Studies on Autolysis Cells Induced by Phosphate Deficiency of the Cyanobacterium *Synechocystis* sp. PCC 6803

November 2018

Ryo ASADA

Studies on Autolysis Cells Induced by Phosphate Deficiency of the Cyanobacterium *Synechocystis* sp. PCC 6803

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

(Doctoral Program in Integrative Environment and Biomass Sciences)

Ryo ASADA

Abbreviations

AP	alkaline phosphatase
BG-11+/−P	BG-11 medium with phosphate sufficient / deficient
Chl*a*	Chlorophyll *a*
CO_2e	CO_2 equivalent
d	day
E. coli	*Escherichia coli*
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LB	Luria-Bertani
lic	lysis inducible cells
OD_{730}	optical density at 730 nm
ORF	open reading flame
PaO	pheophorbide *a* oxygenase
PCC	strain number of Pasteur Culture Collection
PCR	polymerase chain reaction
PHB	polyhydroxybutyrate
PNP	*p*-nitrophenyl phosphate
S. enterica	*Salmonella enterica*
Synechocystis	*Synechocystis* sp. PCC 6803

Abstract

In the cultivation of microalgae for the production of useful compounds, cell disruption to extract the products of interest is a bottleneck and costly process. To establish a cost-effective method to recover these cellular compounds, I developed a method to induce cell lysis via phosphate deficiency in the cyanobacterium *Synechocystis* sp. PCC 6803. In this system, the promoter from the *phoA* gene for alkaline phosphatase regulates the expression of bacteriophage genes encoding the lytic enzymes holin and endolysin, and is induced under phosphate-deficient condition. I observed that 90% of the transformant *lic* (*l*ysis *i*nducible *c*ells) were lysed after 24 h of incubation under phosphate-deficient conditions. I also optimized cell culture conditions to induce cell lysis in highly concentrated cells for the efficient recovery of valuable cellular products and observed over 90% cell lysis after 16 h of incubation under these conditions. Interestingly, I found that the cell size and density of the transformant were larger than the control cells. And the precipitation efficiency was higher, which may be advantageous in cell harvesting process. In this lysis system, transition of growth phase to lysis phase occurs naturally with phosphate consumption by the cells, requiring no additional steps for inducing lysis gene expression. Thus, this inducible lysis system may contribute to decreased cell disruption costs in the algal biotechnology industry.

Keywords:

Cell disruption; Endolysin; Extraction; Holin; Phosphate sensor

General introduction

All vascular plants and algae use light energy to fix carbon dioxide by oxygenic photosynthesis and produce organic substances such as polysaccharides, lipids, proteins, hydrocarbons. Although these substances are used for cell proliferation, studies for using organic matter produced by photosynthesis to industrial use have been conducted (FAO Aquatic Biofuels Working Group. 2010).

Since cyanobacteria, which is a type of algae, produces hydrocarbons and lipids that can be converted to diesel oil, and also ethanol, studies toward industrial production have been vigorously undertaken (Eduardo et al. 2016). Also, the production of isobutanol, which is used for the synthesis of biodiesel, ethylene, which is the basic material of petrochemicals, and polyhydroxybutyrate (PHB), which is biodegradable and substitutes for petroleum-based plastics, has been studied (Matson and Atsumi 2018). In those substances of interest, one of the most demanded product is resource which can be alternative to petroleum resources. Many technologies are dependent on this, and as there are limitation on reserves, the shortage may cause many environmental and economic issues in present and the future.

Synechocystis sp. PCC 6803 (hereafter, *Synechocystis*) is a model cyanobacterium because of the availability of its genomic sequence (Kaneko et al. 1996), its high competence in genetic transformation (Grigorieva and Shestakov 1982), and its utilization of glucose as a carbon source (Williams 1988). Recently, it has been used for applied research widely as a platform for producing useful substances by modifying its metabolic systems genetically. For the goal of production of substances not just at the laboratory level but for industrial use, we need to pay attention not only to production but also to efficient "harvesting" of the products. Since most of the algae including cyanobacteria store the products in the cells, disruption of the cell wall is necessary to harvest the cellular products. The disruption

process is one of the most cost-inefficient bottlenecks in the algal biotechnology industry (Grima et al. 2003; Larena et al. 2004). Various cell disruption methods such as mechanical (e.g., high-speed agitator bead mills and high-pressure homogenizers), chemical (e.g., extraction via organic solvents), and other approaches (e.g., enzymatic cell lysis) have been developed (Gao et al. 2013; Günerken et al. 2015; Barry et al. 2016). However, the extraction process is still a dominant energy-consuming and greenhouse-gas-emitting process. For example, estimations of current energy costs for algal diesel production show that approximately 50% of the total energy required for production is used for the lipid extraction process (Yuan et al. 2015). The extraction process must be either improved or minimized to reduce energy consumption (Passell et al. 2013; Dassey et al. 2014).

An alternative extraction approach is a programmed cell lysis. In nature, cells of microorganisms infected by phage are lysed at the last step of phage domination to release the phage progeny. For example, *Salmonella enterica* phage synthesizes the key lysis protein and enzyme, holin and endolysin using the transcription/translation systems of the host cell (Wang et al. 2000). Holin produces nonselective pores on the plasma membrane, enabling the secretion of endolysin from the cytosol into the periplasmic space (Young 2002). Endolysin then degrades the linkages inside the peptidoglycan layer (Loessner 2005). Besides endolysin, a lysis-associated protein is also involved in cell lysis (Berry et al. 2008, 2012). As a result, the host cell is lysed because of the turgor pressure. By incorporating this phage–lysis system into cyanobacteria, and inducing cell lysis after the target products accumulate, the energy consumption for the extraction stage may be reduced.

I attempted to construct an inducible cell-lysis system which expresses the heterologous lysis enzyme regulated by the nutrient change by genetic modification to reduce the energy required to recover useful substances from cyanobacterial cells. First, in chapter I, I constructed and evaluated the inducible cell lysis system. Then in chapter II, I

characterized further the lysis-inducible cell based on cell size and density compared to wild-type cells.

Chapter I

Inducible Cell Lysis by Phosphate Deficiency

Introduction

The responses of *Synechocystis* cells to changes in environmental conditions have been well studied to advance the understanding of the ways in which photosynthesis acclimates to such changes—e.g., low temperature (Inaba et al. 2003) and high temperature (Slabas et al. 2006), high salinity and osmolality (Paithoonrangsarid et al. 2004), and a specific wavelength of light (Yeh et al. 1997). These environmental changes are perceived by a certain sensory protein, a histidine kinase, which is autophosphorylated under specific conditions and transfers the phosphate group to a cognate response regulator, the transcription factor, which modulates the transcription activity of certain genes in the *Synechocystis* cells (Mizuno et al. 1996). This two-component regulatory signaling pathway regulates the expression of artificially introduced genes under specific conditions (Stock et al. 2000).

In the present study, we developed a novel cell lysis system using the *phoA* promoter for alkaline phosphatase (AP), which is regulated by SphS–SphR, a two-component system that responds to phosphate deficiency (Suzuki et al. 2004). SphS–SphR regulates gene expression for the efficient uptake of inorganic phosphate, including extracellular nuclease, AP, and high-affinity phosphate transporters (Fig. 1) (Hirani et al. 2001). The promoter region of the *phoA* gene in *Escherichia coli* has been used in gene expression such as fusion to bioluminescence genes to develop bacterial sensor cells to assess phosphate bioavailability (Lübke et al. 1995; Dollard and Billard 2003). In this study, I applied the SphS-SphR-regulated promoter of the *sll0654* gene for AP in *Synechocystis* (Hirani et al. 2001; Suzuki et al. 2004) to regulate the target genes by phosphate unavailability, as far as I know this is the first application of this system to regulate expression of the heterologous gene in the *Synechocystis* cells.

In *Synechocystis*, the function of the lytic enzymes has been applied to several two-component systems. Previous studies have successfully induced cell lysis utilizing the regulatory systems that are regulated by different stimuli. The Ni^{2+}-regulated two-component system NrsS–NrsR (López-maury et al. 2002) induces the expression of genes for holin, endolysin, and lysis-associated protein from the *S. enterica* phage P22 (Liu and Curtiss III, 2009). Cell lysis is achieved by the addition of 50 μM Ni^{2+}, to induce the expression of the lysis genes. Liu et al. (2011) improved this lysis system using the *sbtA* promoter—which is induced under CO_2-limited conditions—for the induction of the lysis genes. Another approach that avoids nickel in the medium is the use of the green-light-responding, a two-component system CcaS–CcaR (Hirose et al. 2008) for inducing cell lysis (Miyake et al. 2014). Although these previous lysis strategies have been successfully applied, they require the following: (i) addition of a heavy metal ion, which increases environmental pollution; (ii) light irradiation of a specific wavelength, which does not allow the use of natural sunlight (the most economical light source); and (iii) complete exclusion of CO_2 by sealing the culture, which increases the cost of the algal cultivation process.

Another concern in mass algal cultivation is the application of lysis systems in concentrated algal cell cultures. The cell densities in algal cultures are comparatively lower than those of heterotrophic organisms, e.g., *E. coli* and *Saccharomyces cerevisiae*. This is due to the light required for algal growth and the increased effect of self-shading during cultivation. It is reported that intracellular products such as proteins, DNA, and phycocyanin are secreted into the medium after cell lysis (Liu and Curtiss III 2009). However, the secretion from cells at lower densities makes it difficult to recover the products because of high dilution in the medium. For a more efficient recovery of the products, cell lysis should be applied to concentrated cells.

In this study, I designed the lysis system to be induced under phosphate deficient condition. I fused the promoter of the *phoA* gene and the coding sequences of holin, endolysin, and a lysis-associated protein from *S. enterica* phage P22 and examined cell lysis under phosphate-deficient conditions. I hypothesized that the transcription of the synthetic operon is induced under the phosphate-deficient conditions. I, then, attempted to lyse the cells at a higher cell concentration to demonstrate the practical usefulness of the lysis-inducible strain for the efficient recovery of cell products.

Materials and Methods

2.1. Culture of cyanobacterial cells

A glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 (Williams 1988) was used as a wild-type strain. The cells of *Synechocystis* were cultured in BG-11 medium (Stanier et al. 1971) buffered with 20 mM HEPES–NaOH (pH 7.5). For phosphate-free BG-11, K_2HPO_4 was replaced with KCl. Strains were grown at 34°C under 70 µmol photons m^{-2} s^{-1} using incandescent lamps, with aeration of 1% (v/v) CO_2-enriched air, as previously described (Wada and Murata 1989).

2.2. Construction of cells expressing lysis genes

The genomic DNA of *S. enterica* phage P22 was obtained from National Institute of Technology and Evaluation, NITE Biological Resource Center, Japan, and used to obtain the lytic genes DNA fragments, including the coding regions of the lytic enzymes holin (ORF13), endolysin (ORF19), and lysis-associated protein (ORF15), were amplified via polymerase chain reaction (PCR) using primers 13-F and 15-Kan-R (Table 1). In addition, DNA fragments corresponding to the kanamycin-resistance gene cassette, EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit (Epicentre, Madison, WI), and approximately 1000 bp fragments upstream and downstream of the *phoA* gene of *Synechocystis* were amplified using primer sets Kan-F and Kan-R, phoAup-F and phoAup-13-R, and Kan-phoAdown-F and phoAdown-R, respectively. The resulting PCR products had 15 bp overlapping sequences and were adhered via overlap-extension PCR (Ling and Robinson 1997). The DNA fragment thus generated was introduced into pGEM™-T Easy Vector (Promega,

Madison, WI), and the resulting plasmid was introduced into competent *E. coli* JM109 cells (TaKaRa Bio, Kusatsu, Japan) using heat shock. The transformed *E. coli* were selected on agar-solidified LB medium containing 50 μg mL^{-1} sodium ampicillin and kanamycin sulfate. The DNA sequence of upstream and downstream of *phoA* gene, and the lysis genes was confirmed using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) (Fig. 2).

