Lactic acid and glycogen production from cyanobacteria by metabolic engineering and Utilization of cyanobacterial glycogen for ethanol production

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DOCTORAL DISSERTATION

Lactic acid and glycogen production from cyanobacteria by metabolic engineering and Utilization of cyanobacterial glycogen for ethanol production

代謝改変によるシアノバクテリアからの乳酸・グリコーゲン生産およびシアノバクテリアのグリコーゲンからのエタノール生産

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General Introduction

Background

Biorefinery, the production of bio-fuels and bio-based chemicals from renewable biomass has recently become a topic of intense interest due to increased environmental concerns such as global warming and the depletion of worldwide fossil fuels. This leads to interesting questions and debate over the choice of raw materials for production of new fuels, to replace present petroleum-based fuels [1, 2].

Currently all biofuels and biochemical are derived from starch and sugar-based feedstocks. The sugars in these feedstocks are easy to extract and convert, making large-scale production economical. Ethanol output experienced an increase from16.9 to 72.0 billion liters over the past ten years [3]. This development is retarded due to growing concerns of competition with food, and these plants require high agricultural input in the form of fertilizers and thus attracted attention towards lignocellulosic biomass feedstocks such as crop residues, grasses, sawdust, and wood chips. However, a major concern related to biofuel production from lignocellulosic biomass involves competition for arable land between producers of energy crops and producers of edible crops [4]. These factors have diverged research interests into fuels which are 'non-food', less land and water intensive, which are either integrated into other systems or generate co-products.

Cyanobacteria and Microalgae

Oxygenic photosynthetic microorganisms, including cyanobacteria and microalgae, have recently drawn attention as a new feedstock for 3rd generation biofuels [5]. Algal

biomass has several properties over terrestrial energy crops for biofuels production; they are considered to be among the most efficient organisms regarding the solar energy conversion and they do not compete with food crops, as they can be cultivated with relatively simple requirements in non-arable land [6]. The photosynthetic efficiency of cyanobacteria (3 to 9%) is high compared to that of higher plants (0.25 to 3%) and able to survive with minimal nutrient requirements [7]. Cyanobacteria offer a diverse spectrum of valuable products for the production of biofuels and biochemical [6], However the feasibility of cynobacterial production depends on lowering costs and increasing efficiency in various aspects such as strain improvement, photosynthetic capacity, and harvesting and isolation of products from cyanobacteria [8,9]. Here, we show recent approaches using cyanobacteria as a model organism engineered to convert CO_2 to biochemical and biofuels of commercial interest.

Biochemical production

An efficient alternative strategy for the production of biochemical is to employ photosynthetic bacteria based platform in which photosynthesis and biosynthesis provide the desired product [10]. The Photofermentative chimera approach has been provided for several biochemical feedstock products. In this methodology light driven conversion of CO_2 and water into biofuel can be achieved by combining the elementary reactions of photosynthesis and the Calvin cycle in combination with a fermentative pathway from a chemoheterotrophic microorganism in one single chimera [11-14]. By heterologous expression of pyruvate decarboxylase (PDC) and an alcohol dehydrogenase from *Zymomonas mobilis* provided evidence that ethanol can be formed in this way by *Synechococcus* sp. strain PCC7942. Significantly, the photanol approach is not constrained to ethanol, but rather can be used for a broad range of (fermentation) end products. Any relatively reduced fuel product that can be made from C(3) sugars, like pyruvate, in a limited number of metabolic steps, qualifies [14].

For example, products like lactic acid are interesting products in this respect because of their superior properties and the relatively short (i.e., two to three steps) metabolic routes that are available for their synthesis. Currently, there is an increased demand for lactic acid as a feedstock for the production of biopolymer poly lactic acid (PLA) and has wide applications in food and non-food industries [15]. The recent huge growth of the PLA market will stimulate future demands on lactic acid considerably. Utilization of lactic acid bacteria genes in the cynobacterium *Synechocystis* sp. PCC6803, best-characterized species for genetic engineering, may open new paths for the production of optically pure lactic acid for bio plastic production.

Biofuel production

Several species of cyanobacteria can store significant amounts of energy-rich compounds such as carbohydrates that can be utilized for the production of distinct biofuels [6]. Biomass carbohydrate characterization is crucial for an efficient bioethanol production as not all sugar-forms are fermentable. Glycogen is the primary storage carbohydrate in cyanobacteria [16]. Cultivation and environmental conditions affect significantly the glycogen content. Metabolic analysis and improvement of light utilization efficiency by metabolic engineering will provide a promising strategy for enhancement of glycogen production.

