รายงานวิจัยฉบับสมบูรณ์

โครงการ ระบบกำจัดฟอสฟอรัสแบบใช้แสงโดยไซยาโนแบคทีเรียสายพันธุ์กลายแบบเส้นสาย

โดย นายสุรเชษฐ์ บุรุษอาชาไนย

ธันวาคม ๒๕๕๙ ที่เสร็จโครงการ

สัญญาเลขที่ TRG๕๗๘๐๐๙๔

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ผู้วิจัย นายสุรเชษฐ์ บุรุษอาชาไนย สังกัด
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

รูปแบบ **Abstract (บทคัดย่อ)**

Project Code : TRG5780094

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Project Title : A photosynthetic phosphorus removal system using a genetic modified filamentous cyanobacterium

(ชื่อโครงการ) ระบบกำจัดฟอสฟอรัสแบบใช้แสงโดยไซยาโนแบคทีเรียสายพันธุ์กลายแบบเส้นสาย

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Project Period : 2 years 6 months

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Phosphorus (P) removal from recirculating aquaculture system wastewater was successful in this study. A five-liter photobioreactor was simply operated as bubbling column for this process. A filamentous cyanobacterium *Anabaena* sp. PCC 7120 strain Δall4501 was effective for this removal over the wild type strain. The *all4501* was identified here as the negative regulator for phosphate-sensing system. Inactivation of the *all4501* gene resulted in constitutive expression of Pho regulon genes indicated by activities of alkaline phosphatase and phosphate-specific transport system. Growth curves of wild type and Δall4501 strains were similar, however, the Δall4501 strain took up phosphate in the growth medium faster than the wild type. The initial rate of phosphate uptake of the Δall4501 strain was 8-times higher than the wild type. In addition, the cellular P concentration of the Δall4501 was 2-times higher than the wild type by storing in form of polyphosphate granules. After cells inoculation, the

photobioreactor could be repeatedly used for phosphorus removal up to 10 cycles with the average phosphorus removal efficiency of 57.4 % and 96.9 % for wild type and Δall4501 strains, respectively.

การทดลองนี้ประสบความสำเร็จในการกำจัดฟอสฟอรัสจากน้ำเสียจากระบบเลี้ยงสัตว์น้ำแบบหมุนเวียนได้ โดย ทำการทดลองในถังปฏิกรณ์แบบใช้แสงขนาด 5 ลิตร เดินระบบแบบคอลัมน์ให้อากาศ โดยใช้ไซยาโนแบคทีเรียแบบเส้นสาย *Anabaena* sp. PCC 7120 สายพันธุ์ Δall4501 ซึ่งมีประสิทธิภาพเหนือกว่าสายพันธุ์ดั้งเดิม การทดลองนี้ได้ยืนยันแล้วว่า ยีน *all4501* เป็นตัวควบคุมการตอบสนองเชิงลบ ในระบบตรวจวัดฟอสเฟต การยับยั้งการทำงานของยีน *all4501* นี้ ส่งผลให้มีการแสดงออกของยีนใน โฟ เรคคูลอนตลอดเวลา โดยตรวจวัดจากกิจกรรมของเอนไซม์ แอลคาไลน์ ฟอสฟาเตส และระบบขนส่งฟอสเฟตแบบจำเพาะ การเติบโตของสายพันธุ์กลาย Δall4501 และสายพันธุ์ดั้งเดิมนั้นเหมือนกัน แต่สายพันธุ์กลาย Δall4501 นั้นลดปริมาณฟอสเฟตในอาหารได้รวดเร็วกว่าสายพันธุ์ดั้งเดิม โดยศึกษาอัตราการขนส่งฟอสเฟตเริ่มต้นพบว่า สายพันธุ์ Δall4501 นั้นขนส่งฟอสเฟตได้เร็วกว่าสายพันธุ์ดั้งเดิมถึง 8 เท่า และสายพันธุ์ Δall4501 ยังสะสมฟอสฟอรัสในเซลล์ได้มากกว่าสายพันธุ์ดั้งเดิม 2 เท่า โดยสะสมอยู่ในรูปของ พอลิฟอสเฟต การกำจัดฟอสฟอรัสโดยในถังปฏิกรณ์แบบใช้แสงนี้ สามารถใช้ซ้ำได้ถึง 10 รอบจากการลงเชื้อเริ่มต้นเพียงครั้งเดียว โดยประสิทธิภาพการกำจัดฟอสฟอรัสโดยเฉลี่ยของสายพันธุ์ Δall4501 ได้สูงถึง 96.9 % ในขณะที่สายพันธุ์ดั้งเดิมได้เพียง 57.4 %

Keywords : cyanobacteria, *Anabaena* sp. PCC 7120, negative regulator, phosphorus removal

(คำหลัก) ไซยาโนแบคทีเรีย, *Anabaena* sp. PCC 7120, ตัวควบคุมการตอบสนองเชิงลบ, การกำจัดฟอสฟอรัส

Executive Summary

Recirculating aquaculture systems (RASs) are the environmental friendly and sustainable aquaculture systems. Without water exchange, most nutrients are accumulated in the RAS at very high concentrations, including nitrogen (N) and phosphorus (P). Discharge of the nutrients rich water to water reservoirs results in eutrophication. With our in-house technology, N can be removed out of the RASs via integrating of nitrification and denitrification reactors. By contrast, P is still accumulated in the RASs at very high concentrations which it removal is more complicated. The accumulated P in RAS was mainly in form of soluble inorganic phosphates (Burut-Archanai et al., 2013). Biological P removal using photosynthetic organisms have been widely studied, however, P removal efficiencies were low. Most of them were not able to decrease P concentrations in the wastewater to 0.5 mg P/L which is standard concentration of wastewater allowing for discharge to water resources. A previous study in cyanobacterium *Synechocystis* sp. PCC 6803 strain ΔSphU, lacking negative regulator for phosphate-sensing system, showed that the ΔSphU strain could remove phosphorus in wastewater up to 96%. Removal of P by *Synechocystis* sp. PCC 6803 strain ΔSphU was effective, however, the separation between the cyanobacterial cells and treated water was the main problem. Centrifugation or flocculation induced by chitosan were not practical for large scale wastewater treatment.

Negative regulator for phosphate-sensing system in cyanobacteria and gram-negative bacteria is important for regulation of the Pho regulon gene expression. The Pho regulon genes, such as alkaline phosphatase (*phoA*) and phosphate-specific-transport system (*pstSCAB*), are involved in P metabolism. Under phosphate-sufficient condtions, the Pho regulon expression was repressed. The Pho regulon was highly expressed under phosphate-limiting conditions. These could explain the low efficiency of P removal of cyanobacteria in wastewater containing high concentrations of phosphate. Inactivation of negative regulator resulted in constitutive expression of the Pho regulon. On the other hand, the Pho regulon was still highly expressed at high concentrations of phosphate when negative regulator was inactivated.

Anabaena sp. PCC 7120 is more attractive since the *Anabaena* cells are filamentous and large cell size. Autoflocculation property of the *Anabaena* sp. PCC 7120 could overcome the cell harvesting or separation problem. With simple settle

down method, the *Anabaena* cells were aggregated and settled down to the bottom completely within 1 h. In addition, genome sequence of the *Anabaena* sp. PCC 7120 is also available. Homology analysis of the *Anabaena* sp. PCC 7120 genome revealed that an open reading frame (ORF) "*all4501*" was highly similar (77% similarity) to the negative regulator, *sphU* (*slr0741*), of *Synechocystis* sp. PCC 6803. The *all4501* was target gene in this study to delete out. The deleted *all4501* DNA fragment (Δ*all4501*) was constructed and transformed into *Anabaena* sp. PCC 7120 via triparental conjugative method. The Δ*all4501* fragment was completely segregated into all chromosomal DNA copies of *Anabaena* transformant, yielding *Anabaena* sp. PCC 7120 strain Δall4501. Inactivation of the *all4501* gene did not alter photoautotrophic growth. The growth curves of *Anabaena* sp. PCC 7120 wild type and Δall4501 strains were very similar. The *Anabaena* sp. PCC 7120 strain Δall4501, however, decreased phosphate in the BG-11 medium faster than the wild type strain. From the equal initial cell inoculation $OD_{730\ nm}$ of 0.05 ± 0.01, the *Anabaena* sp. PCC 7120 strain Δall4501 took 3 days for decreasing phosphate concentrations of 5.51 ± 0.17 mg P/L down to 0.51 ± 0.33 mg P/L, while the wild type strain spent 6 days. Rapid decreasing of the phosphate concentrations in the medium of the *Anabaena* sp. PCC 7120 strain Δall4501was co-related with rapid increasing of cellular P content. The cellular P content was measured as total P in whole cells. The *Anabaena* sp. PCC 7120 strain Δall4501 could increased cellular P capacity up to 13.4 ± 2.2 mg P/g DW, while the wild type strain accumulated P at the average of 6.3 ± 0.6 mg P/g DW. However, the cellular P content of the Δall4501 strain was decreased to 9.1 ± 2.4 mg P/g DW on day 4. This decreasing might reflect the phosphate in the medium as during that time the phosphate concentration was only 0.18 ± 0.39 mg P/L. Fluorescence microscopy showed that cellular P in the *Anabaena* sp. PCC 7120 strain Δall4501 was mainly in form of polyphosphate. There were polyphosphate granules accumulated in every cell of *Anabaena* sp. PCC 7120 strain Δall4501. These results clearly showed that phosphate in the medium was taken up and stored inside the cells as polyphosphate.

