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Improvement and screening of astaxanthin producing mutants of newly isolated *Coelastrum* sp. using ethyl methane sulfonate induced mutagenesis technique

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ABSTRACT

Natural astaxanthin is known to be produced by green microalgae, a potent producer of the most powerful antioxidant. To increase the productivity of astaxanthin in microalgae, random mutagenesis has been extensively used to improve the yield of valuable substances. In the presented work, a newly isolated *Coelastrum* sp. was randomly mutagenized by exposure to ethyl methane sulfonate and further screened using two approaches; an approach for high growth mutant and an approach for high astaxanthin producing mutant with a high-throughput screening method using glufosinate. Among these, mutant G1-C1 that was selected using glufosinate showed the highest of total carotenoids (45.48±1.5 mg/L) and astaxanthin (28.32±2.5 mg/L) production, which was almost 2-fold higher than that of wild type. This study indicates that random mutagenesis via chemical mutation strategy and screening using glufosinate successfully expedited astaxanthin production in a mutated strain of a *Coelastrum* sp.

1. Introduction

Astaxanthin which is a naturally occurring carotenoid pigment has been extensively studied because of its high value owing to its powerful antioxidant properties in scavenging the free radical [1, 2]. The powerful antioxidative activity of astaxanthin has possessed a wide range of applications in food, feed additives, pharmaceutical and nutraceutical industries with extensive beneficial effects on human health [3, 4, 5]. Currently, the high demand for the production of natural astaxanthin in the recent market is due to its high capacity of antioxidants compared to synthetic astaxanthin [6].

The natural astaxanthin has been found in several microorganisms, including the bacteria *Mycobacterium lacticola* [7]; fungus *Peniophora* sp. [8]; yeast *Phaffia rhodozyma* [9], and green microalgae *Haematococcus pluvialis* [10]. Astaxanthin production from green microalgae, *H. pluvialis*, is considered as a viable source of natural astaxanthin [11]. However, the yields of astaxanthin in *H. pluvialis* are too low to compete with synthetic astaxanthin as it grows relatively slow with low biomass yield and easy to be contaminated by other fast-growing organisms and consequently hindered the commercial production of natural astaxanthin [12, 13].

Presently, green microalgae that have potential in accumulating natural astaxanthin has received tremendous attention because of its high cost and the possibility of health benefits [14]. Previously, our study reported that astaxanthin production by *Coelastrum* sp. can be a potential strain for producing astaxanthin from a natural source as it is the most comparable to *H. pluvialis* under high light intensity and nitrogen starvation in mixotrophic culture. This further supports the ability of this strain as an astaxanthin producer, which can be the potential alternative to current astaxanthin production [15]. Microalgae with improved growth rate and enhanced carotenoid accumulation make the commercial production of astaxanthin more feasible.

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Therefore, several strategies have been employed for a more economical algae culture in increasing the levels of astaxanthin production in green microalgae. Significant efforts have been undertaken to improve astaxanthin production by selecting high yield strains, optimize cultivation and uses of chemicals as metabolic enhancers of astaxanthin [16,[50] 17]. Nevertheless, the progress of this improvement method has yet to be fully satisfied. Consequently, as an alternative method, the biotechnological methods of genetic modifications and mutagenesis were developed to improve the strain phenotype and accumulation of valuable bio-products in microalgae [18, 19].

Random mutagenesis is an important approach to develop improved microalgae with targeted products [20]. The method of random mutagenesis leads to random changes in the genome by randomly mutating the strain. [21]. The main advantage of random mutagenesis is its simplicity with little knowledge needed on the genes involved in the biosynthetic pathway of the targeted products [22]. There are two classes of random mutagenesis, namely physical mutagenesis using X-rays and UV light, and chemical mutagenesis using ethyl methanesulfonate (EMS) and nitrosomethyl guanidine (NTG) [23, 24]. Chemical mutagenesis is mostly used for creating positive mutants with high carotenoid and lipid content [25, 22]. Among the chemical mutagens, EMS has highest mutagenicity. It has been reported that in microalgae, EMS is the most effective mutagen that has been extensively used to improve traits for commercial applications in creating positive mutants with higher carotenoid content and polyunsaturated fatty acids [26, 27, 28].

EMS can randomly introduce DNA mutations such as nucleotide exchange (substitution), insertion and deletion of one or multiple nucleotides, and subsequently result in amino acid sequence changes. The possibility of microalgae colony that grows after treated with mutagen is believed to be the cell's capability to repair the DNA damage produced by the mutagen [29]. The advantages are the novel mutant phenotypes with altered specific amino acids are generated [30].