Metabolic analysis and engineering to enhance glycogen accumulation

Carbohydrates represent the main energy storage molecules in cyanobacteria, and a broad understanding of primary metabolism is necessary to manipulate electron flux towards these products for bioenergy applications. Under nutrient limitation conditions such as nitrogen starvation unicellular non-diazotrophic cyanobacteria respond to nitrogen limitation

by a process termed chlorosis which leads to degradation of harvesting complexes. This response towards changing levels of combined nitrogen in the environment and physiological adaptation to these conditions is mediated by the NtcA and PII-system which induces alteration of gene expression as well as metabolic adaptation to altered nutrient availability [17]. Microarray analysis have given more insights into the gene expression levels during nitrogen starvation in *Synechosytis* sp. PCC6803 [18], but still now metabolic analysis of mutants defective in response regulator has not yet been analysed. Construction of such mutants and metabolic and gene expression analysis under nitrogen starvation will provide further knowledge for genetic engineering.

Improvement of light utilization efficiency towards glycogen accumulation

Photosynthetic efficiency is directly related to the size of light-harvesting antenna complexes and in response to light intensity antenna size is optimized [14]. When solar energy levels exceed energy demand by the cell, light harvesting complex (LHC) transcription is down-regulated to prevent photodamage, but, low-light levels lead to increased transcription to maximize photon capture efficiency. Thus, the total amount of LHC proteins and their isoform composition is constantly adjusted to the actual demand for light capture and to changing irradiation conditions [19]. *Synechocystis* sp. PCC6803 has evolved to grow well under low light, so when available light exceeds the organism's ability to effectively use that light, wasteful absorption occurs which does not yield photochemistry. While it may seem counter intuitive, reducing the efficiency of light absorption has been proposed as a means to improve the overall efficiency of light utilization Minimizing, or truncating, the chlorophyll antenna size of the photosystems can improve photosynthetic solar energy conversion efficiency and productivity up to 3-fold. Previous studies have shown that under well-defined cultivation conditions antenna mutants are potential candidates for hydrogen production;

however their biomass accumulation or carbohydrate production has not yet been analyzed [20]. Construction of antenna mutants in widely studied *Synechocystis* sp. PCC6803 and optimizing the cultivations condition may enhance the biomass accumulation and their by glycogen production.

Development of biomass conversion technology

Cyanobacteria tend to accumulate carbohydrates during change in cultivation conditions [16]. Cyanobacterial carbohydrate composition can be manipulated by several cultivation techniques. These cultivation techniques mainly related to some of the cultivation and environmental factors, such as nutrients, light, temperature, etc. and which are known that affect the cell growth and their biomass composition. The halophilic cyanobacterium *Arthrospira platensis* is a potential candidate for carbohydrate production, due to its high growth rates, capability to thrive in wastewaters and its filamentous form, making its harvest much easier than unicellular microalgae by filtration due to their larger dimensions [10]. Growth under conditions of high pH and high salinity has the advantage of being resistant to bacterial contamination. *A. platensis* cultivated under phosphorus limitation and the biomass carbohydrates content increased from about 15–20% up to 65% on a dry biomass basis [21], Accordingly, *A. platensis* is expected to be a promising supplier of carbohydrate in the form of glycogen which serves as a feed stock for ethanol production. However the studies dealing with the conversion of this biomass to bioethanol are scarce may be due to the challenge in the conversion of these sugars into biofuel [22]. Exploration of new cost effective methodology will provide benefits in the usage of cyanobacterial biomass as feed stocks.

Constituent of this thesis

Consolidating all the topics above main objective was to produce biochemical and biofuels such as lactic acid and glycogen by genetic engineering and optimization of cultivation conditions and to develop a methodology for the direct conversion of glycogen to ethanol. The contents of each chapter are:

Chapter 1: Utilization of lactic acid bacterial genes in *Synechocystis* sp. PCC 6803 for the production of lactic acid

We investigated lactic acid production in five transformants of *Synechocystis* PCC 6803 in which *ldh* genes that are specific for L-lactic acid production from various lactic acid bacteria (*L. lactis*, *L. plantarum*, and *L. rhamnosus*) were used. In addition, a lactate transporter (*lldp*) gene from *L. plantarum* was expressed with the *ldh* genes in order to secrete lactic acid across the cell membrane.

Chapter 2: Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803

In this study, a Rre37 mutant of *Synechocystis* was constructed and analysis of gene expression and metabolites were carried out to clarify which pathways or genes are regulated by Rre37 in carbon or nitrogen flow.

Chapter 3: Increased biomass production and glycogen accumulation in *apcE* gene deleted *Synechocystis* sp. PCC 6803

In this study, the effect of *apcE* gene deletion on growth of *Synechocystis* sp. PCC6803 was investigated under photoautotrophic condition. Biomass production and the effect of *apcE* gene deletion on glycogen accumulation in *Synechocystis* sp. PCC6803 was investigated particularly under photoautotrophic conditions.

