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Effect of alcohol stress on cell growth and its recovery of *Synechocystis* sp. PCC 6803

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Abstract

Cyanobacteria, classified in the third generation of biofuel resources, enable to promisingly produce bioenergy containing compounds. However, most biofuels, in particular alcohols, are toxic to microbes. The oxidative stress is highly induced by alcohols which dramatically causes cell growth inhibition. Therefore, our objective was to investigate the cellular tolerance response to alcohol stress of *Synechocystis* sp. PCC 6803 via growth and its recovery. Our results showed that ethanol stress for 4 day-treatment, varied in a concentration range of 1.5 – 2.0 % (v/v), obviously decreased *Synechocystis* cell growth by approximately 22 – 65 % while *n*-butanol in a range of 0.2 - 0.3% (v/v) concentrations inhibited growth by 17 – 54 %. Although higher chlorophyll *a* and carotenoid accumulations were induced by alcohols, the O_2 evolution rates were apparently lowered under alcohol stresses. Additionally, the short term stresses for 1 hour-treatment were performed with various high concentrations of either 6 – 21% (v/v) ethanol or 1 – 4.5 % (v/v) *n*-butanol. *Synechocystis* cell growth was surprisingly recovered after re-cultivating for 4 days under 6 - 15% (v/v) ethanol and 1 - 2.5 % (v/v) *n*-butanol conditions. Moreover, transcript levels of genes related to antioxidant enzymes, including *sodB*, *gpx2* and *katG*, were increased under 2.0 % (v/v) ethanol- and 0.3% (v/v) *n*-butanol-stressed conditions whereas SOD enzyme activities were slightly decreased and GPx enzyme activities were not significantly affected.

Introduction

Nowadays, fossil fuel is mainly used for World energy source which ultimately leads to the global climate change crisis and the security crisis of fossil fuel depletion (Lenferna, 2018, Yıldız, 2018). The alternative energy, like biofuel, has become to mitigate these problems which are mainly obtained from biomass, such as alcohol, ether, ester, oil and hydrogen (Ramos *et al*., 2016, Dincer and Zamfirescu, 2014). The third generation of biosources, such as microalgae and cyanobacteria had been continually attempted to develop novel knowledge and applications in field of biotechnology and biofuel production process (Adeniyi *et al*., 2018, Singh and Chanan, 2013). In general, cyanobacterium *Synechocystis* sp. PCC 6803 wide type has a few ethanol production and there is no any recent reports addressed another kinds of alcohol production. Due to alcohol product toxicity, growth of an ethanol producing strain of engineered *Synechocystis* sp. PCC 6803 was lower than wide type and later initiated to decrease at the maximum point of ethanol production (Dexter and Fu, 2009). Many kinds of alcohols, such as ethanol, *n*-butanol and isobutanol, were able to recently produce by genetic modified cyanobacteria. In previous reports, the engineered *Synechocystis* sp. PCC 6803 cells could gain the production up to 5.5 g/L of ethanol (Gao *et al*., 2012) and 0.6 g/L of

isobutanol (Miao *et al*., 2017) whereas the engineered *Synechococcus elongatus* PCC 7942 could produce up to 13.16 mg/L of *n*-butanol (Lan and Liao, 2011).

It was previously reported that *Synechocystis* cells, incubated with exogenous alcohol, had the significant increase of intracellular reactive oxygen species (ROSs) which led further to disturb metabolic processes and membranes (Anfelt *et al*., 2013, Kaczmarzyk *et al*., 2014). The tolerant response to alcohol product toxicity of cyanobacteria encompassed many mechanisms, such as heat shock response, transporter, membrane modification, cell mobility, regulatory system and oxidative stress (Tian *et al*., 2013). In *Synechocystis* sp. PCC 6803, superoxide dismutase (encoded by *sodB*) is an important enzyme to detoxify ROSs in a form of superoxide radical ($\bullet O_2^-$) to hydrogen peroxide (H_2O_2) and continually converted it to water by both glutathione peroxidase (encoded by *gpx1* or *gpx2*) and catalase-peroxidase (encoded by k*atG)* (Gaber *et al*., 2004, Fujisawa *et al*., 2017).

