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Ethyl Methane Sulfonate-Induced *Dunaliella salina* KU11 Mutants Affected for Growth Rate, Cell Accumulation and Biomass

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Abstract*—Dunaliella salina* has great potential as a system for generating commercially valuable products, including beta-carotene, pharmaceuticals, and biofuels. Our goal is to improve this potential by enhancing growth rate and other properties of *D. salina* under optimal growth conditions. We used ethyl methane sulfonate (EMS) to generate random mutants in *D. salina* KU11, a strain classified in Thailand. In a preliminary experiment, we first treated *D. salina* cells with 0%, 0.8%, 1.0%, 1.2%, 1.44% and 1.66% EMS to generate a killing curve. After that, we randomly picked 30 candidates from approximately 300 isolated survivor colonies from the 1.44% EMS treatment (which permitted 30% survival) as an initial test of the mutant screen. Among the 30 survivor lines, we found that 2 strains (mutant #17 and #24) had significantly improved growth rates and cell number accumulation at stationary phase approximately up to 1.8 and 1.45 fold, respectively, 2 strains (mutant #6 and #23) had significantly decreased growth rates and cell number accumulation at stationary phase approximately down to 1.4 and 1.35 fold, respectively, while 26 of 30 lines had similar growth rates compared with the wild type control. We also analyzed cell size for each strain and found there was no significant difference comparing all mutants with the wild type. In addition, mutant #24 had shown an increase of biomass accumulation approximately 1.65 fold compared with the wild type strain on day 5 that was entering early stationary phase. From these preliminary results, it could be feasible to identify *D. salina* mutants with significant improved growth rate, cell accumulation and biomass production compared to the wild type for the further study; this makes it possible to improve this microorganism as a platform for biotechnology application.

Keywords*—Dunaliella salina*, mutant, ethyl methane sulfonate, growth rate, biomass.

I. Introduction

MICROALGAE is a promising organism as a platform for biotechnology application to produce commercially valuable products, pharmaceuticals, and biofuel [1]–[6]. One of several ways to improve microalgae potential is to optimize their growth conditions such as temperature, light source, light intensity and medium compositions, lot of researchers have already published some of these information [7], [8]. Another way is to engineer microalgae via exogenous gene expression requiring such a specific property such as production of nutraceuticals, biomass and biofuels. However, a real mutant identified with a specific phenotype from this technique takes a long time and working with a gene expression has several problems such as transformation fail, low (or undetectable) exogenous gene/protein expression and gene silencing [9], [10]. The other good strategy to receive a better microalgae property is to use physical and chemical mutagen such as UV radiation and EMS to generate random mutants. Using mutagenesis technique as a biotechnology application gives several advantages. For example, the mutagenesis generates a random mutant population faster than a classical way, a selective breeding, and those mutagens (such as UV and/or EMS) induce lot of point mutations on microalgae genome that give rise a various kind of microalgae phenotype [11]–[13]. We can next screen for the desired phenotype, get a number of candidates and select the best strain for a desired phenotype including improved growth and biomass, increased cell size and enhanced some bioproduct or biocompound. The most important reason is that the mutagenesis method is such an easy way to get a better microalgae strain in such a desire phenotype.

From previous studies, several examples have shown promising results from random mutagenesis. For example, a strain of EMS-induced *Chlamydomonas reinhardtii* mutants showed highly abundant lipid bodies and 1.4-fold increased fatty acid methyl ester (FAME) content compared to wild type [14]. Also, *C. reinhardtii* starchless mutant (ADP-glucose pyrophosphorylase mutated) exhibited 10-fold higher of TAG accumulation than wild type [15]. Moreover, UV-induced *D. salina* and *Tetraselmis suecica* mutants had significantly increased in total carotenoid [16]. From these evidences, UV- and EMS-mediated random mutagenesis can generate a better strain for lipid product improvement in microalgae. Thus, we present to use EMS to mutagenize *D. salina* KU11 that possibly get a better growth rate and biomass strain, including some other bioproduct in this study. This could be an advance for a very positive impact on microalgal biotechnology.

In this study, we first set up EMS treatments for *D. salina* KU11 by varying of EMS concentrations (0–1.68%) to generate a killing curve. After that, a number of mutants will be selected to analyze for growth rate, biomass, cell size and possibly including beta-carotene product. Here, we found that 2 of 30 analyzed mutants of 1.44% EMS treatment exhibited increase of growth rate and cell accumulation at the stationary phase up to 1.8 and 1.45 fold, respectively, compared with wild type. Whereas 3 of 30 mutants showed decrease of

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growth rate and number of cell accumulation at stationary phase compared with wild type, the rest of mutants had growth rate similar to wild type. In addition, an improved growth rate mutant showed an increase of biomass accumulation approximately 1.6 fold compared with the wild type strain.