High activities of alkaline phosphatase and phosphate-specific transport system of *Anabaena* sp. PCC 7120 strain Δall4501 even grown in phosphate-sufficient BG-11 medium also confirmed that the All4501 was negative regulator for phosphate-sensing system.

Wastewater treatment using *Anabaena* sp. PCC 7120 strain Δall4501 was performed in 5 L photobioreactor. The concentration of phosphate in recirculating aquaculture system wastewater in this study was 7.9 ± 0.6 mg P/L. The *Anabaena* sp. PCC 7120 strain Δall4501 could decrease phosphate efficiently down to 0.2 ± 0.1 mg P/L or 97% removal within 1 day of treatment. In contrast, the *Anabaena* sp. PCC 7120 wild type strain could remove phosphate only 57%. Separation of the *Anabaena* cells and treated water was simply done by settle down for 1 h. After draining the treated water out the *Anabaena* cells were reused for treatment repeatedly. Removal efficiencies of the *Anabaena* sp. PCC 7120 Δall4501 strain were very high at every cycle of the treatment; at least 94% of phosphate was removed. By contrast, the *Anabaena* sp. PCC 7120 wild type strain could show the best removal only 67% The average of phosphate removal rate of the *Anabaena* sp. PCC 7120 wild type and Δall4501 strain was 4.5 mg P/L/d and 7.7 mg P/L/d, respectively.

Objectives

To modify a new strain of filamentous cyanobacterium with high phosphorus uptake capability.

To develop the phosphorus removal system using filamentous cyanobacteria in photobioreactor for phosphorus treatment in the recirculating aquaculture systems.

Methodology

1. Cyanobacterial growth conditions

Anabaena sp. PCC 7120 was grown in BG-11 medium at 30 ± 3 ºC under constant illumination of 6000 lux. Liquid cultures were grown photoautotrophically in BG-11 aerating with filtered air or shaking 120 rpm on a shaker. For *Anabaena* sp. PCC 7120 strain Δal4501, cells were grown as above with the addition of 25 µg/mL neomycin. The phosphate-limiting BG-11 medium was prepared by replacing of 175 µM KCl instead of 175 µM K_2HPO_4 (Hirani et al, 2001). Cell growth was measured spectrophotometrically at an optical density of 730. Total chlorophyll *a* was extracted by 90% methanol and measured the absorbance at 663 nm (MacKinney, 1941). Cell dry weight was determined by collecting cells on the pre weighted 0.45 µm membrane filter. The cell paste was then dried at 80 ºC 24 h before weight determination.

2. Plasmids and Mutant construction

The genome data of cyanobacteria, including *Anabeana* sp. PCC 7120 is available online from CyanoBase (http://genome.microbedb.jp/cyanobase/Anabaena). A target gene for inactivation is negative regulator for phosphate-sensing system encoded in an open reading frame (ORF) "*all4501*". The *all4501* was deleted between 1 bp upstream of the GTG start codon and 9 bp downstream of the TAA stop codon as described below. The upstream and downstream regions of *all4501* were amplified by PCR using primers shown in table 1. The PCR products were ligated with pGEM-T easy vector. The 1.2 kb of neomycin-resistance (Nm^R) cassette was inserted in pGEM-containg upstream region vector at *the Spe I* site. The DNA fragment of upstream region and Nm^R was cut with Eco RI and then inserted at the *Sph I* site in pGEM-containg downstream region vector, yielding upstream region-Nm^R-downstream region vector or pΔall4501. The Δall4501 fragment was then cut with *Pvu II* and inserted to pRL271 at the *Pst I* site, yielding pRLΔall4501 vector or cargo vector. The

pRLΔall4501 was transformed into *Escherichia coli* HB101 producing *E.coli* cargo strain. The pRLΔall4501 was further transformed into *Anabeana* sp. PCC 7120 via triparental conjugative method (Elhai and Wolk, 1988). Briefly, the *E. coli* cargo strain was mixed with the *E. coli* helper strain (*E. coli* HB101 strain containing pRL623 helper vector). The *Anabaena* cells were then added into the *E. coli* mixture and incubated at 30 ºC under light illumination for 1 h. The mixture was spread on 0.45 µm membrane filter on BG-11 plate without antibiotic and put in the cyanobacterial growth chamber. After 3 days, the filter was transferred on a new BG-11 plate containing 25 µg/mL of neomycin. After the green colonies of tranformants were observed, single colony was restreaked on a new BG-11 plate containing 25 µg/mL of neomycin. The *Anabaena* tranformants were restreaked until the transformed gene of Δall4501 was complete segregation. To confirm complete segregation, colony PCR using "check" primer pairs as shown in table 1 was done.

Table 1 Oligonucleotide sequences for PCR amplification

name	sequence
f Upstream	TCAAAATCTGTCTCTCTCCT
r Upstream	CTTGTAGTCTCAAACGTGAA
f Downstream	GAGTATTTTTAAGCTCATCCCA
r Downstream	GGTAGCTATTTTCAAACATGAG
f Check	AGCTCACGGAGGTTCCATC
r Check	GGGAGGGATGGGATGAGCTT

3. Photobioreactor operation for phosphorus removal

A photobioreactor (PBR) in this study was a clear acrylic cylinder with working volume of 5 L (12 cm diameter and 60 cm height) under 8000 lux continuous illumination on both sides at 30 ± 2 ºC as shown in fig. 1. For P removal, the PBR was operated manually as a bubbling column mode with 22.5 h of continuous aeration, 1 h for settle down, and 0.5 h for water draining and refilling. The treated water was drained out using peristaltic pump (Masterflex C/L 60, USA). The wastewater in this study was

water from a 4000 L recirculating aquaculture system (RAS) indoor tilapia tank operated with bioflocs procedure (Nootong et al., 2011).

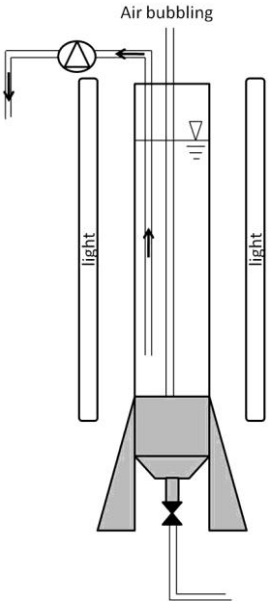

Figure 1. Diagram of a photobioreator for photosynthetic phosphorus removal

4. Measurement of phosphate and total phosphorus

Phosphate concentration was measured spectrophotometrically via ascorbic acid method (APAH, 1998). The mixed reagent of ammonium molybdate, sulfuric acid, ascorbic acid and potassium antimonyl-tartrate was prepared followed by the protocol. The water sample was filtered through GF-C membrane filter (Whatman, USA). The filtrate was added with mixed reagent and incubated at room temperature for 30 min before measured the absorbance at 885 nm. Total phosphorus was digested to phosphate by persulfate autoclave digestion method and analyzed as phosphate (Gross and Boyd 1998). For cellular P content, the *Anabaena* cells were washed twice with phosphate-limiting BG-11 and resuspended in sterile water. The cell suspension was then digested as total phosphorus before measuring as above.

5. Alkaline phosphatase assay

Culture was harvested and resuspended in phosphate-limiting BG-11. The assay buffer of 0.2 M Tris/HCl and 2 mM $MgCl_2$ was prepared at 940 µL and mixed with 30 µL of 120 mM p-nitrophenyl phosphate and 30 uL of cell suspension. The mixture was then incubated at 37 ºC for 20 min. After that, the 150 µL of 4 M NaOH was added to stop the reaction. The mixture was centrifuged at 12000 g for 5 min to remove cell debries. The presence of p-nitrophenol was measured at the absorbance of 400 nm (Hirani et al., 2001).

6. Phosphate uptake assay

Culture in either BG-11 or phosphate-limiting BG-11 was harvested and washed twice with phosphate-free Tris/HCl buffer pH 7.5. The cell concentration was adjusted to an $OD_{730\ nm}$ of 0.3. The uptake experiment was initiated by the addition of K_2HPO_4 solution to the cell suspension under continuous illumination of 6000 lux at room temperature. The residual phosphate was measured in the filtrate through 0.45 μm membrane filter (Burut-Archanai et al., 2011).

