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The Impact of γ-irradiation on Photosynthetic Activity ($^{14}CO_2$-assimilation), Photosynthetic Pigments, Enzymes, and Biomass Productivity of *Chlorella vulgaris*

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ABSTRACT

Recently, γ-irradiation has become more popular as a way to increase total yield and phytochemical components in many plants, including microalgae. A significant increase in the use of γ-irradiation to stimulate biological processes in microalgae have been witnessed. Among functional ingredients identified from marine algae, the three basic classes of natural pigments are chlorophylls, carotenoids, and phycobiliproteins. The γ-irradiated *Chlorella vulgaris* treatment at a dose of 200Gy significantly increased the photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin, and total phycobiliproteins) and photosynthetic enzyme activities (ribulose-1,5-bisphosphate-carboxylase/oxygenase, RUBISCO and phosphoenol-pyruvate carboxylase, PEPCASE), as compared to the control. The results showed a significant increase in photosynthetic activity ($^{14}CO_2$-assimilation), total carbohydrate contents, and total biomass productivity, which in turn increased the total yield of γ-irradiated *C. vulgaris* compared to the control. Meanwhile, for the size of chloroplasts, no significant changes were detected in either the γ-irradiated *C. vulgaris* (200Gy) or control sample. The data revealed that the potential application of γ-radiation as a stimulatory agent to increase the total biomass productivity and nutritive value of *C. vulgaris*, as they increased chlorophylls, carotenoids, and phycobiliproteins contents, may have the potential to be used as a food supplement for treatment of countless diseases.

INTRODUCTION

There has been a growing interest in applying γ-irradiation in recent years to stimulate biological processes in microalgae **(Tale *et al.*, 2018)**. Recent research has proven that gamma rays have economic and efficient applications compared to other ionizing radiation due to their strong penetration and easy availability, which has led to a wide and

useful application in improvement in growth and productivity of various algae and plants **(Tianci *et al.*, 1990; Heidarieh *et al.*, 2012; Moussa *et al.*, 2015)**. **Abomohra *et al.* (2016)** and **Shabana *et al.* (2017)** stated that γ-radiation had a stimulating effect on growth, biomass, pigment content, cellular constituents and some metabolic activities of *A. platensis*. The γ-irradiation can be useful for the alteration of the physiological characters, stimulating the rate of cell division and may increase the enzymatic activity **(Heidarieh *et al.*, 2012)**. Moreover, γ-irradiation could enhance the growth of *Spirulina platensis* **(Moussa *et al.*, 2015)**.

Chlorella vulgaris, an autotrophic microalga that belongs to green algae (Chlorophyta), is rich in chlorophyll, carotenoids, total phycobiliproteins and can be extracted to produce functional foods **(Rani *et al.*, 2018)**. Functional food products from *C. vulgaris* are commercialized in the markets as powder, tablets, or even capsules **(Khairunnisa *et al.*, 2024)**. Natural pigments in algae have a strong role in pigmentation metabolism, photosynthetic process, and also exhibit numerous advantageous biological activities like anticarcinogenic, anti-inflammatory, antiangiogenic, antioxidant, neuroprotective, and antiobesity **(Guedes *et al.*, 2011; Pangestuti & Kim, 2011; Pratita *et al.*, 2024)**. Additionally, microalgae are a natural and safe source for isolating natural materials of great commercial importance in industries such as dietary, pharmaceuticals, and cosmetic products, and these include carbohydrates, and pigments like chlorophylls, carotenoids, and phycobiliproteins **(Obeid *et al.*, 2022; Tounsi *et al.*, 2023)**. Phycobiliproteins are proteins that function as photosynthetic accessory pigments **(Tounsi *et al.*, 2023)**.

Microalgal biomass is commonly used in the food industry as a natural food coloring or as a nutritious additive to improve the nutritional content of traditional foods viz. cookies, bars, and pasta. In the nutraceutical industry, microalgal biomass is widely marketed globally as dietary supplements such as tablets and capsules **(Sahni *et al.*, 2019)**. However, due to their organoleptic properties such as odor, strong color, and taste, the addition of microalgae to food products represents a continuing and ongoing problem **(Lafarga, 2019)**.

