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Evaluating the Effect of Ems as Chemical Mutagen on the Cellular Components of a Micro Green Algae

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ABSTRACT

This study aimed to study the effect of the chemical mutagen ethyl methane sulfate (EMS) on the cellular components of the microgreen alga *Coelasterlla* MH923011. The genus *Coelastrella* has been around for about a century and comprises a small number of species. *Coelastrella MH923011* has a coccoid shape that is elliptical until it becomes citriform. They are found as single-celled microalgae or aggregations of a few cells. Alga was cultivated for 14 days in BG11-modified conditions before being subjected to EMS mutagenesis to determine the effects on biomass and cellular contents such as protein, carbohydrate, lipid, and fatty acid. Mutant one showed that biomass increased from days 7 to 15; the peak growth rate of 0.01g/l on day 13 and subsequent increase by day 15 to 0.03g/l may indicate different physiological responses triggered by mutagens, and showed a rise in protein with 11.22%, Carbohydrate 26.88%, and lipids 26.11% increase. In addition, GC-mass confirmed the presence of seven fatty acids and an increase in mutagenic algae compared to the control group.

Key words: Biofuel, biomass, *Coelastrella MH923011*, fatty acids, proteins.

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INTRODUCTION

Coelastrella MH923011 is a genus of green microalgae that are primarily found in freshwater habitats. It is a member of the Scenedesmataceae family. These unicellular algae are well known for their adaptability and resilience in a variety of biological niches, such as soil, lakes, ponds, and even hostile environments like deserts and snow (Tschaikner et al., 2008). The spheroidal form, green chloroplasts, and abundant biomass that include essential biological components like proteins, carbohydrates, lipids, and pigments like carotenoids and chlorophyll are characteristics of *Coelastrella MH923011* (Borics et al., 2021). Coelastrella's nutritional profile makes it a desirable option for biotechnological uses such as the manufacture of biofuel, aquaculture feed, and nutraceuticals. Specifically, its high protein content and capacity to accumulate lipids offer a way to create sustainable protein and biofuel sources (Ahmad et al., 2021; Mahdi et al., 2021; Razooki et al., 2020). Optimizing the metabolic pathways of *Coelastrella MH923011* to increase its biomass yield and the accumulation of valuable cellular components is becoming more and more popular as the demand for renewable resources rises (Al-Saedi et al., 2019; Mahdi et al., 2021). A promising method for enhancing the metabolic properties of *Coelastrella MH923011* is mutagenesis, which is the process of causing mutations to enhance particular qualities. Chemical mutagens, physical radiation, and even genetic engineering techniques have all been used in the past to increase algal growth. Researchers have created strains with enhanced profiles of critical fatty acids and proteins, higher lipid accumulation, and faster growth rates by using mutagenesis (Chu, 2017; Raghad and Abeer, 2023). For example, research has demonstrated that chemically induced mutations in *Coelastrella MH923011* strains can result in notable increases in lipid content;

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in comparison to their wild-type counterparts, certain variations have exhibited up to a 50% increase in total lipid output (Khoo et al., 2023; Voelker and Davies, 1994). These developments aid in the effective synthesis of biofuel and other beneficial bioactive substances. Additionally, the use of mutagenesis can increase the synthesis of carotenoids, which are significant for their antioxidant qualities and possible health advantages, in addition to improving the lipid and protein content (Divya et al., 2021; Kahl, 1973). This diverse improvement of cellular components using mutagenesis demonstrates how algal strains can be modified to satisfy the increasing need for sustainable resources. Ethyl methanesulfonate (EMS) is regarded as a monofunctional ethylating agent that has demonstrated mutagenic effects across a wide range of genetic test systems, including viruses and mammals. It is regarded as an effective and widely utilised chemical mutagen for inducing point mutations (Till et al., 2004). Thus, the aim of this study was to improve the efficacy effect of chemical mutagen EMS on *Coelastrella MH923011* cellular components.