7. Detection of polyphosphate

Intracellular polyphosphate granules were detected under fluorescence microscopy, staining with 4'6-diamidino-2-phenylindole (DAPI) (Aschar-Sobbi et al., 2008; Burut-Archanai et al., 2013). The DAPI stock solution was prepared at 10 mM. For cell staining, the DAPI solution was added to the cell suspension making the final concentration of 10 μM and incubated in the dark for 5 min. Under the UV light excitation, the complex of DAPI-DNA shows blue fluorescence, while the complex of DAPI-polyphosphate is green.

Results

Characterization of the Anabaena sp. PCC 7120 strain Δall4501

Homozygosity of the *Anabaena* sp. PCC 7120 strain Δall4501 was shown in Fig. 2. A single band of 1.4 kb PCR fragment of *Anabaena* sp. PCC 7120 strain Δall4501 (MT) indicated complete segregation of the *Δall4501* gene into all chromosomal copies of the *Anabaena* sp. PCC 7120 strain Δall4501, while the wild type strain showed a 0.85 kb PCR fragment of the native gene.

The absence of the *all4501* gene was not affected the photoautotrophic growth in BG-11 medium as shown in Fig. 3. The growth curves of Δall4501 and wild type strains were similar at every time points. However, the Δall4501 strain exhibited higher phosphate removal efficiency and storing higher cellular P content than the wild type as shown in Fig. 4. The Δall4501 strain could decrease phosphate concentration in BG-11 medium to 0.5 mg P/L within 3 days, while the wild type strain took longer time of 6 days (Fig. 4A). The cellular P content was analyzed by digesting of the whole cells

shown in Fig. 3B. The cellular phosphorus content of Δall4501 strain was fluctuated, initial content of 7.9 ± 1.1 mg P/ g DW and increased up to 13.4 ± 2.2 1 mg P/ g DW or 1.7 times increased at day 3 and decreased to 9.1 ± 2.4 1 mg P/ g DW afterward. Decreasing of the cellular P content reflected on the phosphate concentrations in the medium. By contrast, the wild type strain showed very low fluctuated phosphorus content at each growth phase with the average of 6.3 mg P/g cell dry weight.

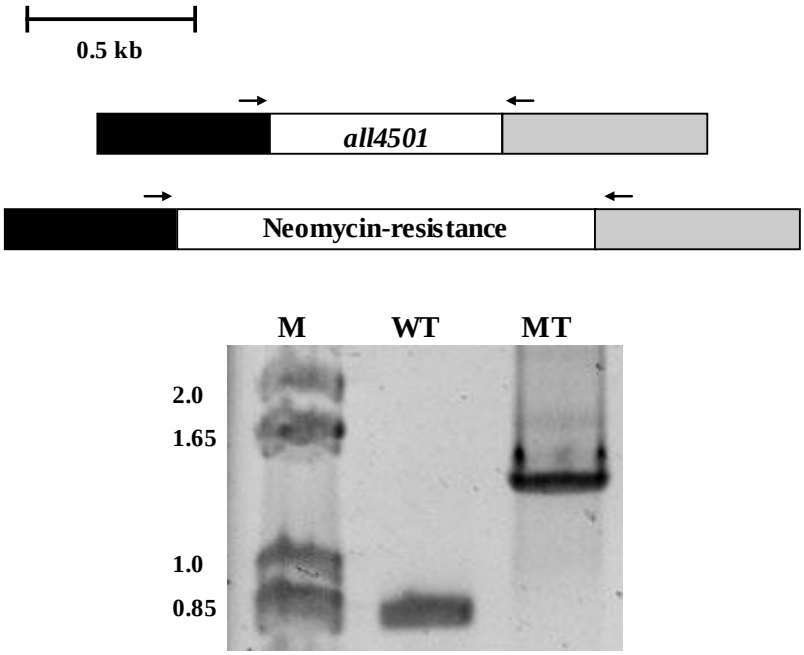

Figure 2. PCR demonstrating complete segregation of the neomycin-resistance cassette in the *Anabaena* sp. PCC 7120 strain Δall4501 (MT) comparing with the wild type (WT) strain. Diagram above shows *all4501* gene in WT and neomycin-resistance cassette in MT strain which black and gray boxes represent upstream and downstream regions of *all4501*. Arrows indicate the position of the PCR primers. Lower panel shows agarose gel electrophoresis of colony PCR of the *Anabaena* sp. PCC 7120 strain wild type (WT) and Δall4501 (MT), with 1 kb Plus DNA ladder (lane M).

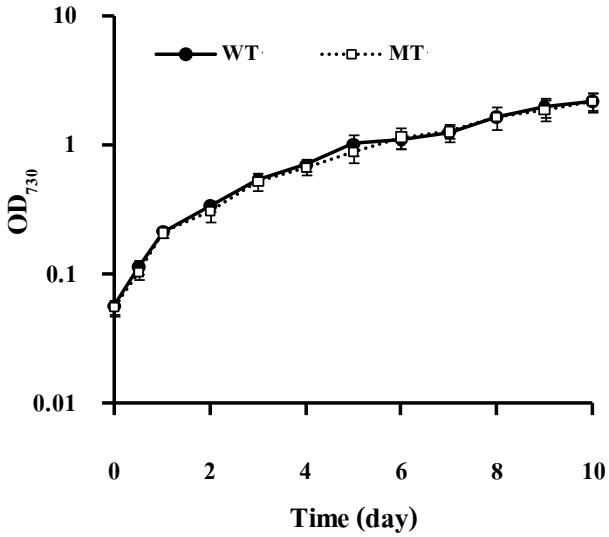

Figure 3. Photoautotrophic growth of the *Anabaena* sp. PCC 7120 wild type (WT, black circles) and Δall4501 (MT, white squares) strains.

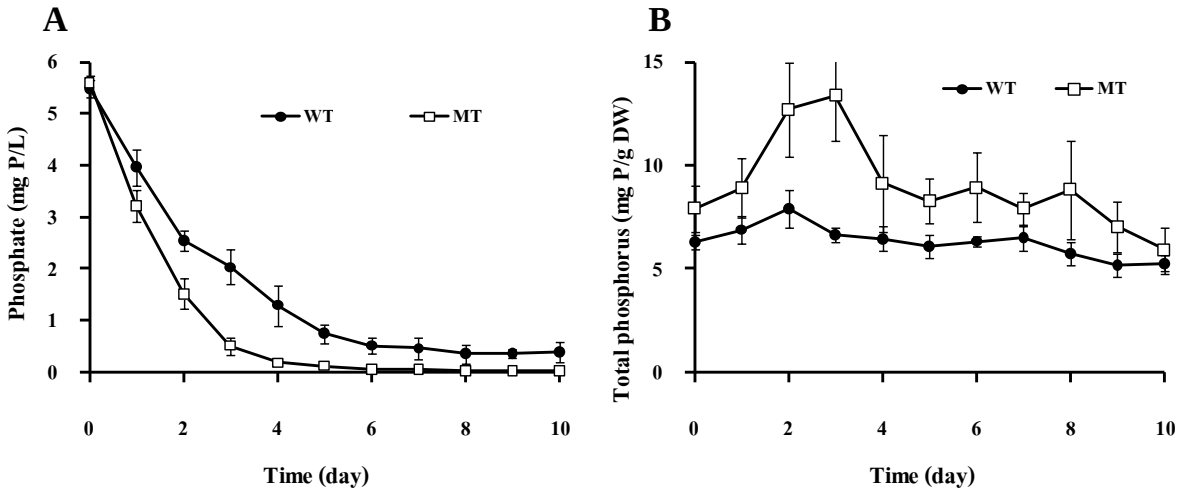

Figure 4. Concentrations of phosphate in the growth medium (A) and cellular phosphorus concentrations (B) of the *Anabaena* sp. PCC 7120 wild type (WT, black circles) and Δall4501 (MT, white squares) strains.

After 2 days cultivation, the *Anabaena* sp. PCC 7120 wild type and Δall4501 strains were stained with DAPI and observed under fluorescence microscopy for polyphosphate content detection. The DAPI-staining results clearly showed that the Δall4501 strain stored large amount of phosphate in form of intracellular polyphosphate granules as shown in Fig. 5. The green fluorescence of DAPI-polyphosphate complex was found at all cells of the Δall4501, whereas it was absent in the wild type strain. Only blue fluorescence of DAPI-DNA complex was found in the wild type strain.

