discovery 2012 software was used for conducting the statistical analysis for the obtained results. The least significant difference

(LSD) was utilized to compare the means at a significance level of 0.05.

RESULTS AND DISCUSSION

Figure 3 shows the growth curve of *C. vulgaris* within various phosphate supplements under light intensity of 268 µE/m²/s, light: dark ratio of 16:8 and temperature of 25 ± 2 °C. It can be noticed from Figure 3 that the different phosphorus treatments resulted in various growth curves. The higher the phosphorous concentration was the higher algal growth was achieved. It can also be noted that the stationary phase period prolonged with increasing phosphorous concentration. The inception of the stationary phase with 6, 4, 2 and 0 g/L addition of phosphorous was recorded at the treatment days of 14, 12, 10 and 6, respectively.

Growth rate for the different treatment scenarios was calculated using the minimum and maximum optical density recorded and the results are presented in Figure 4. The rate was calculated under conditions of 268 µE/m²/s as light intensity, light: dark ratio of 16:8 and 25± 2 °C. There was a significant difference in K value ($p < 0.05$) in all treatment dose except treatment 4 g/L with LSD of 0.019. It can be seen that 0 g/L of phosphorous resulted in the highest growth rate of approximately 0.75 (day^{-1}) followed by the other phosphate treatments. Addition of 2, 4 and 6 g/L resulted in a growth rate of 0.45, 0.43 and 0.42, respectively.

The G value was also calculated for the different treatments as demonstrated in Figure 5. The longest doubling time of about 2.4 days was achieved with 6 g/L phosphorous addition followed by 4 g/L (2.3 days), 2 g/L(2.2 days) and the shortest was observed with 0 g/L (1.35 day). There was a significant difference in G value ($p < 0.05$) in all treatment doses except the treatment 4 g/L with LSD of 0.307.

It can be inferred from the results obtained in this study that decreasing phosphorus concentration increases the dry weight as well as reduces the essential photosynthetic pigments and photosynthetic activity, which was also reported

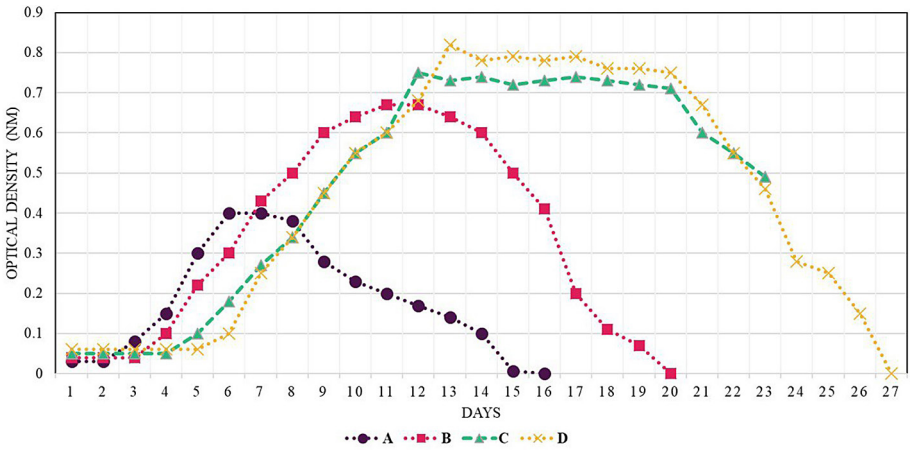

Figure 3. *C. vulgaris* growth curve at different phosphate values (g/L), A = 0 (control); B = 2; C = 4 and D = 6

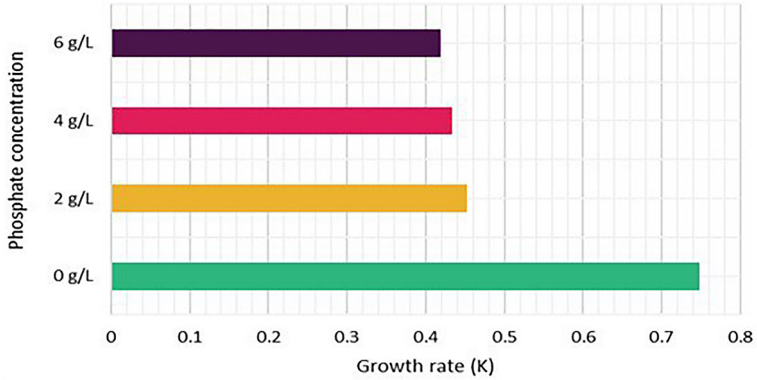

Figure 4. *C. vulgaris* growth rate at different phosphate concentrations (g/L)