Chapter 4: Direct conversion of *Spirulina* to ethanol without pretreatment or enzymatic hydrolysis processes

In the present study, a recombinant *S. cerevisiae* strain secreting α-amylase from *Streptococcus bovis* and displaying glucoamylase from *Rhizopus oryzae* on the cell surface was used for the direct production of ethanol from *A. platensis* glycogen. Additionally, the effect of treating the *A. platensis* cell membrane with lysozyme on enhancing ethanol production was examined. Ethanol was successfully produced from *A. platensis* at high yield without any pretreatment or extraction steps by the combined use of amylase-expressing yeast and lysozyme addition

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Synopsis

Chapter 1

Utilization of lactic acid bacterial genes in *Synechocystis* sp. PCC 6803 for the production of lactic acid

Metabolic pathway engineering of cyanobacteria for the production of industrially important chemicals from atmospheric CO_2 has recently generated interest. Here, we engineered *Synechocystis* sp. PCC 6803 to produce lactic acid using a lactate dehydrogenase (*ldh*) gene from different lactic acid-producing bacteria, namely, *Lactococcus lactis* (*ldh*B and *ldh*X), *Lactobacillus plantarum* (*ldh*L and *ldh*), and *Lactobacillus rhamnosus* (*ldh*L), and this lactic acid was secreted outside the cell using a transporter (*lldp*) gene from *L. plantarum*. Expression of each *ldh* in *Synechocystis* sp. PCC 6803 was ascertained by reverse-transcriptase polymerase chain reaction. Five transformants led to the production of L-lactic acid. Co-expression of *lldp* with *ldhB* from *L. plantarum* or *ldhL* from *L. rhamnosus* led to the secretion of lactic acid into the medium with concentration of 0.17 ± 0.02 or 0.14 ± 0.02 mM after 18 days cultivation.

Chapter 2

Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803

Rre37 (sll1330) in a cyanobacterium *Synechocystis* sp. PCC 6803 acts as a regulatory protein for sugar catabolic genes during nitrogen starvation. Low glycogen accumulation in Δ*rre37* was due to low expression of glycogen anabolic genes. In addition to low 2-oxoglutarate

accumulation, normal upregulated expression of genes encoding glutamate synthases (*gltD* and *gltB*) as well as accumulation of metabolites in glycolysis (fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate) and tricarboxylic acid (TCA) cycle (oxaloacetate, fumarate, succinate, and aconitate) were abolished by *rre37* knockout. Rre37 regulates 2-oxoglutarate accumulation, glycogen accumulation through expression of glycogen anabolic genes, and TCA cycle metabolites accumulation.

Chapter 3

Increased biomass production and glycogen accumulation in *apcE* gene deleted *Synechocystis* sp. PCC 6803

The effect of phycobilisome antenna-truncation in the cyanobacterium *Synechocystis* sp. PCC 6803 on biomass production and glycogen accumulation have not yet been clarified. To investigate these effects here, the *apcE* gene, which encodes the anchor protein linking the phycobilisome to the thylakoid membrane, was deleted in a glucose tolerant strain of *Synechocystis* sp. PCC 6803. Biomass production of the *apcE*-deleted strain under photoautotrophic and atmospheric air conditions was nearly double that of strain PCC 6803 (1.24 ± 0.03 versus 0.75 ± 0.05 g cell-dry weight L^{-1}, respectively) after 12 days of cultivation. In addition, the glycogen content of the *apcE*-deleted strain (24.2% ± 0.7%) was also higher than that of strain PCC 6803 (11.1% ± 0.3%). Together, these results demonstrate that antenna truncation by deleting the *apcE* gene was effective for increasing biomass production and glycogen accumulation under photoautotrophic and atmospheric air conditions in *Synechocystis* sp. PCC 6803.

Chapter 4

Direct conversion of *Spirulina* to ethanol without pretreatment or enzymatic hydrolysis processes

Oxygenic photosynthetic microorganisms such as cyanobacteria and microalgae have attracted attention as feedstocks for next-generation biofuels. To date, however, there are no reports of efficient bioethanol production from cyanobacterial glycogen by yeast fermentation. Additionally, multiple pretreatment and enzymatic hydrolysis steps of polysaccharides are required for conventional ethanol production from agricultural crops and microalgae. Here, we investigated direct ethanol production from *Arthrospira* (*Spirulina*) *platensis*, a fast-growing halophilic cyanobacterium that accumulates large amounts of glycogen, using lysozyme and a recombinant amylase-expressing yeast strain to eliminate the need for biomass pretreatment and amylase hydrolysis. In the direct conversion process from *A. platensis* to ethanol, 6.5 g L^{-1} (ethanol productivity of 1.08 g L^{-1} d^{-1}) ethanol was produced. The total ethanol yield based on glycogen consumption was 86% of theoretical yield, which to our knowledge, is the highest yield of bioethanol from an oxygenic photosynthetic microorganism. The present findings indicate that *A. platensis* is a remarkable carbohydrate feedstock in the form of glycogen, which is a promising material for the production of bioethanol and various other commercially valuable chemicals.

