Tolerance Level to Alcohol Toxicity of Cyanobacterium *Synechocystis* SP. PCC 6803 Mutant

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Abstract

The imbalance of increased energy consumption has occurred while the fossil fuel resource has been decreasing. Therefore, finding of renewable energy is important and challenging for our World energy. The third generation of bioresources comes from cyanobacteria that can produce bioenergy compounds, such as ethanol, alkanes and fatty alcohol, via their atmospheric CO_2 fixation. However, the main limited productivity is cell and membrane damages by free radicals-generating alcohol products. In this study, one of tolerant mechanisms to alcohol stress is focused on phospholipid modification. We aimed to monitor alcohol tolerance level in cyanobacterium *Synechocystis* sp. PCC 6803 with overexpressing *plsX* (*slr1510*) gene, encoding putative acyltransferase involved in phospholipid synthesis (hereafter OX_*plsX*). Cell growth of OX_*plsX* was higher than WT and control WT under both normal and alcohol stressed conditions, as well as their oxygen evolution rates. The cell growth recovery of OX_*plsX* showed highest level at day 3 to day 5 of recovery duration after 24h-treatment of 8% (v/v) ethanol. Also, the color of OX_*plsX* cell culture showed darkest green when compared to other strains. Interestingly, the morphology of OX_*plsX* was least aggregated under alcohol treatment when compared with WT and control WT. Then, our findings suggested that OX_*plsX* strain had higher tolerance level to alcohol stress, as well as its growth recovery from alcohol treatment, especially at 8% (v/v) ethanol concentration.

Keywords: *Synechocystis, Alcohol toxicity, Ethanol stress, Putative acyltransferase, PlsX, Growth recovery*

1. Introduction

In 2018, the energy consumption was grown in 2.9% of primary energy and its carbon emission was about 2.0% of all energy use. The increase of energy consumption is in opposite with decreased residual resource, as recently known that the fossil fuel was gradually decreased. For the alternative aspect, the renewable energy from cyanobacteria is practically important route for energy production and, especially, could reduce CO_2 from the atmosphere. The first generation of renewable energy sources was the food crops, such as soybean and sugarcane which later concerned to compete with food availability, used large area and freshwater supply. Second generation of renewable energy was energy crops and lignocellulosic materials which gained laborious and costly technologies (Ho et al., 2013). At present, the third generation of renewable energy has emphasized on microalgae and cyanobacteria which can reduce problems of arable land usage. Energy carriers or biofuel products produced by cyanobacteria are such as, bioethanol, biodiesel, biohydrogen. In this study, we used one model of cyanobacteria, namely *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) which normally could produce bioethanol. *Synechocystis* is unicellular, freshwater cyanobacterium that has complete genome sequence. On the other hand, the limitation of alcohol production in microorganisms occurred as such due to the toxicity of alcohol products. Ethanol is organic solvent which is highly toxic to cells and cell membranes by generated free radicals. Generally, cyanobacteria have common tolerant response to alcohol stress such as, increased activities of multiple membrane transporters, enhanced cell mobility, and modification of cell membrane and envelope (Qiao et al., 2012). The main effect is directed to membrane modification including changes on protein translocation, envelop proteins and lipid/phospholipid synthesis (Tian et al, 2013). Lipid or phospholipid synthesis consists of many steps of reactions such as FASII. In this study, we focused on *plsX* gene which encodes putative acyltransferase enzymes or fatty acid/ phospholipid synthesis protein. Previously, it was found in bacteria that *PlsX* (*slr1510*)

could inactivate membrane lipid production when it was deleted (Kaczmarzyk et al., 2018). On the other hand, *plsX* overexpression increased the total lipid content which mainly belongs to the composition of membrane lipids (Towijit et al., 2018).

Therefore, the objective of this study was to investigate the alcohol tolerance level, in particular ethanol, of the *plsX*-overexpressing *Synechocystis* strain via measuring growth and its growth recovery, when compared with *Synechocystis* wildtype (WT) and *Synechocystis* control wildtype (control WT) which contained antibiotic-resistance gene.