The synthetic operon including the lysis gene cassette was introduced into the chromosome of *Synechocystis* by double homologous recombination based on a method partially modified from that reported by Williams (1988). Wild-type cells were cultured in BG-11 medium (Stanier et al. 1971) until the logarithmic growth phase, and the collected cells were resuspended in fresh BG-11 medium. The 100 μL of the culture was mixed with the 1 μg of the plasmid, and then incubated overnight with shaking at 30°C under 20 μmol photons m^{-2} s^{-1}. The cell suspension was spread onto agar-solidified BG-11 medium supplemented with 5 μg mL^{-1} of kanamycin sulfate. Then, kanamycin-resistant colonies were obtained and transferred to BG-11 medium supplemented with 25 μg mL^{-1} kanamycin sulfate. The transferred cells were named *l*ysis *i*nducible *c*ells, hereafter referred to as *lic* cells (Fig. 3A). As a negative control, a *phoA*-deletion strain (Δ*phoA*), in which the coding sequence of the *phoA* gene was replaced with the kanamycin-resistance gene, was also prepared.

2.3. Induction of cell lysis

To evaluate the lysis response of the strains cultured under the phosphate-deficient conditions, the precultured cells were washed thrice with phosphate-free BG-11 (BG-11 (−P)) and used to inoculate fresh BG-11 (−P), with phosphate-sufficient BG-11 (BG-11 (+P), 0.18 mM K_2HPO_4) as a control. The cell density was adjusted to a final optical density of

0.1 at 730 nm (OD_{730}), and the cells were cultured for 3 d under 70 µmol photons m^{-2} s^{-1} at 34°C and aeration with 1% (v/v) CO_2-enriched air.

To evaluate cell lysis under high-cell-density culture conditions, I utilized two different strategies. Firstly, the wild-type and *lic* cells cultivated under phosphate-sufficient conditions were concentrated to an OD_{730} between 4.2 and 4.4 and suspended in the same medium. The phosphate in the medium was subsequently consumed by the growing cells. Secondly, I prepared both strains in the culture and allowed them to grow until they consumed the phosphate in the medium, and then concentrated the cells to the same OD_{730}. The cultures were incubated under 300 µmol photons m^{-2} s^{-1} at 34°C and aeration with 1% (v/v) CO_2-enriched air. Microscopic observation after staining with SYTOX Green (Life Technologies Japan, Tokyo, Japan) was used for the detection of cell lysis, and AP activity in the wild-type cells was measured for evaluation of the response to the phosphate-deficient conditions.

2.4. Evaluation of cell lysis

Cell turbidity was measured by OD_{730}. Chlorophyll *a* (Chl*a*) was extracted with 90% methanol, and the concentration was calculated from the absorbance of the supernatant at 665 nm (Tandeau de Marsac and Houmard 1988). An ultraviolet-visible spectrophotometer, UV-1700 Pharma Spec (Shimadzu, Kyoto, Japan), was used for the spectrum measurements.

2.5. Other analysis methods

A fluorescent microscopic observation was applied to count the live and dead cells after mixing with SYTOX Green, which stains the nucleic acids of only the dead cells, whose

plasma membrane were disordered. Image processing and analyzing software (Image J, National Institutes of Health, Bethesda, MD) was used for counting the cell numbers.

The phosphate concentration in the supernatant of the culture medium was measured using the molybdenum blue method (Holman 1943). Briefly, 1 mL supernatant was mixed with 20 µL acid molybdate solution (2.5% ammonium molybdate, 0.1% potassium antimonyl tartrate sesquihydrate, and 3.15 M H_2SO_4), and 20 µL of acid ascorbate solution (10% ascorbic acid and 2.25 M H_2SO_4) was added. Then, the mixture was incubated at room temperature for 15 min, and the absorbance at 883 nm of the sample was measured.

The protein concentrations in the culture supernatant were measured using a solution of bovine serum albumin as a standard by DC Protein Assay Kit (Bio-Rad, Hercules, CA), which uses a colorimetric assay based on the Lowry method.

AP activity was assayed by observing the degradation rate of *p*-nitrophenyl phosphate to *p*-nitrophenol (Aiba et al. 1993) and normalized by the cellular concentration of Chl*a*.

Results and Discussion

3.1. Confirmation of gene insertion in the transformants

The genomic insertions including the native *phoA* gene, lysis gene operon, and, kanamycin-resistance gene, were confirmed via PCR using the primer pairs phoAup-F and phoAdown-R (Table 1). A 6.0 kb fragment, a 4.0 kb fragment, and a 2.8 kb were obtained when the genomic DNAs from the wild-type, *lic* strain, and Δ*phoA* respectively, were used as templates of PCR (Fig. 3B).

3.2. Growth and cell lysis in lysis-gene-introduced cells

To investigate the cell response to the BG-11 (+P) and BG-11 (−P) cultures, I measured OD_{730} of the cell culture (Fig. 4A). In the BG-11 (+P) culture, the cells of the wild-type, Δ*phoA*, and *lic* strains reached an OD_{730} of 6.2 ± 0.07, 6.7 ± 0.09, and 6.9 ± 0.16, respectively, after 3 d. The maximum growth rates (d^{-1}) of the wild-type, Δ*phoA*, and *lic* strains in the BG-11 (+P) medium were 2.3 ± 0.01, 2.0 ± 0.05, and 2.2 ± 0.05, respectively. Because the *lic* cells showed similar growth rates and Chl*a* contents under BG-11 (+P) culture conditions (Fig. 4B), I concluded that the cell viability is nearly equal between the three strains.

In the BG-11 (−P) culture, considerable growth inhibition was observed (Fig. 4A). The cells of wild-type, Δ*phoA*, and the *lic* strains reached an OD_{730} of 1.7 ± 0.07, 1.8 ± 0.09, and 1.7 ± 0.16, respectively, after 3 d, and the maximum growth rates (d^{-1}) of the strains were 1.8 ± 0.06, 1.6 ± 0.08, and 1.6 ± 0.05, respectively. Chl*a* contents in the cells also showed trends similar to that of the growth inhibition (Fig. 4B). Phosphate starvation is

widely known to be a major limiting factor for cell growth in cyanobacteria (Schindler 1977), and the growth rates of all strains monitored via optical density were similarly reduced in BG-11 (−P). AP activity in the wild-type cells was induced when cultivated in BG-11 (−P) (Fig. 4C), indicating retardation of growth due to the limitation of phosphate availability.

To evaluate cell lysis, the wild-type, Δ*phoA*, and *lic* cells were grown in BG-11 (+P) medium and then transferred into BG-11 (−P) medium when the OD_{730} reached 0.1 (Fig. 4F). Dead cells were distinguished under fluorescence microscopy after staining with SYTOX Green, a nucleic-acid-staining reagent that does not readily penetrate the intact cell membranes (Fig. 5). Notably, under phosphate deficient condition, after 1 d of cultivation, many of the *lic* cells exhibited fluorescence, possibly caused by the activity of lysis enzymes damaging the peptidoglycan layers and plasma membranes of the *lic* cells. A count of the dead (stained) cells indicated that 90% of the *lic* cells were lysed after 1 d of cultivation under the BG-11 (−P) culture, whereas the extent of staining in wild-type cells and Δ*phoA* cells was negligible (Figs. 4D and 5). In the phosphate sufficient culture, the cell lysis activity was not observed in any strains (Fig. 4E). In a previous study, Miyake et al. (2014) developed cells in which lysis was induced by irradiation with green light (520 nm) and observed that 40% of the cells were lysed after 64 h of irradiation. In addition, Liu and Curtiss (2009) produced cells induced via the administration of Ni^{2+} in the medium and observed complete cell lysis in half a day following Ni^{2+} administration. My result of more than 90% lysis in 1 d was similar to the results of these studies. However, this lysis system utilizes natural sunlight for cultivation and does not require any toxic heavy metal ions, which may cause environmental contamination.

After 1 d under phosphate-deficient conditions, the number of viable *lic* cells recovered in the succeeding days. There is a possibility that phosphate and/or phosphorus compounds released from the damaged or lysed cells may have been utilized by the surviving

cells as phosphorus sources, which could have supported the growth of viable cells. In addition, the release of phosphorus compounds into the medium from the cells that undergo earlier lysis may repress the expression of the lysis genes from the *phoA* promoter. Thus, not all the cells were lysed in this system, but the recovered cells may be utilized in subsequent cultivations. The total phosphate concentration was negligible in the medium after removal of the *lic* cells (Fig. 4D), the phosphorous compounds released from the dead cells might immediately recover by the surviving cells. Meanwhile, several types of active transposons are reported in the chromosome of *Synechocystis* and the harmful DNA regions are inactivated by the insertion of these transposons (Okamoto et al. 1999). This result indicated that the resume of the cell growth in Fig. 4F might be the recovery of the phosphate by the surviving cells.

The *lic* cells cultured under phosphate-deficient conditions exhibited a considerable increase in proteins in the culture medium supernatant (Fig. 4G). Although the cell lysis was clearly induced in the first 1 d (Fig. 4F), the protein concentrations gradually increased for the first 3 d of cultivation. Referring to the results of microscopic observation, some cells stained with SYTOX Green also exhibited red fluorescence from Chl*a*, which decreased as time passed (Fig. 6). These results suggest that the intracellular compounds were gradually released following cell lysis. Liu and Curtiss (2009) also noted that the timing of the leakage of cell content after cell lysis differed between pigment, DNA, and proteins. I observed minimal SYTOX-Green-stained cells from the cultures of wild-type and Δ*phoA* cells, indicating that the cell walls and plasma membranes were not damaged by the phosphate deficiency in these strains (Fig. 5).

Almost no cells of the all strains were stained with SYTOX Green during the 3 d culture period when cultured in BG-11 (+P) medium (Fig. 4E), suggesting that the expression of the lysis genes was completely suppressed in the *lic* cells, and their viability

was similar to those of the wild-type and Δ*phoA* cells. Under phosphate-deficient conditions, the expression levels of the *phoA* gene, which was replaced by lysis genes in this study, were approximately 70-fold higher than under phosphate-sufficient conditions (Suzuki et al. 2004). Because the *phoA* promoter induces gene expression only during phosphate deficiency, expression of the lytic genes is precisely regulated by the absence of phosphate. Thus, an inducible native promoter such as the *phoA* promoter may be used for precise switching of gene expression.

3.3. Lysis of concentrated cells

As compared with cultures of heterotrophic microorganisms, cell concentrations in microalgal cultures are much lower because the growth of the microalgal cells requires light, which is frequently limited as a result of self-shading. Thus, the concentration of the products is comparatively low in the microalgal cultures. To address this, the algal cells should be concentrated before the induction of lysis to avoid dilution of the cellular products into the culture medium. Also, exchanging the culture medium to remove phosphate and induction of the expression of the lysis genes should be avoided, so as not to add extra processes and energy costs. In the present study, I attempted to achieve cell lysis via the induction of phosphate deficiency at a higher cell concentration without the exchange of medium.