MATERIALS AND METHODS

Preparation of the Broth Culture: The experimental work was conducted using a specific type of microalga strain that was recently isolated from the Tigris River and recorded under accession number MH923012 by (Abed et al. 2019). The algae specimen (*Coelastrella spp.*) was acquired and identified as *Coelastrella MH923011* in the Biology Department of the University of Baghdad. The culture broth BG11 (Himedia-India) was prepared by adding 3.2 grams of powder to each 2 litres, with a recommended pH adjustment to 7.1, and sterilized via autoclaving at 121°C for 15 minutes. The medium was then allowed to cool to room temperature (Hassan et al., 2021; Razooki et al., 2020).

Preparation of EMS for Chemical Mutagenesis: At pH 7, ethyl methanesulfonate (EMS) was prepared at a concentration of 0.5% (v/v) in 100 mL of phosphate buffer solution (Meng et al., 2024).

EMS-Induced Mutagenesis: Random mutagenesis was conducted on cell suspensions of the microalga *Coelastrella* MH923011 during the exponential phase at a concentration of 10^7 cells/mL by exposing them to a 0.5% EMS solution (Himedia-India) for one hour in darkness at room temperature, with vigorous agitation at 300 rpm. After incubation, the treated cultures underwent centrifugation at 8000 rpm for 10 minutes. The resulting pellets were subsequently washed twice with an autoclaved 10% sodium thiosulfate solution (w/v) to remove any excess EMS. The microalgae were re-suspended in 10 mL of sterile BG11 medium and incubated in the dark overnight for further selection. The cultures were then incubated for three weeks at 25°C (Al-Saedi et al., 2019).

Assessment of Microalgal Biomass: The growth rate was assessed based on the method described by (Mackinney, 1941) as an indirect method for chlorophyll quantification. The pigments are completely extractable in acetone and exhibit a specific absorbance at a wavelength of 663 nm. Ten millilitres of microalgae cultures were subjected to centrifugation at 5000 rpm for 10 minutes. The pellet was re-suspended in 80% acetone and subjected to vigorous vortexing. The tubes were incubated in a water bath at 60°C in darkness for 1 hour, with periodic agitation. Following incubation, the solution was centrifuged once more, and the supernatant was stored in darkness. The procedure for the pellet was repeated to ensure complete chlorophyll extraction. The absorbance of the supernatant was recorded at 663 nm using a UV-Vis spectrophotometer, with 80% acetone as the blank reference. Growth was evaluated spectrophotometrically every two days, and the growth curve was constructed by graphing absorbance versus time.

Quantification of Cellular Constituents

Protein: The Kjeldahl method was employed to ascertain the protein percentage in the samples, following the procedure outlined by (Van Dijk and Houba, 2000). A known mass of the sample (0.2 g) was placed in a digestion flask, and 5 mL of concentrated sulfuric acid (H_2SO_4), potassium sulfate (K_2SO_4), and copper sulfate ($CuSO_4$) were added. The mixture was subsequently heated to facilitate complete digestion. The mixture transformed

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into a transparent, blue-hued liquid upon digestion. The digested liquid was quantitatively transferred to the distillation flask of the Kjeldahl apparatus, which was attached to a flask containing a 40% concentrated sodium hydroxide (NaOH) solution. A test tube was submerged in a receiving flask containing a specified volume of 20% boric acid (H_3BO_3), together with drops of methyl red and bromocresol blue indicators, at the conclusion of the distillation. The flask was subsequently heated until the collected liquid from the distillation reached approximately 25 mL, after which it was titrated with 0.1 N hydrochloric acid (HCl). A control solution (blank) was prepared with the aforementioned components, excluding the sample. The protein percentage was calculated using the following equation:

Protein (%) = [(Volume of HCl consumed by sample – Volume of HCl consumed by blank) × 0.014 × 6.25 × 100] / (Sample weight in grams)

