The Optimal Mutagen Dosage to Induce Point-Mutations in *Synechocystis* sp. PCC6803 and Its Application to Promote Temperature Tolerance

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**Abstract**

Random mutagenesis is a useful tool to genetically modify organisms for various purposes, such as adaptation to cultivation conditions, the induction of tolerances, or increased yield of valuable substances. This is especially attractive for systems where it is not obvious which genes require modifications. Random mutagenesis has been extensively used to modify crop plants, but even with the renewed interest in microalgae and cyanobacteria for biofuel applications, there is relatively limited current research available on the application of random mutagenesis for these organisms, especially for cyanobacteria. In the presented work we characterized the lethality and rate of non-lethal point mutations for ultraviolet radiation and methyl methanesulphonate on the model cyanobacteria *Synechocystis* sp. PCC6803. Based on these results an optimal dosage of 10–50 J/m² for UV and either 0.1 or 1 v% for MMS was determined. A *Synechocystis* wildtype culture was then mutagenized and selected for increased temperature tolerance in vivo. During the second round of mutagenesis the viability of the culture was monitored on a cell by cell level from the treatment of the cells up to the growth at an increased temperature. After four distinct rounds of treatment (two with each mutagen) the temperature tolerance of the strain was effectively raised by about 2°C. Coupled with an appropriate in vivo screening, the described methods should be applicable to induce a variety of desirable characteristics in various strains. Coupling random mutagenesis with high-throughput screening methods would additionally allow to select for important characteristics for biofuel production, which do not yield a higher fitness and can not be selected for *in vivo*, such as fatty acid concentration. In a combined approach with full genome sequencing random mutagenesis could be used to determine suitable target-genes for more focused methods.

With the advent of next generation sequencing techniques it has become economically feasible to use random mutagenesis strategies first and then re-sequence various generated mutants. Genes of interest identified this way, could then be modified by more targeted methods.

Random mutagenesis also has a strong, but doubtful, advantage of not being classified as a method producing genetically modified organisms. In many places such as the EU this imposes fewer regulatory hurdles.

In general, mutations are classified by the type of mutation taking place and/or by the method of action by which the mutation occurs. Types of mutations are insertions, deletions and substitutions. The latter is further divided into transitions (exchange of purine to another purine or a pyrimidine to another pyrimidine), and transversions (purine for a pyrimidine or vice versa). The mode of action is divided into two main categories: Direct mutations and indirect mutations. Direct mutations occur as a direct consequence of the mutagen, such as a miss-pairing of O²-Methylguanine with Thymine after treatment with Methyl methanesulphonate (or a similar alkylating agent). Indirect...
Mutagenesis in *Synechocystis* for Thermotolerance

Meireles et al. [23] used UV mutagenesis in order to increase the yield of metabolites of interest in the microalga *Pavlova lutheri*, however the optimal dosage was not empirically determined and the described conditions are difficult to reproduce since the UV exposure was measured in time. Ong et al. [24] used EMS (an alkylating mutagen) to create microalgal mutants with greatly improved growth rates at increased temperature. Chaturvedi et al. [25] also used EMS to generate mutant microalgae. While their experiments and screening were designed to increase the content of eicosapentaenoic acid within the algae, they noted that the generated mutants demonstrated better thermotolerance compared to the WT strain. Both of these studies demonstrate the viability of using random mutagenesis to increase thermotolerance of phototrophic organisms.

For cyanobacteria the published literature on mutagenesis is far more limited. Lambert et al. [16] however did a comprehensive study comparing the effect of various mutagens on the unicellular cyanobacterium *Gloeocapsa alpicola*. They characterized each mutagen by plating cultures exposed to different dosages of mutagen on agar plates to determine the lethality and on streptomycin selection plates to determine the mutation rate.

Streptomycin inhibits cellular replication by interfering with protein biosynthesis and has been shown to directly interact with the small ribosomal subunit [26]. Resistance to Streptomycin can be conferred through various mutations in the small or the large subunit [26,27,28]. Within the scope of this work Streptomycin resistance is used as a marker to measure the relative rate of point mutations without discriminating the exact mutation conferring the resistance.