Generally, there are two stages of the experimental strategy included in random mutagenesis. The first stage is to generate random mutants by mutagenesis and followed by a screening strategy in selecting the positive mutants in the second stage [31]. After mutated strains have been created, mutants with a positive effect can be efficiently and effectively isolated by using an effective screening strategy [21]. Typically, screening the mutant strains with high carotenoid content required manual inspection of every colony which is inefficient.

Therefore, the combination of an efficient screening of desired phenotypes to generate random mutation using high-throughput screening is an essential step for successful random mutagenesis [32]. The chemical inhibitors that act as herbicide-resistant mutants can be used as efficient screening tools in creating high-throughput screening to effectively screen the mutated strains with a high yield of targeted pigment [22].

Herbicides can affect the primary metabolic reaction essential for plant and algal cells. Cells resistant to herbicides have been generally used in mutagenesis experiments to screen the mutants with higher yields of targeted products [33, 34]. An example of an inhibitor (herbicide) is glufosinate or called as phosphinothricin [35]. Glufosinate is an inhibitor of the enzyme glutamine synthetase (GS) that can efficiently block the activity of enzyme GS which is a crucial enzyme in ammonia assimilation [36, 37]. As a result, this leads to the intracellular accumulation of ammonia originating either from exogenous nitrate reduction or endogenous catabolic source [38]. This accumulation may eventually cause cell death [39]. Inhibition of GS by glufosinate is metabolically equivalent to nitrogen starvation. The deficiency or lack of nutrient elements is a key factor in stimulating the accumulation of secondary carotenoids [34]. As a result, glufosinate impairing nitrogen assimilation by inhibiting GS leads to the induction of astaxanthin accumulation [35].

To date, mutant strains of *Coelastrum* sp. has not been reported for commercial production of astaxanthin. This study intends to provide improvement of newly isolated *Coelastrum* sp. strain in the production of astaxanthin, aiming at creating a high-throughput method to increase the efficiency of screening by selecting astaxanthin producing strains from chemical mutagenesis. Mutagen EMS was first applied to create the mutated strains followed by comparing the screening method in selecting the positive mutants using glufosinate for high-throughput screening method.

2. Materials and methods

2.1. Algal strain and culture

The green microalgae *Coelastrum* sp. isolated from a sampling site at Hulu Langat river, Kuala Selangor, Malaysia was cultured in AF-6 medium comprising 0.14 g/L $NaNO_3$, 22 mg/L NH_4NO_3, 30 mg/L $MgSO_4 \bullet 7H_2O$, 10 mg/L $CaCl_2 \bullet 2H_2O$, 2 mg/L Fe-citrate, 2 mg/L Citric acid, 10 mg/L KH_2PO_4, 5 mg/L K_2HPO_4, trace metal solution (0.98 mg/L $FeCl_3 \bullet 6H_2O$, 0.18 mg/L $MnCl_2 \bullet 4H_2O$, 0.11 mg/L $ZnSO_4 \bullet 7H_2O$, 0.02 mg/L $CoCl_2 \bullet 6H_2O$, 0.0125 mg/L $Na_2MoO_4 \bullet 2H_2O$, 5.0 mg/L $Na_2EDTA \bullet 2H_2O$) and a mixture of vitamins (2 μg/L Biotin, 1 μg/L Pyridoxine and10 μg/L Thiamine) according to media recipe available in the Microbial Culture Collection National Institute for Environmental Studies (NIES-collection), Japan [40].

Coelastrum sp. was cultured in two-stage production, the first stage for vegetative growth and the second stage for astaxanthin production. During the early stage, cultures were grown under controlled laboratory conditions at 25±1°C with continuous aeration and enriched with 1% CO_2. It was illuminated at a continuous light intensity with fluorescence light at standard photon flux densities (PFDs) of 70 μmol photons m^{-2} s^{-1} until microalgae cultures reach exponential growth phase for five days. Cell growth was observed by measuring absorbance at 750 nm using a spectrophotometer (UV-1600, Shimadzu, Japan).

For the induction of astaxanthin biosynthesis in the second stage, the biomass of microalgae was harvested and various supplements were added according to optimize conditions in accumulating astaxanthin in *Coelastrum* sp. with details described in our previous work [16]. Sodium acetate, sodium chloride and sodium nitrate were used at a final concentration of 0.5 g/L, 3 g/L and 0.1 g/L, respectively. Microalgal cells were subsequently exposed to continuous high PFDs with 250 μmol photon m^{-2} s^{-1}. The cells were then subjected to astaxanthin extraction. All the experiments were carried out in triplicates.

The growth of mutants in microplate cell culture was monitored by counting the cell using a hemocytometer. The selected mutants were then grown with 30 mL optimal *Coelastrum* sp. medium in 50-mL flasks under 70 μmol photons m^{-2} s^{-1} and 25±1°C with 1% CO_2. The cell density of each culture was determined by measuring OD_{750} using a UV-vis spectrophotometer. Biomass determination were performed as described by Boussiba and Vonshak (1991) and expressed as g/L [41]. Specific growth rate (μ, day^{-1}) was calculated according to the equation $\mu\ (day^{-1}) = (\ln X_2 - \ln X_1)/ (t_2 - t_1)$, where X_2 and X_1 are the cell dry weight concentration (g/L) at time t_2 and t_1, respectively.