The timing of the cell concentration (before or after phosphate consumption) severely affected the efficiency of cell lysis. As shown in Fig. 7B and 7D, I concentrated the cells in culture to adjust the OD_{730} to approximately 4 and allowed the cells to consume the remaining phosphate in the medium (~45 μM phosphate). Phosphate in the medium was almost completely removed in 3 h in both the wild-type and *lic* cultures, as shown in Fig. 7B and 7D, respectively. The rates of phosphate uptake were 2.7 ± 0.1 and 2.8 ± 0.2 μmol

OD_{730}^{-1} h^{-1} in the wild-type and *lic* cells, respectively. Although I speculated that lysis of the *lic* cells was induced after the consumption of phosphate in the media, considerable numbers of dead cells, which were stained by SYTOX Green, did not appear until day 4 and reached only 29% of the total cells after 5 d. To estimate the induction of *phoA* promoter activity, I assayed the AP activity of wild-type cells; the AP activity was detected at 3 μmol PNP mg^{-1} Chl*a* h^{-1} on 2 d, and these levels were maintained until 5 d (Fig. 7B). In the case of cell lysis induction shown in Fig. 4C, the wild-type cells in the exponential growth phase showed an induced AP activity of over 17 μmol PNP mg^{-1} Chl*a* h^{-1} when cultivated under phosphate-deficient conditions for 1 d, and a large number of the *lic* cells were lysed during this incubation (Fig. 4F). Thus, I concluded that this way of induction was unable to fully induce the *phoA* promoter. In this case, the light inhibition by high cell concentration may be the cause of low level induction of the lysis. The *lic* culture reaching OD 4.0 consumed all phosphate, but the cell lysis was not induced (Fig. 4D and 4E). Previous studies also reported that light irradiation is necessary for cell lysis, and lysis efficiency is decreased by the high concentration of the culture (Liu et al. 2011), and the regulation of gene expression in the cyanobacterial cells are known to stimulate under the properly illuminated conditions (Mironov et al. 2012). It is also reported that the induction of AP activity under the phosphate-deficient environment is lowered in cells of the cyanobacterium *Anabaena oryzae* incubated under the dark condition (Singh and Tiwari 2000). Thus, I speculated that, in addition to phosphate deficiency, light irradiation may be necessary to induce cell lysis.

For the second attempt, I concentrated the cells after they had consumed the phosphate from the media. I cultured the wild-type and *lic* cells in phosphate-sufficient media until the phosphate was completely consumed (Fig. 7A and 7C). The rates of phosphate uptake were 5.8 and 4.0 μmol OD_{730}^{-1} h^{-1} for the wild-type and *lic* cells, respectively. These results indicate that the cells before concentration possessed higher

phosphate uptake activities than those after concentration, as shown in Fig. 7B and 7D. The cells were then further cultured for 5 h after all the phosphate had been eliminated from the media to allow the induction of lysis genes from the *phoA* promoter; in the previous study, the induction of *phoA* gene expression was initiated after 1 h and reached its maximum level after 4–8 h of incubation in phosphate-deficient medium (Suzuki et al. 2004). During 5 h of incubation under phosphate-deficient conditions, the cells may induce the expression of lysis genes from the *phoA* promoter. Then, I collected the cells of the wild-type and the *lic* cultures by centrifugation and resuspended the precipitated cells in fresh media to an OD_{730} of approximately 4.0 (Fig. 7A and 7C). The lysis rate of the concentrated *lic* cells, which were stained with SYTOX Green, rapidly increased and reached over 90% after 16 h of incubation following the concentration of the cells (Fig. 7C), whereas only 2% of the wild-type cells were stained by SYTOX Green under the same culture conditions. When I concentrated the cells cultivated for 5 h following phosphate consumption, the wild-type cells exhibited 5.2 μmol PNP mg^{-1} Chl*a* h^{-1} of AP activity, and this activity steeply increased up to 16.8 μmol PNP mg^{-1} Chl*a* h^{-1} until 16 h after the cell concentration (Fig. 7A). The increase in AP activity in the wild-type cell culture and the increase in the percentage of lysed cells in the *lic* cell culture were very well correlated (Fig. 7A and 7C); the AP activity and the lysed cells were much higher with this method than with the former method (Fig. 7B and 7D). I speculate that the concentration of cells before the complete consumption of phosphate in the media suppressed the induction of AP activity due to inefficient irradiation of the cells. Thus, AP activity in the wild-type cultures and cell lysis in the *lic* cultures were highly induced when the cells were incubated for 5 h after the complete consumption of phosphate and then concentrated.

I demonstrated that proteins accumulated in the culture medium of the *lic* cells following phosphate deficiency (Fig. 4G). When I left the concentrated cultures without

mixing after the experiment, the color of the *lic* culture supernatants differed from that of the wild-type culture (Fig. 8). As the *lic* cells sedimented, unidentified brown components appeared near the surface of the culture, and a blue component was observed near the precipitated cells. The brown and blue compounds may be derivatives of chlorophyll and phycocyanin, respectively. In *Synechocystis*, chlorophyllide and pheophorbide are identified as intermediates of chlorophyll degradation (Vavilin et al. 2005), and *slr1747* in *Synechocystis* is considered a putative pheophorbide *a* oxygenase (PaO) (Gray et al. 2004). Because PaO degrades pheophorbide to a red chlorophyll catabolite, the brownish color may be the degraded chlorophyll. An antenna complex phycobilisome, including phycocyanin, is a high-molecular-weight proteinous complex in cyanobacterial cells; hence, its leakage may require more time than the small-molecular-weight derivatives of chlorophyll.

This cell lysis system, induced by the deficiency of an essential nutrient, is a relatively simple and applicable system in the culture of photoautotrophic organisms. In order to operate this lysis system successfully, the amount of phosphate in the medium, which is enough to increase biomass and produce target compounds, should be precisely adjusted, because the switching of the phosphate-sufficient stage to starvation stage is one of the key factors. The phosphate deficiency occurs as the cells grow and consume nutrients; therefore, the addition of inducers is not required as in previous studies (Liu and Curtiss III 2009; Miyake et al. 2014). For cell lysis in the concentrated culture, preinduced expression of the lysis genes under the phosphate-deficient conditions prior to cell concentration is of great importance. Although the cell lysis induced by CO_2 limitation also does not require inducers (Liu et al. 2011), this system requires complete exclusion of CO_2 from the culture vessel to induce the cell lysis, making it unsuitable for large-scale cultivation.

Conclusion

In the present study, I constructed a cell lysis system in *Synechocystis* induced by phosphate deficiency. The efficiency of cell lysis in this system is comparable to previous studies on cyanobacterial lysis systems and does not require any external inducer, because the system is induced by deficiency or consumption of the nutrients in the medium. In addition, preinduction of the lytic gene achieves a high rate of lysis under high-cell-density culture conditions. These results may contribute to efficient cell lysis and energy cost savings in the algal industrial scene.

Chapter II

Simulation of Application of the *lic* Cells for the Large-Scale Cultivation and its Unique Phenotype for the Application

Introduction

Many material productions use autotrophic organisms which can use sunlight, as a natural energy source. Especially, using algae which require less cultivation area compared to the land plants, because they can be cultured stereoscopically.

The production of algal compounds requires energy consumption for the cultivation, collection, extraction, and purification. And the extraction process, cell disruption, is necessary because most of the algae accumulate the cellular products inside the cells. This process is the cause of the cost increase, because recent technologies are still dependent on mechanical or chemical extraction (Gao et al. 2013; Günerken et al. 2015; Barry et al. 2016). To reduce the energy costs, I constructed auto cell lysis system in cyanobacterial genome which can be induced arbitrary, as I described in the chapter I. The cell lysis is induced by the lack of a nutrient in the growth medium; phosphoric acid, and the lysis automatically starts by phosphate consumption by the cell growth.

In chapter I, my main objective was to develop the *l*ysis-*i*nducible-*c*ells (*lic*) which were evaluated for their growth and lysis ability. The lysis system which was constructed in this study does not affect cell growth during the biomass production stage under the phosphate-sufficient conditions, because the *lic* cells proliferated as well as the wild-type cells in the BG-11 (+P) culture medium. And I developed a system for the induction of the cell lysis in the concentrated culture. The concentration of the culture is very important to prevent the dilution of the product into the medium. Generally, in the laboratory scale, for the concentration of the cells, the centrifugation is applied, but this process is a very energy consuming process. Because the application of the centrifugation for concentrating the cell form the culture costs very high amount of energy (Passell et al. 2013; Dassey et al. 2014), it is better to concentrate the cells by natural precipitation. To achieve the rapid natural

precipitation, the cells should be large and have high density. In this chapter, I investigated the characteristics of the *lic* cells from the view of these points.

Materials and Methods

2.1. Culture of cyanobacterial cells

The cells of *Synechocystis* — wild-type, Δ*phoA*, and the *lic* cells were cultured in BG-11 medium (Stanier et al. 1971) buffered with 20 mM HEPES–NaOH (pH 7.5). Strains were grown at 34°C under 70 µmol photons m^{-2} s^{-1} using incandescent lamps, with aeration of 1% (v/v) CO_2-enriched air (Wada and Murata 1989). Cell growth was measured with the optical density at 730 nm (OD_{730}). Kanamycin sulfate as a selective antibiotic for mutants was not added to cultures for avoiding any sub-effect on the cells. To observe the individual difference of the cells, Δ*phoA* and the *lic* cells were prepared from independent colonies named as Δ*phoA* 3-1-2, 3-2-1, 3-2-2, and *lic* 1-1, 1-2, 1-3, 1-4, 1-5, 5-1, 5-2, 5-3, 5-4, 5-5, respectively. Unless otherwise specified, wild-type, Δ*phoA* 3-1-2, and *lic* 1-1 were selected for a series of experiments.

2.2. Cell size

Cell size was measured with particle counting analyzer CDA-1000 (Sysmex, Kobe, Japan), and the average cell size was calculated from more than 3000 cells.

2.3. Morphological cell observation

Bright field microscopy was applied to observe the morphology of the cells in the 1 d cultures with the phosphate-sufficient medium. Also, determination of dead cells was observed by fluorescence microscopy using SYTOX Green.

2.4. Cell density

First, several solutions of polyethylene glycerol (PEG) (average molecular weight: 20000 ± 5000) (FUJIFILM Wako Pure Chemical, Osaka, Japan) were prepared with densities of 0.9955 to 1.1021 g cm^{-3}. Then, cells were suspended in these PEG solutions, and centrifuged under 5000 g × 1 min. Then, OD_{730} of the supernatants was measured. To determine the density of the cells, I plotted OD_{730} versus density to show at which point is the cell lighter than the density of the PEG solutions.

2.5. Precipitation efficiency

The strains grown 1 d under phosphate-sufficient medium with OD_{730} relatively equal to 1.0 were centrifuged at different speeds (100 to 500 × g × 1 min). Then OD_{730} of the supernatants was measured. To evaluate cell precipitation rate, I plotted OD_{730} versus centrifugal speed to observe at which speed does the cells show different precipitation efficiency.