Our objective of this study was to investigate the effects of ethanol and *n*-butanol on cell growth, photosynthetic efficiency, transcript levels of *sodB*, *gpx1*, *gpx2* and *katG* genes, and antioxidant enzymatic activities including superoxide dismutase (SOD) and glutathione peroxidase (GPx) of *Synechocystis* sp. PCC 6803 wild type.

Methodology

Synechocystis growth conditions and alcohol treatments

Synechocystis sp. PCC 6803 cells were grown in BG_{11} medium under normal growth condition at 28°C with continuous illumination of 50 μE/m^2/s on a shaker at 160 rpm. The cell density was determined for growth using spectrophotometer. Cell culture with mid-logarithmic growth phase (OD_{730} ~ 0.8-1.0) was used for alcohol experiment. Various ethanol or *n*-butanol concentrations were added into BG_{11}. Cells were cultured for a long term-4 days whereas in a short term treatment, cells were incubated with various ethanol or *n*-butanol concentrations for 1 hour before washing cell pellets with new BG_{11} medium and further cultured for 4 days.

Determinations of chlorophyll a and carotenoid contents

For each sample, 1 ml of cell culture was harvested by centrifugation at 10,000 rpm (17,507×g) for 10 min and discarded the supernatant. Intracellular pigments containing chlorophyll *a* (chl *a*) and carotenoids of cell pellet were extracted by adding *N, N*-dimethylformamide (DMF) and incubated for 10 min under darkness. Then, the sample was centrifuged at 10,000 rpm (17,507×g) for 10 min. The yellowish supernatant fraction was measured its absorbance at 461, 625 and 664 nm which used later to calculate chlorophyll *a* and carotenoids contents (Chamovitz *et al*., 1993, Moran, 1982).

Chlorophyll *a* content (μg) = $(12.1 \times OD_{664}) - (0.17 \times OD_{625})$

Carotenoid content (μg) = $(OD_{461} - (0.046 \times OD_{664})) \times 4$

Determination of oxygen evolution rate

For each sample, 2 ml of cell culture was collected and incubated under darkness for 30 min. Then, measured the oxygen evolution using a Clark-type Oxygen electrode (Hansatech Instruments, US) under a saturated white light source at 25°C. The unit of oxygen evolution rate represented as μmol O_2/mg chl *a*/h.

RT-PCR

For each sample, 15 ml of cell culture at day 4 was collected and total RNA was isolated with TRI reagent® (MRC, USA) following by a hot-phenol extraction method adapted from Wilhelm and Martin (2017) and Kingston *et al*. (1996). DNase treatment was performed by incubating at 37°C for 20 min with DNaseI and stopped the reaction by incubating at 65°C for 10 min with with EDTA. Approximately 5 μg of total RNA was synthesized to cDNA using the SuperScript® III First-Strand System (Invitrogen, USA). Each cDNA sample was used as

a template for PCR. The transcript level of each gene (*sodB*, *gpx1*, *gpx2* and *katG*) was quantifiably normalized with *16s rRNA*. The GelQuant.NET software (biochemlab solutions.com) was used to measure the band intensity.

Determinations of antioxidant enzymatic activities

For each sample, 50 ml of day 4-cell culture was harvested by centrifugation at 5,500 rpm (3,505×g), 20 °C for 10 min and washed the pellet with 20 ml of the solution containing 10 mM HEPES-NaOH pH 7.5, 5 mM NaCl and 2 mM Na_2EDTA. Then, centrifuged and re-suspended the pellet with 2 ml of solution containing 10 mM HEPES-NaOH pH 7.5 and 20% (w/v) sucrose. French pressure cell press was used for disrupting cells under cold temperature using 1000 psi of pressure. Finally, the crude enzyme sample was centrifuged at 14,000 rpm (24,510×g), 4°C for 30 min, collected the supernatant (total protein) and kept at -20°C not longer than 2 days. Approximately 10 μg of total protein was used to determine the enzymatic activities. The superoxide dismutase (SOD) activity was measured using Superoxide dismutase (SOD) assay kit (Elabscience, USA) whereas the glutathione peroxidase (GPx) activity was measured using Glutathione peroxidase (GSH-PX) assay kit (Elabscience, USA).