2.2. Morphology observation

Algal morphology was observed by light microscopy using an Olympus BX41 microscope (Olympus Co. Ltd., Tokyo, Japan) equipped with digital camera, aiming to compare the cell morphology of the wild type and mutant *Coelastrum* sp.

2.3. Random mutagenesis by ethyl methane sulfonate (EMS)

One mL wild type culture of *Coelastrum* sp. taken from the logarithmic growth phase (1×10^6 cells/mL) was washed with 0.2 M phosphate buffer (pH 7). Then, cells were treated at different EMS concentrations (0.1 M, 0.2 M and 0.4 M) for 15 min, 30 min and 60 min. After treatment with EMS, the treated cells were washed twice with 0.2

M phosphate buffer (pH 7) and centrifuged at 2000 × *g* for 10 min at 25°C to discard the supernatant. The treated cells were stored overnight at 7±1°C in the dark. Later, the treated cells were re-suspended and washed twice with 10% (w/v) sodium thiosulfate for decontamination of EMS. Serial dilutions of each culture were prepared and plated on 2% agar plate made by supplementation of AF-6 medium. Plates were then maintained in a growth chamber under controlled laboratory conditions at 25±1°C and illuminated at a continuous 70 µmol photons m^{-2} s^{-1} of light intensity. The colonies that were visible within 20–25 days were counted. The survival rate and the mortality rate were calculated for each EMS concentration and exposure time, and compared with non-treated EMS culture. The survival rate and mortality rate were calculated using the following formulae:

$$\text{Survival rate}(\%) : \frac{\text{Total number of survived cells} \times 100}{\text{Total number of cells(control)}}$$

$$\text{Mortality rate}\,(\%) : \frac{(\text{Control} - \text{Total number of survived cells}) \times 100}{\text{control}}$$

2.4. Screening of mutants

After mutated cells have been created, efficient screening of desired phenotypes from thousands of strains is the critical step for successful mutation breeding. In the presented study, two screening approaches were applied to obtain mutants with high astaxanthin content. One is an approach to screen for high growth mutant, and the other is an approach of screening high astaxanthin content mutant with a high-throughput screening method using glufosinate.

2.4.1. Approach of screening astaxanthin producing mutant with high growth

The large and green colonies of mutant cells that were visible under the treatment of selected mutagen dose were picked out with a toothpick and inoculated into microplate cell culture (12 well x 2 mL). All the mutants were monitored by counting cell number using a hemocytometer. Then, mutants with a higher number of cells were selected and transferred to a larger volume medium for cell propagation (30 mL optimal *Coelastrum* sp. medium in 50-mL flasks). Mutant cells with higher growth were compared with the wild type and were chosen to be further analyzed based on the amount of astaxanthin content.

2.4.2. Approach of screening astaxanthin producing mutant with high-throughput screening

A herbicide, glufosinate, was used as an inhibitor for the rapid screening of highly improved astaxanthin-producing mutants. To find the optimal concentration of glufosinate resistant to *Coelastrum* sp., the vegetative cells were first screened by adding glufosinate with various concentrations (25, 50, and 100 µM) to *Coelastrum* sp. culture on a solid agar plate. Then, the wild type *Coelastrum* sp. treated with a selected mutagen dose of EMS was spread on agar plates containing glufosinate. The cells were then incubated in a growth chamber at 25±1°C and illuminated at a continuous light intensity with fluorescence light at 70 µmol photons m^{-2} s^{-1}. The large colonies were selected and used to inoculate in a liquid medium for growth and astaxanthin analysis.

2.5. Extraction and analysis of total carotenoid and astaxanthin

To measure total carotenoid and astaxanthin content, 15 mL volume of wild type and mutant culture was centrifuged at 2000 × *g* for 10 minutes at 4 °C. The pellet was lyophilized using a freeze dryer (Lyphlock 6; Labconco, USA) for 8 hours. Then, the cells were homogenized with acetone and kept in a water bath at 70°C for 10 min followed by vortexing for 5 minutes. The mixture was centrifuged at 2000 × g for 10 min and the supernatant was collected. Supernatant collections were conducted repeatedly until the cells were faded. The concentration of total carotenoid was estimated by measuring at absorbance 470 nm and calculated using the Lichtenthaler (1987) equations [42]. The astaxanthin concentration was then measured by the spectrophotometric method and calculated with the equation, c (mg/L) = 4.5 × A_{480} × (V_a / V_b) × f. Where c is the astaxanthin concentration, V_a (mL) is the volume of solvent, V_b (mL) is the volume of algal sample, and f is the dilution ratio. 480 nm was the absorption peak of astaxanthin. A_{480} was determined by measuring the absorbance at 480 nm. Acetone was used as blank for the measurement.