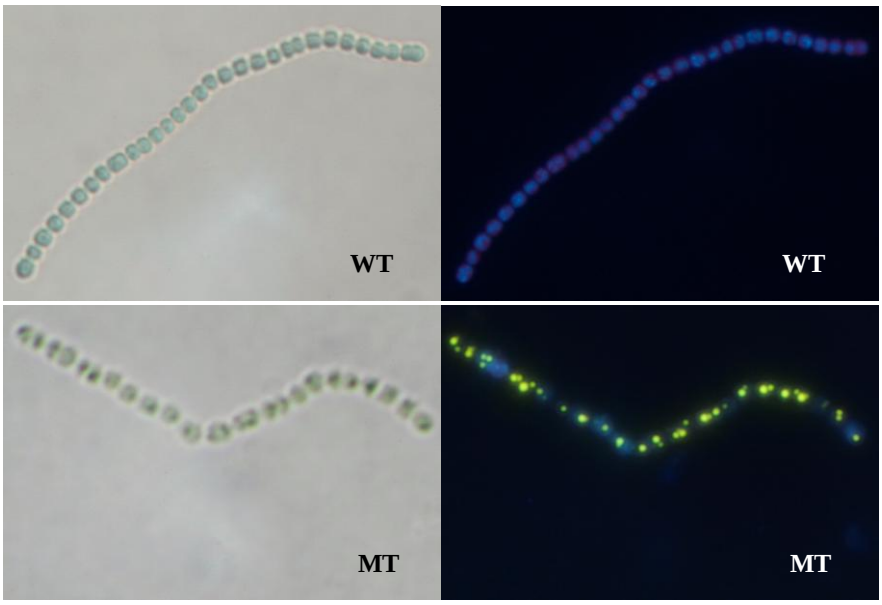

Figure 5. Microscopic images of *Anabaena* sp. PCC 7120 wild type (WT, upper panel)/ and Δall4501 (MT, lower panel) strains with DAPI staining under visible light (left panel) and UV light (right panel). Green fluorescence indicates intracellular polyphosphate granules in the *Anabaena* sp. PCC 7120 strain Δall4501.

The Pho regulon, including alkaline phosphatase (PhoA) and phosphate-specific transport (Pst) system, expression was also determined in the *Anabaena* sp. PCC 7120 wild type and Δall4501 strains. For wild type strain, the Pho regulon expression is repressed under phosphate-sufficient conditions, and highly expressed under phosphate-limiting conditions (Hirani et al., 2001; Suzuki et al., 2004; Juntarajumnong et al., 2007). Both strains of *Anabaena* were grown in either BG-11 or phosphate-limiting BG-11 for 2 days before measuring of alkaline phosphatase activity. Figure 6 shows the activities of alkaline phosphatase of both strains. The wild type strain well regulated the alkaline phosphatase as mentioned above. In contrast, the *Anabaena* sp. PCC 7120 strain Δall4501 were constitutively expressed alkaline phosphatase, a member of Pho regulon, even cells were in phosphate-sufficient condition.

The activity of Pst system was also measured and shown in Fig. 7. Under phosphate-sufficient condition, phosphate uptake of wild type was monophasic with very low activity. The biphasic phosphate uptake curve was found when cells grown under phosphate-limiting condition with high rate of phosphate uptake during 10 min of experiment and lower rate of phosphate uptake afterward, indicating the different activities of 2 phosphate transport systems. In contrast, the Δall4501 strain had similar biphasic phosphate uptake pattern under either phosphate-sufficient or phosphate-limiting conditions. These results clearly showed that the Pho regulon genes were constitutively expressed in the Δall4501 strain. On the other hand, the *all4501* functions as negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120.

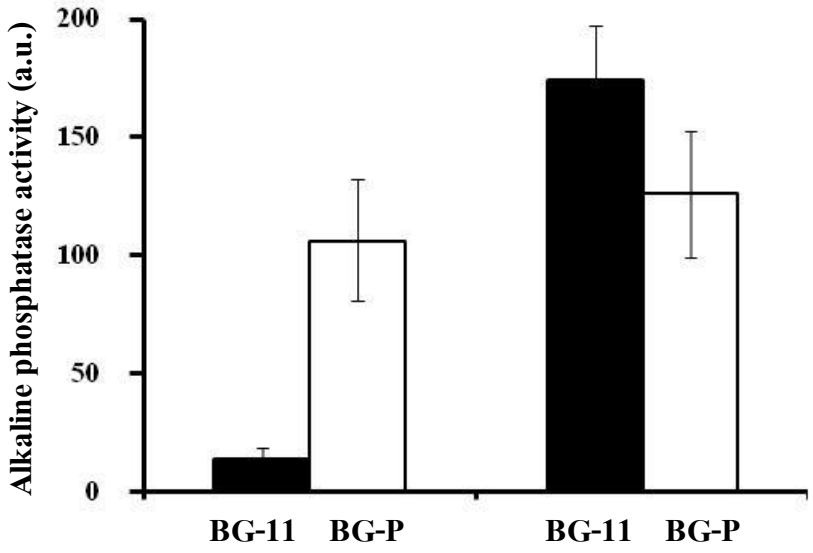

Figure 6. Alkaline phosphatase activity of *Anabaena* sp. PCC 7120 wild type (WT) and Δall4501 (MT) strains under phosphate-sufficient (BG-11, black bars) and phosphate-limiting (BG-P, white bars) conditions.

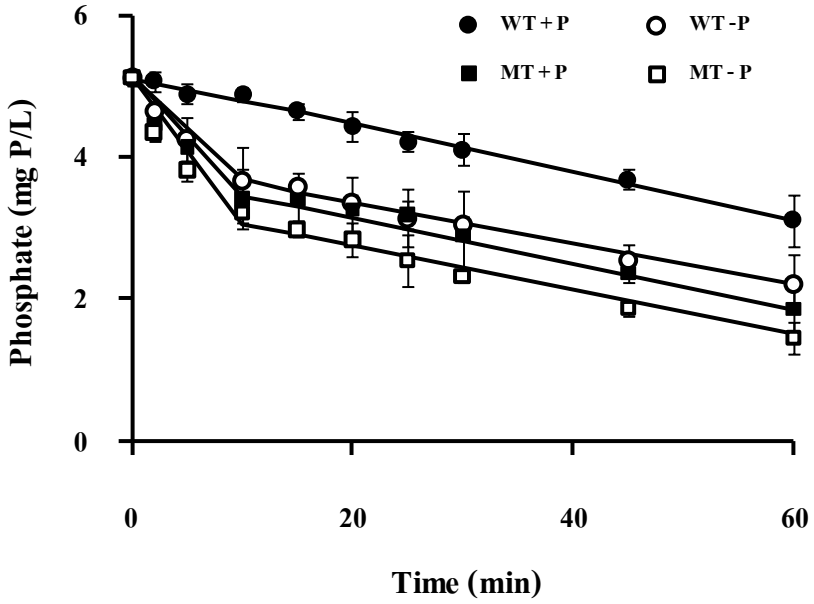

Figure 7. Phosphate uptake activity of *Anabaena* sp. PCC 7120 wild type (WT, circles) and Δall4501 (MT, squares) strains under phosphate-sufficient (black symbols) and phosphate-limiting (white symbols) conditions.

Autoflocculation property of the Anabaena sp. PCC 7120

The main problem in algal/cyanobacterial biotechnology is separation of the biomass and water body. Using *Anabaena* sp. PCC 7120 could overcome this problem as the *Anabaena* cells are large with the diameter of 3-4 µm and filamentous (Flores and Herrrero, 2010; Chen et al., 2014). With simple settle down method, the filaments were aggregated and down to bottom completely within 1 h as shown in Fig. 8.

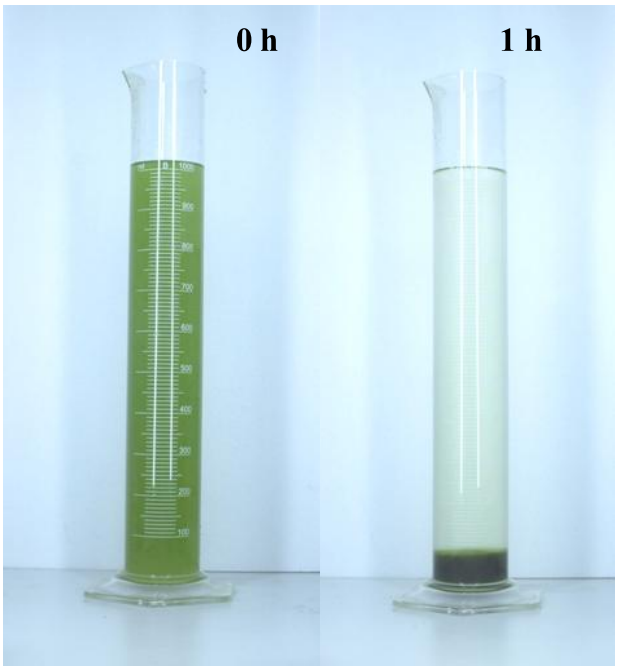

Figure 8. Autoflocculation process of the *Anabaena sp. PCC 7120*

Wastewater treatment by Anabaena sp. PCC 7120 Δall4501 strain in photobioreactor

The *Anabaena* sp. PCC 7120 wild type and Δall4501 strains were initially grown in the photobioreactor (PBR) with BG-11 medium until the cell concentration of 5 mg chl *a*/L (~OD_{730nm} of 0.4). After that, the medium was drained out of the PBR and refilled with wastewater. The wastewater in this experiment was water from 4000 L recirculating aquaculture tank. The treatment result was shown in Fig. 9 which clearly showed that the *Anabaena* sp. PCC 7120 Δall4501 strain could efficiently remove phosphate from average of 7.9 mg P/L to lower than 0.25 mg P/L within 1 day. The wild type strain also showed the activity of phosphate removal but at the lower extent. Both strains could be reused for phosphate treatment up to 10 cycles, after that the cells became yellow-brown attaching on the photobioreactor wall. The average of phosphate removal of the wild type and Δall4501 strain was 57.4 % and 96.9 %, respectively. On the other hand, the wild type could decrease phosphate concentrations down to 3.3 ± 0.6 mg P/L, while the Δall4501 strain did 0.2 ± 0.1 mg P/L.