For this study we used *Synechocystis* sp. PCC6803 as the target organism, as it is a widely used model organism for phototrophic organisms and cyanobacteria in particular. Being from the order Chroococcales it is unicellular and officially classified as a fresh water strain, though it is highly tolerant to salt and marine media [29]. The *Synechocystis* genome was sequenced in 1996 [30], as the first genome from a photosynthetic organism.

Ultraviolet light (UV) and MMS were chosen for initial characterization with *Synechocystis* as they were determined as the best mutagens by Lambert et al. [16].

First the lethality and mutation induction of both mutagens on *Synechocystis* were characterized. The thus determined optimal dosages were then used for applied mutagenesis coupled with *in vivo* selection.

One big issue for the optimization of culture conditions is the temperature range, in particular when considering desert areas for outdoor cultivation. Thus we chose tolerance to high temperature as an initial target for our optimization strategy – also since it allows a relatively simple experimental setup for *in vivo* selection.

Methods discussed in this paper should be easily applicable to the optimization of other characteristics however. Ther have been previous studies which resulted in increased thermal tolerance of *Synechocystis*. This was mainly achieved by increasing the expression of heat shock proteins. Nakamoto et al. [31] demonstrated that the inactivation of the *hrcA* repressor in *Synechocystis* led to an increased ex of heat shock proteins and slight increase of maximal cell density when culturing at 42°C. However the ΔhrcA mutant did not have a fully induced expression of these proteins, which they speculated was due the presence of a further regulation mechanism. Suzuki et al. [32] later identified *hik34* as an additional regulator for heat shock proteins. *Synechocystis* Δhik34 mutants, exhibited a strongly increased expression of these proteins, thus resulting in an increased thermal tolerance (surviving 3 h at 47°C whereas the wt only survived 2 h).
The random mutagenesis approach used in this paper has the advantage of not being constrained to the limited improvement of thermal tolerance achievable through increased heat shock protein expression. Thus opening the possibility to increase the thermal tolerance further than has been previously demonstrated.

Materials and Methods

Organism
Synechocystis sp. PCC 6803 salt adapted wild type, obtained from Cyano Biofuels GmbH.

Media
All strains were cultivated in sterile mBG11 Media (30 g/l Instant Ocean®, 17.65 mM NaNO₃, 0.18 mM K₂HPO₄, 0.03 mM Citric acid, 0.003 mM EDTA (disodium magnesium), 0.19 mM Na₂CO₃, 0.03 mM Ferric ammonium citrate and trace metals).

UV Mutagenesis
Synechocystis culture (in the logarithmic growth phase) with 5*10⁷ cells/ml was placed on a petri dish (either 1 ml in a 40 mm dish, or 35 ml in a 230 mm² dish), homogeneously spread, and irradiated with up to 300 J/m² UV light. Irradiation was performed in a UVC 500 crosslinker (Hoefer, San Francisco) with only one lamp (Sankyo Denki G8T5; 8W) for a more precise dosing at a wavelength of 520 nm. Irradiation time was automatically adapted by the devices integrated sensor. Cells were then either used for lethality and mutation characterization or cultivated under temperature tolerance selective conditions. Great care was given to not expose cells to any light with wavelengths <520 nm after mutagenesis, by covering cultures with an Asmetec SFG10 filter, to prevent photolyase reactivation.

MMS Mutagenesis
Synechocystis culture (in the logarithmic growth phase) with 5*10⁷ cells/ml was incubated with up to 5 v% MMS (99% purity, Sigma) for 1 minute (in either a 1.7 ml reaction tube or a 500 ml centrifugation vessel). Cells were then centrifuged at 3.500 g for 1 min, the supernatant removed, and resuspended in mBG11. After a second washing step, cells were diluted and either used for lethality and mutation rate determination or cultivated under temperature tolerance selective conditions. Great care was given to not expose cells to any light with wavelengths <520 nm after mutagenesis, by covering cultures with an Asmetec SFG10 filter, to prevent photolyase reactivation.