Phycobiliprotein fractions (C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins) are bioactive compounds in microalgae with quite diverse amounts and variations. Microalgae consist of chlorophyll, carotenoids, and phycobiliproteins **(Andrade, 2018; Khairunnisa *et al.*, 2024)**. They have antiradical activity and can be used as natural dyes. However, phycobiliprotein is not as widely studied as carotenoids and chlorophyll **(Karseno *et al.*, 2013)**. Phycobiliprotein is a pigment-protein complex with a water-soluble, bright color, and works as a light catcher to assist in the ongoing process of photosynthesis. Green phycobiliproteins are classified into three groups: phycoerythrin (red pigment), phycocyanin (blue pigment), and allophycocyanin (blue pigment) **(Su *et al.*, 2014)**. This pigment is known to have acted as a natural dye, antiinflammatory properties, an anticancer, antiradical, and antimicrobial **(De Morais *et al.*, 2018)**. The

phycobiliproteins molecule in *C. pyrinoidosa* has the potential to be employed as a dietary supplement due to its antioxidant action **(Pratita *et al.*, 2024)**. According to earlier studies, *C. vulgaris* is one of the numerous kinds of microalgae that contain phycobiliproteins pigments **(Djunaedi *et al.*, 2017)**.

The objectives of this research was to investigate the role of γ-irradiation treatment in *C. vulgaris* for increasing the nutritional, commercial value, and some vital growth metabolites and photosynthesis profile of photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins), photosynthetic activity ($^{14}CO_2$-assimilation), and photosynthetic enzyme activities (phosphoenolpyruvate carboxylase, PEPCASE and ribulose-1,5-bisphosphate-carboxylase/oxygenase, RUBISCO), total carbohydrate contents and total biomass productivity.

MATERIALS AND METHODS

Growth medium, growth conditions, and γ-irradiation treatment of *C. vulgaris*

The algae used in this study, *C. vulgaris*, were obtained from the National Institute of Oceanography and Fisheries, hydrobiology laboratory, Qanater Branch, Egypt, and cultured in BG-11 media **(Helal *et al.*, 2023; Al-Habeeb *et al.*, 2024)**. The chemical contents of the BG-11 media used are illustrated in Table (1).

The cultural medium was autoclaved at 120°C for 20 minutes before inoculation, and the required illumination was provided by sunlight. The solution was continually mixed by an aerator at a temperature of 30±2°C, pH 7.5, along with the photoperiod being 16/8h of a day/night cycle. Volumes of 500mL of *C. vulgaris* after 20 days of growth were subjected to the optimum dose of 200Gy of γ-irradiation **(Helal *et al.*, 2023; Al-Habeeb *et al.*, 2024)**. The γ-irradiation was produced using a Co^{60} source in Nasr City, Egypt, at the Egyptian Atomic Energy Authority for 3.9h (0.84 Gy min^{-1} exposure rate).

Photosynthetic pigments

Chlorophyll a, b and carotenoids were estimated by the method of **Bazarnova *et al.* (2024)**. Estimation of phycobiliproteins fractions (C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins) was carried out according to **Lamela and Márquez-Rocha (2000)** and **Moussa *et al.* (2015)**.

Separating the chloroplasts

The process of chloroplast isolation from *C. vulgaris* utilized the technique of chloroplast isolation buffer with 60 mM Tris–HCl, 0.35 M sorbitol, 6 mM EDTA, and pH 7.5 **(Moussa, 2011; Tirado & Combariza, 2024)**. Crude chloroplasts were purified by centrifugation using 50-75% Percoll gradient. The intact chloroplasts were obtained from the gradients, diluted three to four times, and centrifuged at 3000x*g* for 4min.

Subsequently, chloroplasts were resuspended in the isolation buffer and were kept in darkness until future use. All procedures were carried out at 0–5°C.

