A novel cell lysis system induced by phosphate deficiency in the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

In the cultivation of microalgae for the production of useful compounds, cell disruption to extract the products of interest is a bottleneck process. To establish a cost-effective method to recover these cellular compounds, we developed a method to induce cell lysis via phosphate deficiency in the cyanobacteria *Synechocystis* sp. PCC 6803. In this system, the promoter from the *phoA* gene for alkaline phosphatase expressed bacteriophage genes encoding the lytic enzymes holin and endolysin, thus the cell lysis is induced under phosphate deficient condition. We observed that 90% of the cells, introduced these bacteriophage gene, were lysed after 24 h of incubation under phosphate-deficient conditions. We also developed a method 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. This inducible lysis system may contribute to decreased cell disruption costs in the algal biotechnology industry.

Keywords

Cell disruption; Endolysin; Extraction; Holin; Phosphate sensor

1. Introduction

Microalgae produce organic compounds through photosynthesis, and they are commercially cultivated as platforms for the production of valuable materials such as lipids, carotenoids, and proteins in a carbon-neutral manner. Because most algae store these products inside their cells, disruption of the plasma membrane 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, for which algae with a 20% lipid content are cultivated in a one-acre pond, 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 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 enzymes 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.

The function of the lytic enzymes has been applied to the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*). *Synechocystis* is often used as a model cyanobacterium because of the availability of its genomic sequence (Kaneko et al. 1996), its high competency of genetic

transformation (Grigorieva and Shestakov 1982), and its utilization of glucose as a carbon source (Williams 1988). The responses of *Synechocystis* cells to changes in environmental conditions have also been well studied to advance our 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 the certain sensory protein 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).

Previous studies have successfully induced cell lysis utilizing two-component systems that are regulated by different stimuli. The Ni^{2+}-regulating, 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 was achieved by the addition of 50 μM Ni^{2+}, inducing the expression of the lysis genes. Liu et al. (2011) improved this lysis system using the *sbtA* promoter—which is induced under CO_2-deficient conditions—for induction of the lysis genes. Another approach that avoids nickel in the medium is the use of the green-light-responding, two-component system CcaS–CcaR (Hirose et al. 2008) for inducing cell lysis (Miyake et al. 2014). Although these lysis strategies have been successfully applied, they require the following: (i) addition of heavy metal ions, which increases environmental pollution; (ii) the cultures can be directly illuminated by the sunlight (the most economical light source), when suitable optical filters, which can pass the specific wavelength of the light is applicable; 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., *Escherichia 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 protein, DNA, and phycocyanin were 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 product, cell lysis must be applied to concentrated cells.

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 (Hirani et al. 2001). The promoter region of alkaline phosphatase gene in *Escherichia. coli* has been used gene expression such as fusion to bioluminescence genes to develop bacterial sensor cells to assess phosphate bioavailability (Lübke et al. 1985; Dollard and Billard 2003). In this study, we applied the SphS-SphR-regulated promoter of the *sll0654* gene for alkaline phosphatase in *Synechocystis* (Hirani et al. 2001; Suzuki et al. 2004) to regulate the target genes by phosphate unavailability, as far as we know this is the first application of this system to regulate expression of the heterologous gene in the cyanobacterial cells.

We 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. We hypothesized that the transcription of the synthetic operon is induced under phosphate-deficient conditions. We 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.

2. 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).

124 *2.2. Construction of cells expressing lysis genes*

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126 The genomic DNA of *S. enterica* phage P22 (National Institute of Technology and Evaluation, NITE
127 Biological Resource Center, Japan) was used as the lytic gene template. DNA fragments, including the
128 coding regions of the lytic enzymes holin (ORF13), endolysin (ORF19), and lysis-associated protein
129 (ORF15), were amplified via polymerase chain reaction (PCR) using primers 13-F and 15-Kan-R
130 (Supplementary table 1). In addition, DNA fragments corresponding to the kanamycin-resistance gene
131 cassette, EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit (Epicentre, Madison, WI), and approximately
132 1000 bp fragments upstream and downstream of the *phoA* gene of *Synechocystis* were amplified using
133 primer sets Kan-F and Kan-R, phoAup-F and phoAup-13-R, and Kan-phoAdown-F and phoAdown-R,
134 respectively. The resulting PCR products had 15 bp overlapping sequences and were adhered via overlap-
135 extension PCR (Ling and Robinson 1997). The DNA fragment thus generated was introduced into
136 pGEM™-T Easy Vector (Promega, Madison, WI), and the resulting plasmid was introduced into
137 competent *E. coli* JM109 cells (TaKaRa Bio, Kusatsu, Japan) using heat shock. The transformed *E. coli*
138 were selected on LB agar medium containing 50 μg mL^{-1} sodium ampicillin and kanamycin sulfate. The
139 DNA sequence of the introduced plasmid was confirmed using a 3130 Genetic Analyzer (Applied
140 Biosystems, Foster City, CA).

