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Influence of Two Nutritional Factors (Nitrate and Phosphate) on the Lutein Composition of *Coelastrella saipanensis* Alga and Estimation of Its Antioxidant Property

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ABSTRACT

Lutein pigment is one of the valuable carotenoids found in many algae and plants, known for its antioxidant properties and its therapeutic role, especially in eye health and its use as a natural colorant in food and in many pharmaceutical uses. The current study aimed to determine the effect of nitrate and phosphate on the amount of lutein produced by algae to evaluate the effectiveness of the resulting lutein as an antioxidant to protect cells from free radicals. The results of the present study indicated that the highest value of lutein in *Coelastrella saipanensis* was 0.562µg/ mg at 0.01 and 0.02g/ L concentration of nitrate and phosphate, respectively, while the lowest value of lutein was 0.1303 and 0.13µg/ mg without adding nitrate and phosphate to the medium (0g/ L). The results of statistical analyses were supported at $P<0.05$, indicating differences in lutein production. The antioxidant activity results using the DPPH assay indicated that lutein had high antioxidant activity, with the lowest antioxidant activity of 18% recorded at lutein concentration of 10µg/ mL, while the highest antioxidant activity was recorded at lutein concentration of 35µg/ mL, reaching 48%. The results showed the importance of controlling nutrients, especially nitrate and phosphate, and providing appropriate growth conditions and concentrations in the growth medium to increase the production of lutein and also indicated the properties of this pigment as an antioxidant against free radicals and thus its potential use in many natural pharmaceutical formulations.

INTRODUCTION

The biomass of microalgae is self-sustaining and can be produced using carbon dioxide, light, and inorganic nutrients. Microalgae are highly rich in primary substances, including fats, carbohydrates, proteins, and pigments. Beyond these, algae can produce a number of high-value compounds, such as sugars, polyunsaturated fatty acids, carotenoids (lutein, zeaxanthin, and astaxanthin), and vitamins with great nutritional and pharmaceutical value **(Markou & Nerantzis, 2013)**. Among the many carotenoids produced by algae, lutein is one of the most sought-after in the market **(Li *et al.*, 2011)**. Lutein is a yellow pigment of plants and an oxidized xanthophyll that is highly concentrated in higher plants and green algae **(Dall'Osto *et al.*, 2006; Ceron *et al.*,**

2008). Algae, and most specifically the genus *Chlorella*, have been pointed out as one of the best commercial sources of lutein production due to their fast-growing growth and high content of this pigment **(Leong & Chang, 2023)**. Lutein is a very potent functional compound that has gained much interest in human health because of its various biological properties. Drugs and dietary supplement forms are using lutein widely since it has so many benefits for the human body. Since lutein can only be obtained through diet, its supplements are useful to ensure adequate lutein supply **(Fuad *et al.,* 2020)**. Also known as the "eye vitamin", lutein protects eye tissues from ultraviolet radiation and oxidative damage due to its antioxidant properties **(Roberts *et al.,* 2009)**. In addition to its antioxidant properties, lutein also has anti-cancer effects and promotes neural development in infants **(Hu *et al.,* 2018)**. Moreover, lutein plays a protective role in delaying chronic diseases **(Blanco *et al.,* 2007)**.