Chloroplasts size determination

Chloroplast size distribution was determined by dynamic light scattering technique (Beckmann, Coulter N4 Plus apparatus, Midland, Canada). The scattering angle was equal to 90°. The mean particle size was calculated under the assumption of a unimodal distribution **(Tirado & Combariza, 2024)**.

Table 1. Chemical composition of BG-11 media used

Chemicals (g/L)	BG-11 media
$NaNO_3$	1.6
K_2HPO_4	3.050
$MgSO_4.7H_2O$	7.500
$CaCl_2.2H_2O$	3.600
Citric acid. $1H_2O$	0.600
Ammonium ferric citrate	0.600
EDTA (disodium salt)	0.100
Na_2CO_3	0.020
Trace metal	1 ml
H_3BO_3	2.860
$MnCl_2.4H_2O$	1.810
$ZnSO_4.7H_2O$	0.225
$Na_2MoO_4.2H_2O$	0.390
$CuSO_4.5H_2O$	0.076
$Co(NO_3)_2.6H_2O$	0.049
Distilled water	1.0 L
pH	7.5±0.2

Photosynthetic activity ($^{14}CO_2$-assimilation)

Photosynthetic efficiency ($^{14}CO_2$ fixation) was estimated at the Radioisotope Department, Egyptian Atomic Energy Authority (**Moussa *et al.*, 2015, 2016)**. Each treatment's flask was placed under the Bell Jar, which served as a chamber for photosynthetic activity. Inside the chamber, a reaction was initiated between 15% HCl and 50µCi $NaH^{14}CO_3$ with 100mg Na_2CO_3 as a carrier, and the illumination was afforded by a tungsten lamp, resulting in the generation of radioactive $^{14}CO_2$. After 35 minutes, which was considered zero time, the excess $^{14}CO_2$ was trapped by 1.0 N NaOH. The samples were taken out of the chamber and were allowed to absorb CO_2 for two hours in normal air, and then frozen for five minutes to stop the photosynthetic chemical reactions. After that, the samples were extracted using 75% hot ethanol. The Bray Cocktail was used to measure the

^{14}C in soluble components from the ethanolic extracts **(Bray, 1960)**, and counting was performed using the Liquid Scintillation Counter (Nuclear Enterprises, Edinburg, UK; LSC2-Scaler Rate meter SR7).

Enzymes assay

Enzyme activities of phosphoenolpyruvate carboxylase (EC 4.1.1.31) and ribulose-1,5-bisphosphatecarboxylase/oxygenase (EC 4.1.1.39) was estimated by **Warren *et al.* (2000)** and **Moussa *et al.* (2015)**.

Biomass estimation

After 20 days of growth, aliquots of one liter was centrifuged at 5000 x*g* for 20 min. The biomass after centrifugation was dried at 100°C to constant weight, cooled, and weighed. The results were calculated and presented in gL^{-1} (**Yoo *et al.*, 2010; Ratomski & Hawrot-Paw, 2021)**.

Total carbohydrate content

Total carbohydrate content was estimated by the method of **Singh *et al.* (2019)**.

Statistical analysis

The statistical software program (SPSS version 17, SPSS Incorporated Company, Illinois, USA) was used to conduct analytical statistics. The mean ± standard deviation (SD) of three replicates was used to present the results. To ascertain the level of significance, the collected data were statistically examined using one-way analysis of variance (ANOVA) at $P \leq 0.05$. Using Tukey's analysis, treatment means were compared at $P \leq 0.05$ **(Abdel-Alim *et al.*, 2023)**.

RESULTS AND DISCUSSION

The data for photosynthetic pigments (chlorophyll *a*, *b*, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins), and size of chloroplasts in *C. vulgaris* and/or γ-irradiation after 20 days of growth are listed in Table (2). The results revealed that in γ-irradiated *C. vulgaris* (200Gy), all photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins) increased significantly ($P < 0.05$) compared to the control. Meanwhile, average sizes of chloroplasts (812nm) isolated from control was not significantly different from the size of the chloroplasts (805nm) obtained from γ-irradiated *C. vulgaris* (Table 2).