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

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153 *2.3. Induction of cell lysis*

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155 To evaluate the lysis response of the strains cultured under phosphate-deficient conditions, the
156 precultured cells were washed thrice with phosphate-free BG-11 (BG-11 (−P)) and used to inoculate fresh
157 BG-11 (−P), with phosphate-sufficient BG-11 (BG-11 (+P), 0.18 mM K_2HPO_4) as a control. The cell
158 density was adjusted to a final optical density of 0.1 at 730 nm (OD_{730}), and the cells were cultured for 3 d
159 under 70 μmol photons m^{-2} s^{-1} at 34°C and aeration with 1% (v/v) CO_2-enriched air.

160 To evaluate cell lysis under high-cell-density culture conditions, we utilized two different strategies.
161 Firstly, the wild-type and *lic* cells cultivated under phosphate-sufficient conditions were concentrated to
162 an OD_{730} between 4.2 and 4.4 and suspended in the same medium. The phosphate in the medium was
163 subsequently consumed by the growing cells. Secondly, we prepared both strains in the culture and
164 allowed them to grow until they consumed the phosphate in the medium, and then concentrated the cells
165 to the same OD_{730}. Microscopic observation after staining with SYTOX® Green (Life Technologies
166 Japan, Tokyo, Japan) was used for the detection of cell lysis, and AP activity in the wild-type cells was
167 measured for evaluation of the response to the phosphate-deficient conditions. The cultures were further
168 incubated for 5 d under 300 μmol photons m^{-2} s^{-1} at 34°C and aeration with 1% (v/v) CO_2-enriched air.

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170 *2.4. Evaluation of cell lysis*

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

176 Fluorescent microscopic observation was applied to count the live and dead cells after mixing with
177 SYTOX Green, which stains the nucleic acids of only dead cells. Image processing and analyzing
178 software (Image J, National Institutes of Health, Bethesda, MD) was used for counting the cell numbers.

179 The phosphate concentration in the supernatant of the culture medium was measured using the
180 molybdenum blue method (Holman 1943). Briefly, 1 mL supernatant was mixed with 20 μL acid
181 ascorbate solution (2.5% ammonium molybdate, 0.1% potassium antimonyl tartrate sesquihydrate, and

3.15 M H_2SO_4), and 20 μL of acid molybdate 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 of the sample was measured at 883 nm.

The protein concentrations in the culture supernatant were measured with a 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 amount of Chl*a*.

3. Results and discussion

3.1. Confirmation of gene insertion in the transformants

The genomic insertions including the native *phoA gene* operon, lysis gene operon, and, kanamycin-resistance gene operon, were confirmed via PCR using the primer pairs phoAup-F and phoAdown-R (Supplementary 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 (Supplementary fig. 1B).

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, we measured the cell culture OD_{730} (Fig. 1A). 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 (data not shown), we suggest that the cell viability is nearly equal between the three strains.