Results and Discussion

3.1. Simulation of application of the lic *cells to the practical scale culture*

The advantage of the *lic* cells is that they may be used for large-scale designed cultivation in the algal industry (Fig. 9). The *lic* cell is grown under phosphate-sufficient medium, and after it consumed phosphate completely and inducing cell lysis, the cell is concentrated. In this cultivation stage, adjusting the amount of phosphate for appropriate timing for cell lysis is important to induce efficient cell lysis. Phosphate must be consumed before the cell density reached high because light irradiation should not inhibit the induction of lysis genes. The cells are naturally precipitated, and the supernatant is transferred into the next culture tank, and then the cells in the concentrated culture are lysed. After the cell lysis in concentrated culture, the substrates of interest are extracted, and the survived cells, which is around 10 %, can be used to another culture. Also, nutrients especially phosphate is resupplied to the supernatant of the culture and reused for another culture.

3.2. Cell size and density

The trends of cell size of the wild-type, Δ*phoA*, and the *lic* cells were almost constant throughout the culture period (Fig. 10A). It is suggested that the size of the cell is possibly an innate characteristic, independent of growth phase. In this experiment, the culture of each strain was started at OD_{730} of 0.2, and the cell number in the *lic* cells at this point was relatively lesser than control cells (Fig. 10B). This discrepancy may be the larger cell size of the *lic* cells. Indeed, observations of the bright field microscopy revealed larger cell size of the *lic* cells (Fig. 11B). The size of the *lic* cell may not be related to the lack of coding region

of alkaline phosphatase, because there was no difference in the cell size between wild-type and Δ*phoA*. Only the *lic* strain has the insertion of lysis genes, and it may affect the cell construction especially cell wall structure because it can degrade the peptide glycan layer in the cell wall. However, the *lic* cells cultured with the phosphate-sufficient medium did not express the lysis genes and I did not observe lysed cells in the culture. To further investigate the mechanism for the cell enlargement, I should delete the genes in the lysis operon one by one.

The density of each PEG solutions was observed (Fig. 12A). A mixture of each culture and the PEG solution was centrifuged with a series of gravity and investigated the density of the cell (Fig. 12B). The density of wild-type and Δ*phoA* were 0.9965 to 0.9974 g cm^{-3} and, the *lic* cells were 0.9979 to 0.9985 g cm^{-3}.

3.3. Precipitation efficiency

When centrifugation was applied to each culture, the efficient precipitation was observed in the *lic* cells. Especially, when 200 × *g* × 1 min of centrifugation was applied, the rate of non-precipitated cell, cells remaining in the media, of the *lic* strain was 47%, while other strain showed 76 to 77 % (Fig. 13). This may be due to the higher density and larger cell size of the *lic* cells.

In the *lic* cells, the *phoA* region was replaced with lysis genes — holin, endolysin, and lysis associated protein, and the kanamycin-resistance gene. The expression of the lysis gene is suppressed under the phosphate-sufficient condition, and the *phoA* promoter is only activated under phosphate-deficient (Suzuki et al. 2004). Still, there is a possibility that the gene expression was leaky, and it affected the structure of the cell wall, resulting in enlargement of the cell. However, there are no previous reports to support this hypothesis.

If there is lysis genes expression, still the expression level may not be enough to lyse the *lic* cells, because I observed almost no cell was lysed in the enlarged cells (Fig. 11C) The insertion of the kanamycin-resistance gene did not affect the cell size, because cell size and density of the Δ*phoA* was similar with wild-type.

For further analysis, gene expression of the lysis genes under phosphate-sufficient condition may clarify to classify the cause of the cell enlargement. Also, it is reported that several genes' expression is related to cell size (Marbouty et al. 2009), and it is recommended to measure the expression level of these genes. In addition, morphological observation by electron microscopy may reveal the difference of the structures of cells.

Conclusion

Interestingly, I observed that the cell size and the density of the *lic* cells were larger than wild-type and Δ*phoA*. The growth rate of the *lic* cells was not inferior to the control cells. In addition, the sedimentation efficiency of the *lic* cells was higher than the control cells.

In the cell concentration process of algal material production, the *lic* cells' characteristic of fast precipitation or sedimentation are desirable traits for lesser energy operation of the cell collection. Also, this precipitation is preferable to be done with natural precipitation, not to add energy cost by centrifugation. Hopefully, I will also be able to determine the underlying causes of these differences through future experiments such as transcriptome analysis and electron microscopy viewing of the cell walls.

General Discussion

In this study, I successfully constructed the auto lysis system induced by phosphate deficiency in *Synechcystis*. The lysis induction was accurately switchable with the lack of phosphate. Also, cell lysis at concentrated culture, which is preferable for the efficient material collection after cell lysis, was performed. The key to efficient lysis is the timing of the induction with enough light irradiated to the cells. Thus, lysis induction should be conducted before cell concentration.

Other advantages of the *lic* cells were also observed. The size and density of the *lic* cells were larger than the control cells, and it required less gravity for the cell sedimentation. The characteristic of the *lic* cells such as efficient sedimentation which may be derived from its cell size is advantageous in the recovery process in algal material production. Noteworthy, even with the larger size of the cell, the growth ability was similar to the control cells. Still, the mechanism of the cell enlargement needs further research.

To address the cost-competitive problem in algal material production especially algal biofuels, there are several challenges to maximize the lysis system for mass cultivation. First, the timing of the cell lysis induction may be crucial for the practical application of the *lic* cell. It can be easy to control the phosphate amount in laboratory scale culture. But in large scale culture, the stirring of the culture is not enough, it may lead to localized lysis. The lysis efficiency depends on the cell concentration and the timing of phosphate consumption. Thus, the adjustment of phosphate amount in the medium, and the cell concentration should be controlled. Also, the culture condition for efficient lysis should be optimized. If natural sunlight is applied for the culture, it can induce cell lysis at day time, but not night. Because light may be necessary for lysis induction, the lysis stage should be conducted under light illumination.

This lysis system is possible to apply in other gram-negative bacteria which possess peptidoglycan layer, such as *E. coli* or *Pseudomonas* species. Also, if the introduction of the lysis gene leads to the enlargement of the cell size as seen in this study, it can contribute to reduce the energy requirement for cell collection.

The water requirement for algal culture should be reduced for sustainable production, and recycling of water contributes not only to reduce the water footprint but to also minimize the energy requirement for the cultivation if reuse of the remaining nutrients in the medium is enabled (Farooq et al. 2015). Thus, the culture strategy based on this lysis system may contribute to saving resources and energy. This system can be fit to the life cycle assessment presented by Yuan et al. (2015) which incorporated the recycling and recovering the growth media and biomass residue. In their best scenario, energy requirements and greenhouse gas emission for producing 1 MJ of algal biodiesel were 1.02 MJ and 71 g CO_2e (CO_2 equivalent), respectively. In this scenario, bioflocculation and dissolved air flotation enable 90% harvesting efficiency. The characteristic of the *lic* cell which is higher efficiency of precipitation may improve this efficiency. Also, oil extraction efficiency from 1 kg wet biomass using hexane was 73.6 %, and this efficiency can be enhanced by applying cell lysis system which can make cell walls fragile. In addition, the energy requirements of cell disruption for hexane extraction (0.59 MJ kg^{-1} biomass) can be reduced.

At last, the material production using algae including oil, chemical compounds, and foods is very promising. In addition to the variety of the products, the production is carbon neutral. The blueprint of Sustainable Development Goals for 2030 by United Nations has 17 goals such as affordable and clean energy, climate action, and zero hunger. I believe that algal biotechnology has the capacity to contribute solutions for these goals. Still, there are problems to solve for the practical use of algae in industrial scale, we researchers should not stop in taking on the challenge enthusiastically.

Tables and Figures

Table 1. Primers used for constructing *lic* and Δ*phoA* strains.

Primer name	Sequences (5´ to 3´)
Construction of *lic* strain	
phoAup-F	GATCTACTAGCTTCTGCCAG
phoAup-13-R	ATGTTTTTCTGGCATAATTGCTTTAGAAATTTCTC
13-F	ATGCCAGAAAAACATGATCT
15-Kan-R	ATGGTTGAGATCTTCTTATTTTAAGCACTGACTCC
Kan-F	ATCTCAACCATCATCGATGAATTG
Kan-R	AAAGCCGCCGTCCCGTCAAG
Kan-phoAdown-F	GGCTTTAGATCTTCTCATCAAAACGATTAGAGCC
phoAdown-R	ATAGATTGGCTTGGCGTAGC
Construction of Δ*phoA*	
phoAup-F	GATCTACTAGCTTCTGCCAG
BamHI-phoAup-R	GGATCCAATTGCTTTAGAAATTTCTC
phoAup-BamHI-phoAdown-F	ATTTCTAAAGCAATTGGATCCTCATCAAAACGATTAGAGCC
phoAdown-R	ATAGATTGGCTTGGCGTAGC

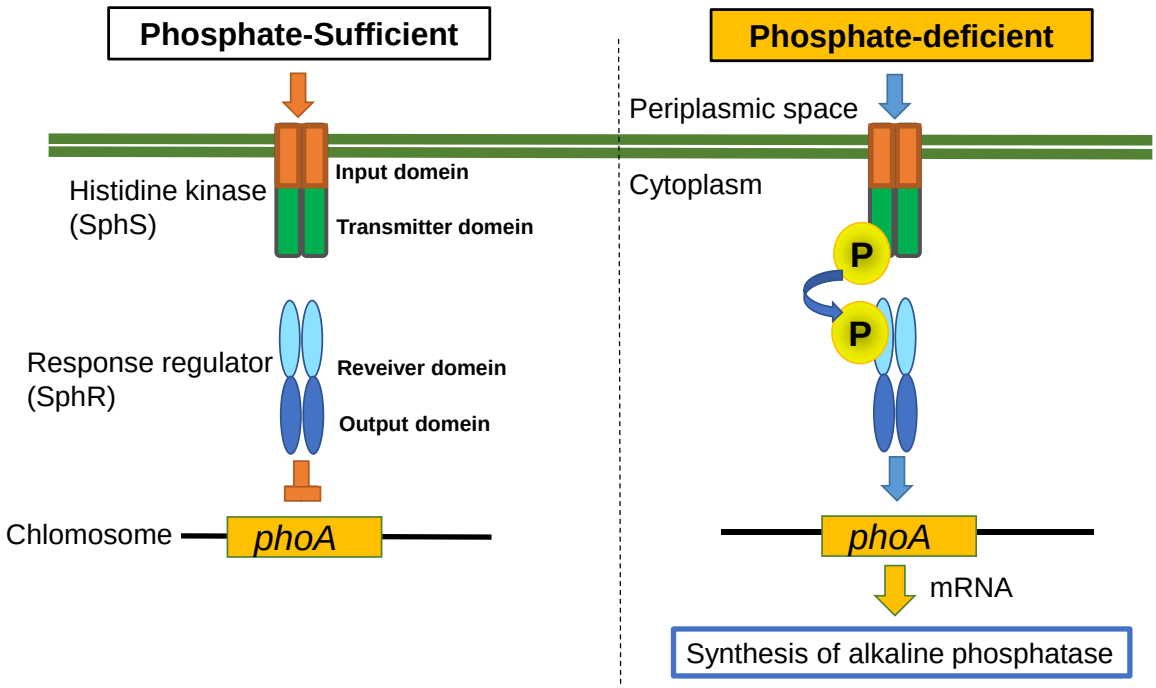

Figure 1. Gene expression regulated by SphS-SphR two-component system. Left and right figure indicate regulation of gene expression under phosphate sufficient and deficient, respectively. The P indicates transphosphorylation. Synthesis of alkaline phosphatase is only induced under phosphate-deficient condition.