Results and Discussion

Effects of exogenous alcohols on Synechocystis growth inhibition and cell growth recovery

Synechocystis sp. PCC 6803 cells were investigated the effects of exogenous alcohols in both long term and short term treatments (Figure 1). In long term 4 days-culture, *Synechocystis* cells were grown in BG_{11} medium with and without alcohol additions. Under ethanol stress shown in Figure 1A, cell density (OD_{730}) under 1.5% and 2.0% (v/v) of EtOH was decreased around 22% and 65% reduction, respectively, when compared to that under normal BG_{11} condition. In Figure 1B, BuOH treatments at 0.2% and 0.3% (v/v) concentrations inhibited around 17% and 54% when compared to that of normal control. These results indicated that exogenous 1.5 - 2% (v/v) EtOH concentrations and 0.2 - 0.3% (v/v) BuOH concentrations were in moderate severity stage of solvent stress on cell growth. Additionally, 2.5% (v/v) of EtOH and 0.4% (v/v) of BuOH affected in a zone of high severity stage with approximately 81% and 73% inhibition, respectively. When compared to previous reports, *Synechocystis* cell density showed a half decrease when culture medium was supplied with 1.5%, 1.8% and 1.9% (v/v) of EtOH (Qiao *et al*., 2012, Zhang *et al*., 2015, Zhu *et al*., 2015). Moreover, glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 were completely inhibited (100%) with 4% (v/v) of EtOH and slightly decreased on chlorophyll *a* content (in term of μg/ml) for 6 days (Vidal, 2017). In this study, we also found that 3.5%, 4.0% and 4.5% (v/v) of EtOH concentrations inhibited cell growth for approximately 92%, 94% and 100% inhibition, respectively. For BuOH stress, there were coincided with previous reports which demonstrated that 0.2% of BuOH treatment significantly decreased cell growth with 50% reduction compared to normal condition at 24 h (Tian *et al*.,2013) whereas another report showed that cell growth rate was about 50% decreased by 4 g/L (or 0.4% (v/v) of *n*-butanol) for 7 days (Anfelt *et al*.,2013).

For short term stress shown in Figure 2, *Synechocystis* cells were incubated with various alcohol concentrations for 1 h and investigated cell growth recovery by 4 days re-cultivation. The determined OD_{730} was relatively normalized with OD_{730} of cells grown under BG_{11} medium without alcohol treatment. Results from Figure 2A and B showed that the growth recovery was capable to maintain after cells were 1 h-treated with about 6 – 12% (v/v) of EtOH and 1 – 2% (v/v) of *n*-butanol. Interestingly, 1 h-treated cells with 15% (v/v) EtOH and 2.5 % (v/v) *n*-butanol conditions demonstrated approximately 50% growth recovery. Moreover, the lower efficiency of cell growth recovery was observed when cells were incubated with high concentrations of more than 16% (v/v) EtOH and 3% (v/v) *n*-butanol. However, the severity of 1 h-2.5 % *n*-butanol treatment killed over 99% of *Synechocystis* cell growth (Kaczmarzyk

et al., 2014). It was consistent with Anfelt and co-workers (2013), the percentage of viability by spotting alcohol treated-cells on BG_{11} agar plate, and defined as colony-forming-units (c.f.u). Thus, we proposed that the re-culture to new BG_{11} liquid medium might help cells to uniformly refresh and highly recover than re-culture on agar plate.