2. Objectives

To investigate the tolerance level of *Synechocystis* sp. PCC 6803 mutant to alcohol stress by measuring growth, oxygen evolution rate, and contents of intracellular pigments compared to wild type

3. Materials and Methods

3.1 Cyanobacterial strain

Synechocystis sp. PCC 6803 wild type (WT), kindly provided by Prof. Peter Lindblad from the Photochemistry and Molecular Science, Department of Chemistry- Ångström Laboratory, Uppsala University, Sweden. The *plsX*-overexpressing *Synechocystis* strain, or OX_*plsX* was constructed by Miss Nutchaya Songruk from Department of Biochemistry and Molecular Biology – Cyanobacterium Laboratory, Chulalongkorn University, Thailand (Towijit et al., 2018).

3.2 Culture conditions

In the previous study, to overexpress *plsX* gene in *Synechocystis* genome, we used the pEERM expression plasmid containing chloramphenicol resistance gene as a vector (Towijit et al., 2018). Then, the culture of native wild type cells was grown in normal BG_{11} medium whereas the control wild type (*Synechocystis* wild type containing chloramphenicol resistance gene from pEERM expression vector) and OX_*plsX* cells could be grown in BG_{11} medium containing 30 µl/ml of chloramphenicol. The culture condition was performed at 28 °C under a light intensity of approximately 40-50 µmol photons/m^2/s in a shaking incubator with speed of 160 rpm.

3.3 Growth determination

Cultured cells with OD_{730} of about 0.8-1.0 were harvested by centrifugation at 6000 rpm for 10 min at 4 °C and then used as a stock culture in 100 ml BG_{11} medium in a 250 ml flask. One ml of culture sample was collected and measured growth using spectrophotometer at OD_{730} every 24 h. The experiments were repeated at least three times.

3.4 Intracellular pigment determination

One ml of cell culture was harvested by centrifugation at 10000 rpm (17507*xg*) for 10 min and discarded the supernatant. The cell pellet was extracted by N, N-dimethylformamide (DMF) and incubated for 10 min under darkness. After that, the supernatant was separated by centrifugation at 10000 rpm (17507*xg*) for 10 min. And then, the obtained yellow supernatant was measured absorbances by spectrophotometer at 461, 625 and 664 nm and calculated for chlorophyll *a* and carotenoid contents (Towijit et al., 2018).

3.5 Ethanol treatment

Cultured cells were harvested by centrifugation at 6000 rpm and then adjusted OD_{730} to 0.5 in 100 ml BG_{11} medium containing 0%, 2%, 4%, 8% and 16% (v/v) ethanol concentrations, respectively. Samples were incubated for 24 h and washed by new BG_{11} medium. After adjusting alcohol-treated cells to OD_{730} about 0.1 in 100 ml BG_{11} medium (no ethanol), cell growth and pigment contents were measured using spectrophotometer at OD_{730} every 24 h.

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3.6 Oxygen evolution rate determination

Oxygen evolution rate represents the photosynthetic efficiency. Two ml of cell culture was harvested by centrifugation and incubated under darkness for 30 min. The oxygen evolution rate was measured by using a Clark-type Oxygen electrode at 25 °C. The unit of oxygen evolution rate was µmol/mg Chl *a*/h.

4. Results and Discussion

Growth and pigment contents under normal condition

Cells were cultured in BG_{11} medium under normal condition. Cell growth was measured for 15 days of cultivation. Growth of OX_*plsX* showed the highest level from day 9 to day 15 of cultivation among all strains studied (Figure. 1a). Also, chlorophyll *a* content of OX_*plsX* was highest from day 7 - 15 (Figure 1b) whereas carotenoid content of OX_*plsX* was less than WT from day 5 – 15 (Figure 1c). Previous study (Towijit et al., 2018) revealed that growth of OX_*plsX* was higher than those of WT and WTc (control wild type) strains from day 10 to 16 which was consistent with our study. However, the pigment contents accumulated in OX_*plsX* including chlorophyll *a* and carotenoids occurred in different patterns from our results. Their results revealed the insignificant differences of chlorophyll *a* and carotenoid contents in OX_*plsX* at 16 days-normal cultivation when compared to those of WT and WTc.