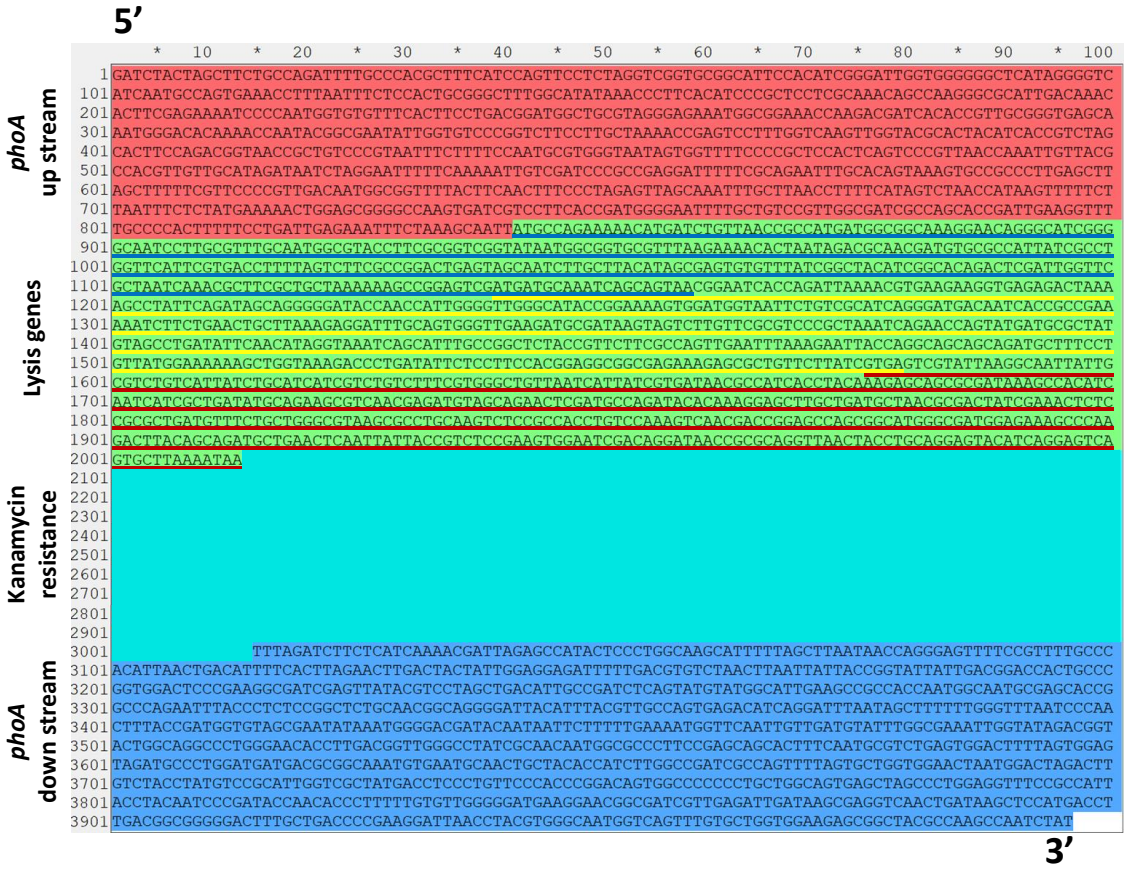

Figure 2. The DNA sequence of the inserted genes introduced into *Synechocystis*. The sequences of upstream and downstream of *phoA* gene, and lysis genes; holin (blue under-bar), endolysin (yellow under-bar), and lysis associated protein (red under-bar), are labeled.

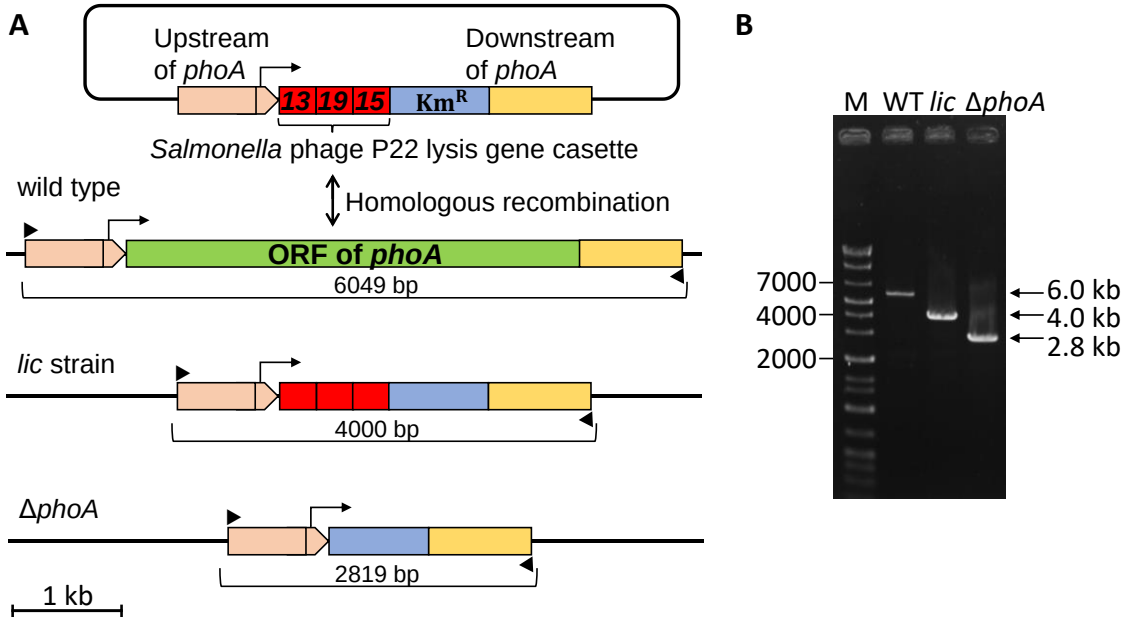

Figure 3. (A) Strategy of *lic* strain construction. The genomic region of the alkaline phosphatase gene (*phoA*) promoter; the coding sequences of holin (ORF13), endolysin (ORF19), and lysis-associated protein (ORF15); and a kanamycin-resistance gene cassette were flanked by 1 kbp upstream and downstream sequences of the *phoA* gene. The plasmid was used for the substitution of the native *phoA* coding region by homologous recombination. The *phoA*-deletion strain (Δ*phoA*), in which the coding sequence of the *phoA* gene was replaced with the kanamycin-resistance gene, was prepared as the negative control. (B) Agarose-gel electrophoresis of the amplified DNA fragments. Genomic regions in the wild-type and *lic* strains were amplified using the following primers: phoAup-F and phoAdown-R. M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA).

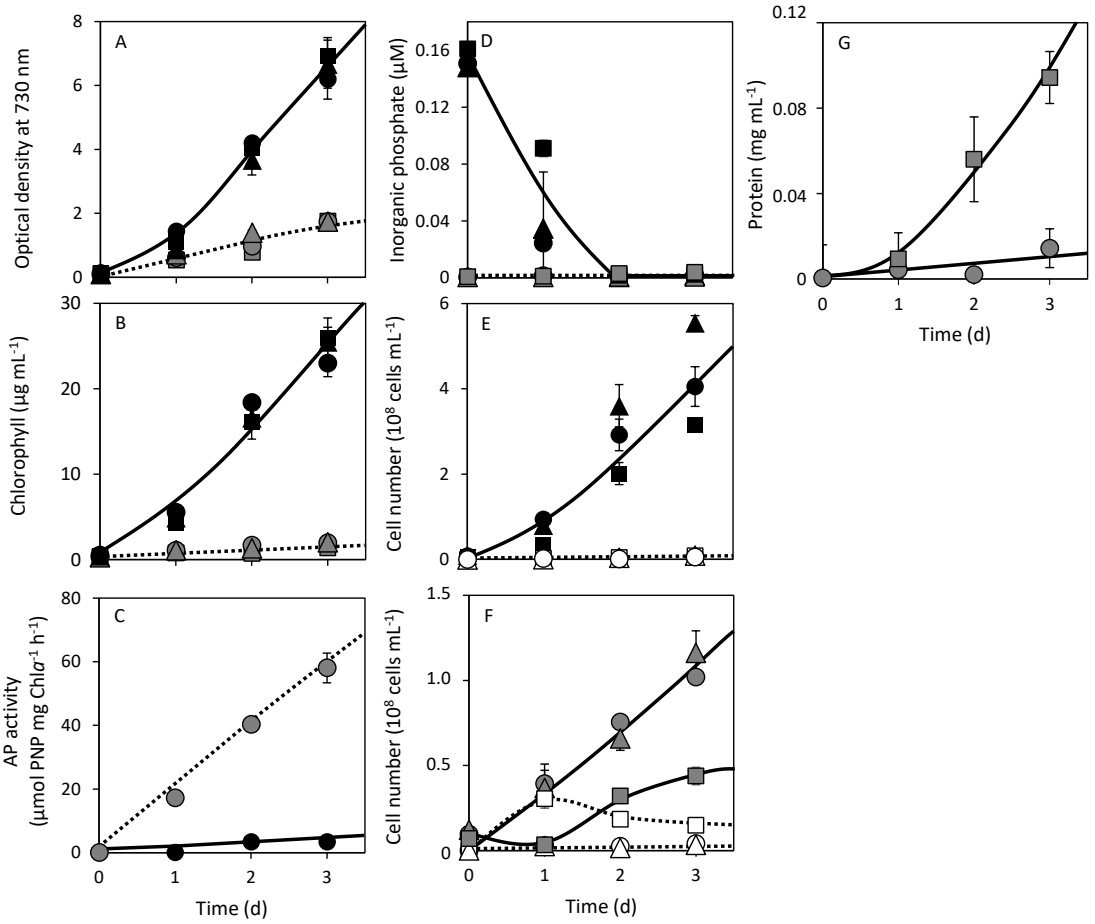

Figure 4. Optical densities (A) and chlorophyll contents (B) of the cultures of wild-type cells (circles), Δ*phoA* cells (triangles), and *lic* cells (squares) under phosphate-sufficient (black) or phosphate-deficient (gray) conditions. The activity of alkaline phosphatase in the wild-type cells (circles) under phosphate-sufficient (black) or phosphate-deficient (gray) conditions (C). The amount of phosphate inside the supernatant of wild-type cells (circles), Δ*phoA* cells (triangles), and *lic* cells (squares) under phosphate-sufficient (black) or phosphate-deficient (gray) conditions (D). The number of live (gray) and dead (white) cells of the wild-type (circles), Δ*phoA* (triangles), and *lic* (squares) cells under phosphate-sufficient (E) and deficient conditions (F). Protein contents in the phosphate-deficient cultures of wild-type cells (circles) and *lic* cells (squares) (G). Values represented are the means of three independent biological replicates ± standard deviations.