Cultivation Conditions
For the mutagen characterization experiments all cultures were kept in culture flasks (75 cm²). Cultures were exposed to 4.5 µE*m⁻²*s⁻¹ of light with wavelengths of >520 nm (filtered through Asmetec SFG10 filter). For the temperature adaptation cells were cultured in a 11 bioreactor with pH-controlled 10% CO2/air addition. During a 12 h daylight phase, pH was kept at 7.3+/−0.05, light at about 125 µE/m²*s (170 from one side and 80 µE/m²*s from the other) and the culture was actively tempered. During the night phase cultures were bubbled with 10 ml/min air, and (passively) cooled down to 23–26°C. Cultures were diluted with fresh media so they stayed within an OD₇₅₀ between 1 and 2 (measured with a Shimadzu UV-1800 photometer), thereby keeping them in the logarithmic growth phase.

Determination of Lethality
The amount of viable cells after treatment was determined by plating 5*10⁴ cells on mBG11 agar immediately after the mutagenic treatment. Cells were grown with 2.2 µE*m⁻²*s⁻¹ under a SFG10 filter (to inactivate photo-reactivation) for about 2 weeks before being counted. For MMS dosages 1% and up 5*10⁷ cells were additionally plated and grown under the same conditions to increase the sensitivity of the lethality determination.

Mutation Rate Determination
The rate of non-lethal mutations was determined by diluting the treated cells 1:100 and cultivating them as previously described for 3 weeks, and then plating 5*10⁵ cells on selection plates (mBG11, 2 µg/ml Streptomycin). After growth on the plates for about 2 weeks with 5 µE*m⁻²*s⁻¹ (without SFG10 filter), colonies were counted to determine the amount of viable cells with the ribosomal point mutation allowing for streptomycin resistance.

Cell Counting and Viability Determination
Cells were counted either manually under a stereo microscope (SterEO Discovery V12; Zeiss, Hamburg), or using an automated procedure using the autofluorescence of phycocyanin. Fluorescence pictures of the plates where taken with a Keyence BZ9000 microscope and a phycocyanin fluorescence filter set (excitation: 600/37 nm; beam splitter: 625 nm; emission: 655/40 nm). Using the automated scanning module of the Keyence Software, pictures of an area of 25 cm² where taken. Automated counting was done by an ImageJ Plugin which uses histogram thresholding for segmentation and the integrated Analyze Particles function for the counting of the colonies. The Plugin is available as Additional File S1.

To determine the viability of the cultures after mutagenesis, cells were analyzed using a Chlorophyll viability analysis as described by Schulze et al. [33].

Temperature Tolerance Selection
Mutagenesis and cultivation were performed as described above. For UV and MMS mutagenesis the dosages 50 J/m² and 0.1 v% and 1 v% (half the culture volume per dosage) were used respectively, as they were determined to be optimal. Mutagenesis was performed in discrete “rounds” of treatment, each consisting of three phases: exposure/selection, recovery, and determination of maximum temperature tolerance. After each round of mutagenesis three cultures were cultivated in parallel: Backup (previous best culture under conditions which allows good growth); Mutant (Mutagenized previous best culture cultivated under selective conditions) and Control (previous best culture, cultivated under the same selective conditions as Mutant culture). For UV mutagenesis cultures were covered with SFG10 filters for a week following the initial irradiation.

The Mutant and Control cultures were kept under highly selective conditions until the Mutant culture showed a sharp decrease in cell density (OD), spectra peaks and cell viability. Both cultures were then kept at non-selective conditions to allow surviving cells to recover. Temperature was then slowly ramped up to the new tolerated maximum-temperature. Typically the control culture did not recover and its reactor was used to test the mutagenized culture at two temperatures in parallel. A culture was considered to be growing stably at a given temperature, if it grew consistently at that temperature for at least two weeks.
Results

Mutagen Characterization

Characterization of UV as a mutagen for *Synechocystis* PCC6803 clearly shows an exponential decrease in the survival rate from 100% viable cells at 0 J/m², to about 10% at 50 J/m², and almost 0% at 100 J/m² (fig. 1). The amount of viable cells on selection plates, which is equivalent to the rate of non-lethal point mutations, peaks early at 10–50 J/m², indicating a high mutation rate, and then steadily drops with increased dosage.

MMS as a mutagen for *Synechocystis* PCC6803 shows a similar exponential drop of the survival rate with increasing dose. 10% survival under our experimental condition was observed at 1 v%. MMS (fig. 2).