Table 1
Survival rate of *Coelastrum* sp. mutant

Concentration \ Time	0.1 M	0.2 M	0.4 M
15 min	57.97 %	28.99 %	25.44 %
30 min	31.88 %	32.61 %	18.34 %
60 min	47.83 %	26.04 %	12.43 %

Table 2
Mortality rate of *Coelastrum* sp. mutant

Concentration \ Time	0.1 M	0.2 M	0.4 M
15 min	42.03 %	71.01 %	74.56 %
30 min	68.12 %	67.39 %	81.66 %
60 min	52.17 %	73.96 %	87.57 %

3. Results and discussion

3.1. Isolation of Coelastrum sp. mutants

The development of commercial cultures as an astaxanthin source requires a highly productive strain. Random mutagenesis using chemicals such as EMS is an effective strategy since it is a promising method to genetically improve astaxanthin production as it has been successfully applied in the past to enhance the productivity of various microalgal species [43, 44]. EMS mutagenesis is a most widely used chemical mutagenesis technique as it has high mutagenicity compared to those mutants obtained by physical mutation using electromagnetic radiation such as X-rays and UV light [25]. Therefore, in this study, chemical mutagenesis was applied to mutate the wild type of *Coelastrum* sp. strain to improve astaxanthin productivity genetically.

To decide the concentration of mutagen and the contact time, many trials were carried out to obtain a satisfactory survival rate after treatment with the mutagen. Random mutagenesis was controlled by changing mutagen parameters, such as concentration and incubation time of EMS. The mutagen dose of EMS was chosen based on the optimum dose that was able to induce the minimum mortality rate at approximately 85%. This selection was performed because the higher the mortality rate, the higher the potential of the survivor cells were to be potentially mutated. However, too high mortality rate might decrease the potential of mutant cells to survive [31]. The result showed that the survival rate was found to be concentration-dependent. Lower EMS concentrations in any exposure time were unable to induce the required minimum mortality. The mutant isolated under 0.4 M of EMS with 60 min exposure time was observed to have the lowest survival rate (12.43%, Table 1) and highest mortality rate (87.57%, Table 2). This survival rate was found to decrease with increasing concentration of EMS. Subsequently, the treatment of cells by 0.4 M of EMS and 60 min exposure time was selected for a mutant generation as it can provide the highest mortality by creating potentially mutated strains.

3.2. Selection of highly productive mutants

Conventionally screening mutated strains with high carotenoid

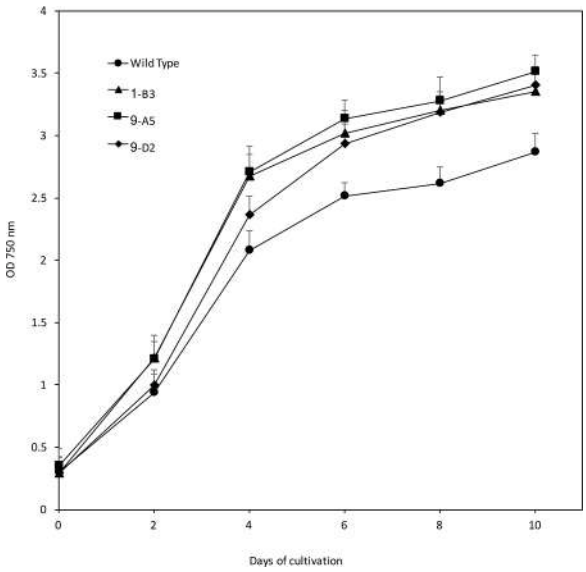

Fig. 1. Growth curve of mutants selected from the approach of screening astaxanthin producing mutant with high growth without using glufosinate and compared with the wild type. Data represent an average of 3 replications and error bars indicate mean ±SD

Table 3
Specific growth rate of mutants selected from the approach of screening astaxanthin producing mutant with high growth without using glufosinate and compared with the wild type.

Microalgae	Specific Growth Rate, μ Day^{-1}
Mutant 1-B3	0.73±0.24
Mutant 9-A5	0.75±0.14
Mutant 9-D2	0.68±0.15
Wild Type	0.57±0.11

content was quite time-consuming, laborious, and inefficient [32]. After mutated strains have been created, an efficient method of screening mutants with desired phenotypes was the critical step for a successful mutagenesis approach. To overcome the drawback, herbicide can be used as a breakthrough to find high-throughput strains [45]. This study aimed to compare the approach of astaxanthin producing mutant with and without the inhibitor using herbicide, glufosinate. The reason for this comparison is to compare the efficiency of selected mutant strains to efficiently screen the mutants of the desired phenotypes.