Serratos *et al.* (2021) reported that chlorophyll *a*&*b* are the main pigment components necessary for photosynthetic activity found in *C. vulgaris*. **Moussa (2011)** reported that γ-irradiation (20Gy) had no significant effect on chloroplasts size of soybean.

C. vulgaris is known to have the highest protein content compared to other microalgae. One of the proteins which has the potential to be developed as a food supplement is phycobiliproteins **(Pratita *et al.*, 2024)**. Gamma radiation treatment induce stressed cells which accelerate the release of pigments **(Rahmawati *et al.*, 2017; Pratita *et al.*, 2024)**. The main function of chlorophyll is that it acts as a pigment involved in photosynthetic processes. The health benefits of chlorophyll make it one of the pigments targeted for use in functional foods. Diabetes, asthma, and haemophilia are known to be prevented by chlorophyll **(Khairunnisa *et al.*, 2024)**.

The data for photosynthetic efficiency ($^{14}CO_2$-assimilation), and photosynthetic enzyme activities (ribulose-1,5-bisphosphate-carboxylase/oxygenase and phosphoenol-pyruvate carboxylase), total carbohydrate content, and biomass productivity in *C. vulgaris* and/or γ-irradiation after 20 days of growth are listed in Table (3). The results revealed that in γ-irradiated *C. vulgaris* (200Gy), photosynthetic activity ($^{14}CO_2$-fixation), and photosynthetic enzyme activities (phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate-carboxylase/oxygenase), total carbohydrate and biomass productivity content increased significantly ($P< 0.05$) compared with the control. Findings of total carbohydrates and photosynthetic activity revealed that gamma irradiation can influence photosynthesis of *C. vulgaris* cells and can stimulate the $^{14}CO_2$ fixation rate of the cell, thereby improving algal growth **(Agarwal *et al.*, 2008; Moussa *et al.*, 2015)**. The enhancement of photosynthetic activity may be explained on the basis of the increased pigments, carotenoids and phycobiliproteins contents of γ-irradiated *C. vulgaris* cells. However, low doses of gamma rays significantly increased the soluble sugars, total chlorophyll level, photosynthetic activity, and the carbohydrate contents **(Nouri & Toofanian, 2001; Khodary & Moussa, 2003; Moussa, 2011; Moussa *et al.*, 2015)**.

The enhancement in the carboxylating enzyme activities of PEPCASE and RUBISCO in γ-irradiated *C. vulgaris* may reflect the efficiency of photosynthetic process and/or the carbon dioxide fixation **(Moussa, 2001; Agarwal *et al.*, 2008; Moussa *et al.*, 2015; Moussa & Hassen, 2017)**. Moreover, **Moussa (2011)** reported that γ-radiation treatment (20Gy) could stimulate the enzyme activities of RUBISCO and PEPCASE of drought-stressed soybean plants. **Zelle *et al.* (2011) and Zhang *et al.* (2012)** argued that the increase in phosphoenol pyruvate carboxylase activity may further increase ribulose-1,5-bisphosphate carboxylase/ oxygenase activity by anaplerotic carbon flow (the reversible oxidative decarboxylation of malate to pyruvate and CO_2) and may promote carbohydrate synthesis. *C. vulgaris* treated with γ-irradiation at a dose of 200Gy after 20 days of growth induced higher biomass productivity (2.4 gL^{-1}) compared to the control ($1.2gL^{-1}$). The production of microalgae biomass should be carried out before starting the large-scale production process **(Ratomski & Hawrot-Paw, 2021)**.