Surprisingly, the amount of viable cells on selection plates exhibits two distinct peaks, one at 0.1 v% and a second one at 1 v% with a significant drop in between. Beyond 1 v% the amount of colonies also steadily drops. A repetition of this experiment (data not shown) confirmed this unexpected shape of the curve.

Applied Mutagenesis

Mutagenesis was performed in discrete “rounds” of treatment, each consisting of three phases. Within the scope of this work a round of mutagenesis is defined as the period starting with mutagen exposure of the cells and ending with the determination of the new maximum-temperature tolerated by the mutagenized culture.

Each round of mutagenesis followed the same procedure. After the mutagenesis, there was a selection phase at high temperature, where most cells were killed off. This was followed by a recovery phase at lower temperature. Once the mutagenized culture began to recover the temperature was gradually ramped up and the new tolerated maximum-temperature was determined.

In total, four rounds of mutagenesis were performed, first two rounds of UV (50 J/m²) and then two of MMS (0.1 v% and 1 v%). After each round of mutagenesis the temperature tolerance of the mutagenized culture was effectively increased, while the control-culture was not able to recover from the selective conditions. A summary of the applied conditions and obtained temperature tolerances can be found in table 1. In total an improvement in temperature tolerance of about 2°C could be achieved.

The final culture is now able to stably grow at 45°C and 23–26°C in a 12 h day/night cycle, whereas the starting wildtype was not able to tolerate 43°C day temperature under the given conditions. The improved culture does not show an aberrant phenotype in any other aspect and most cells are still able to grow on agar plates.

At the second round of mutagenesis (UV), the viability of all three cultures (Backup, Mutant & Control) was monitored on a cell by cell basis from initial exposure until the end of the recovery phase.

Cell viability was monitored with a novel method developed by Schulze et al. [33] which uses the Chlorophyll autofluorescence and a green fluorescence to microscopically determine the status of each cell individually. As with each round of mutagenesis, all three cultures were inoculated from the same starting culture and began with about 90% viable cells. Under the selective conditions the percentage quickly dropped off for

![Figure 1. Characterization of UV.](https://example.com/figure1.png)
both cultures. However during the recovery-phase at reduced temperature (42°C) the mutagenized culture was able to recover in about 5 days, whereas the amount of viable cells in the control culture stayed near 0%. As the percentage of viable cells continued to increase in the mutagenized culture the cultivation temperature was also increased in various steps (fig. 3). After growing stably at 43.5°C for 10 days the temperature was continued to be incrementally increased until the new maximum-temperature tolerance of 43.8°C was determined and the next round of mutagenesis could be performed (data not shown). The backup culture, meanwhile was constantly kept at 43.1°C (well tolerated temperature after first round of mutagenesis) and the percentage of viable cells as determined by the chlorophyll fluorescence was always between 80 and 100% (data not shown).

The course of the culture viability and temperature progression shown is representative for all rounds of mutagenesis (except the 3rd).

Discussion

To characterize the mutagens MMS and UV, cells treated with varying dosages were each plated on regular agar plates to determine the survival rate and on selection plates to determine the mutation rate.

Table 1. Summary of the conditions and results of each round of mutagenesis.

| Mutagenesis Round | Mutagen used | Selection temperature | Recovery temperature | Maximum tolerated temperature (day) |
|-------------------|--------------|-----------------------|----------------------|-------------------------------------|
| 0 (wt)            | none         |                       |                      | <43                                 |
| 1                 | UV           | 44                    | 41                   | 43.3                                |
| 2                 | UV           | 44.5                  | 42                   | 43.8                                |
| 3*                | MMS          | 45 (24 hours)         | 40                   | 44                                  |
| 4                 | MMS          | 45                    | 43                   | 45                                  |

*Note that in the 3rd round of mutagenesis there was a technical problem with the temperation, and cultures were also tempered during the night cycle, leading to many more cells dying than usual. A recovery at lower temperature was used to rescue surviving cells.

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The observed exponential drop off in the survival rate for both mutagens was in line with our expectations. For both mutagens the cytotoxic effects due to DNA lesions or other chemical reactions increase in proportion with the exposure of the cells, explaining the increased lethality.