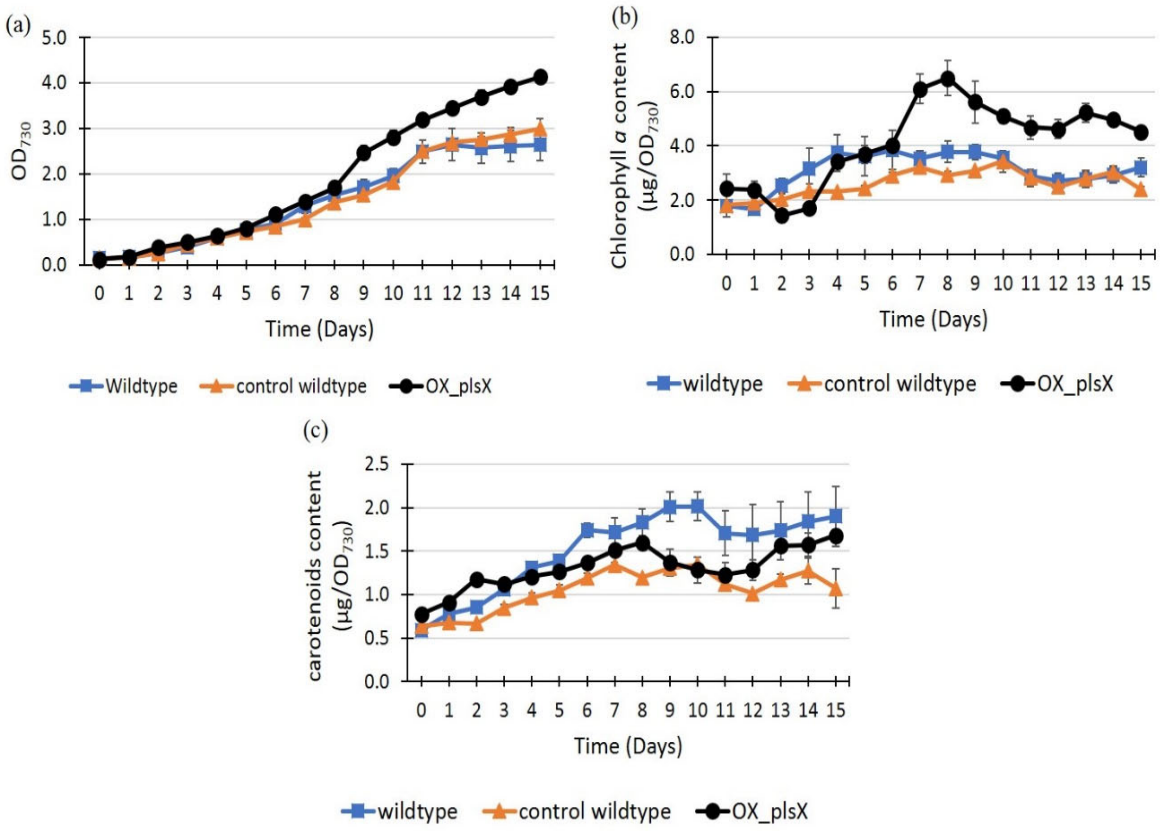

Figure 1 Growths and pigment contents of three *Synechocystis* sp. PCC 6803 strains grown under normal condition in 15 days of cultivation. (a) growth (b) chlorophyll *a* content, and (c) carotenoid content.

Ethanol treatment and cell growth recovery

Initially, the morphology of cells was microscopically monitored under each alcohol treatment. It was found that cell distribution was changed when compared between before and after ethanol stress, as

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shown in Figures. 2 – 4. *Synechocystis* cells after 24 h-ethanol stress were apparently aggregated, in particular under 8% and 16% (v/v) ethanol conditions, as coincident with previous works under ethanol (Qiao et al., 2012) and butanol (Tian et al., 2013) treatments. Interestingly, the aggregation of OX_*plsX* cells was least when compared to other strains.

After treating alcohol in various concentrations for 24 h, the growth recovery in normal BG_{11} medium was monitored. In Figure. 5, the growth recovery gave lower OD_{730} after treating with high ethanol concentration. This result suggested the severe alcohol effect of dose-dependent level on growth recovery. However, the cell growth recovery of three strains were not significantly different under 0%, 2%, 4% and 16% (v/v) ethanol conditions (Figure 5a – c, and e), although the slightly higher level of OX_*plsX* was observed. Interestingly, the highest growth recovery of OX_*plsX* was clearly shown at day 5 after treating 8% (v/v) ethanol condition (Figure 5d). In Figure. 6, the color of cell culture was consistent with the cell growth at day 0 (Figure. 6a – c) and day 5 (Figure. 6d – f). At day 5 of recovery duration, OX_*plsX* cells treated by 8% (v/v) ethanol presented dark green more than other two strains. Our results suggested that this darker green color of cell culture represented the higher growth of OX_*plsX* which could recover itself from alcohol stress better than wild type corresponding to their OD_{730} in Figure 5.