Total Carbohydrates: The total carbohydrate content was determined using the method described by (McCluer, 1963). A 0.2 g sample was mixed with 25 mL of 1N perchloric acid ($HClO_4$) in a test tube, which was subsequently placed in a water bath at 60°C for 30 minutes. The mixture was then filtered using filter paper. A 1 mL aliquot of the filtrate was collected in a volumetric flask, and 9 mL of distilled water was added, resulting in a total volume of 10 mL. Then, 1 mL of this diluted solution was mixed with 5 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid (H_2SO_4). The mixture was allowed to cool. A spectrophotometer was used to measure the absorbance at a wavelength of 490 nm. Various concentrations of glucose (0, 0.2, 0.3, 0.4, and 0.5 mg/mL) were prepared, and their absorbance values were recorded to construct a calibration curve. The absorbance of the sample was subsequently measured and plotted onto the calibration curve, allowing for the calculation of concentration. The carbohydrate percentage was calculated using the following equation:

Carbohydrate (%) = [Concentration derived from the calibration curve (mg/mL) × Dilution factor (10 mL) × 25 mL × 100] / (Sample weight in grams × 1000).

Lipid: The lipid content was quantified utilizing the (Pérez-Palacios et al., 2008) methodology. A 10 g aliquot of the desiccated sample was encapsulated in filter paper and subsequently positioned within the thimble of a Soxhlet extraction apparatus. Hexane was then introduced into the 250 mL flask of the apparatus, and the extraction process commenced. The extraction required approximately 5 hours. The solvent was then collected from the apparatus and evaporated from the flask. To assist with the evaporation of residual solvent and to remove any fatty materials, the flask was placed in an electric oven set at 80°C for 30 minutes. The flask was then removed from the oven, allowed to cool in a desiccator, and weighed. The fat percentage was calculated using the following formula:

Lipid (%) = [(Weight of the flask with lipid – Weight of the empty flask) / Weight of the sample] × 100

Estimation of Fatty Acids: Lipid extraction was conducted using the (Pérez-Palacios et al., 2008) technique, utilizing the Soxhlet lipid extraction apparatus. Lipid Esterification: The sample was prepared using the AOAC-approved procedure. The method involves reacting lipids with methanolic potassium hydroxide to esterify fatty acids. To prepare the methanolic KOH solution, 11.2 g of potassium hydroxide (KOH) was dissolved in 100 mL of methanol, and 8 mL of this methanolic KOH solution was added to 1 g of fat. The mixture was vigorously agitated with 5 mL of hexane for 30 seconds and then allowed to separate into two distinct layers. The esterified fatty acids (fatty acid methyl esters, FAMEs) were extracted from the hexane layer and transferred into a vial for analysis. GC Analysis: The fatty acid methyl esters were analyzed using a Japanese gas chromatography system (GC-2010) equipped with a flame ionization detector (FID) and a capillary column (SE-30) that was 30 meters long and 0.25 mm in internal diameter. The fatty acid compounds were identified and quantified as detailed below (Table 1).

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Table1. Gas chromatography system (GC-2010)

Paragraph name	Temperature
Injection area temperature	280 C
Detector temperature	310 C
Separation column temperature	120 – 290 (10 C / MIN)
Gas Flow Rate	100 Kpa