Table 2. Photosynthetic pigments (chlorophyll *a*, *b*, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins), and size of chloroplasts in *C. vulgaris* and/or γ-irradiation after 20 days of growth

Parameter	γ-irradiated *C. vulgaris* (Gy)	
	0.0	**200**
Chlorophyll *a* (mg g^{-1}FW)	17.8±0.89[b]	23.6±0.78[a]
Chlorophyll *b* (mg g^{-1}FW)	13.6±0.95[b]	18.3±0.74[a]
Total chlorophyll (*a*+*b*) (mg g^{-1}FW)	31.4±1.82[b]	41.9±2.41[a]
Carotenoid (mg g^{-1}FW)	64.7±6.31[b]	76.9±5.38[a]
C-phycocyanin (mg g^{-1}FW)	12.9±0.90[b]	17.5±1.22[a]
Allophycocyanin (mg g^{-1}FW)	10.7±0.86[b]	16.8±1.18[a]
Phycoerythrin (mg g^{-1}FW)	8.5±0.34[b]	14.2±0.85[a]
Total phycobiliprotein (mg g^{-1}FW)	32.1±2.76[b]	48.5±2.91[a]
Size of chloroplasts (nM)	812 ±97.44[a]	805±88.65[a]

Values are represented as the mean ± SD of triplicate. Means marked with the same superscript letters in the same row are not-significant (P>0.05), whereas others with different superscript letters are significant (P<0.05).

The increased of total biomass productivity which, in turn, increased the total yield is an inevitable result of the increased photosynthetic pigments (chlorophyll *a*, *b*, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins), photosynthetic activity ($^{14}CO_2$-assimilation). The photosynthetic activity and associated biomass production are significantly higher, as compared to land plants **(Moussa & Mohamed, 2011; Moussa *et al.*, 2015; Ratomski & Hawrot-Paw, 2021)**. Microalgae biomass has been widely used in many industries like food, animal feed, pharmaceutical, biofuel production, and water purification **(Spolaore *et al.*, 2006; Ratomski & Hawrot-Paw, 2021)**. **Sahni *et al.* (2019)** investigated that microalgal biomass is widely commercialized worldwide in the nutraceutical sector as food supplements (tablets and capsules), while in the food market they are normally incorporated as a natural food colorant

or as a healthy supplement, able to enhance the nutritional value of conventional food products (pasta, bars, and cookies).

Table 3. Photosynthetic efficiency ($^{14}CO_2$-assimilation), and photosynthetic enzyme activities (ribulose-1,5-bisphosphate-carboxylase/oxygenase and phosphoenolpyruvate carboxylase), total carbohydrate content and biomass productivity in *C. vulgaris* and/or γ-irradiation after 20 days of growth

Parameter	**γ-irradiated *C. vulgaris* (Gy)**	
	0.0	**200**
Photosynthetic activity ($^{14}CO_2$-assimilation) (Kilo Becquerel mg^{-1}FW)	9.8±0.39^{b}	14.3±0.57^{a}
Ribulose-1,5-bisphosphate-carboxylase/oxygenase (μM mg^{-1}protein min^{-1})	98.7±8.14^{b}	115.8±9.16^{a}
Phosphoenolpyruvate carboxylase (μM mg^{-1}protein min^{-1})	62.7±5.02^{b}	72.5±6.12^{a}
Total carbohydrate content (mg glucose g^{-1}DW)	198.8±15.21^{b}	278.6±17.23^{a}
Biomass production Dry weight (g L^{-1})	1.2±0.05^{b}	2.4±0.04^{a}

Values are represented as the mean ± SD of triplicate. Means marked with the same superscript letters in the same row are not-significant (P>0.05), whereas others with different superscript letters are significant (P<0.05).

CONCLUSION

Using γ-irradiation treatment of *C. vulgaris* at a dose of 200Gy induced a significant increase (P< 0.05) in some important metabolites, including chlorophyll *a*, *b*, total chlorophyll, carotenoids, C-phycocyanin, allophycocyanin, phycoerythrin, and total phycobiliproteins, having high nutritive value and the potential to be used as a strong food supplement. Besides, γ-irradiation significantly increased photosynthetic efficiency ($^{14}CO_2$-assimilation), and photosynthetic enzyme activities (phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate-carboxylase/oxygenase), total carbohydrate content, and total biomass productivity, which in turn increased the total yield of *C. vulgaris* that has the potential to be used as a food supplement for treatment of many diseases.

"Declarations"

Consent for publication

All authors agree to publish in the journal.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Conflicts of interest

We have no conflicts of interest to disclose.

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