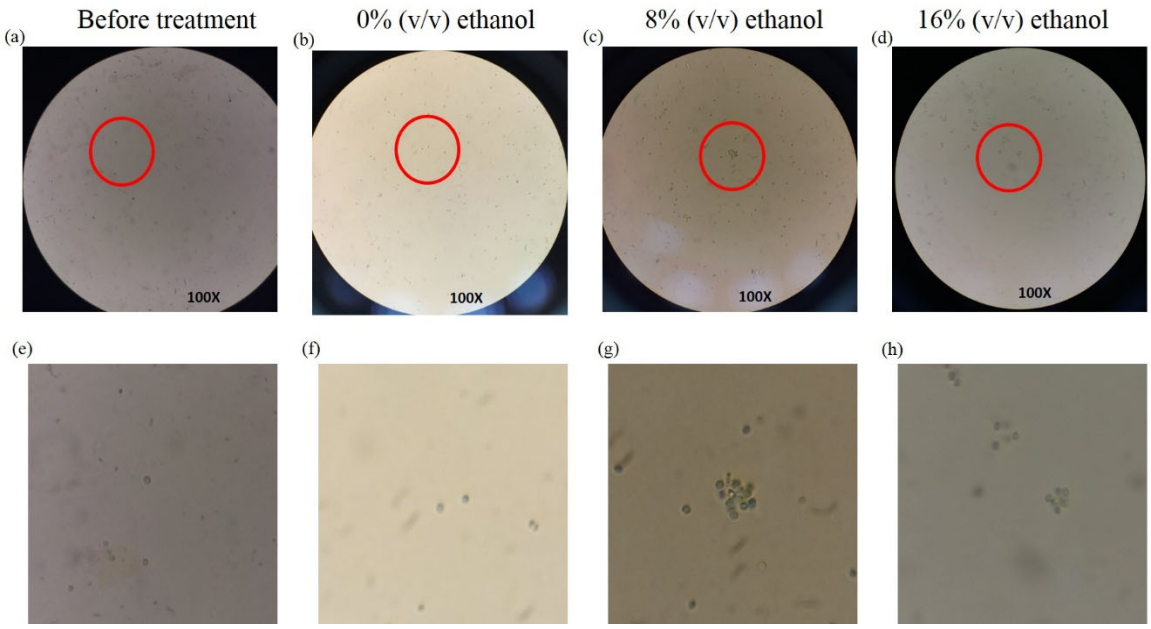

Figure 2 Morphology of WT under microscope (100X). WT cells were treated with 0, 8 and 16 % (v/v) ethanol for 24 h. The red circles in (a), (b), (c), (d) are enlarged in (e), (f), (g) and (h), respectively.