Total phosphorus removal from single inoculation of the *Anabaena* sp. PCC 7120 wild type and Δall4501 strain in the photobioreactor was 2.0 g P and 3.5 g P, respectively. The average of phosphate removal rate of the wild type and Δall4501 strain was 4.5 mg P/L/d and 7.7 mg P/L/d, respectively.

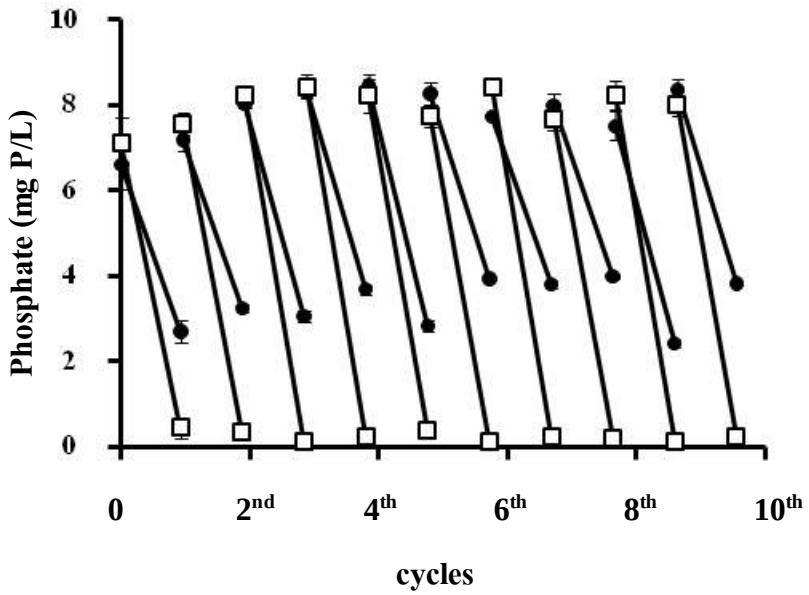

Figure 9. The concentrations of residual phosphate in photobioreactor during phosphate treatment by *Anabaena* sp. PCC 7120 wild type (black circles) and Δall4501 (white squares) strains.

Discussion

A target gene for deletion mutagenesis of the *Anabaena* sp. PCC 7120 in this study was all4501, which was replaced with the 1.2 kb of neomycin-resistance cassette. The all4501 gene of Anabaena sp. PCC 7120 shares strong similarity with the slr0741 (sphU) of Synechocystis sp. PCC 6803 to 77% in deduced amino acid sequences (64% identity). The sphU gene was previously identified as a negative regulator for two-component phosphate sensing system (Juntarajumnong et al., 2007). Inactivation of this gene results in constitutive expression of the genes in Pho regulon, including alkaline phosphatase (*phoA*), phosphate specific transport system (*pstSCAB*), either *in Escherichia coli* or *Synechocystis* sp. PCC 6803 (Juntarajumnong et al., 2007; Lamarche et al., 2008; Burut-Archanai et al., 2009). In this study, it was shown that the all4501 is the negative regulator for phosphate sensing system in *Anabaena* sp. PCC 7120. Inactivation of the *all4501* in *Anabaena* sp. PCC 7120 strain Δall4501 resulted in constitutive expression of the Pho regulon genes, alkaline phosphatase (*phoA*) and phosphate specific transport system (*pstSCAB*), detected by their activities, without any effect on the growth (Fig. 2-7). The Δall4501 strain was also capable to store phosphate 2 times higher than the wild type, in form of intracellular polyphosphate (Fig. 4 and 5). It might be explained that the genes for polyphosphate metabolism, *ack* and

pta, were constitutively expressed in the Δall4501 strain (Juntarajumnong et al., 2007; Burut-Archanai et al., 2013).

Phosphorus removal using photosynthetic organisms have been widely studies (Chevalier et al., 2000; Martinez et al., 2000; Hernandez et al., 2006; Fierro et al., 2008; Zhang et al., 2008; Burut-Archanai et al., 2013). They performed high phosphorus removal efficiency at low concentrations of phosphate (~1 mg P/L). At high phosphate concentration (over 5 mg P/L), none of the wild type strain could remove phosphate down to 0.5 mg P/L. This manner was a result of phosphate specific transport system (pstSCAB), a member of Pho regulon, was repressed under high concentration of phosphate (Suzuki et al., 2004; Lamarcheet al., 2008). High performance phosphate removal have been shown in *Synechocystis* sp. PCC 6803 strain ΔSphU (Burut-Archanai et al., 2013) and the *Anabaena* sp. PCC 7120 strain Δall4501 (Fig. 4A and 9). Comparing the cell capacity for phosphate storing, the *Anabaena* sp. PCC 7120 strain Δall4501 could store phosphate 20 times higher than the *Synechocystis* sp. PCC 6803 strain ΔSphU (Burut-Archanai et al., 2013). In addition, cell harvesting and separation between *Anabaena* biomass and water medium were able to perform spontaneously by gravitational settle down, so called “auto-flocculation process” (Chen et al., 2014). For these reasons, the *Anabaena* sp. PCC 7120 strain Δall4501 are very interesting for developing large scale photobioreactor with simple sedimentation for cell harvesting. Phosphorus removal form RAS using *Anabaena* in this study was able to repeat 10 cycles of the treatment (Fig. 9). After that, the *Anabaena* cells were aggregated and unable to be resuspended homogeneously. This event reflected RAS pond, which was operated with bioflocs technology. The microorganisms in bioflocs produced various flocculating agents into the water medium (De Schryver et al, 2008). The presence of flocculating agents in RAS water might induce floc formation of *Anabaena* cells in the photobioreactor.

Future perspectives

Negative regulator for phosphate-sensing system is an ideal target gene for developing biological phosphorus removal system. Inactivation of this gene enhanced phosphate uptake activity and capacity for P storage without negative effect on cell growth. This P removal photobioreactor was simply designed and operated which was economic and laborless. The further study might integrate this photobioreactor with the recirculating aquaculture system. In addition, this knowledge might be applied to others

cyanobacteria those are able to grow under desired conditions for wastewater treatment. Inactivation of the negative regulator also increased P content of biomass, which might be used for high P feed or fertilizer. Using this cyanobacterium as feed could recycle the nutrients back to aquatic organisms.

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Abstract: A negative regulator for phosphate-sensing system in Anabaena sp. PCC 7120 is encoded by all4501. The phosphorus availability and total cellular phosphorus content were enhanced in the (delta)all4501 strain lacking this negative regulator, while, growth curve was similar to the wild type. The initial rate of phosphate uptake and cellular phosphorus content of the (delta)all4501 strain were 8-times and 2-times higher than the wild type strain. Increasing of cellular phosphorus content was clearly shown that phosphorus was stored as polyphosphate granules. Phosphorus removal from recirculating aquaculture system using the Anabaena sp. PCC 7120 strain (delta)all4501 was performed in a 5 L photobioreactor. Separation of treated water and cyanobacterial cells could be achieved spontaneously via simple settle down method. With single starter cell inoculation, the photobioreactor could be repeatedly used for phosphorus removal up to 10 cycles with the average phosphorus removal efficiency of 57.4 % and 96.9 % for wild type and (delta)all4501 strains, respectively.

Cover Letter

October 12, 2016

Dear Editor

Please find attached the electronic submission of our manuscript entitled “Identification of negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120: A target gene for developing phosphorus removal” for the consideration of publication as a short communication. Surachet Burut-Archanai and Sorawit Powtongsook mutually agree for submitting this manuscript to “Biochemical Engineering Journal” under classification of “40.090: other environmental bioengineering”. Data from this manuscript is our original work and it has never been published elsewhere.

The paper demonstrates that an open-reading frame *all4501* of *Anabaena* sp. PCC 7120 is a negative regulator for phosphate-sensing system. Inactivation of the *all4501*, yielding the Δall4501 strain, results in constitutive expression of Pho regulon genes, indicated by alkaline phosphatase and phosphate uptake activities. The Δall4501 strain removes phosphate effectively by taking up phosphate and storing as intracellular polyphosphate granules. The Δall4501 strain shows high potential for phosphate removal as the phosphate concentration of water from aquaculture system could be decreased down to 0.2 mg P/L by this strain or 13-times lower than the wild type strain. In addition, separation between treated water and cyanobacterial cells which is the main problem in microalgae utilization, could simply achieve via settle down method. With single inoculation of the cells into a 5 liters photobioreactor, the phosphate removal could be repeatedly performed up to 10 cycles. The concentrations of phosphate from aquaculture system water could be removed 96.9 % by the Δall4501 strain.