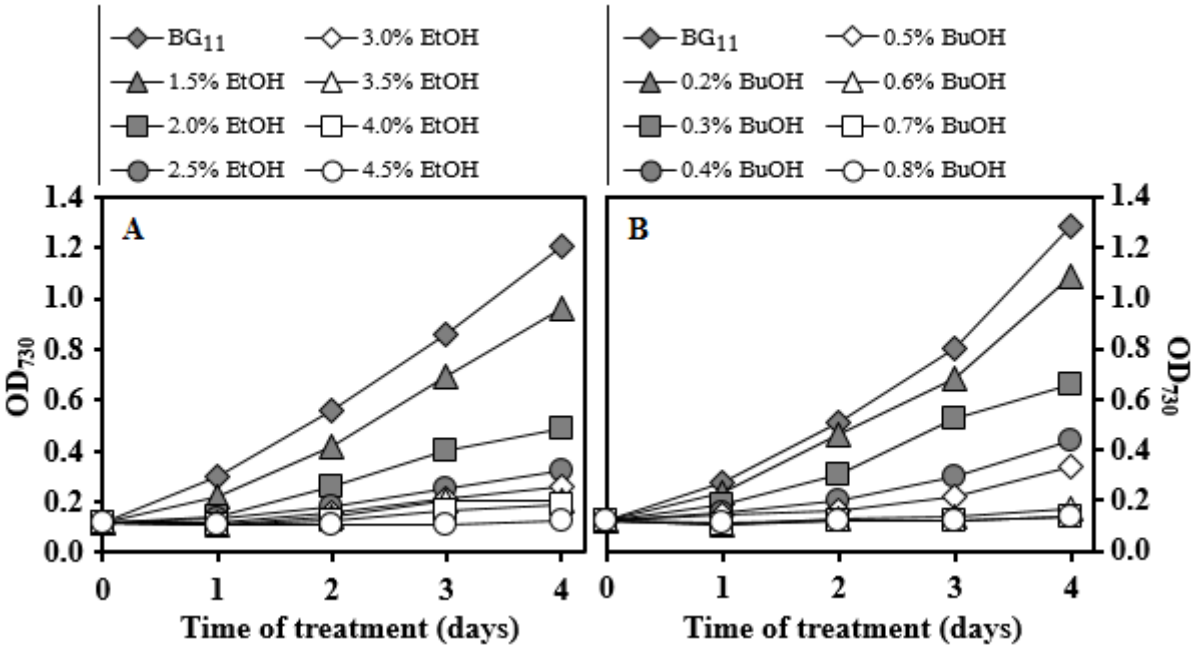

Figure 1. Growth curves of *Synechocystis* sp. PCC 6803 under various concentrations of ethanol (A) and *n*-butanol (B) treatments for long term-4 days.

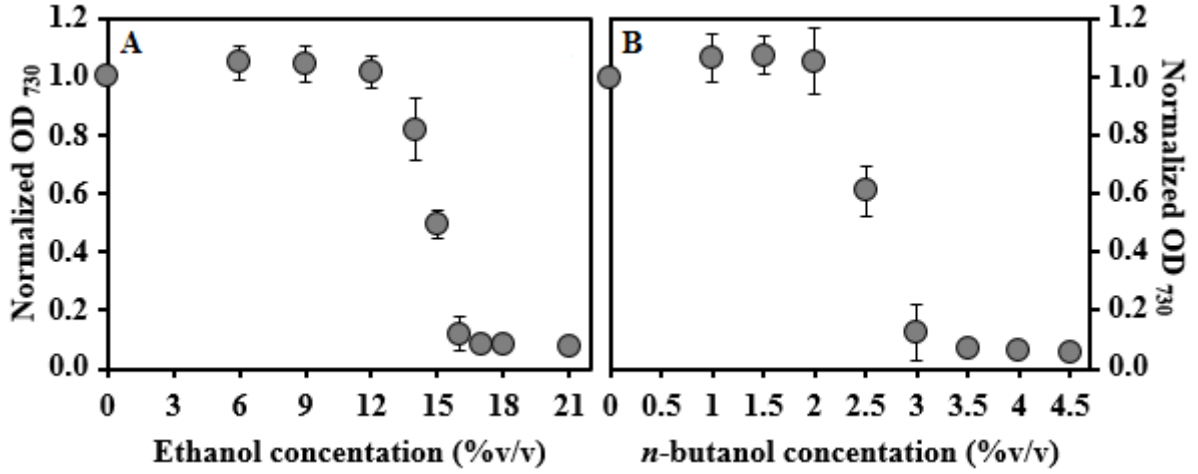

Figure 2. Growth recovery for 4 days of *Synechocystis* sp. PCC 6803 after various 1 h-alcohol treatments including ethanol (A) and *n*-butanol (B). Data represent mean ± S.D., (n=3).

Intracellular pigments and oxygen evolution rate under long term alcohol stress

In Figure 3A and B, there were interesting that alcohol treatments with 2% (v/v) EtOH and 0.3% (v/v) BuOH significantly induced higher accumulations of chlorophyll *a* and carotenoids than those under normal BG_{11} condition. However, oxygen evolution rates of both alcohol treated cells were decreased (Figure 3C). There was a previous report demonstrated that contents of phycocyanin and chlorophyll pigments were increasable by 4 g/L (or 0.4% (v/v)) of *n*-butanol for 1 h-treatment in cyanobacteria (Anfelt *et al*., 2013). On the other hand, 0.5 M (or 2.3 % (v/v)) of ethanol treatment could reduce cyanobacterial photosynthetic yield (Ruffing *et al*., 2014) which related to more explanation that the reduced oxygen evolution rate

indicated the reduction of PSII activity and cell growth (Campbell *et al*., 1998). Moreover, carotenoids protected cyanobacteria against photo-oxidative damage (Hirschberg and Chamovitz, 1995) by acting as antioxidants. Then, high carotenoid accumulation under oxidative stress suggested the mechanical adaptation to stress (Zuluaga *et al*., 2017).