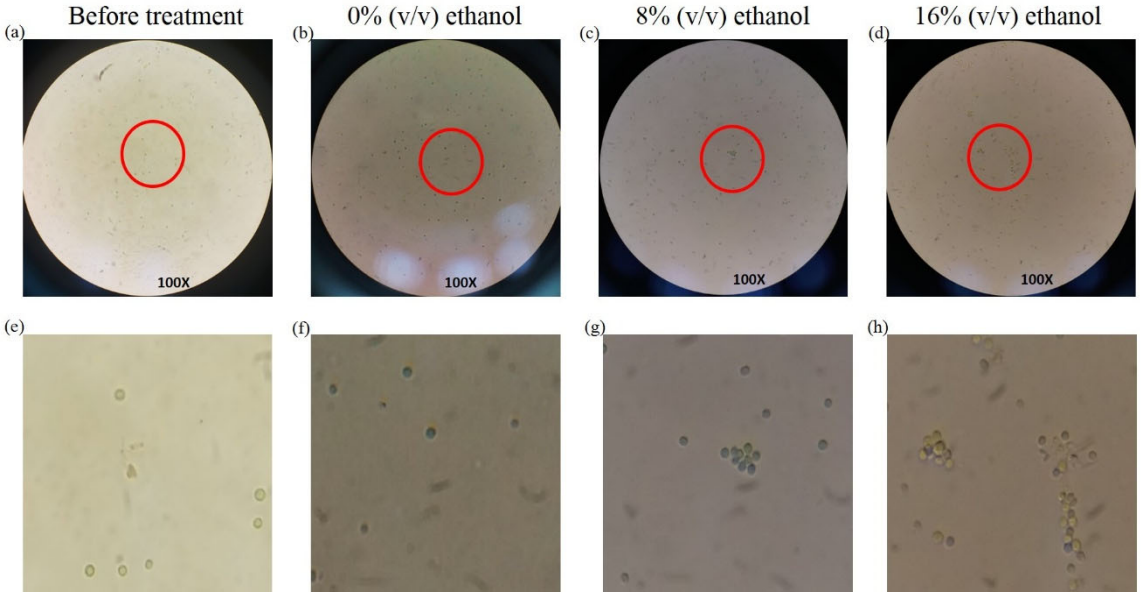

Figure 3 Morphology of control WT under microscope (100X). Control WT cells were treated with 0, 8 and 16 % (v/v) ethanol for 24 h. The red circles in (a), (b), (c), (d) are enlarged in (e), (f), (g) and (h), respectively.

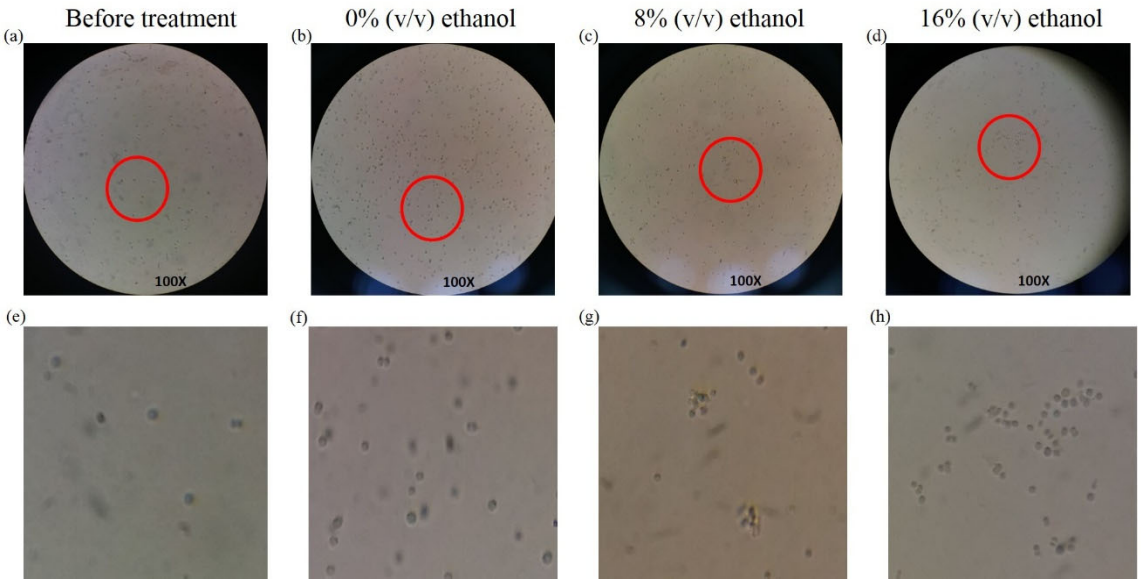

Figure 4 Morphology of OX_*plsX* under microscope (100X). OX_*plsX* cells were treated with 0, 8 and 16 % (v/v) ethanol for 24 h. The red circles in (a), (b), (c), (d) are enlarged in (e), (f), (g) and (h), respectively.