Nutrients are one of the most vital environmental factors affecting carotenoid production and at the same time directly affecting growth. Nitrogen deficiency is one of the stress conditions caused by nutrients and is one of the most important elements present in the cell due to its presence in proteins and enzymes and also plays a direct role in growth **(Faraloni & Torzillo, 2017)**. Nitrogen is a factor that plays an important role in the accumulation of carotenoids inside cells in autotrophs **(Cordero *et al.,* 2011; Přibyl *et al.,* 2016)**. In addition, nitrogen is one of the nutritional factors affecting growth and biomass production in algae and may cause serious changes in cellular metabolic rates **(Sarsekeyeva *et al.,* 2024)**. Nitrogen is one of the main nutrients that affects the biosynthesis of microalgae and the lutein pigment **(Dineshkumar *et al.,* 2016; Xie *et al.,* 2019)**. In addition, microalgae can absorb various sources of nitrogen, including nitrate, ammonium, urea, yeast extract, and sometimes amino acids. Nitrate is not only essential for the growth of microalgae cells but also plays an important role in enhancing the lutein pathway. The highest accumulation of lutein occurs at the onset of nitrogen starvation, as revealed by many studies **(Del Campo *et al.,* 2000; Ho *et al.,* 2015; Chen *et al.,* 2019)**. According to **Chen *et al.* (2011)**, high levels of nitrate have no influence on growth, but even low levels of ammonium can induce a remarkable decline in *D. tertiolecta* growth.
In addition, compared to nitrogen, phosphorus is one of the main growth-limiting nutrients in the environment for microalgae **(Sun *et al.,* 2019)**. **Kozłowska-Szerenos and Zieliński (2000)** indicated that phosphorus deficiency or limited phosphorus had a significant influence on the growth of *Chlorella vulgaris* algae; however, it did not have any effect on chlorophyll content compared to control groups. Therefore, the goal of this study was to evaluate the effect of different nitrate and phosphate concentrations on lutein production by *Coelastrella saipanensis* algae.

MATERIALS AND METHODS

Algae cultivation and biomass production

Pure and free-of-contamination cultures of *Coelastrella saipanensis* algae were obtained from the Advanced Environmental Laboratory in the Biology Department/College of Education/Qadisiyah University. To ensure these cultures are free of fungi and bacteria, a sample of the algal culture was cultured on a solid Nutrient agar medium specific for bacterial testing incubated at 37°C for 72 hours to ensure its purity **(Anderson, 2005)**. For biomass production, the algal culture was transferred to a 500ml sterile Erlenmeyer flask containing 400ml of Chu10 medium **(Chu, 1942)**, which was modified by **Kassim *et al.* (1999)**. To grow and multiply the green algal cultures, they were incubated in an incubator at 25°C and a light intensity of 80µmol m^{-2} s^{-1}. The algal samples were incubated until growth decreased and reached the death phase **(Tredici, 2004)**.

Effect of nitrate and phosphate on lutein production

The algal cultures under study were cultured with different concentrations of nitrate and phosphate. They were treated with different concentrations of nitrate (0, 0.01, 0.015, and 0.02g/ L) and different concentrations of phosphate (0, 0.02, 0.03, and 0.04g/ L) in the Chu10 medium to evaluate their effect on lutein production by *Coelastrella saipanensis*. The culture conditions included the light intensity of 80µmol m^{-2} s^{-1}, temperature at 28°C, pH 7.2, and photoperiod at light; darkness 16:8 hr. Samples were then drawn and analyzed using high-performance liquid chromatography.

Antioxidant activity of lutein

The antioxidant activity of lutein was determined as electron-donating ability of the prepared samples. Vitamin C was used as a standard by measuring its ability to decolorize the purple alcoholic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric test uses the stable radical dye 2,2-diphenyl-1-picrylhydrazyl as a reagent **(Marinova & Batchvarov, 2011)**. The lutein pigment extracted from *Coelastrella saipanensis* was used in different concentrations according to the method of **Sivathanu and Palaniswamy (2012)**. It was extracted and purified by a German Knauer HPLC using the C18 column, as illustrated in Fig. (1).

Statistical analysis

Statistical analysis has been done using SPSS version 26, the one-way ANOVA, LSD computation having been performed to compare treatments subjected to different concentrations of nitrate and phosphate on *Coelastrella saipanensis* regarding lutein production. All experiments were done in three parallel sets **(Mustafy & Rahman, 2024)**.

