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Impact of the Chemical Mutagenesis on Biomass and Productivity of some Micro Green Algae

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Abstract: Mutant microalgae Nanochloropsis oculata. and Chlorella sp included in this study exhibited a high potential for energetic lipid storage as well as high growth rates comparing to the un mutants (standard), therefore, considered promising candidates for advancing the biofuel production. In addition to the exponentially increase in biomass carbohydrates, proteins and fatty acids remarkably increased after these strains were mutated by ethyl methane sulphonate (EMS), while total lipids decreased. Biochemical compositional analysis was carried out by using gas chromatography to find out concentrations of five major fatty acids: Palmitic acid, Lenolenic acid, Stearic acid, Oleic acid and Linoleic acid. In mutant N. oculata and Chlorella sp. Lenolenic acid is the fatty acid with high percentage (11.7 and 4.8 %) respectively compared with un- mutant (0.241 and 1.824) %. Five fatty acids were selected to assess effect of the mutation, which revealed that initial concentrations of all the fatty acids before the mutation were multiplied about 48, 16, 14, 7 and 6 fold of Lenolenic, Palmitic, Oleic, Stearic and Linoleic acids respectively in N. oculata, while slightly changes occurred in the concentrations of Chlorella sp fatty acids.

Keywords: EMS, Microalgae, Biofule, Mutant

Introduction

There are many potential advantages of using biofuel, the emissions from such fuels are far lower than those of fossil fuels and biofuels crucially biodegrade much faster also non-toxic comparing to the catastrophic impacts on atmosphere and biosphere result from biofuel are far less than the horror accompanies from continuing urge of using fossil fuel, although one drawback needs work in fossil fuels is that burning it releases nitrogen oxide into the air (Muto et al., 2013). Micro green algae are known as the oldest life forms, they are the large and diverse group of aquatic organisms that lack the complex cell structures found in higher plants, moreover the algae presence and populated approximately all types of environments, some species thriving in freshwater, others in saline conditions, hot springs, and seawater (Brennan and Owende, 2010). The current depletion of world oil resources and pollution of our planet create a dilemma intriguing minds of scientists to confront this dilemma, therefore, efforts have been made to produce an ecologically pure fuel based on renewable bioresources (biofuel) and the treatments of biological waste through anaerobic digestion have many benefits; biogas leads not only to cleaner and healthier environment but also produces a renewable energy sources such as methane (Balat and Balat, 2009).

Micro-algae have received considerable interest as a potential feedstock for biofuel production because they have higher co_2 fixation rates than terrestrial plants and can thus utilize co_2 from flue gas to produce biomass depending on the species and cultivation conditions, they can produce useful quantities of polysaccharides and triacylglycerides, which are raw materials for producing bioethanol and biodiesel (Chien-Ya et al., 2011). Microalgae synthesize fatty acids as building blocks to form various lipid compounds, such as polarized

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structural and functional lipid such as phospholipid and glycolipid together with neutral lipids such as monoglyceride, diglyceride, and triglyceride, free fatty acid, hydrocarbon and pigment. Limited polarized lipid is located in organelle membrane within the cell, such as lactide in the chloroplast.

Triglyceride is a neutral lipid stored in the vacuole inside the cell. High C18:0 and C18:1content inside microalgae is the precondition in biodiesel production for their oxidative stability and high adaptation ability potential for industrial-scale production (Olofsson et al., 2012). C16:0 is also proposed as the suitable fatty acid in renewable biodiesel production. Both are rarely found in *Nannochloropsis* sp. cells, these microalgae species are became a potential biofuel material species. Researchers on microalgae mutated by ethyl methane sulfonate are still rare. Chemical mutagens induced mutagenesis may be particularly biased in some cases, as they may increase the mutation levels, particularly in some genomic regions with high GC-content. It is expected that some mutagenesis experiments may not result in desired phenotypes due to limited mutant pools. Therefore, it is important to understand the biases before selection of a chemical mutagen for mutagenesis experiments. Ethyl methanesulfonate (EMS) is another popular chemical mutagen that has been proven to be effective and efficient in mutagenesis (Jabeen and Mirza, 2004).