In the BG-11 (−P) culture, considerable growth inhibition was observed (Fig. 1A). Cells of the wild-type, Δ*phoA*, and *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 (data not shown). 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. 1B), 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. 1C). Dead cells were distinguished under fluorescence microscopy after staining with SYTOX Green, a nucleic-acid-staining reagent that does not readily penetrate intact cell membranes (Fig. 2). Notably, after 1 d of cultivation, many of the *lic* cells exhibited fluorescence, possibly caused by lysis enzyme activity 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. 1C and 2). 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. Our result of more than 90% lysis in 1 d is similar to the results of these studies. However, our 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 (data not shown), the phosphorous compounds released from the dead cells might immediately recover by the surviving cells. Meanwhile, several 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). We confirmed that length of the DNA region containing the lysis genes before and after the recovery from the phosphate-deficiency was not altered and the recovered cells were lysed again by subsequence exposure of the phosphate-deficiency (data not shown). These results indicated that the resume of the cell growth in Fig. 1C 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. 1D). Although cell lysis was clearly induced in the first 1 d (Fig. 1C), the protein concentrations gradually increased for the first 3 d of cultivation (Fig. 1D). Referring to the results of microscopic observation, some cells stained with SYTOX Green also exhibited red fluorescence from Chl*a* (Fig. 2), which decreased as time passed (data not shown). These results suggest that the intracellular compounds were gradually released following cell lysis. Liu and Curtiss (2009) also noted that the timing of cell content leakage after cell lysis differed between pigment, DNA, and other proteins. We 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. 2).

Almost no cells were stained with SYTOX Green during the 3 d culture period when cultured in BG-11 (+P) medium (Fig. 1C), 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 microalgal cells require light, which is frequently limited as a result

of self-shading. Thus, the concentration of the products is comparatively low in microalgal cultures. To address this, the algal cells should be concentrated before the induction of lysis to avoid dilution of the cellular products in the culture medium. Also, exchanging the culture medium to remove phosphate and induce the expression of the lysis genes should be avoided, so as not to add extra processes. In the present study, we 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. 3B and 3D, we 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. 3B and 3D, 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 we 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, we assayed the AP activity of wild-type cells; the AP activity was around 3 μmol PNP mg^{-1} Chl*a* h^{-1} at 2 d, and these levels were maintained until day 5 (Fig. 3B). In the case of cell lysis induction shown in Fig. 1C, 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 (Fig. 1B), and a large number of the *lic* cells were lysed during this incubation (Fig. 1C). Thus, we concluded that this method of induction was unable to fully induce the *phoA* promoter. Previous studies 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, we speculated that, in addition to phosphate deficiency, light irradiation may be necessary to induce cell lysis.

For the second attempt, we concentrated the cells after they had consumed the phosphate from the media. We cultured the wild-type and *lic* cells in phosphate-sufficient media until the phosphate was

298 completely consumed (Fig. 3A and 3C). The rates of phosphate uptake were 5.8 and 4.0 μmol OD_{730}^{-1} h^{-1}
299 for the wild-type and *lic* cells, respectively. These results indicate that the cells before concentration
300 possessed higher phosphate uptake activities than those after concentration, as shown in Fig. 3B and 3D.
301 The cells were then further cultured for 5 h after all the phosphate had been eliminated from the media to
302 allow the induction of lysis genes from the *phoA* promoter; in our previous study, the induction of *phoA*
303 gene expression was initiated after 1 h and reached its maximum level after 4–8 h of incubation in
304 phosphate-deficient medium (Suzuki et al. 2004). During 5 h of incubation under phosphate-deficient
305 conditions, the cells may induce the expression of lysis genes from the *phoA* promoter. Then, we
306 collected the cells of the wild-type and the *lic* cultures by centrifugation, removed the cell-free media, and
307 resuspended the precipitated cells in fresh media to an OD_{730} of approximately 4.0 (Fig. 3A and 3C). The
308 lysis rate of the concentrated *lic* cells, which were stained with SYTOX Green, rapidly increased and
309 reached over 90% after 16 h of incubation following the concentration of the cells (Fig. 3C), whereas only
310 2% of the wild-type cells were stained by SYTOX Green under the same growing conditions. When we
311 concentrated the cells cultivated for 5 h following phosphate consumption, the wild-type cells exhibited
312 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}
313 Chl*a* h^{-1} until 16 h after the cell concentration (Fig. 3A). The increase in AP activity in the wild-type cell
314 culture and the increase in the percentage of SYTOX-Green-stained cells in the *lic* cell culture were very
315 well correlated (Fig. 3A and 3C); the AP activity and the lysed cells were much higher with this method
316 than with the former method (Fig. 3B and 3D). We speculate that the concentration of cells before the
317 complete consumption of phosphate in the media suppressed the induction of AP activity due to
318 inefficient irradiation of the cells. Thus, AP activity in the wild-type cultures and cell lysis in the *lic*
319 cultures were highly induced when the cells were incubated for 5 h after the complete consumption of
320 phosphate and then concentrated.