II. Materials and Methods

A. Strain and Culture Condition

Dunaliella salina wild type (WT) strain KU11 was received from the laboratory of Assoc. Prof. Niran Juntawong, Kasetsart University. For *Dunaliella* stock maintenance, standard growth curve, and growth rate analyses, *Dunaliella* cultures (WT and mutants) were grown with Ramaraj medium [7], pH 7.2, at 25 °C with continuous light (~30-50 μE m^{-2} s^{-1}) on a rotary platform shaker at 140 rpm in an algae room.

B. Random Mutagensis by EMS and Mutant Screening

Dunaliella salina KU11 was grown to mid-exponential phase (5-6 x 10^6 cells/mL) and 5 mL of culture was harvested by centrifuging at 1250 x g. After that, cells were resuspended in 500 μL of PN buffer (0.1 M sodium phosphate pH 7.0; 1.5 M NaCl) before treating with various concentrations of EMS as described in [17]. Briefly, cell suspensions at 25-30 x 10^6 cells/mL were treated with 0, 0.8, 1, 1.2, 1.44 and 1.68% (w/v) of EMS (Sigma-Aldrich, USA) concentration for 1 hr with gently agitating in dark. After that, EMS-treated samples were inactivated with 500 μL of fresh-made sterile 10% (w/v) sodium thiosulfate, washed 1 time with 1 mL of PN buffer, resuspended in 1 mL of fresh Ramaraj medium, and kept in dark for 24 h. Then equal cell numbers of each treatment was spread uniformly on Ramaraj agar plates. Colonies appeared in 14 days. The EMS killing curve was generated and cells treated with 1.44% (w/v) of EMS were used for the further experiment as this concentration gave rise of isolate mutant colonies.

After 7 days, 30-isolated colonies from *Dunaliella* cells treated with 1.44% (w/v) of EMS were transferred from agar plates into two of 25-well plates containing 3 mL of Ramaraj medium to make a stock of liquid cell culture. After 4 days of cultivation, each colony was subcultured into fresh Ramaraj medium into two of 25-well plates for another round of growth observation beginning with the same number of cell inoculation for the first screening of cell growth in each strain. Cell growth of each sample was observed by a greener color and a number of cells using hemacytometer compared to wild type. The greener strain compared to wild type was selected to measure its growth rate, cell size and biomass in the further analyses.

C. Growth Rate and Cell Size Analyses

Algal cell counts and cell size measurements were determined into the ambient air condition in Ramaraj medium. All cultures were grown in 125-mL flasks that were agitated on a rotary shaker, under continuous light (~30 μE m^{-2} s^{-1}) and at 25 °C in a control algae room.

For each growth sample measurement, cultures were started in the following way. *Dunaliella* mutants with an initiate cell number of 5 x 10^5 cells/mL were inoculated into 50 mL of Ramaraj medium and grown for 4 days. After that cells were transferred into a 100-mL-experimental flask to make 5 x 10^5 cells/mL in a final culture volume of 50 mL. Cells were counted with a hemacytometer every 24 hrs to plot a growth curve until entering stationary stage (~ 7-10 days).

For cell size measurement, *Dunaliella* cultures were grown same as growth experiment. In day 4, 20 cells in each sample were randomly picked and then cell length (l) and width (w) were measured by using ocular and stage micrometers under the light microscope. The cell size of each sample was averaged and the standard error of the mean was calculated using Microsoft Excel. The data was plotted and interpreted as column chart.

D. Biomass Measurement

Algal biomass measurements were determined into the ambient air condition in Ramaraj medium. All cultures were grown in 500-mL flasks that were agitated on a rotary shaker, under continuous light (~50 μE m^{-2} s^{-1}) and at 25 °C in an algae room.

For each biomass measurement, two separate experiments were set up. One was used to determine biomass in log phase and the others were used to determine biomass in stationary phase. An initial cell number of 3 x 10^5 cells/mL of *D. salina* mutant strain #24 and the wild type were inoculated into 250 mL of Ramaraj medium. In log phase experiment, cells were collected at day 3, 4 and 5 after inoculation (mid- and late-exponential, and early-stationary phase, respectively). In stationary phase experiment, cells were collected at day 5, 6, 7 and 8 after inoculation (early-, mid- and late-stationary phase, respectively). Cells were collected by filtration onto pre-weighed glass microfiber filters (GF/F Φ 47 nm, GE Healthcare Life Science) and the dry weight determined after drying overnight in a 70 °C oven.