Sincerely,

Surachet Burut-Archanai

***Highlights (for review)**

HIGHLIGHT

- The ORF *all4501* encodes negative regulator for phosphate-sensing system.
- Inactivation of all4501 resulted in constitutive expression of Pho regulon genes.
- Phosphate uptake and cellular phosphorus content were increased in Δall4501 strain.
- The Δall4501 strain could remove phosphate from raw RAS water repeatedly.

Identification of negative regulator for phosphate-sensing system in Anabaena sp. PCC 7120: A target gene for developing phosphorus removal

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ABSTRACT

A negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120 is encoded by *all4501*. The phosphorus availability and total cellular phosphorus content were enhanced in the Δall4501 strain lacking this negative regulator, while, growth curve was similar to the wild type. The initial rate of phosphate uptake and cellular phosphorus content of the Δall4501 strain were 8-times and 2-times higher than the wild type strain. Increasing of cellular phosphorus content was clearly shown that phosphorus was stored as polyphosphate granules. Phosphorus removal from recirculating aquaculture system using the *Anabaena* sp. PCC 7120 strain Δall4501 was performed in a 5 L photobioreactor. Separation of treated water and cyanobacterial cells could be achieved spontaneously via simple settle down method. With single starter cell inoculation, the photobioreactor could be repeatedly used for phosphorus removal up to 10 cycles with the average phosphorus removal efficiency of 57.4 % and 96.9 % for wild type and Δall4501 strains, respectively.

Keywords; recirculating aquaculture system; phosphorus removal; cyanobacteria; negative regulator

1. Introduction

Recirculating aquaculture systems (RASs) are environmental friendly with high production yield and less water demand. Without water exchange, RAS technology requires the appropriate technology to minimize solids and nutrients accumulation in the water [1-3]. The removals of either solids or nitrogenous compounds in the RASs have been achieved via several treatment technologies [1-4]. In contrast, removal of phosphorus from the RAS is more complex, the practical phosphorus removal technology is not yet available [2]. Phosphorus is the major nutrient causing eutrophication [5]. Discharge of phosphorus into the natural water resources must be well regulated. Previous study on phosphorus accumulation in the RAS shown that over 94% of phosphorus was in form of inorganic phosphates [6]. Traditional chemical phosphorus removal process is not suitable with the RAS due to high toxicity of heavy metal for chemical precipitation processes that may harm to fish. Biological phosphorus removal using heterotrophic polyphosphate accumulating organisms (PAOs) have been extensively studied [7, 8]. They require a closed reactor for anaerobic process incorporating with organic carbon addition. Although, the removal efficiency is not stable as the microorganisms

responsible for PAOs are uncultured, and the whole mechanisms of phosphorus removal especially ecology of microbial populations are still unclear [9, 10].

Alternatively, phototrophic organisms such as algae and cyanobacteria are more attractive for phosphorus removal [6, 11, 12]. The photosynthetic phosphate removal process requires neither addition of carbon source, nor enclosed system for anaerobic condition. They take up phosphate directly and massively store in the cells as polyphosphate granules. The cyanobacterial phosphate specific transport system (Pst system) was previously studied and clearly shown that this process was energy-dependent requiring only light energy [13]. In addition, the affinity for phosphate of Pst system was very high with the transport constant (K_s) of 4 μg P/L [13]. In prokaryotic organisms, two-component phosphate sensing system is used for monitoring the extracellular phosphate concentration [14]. Inactivation of a negative regulator for phosphate sensing system (PhoU or SphU) resulted in constitutive expression of genes involving in phosphorus metabolism, for example, alkaline phosphatase and phosphate specific transport system [14, 15]. A previous study has shown that the cyanobacterium *Synechocystis* sp. PCC 6803 strain ΔSphU efficiently removed phosphate in the RAS [6]. However,

the separation of the treated water and cyanobacterial cells with less than 2 μm in size was complicated as it cannot be separated by regular filtration device. In this study, the filamentous *Anabaena* sp. PCC 7120 was selected to overcome the cell separation problem of the unicellular cyanobacterium. With the auto-flocculation property of the *Anabaena* sp. PCC 7120, cell separation was able to simply perform via settle down method [16]. The *Anabaena* genome also shares very high sequence similarity in phosphate sensing system with the well studied *Synechocystis* sp. PCC 6803. An open reading frame of *all4501,* sharing strong similarity with the *slr0741* (*sphU*) of *Synechocystis* sp. PCC 6803 to 77% in deduced amino acid sequences (64% identity), was a target for mutagenesis in this study.

2. Materials and Methods

2.1 Culture conditions and strain construction

Anabaena sp. PCC 7120 was grown in 250 mL flask containing 100 mL BG-11 medium under continuous shaking at 120 rpm and white light illumination at 6000 lux under room temperature 30 ± 3 ºC. Cell density was measured at an optical density of 730 nm (OD_{730}), and total chlorophyll *a*

concentration was determined by spectrophotometer after extracted with 90% methanol [17]. The target gene for deletion mutagenesis was *all4501* encoding a putative negative regulator for phosphate sensing system. The 1.2 kb of neomycin-resistance cassette was replaced between 1 bp upstream of the GTG start codon and 9 bp downstream of the TAA stop codon of the 0.67 kb of *all4501* gene via ligation of neomycin-resistance cassette with upstream and downstream regions of *all4501*. Both upstream and downstream regions of *all4501* were amplified by PCR using primers shown in table 1. The Δ*all4501:Nm* was then transformed into *Anabaena* sp. PCC 7120 via triparental conjugation [18]. The transformants were screened on BG-11 plate containing 20 μg/mL neomycin. The complete segregation of the Δ*all4501* gene in genomic DNA was confirmed by colony PCR with specific primers shown in table 1. For phosphate-limiting BG-11 medium, K_2HPO_4 was replaced by KCl at the same concentration [15, 19].

2.2 Analytical methods

Phosphate concentration was measured spectrophotometrically via ascorbic acid method [20]. Total phosphorus was digested to phosphate by persulfate autoclave digestion method and analyzed as phosphate [21]. Intracellular polyphosphate granules were detected under fluorescence microscopy, staining with 4'6-diamidino-2-phenylindole (DAPI) [6, 22]. Alkaline phosphatase activity was determined by the hydrolysis of *p*-nitrophenyl phosphate [15, 19]. Phosphate uptake study was done according to Burut-Archanai et al. [13].

2.3 Phosphate removal in a photobioreactor

A photobioreactor in this study was a clear acrylic cylinder with working volume of 5 L (12 cm diameter and 60 cm height) operated under 6000 lux continuous illumination at 30 ± 2 ºC. Starter culture of *Anabaena* in BG-11 medium was inoculated to the initial concentration of 1.8 mg Chl *a*/L. The photobioreactor was operated manually as a bubble column mode with 23 h of continuous aeration. Once a day, the aeration was paused for 1 h allowing 45 min of cell settling and 15 min of water draining and refilling with the water from RAS. The RAS in this study was a 4000 L indoor tilapia tank

operated with bioflocs procedure [23]. To evaluate phosphate removal efficiency, 7 L of the raw water containing high phosphate concentration from the RAS was withdraw to 10 L settling tank and left for 1 hour settlement. Water from upper layer was then transferred into the photobioreactor. The cells density and phosphate concentration in the photobioreactor was measured using water sampling from the center of the reactor. The residual phosphate in the water was measured after the sample filtration through 0.45 μm membrane filter.

3. Results and discussion

3.1 Growth and cellular phosphorus content of the Anabaena sp. PCC 7120 strain Δall4501

Complete segregation of the *Δall4501:Nm* gene in the genomic DNA of *Anabaena* sp. PCC 7120 was demonstrated by colony PCR using "Check" primer pairs shown in table 1. In Fig. 1A, a single band of 1.4 kb PCR fragment of *Anabaena* sp. PCC 7120 strain Δall4501 (MT) indicated homozygous genotype of this strain, while the wild type strain showed a 0.85 kb PCR fragment. The absence of the *all4501* gene was not affected the photoautotrophic growth in BG-11 medium as shown

in Fig. 1B. Inactivation of the negative regulator for phosphate sensing system (sphU) in *Synechocystis* sp. PCC 6803 also had no negative effect on photoautotrophic growth [6, 15]. However, the Δall4501 strain exhibited higher phosphate removal efficiency and capacity as shown in Fig. 1C and 1D, respectively. To decrease the phosphate concentration in BG-11 medium to 0.5 mg P/L, the Δall4501 strain took 3 days while the wild type strain took longer time of 6 days. Total phosphorus concentration was analyzed in whole cells to indicate the cellular phosphorus content of each strain. The wild type strain showed very low fluctuated phosphorus content at each growth phase with the average of 6.3 mg P/g cell dry weight. In contrast, the cellular phosphorus content of Δall4501 strain was more fluctuated with an initial concentration of 7.9 mg P//g cell dry weight and increased up to of 13.4 mg P/g cell dry weight within 3 days. Afterward, the phosphorus content was decreased to an average of 8.0 mg P/g cell dry weight. Decreasing of the phosphorus content of the Δall4501 strain after 3 days might reflect the phosphorus availability in the medium as the phosphate concentrations were lower than 0.17 mg P/L. Noted that, the maximum capacity for cellular phosphate storing of the *Anabaena* sp. PCC 7120 strain Δall4501 was 20 times higher than the *Synechocystis* sp. PCC 6803 strain ΔSphU [6].