3.2.1. Screening astaxanthin producing mutant with growth speed

The conventional method for screening the mutated strains with high astaxanthin content required manual inspection of each colony that appeared on the plate. A total of 120 colonies grown under treatment with selected mutagen dose (0.4M EMS with 60 min exposure time) on the agar plate were chosen randomly based on their color and colony characteristics. The selected colonies were inoculated and transferred into microplate cell culture and were allowed to grow under normal growth conditions. After 20 days of inoculation, 20 mutants with the highest number of cells were chosen and transferred to a more substantial volume of liquid medium for cell propagation. The selected 20 mutant cells were first screened in terms of their biomass and growth rate because the adverse impact of astaxanthin productivity can be greatly influenced by the productivity of algal biomass [46]. The mutant cells with an excellent growth were selected to further screening the higher astaxanthin productivity strain.

The growth curve of the selected mutant as shown in Fig. 1 showed that the top three mutants (1-B3, 9-A5 and 9-D2) with the highest cell

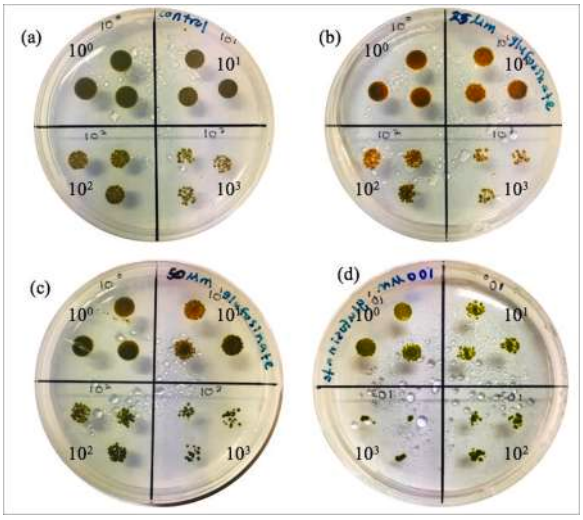

Fig. 2. The effect of glufosinate on the growth of *Coelastrum* sp. after one month incubation. 10 μl of cell suspension with different cell conditions were applied under different glufosinate concentration **(a)** 0 μM (Control); **(b)** 25 μM; **(c)** 50 μM; **(d)** 100 μM

density were found to be comparatively higher than the wild type and were analyzed further. Among these mutants, the cultivation of 9-A5 and 1-B3 mutants showed the highest specific growth rate with 0.75 day^{-1} and 0.73 day^{-1}, respectively, on day 4 (Table 3). The specific growth rate of mutant 9-D2 shown in Table 3 was 0.68 day^{-1}. The values of the specific growth rates of these mutants were somehow more elevated than the specific growth rate of the wild type, which was 0.57 day^{-1} on day 4. These results showed that the mutants cultivation was able to produce higher cell density with a higher growth rate.

3.2.2. Screening astaxanthin producing mutant using glufosinate

Therefore, in the presented study, glufosinate was used as a high-throughput method to increase the screening efficiency by selecting astaxanthin producing mutant from mutagenesis. Glufosinate, as an inducer of astaxanthin, was first applied and tested for its effects on growth of *Coelastrum* sp. The appropriate concentration of glufosinate was required for screening the mutants by checking the minimum inhibitory concentration. In this study, the effect of glufosinate on actively growing cells was first screened under a wide concentration of glufosinate at 25, 50, and 100 μM to identify the minimum inhibitor of resistant cells towards herbicide. It was found that, in the presence of glufosinate, *Coelastrum* sp. (wild type) showed efficient carotenoid inducibility with a rapid color change from green to an orangish color.

Fig. 2 demonstrates that the colonies showed rapid color changes by turning to orangish color almost entirely after one month with 25 μM of glufosinate. This fast color change might be due to the effect of glufosinate that inhibits the activity of enzyme glutamine synthetase and initiates the accumulation of oxidant, thereby triggered the production of carotenoids with color changes. When glufosinate was applied in 50 μM, only a part of the colonies remained green and partially turned to orangish color with a lesser number of colonies. Notably, the cells were still growing when glufosinate concentration was at 100 μM, but the cell numbers declined and did not show any signs of color change. At a higher concentration of glufosinate, the mortality of cells increased, thereby inhibiting and slowing the production of carotenoids. As a result of glufosinate treatment, the most effective concentration for glufosinate was found to be in the range of 25 μM. Consequently, 25 μM glufosinate was chosen for the subsequent screening experiments as it showed rapid color changes and was found to have the minimum inhibitory towards the *Coelastrum* sp. cell.