Gene expression under alcohol treatments

Transcript levels (Figure 4) of genes related to antioxidant enzymes including *sodB* (or *slr1516*) encoding superoxide dismutase (SOD), *gpx1* (or *slr1171*) and *gpx2* (or *slr1992*) both encoding glutathione peroxidase (GPx) and *katG* (or *sll1987*) encoding catalase-peroxidase (KatG) were investigated at day 4-long term treatment under 2.0% (v/v) of ethanol (E+) and 0.3% (v/v) of *n*-butanol (B+). Results were shown in a fold change value when compared to that transcript band intensity under normal BG_{11} condition (Figure 4, left-hand side). Under ethanol (+E) stress, those four genes were up-regulated for approximately 1.2 - 1.3-fold increases of fold change whereas there were approximately 1.2 - 1.6-fold increases under *n*-butanol (B+) treatment. It was similar with the previous report, *sodB* and *gpx2* transcript levels were up-regulated by approximately 2-fold under 40 mg/L (or 0.49 % (w/v) of *n*-butanol treatment for 12 h. After constructing *sodB*-overexpressing *Synechocystis* strain, they found the increase on growth compared to wide type under the same condition (Anfelt *et al*., 2013). On the other hand, *Synechocystis* sp. PCC 6803 contains two types of glutathione peroxidase-like proteins (GPX-1 and GPX-2) from *gpx1* and *gpx2* transcripts that have different responses to stress conditions (Gaber *et al*., 2004). In our result, the *gpx2* transcript level was normally higher than that of *gpx1* (Figure 4, right-hand side). Both E+ and B+ treatments could induce both *gpx1* and *gpx2* transcript levels in equal proportion. Oxidative stress response was then addressed as one of crucial factors for alcohol tolerance mechanism of *Synechocystis* sp. PCC 6803 cells (Tian *et al*., 2013).

Effect of alcohol stress on SOD and GPx activities

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities were assayed at day 4 of long-term treatment under 2% (v/v) of ethanol (E+), 0.3% (v/v) of *n*-butanol (B+) compared with normal condition (BG_{11}) (Figure 5). Our results indicated that SOD activities under both E+ and B+ conditions were significantly decreased when compared with normal condition (Figure 5A and B). It was interesting that SOD activities were down-regulated which was not corresponded to up-regulated *sodB* transcript levels. We noted the different SOD enzyme expressions among other organisms, such as *Microcystis aeruginosa* faced oxidative stress by exposing to Ni metal showed no differences of SOD activity when compared with normal condition (Berenice *et al*., 2016). In plant *Jatropha curcas*, it was reported that mild drought stress did not affect SOD activity when compared with control plant while malondialdehyde (MDA) content was significantly higher than control plant (Arcoverde *et al*., 2011). Additionally, cassava leaves under oxidative stress had a significant decrease of SOD activity compared to that pre-treatment control whereas MDA content was significantly enhanced (Xu *et al*., 2013). In animal, rats fed with alcohol showed a significant decrease of SOD activity when compared with control group (George and Chaturvedi, 2016). On the other hand, the GPx activity in this study was not significantly changed (Figure 5B) although there were slight decreases observed under E+ and B+ treatments when compared to normal BG_{11} condition. It was explained in a recent report that GPx activity response to stresses including both GPX-1 and GPX-2 were also different, such as the increased activity was observed only in GPX-1 by high light treatment whereas the GPX-2 activity was not changed in *Synechocystis* sp. PCC 6803 (Gaber *et al*., 2004).