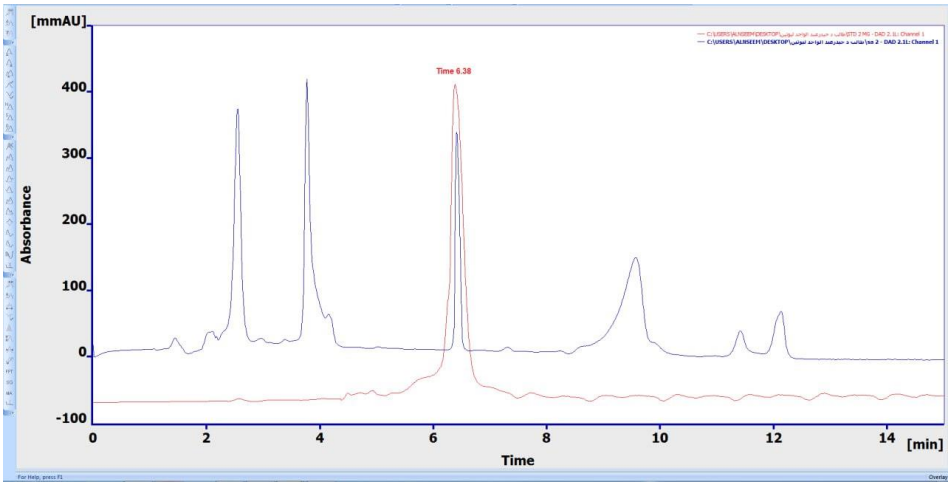

Fig. 1. Lutein obtained from *Coelastrella saipanensis* and in response to the lutein standard with a red color that was at a retention time of 6.38

RESULTS

Results on the use of different concentrations of nitrate and phosphate in treating the studied algae indicated that they had some influence on lutein concentration. The results of this study showed that the highest amount of lutein in *Coelastrella saipanensis* was 0.562µg/ mg, which was obtained with a treatment of 0.01 and 0.02g/ L nitrate and phosphate concentrations, while the lowest amount of lutein was observed at 0.1303 and 0.13µg/ mg without adding nitrate and phosphate to the culture medium (0g/ L) (Table 1 & Fig. 2). In the meantime, the results of statistical analysis also confirmed these differences in lutein production at a significance level of $P<0.05$.

The results of the antioxidant activity using the DPPH assay showed that lutein had high antioxidant activity, and this antioxidant activity increased with increasing pigment concentration. The lowest oxidation activity of 18% was recorded when the concentration of lutein was 10µg/ ml, while the highest oxidation activity reached 48% at a concentration of 35µg/ ml of lutein (Fig. 3).

Table 1. Effect of nitrates and phosphates on Lutein content in the alga *Coelastrella saipanensis*

Nitrate g/l	Lutin µg/mg	Phosphate g/l	Lutin µg/mg
	Mean±SE		Mean±SE
0	0.1303±0.02074 B	0	0.13±0.02082 B
0.01	0.562±0.06245 A	0.02	0.562±0.06245 A
0.015	0.1667±0.02848 B	0.03	0.1857±0.00338 B

0.02	0.148±0.00265 B	0.04	0.1453±0.00328 B
LSD	0.094	LSD	0.0809

Letters indicate significant differences between nitrate and phosphate concentrations