321 We demonstrated that proteins accumulated in the culture medium of the *lic* cells following
322 phosphate deficiency (Fig. 1D). When we left the concentrated cultures without mixing after the
323 experiment, the color of the *lic* culture supernatants differed from that of the wild-type culture
324 (Supplementary fig. 2). As the *lic* cells sedimented, unidentified brown components appeared near the
325 surface of the culture, and a blue component was observed near the precipitated cells. The brown and blue
326 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 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). 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.

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. This lysis system does not affect cell growth during the biomass production stage under phosphate-sufficient conditions, because the *lic* cells proliferated as well as the wild-type cells in the BG-11 (+P) culture medium (Fig. 1A). The advantage of the *lic* cells is that they may be used for high-scale designed cultivation in algal industry (Supplementary fig. 3). In addition, the timing of lysis may be controlled by the initial concentrations of inorganic phosphate in the media; after cell growth consumes the phosphate, the cells are precipitated and cell lysis is induced. This precipitation is preferable to be done with natural sedimentation not to add energy cost by centrifugation. The supernatant is transferred into a new culture tank and is reusable for repetitive culture after the addition of phosphate and other nutrients. The water requirement for this method of algal culture should be reduced for sustainable production, and recycling of water contributes not only to reduce the water footprint but also to 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.

4. Conclusion

In the present study, we 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. Our results may contribute to efficient cell lysis and energy cost savings in the algal industrial scene.

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Figure captions

Fig. 1 Optical densities 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 (A). Activity of alkaline phosphatase in the wild-type cells (circles) under phosphate-sufficient (black) or phosphate-deficient (gray) conditions (B). Number of live (gray) and dead (white) cells of the wild-type (circles), Δ*phoA* (triangles), and *lic* (squares) cells under phosphate-deficient conditions (C). Protein contents in the phosphate-deficient cultures of wild-type cells (circles) and *lic* cells (squares) (D). Values represented are the means of three independent biological replicates ± standard deviations.

Fig. 2 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.

Fig. 3 Induction of cell lysis after concentration of the cells. A and C show the results of concentration of the cells after complete consumption of phosphate; B and D show the results of concentration of the cells before consumption of phosphate. A and B 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.

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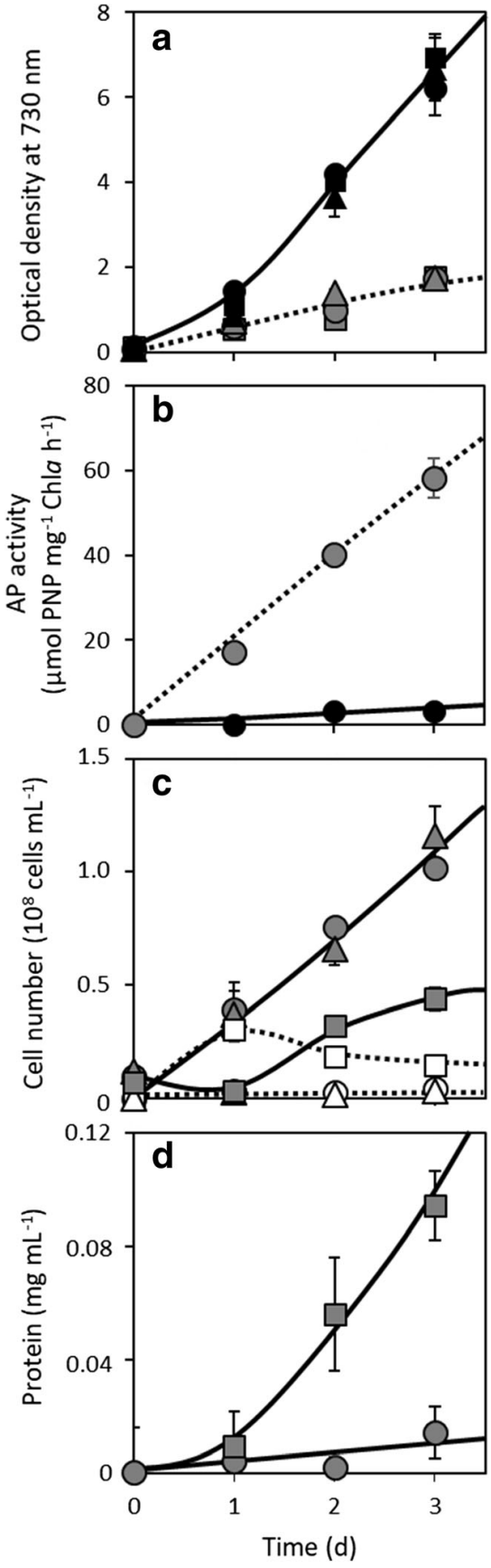
Fig. 1