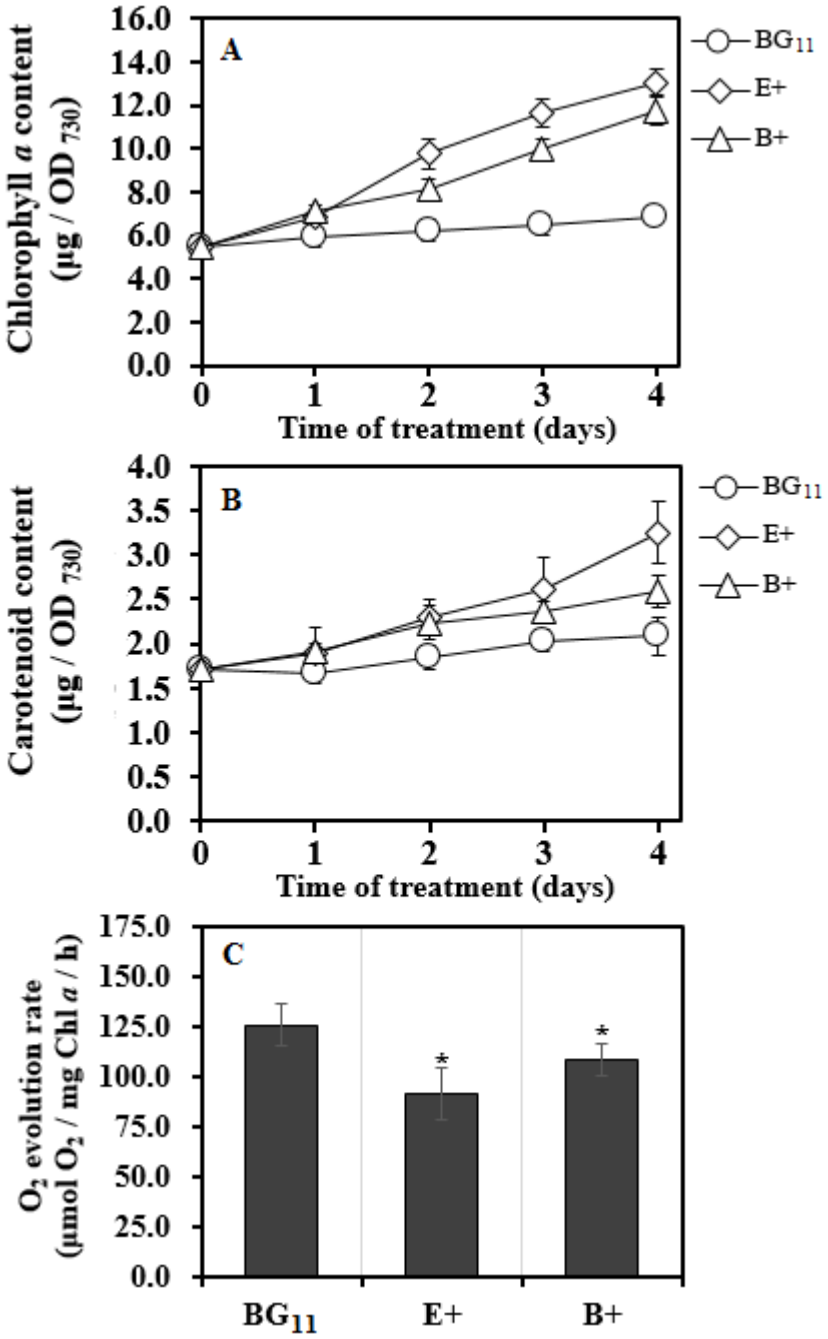

Figure 3. Pigment contents of chlorophyll *a* (A) and carotenoids (B) and oxygen evolution rate (C) at day 4 of long term culture of *Synechocystis* sp. PCC6803 under alcohol treatments including 2% (v/v) EtOH (opened diamond [E+]) and 0.3% (v/v) BuOH (opened triangle [B+]), compared to normal condition (opened circle). Data represent mean ± S.D., (n=3). *$p<0.05$ as a significant difference with value under BG_{11} control.