3.2 Detection of polyphosphate, alkaline phosphatase and phosphate uptake

After 2 days cultivation, the *Anabaena* sp. PCC 7120 wild type and Δall4501 strains were stained with DAPI and observed under fluorescence microscopy as shown in Fig. 2. The results clearly showed that the Δall4501 strain stored large amount of phosphate in form of intracellular polyphosphate granules. The green fluorescence of polyphosphate-DAPI complex was found at all cells of the Δall4501, whereas it was absent in the wild type strain. This result clearly supported the above result that higher phosphorus content of the Δall4501 over wild type strain. It might be explained that the genes for polyphosphate metabolism were constitutively expressed in the Δall4501 strain [15, 24].

The phosphate uptake and alkaline phosphatase activities were observed in both strains under either phosphate-sufficient or phosphate-limiting conditions and shown in Fig. 3A. The activity of phosphate uptake of wild type strain under phosphate-sufficient condition was monophasic with very low activity. The biphasic phosphate uptake curve was found when cells grown under phosphate-limiting condition with high rate of phosphate uptake during 10 min of experiment and lower rate of

phosphate uptake afterward, indicating the different activities of 2 phosphate transport systems. In contrast, the Δall4501 strain had similar biphasic phosphate uptake pattern under either phosphate-sufficient or phosphate-limiting conditions. These results suggested that the high affinity phosphate transport system, a member of Pho regulon, was constitutively expressed in the Δall4501 strain. The constitutive expression of the Pho regulon of the Δall4501 strain was also confirmed by the alkaline phosphatase activity. The alkaline phosphatase activity of the Δall4501 strain was found under either phosphate-sufficient or phosphate-limiting conditions, while it was repressed in the wild type strain grown under phosphate-sufficient conditions (Fig. 3B). These results indicated that all genes in Pho regulon, such as, alkaline phosphatase, phosphate specific transport system, and polyphosphate kinase were constitutively expressed in the Δall4501 strain.

3.3 Removal of phosphate from aquaculture water

The *Anabaena* cells were initially grown in the photobioreactor with BG-11 medium until the cell concentration reached 5 mg chl *a*/L (~OD_{730nm} of 0.4). After cells sedimentation, the medium was

drained out and the photobioreactor was refilled with RAS water. The treatment result was shown in Fig. 4 which clearly showed that the *Anabaena* sp. PCC 7120 Δall4501 strain could efficiently remove phosphate from average of 7.9 mg P/L to lower than 0.25 mg P/L within 23 h. The wild type strain also showed the activity of phosphate removal but at the lower extent. With simple settle down method, over 95% of the cells could be retained in the photobioreactor as similar to previous report [16]. The phosphate removal using photobioreactor could be performed up to 10 cycles from single inoculation with the average of phosphate removal efficiency of 57.4 % and 96.9 % via wild type and Δall4501, respectively. Total phosphorus removal of the *Anabaena* sp. PCC 7120 wild type and Δall4501 strain in the photobioreactor was 2.0 g P and 3.5 g P, respectively. Both strains could be reused for phosphate treatment up to 10 cycles, after that the cells were aggregated, attaching on the photobioreactor wall and unable to be resuspended homogeneously. This finding might be related with the RAS with bioflocs procedure. The microorganisms in bioflocs produced various natural flocculating agents into the water medium [25] which might induce flocculation of *Anabaena* cells in the photobioreactor.

4. Conclusion

Inactivation of *all4501* resulted in constitutive expression of the Pho regulon genes without negative effect on photoautotrophic growth, suggesting the *all4501* encoding for a negative regulator for phosphate sensing system. The Δall4501 strain removed phosphate 2 times faster and stored cellular phosphorus 2 times higher than the wild type strain. The Δall4501 strain removed phosphate from raw RAS water from 7.9 mg P/L to lower than 0.25 mg P/L within 23 h which equivalent to 96.9 % removal efficiency. In contrast, the wild type strain performed only 57.4 % phosphate removal.

Acknowledgments

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Figure legend

Fig 1. Characterization of *Anabaena* sp. PCC 7120 wild type (WT) and Δall4501 (MT) strains. (A) PCR demonstrating complete segregation of the neomycin-resistance cassette in the MT strain comparing with WT strain. Diagram above shows *all4501* gene in WT and neomycin-resistance cassette in MT strain which black and gray boxes represent upstream and downstream regions of *all4501*. Arrows indicate the position of the PCR primers. (B) Photoautotrophic growth, (C) the concentrations of residual phosphate in the medium, and (D) the cellular phosphorus content of WT (black circle symbols) and MT (white square symbols) strains grown in BG-11 medium.

Fig 2. Microscopic images of *Anabaena* sp. PCC 7120 wild type (WT) and Δall4501 (MT) strains with DAPI staining under visible light (left panel) and UV light (right panel). Green fluorescence indicates intracellular polyphosphate granules in the mutant strain.

Fig 3. Phosphate uptake and alkaline phosphatase activity of *Anabaena* sp. PCC 7120 wild type and Δall4501 strains under phosphate-sufficient and phosphate-limiting conditions. (A) Phosphate uptake

of wild type (circle symbols) and Δall4501 (square symbols) strains grown under phosphate-sufficient (black symbols) and phosphate-limiting (white symbols) conditions. (B) Alkaline phosphatase activity of wild type (WT) and Δall4501 (MT) strains grown under phosphate-sufficient (black bars) and phosphate-limiting (white bars) conditions.

Fig 4. The concentrations of residual phosphate in photobioreactor during phosphate treatment by *Anabaena* sp. PCC 7120 wild type (black circle symbols) and Δall4501 (white square symbols) strains.

Table 1 Oligonucleotide sequences for PCR amplification

name	sequence
f Upstream	TCAAAATCTGTCTCTCTCCT
r Upstream	CTTGTAGTCTCAAACGTGAA
f Downstream	GAGTATTTTTAAGCTCATCCCA
r Downstream	GGTAGCTATTTTCAAACATGAG
f Check	AGCTCACGGAGGTTCCATC
r Check	GGGAGGGATGGGATGAGCTT

Figure

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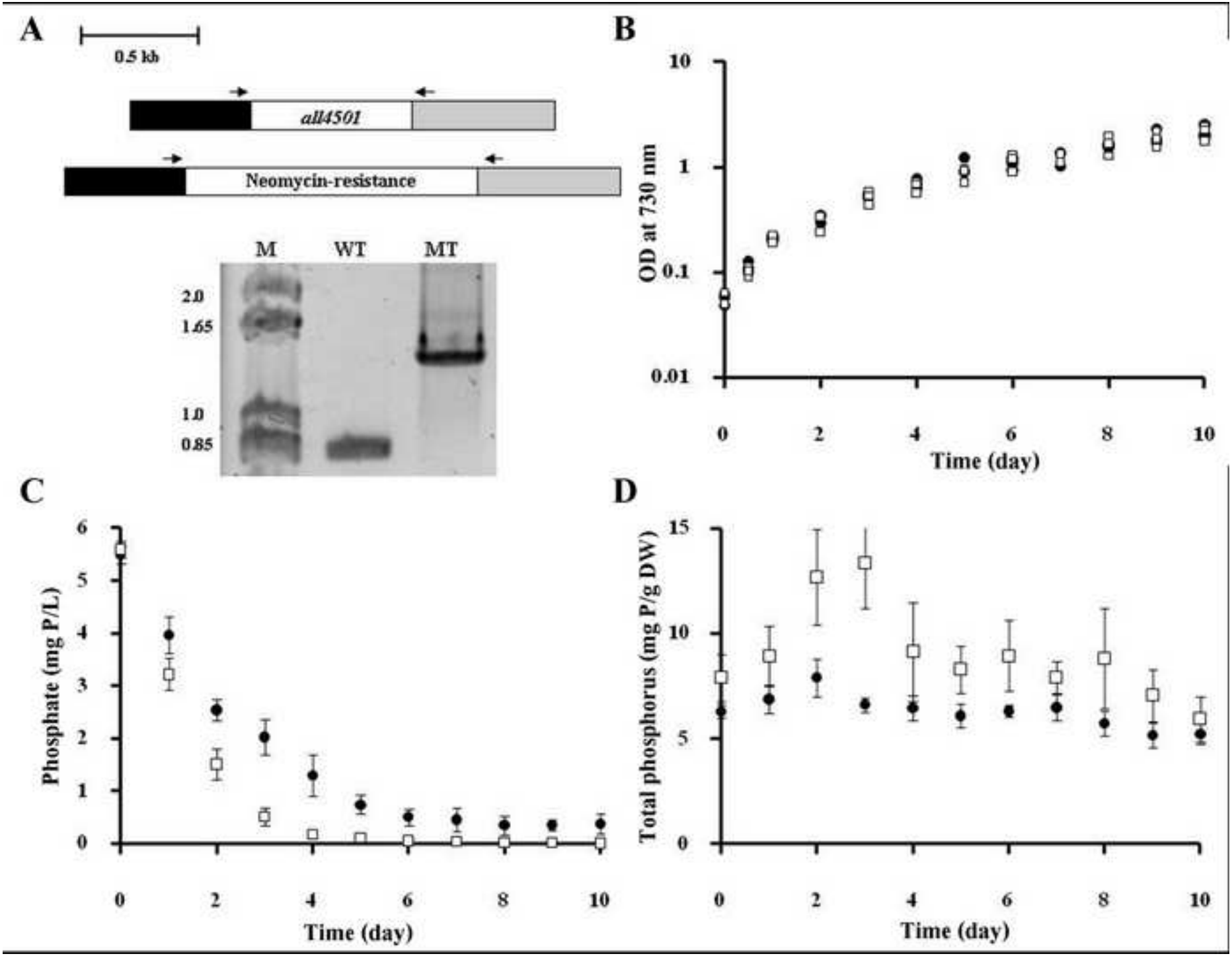