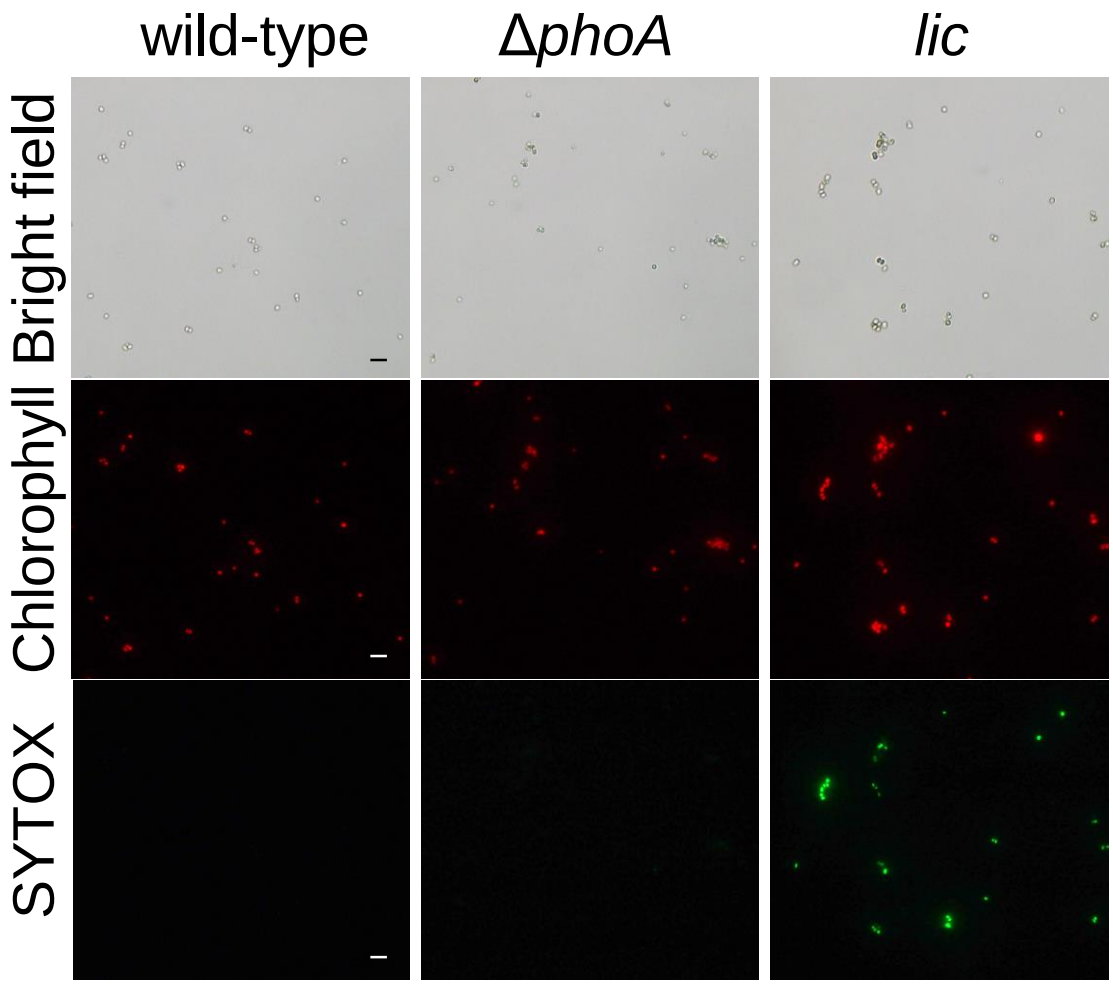

Figure 5. Images of wild-type, Δ*phoA*, and *lic* cells cultured for 1 d under phosphate-deficient conditions. Cell images were observed under bright-field microscopy, and the dead cells stained with SYTOX Green and Chl*a* fluorescence were observed under fluorescence microscopy. Bars indicate the 10 μm scale.

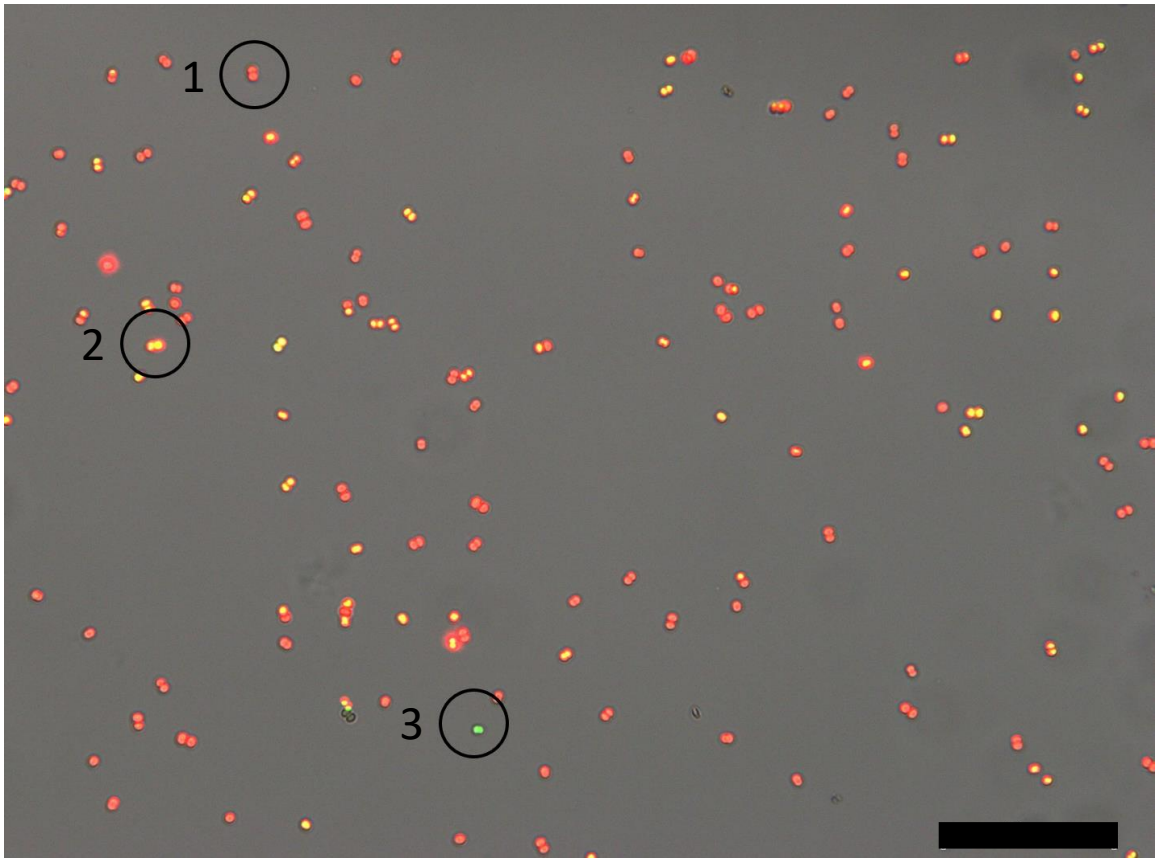

Figure 6. Merged picture of bright field and fluorescence microscopy observation of *lic* cells. (1) Cell possessed only Chl*a* fluorescence (live cell). (2) Cell possessed Chl*a* and SYTOX fluorescence (lysed cell). (3) Cell possessed only SYTOX fluorescence (lysed cell). Bars indicate the 50 µm scale.

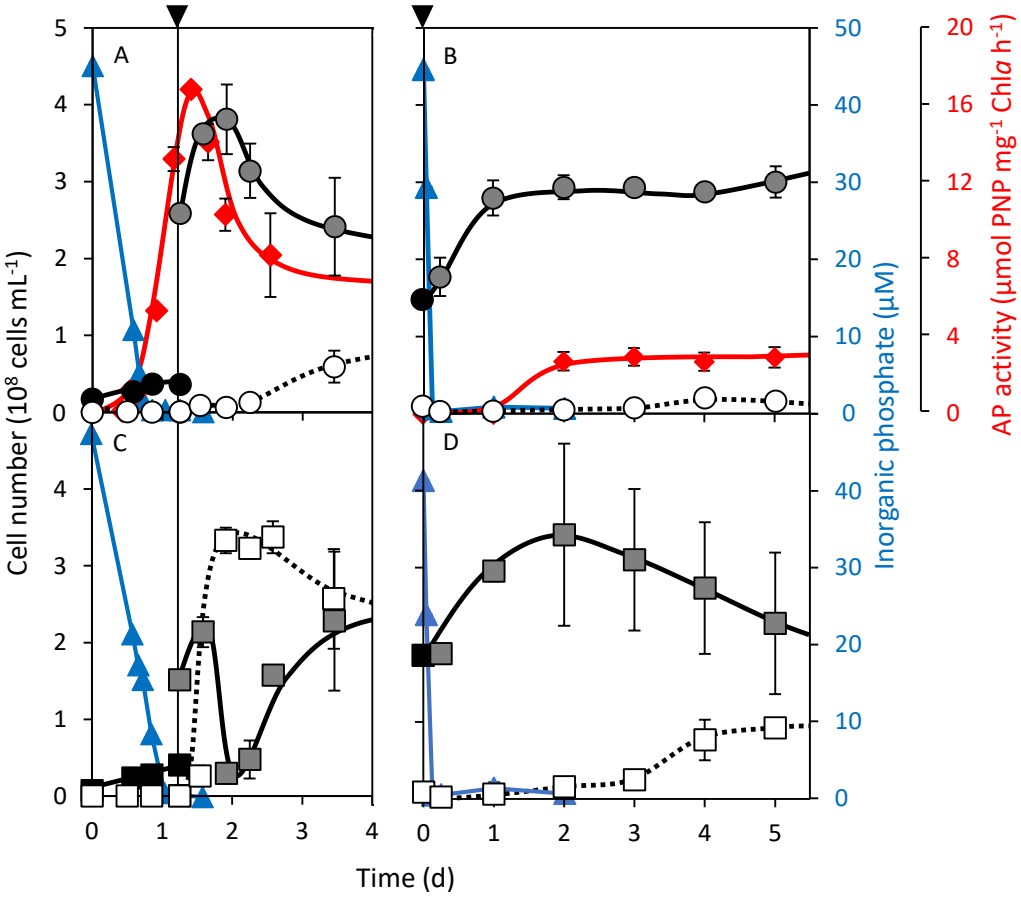

Figure 7. Induction of cell lysis after concentration of the cells. A and C show the results of the concentration of the cells after complete consumption of phosphate; B and D show the results of the concentration of the cells before consumption of phosphate. A and C indicate the wild-type cell results; C and D indicate the *lic* cell results. Black and gray symbols and lines indicate living cells under phosphate-sufficient and phosphate-deficient conditions, respectively; white symbols and dotted lines indicate the dead cells. Circles and squares indicate the wild-type and *lic* cells, respectively. Blue triangles and lines indicate the concentration of phosphate in the media, and red diamonds and lines indicate the activity of alkaline phosphatase. Arrowheads above the graphs and vertical lines indicate the time of concentration of the cells. Values represented are the means of three independent biological replicates ± standard deviations.

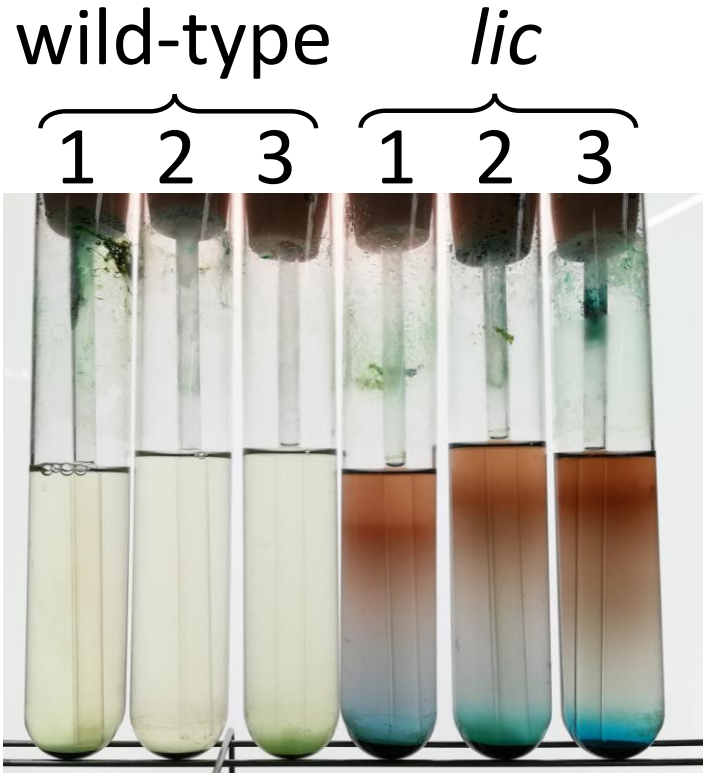

Figure 8. Cultures of wild-type cells and *lic* cells after cultivation. The tubes shown were left at room temperature for 10 d.