As expected, the observed rate of non-lethal point mutations induced by both mutagens was considerably increased compared to the control for both mutagens at most tested dosages. With increasing dosage however the rate of non-lethal point mutations dropped, at high dosages even below the non-mutagenized control. This can be easily explained: During cultivation in liquid media, there already was a selection for cells able to reproduce in liquid environment. Plating on selection plates additionally screens for cells still able to grow on solid media, which additionally also have a ribosomal mutation inducing a streptomycin resistance. The probability of carrying such a ribosomal mutation is increased in proportion with the applied dosage. However with increasing dosages this effect is gradually canceled out by other accumulated disadvantageous mutations which might be lethal on agar cultivation.

For UV mutagenesis the increased point mutation rate reaches its optimum for inducing non-lethal mutations between 10–50 J/m². After this point the number of colonies starts dropping again, as the increased number of mutation leads to some cells unable to grow on plates at all. The rate of non-lethal point mutations for mutagenized cells however, does not drop off below the non-mutagenized control until a dosage between 200–300 J/m² is reached, at which point the general survival rate was already well below 1% (fig. 1).

For MMS the number of colonies on the selection plates showed two peaks: one at 0.1 v% and one at 1 v% (fig. 2). The shape of the curve may indicate two superimposed processes. Dosages up to 0.1 v% are probably not high enough to trigger a strong response of the cellular repair mechanisms (based on MGMT), especially considering the short exposure time. As the expression of MGMT remains low, the induced DNA lesions remain unrepaired, allowing effective mutagenesis to take place. For dosages above 0.2 v%, MGMT appears to have a significant effect, lowering the overall mutation rate. At 1 v% a second local maximum is reached; the increased amount of alkylations appears to surpass MGMT’s ability to repair them. After 1 v% the number of colonies again steadily drops off due to mutations which inhibit growth on plates, and falls below the non-mutagenized control at a dose of 2–3.5 v%.

The graph for UV does not show a similar progression, as the cells specific response to UV irradiation (photolyase) has been effectively disabled by cultivating cells under a SFG-10 filter.

We conclude that the optimal dosages for the nonlethal induction of point mutations for UV is in the range of 10–50 J/m² and either 0.1 or 1 v% for MMS. These dosages induce the maximal rate of non-lethal point mutations, increasing it well above its natural level without creating strong aberrations from the
wildtype, such as the loss of the ability to grow on plate. These
dosages should therefore be optimal for applications involving
directed evolution methods, such as adaption of strains to new
culturing conditions.

These determined optimal dosages were then used to effectively
increase the thermal tolerance of *Synechocystis* PCC6803. In four
rounds of mutagenesis the temperature tolerance under given
cultivation conditions was increased from <43°C to 45°C. As the
control-culture was not exposed to the mutagens and was not able
to show similar increases, it is clear that the increased rate of
mutation induced by these mutagens is necessary for such fast
achievements.

Two different mutagens were used, which as discussed earlier
have differing probabilities to induce the various possible
mutations. By using two different mutagens the inherent bias to
each mutagen for the induction of certain mutations is reduced,
and the chance to induce the optimal mutations should be
increased.

A viability analysis of cultures after mutagenesis (fig. 3) clearly
shows that from an equal starting pool of healthy cells the non-
mutagenized ones died faster and in greater amount than the
mutagenized cells when cultivated under selective conditions. This
is especially relevant when considering, that at the applied dosage
the mutagen itself already induced a high rate of lethality to the
culture. For UV the determined survival rate (on plates) at the
applied dosages is about 10%, giving a lethality of 90%. Still, the
mutagenized cells were faster to recover from the selective
conditions than the untreated control, showing that the positive
effects of some point mutations outweighed the negative effects
such as lethal- and other disadvantageous mutations. The lowest
percentage of viable cells reached for the culture was 4%; 19 days
after mutagenesis. Of the around 10% of cells which did not
develop a lethal mutation because of the treatment, probably only
very few have a selective advantage at the increased temperature.
These however have had ample time to reproduce until they
constitute 4% of the total cells at day 19. Note that the actual
number of viable cells in liquid media might be somewhat higher,
as lethality was determined by plating.