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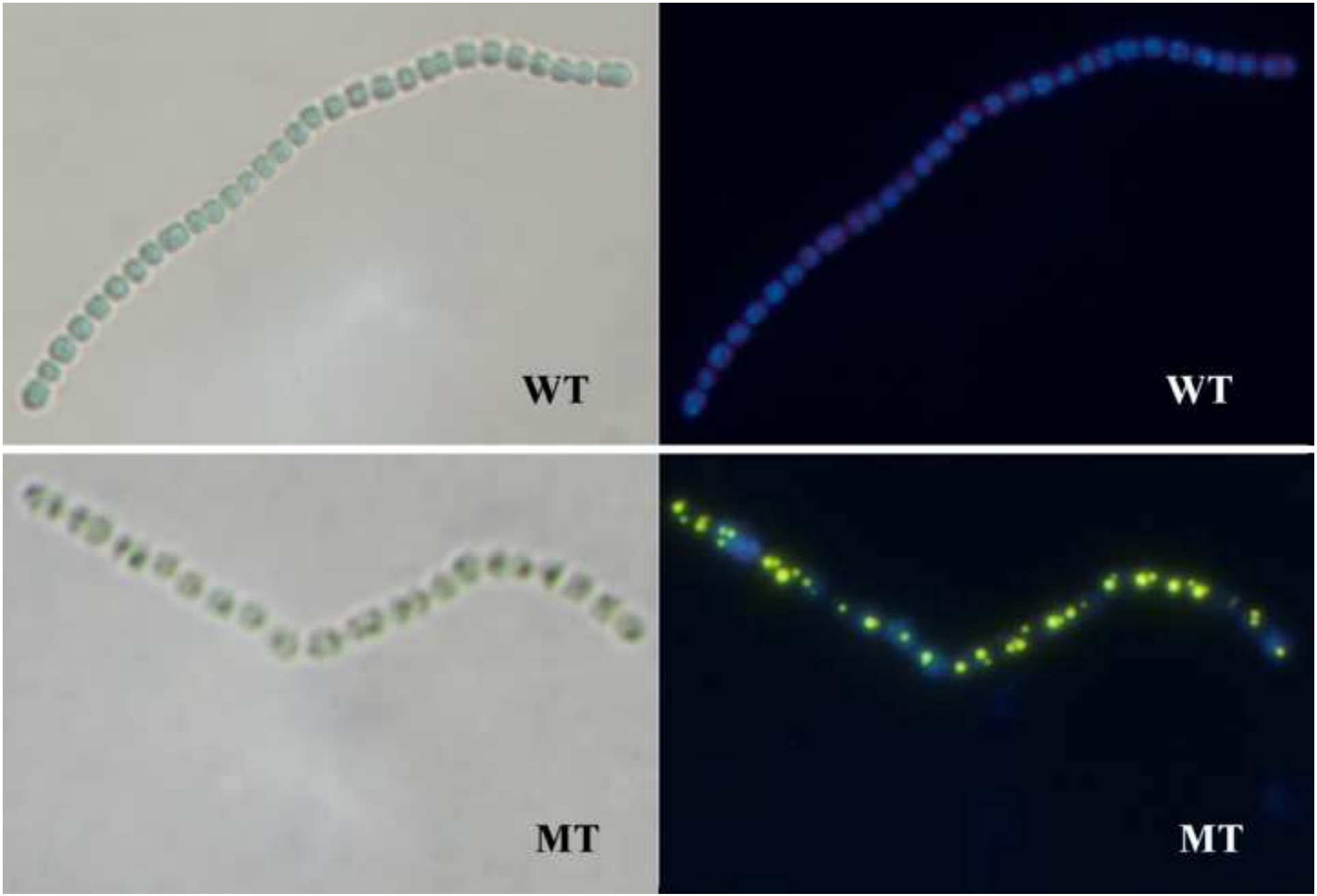

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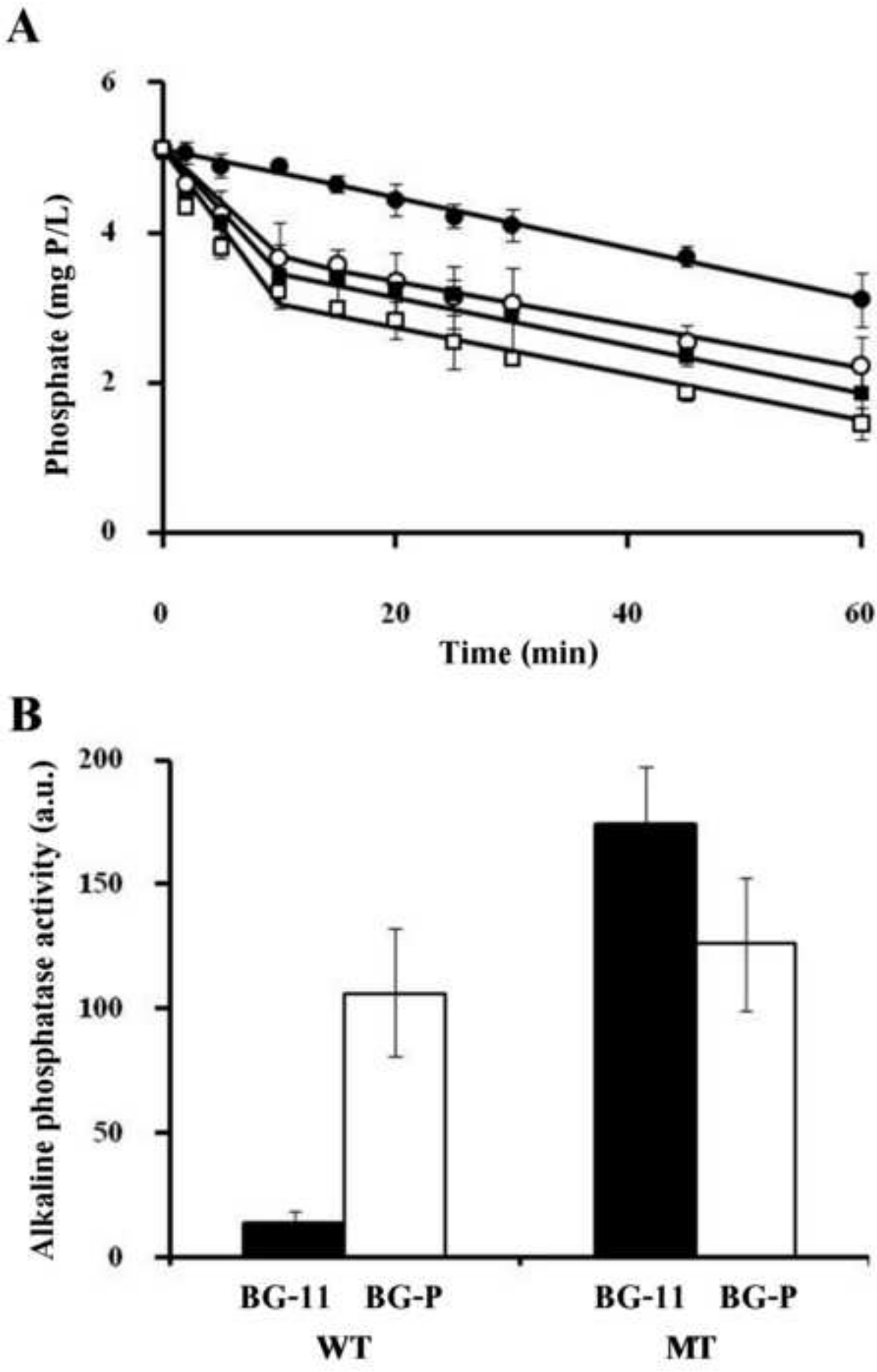

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