a
b
c
d
Optical density at 730 nm
AP activity
(µmol PNP mg-1 Chla h-1)
Cell number (10^8 cells mL-1)
Protein (mg mL-1)
Time (d)
0
2
4
6
8
20
40
60
80
0.5
1.0
1.5
0.04
0.08
0.12
1
2
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548 Fig. 2

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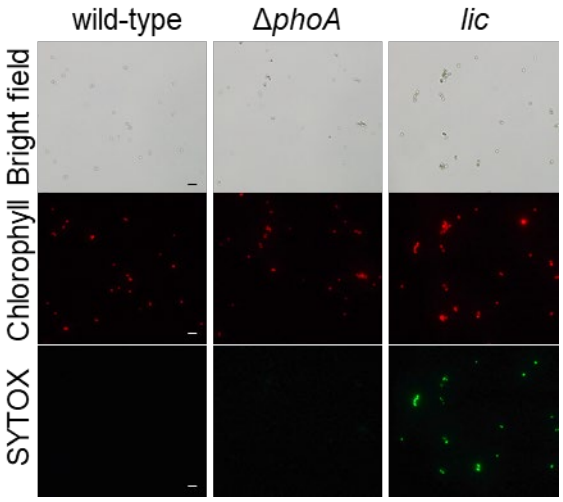

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554 Fig. 3

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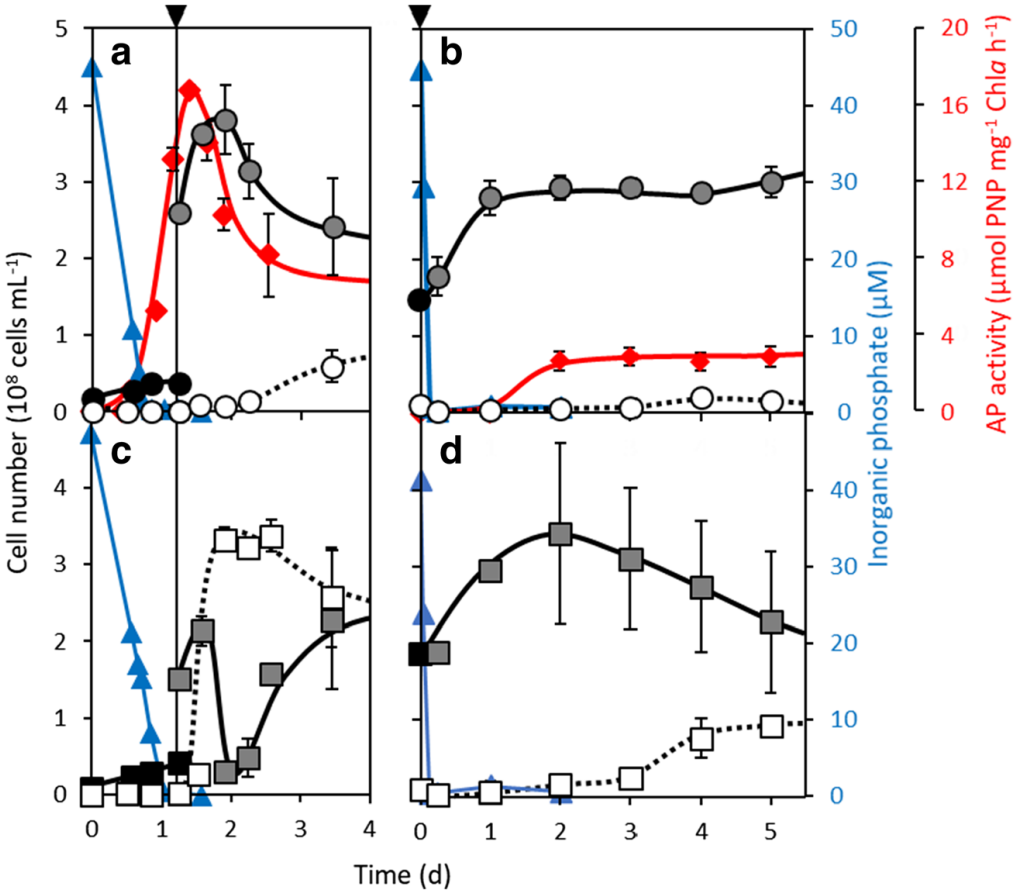