Successful mutagenesis has been performed using EMS to create algal mutants with increased lipid accumulation, for example *Chlamydomonas reinhardtii* was randomly mutagenized by EMS for enhanced lipid production (Chaturvedi. and Fujita, 2006). Chemical mutagenesis has long been employed to improve the biomass of microalgae lipid productivity such as *Nannochloropsis* sp. and *Chlorella* sp. (Doan and Obbard, 2012; Shin et al., 2016). It has been demonstrated that the attenuation of the light-harvesting property by mutation could improve the photosynthesis efficiency of microalgae by decreasing the cell shading effect, allowing an increase in biomass and lipid productivities (Polle et al., 2002). Chemical mutagenesis method using ethyl methane sulphonate (EMS) which was used in this research is relatively new and complicated. This study, aims to demonstrate impact of chemical mutagenesis to generate mutant in two water surface- floating microalgae to identify proximate cell composition (carbohydrate, fatty acid, and protein) in *Nannochloropsis oculata*. and *Chlorella sp*.

Materials and Methods

Isolation and Purification of Algae

The microalgae samples were collected from the water cannels around the University of Baghdad- Al-Jadriah, uni-algal cultures of *Chlorella sp* and *N. oculata* were obtained using serial dilution method with 1ml of sample inoculated into 9 ml of Chu-10 nutrient solution. The procedures were repeated with microscopic examination until one species of both microalgae were obtained.

Chu-10 Media Preparation

Stock solution (250 mL) from the following salts were prepared: MgSO4.7H2O, K2HPO4, NaNO3, CaCl2, FeCl3, EDTA-Na, NaCl, Na2CO3, MnCl2.4H2O, (NH4) 6Mo7O24.4H2O, ZnSO4.7H2O, CuSO4.5H2O, CoCl2.6H2O, H3BO3, and Na2SiO3. 2.5 ml of each stock were taken and completed the volume to one liter of distilled water according to Kassim and Salman (1999), while the medium was autoclaved and used to culture *Chlorella sp.* and *N. oculata*

Chemical Mutagenesis

Preparation of Ethyl Methane Sulphonate (EMS)

EMS with 0.5 % (v/v) in 100 ml phosphate buffer solution at (pH7) was prepared (Rasha and Kadium, 2017).

EMS Mutagenesis

Cell suspensions of both microalgae *N. oculata* and *Chlorella sp*. in exponential phase at 107cells/ml were subjected to random mutagenesis via exposure to EMS concentration (Himedia-India) 0.5% for 1 hour in darkness at room temperature with vigorous agitation at 300 rpm. Following incubation, treated cultures were

centrifuged at 8000 rpm for 10 min., pellets were then washed twice with the autoclaved solution of 10% (w/v) sodium thiosulfate to remove excess EMS. Micro algae were re-suspended into 10 ml sterile Chu-10 medium and kept in darkness overnight to grow for further selection. After three weeks incubation at a temperature 25 Cº (Kawaroe et al., 2015).

Estimation of Micro Algal Biomass

The growth rate was estimated according to Mackinney, G. (1941) as an indirect method for chlorophyll estimation. The pigments can be completely extractable in acetone and exhibit characteristic absorbance at 663 nm wavelength. 10 ml of microalgae cultures were centrifuged at 5000 rpm for 10 minutes. The pellet was re-suspended in 80% acetone and vortexes thoroughly. The tubes were incubated in water bath at 60 °C in dark for 1 hr. with occasional shaking. After 1 hr. incubation, the suspension was centrifuged again and the supernatant was stored in dark. The procedure was repeated for the pellet to ensure complete chlorophyll extraction. An absorbance of the supernatant was read at 663 nm in UV-Vis spectrophotometer using 80% acetone as blank. The growth was estimated spectrophotometrically every 2 days, and the curve was plotted using the absorbance versus time.

Evaluation Effects of the Mutation on Major Biochemical Composition

Carbohydrates Extraction

The carbohydrates were extracted using anthrone method Raunkjær, et al. (1994), which is a simple colorimetric method based on a reaction between the carbohydrates and anthrone reagent developing a green color. A solution with 0.1 of anthrone was prepared in 75% H2SO4; the reagent must be prepared freshly at the day of measurement. Glucose standard curve was prepared with final concentration 100 mg/L. 100 mg of freeze-dried algal biomass was weighed and transferred to COD tube and placed on ice, 2 mL of 75% H2SO4 were added to the samples and vortexed thoroughly to mix the acid and the biomass.