E. Statistical Analysis and Experimental Replication

Biological/technical replicates and statistical analyses of data generated in this study were as follows. Growth curve analyses were performed on three biological replicate cultures for each strain. Each growth curve of each biological replicate was generated with three technical replicates obtained and averaged for each data point. The standard error of the mean was calculated for each biological replicate data point using Microsoft Excel. One biological replicate was used as a representative because overall growth curves had shown the same trend (data for two biological replicates are not shown).

Biomass analyses were performed on two biological replicate cultures for each strain (wild type and the mutant #24). One biological replicate was used a representative since overall biomass measurement had shown the same trend.

III. Results

A. The Standard Growth Curve of D. salina KU11

To determine the growth phrase of *D. salina* KU11 under laboratory conditions, we set the experiment by using an initial cell number of *D. salina* KU11 at 50 x 10^4 cells/ mL

and count for every 24 hr for 7 days. We found that *D. salina* KU11 started entering early log, mid-log through stationary phase at day 2, 3 and 4, respectively and accumulating cell number approximately at 200, 600, 1000 x 10^4 cells/mL, respectively, as shown in Fig. 1.

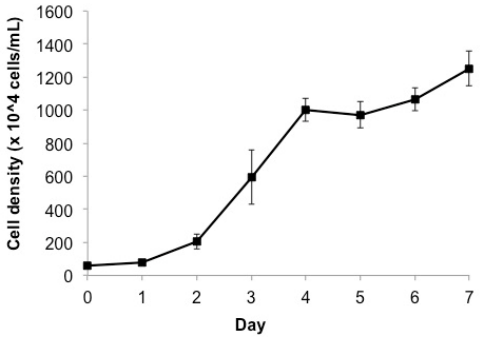


Fig. 1 Growth phase analysis of *D. salina* KU11 (wild type strain)

B. Generating of The EMS Killing Curve

To determine percentages of survivors and an appropriate EMS concentration for EMS mutagenesis, we treated *D. salina* KU11 cells with a varying of EMS concentration (0, 0.8, 1, 1.2, 1.44 and 1.68%) and found that an increase of EMS concentration resulted in a decrease of cell survivors. 0% of EMS appeared too many colonies to count and 0.8, 1, 1.2, 1.44 and 1.68% of EMS concentration gave rise of survivor colonies approximately 600, 500, 500, 300, 25 colonies, respectively, as shown in Fig. 2. From this result, we decided to use mutant colonies of 1.44% of EMS because most of 300 survivor colonies appeared an individual colony that was easy to pick and we were confident to determine each colony that exhibited an individual phenotype.

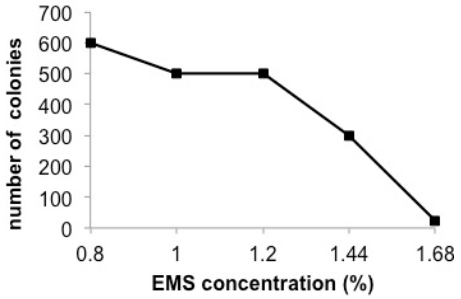
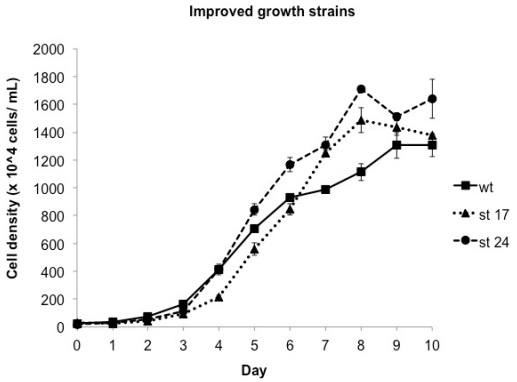


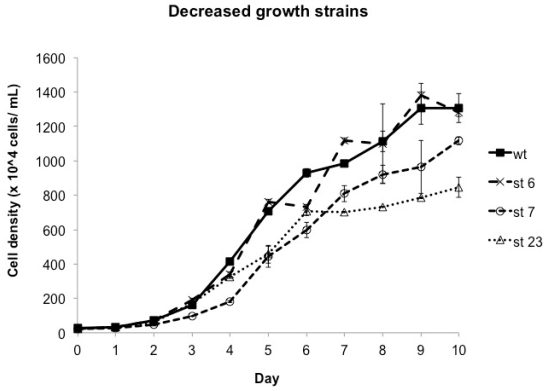
Fig. 2 EMS killing curve of *D. salina* KU11