For screening the mutant with a high-throughput of astaxanthin,

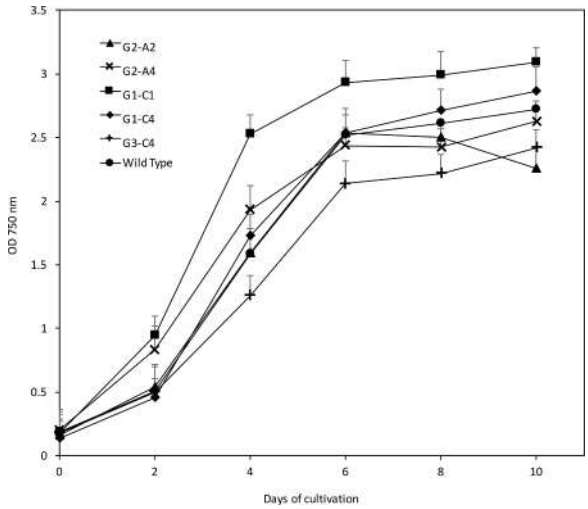

Fig. 3. Growth curve of mutants selected from the approach of screening astaxanthin producing mutant with high throughput screening using glufosinate and compared with the wild type. Data represent an average of 3 replications and error bars indicate mean ±SD

Table 4
Specific growth rate of mutants selected from the approach of screening astaxanthin producing mutant with high throughput screening using glufosinate and compared with the wild type

Microalgae	Specific Growth Rate, μ Day^{-1}
Mutant G2-A2	0.53±0.16
Mutant G2-A4	0.55±0.19
Mutant G1-C1	0.79±0.15
Mutant G1-C4	0.64±0.20
Mutant G3-C4	0.38±0.16
Wild Type	0.56±0.12

Coelastrum sp. treated with 0.4 M EMS and 60 min exposure time was spread on an agar plate containing 25 μM of glufosinate. Screening of mutants using glufosinate relies on identifying colonies that are capable of surviving in the presence of inhibitory concentrations of glufosinate. There were 37 colonies that appeared under EMS and glufosinate treatment, which were then transferred into microplate cell culture and allowed to grow under normal growth conditions. After 20 days of inoculation, 20 mutants with the highest cell densities were selected from 37 mutant strains and were grown in a larger volume of liquid medium culture to monitor the growth of mutant cells. Out of the total number of mutant colonies that appeared with glufosinate treatment, five mutants with the highest number of cells after growing from a single colony were selected to compare with the wild type. Fig. 3 shows the growth phase occurrence treated in EMS with glufosinate for the mutants and wild type cultivation. Among these mutants, two mutants (G1-C1 and G1-C4) had higher cell densities than the wild type at day 10.

These mutants have been found to be more stable and comparatively higher than the wild type. Cultivation of mutant G1-C1 at day 10 had the highest cell density between these two mutants. For the specific growth rate shown in Table 4, the cultivation of mutants G1-C1 (0.79 day^{-1}) and G1-C4 (0.64 day^{-1}) had the highest value on day 4 of the logarithmic phase. This value was higher than the specific growth rate of wild type cultivation (0.56 day^{-1}). Nevertheless, mutants G2-A2, G2-A4 and G3-C4 that were screened using glufosinate were deselected due to lower cell density and lower specific growth rate of 0.53 day^{-1}, 0.55 day^{-1} and 0.38 day^{-1} respectively, compared to the wild type. Subsequently, the two selected mutants (G1-C1 and G1-C4) were further analyzed and compared based on their pigment production.

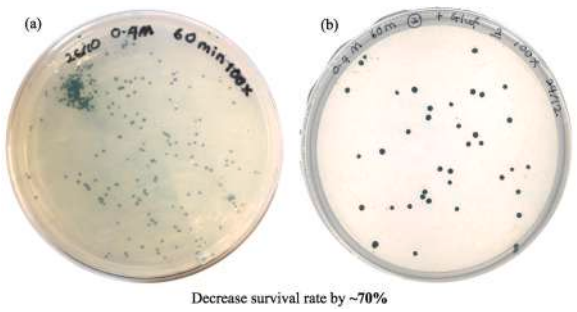

Fig. 4. Comparison of mutant cells that are visible after treated with **(a)** only EMS and **(b)** mutant cells visible after treated with EMS in the presence of inhibitor, glufosinate