RESULTS AND DISCUSSION

Assessment of *Coelasterlla* MH923011 biomass: A useful indirect metric for determining the biomass concentration in microalgal cell suspensions is optical density. A suitable standard curve establishes a direct correlation between a suspension's light absorption and its chlorophyll concentration. However, when the cells' pigment content varies, errors could occur (Jiménez-Lao et al., 2021). The mutant *Coelastrella MH923011* did not attain the stationary phase after 15 days, in contrast to the un-mutated culture, when grown in BG11 media under a light intensity of 167.48 µE m^{-2} s^{-1}, a temperature of 24 ± 2°C, and an initial pH of 7, with constant agitation. The growth curve is illustrated in Table 2 and Figures 1 and 2. Under the aforementioned culture conditions, during the 15-day culturing period, the pigment content of the un-mutated *Coelastrella MH923011* ranged from 0 to 0.02 g/L, with an increase in algal growth observed between days 11 and 15. The maximum growth rate reached 0.04 g/L on day 13, declining to 0.02 g/L by day 15, as illustrated in Table 2 and Figures 1 and 2. In contrast, the mutated *Coelastrella MH923011* exhibited pigment content varying from 0.002 to 0.03 g/L, with growth enhancement noted between days 7 and 15. The peak growth was recorded at 0.01 g/L on day 13, followed by an increase to 0.03 g/L on day 15. The initial rapid development presumably signifies excellent conditions for *Coelastrella MH923011*, including favorable light, nutritional availability, and temperature, which are essential for photosynthetic organisms (Thepsuthammarat et al. 2023; Torzillo and Vonshak, 2013). The maximum growth observed on day 13 indicates that *Coelastrella MH923011* thrived under ideal cellular division and resource distribution conditions. The subsequent decline in growth rate may result from various factors, including nutrient exhaustion or heightened waste accumulation, which is typical in algal cultures nearing the stationary phase (Chiu-Mei et al., 2017; Jiang et al., 2022). The treatment with mutagens can induce stress responses in algal cells. These chemicals may activate adaptive mechanisms or impede growth by damaging cellular components (Razooki et al., 2020; Trovão et al., 2022). The observation that the growth reached a lower peak rate (0.01 g/L) compared to 0.04 g/L in the un-mutated culture indicates that although mutagens might elicit stress that occasionally stimulates development (e.g., via adaptive responses), they typically do not facilitate optimal growth rates (Gutiérrez et al., 2021; Nayaka et al., 2017; Till et al., 2004). This corresponds with data indicating that mutagens might induce stasis or reduced development with prolonged exposure (Le et al., 2024; Zhang et al., 2023). The growth observed on day 15 in the mutagen-treated culture may indicate an acquired resistance or adaptation to the mutagen, implying that *Coelastrella MH923011* can endure the mutagenic environment following initial stress, thereby reinforcing the notion of resilience in microalgae (Markou et al., 2014; Mingmin et al., 2021; Passy and Larson, 2011).

Table 2. *Coelasterlla MH923011* cultivation growth rate before and after 0.5% EMS treatment

Duration Day	O.D 663nm reading for *Coelasterlla* MH923011 before mutagenesis	O.D 663nm reading for *Coelasterlla* MH923011 after mutagenesis
Zero	0	0
1	0	0
3	0	0
7	0	0.001
9	0	0.003
11	0.02	0.004
13	0.04	0.01
15	0.02	0.03

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Figure 1

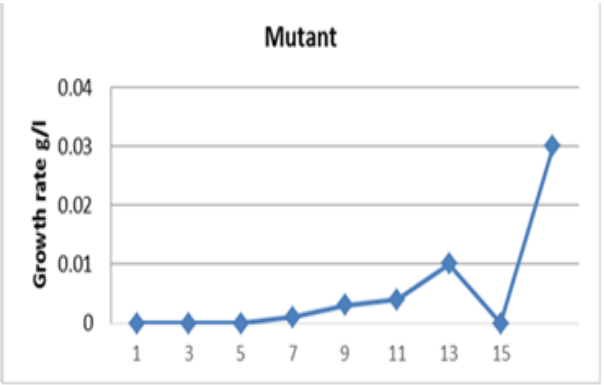

Figure 2

Figure 1,2. Variations in biomass in the un mutant and the mutant *Coelasterlla* MH92301

Assessment of the mutation's impact on the main biochemical composition

Protein Content: The protein content of the mutated *Coelastrella MH923011* increased to 43.6%, representing an 11.224% increase compared to the un-mutated culture (39.2%). This implies that the genetic alteration induced by EMS mutagenesis may promote protein biosynthesis or storage, thereby enhancing the mutant's capacity for protein synthesis or accumulation. Improvements in growth rate, metabolic efficiency, or modifications to metabolic pathways that support protein production are frequently associated with increases in protein content (Galasso et al., 2019; Alshididi et al., 2024) (Figure 3).