C. Growth Rate, Cell Accumulation and Cell Size Analyses in Six D. salina KU11 Mutants

We next set out to determine whether or not EMS-induced mutants would improve growth rate compared to wild type strain (*D. salina* KU11). As an initial test of mutant screen, we first started selecting total 30 mutants from 300 mutants survived in cell-treated EMS at 1.44%. Each individual colony (of total 30 colonies) was randomly selected and grew under a condition as described in Materials and Methods section. We found that mutant colony #17 and #24 showed an improvement of growth rate and cell accumulation at the stationary phase ~1.8 and 1.45 fold, respectively, compare with wild type (Fig. 3 (a)). On the other hand, mutant colony #6 and #23 had declined their growth rate and cell accumulation at the stationary phase down to ~1.4 and 1.3 fold, respectively, compared with wild type (Fig. 3 (b)). For the rest of 26 mutant colonies, they showed no significant difference in growth rate when compared to wild type (data not show). The results suggested that there were about 6.67% of 30 mutants improving growth rate for the initial screen and we might be able to possibly get approximately 20 mutants from total of 300 mutants. In addition, different growth rate phenotypes of 30 mutants analyzed could be interpreted due to random point mutations on *Dunaliella* genome via EMS mutagenesis.



(a)



(b)

Fig. 3 Analyses of six EMS mutants growth measurement compared with wild type strain. Representative of (a) Improved growth strains; #17, #24 (b) Decreased growth strains; #6, #7, #23

From the growth rate results, we hypothesized that whether or not the growth rate changed was possibly related to cell size changed in each strain. We then observed cell size for mutant colony #17 and #24 (represents of improved-growth strains), #6 and #23 (represents of reduced-growth strains) including wild type strain by randomly measuring width length of 20 cells. The result showed that all strains had similar cell size with no significant different (Fig. 4). This result suggested that

growth rate changed in each strain was not related to cell size and it might come from genetically change (point mutations) in those mutant strains induced by EMS mutagen.

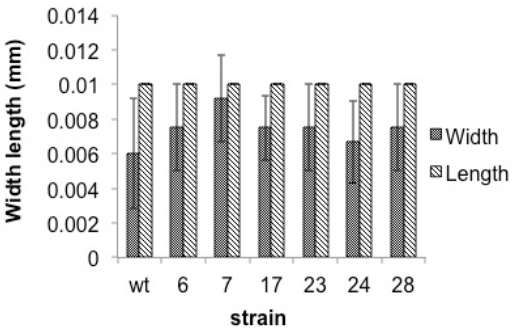
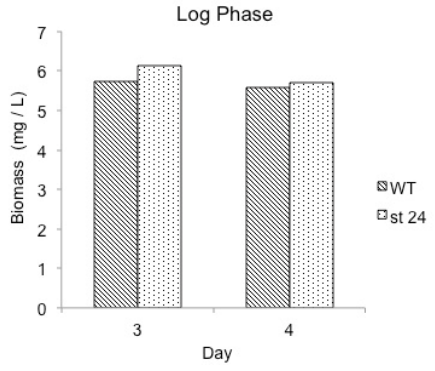


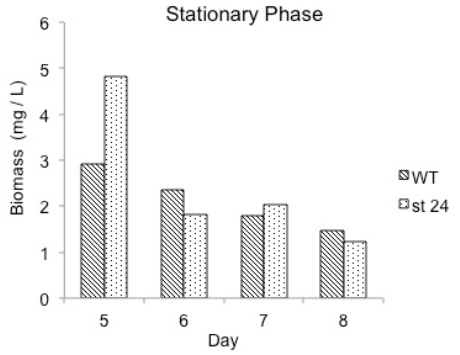
Fig. 4 Cell size measurements of wild type and six-mutant strains