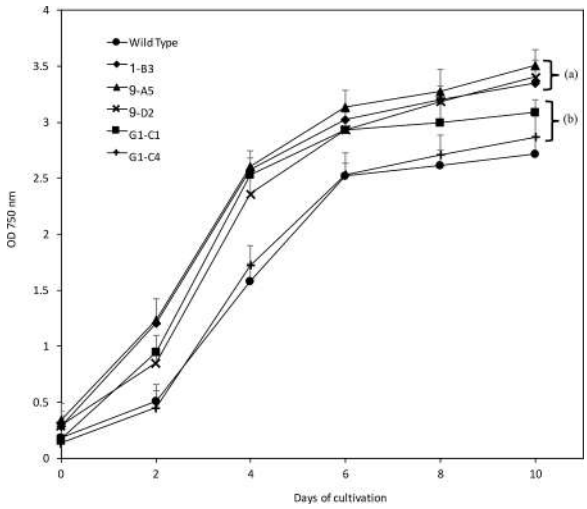

Fig. 5. Combine growth curve of **(a)** top mutants selected from the approach of screening astaxanthin producing mutant with high growth without using glufosinate and, **(b)** top mutants selected from the approach of screening astaxanthin producing mutant with high throughput screening using glufosinate. Data represent an average of 3 replications and error bars indicate mean ±SD

3.3. Comparison of mutants selected by different methods

Fig. 4 shows the comparison of mutant cells visible on the agar plate after EMS treatment with and without the inhibitor, glufosinate. Based on the results, the number of mutant colonies that appeared in the presence of glufosinate was found to be fewer than mutants grown without the use of glufosinate. The survival rate of the mutants obtained from the inhibitor groups decreased 70% with lower number of colonies compared to screening without the use of the inhibitor. The decrease in the survival rate of the mutant in the inhibitor (glufosinate) because the mutant that survived was resistant to this inhibitor. The survived mutants might be expected to possess mutated enzymes with an altered expression that enables the synthesis of desired pigments in the presence of inhibitors.

These findings suggest that inhibiting anti-oxidative enzymes in vegetative cells could be an effective way to shorten the induction phase and hence facilitate astaxanthin synthesis. In *Coelastrum* sp., glufosinate acts as an artificial stress inducer for carotenogenesis, accelerating the synthesis of astaxanthin which enables the finding of the astaxanthin hyper-producers.

3.4. Growth and astaxanthin production by top mutants and wild type

Fig. 5 shows the growth curve of the top mutants that were comparatively higher than the wild type, which was selected from the

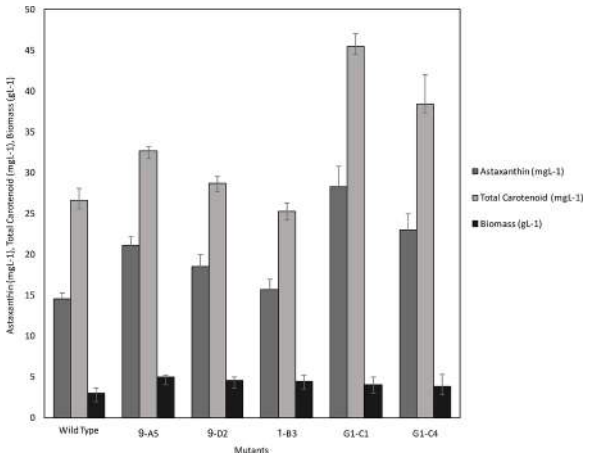

Fig. 6. Comparison of biomass, total carotenoids and astaxanthin content in the wild type and top mutants. Data represent an average of 3 replications and error bars indicate mean ±SD

screening with and without using glufosinate. These results showed that the selection of high biomass strains without the use of glufosinate could enhance cell numbers as it tends to have higher growth compared to the mutants selected using glufosinate. The use of inhibitor, herbicide-glufosinate might disrupt the metabolic processes essential for algal cells by decreasing the survival rate of cells and affecting the quality growth of the mutant [33, 34].

All the selected mutants were then further analyzed and compared based on their total carotenoid and astaxanthin production. Fig. 6 demonstrates that unlike other mutants, the total carotenoid and astaxanthin content of mutants selected under conditions of glufosinate (G1-C1 and G1-C4) were higher than those without the inhibitor. Despite the growth profile of these mutants was lower than that of mutants selected in the absence of the inhibitor, the result showed that glufosinate was efficient in screening the mutants as it was able to provide strains with a higher producing capacity of pigment contents. Besides, from the viewpoint of selective efficiency, the glufosinate-based screening is much higher than the growth-based screening because two and three candidates of mutant strains were selected from 37 and 120 mutants, respectively.

According to the screening result, mutant G1-C1 was chosen for its high total carotenoid and astaxanthin content and further compared with the wild type. The selected mutant G1-C1 acquired 28.32 mg/L of astaxanthin content, which was approximately 2-folds more astaxanthin than the wild type strain (14.5 mg/L). Study by Kamath et al. (2008) also reported that the growing culture of green alga *H. pluvialis* exposed to mutagens EMS over herbicide glufosinate had increased the total carotenoid and 2.2 to 3.8 % w/w of astaxanthin contents creating hyper-production of astaxanthin [22].