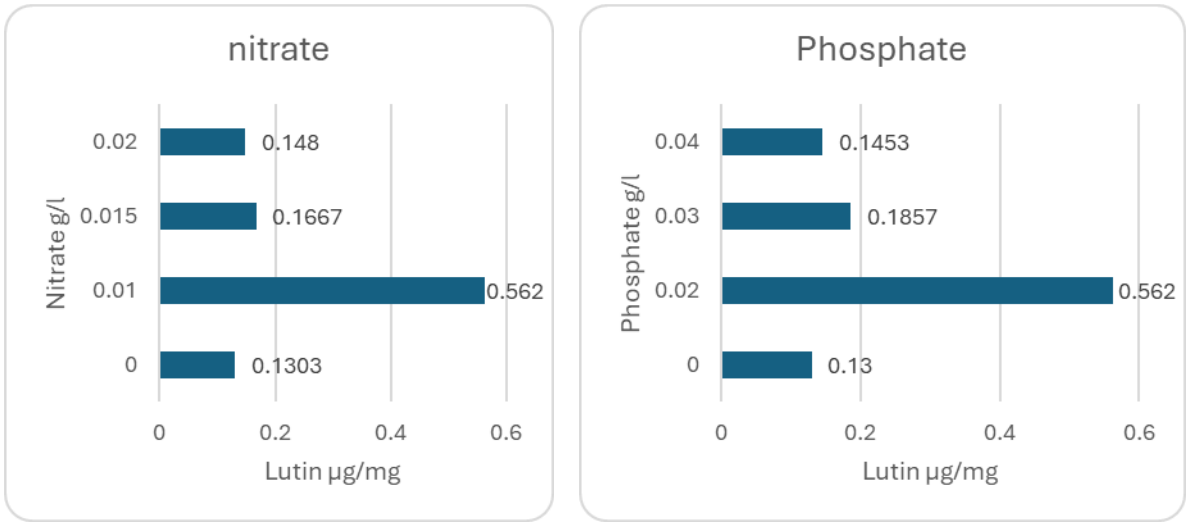

Fig. 2. Effect of nitrates and phosphates on lutein content in the *Coelastrella saipanensis*

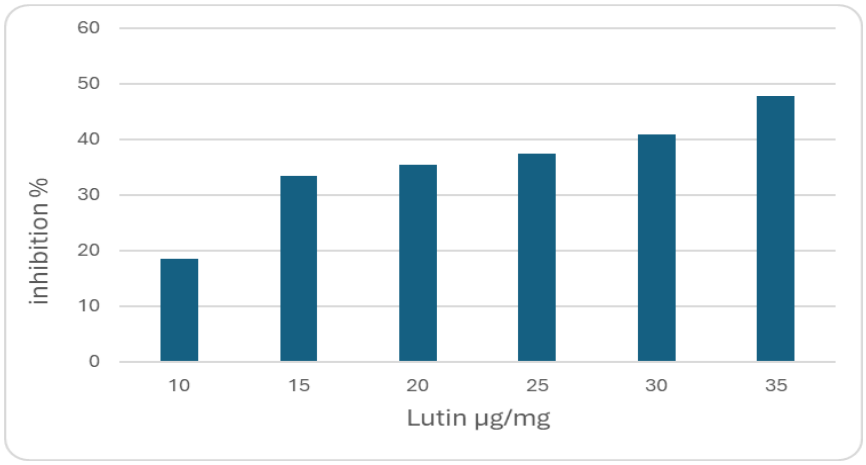

Fig. 3. Antioxidant activity of lutein purified from *Coelastrella saipanensis* using DPPH assay

DISCUSSION

This study showed that *Coelastrella saipanensis* algae have the highest lutein production with nitrate and phosphate at 0.01 and 0.02g/ L, respectively. These conditions fall under the optimum concentration of Chu10 medium; hence, the availability of nitrate and phosphate as primary nutrients favors algal growth and production of the lutein pigment. This finding is in agreement with the work of **Hong *et al.* (2023)**, where an improvement in the culture conditions of *Mychonastes* sp. algae

resulted in 2 to 2.6-fold higher lutein and zeaxanthin production compared to the improved medium and standard medium, respectively.