Lipid Content: The lipid content of the mutated *Coelastrella MH923011* was 25.6%, representing a 26.108% increase compared to the control (20.3%). This suggests that the mutant may have altered metabolic pathways that favor lipid accumulation or increased lipid production. Changes in fatty acid biosynthesis pathways or energy storage systems may be linked to elevated lipid content. Additionally, changes in cellular energy stores and stress response pathways are frequently associated with elevated lipid levels (Jiang et al., 2022; Khoo et al., 2023; Zhang et al., 2023) (Figure 3)

Carbohydrate Content: The carbohydrate content increased from 18.6% in the un-mutated culture to 23.6% in the mutated culture, representing a 26.881% increase. This could suggest that the mutant's carbohydrate metabolism has been altered, potentially improving the production or storage of polysaccharides such as starch or glycogen. Additionally, the accumulation of carbohydrates may reflect improved energy storage capacity or modifications in photosynthetic efficiency or carbon fixation in the mutant (Li et al., 2019a and b), as shown in Table 3 and Figures 3 and 4.

Table 2. Chemical makeup of mutant and unmutant *Coelasterlla MH923011* differs in terms of lipids, proteins, and carbohydrates

Name	Protein %	Lipid %	CHO %
Un mutant	39.2	20.3	18.6
Mutant	43.6	25.6	23.6
Increasing %	11.224	26.108	26.881

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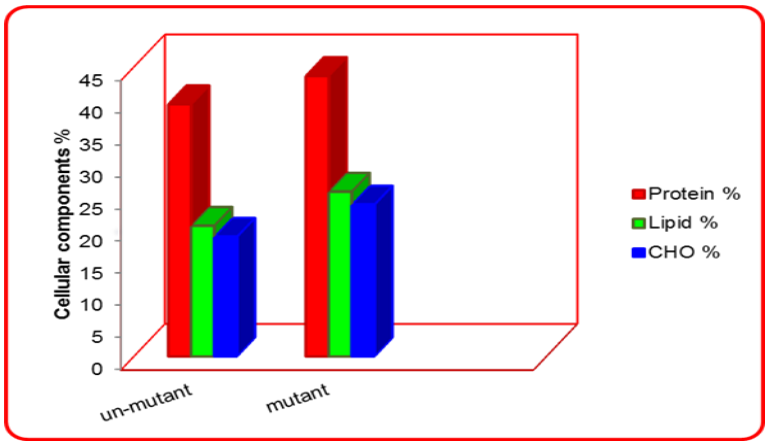

Figure 3. Differences between mutant and unmutant *Coelasterlla* MH923011 in terms of their chemical makeup (lipids, proteins, and carbohydrates).

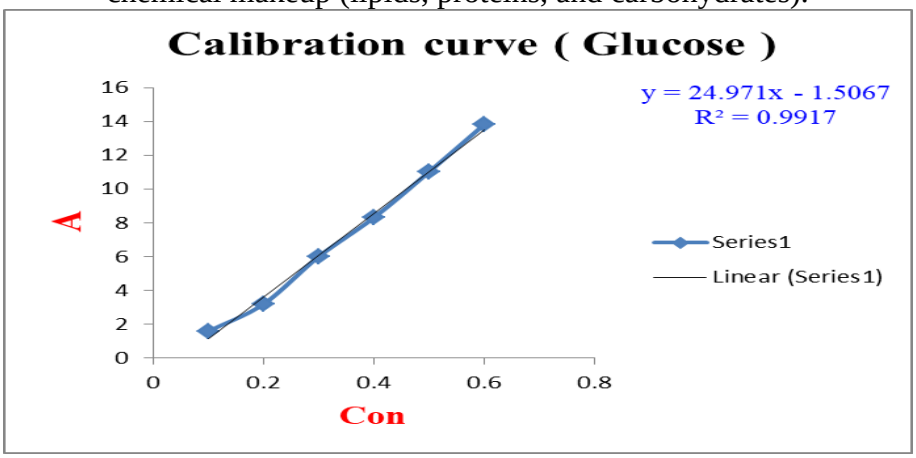

Figure 4. Variations in glucose concentration in mutant *Coelasterlla MH92301*

Fatty acids

Palmitic Acid (C16:0): The amount of palmitic acid in the mutant increased by 9.31% (from 6.12% to 6.69%). Palmitic acid is a crucial saturated fatty acid that plays an essential role in the synthesis of phospholipids and triglycerides. An increase in palmitic acid may indicate improved fatty acid synthesis, which could be caused by changes in the genes encoding fatty acid synthase or by an increase in the substrate available for its synthesis (Chu, 2017; Elshobary et al., 2022). It is also possible that the mutant's increased palmitic acid indicates a shift toward a more saturated lipid profile.