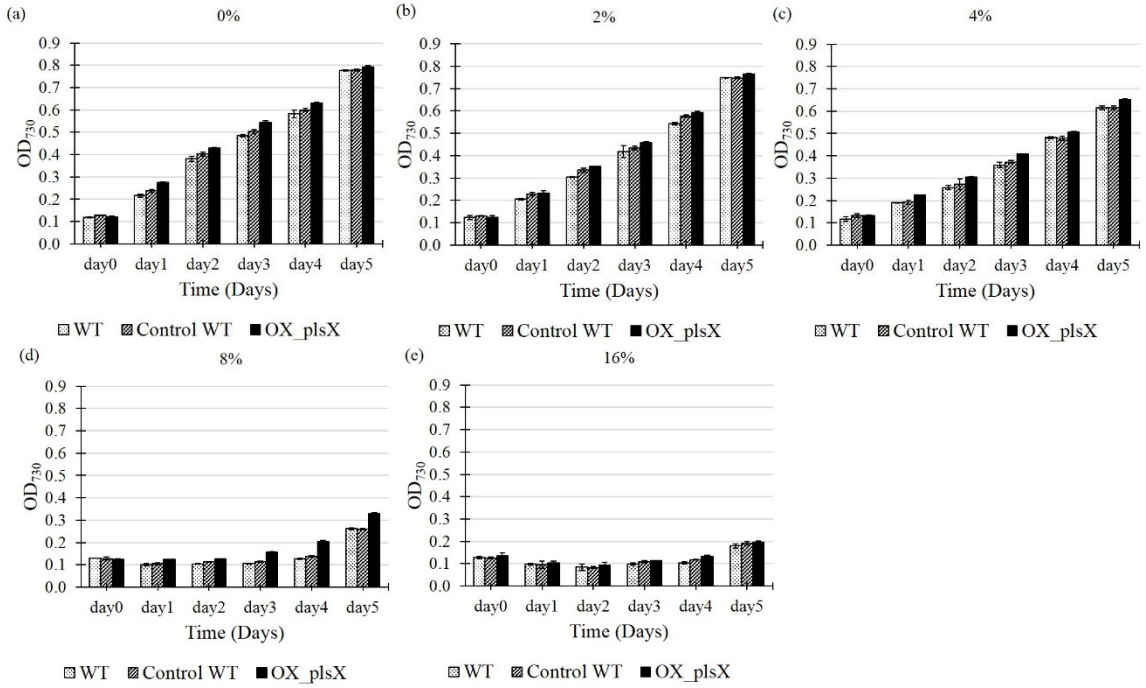

Figure 5 Growth recovery of three *Synechocystis* strains after various 24 h-alcohol treatments. The 24 h-alcohol treatment was varied in (a) 0% (v/v) ethanol concentration, (b) 2% (v/v) ethanol concentration, (c) 4% (v/v) ethanol concentration, (d) 8% (v/v) ethanol concentration, and (e) 16% (v/v) ethanol concentration.

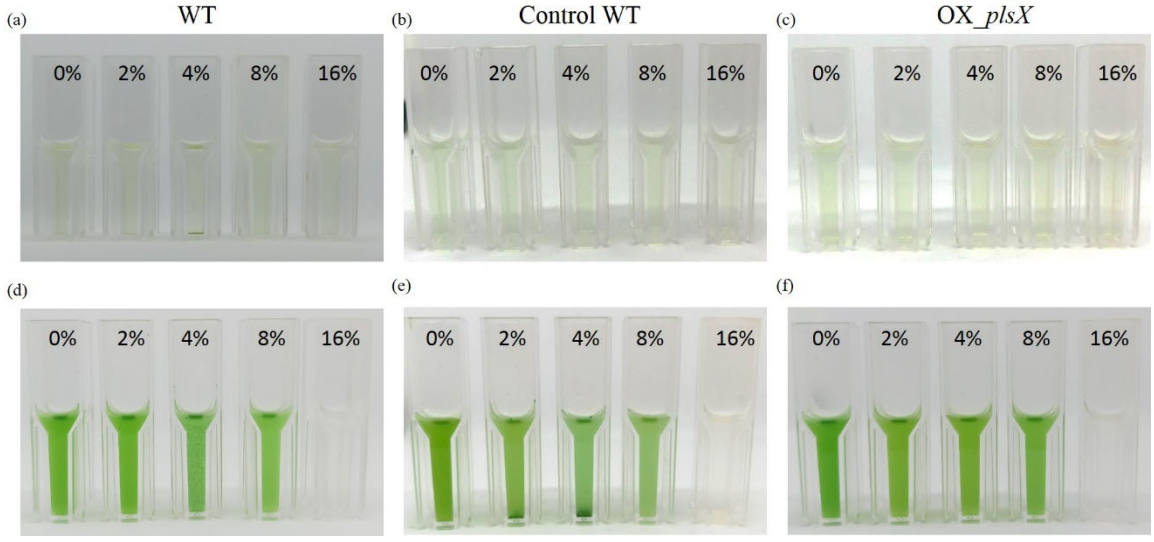

Figure 6 Cell color at day 5 of recovery duration of three *Synechocystis* strains after various 24 h-ethanol treatments (0%, 2%, 4%, 8% and 16% (v/v) ethanol concentrations). (a – c): cell on day 0 of recovery duration after 24 h-alcohol treatments, (d – f): cell on day 5 of recovery duration after 24 h-alcohol treatment.