Anthrone reagent was added to all the tubes including glucose tubes (4 mL) and vortexed again to ensure the mixing of the reagent with the carbohydrates. Tubes were placed in water bath 100 °C for 15 minutes and then cooled down to room temperature. The absorbance was measured at 625 nm for all the tubes and calibration curve was drawn from the glucose concentration and the total carbohydrates were obtained using this calibration curve.

Lipids Extraction

The lipids were extracted according to Bligh, (1959). For 1 gm of a dried algal sample, 2 mL of methanol and 1 mL of chloroform were added, the mixture was kept at room temperature for 24 hours, and the mixture was then vortexed for 2 minutes. 1 ml of chloroform was added again and mixed well. Then 1.8 ml of distilled water was added. The mixture was vortexed again for 2 minutes followed by centrifugation for 10 min at 2000 rpm. Upper layer was discarded and the lower layer was filtered with Whatman No. 1 filter paper. The vial was weighed and recorded as (weight 1). The vial was put in a water bath until evaporation; the vial was weighed again and expressed as (weight 2). The lipid yield was calculated as w2-w1 and the result was expressed as % dcw gm/ L.

Results and Discussion

Estimation of Microalgal Biomass

Optical density is used as a convenient indirect measurement of biomass concentration in microalgal cell suspensions. The absorbance of light by a suspension can be related directly to chlorophyll concentration using a suitable standard curve. However, in accuracies can be introduced when the pigment content of the cells changes. Mutant *Chlorella* sp. and *N. oculata* reached stationary phase after 17 day comparing to the un-mutated culturing in Chu-10 media under light intensity 167.48 µE m−2s−1 and tmperature (24±2) and initial pH 7 with continuous shaking. The growth curve is shown in (Figures 1, 2, 3, 4). Under the pre mentioned culture conditions and during 17 day of culturing pigment content of the un- mutated *Chlorella* sp. varied between 0.055 and 1.329 g/l while mutated *Chlorella* sp. varied between 0.076 and 1.462 g/l. In un-mutated *N. oculata* pigment

content varied between 0.094 and 0.848 g/l, while 0.024 and 0.977 g/l in the mutated. The highest $O.D_{663}$ reading was in 17 days about 1.462 g/l in the mutated *Chlorella* sp. while the highest $O.D_{663}$ reading of mutated *N. oculata* was 0.977g/l as shown in Table 1.

Evaluation Effects of the Mutation on Major Biochemical Composition

As with biomass, lipid content was also measured, lipid content decreased after mutation in both algae. Protein concentration in mutated *N. oculata* was higher than that in mutated *Chlorella sp.* and un mutated *N. oculata*, in bottom line production of protein decreased after mutation in *C. kesslari* while increased in *N. oculata* Highest carbohydrates contents occurred in mutated *Chlorella sp.* comparing to its concentration in mutated *N. oculata* Mutation caused increased in carbohydrates in both algae. A summary production of lipids and carbohydrates increased after mutation in *Chlorella sp.* except protein production decreased comparing to their production levels in *N. oculata* as shown in Figure 5.

Table 1. The growth rate of *N. oculata* and *Chlorella sp.* Cultivation before and after the treatment with 0.5% EMS.

Duration	**$O.D_{663}$ reading for *Chlorella sp.***		**$O.D_{663}$ reading for *N. oculata***	
Day	**Before**	**After**	**Before**	**After**
Zero	0.055	0.076	0.094	0.024
2	0.102	0.124	0.053	0.127
4	0.068	0.185	0.053	0.158
6	0.091	0.226	0.093	0.143
8	0.272	0.42	0.187	0.234
10	0.359	0.483	0.233	0.209
12	0.595	0.866	0.63	0.458
17	1.32	1.462	0.848	0.977