It is important to note that the improved culture is a mixture of
various new strains with an as of yet unknown genetic makeup.
This mix however does not have a strongly aberrant phenotype
(aside from the thermal tolerance), and many cells are still able to
grow on plates. In comparison to methods where single cells are
picked directly after mutagenesis before being characterized.
This was successfully demonstrated by raising *Synechocystis*
temperature tolerance by about 2°C in four rounds of mutagen-
esis. Coupled with an appropriate screening, the described
methods should be applicable to induce a variety of desirable
characteristics in various strains. These methods are best suited to
adaptations which allow an in vivo selection, such as adaptations to
cultivation conditions (e.g. toxic substances, salt concentration,
mixing conditions … ). This is especially attractive for systems
where it is not obvious which genes require modifications.

Coupling random mutagenesis with high-throughput screening
methods would additionally allow to select for strains with
characteristics which do not yield a higher fitness and can not be
selected for in vivo (e.g. fatty acid concentration).

At the very least, random mutagenesis methods can be used as a
first step in a combined approach with full genome sequencing
(which is now cheaply available through next-generation tech-
niques), to determine suitable target-genes for more focused and
comprehensive methods.

Supporting Information

Additional File S1 ImageJ plugin for automated colony
counting and example pictures. The plugin can be used for
automated counting of cyanobacterial colonies on agar plates
using phycocyanin fluorescence. ImageJ is required and can be
downloaded from http://rsweb.nih.gov/jj/download.html. For
an installation of the plugin extract the.jar file into the plugin
folder and restart ImageJ. The plugin can be found under Plugins
> CountCyanoplate. Example images for the plugin can be found in
the folder ExampleImages. The plugin is also hosted at Github
at: https://github.com/KatjaSchalke/CyanoColonyCounter
(ZIP)

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Author Contributions

Conceived and designed the experiments: UMT UD MF. Performed the
experiments: UMT UD KS. Analyzed the data: UMT SL KS. Wrote the
paper: UMT UD KS MF. Designed and wrote the software used to count
the number of cyanobacteria colonies: KS.



References

1. Mascarelli AL (2009) Gold rush for algae. Nature 461(7262): 460–461.
2. Haag AL (2007) Algae bloom again. Nature 447(7144): 520–521.
3. Miller J (1985) Mutagenic specificity of ultraviolet light. J Mol Biol 182(1): 45–
43.
4. Witkin EM (1976) Ultraviolet mutagenesis and inducible DNA repair in
*Escherichia coli*. Bacteriological reviews 40(4): 869–907.
5. Miller J (1985) Mutagenic specificity of ultraviolet light. J Mol Biol 182(1): 45–
65.
6. Miller J (1985) Mutagenic specificity of ultraviolet light. J Mol Biol 182(1): 45–
65.
7. With TD (1996) Ultraviolet mutagenesis and inducible DNA repair in
*Escherichia coli*. Bacteriological reviews 40(4): 869–907.
8. Foster IL (1991) In vivo mutagenesis. Methods Enzymol 204: 114–125.
9. Minda R, Ramachandran J, Joshi VP, Bhattacharjee SK. (2005) A homozygous
reca mutant of *Synechocystis PCC6803* construction strategy and characteristics
eliciting a novel RecA independent UVC resistance in dark. Mol Genet Genomics 274(6): 616–624.

10. Ries G, Buchholz G, Frohmeyer H, Hohn B (2000) UV-damage-mediated induction of homologous recombination in Arabidopsis is dependent on photosynthetically active radiation. Proc Natl Acad Sci U S A 97(24): 13425–9.

11. McCready S, Müller JA, Boubriak I, Berquist B, Woon Loon Ng, et al. (2005) UV irradiation induces homologous recombination genes in the model archaeon, Halobacterium sp. NRC-1. Saline systems 1: 3.

12. Todd P, Moritz-Bragadin C (1979) MMS mutagenesis in strains of Escherichia coli carrying the R46 mutagenic enhancing plasmid: phenotypic analysis of Arg+ revertants. Mutat Res 62: 227–237.