The optimum growth of various species has also been argued to be dependent on appropriate concentrations of nutrients, especially nitrate and phosphate **(Pengadeth *et al.*, 2024)**. Optimal physical and nutritional conditions of the culture medium, such as pH, temperature, nitrogen and phosphorus source, salinity, and light conditions, thus allow for better growth of the microalgae **(Benavente-Valdés *et al.*, 2016; Khan *et al.*, 2018)**. This is in agreement with a number of similar studies. According to **Chen *et al.* (2017)**, controlling the availability of preferential nitrogen with an appropriate concentration of the primary nitrogen source is crucial in the microalgae cultivation process for stimulating lutein accumulation in microalgae cells. Moreover, an optimum nitrate concentration is also needed to obtain the highest lutein production efficiency within the shortest time of algal cell harvest. In this study, lutein production by *C. sorokiniana* Mb-1 at different concentrations of sodium nitrate was measured as 0, 0.5, 1.0, 1.5, 2, 2.5, and 3g/ L. It showed a maximum lutein production of 3.6mg/ L/ day at a nitrate concentration of 1.5-2.0g/ L. Hence, these results indicated that sufficient nitrogen in the culture medium is required to enhance lutein accumulation in the *Chlorella sorokiniana* Mb-1 strain. This finding is in agreement with **Xie *et al.* (2013)**, who studied the strain *Desmodesmus* sp. F51 for its ability to produce lutein under conditions of nitrate concentration and light intensity manipulation. They found that there was a need for a sufficiently high nitrogen concentration for the accumulation of lutein, whereas high light intensities, though enhancing the growth of cells, tend to decrease the content of lutein. The best cell growth and lutein production occurred when light intensity and initial nitrate concentration were 600µmol m^{-2} s^{-1} and 8.8 mM, respectively.

Faraloni and Torzillo (2017) also showed that beta-carotene and lutein accumulate under optimal cell growth conditions, in line with their role in the photosynthesis process. The results of the present study are consistent with the findings of **Wang *et al.* (2010)**, which showed that the availability of high levels of nitrogen and phosphorus provides optimal growth conditions, while phosphorus deficiency is associated with slow growth rates and low cell density. Also, the rate of cellular metabolism can be affected by different forms of nitrogen **(Chen *et al.*, 2011)**. This is in agreement with the study of **Coulombier *et al.* (2020)**, who investigated the effect of nitrogen availability on the peroxyl radical scavenging activity and carotenoid content of *Nephroselmis* sp. under different nitrogen conditions: increased nitrogen, nitrogen limitation, and nitrogen starvation. They found that nitrogen availability significantly affected antioxidant activity in *Nephroselmis* sp. and the extract was more active under nitrogen saturation conditions than under conditions of nitrogen limitation or starvation. It was observed that the contents of lutein and xanthophyll cycle pigments such as violaxanthin, zeaxanthin, and antheraxanthin were high under increased nitrogen conditions, showing that nitrogen-rich environments enhance carotenoid synthesis, thus increasing the antioxidant capacity in

Nephroselmis sp. Since phenolic and carotenoid compounds gave the same trend in response to nitrogen levels, it may be assumed that these were the major compounds contributing to the antioxidant activity. **Goiris *et al.* (2012)** also reported that antioxidant activities were between 3- and 10-fold higher in nitrogen-sufficient conditions compared to nitrogen-deficient conditions in the following three microalgae: *Chlorella vulgaris*, *Tetraselmis suecica*, and *Phaeodactylum tricornutum*. While **Çakmak *et al.* (2015)** and **Aremu *et al.* (2016)** presented the negative effects of nitrogen starvation on the antioxidant activity of *Chlamydomonas reinhardtii* and *Chlorella* strains, respectively.