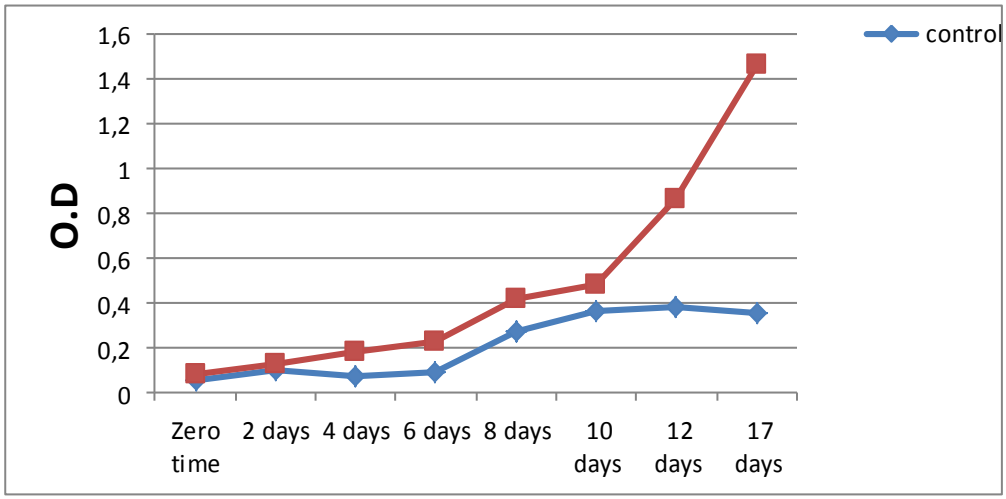

Figure 1. Variations in biomass between mutant *Chlorella sp* and the control (un mutant)

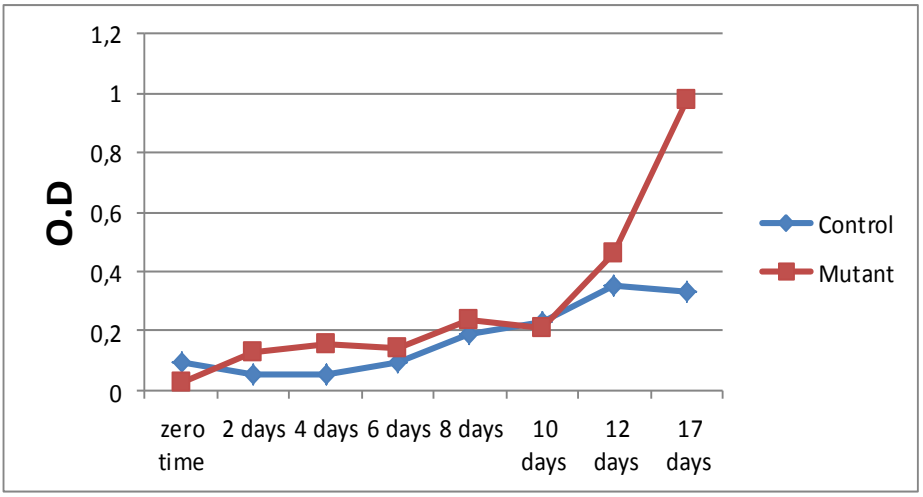

Figure 2. Variations in biomass between mutant *N.oculata* and the control (un mutant)