In previous study, the growth of *Synechocystis* cells were 50% decreased by 1.5% (v/v) ethanol treatment for 24 h (Qiao et al., 2012; Zhang et al., 2015; Wang et al., 2012). For butanol treatment, the 50%

decrease of *Synechocystis* growth occurred in 0.2% (v/v) butanol treatment for 24 h (Tian et al., 2013). In addition, the 0.25% (v/v) 1-butanol treatment gave lower growth of about 75% decrease for 72 h than under normal BG_{11} medium (Gao et al., 2017). *Synechocystis* cells were killed over 99% under 2.5% (v/v) n-butanol treatment for 1 h (Kaczmarzyk et al., 2014). Furthermore, the 50% decrease of *Synechocystis* growth under 1.5% (v/v) ethanol condition, 0.2% (v/v) butanol and 0.8% (v/v) hexane occurred for 48 h treatment (Pei et al., 2017). However, our finding demonstrated that *Synechocystis* cells treated with 8% and 16% (v/v) ethanol conditions for 24 h could recover themselves from stress about 62.5% and 75%, respectively, for 5 days when compared to normal BG_{11} without ethanol.

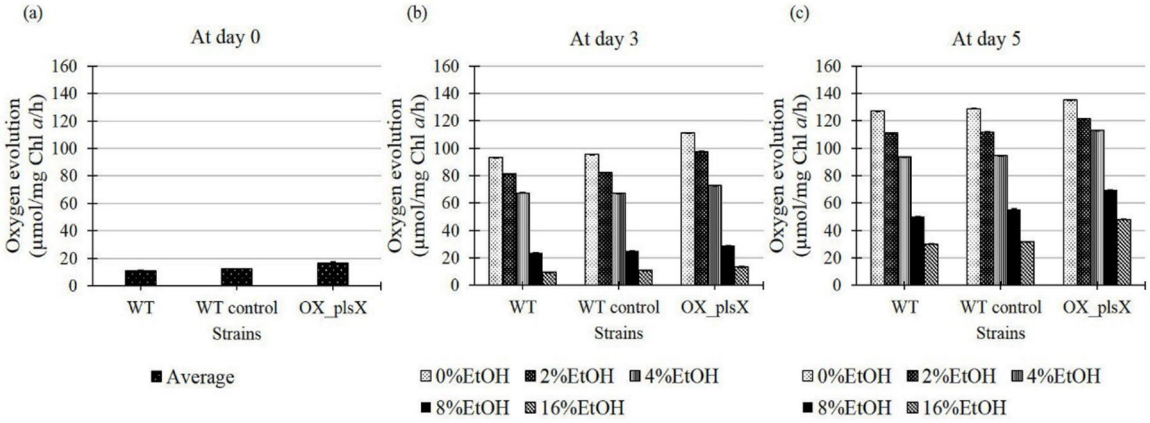

Figure 7 Oxygen evolution rates of three *Synechocystis* strains under growth recovery duration after 24h-alcohol treatments. (a) at day 0 of recovery duration (b) at day 3 of recovery duration, and (c) at day 5 of recovery duration.

Oxygen evolution of recovered cells

Oxygen evolution rate represents the photosynthetic efficiency which showing oxygen yield during light reaction of photosynthesis. In Figure. 7, the oxygen evolution rates of cells during recovery duration were significantly increased up to day 5. Our results demonstrated that oxygen evolution rates of OX_*plsX* cells were increased in highest level when compared to WT and control WT at both day 3 and day 5. However, it was confirmed that the increased ethanol concentration had harmful effect on high reduction of photosynthetic efficiency of *Synechocystis* cells. From previous study, the photosynthetic efficiency reported in term of photosynthetic yields (F_v'/F_m') of *Synechocystis* cells after treating with 0.5 M (or 2.3% (v/v)) ethanol gave lowest value near zero within 10 h (Ruffing, & Trahan, 2014).

5. Conclusion

Our findings demonstrated that *plsX*- overexpressing *Synechocystis* strain had higher tolerance ability to ethanol stress when compared to *Synechocystis* wild type and control wildtype, as evident by the higher levels of growth recovery and oxygen evolution rates. Thus, the higher expression of one phospholipid synthetic gene, herein *plsX*, would be one survival mechanism of tolerance responses to alcohol toxicity in *Synechocystis* sp. PCC 6803.

6. Acknowledgements

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7. References

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