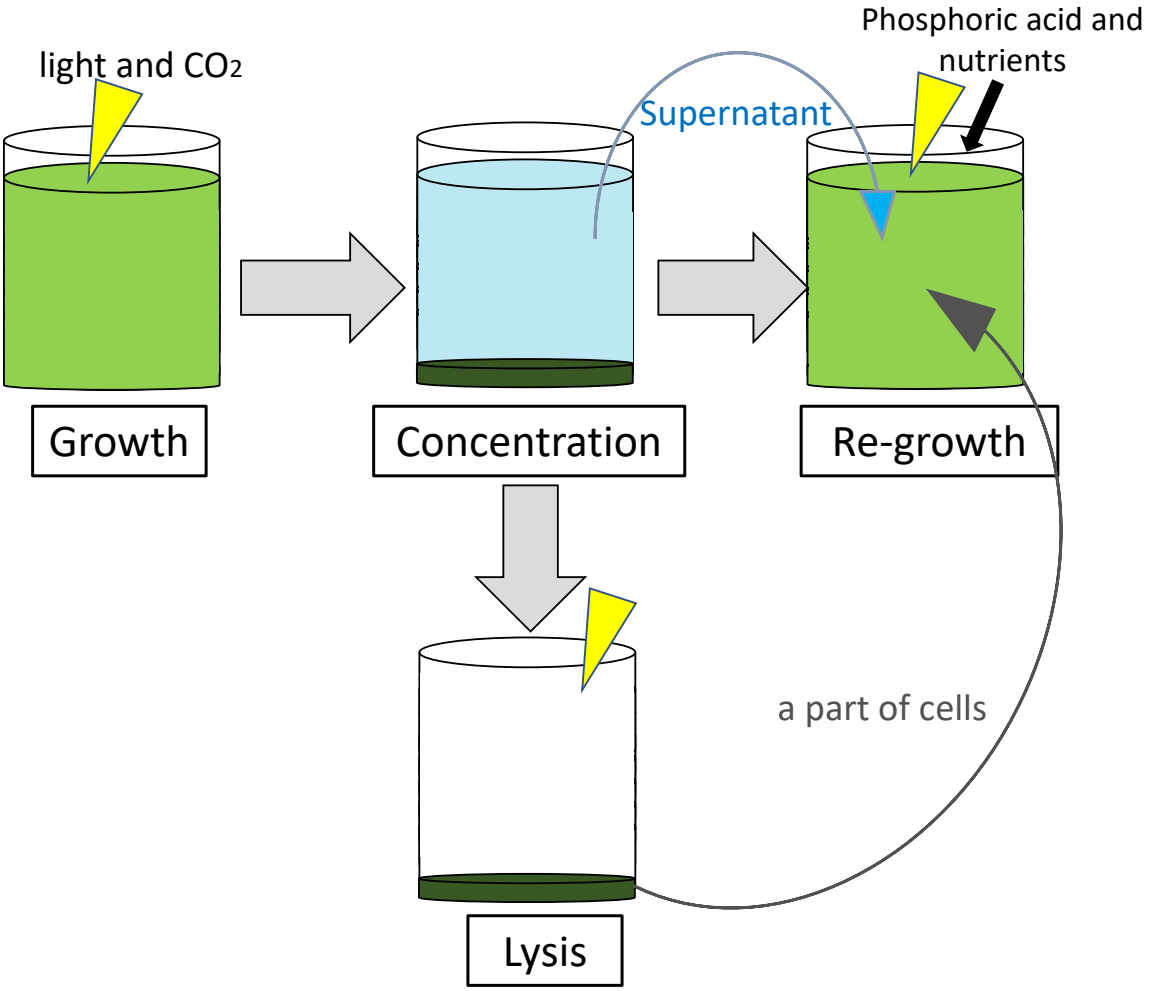

Figure 9. Application of the *lic* strain for large-scale cultivations. The *lic* strain grows and produces the target compounds while consuming phosphate. After the cells consume all the phosphate leading to phosphorus starvation, the mixing of the culture is halted, cells are precipitated, and cell lysis is induced. The supernatant of the medium is transferred into a new culture tank, the nutrient concentration is adjusted, and the medium is reused for the next culture. The remaining living cells may be used to seed the next culture.

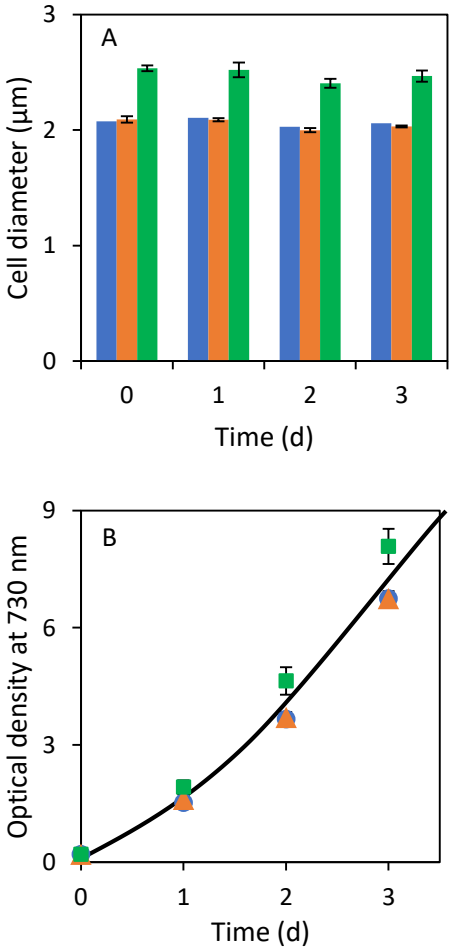

Figure 10. Cell diameter and growth of wild-type (blue bars or circles), Δ*phoA* (orange bars or triangles), and the *lic* cells (green bars or squares) under 3 d culture in phosphate-sufficient medium. (A) Cell diameter of each strain during the growth. (B) Optical density of each strain which represents cell growth. Values for Δ*phoA* and the *lic* cells represented are means of three and ten independent biological replicates ± standard deviations, respectively.

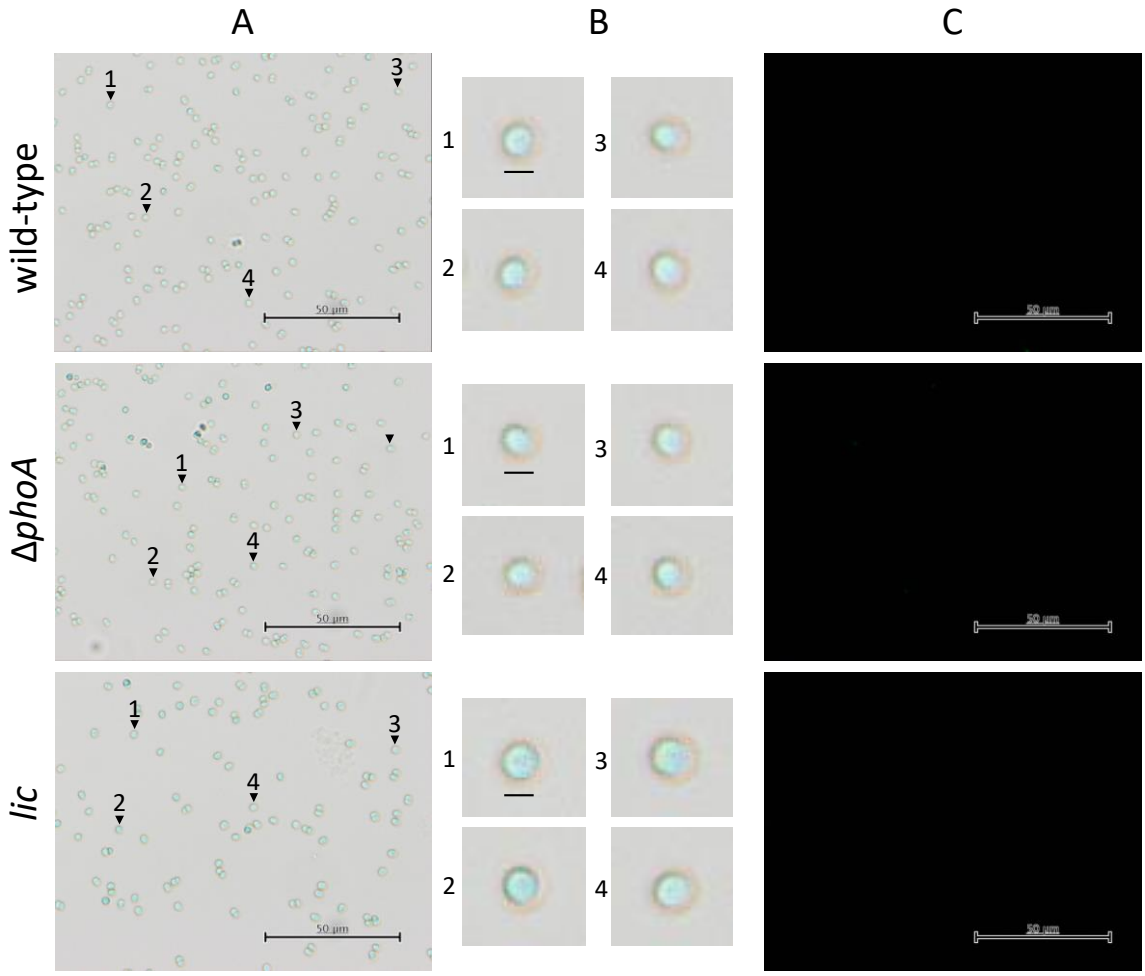

Figure 11. Bright field (A), enlarged images (B) and SYTOX fluorescence cell images (C) of wild-type, Δ*phoA*, and the *lic* cells after 1 d of culture with the phosphate-sufficient medium. Bars in left and right pictures indicate a scale of 50 μm, and bars in middle pictures indicate the scale of and 2 μm. Numbers with arrows from (A) represent enlarged cell views in (B).