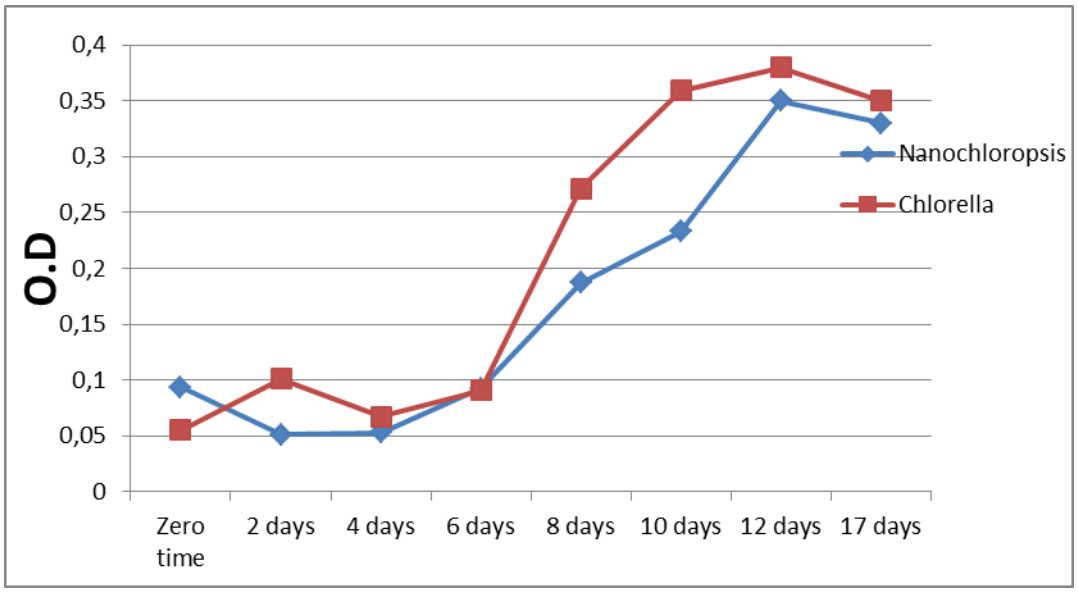

Figure 3. Variations in biomass between *N.oculata* and *Chlorella sp* before mutagenicity

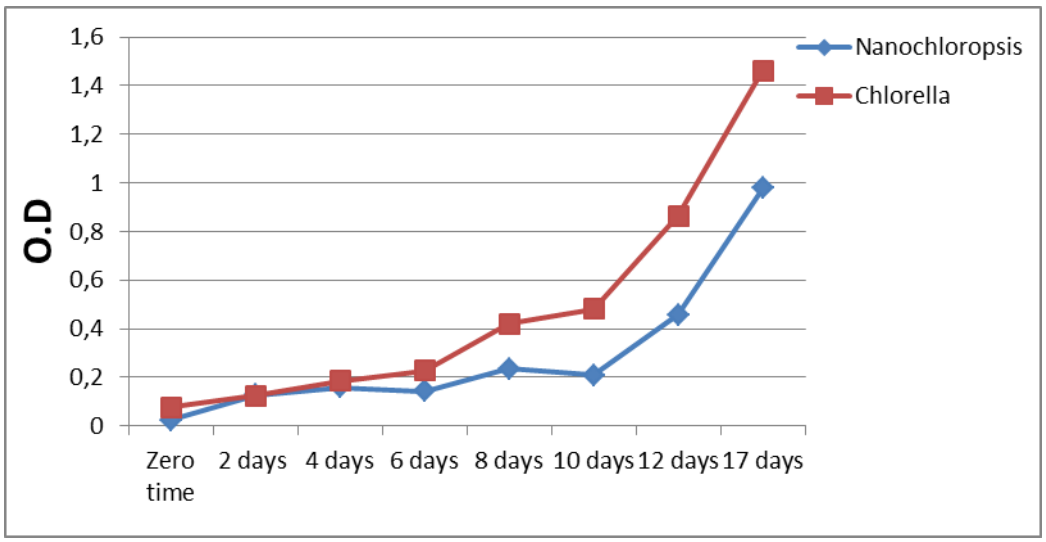

Figure 4. Variations in biomass between *N. oculata* and *Chlorella sp* after mutagenicity

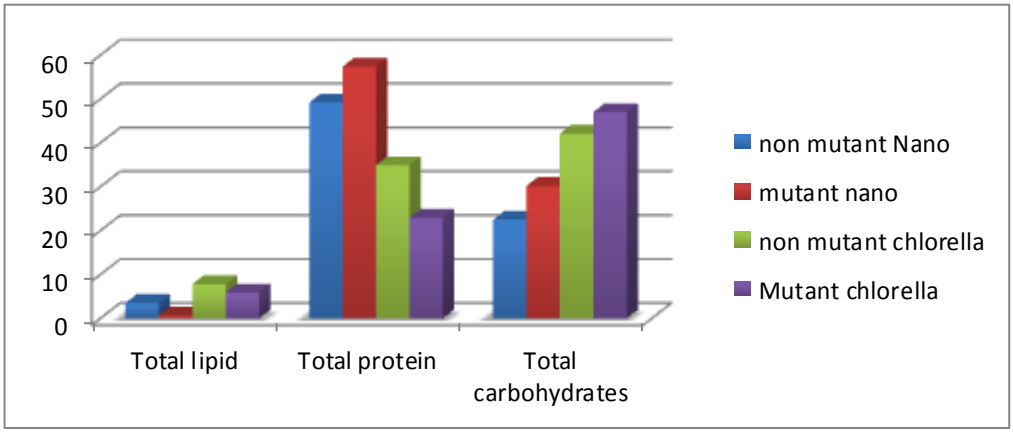

Figure 5. Variations in chemical composition (Lipids, proteins and carbohydrates) between mutant and un mutant *Chlorella sp* and *N. oculata*