D.Biomass Analysis of Mutant #24 and Wild Type Strain

We next set out to see if the mutant strain #24, the improved growth rate strain, could improve its biomass production compared with wild type strain or not. The two separate experiments were set up to analyze biomass accumulation in log phase (Fig. 5 (a)) and stationary phase (Fig. 5 (b)). The mutant #24 and wild type were grown under laboratory condition as described in Materials and Methods section. The result of biomass analysis in log phase (Fig. 5 (a)) showed while entering day 3 through day 4, the mutant #24 (5.6 - 6.1 mg/L) had slightly increased of biomass accumulation ~ 1 fold compared with wild type (5.5-5.7 mg/L). When cultures were entering into stationary phase at day 5, biomass of mutant #24 was still high at ~ 4.8 mg/L and dramatically declined on day 6, 7 and 8 that was 1.8, 2.0 and 1.2 mg/L, respectively (Fig. 5 (b)). Whereas, biomass accumulation of wild type strain began dropping sharply on day 5 (early stationary phase), less than the mutant #24 ~ 1.7 fold, 6, 7 through day 8 that was 2.4, 1.8 and 1.5 mg/L, respectively (Fig. 5 (b)). This result suggested that the mutant strain #24 was a promising strain accumulating high biomass, especially on day 5 (early stationary phase), that its biomass was still close to day 3 when compared with wild type strain.



(a)



(b)

Fig. 5 Biomass measurements of wild type and a mutant strain #24. (a) In log phase (b) In stationary phase

IV. Discussion

In this study, we set out to create *Dunaliella salina* KU11 mutants by EMS mutagenesis. Several mutants were screened for a better strain that improved growth rate, cell accumulation and biomass. We got a mutant strain # 17 and #24 that showed a promising phenotype on growth rate, cell accumulation at the stationary phase and biomass.

Under lab conditions, we first set out an experiment to analyze *D. salina* KU11 (wild type strain) growth rate and we found that 4-6 x 10^6 cells/ mL was *D. salina* KU11 log phase corresponding to Sathasivam's study [7]. Then, we used mid-log phase (6 x 10^6 cells/ mL) to test with EMS treatments.

We used EMS to mutaginize *Dunaliella* cells because 1) it is a powerful and easy technique to generate a number of mutants, 2) we can create a number of candidate mutants that are from various types of random mutations by modulating A-T to G-C in DNAs on microalgae genome as described in many successful reports in several microorganisms [12], [14], [18]. For instance, *C. reinhardtii* starchless mutants (ADP-glucose pyrophosphorylase mutated) exhibited 10-fold increase of TAG accumulation compared to wild type by using EMS mutagenesis [15], [19]. 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 [20]. For this experiment, we first had to know what the range of EMS concentrations to treat with *D. salina* KU11 strain is. We then generated a killing curve by growing *Dunaliella* cells in varying of 0 – 1.68% EMS as previous report [17], [21]. We used mutants from 1.44% EMS concentration that permitted a 30% of survival lines because this concentration gave raise an optimal number (~ 300 survival candidates) of isolated colonies. We used only 10% (30 lines) of those 300 survival lines for an initial screening and found 2 strains of those 30 mutants improving their growth rates, cell number and biomass. It is necessary to do more mutant screening (~ 100 - 200 mutants) to get more candidates and it is possible that we can get an improving growth rate and biomass strain better than the ones we have (mutant # 17 and 24). As described before, using EMS mutagenesis is necessary to do a large

number of mutant screening to get the best strain for a desired phenotype. Moreover, *Dunaliella* genus has a wide range on its growth condition. Some *Dunaliella* specie can grow under subzero temperature or high light intensity or very acidic environment [22]. For instance, *D. salina* could grow under low to high light intensity (30 - 1,700 μE m^{-2} s^{-1}), temperature (20-50 °C) and salt concentration (0.5-2.5) and Wu et al. found that the highest condition giving the highest level of beta-carotene production was 245 μE m^{-2} s^{-1} at 22 °C and an optimal of KNO_3, $CO(NH_2)_2$ and $NaHCO_3$ concentration for *D. salina* growths were 0.5, 0.36 and 1.5 g L^{-1}, respectively [8]. In our growth and biomass measurement, we grew and analyzed the mutant #17 and #24 under a specific lab condition (Materials and Methods section). Thus, it can be better to find an optimal growth condition for these mutant strains resulting in a better growth rate and biomass production and apply the strains on economically large scale production by culturing in either a bioreactor or an open pond outdoor.

V. Conclusion

In this experiment, the genetic variation was created by random mutagenesis (EMS mutagen). We did an initial screening by 10% of total mutants and found 2 mutant strains (#17 and #24) have improved growth rate and biomass. From their growth rate, cell accumulation and biomass found that the mutant #24 was the best strain in our current experiment. We plan to do more screening on the rest of those 300 mutants to see if we can find the best strain of EMS-induced *D. salina* KU11 mutants compared to wild type. Moreover, we also would like to do the further study to find an optimal growth condition outdoor on a large scale by either an open pond or a bioreactor to develop our finding strain as an economically strain from biotechnology application.

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