The growth and total carotenoid content of mutants generated with and without herbicide, glufosinate screening differed which could be attributed to alterations in the photosystem. Differences in the transcript levels of carotenogenic genes in different mutants were suggested to support the aforementioned statement [22]. In microalgae, the genes involved in carotenoid and astaxanthin biosynthesis are regulated by a series of carotenogenic genes [47, 48]. To explore the molecular mechanisms of higher astaxanthin contents in the mutant, the differences of gene expression in carotenoid biosynthesis between mutant and wild type strains would provide strong evidence of the exact mechanisms responsible for altering astaxanthin production in *Coelastrum* sp. mutant. The mechanism of why mutant G1-C1 produced more astaxanthin is expected to be investigated in detail.

Besides that, there was also a considerable difference in the morphology of *Coelastrum* sp. between the WT and mutant under observation using a light microscope (Fig. 7). For the wild type, the cells were grown in a cluster of cells. However, the mutant exhibited curious morphological features as the cells were separating and became single cells. The separation of cells in the mutant strain might be the reason of the higher number of cells and growth of the mutant compared to the wild type. This might make it easier for the mutant cells to accumulate the pigments as it was in the form of single cells.

Results indicated prominently higher efficiency of screening the mutants with desired properties by the inhibition of glutamine synthetase using glufosinate for a successful mutagenesis approach. This report provides the rapid selection of positive mutants from a fewer number of cells to obtain astaxanthin hyper-producing mutants of *Coelastrum* sp. using a simple and efficient high-throughput screening method. The introduction of glufosinate was proven to be an effective screening indicator with which the astaxanthin production was improved observably.

H. pluvialis which is known to be the richest source of astaxanthin production is facing difficulty scaling up as the microalga growth rate is slow and more susceptible to contamination. Although the astaxanthin content is high, the biomass is relatively low, hence limits the rate of commercial-scale production of astaxanthin [49]. In this study, *Coelastrum* sp. mutant was identified for its ability to enhance astaxanthin content. Previously, the total biomass yield of a newly isolated *Coelastrum* sp. strain and *H. pluvialis* was studied by **Tharek et al. (2020)**. They found that the total biomass of a locally isolated strain, *Coelastrum* sp. surpassed *H. pluvialis* which could be viewed as a useful advantage in a real production process [15]. The uses of local strains are possibly more productive and desirable for adapting to climatic conditions for more sustainable and less energy-consuming processes for astaxanthin production.

4. Conclusion

The findings of this study revealed that the genetic improvement of newly isolated *Coelastrum* sp. by random mutagenesis have altered

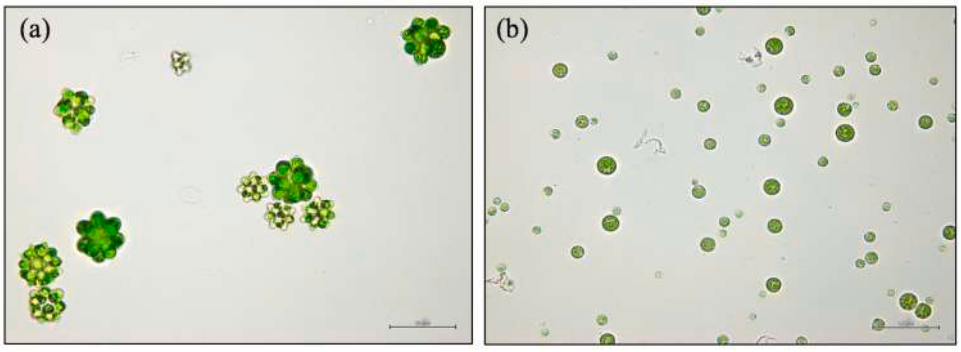

Fig. 7. Light microscopic images of *Coelastrum sp.* morphology of **(a)** wild type and **(b)** mutant G1-C1. Scale bars = 50 μm (x 20 magnification)

certain biochemical characteristics of the wild type. The altered biochemical properties of *Coelastrum* sp. mutants were demonstrated to be a successful strategy to increase the content of astaxanthin. In the current study, mutagenesis using chemical mutagen of EMS was attempted to increase the microalgae biomass and carotenoids production in *Coelastrum* sp. In concurrence with this study, the results revealed that the selected mutant G1-C1 using inhibitor-glufosinate screening exhibited an increase of astaxanthin content with almost 2-fold higher compared to the WT. This efficient method for generating random mutation and screening using glufosinate allowed rapid high-throughput screening, leading to a successful mutagenesis approach for improving *Coelastrum* sp. strains. Besides that, the data in this study might provide scope to further studies on molecular aspects to help for a better understanding of astaxanthin profile that corresponds to the expression profile of carotenogenic genes in *Coelastrum* sp. mutant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00673.

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