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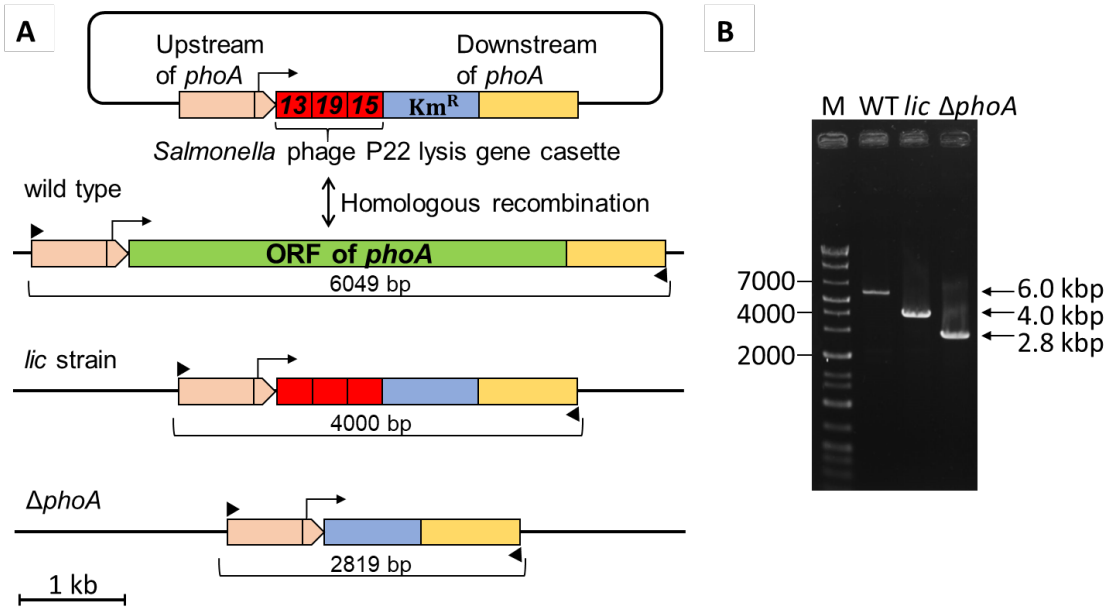

Supplementary fig. 1 (A) Strategy of *lic* strain construction. The genomic region of the alkaline phosphatase gene (*phoA*) promoter; the coding sequences of holin (ORF13), endolysin (ORF19), and lysis-associated protein (ORF15); and a kanamycin-resistance gene cassette were flanked by 1 kbp upstream 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).

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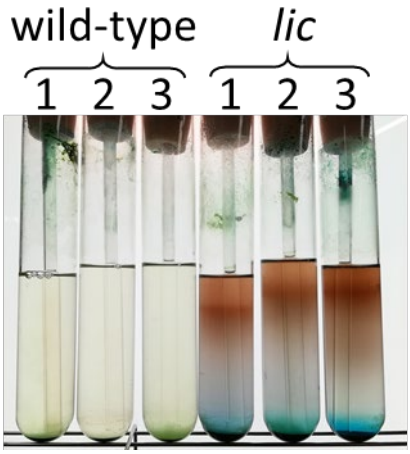

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579 **Supplementary fig. 2** Cultures of wild-type cells and *lic* cells after cultivation. The tubes shown were left

580 at room temperature for 10 d.