Oleic Acid (C18:1): The amount of oleic acid, a monounsaturated fatty acid, in the mutant rose by 3.62% (from 12.14% to 12.58%). Better nutritional and functional qualities, such as increased bioavailability and excellent oxidative stability, are frequently associated with oleic acid. According to (Zhu et al., 2022), the slight increase suggests that the mutation may be adjusting lipid unsaturation, which could change cell membrane fluidity and signaling pathways.

Linoleic Acid (C18:2): The mutant exhibited a minor 2.08% increase in this polyunsaturated fatty acid (from 14.89% to 15.20%). This modification may indicate a slight shift in desaturase activity, specifically in the enzymes that add double bonds to fatty acid chains. Linoleic acid is essential for the synthesis of eicosanoids, signaling molecules implicated in inflammation and cell proliferation (de Jonge et al., 1996).

Stearic Acid (C18:0): Stearic acid increased by 2.45% in the mutant (from 4.89% to 5.01%), a relatively modest shift compared to other fatty acids. Stearic acid is a saturated fatty acid that often serves as a precursor to oleic acid in the biosynthesis pathway. The increase in stearic acid may result from altered activity of enzymes involved in fatty acid elongation (Elshobary et al., 2022).

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Linolenic Acid (C18:3): Linolenic acid exhibited a remarkable 9.46% increase (from 0.74% to 0.81%). This polyunsaturated fatty acid is crucial for human health as it maintains cellular membranes and possesses anti-inflammatory properties. The increase reflects greater activity of desaturase enzymes or a change in the regulation of lipid metabolism pathways (Harris and James, 1965).

Butyric Acid (C4:0): The most substantial increase was reported for butyric acid, which jumped by 14.59% in the mutant (from 2.33% to 2.67%). Butyric acid is a short-chain saturated fatty acid produced through the fermentation of dietary fibers and is beneficial for intestinal health and inflammation regulation. Given the increase in butyric acid, it is possible that the mutation impacted the pathways that produce short-chain fatty acids, potentially increasing the activity of the enzymes responsible for their production (den Besten et al., 2013).

Arachidic Acid (C20:0): Arachidic acid rose from 2.10% to 2.39%, representing a 13.81% increase in the mutant. Arachidic acid is a long-chain saturated fatty acid found in lipids such as phospholipids and sphingolipids. Changes in lipid synthesis pathways that encourage the synthesis of longer-chain fatty acids may cause an increase in arachidic acid, which could enhance the structural integrity of cellular membranes (de Jonge et al., 1996).

Table 3. Fatty acid concentration differences (%) between the control and mutant *Coelasterlla* MH923011

Fatty acids 5	Control%	Mutant%	%Percentage of increasing
Palmatic %	6.12	6.69	9.313
Oleic %	12.14	12.58	3.624
Linoleic %	14.89	15.20	2.081
Stearic %	4.89	5.01	2.453
Linolenic %	0.74	0.81	9.459
Butyric acid %	2.33	2.67	14.592
Arachidic %	2.10	2.39	13.809