The results of the present study showed that minimum lutein was recorded with low and high concentrations of nitrate and phosphate, which is due to stress conditions at higher and lower concentrations of the present study, which supports the findings of **Xie (2015)**. Lutein and chlorophyll under different stress conditions other than high and low nitrogen conditions resulted in reduced biomass production under high and low nitrogen conditions. This is because, under nitrogen deficiency conditions, available nitrogen may inhibit the synthesis of light-harvesting complexes (LHC) and lead to the degradation of chlorophyll for nitrogen recycling, which reduces lutein and chlorophyll content. In addition, previous studies **(Minhas *et al.*, 2016)** have shown that primary carotenoids such as lutein are degraded under stress conditions and therefore their concentration decreases. This decline may also be due to the reduced supply of nutrients, promoting a chain reaction. As nutrient content in the cell drops, light-harvesting substances and carbon dioxide become scarce, which further limits protein synthesis and reduces growth potential. Consequently, this results in a decrease share by the cell in both pigments and photosynthetic proteins to a new steady rate of growth. Limiting nitrogen and phosphorus affect both the concentrations of large molecules and the specific activities of enzymes and ribosomes **(Bora *et al.*, 2024)**. Moreoveer, the results of the present study are consistent with the decrease in lutein content with decreasing nitrogen concentration, as **Del Campo *et al.* (2000)** also found that the lutein content in *Muriellopsis* sp. increases with the increase in the number of cells during the exponential growth phase and reaches its maximum value in the early stationary phase and then the level of lutein decreases when nitrogen is limited. Similarly, **Bar *et al.* (1995)** reported a decrease in chlorophyll and primary carotenoid (beta-carotene and lutein) content in *C. zofingensis* under combined conditions of high light intensity and nitrogen deficiency.

The results of the present study confirm the finding of **Ali *et al.* (2022)** that maximum concentration of beta-carotene and lutein were found in *Coelastrella* sp. algae at optimal growth conditions, which is concurrent with the nitrogen and phosphorus-rich BG-11 medium. In contrast, under nitrogen and phosphorus-deficient conditions, the minimum concentrations of beta-carotene and lutein were obtained. Therefore, the limitation of phosphorus availability in the culture medium will have a negative effect on cell growth and the energy consumed in cell division will affect the carotenoid formation process **(Faraloni & Torzillo, 2017)**. **Li *et al.* (2014)** showed that in the green alga

Chlorella pyrenoidosa, there is no significant correlation between lutein and chlorophyll content under both high and low nitrogen conditions under heterotrophic culture.

Results on the antioxidant activity of lutein in this study can be attributed to the anti-inflammatory and antioxidant effects of lutein and its special structure, especially the presence of conjugated double bonds and hydroxyl groups **(Zhang *et al.*, 2018)**. Conjugated double bonds act as a strong antioxidant and react with free radicals by donating electrons to form a more stable product. This structural feature may also affect its absorption efficiency by modulating the carotenoid polarity and flexibility **(Ahn & Kim, 2021)**. **Sindhu *et al.* (2010)** showed that lutein, due to its polar nature and an extended system of conjugated double bonds, has a high free radical scavenging ability. *In vitro*, lutein was capable of superoxide radical, hydroxyl radical scavenging, and inhibition of lipid peroxidation. The IC50 values against these radicals were 21, 1.75, and 2.2µg/ ml, respectively. Furthermore, lutein showed a potent scavenging on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (IC50 35µg/ ml) and nitric oxide radicals (IC50 3.8µg/ ml), while higher doses were required for the inhibition of 2,2-azobis-3-ethylbenzthiozoline-6-sulfonic acid radicals. Lutein also presented a 50% iron (III) reduction capacity at a dose of 0.3µmol/ ml FeSO4. In addition, there was inhibition of superoxide production in macrophage from oral administration of lutein at doses of 50, 100 and 250mg/ kg body weight to be inhibited at 34.18, 64.32 and 70.22%, respectively. Furthermore, during one month on the supplement test of mice with oral lutein, the enzymatic activity of catalase, superoxide dismutase, glutathione reductase, glutathione in blood and liver increased significantly. Meanwhile, activity against glutathione peroxidase and glutathione-S-transferase in liver also increased.

CONCLUSION

This study found that variation in nitrate and phosphate concentrations would affect lutein production in a *Coelastrella saipanensis* microalgae. The main result of the present study showed that nutrient availability, especially nitrate and phosphate, is the most critical parameter to optimize growth conditions and to enhance lutein production. In addition, it estimated the optimal amount of nitrate and phosphate at 0.01 and 0.02g/ l, respectively, which is capable of enhancing lutein production. This study confirmed that lutein has strong antioxidant activity against free radicals.

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