Fatty Acids

Biochemical compositional analysis was carried out by using gas chromatography to find out concentrations of five major fatty acids: Palmitic acid, Lenolenic acid, Stearic acid, Oleic acid and Linoleic acid. In mutant *N. oculata* and *Chlorella sp.* Lenolenic acid is the fatty acid with high percentage (11.7 and 4.8 %) respectively compared with un- mutant (0.241 and 1.824) % as shown in table 2. These results showed that fatty acids increased in mutant algae 11 folds than un- mutant. In other hand mutant *N. oculata* showed increased in all fatty acids percentage that detected compared with mutant *Chlorella sp.* as shown in Table (2).

Table 2: Variations in fatty acids concentrations (%) between mutant and the control (un -mutant) *Chlorella sp* and *N. oculata.*

Algae	Palmitic acid	Lenolenic acid	Stearic acid	Oleic acid	Linoleic acid
Un-mutant *Nanochloropsis*	0.19	0.241	1.08	0.29	1.03
Mutant *Nanochloropsis*	3.1	11.7	8.17	4.13	6.01
Un-mutant *C. kesslari*	0.574	1.824	1.04	0.621	0.94
mutant *C. kesslari*	0.61	4.8	0.83	0.437	0.82

Discussion

Ong et al. (2010) stated that *C. kesslari* mutated with 0.1 M EMS and cultivated in higher temperature from the original species has higher specific growth rate, this came along with the results of this study, on the other hand *Nannochloropsis* sp. treated with the same EMS concentration gave higher growth rate value comparing with the un mutated. Also, higher lipid productivity of the mutated species cultivated with high temperature is higher compared to its original form. Results range between 0.6 - 1.2 g/l for mutated *Chlorella* sp. with 0.1 M EMS and 0.4 - 0.9 g/l for un mutated with 40°C cultivation temperature.

A few notable studies in this scientific field such as Chaturvedi and Fujita (2006) which included EPA fatty acid compound of mutated *Nannochloropsis* sp., also Doan and Obbard (2012) figured out that lipid compound of *Nannochloropsis* increased after the mutation From another research conducted by Chaturvedi and Fujita (2006), it is proven that EPA type fatty acid can be boosted by applying mutagenesis on *Nannochloropsis oculata*. Research by Doan and Obbard (2012) that tested lipid content increase of *Nannochloropsis* sp., mutated microalgae species can produce more fatty acid compared to its original state, both in exponential and stationery phase.

In this research EMS used to mutaginize *Chlorella sp.* and *N. oculata* cells because it is a powerful and easy technique to generate a number of mutants, and we can create a number of candidate mutants that are from various types of random mutations by modulating AT to G-C in DNAs on microalgae genome as described in many successful reports in several microorganisms (Kim et al., 2006; Sandesh et al., 2008; Lee et al., 2014). For instance, *Chlamydomonas reinhardtii* mutants (ADP glucose pyrophosphorylase mutated) exhibited 10-fold increase of TAG accumulation compared to wild type by using EMS mutagenesis (Radakovits et al., 2010 and work et al., 2010).

Other EMS application was also used in other eukaryotic organism such as *Saccharomyces cerevisiae*, *Cyclotella* sp. and *Arthrospira platensis* (cyanobacterium) to find a higher bioethanol producer (Mobini-Dehkordi et al., 2008). Fatty acids (FAs) are one of the primary metabolites of microalgae, which enrich their utility both in the form of food and fuels. Palmitic acid (C16:0) and oleic acid (C18:1n9c) were found to be dominant among the members of Cyanophyceae whereas members of Chlorophyceae were rich in palmitic acid (C16:0), oleic acid (C18:1n9c) and linoleic acid (C18:2n6). (Sahu et al., 2013). *Nannochloropsis* sp. have attracted sustained interest from algal biofuels researchers owing to their rapid growth, high amounts of triacylglycerol (TAG) and high-value polyunsaturated fatty acid (FA) and their successful cultivation at large scale using natural sunlight by multiple institutes and companies (Ajjawi et al., 2017; Wei H et al., 2017; Zienkiewicz et al., 2017).

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