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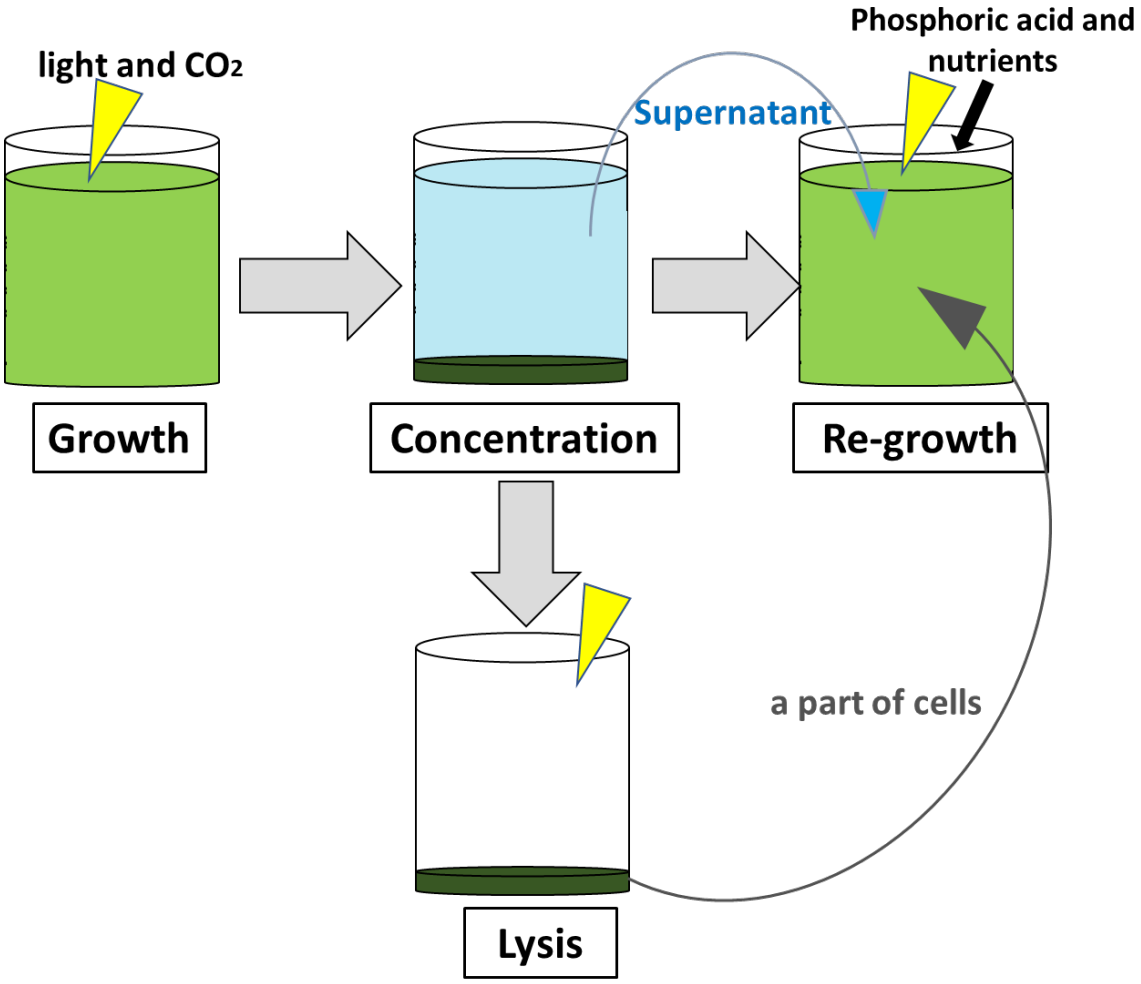

Supplementary fig. 3 Application of the *lic* strain for large-scale cultivations. The *lic* strain grows and produces the target compounds, and consumes phosphate. After the cells consume all the phosphate leading phosphorus starvation, the mixing of the culture is halted, cells are precipitated, and 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.

Supplementary 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	ATTTCTAAAGCAATTGGATCCTCATCAA
	AACGATTAGAGCC
phoAdown-R	ATAGATTGGCTTGGCGTAGC