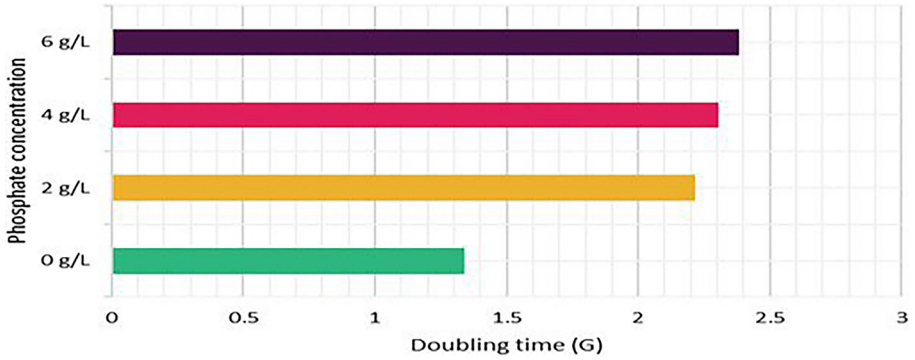

Figure 5. *C. vulgaris* doubling time at different phosphate concentrations (g/L)

in (White *et al.,* 2011; Salman *et al.,*2023a). In the photosynthesis, the reduction of light energy results in membrane damage by an increased excessive photo-oxidation process. To solve this issue, a reduction of thylakoid membranes might be compensated (Wanner *et al.,* 1986; Abdul-Adel and Salman, 2019). Salman *et al*. (2023 b) published a study on green algae *C. kessleri* which concluded that there was a growth increase with phosphate starvation which in turn support the findings of the current work.

Effect of phosphorus on carbohydrate and ethanol

Figure 6 shows the ethanol and carbohydrate content of *C. vulgaris* within different phosphate concentrations (g/L) with LSD of 0.335 and 0.729 for ethanol and carbohydrate, respectively. The findings indicated that there was an increase in the carbohydrate content of *C. vulgaris* with decreasing phosphate concentration. Literature were reported that if the green algae subjected to phosphorus shortage, the newly fixed carbon separates to the synthesis of non-phosphorylated storage polyglucans or ucrose with less photosynthate directed toward respiratory metabolism and other biosynthetic pathways (Sicher and Kramer, 1988; Markou *et al.,* 2012). In addition, Duff and his co-worker have stated that phosphorus starvation induces glycolytic »bypass« reactions in heterotrophic *B. nigra* (Duff *et al.,* 1989). In their work on Ankistrodesmusfalcatus alga, Kilham et al. (1997) noticed that under the shortage of phosphate content, the carbohydrate value increased. Another study done by Markou *et al.* (2012) showed that limiting phosphorus content in a cyanophyta A. Platensis accumulated the carbohydrate between 60% and 65% of dry weight. The current work utilized the microalga-based carbohydrate to produce ethanol, as also reported by other studies (Skjånes *et al.,* 2007; John *et al.,* 2011). As represented in Figure 6, at 0 g/L treatment, there was a highest bioethanol yield. Significant differences were obtained in all treatment as well as control except 6 g/L treatment. This could be clarified, as there is an increase in a carbohydrate content when phosphate concentration decreases. This resulted in bioethanol yield increase due to the fact that bioethanol production

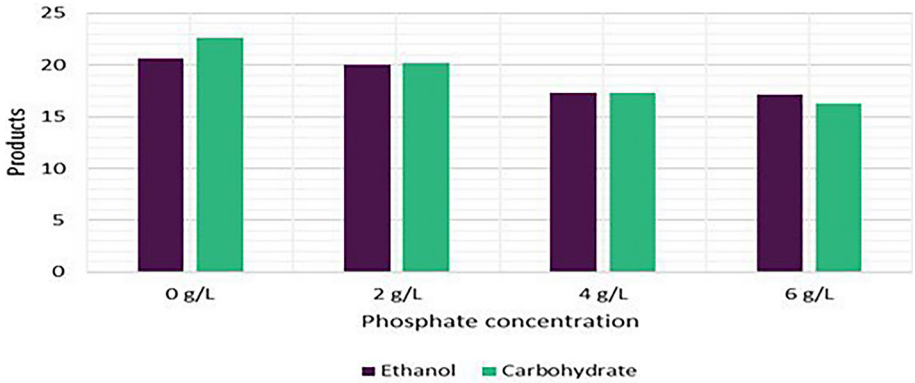

Figure 6. Both Ethanol and carbohydrate content in microalgae *C. vulgaris* at various phosphate concentrations (g/L)

is produced from carbohydrates substrate by the process of fermentation (Nguyen *et al.,* 2009; Choi *et al.,* 2009).

CONCLUSIONS

The algae capable of accumulating high starch can serve as an excellent alternative to food crops for bioethanol production, green fuel for a sustainable future. It was found that the phosphorus content of growth media had an impact on growth rate and behavior of the growth curve. Zero addition of phosphate supplement resulted in higher growth rate compared to treatments with phosphorous addition. The higher the phosphorous concentration, the lower the growth rate was. This results in an opposite trend for doubling time as the higher the phosphorous concentration the higher the doubling time was. Starving *C. vulgaris* from phosphorous resulted also in an increase in carbohydrate content and bioethanol yield.

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