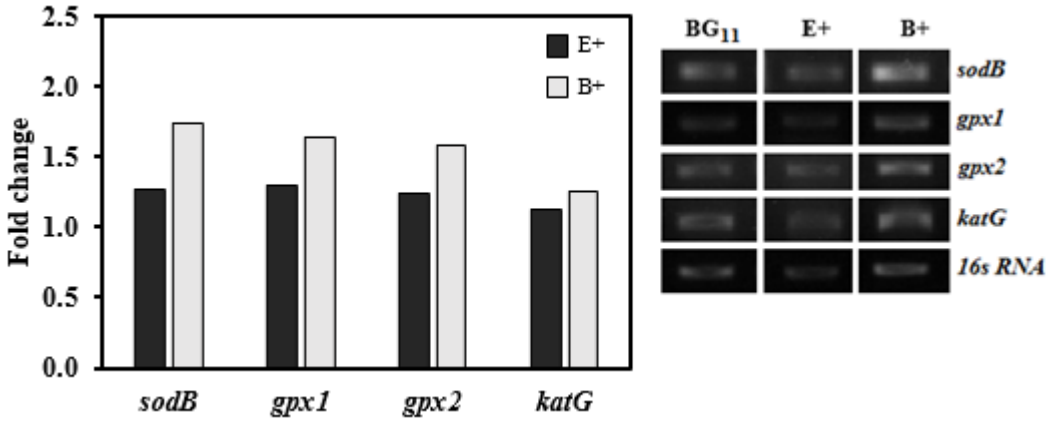

Figure 4. Fold change of transcript band intensity (left) under each treatment divided by that of normal BG_{11} condition and agarose gel electrophoresis (right) of RT-PCR products including *sodB*, *gpx1* and *gpx2* transcripts under alcohol treatments. Cells were treated with 2% (v/v) ethanol (E+) and 0.3% (v/v) *n*-butanol (B+) for 4 days of long-term cultivation.

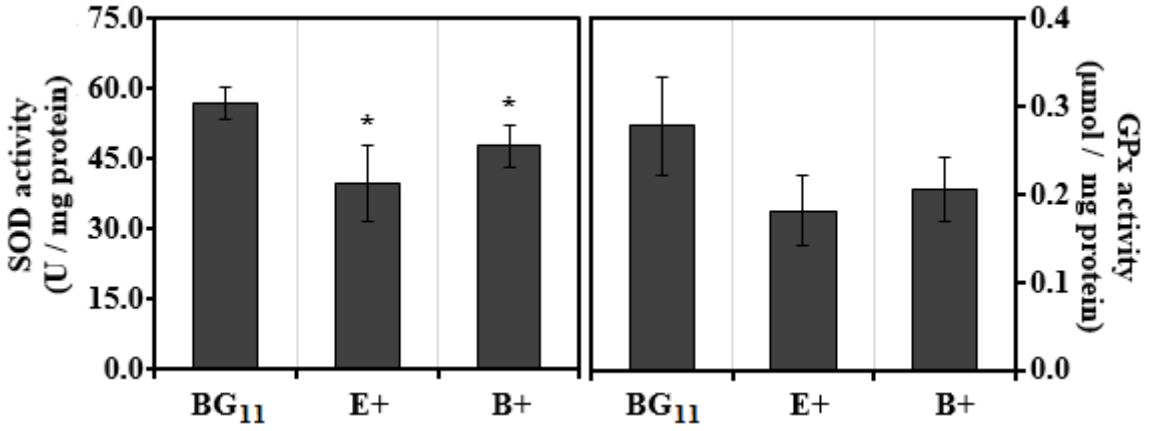

Figure 5. Enzymatic activities of SOD (A) and GPx (B) at day 4-long term treatment of *Synechocystis* sp. PCC 6803 under alcohol treatments including 2% (v/v) ethanol (E+) and 0.3% (v/v) *n*-butanol (B+). Data represent mean ± S.D., (n=3). *$p<0.05$ as significant difference when compared to that under normal BG_{11} condition.

Conclusion

Synechocystis sp. PCC 6803 had a tolerant adaptability to moderate toxicity of alcohol stress, under 2% of ethanol and 0.3% of *n*-butanol, by enhancing chlorophyll *a* and carotenoids although growth and oxygen evolution rate were reduced. Additionally, the amounts of gene transcripts related to the detoxification system consisted of *sodB*, *gpx1*, *gpx2* and *katG* were obviously induced under alcohol stresses. In the aspect of cell growth recovery, *Synechocystis* cells could be 4 days-recovered after treating with high concentrations of alcohols for 1 h, especially under 6 – 15% (v/v) of ethanol and 1 – 2.5 % (v/v) of *n*-butanol. Altogether, we obtained the basic knowledge data that useful for further improving the metabolic adaptability for alcohol tolerance in engineered cyanobacteria with alcohol tolerable efficiency.

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