13. Swann PF (1990) Why do O6-alkylguanine and O4-alkylthymine miscode? The relationship between the structure of DNA containing O6-alkylguanine and O4-alkylthymine and the mutagenic properties of these bases. Mutat Res 233(1–2): 81–94.

14. Shevell D, Friedman B (1990) Resistance to alkylation damage in Escherichia coli: role of the Ada protein in induction of the adaptive response. Mutat Res 233: 53–72.

15. Lucas-Lledó JI, Lynch M (2009) Evolution of mutation rates: phylogenomic analysis of the photolyase/cryptochrome family. Mol Biol Evol 26(5): 1143–53.

16. Lambert JAM, Williams E, O’Brien PA, Houghton JA (1980) Mutation Induction in the Cyanobacterium Gloeocapsa alpica. J Gen Microbiol 121(1): 213–219.

17. Levine E, Thiel T (1987) UV-inducible DNA repair in the cyanobacteria Anabaena spp. J Bacteriol 169(9): 3988–3993.

18. Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y (1988) Regulation and expression of the adaptive response to alkylating agents. Annu Rev Biochem 57: 133–57.

19. Demple B, Sedgwick B, Robins P (1985) Active site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis. Proc Natl Acad Sci U S A 82(9): 2688–92.

20. Currier T, Haury J, Wolk C (1977) Isolation and preliminary characterization of auxotrophs of a filamentous Cyanobacterium. J Bacteriol 129(3): 1556–1562.

21. Wolk CP, Cai Y, Cardemil L, Flores E, Hohn B, et al. (1988) Isolation and complementation of mutants of Anabaena sp. strain PCC 7120 unable to grow aerobically on dinitrogen. J Bacteriol 170(3): 1239–44.

22. Floriano R, Herrera A, Flores E (1992) Isolation of arginine auxotrophs, cloning by mutant complementation, and sequence analysis of the argC gene from the cyanobacterium Anabaena species PCC 7120. Mol Microbiol 6(13): 2083–94.

23. Metreux LA, Guerdes AC, Malcata FX (2003) Increase of the yields of eicosapentaenoic and docosahexaenoic acids by the microalga Phaeodactylum tricornutum following random mutagenesis. Biotechnol Bioeng 81(1): 50–55.

24. Ong SC, Kao CY, Chiu SY, Tsai MT, Lin CS (2010) Characterization of the thermal-tolerant mutants of Chlorella sp. with high growth rate and application in outdoor photobioreactor cultivation. Bioresource Technol 101(8): 2890–3.

25. Chaturvedi R, Fujita Y (2006) Isolation of enhanced eicosapentaenoic acid producing mutants of Anabaena variabilis ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level. Physiological Res 54(3): 209–219.

26. Carter AP, Clemens WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, et al. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407(6932): 340–9.

27. Springer B, Kudan Y, Pramananman T (2001) Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. Antimicrob Agents Chemother 45(10): 2877–2884.

28. Agarwal D, Gregory ST, O’Connor M (2011) Error-prone and error-restrictive mutations affecting ribosomal protein s12. J Mol Biol 410(1): 1–9.

29. Marin K, Hukau J, Fulda S (2002) Salt-dependent expression of glycosylglycerol-phosphate synthase, involved in osmolyte synthesis in the cyanobacterium Synechocystis sp. strain PCC6803. J Bacteriol 184(11): 2970–2.

30. Kaneko T, Sato S, Kotsui H, Tanaka A, Asamizu E, et al. (1996) Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). DNA Res 3(3): 185–209.

31. Nakamoto H, Suzuki M, Kojima K (2003) Targeted inactivation of the hrcA repressor gene in cyanobacteria. FEBS Letters 549(1–3): 57–62.

32. Suzuki I, Kanesaki Y, Hayashi H, Hall JJ, Simon WJ, et al. (2003) The histidine kinase Hik34 is involved in thermotolerance by regulating the expression of heat shock genes in synechocystis. Plant physiology 130(3): 1409–21.

33. Schulze K, López DA, Tilmich UM, Frohmeyer H (2011) A simple viability analysis for unicellular cyanobacteria using a new autofluorescence assay, automated microscopy, and ImageJ. BMC Biotechnol 11(1): 118.