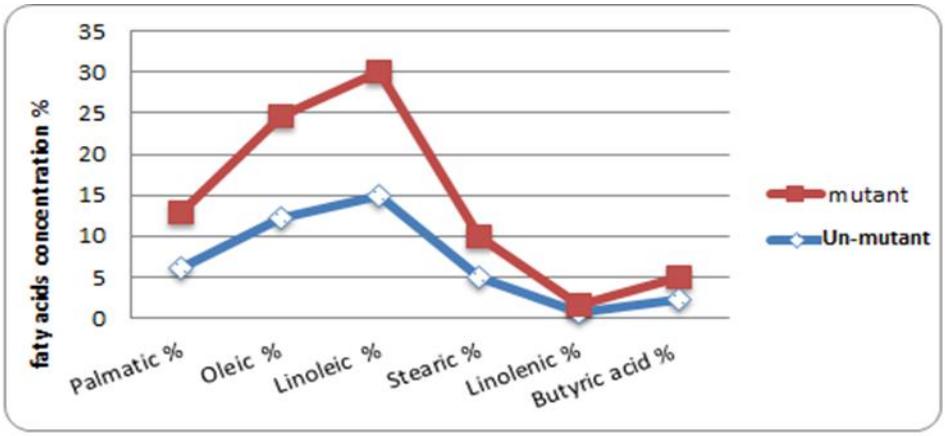

Figure 6. Fatty acid concentration differences (%) between the un -mutant and mutant *Coelasterlla* MH923011

CONCLUSION

The growth patterns of *Coelasterlla* MH923011 that have been observed indicate that experimental and environmental factors greatly influence algal growth dynamics. The divergent reactions to light and nutrient availability in the control and stress response pathways in the presence of mutagens reveal algal resilience and adaptation. The findings highlight how intricate algal growth behavior is, especially when biochemical stresses are present. The mutant's observed increases in protein, fat, and carbohydrate content may have significant ramifications for its possible uses. Due to its improved nutritional profile, the mutant may be more suited for industrial or agricultural applications where greater yields of these nutrients are needed. Based on the observed increases in the quantity of fatty acids in the mutant, it is possible that the

mutation altered the pathways involved in lipid biosynthesis, boosting the creation of long-chain and short-chain fatty acids as well as saturated and unsaturated fatty acids. The mutant may benefit from these modifications in terms of improved membrane characteristics, changed energy storage, or increased stress tolerance. More research is required to identify the precise genetic and enzymatic alterations causing these changes in the composition of fatty acids. For example, macronutrients are needed when producing food, biofuel, or animal feed.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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تقييم تأثير الاثيل ميثان سلفونيت كمطفر كيميائي على المكونات الخلوية للطحالب الخضر الدقيقة

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المستخلص

هدفت هذه الدراسة الى دراسة تأثير المطفر الكيميائي الاثيل ميثان سلفونيت على المكونات الخلوية لطحلب الكوليسترلا الخضراء الصغيرة .جنس الكوليسترلا موجود منذ حوالي قرن ويضم عددا صغيرا من الأنواع. الانواع التابعة لهذا الجنس لها شكل كروي بيضاوي الشكل حتى يصبح شجيري الشكل. توجد على شكل طحالب دقيقة وحيدة الخلية أو كتجمعات لعدد قليل من الخلايا. وتمت زراعة الطحلب لمدة 14 يوما في ظروف معدلة على وسط BG11 قبل إخضاعها للمطفر الكيميائي الاثيل ميثان سلفونيت لتحديد تاثيره على الكتلة الحيوية,والمحتويات الخلوية مثل البروتينات والكربوهيدرات والدهون والأحماض الدهنية. وأظهرت الطحالب المطفرة زيادة بالكتلة الحيوية من الأيام 7 إلى 15 ، ومعدل النمو الأقصى البالغ 0.01غم/لتر في اليوم 13 والزيادة اللاحقة بحلول اليوم 15 الى 0,03 غم/لتر ,وقد تشير إلى استجابات فسيولوجية مختلفة تسببها الطفرات ، وأظهرت ايضا ارتفاعا في نسبة البروتينات بنسبة 11,22% والكاربوهيدرات بنسبة 26,88% والدهون 26.11%. بالإضافة إلى ذلك أكدت قياسات كروموتوغرافيا الغاز وجود سبعة أحماض دهنية والتي ازدادت في الطحالب المطفرة قياسا بمجموعة السيطرة.

الكلمات المفتاحية: الوقود الحيوي ,الكتلة الحيوية , الكوليسترلا, الاحماض الدهنية, البروتينات