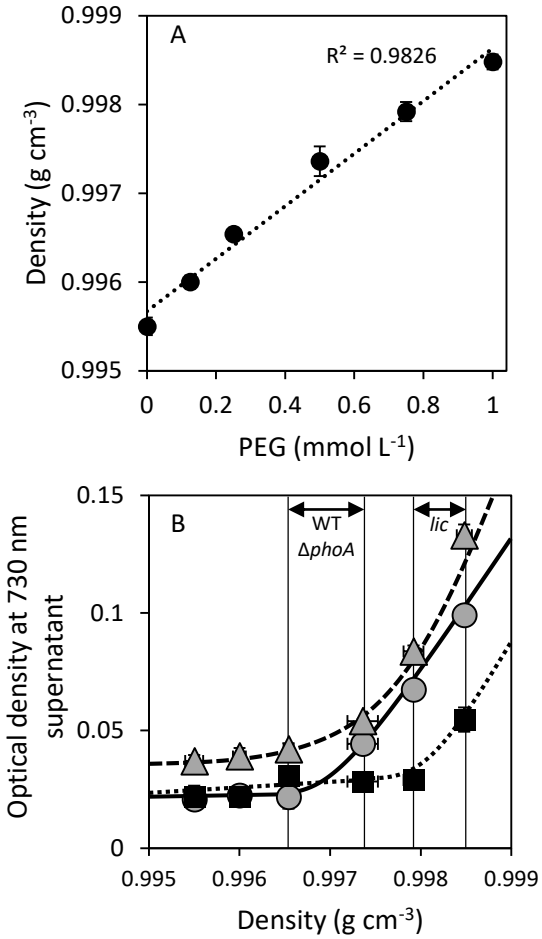

Figure 12. The density of PEG and each strain. (A) The concentration of PEG solution and their density. Values represented are the means of five independent technical replicates ± standard deviations. (B) The optical density of cells remaining in the supernatant after centrifugation in various concentration of PEG solutions vs. the density of the respective PEG solutions. The arrows mean predicted range of each cell density. Each plot represents wild-type (circles), Δ*phoA* (triangles), and the *lic* cells (squares) after 1 d culture with the phosphate-sufficient medium. Values represented are the means of three independent biological replicates ± standard deviations.

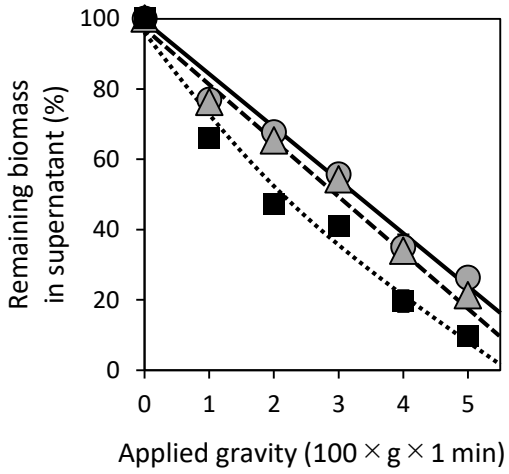

Figure 13. Cell precipitation efficiency of wild-type (circles), Δ*phoA* (triangles), and the *lic* cells (squares) after 1 d culture with the phosphate-sufficient medium. Series of gravity was applied to the cultures. Values represented are the means of three independent biological replicates ± standard deviations.

References

Aiba H, Nagaya M, Mizuno T (1993) Sensor and regulator proteins from the cyanobacterium *Synechococcus* species PCC7942 that belong to the bacterial signal-transduction protein families: implication in the adaptive response to phosphate limitation. Mol Microbiol 8: 81–91.

Barry A, Wolfe A, English C, Lambert D (2016) National algal biofuels technology review, U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office.

Berry J, Rajaure M, Pang T, Young R (2012) The spanin complex is essential for lambda lysis. J Bacteriol 194: 5667–5674.

Berry J, Summer EJ, Struck DK, Young R (2008) The final step in the phage infection cycle: The Rz and Rz1 lysis proteins link the inner and outer membranes. Mol Microbiol 70: 341–351.

Dassey AJ, Hall SG, Theegala CS (2014) An analysis of energy consumption for algal biodiesel production: comparing the literature with current estimates. Algal Res 4: 89–95.

Dollard MA, Billard P (2003) Whole-cell bacterial sensors for the monitoring of phosphate bioavailability. J Microbiol Methods 55: 221–229.

Eduardo C, Silva DF, Bertucco A (2016) Bioethanol from microalgae and cyanobacteria : a review and technological outlook. Process Biochem 51: 1833–1842.

Farooq W, Suh WI, Park MS, Yang J (2015) Water use and its recycling in microalgae cultivation for biofuel application. Bioresour Technol 184: 73–81.

Gao Y, Feng X, Xian M, Wang Q, Zhao G (2013) Inducible cell lysis systems in microbial production of bio-based chemicals. Appl Microbiol Biotechnol 97: 7121–7129.

Gray J, Wardzala E, Yang M, Reinbothe S, Haller S, Pauli F (2004) A small family of LLS1-related non-heme oxygenases in plants with an origin amongst oxygenic photosynthesizers. Plant Mol Biol 54: 39–54.

Grigorieva G, Shestakov S (1982) Transformation in the cyanobacterium *Synechocystis* sp. 6803. FEMS Microbiol Lett 13: 367–370.

Grima EM, Belarbi EH, Fernández FGA, Medina AR, Chisti Y (2003) Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv 20: 491–515

Günerken E, Hondt ED, Eppink MHM, Garcia-Gonzalez L, Elst K, Wijffels RH (2015) Cell disruption for microalgae biorefineries. Biotechnol Adv 33: 243–260.

Hirani TA, Suzuki I, Murata N, Hayashi H, Eaton-Rye JJ (2001) Characterization of a two-component signal transduction system involved in the induction of alkaline phosphatase under phosphate-limiting conditions in *Synechocystis* sp. PCC 6803. Plant Mol Biol 45: 133–144.

Hirose Y, Shimada T, Narikawa R, Katayama M., Ikeuchi M (2008) Cyanobacteriochrome CcaS is the green light receptor that induces the expression of phycobilisome linker protein. Proc Natl Acad Sci USA 105: 9528–9533.

Holman WIM (1943) A New Technique for the Determination of Phosphorus by the Molybdenum Blue Method. Biochem J 37: 256–259.

Inaba M, Suzuki I, Szalontai B, Kanesaki Y, Los DA, Hayashi H, Murata N (2003) Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. J Biol Chem 278: 12191–12198.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M,

Yasuda M, Tabata S (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. DNA Res 3: 109–136.

Larena I, De Cal A, Melgarejo P (2004) Solid substrate production of Epicoccum nigrum conidia for biological control of brown rot on stone fruits. Int J Food Microbiol 94: 161–167.

Ling MM, Robinson BH (1997) Approaches to DNA mutagenesis: an overview. Anal Biochem 254: 157–178.

Liu X, Curtiss III R (2009) Nickel-inducible lysis system in Synechocystis sp. PCC 6803. Proc Natl Acad Sci U S A 106: 21550–21554.

Liu X, Fallon S rah, Sheng J, Curtiss III R (2011) CO_2-limitation-inducible Green Recovery of fatty acids from cyanobacterial biomass. Proc Natl Acad Sci U S A 108: 6905–6908.

Loessner MJ (2005) Bacteriophage endolysins — current state of research and applications. Curr Opin Microbiol 8: 480–487.

López-maury L, García-domínguez M, Florencio FJ, Reyes JC (2002) A two-component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol Microbiol 43: 247–256.

Lübke C, Boidol W, Petri T (1995) Analysis and optimization of recombinant protein production in *Escherichia coli* using the inducible *pho A* promoter of the *E. coli* alkaline phosphatase. Enzyme Microb Technol 17: 923–928.

Mironov KS, Sidorov RA, Trofimova MS, Bedbenov VS, Tsydendambaev VD, Allakhverdiev SI, Los DA (2009) Characterization of the *Synechocystis* strain PCC 6803 penicillin-binding proteins and cytokinetic proteins FtsQ and FtsW and their network of interactions with ZipN. J Bacteriol 191: 5123–5133.

Mironov KS, Sidorov RA, Trofimova MS, et al (2012) Light-dependent cold-induced fatty acid unsaturation, changes in membrane fluidity, and alterations in gene expression in *Synechocystis*. Biochim Biophys Acta 1817: 1352–1359.

Miyake K, Abe K, Ferri S, Nakajima M, Nakamura M, Yoshida W, Kojima K, Ikebukuro K, Sode K (2014) A green-light inducible lytic system for cyanobacterial cells. Biotechnol Biofuels 7: 56.

Mizuno T, Kaneko T, Tabata S (1996) Compilation of all genes encoding bacterial two-component signal transducers in the genome of the cyanobacterium, *Synechocystis* sp. strain PCC 6803. DNA Res 3: 407–414.

Okamoto S, Ikeuchi M, Ohmori M (1999) Experimental analysis of recently transposed insertion sequences in the cyanobacterium *Synechocystis* sp. PCC 6803. DNA Res 6: 265–273.

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, Murata N (2004) Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. J Biol Chem 279: 53078–53086.

Passell H, Dhaliwal H, Reno M, Wu B, Ben Amotz A, Ivry E, Gay M, Czartoski T, Laurin L, Ayer N (2013) Algae biodiesel life cycle assessment using current commercial data. J Environ Manage 129: 103–111.

Schindler DW (1977) Evolution of Phosphorus Limitation in Lakes. Science 195:260–262.

Singh SK, Tiwari DN (2000) Control of alkaline phosphatase activity in *Anabaena oryzae* Fritsch. J Plant Physiol 157: 467–472.

Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ (2006) Proteomic analysis of the heat shock response in *Synechocystis* PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6: 845–864.

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (Order *Chroococcales*). Bacteriol Rev 35: 171–205.

Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215.

Suzuki S, Ferjani A, Suzuki I, Murata N (2004) The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in *Synechocystis*. J Biol Chem 279: 13234–13240.

Tandeau de Marsac N, Houmard J (1988) Complementary chromatic adaptation: physiological conditions and action spectra. Methods Enzymol 167: 318–328.

Vavilin D, Brune DC, Vermaas W (2005) ^{15}N-labeling to determine chlorophyll synthesis and degradation in *Synechocystis* sp. PCC 6803 strains lacking one or both photosystems. Biochim Biophys Acta 1708: 91–101.

Wada H, Murata N (1989) *Synechocystis* PCC6803 mutants defective in desaturation of fatty acids. Plant Cell Physiol 30: 971–978.

Wang I, Smith DL, Young R (2000) Holins: the protein clocks of bacteriophage infections. Annu Rev Microbiol 54: 799–825.

Williams JGK (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol 167: 766–778.

Yeh K-C, Wu S-H, Murphy JT, Lagarias JC (1997) A Cyanobacterial Phytochrome Two-Component Light Sensory System. Science 277: 1505–1508.

Young R (2002) Bacteriophage holins: deadly diversity. J Mol Microbiol Biotechnol 4: 21–36.

Yuan J, Kendall A, Zhang Y (2015) Mass balance and life cycle assessment of biodiesel from microalgae incorporated with nutrient recycling options and technology uncertainties. GCB Bioenergy 7: 1245–1259.

Acknowledgments

First, I would like to express my sincere gratitude to Professor Dr. Iwane Suzuki, for his great guidance and support during my research life in University of Tsukuba.

I am also thankful to Professor Dr. Yoshihiro Shiraiwa, for his support.

I would like to express my thanks to Juntila Darryl Joy for her many contributions to my work.

I appreciate to Dr. Yohei Shimura for suggestions and support to my work.

I also appreciate and thank all members of the Laboratory of Plant Physiology and Metabolism of the University of Tsukuba.

I am grateful to my family, especially my father Koji Asada and my mother Rieko Asada, for their continuous encouragement and empowerment.

Lastly, I would like to express my deep appreciation to Dr. Daisaku Ikeda, the founder of Soka University and my mentor in life for giving me inspiration, strength and courage.

Upon receiving this Doctor of Philosophy, I remember this statement “For what purpose should one cultivate wisdom? May you always ask yourselves this question!” and try my best to contribute in making this society a better place for many people.