Chapter 1

Utilization of lactic acid bacterial genes in *Synechocystis* sp. PCC 6803 for the production of lactic acid

Introduction

Increases in atmospheric CO_2 and global warming have created interest in transforming CO_2 into high-value products using photosynthetic microorganisms [1,2]. Cyanobacteria are useful microorganisms because they can convert sunlight and atmospheric CO_2 into carbon-based compounds, grow in water with minimal salts and minerals, and have transformability for genetic engineering [2,3]. Using cyanobacteria, various energy sources such as ethanol, fatty acids, and isobutanol have been produced.[3–6] *Synechocystis* sp. PCC 6803 used in this study was the first cyanobacterium whose genome was sequenced [7].

Lactic acid is a highly useful chemical and is used in the food, textile, pharmaceutical, and chemical industries [8,9]. Therefore, we metabolically engineered *Synechocystic* sp. PCC 6803 in this study for the production of lactic acid. Many lactic acid bacteria are naturally quite optimal lactic acid producers[10]. However, the expression of lactate dehydrogenase (*ldh*) genes from lactic acid bacteria in *Synechocystis* sp. PCC 6803 has not been previously carried out, although the expression of *ldh* genes from *Bacillus subtilis* and *Escherichia coli* has been investigated [11,12]. L-lactic acid can be produced by species such as *Lactobacillus rhamnosus*, *Lactococcus lactis*, and *Lactobacillus plantarum* by converting pyruvate [13,14]. In addition, lactic acid bacteria can produce lactic acid with high yield and high productivity [15]. Here, we investigated lactic acid production in five transformants of *Synechocystis* PCC 6803 in which *ldh* genes that are specific for L-lactic acid production from various lactic acid bacteria (*L. lactis*, *L. plantarum*, and *L. rhamnosus*) were used. In addition, a lactate transporter (*lldp*) gene from *L. plantarum* was expressed with the *ldh* genes in order to secret lactic acid across the cell membrane.

Materials and Methods

Strains and growth conditions.

A glucose tolerant strain of *Synechocystis* sp. PCC 6803 was obtained from Prof. Ikeuchi. *Synechocystis* sp. PCC 6803 was cultured in BG11 medium [16] at 30°C (pH 7.0) under continuous light illumination of approximately 50 μmol photons m^{-2} s^{-1}. *L. lactis* IL1403 having *ldhB* and *ldhX*; *L. plantarum* NCIMB8826 having *ldhL*, *ldh*, and *lldp*; and *L. rhamnosus* GG ATCC53103 having *ldhL* were grown in MRS medium (Difco Laboratories, Detroit, MI) with continuous shaking of 150 rpm at 37°C.

Cloning and transformation.

ldh genes that produce L lactic acid were isolated from the above lactic acid bacteria by using the primers listed in Table 1. Primers were designed with *NdeI* restriction sites on both ends. pTCP2031V vector (a kind gift from Prof. Ikeuchi), which has chloramphenicol resistance and a psbA2 promoter, was used for cloning. *ldh* genes were ligated into *NdeI* restriction sites of pTCP2031V using the In-fusion Cloning Kit (Clonetech, Takara Bio. Inc., Shiga, Japan). Five clones of the plasmid harboring each *ldh* gene were used to transform *Synechocystis* sp. PCC 6803 by homologous recombination [17, 18] to obtain 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL). Positive clones were selected based on PCR analysis. The five transformants were grown on modified BG11 solid medium containing 34 mg L^{-1} chloramphenicol. Later, wild-type and transformants were grown in 200 ml flasks with 75 ml BG11 medium at 30°C with continuous light illumination of 50 μmol photons m^{-2} s^{-1} with shaking at 100 rpm. Growth of the 5 transformants and wild-type were monitored turbidimetrically by measuring

the optical density at 750 nm (OD_{750}) at 0, 3, 6, 9, and 12 days. The specific growth rate (μ) was calculated from the slope of OD_{750} from 6 to 12 days versus time as follows: $\mu = \Delta \ln OD_{750} / \Delta t$, where t is the cultivation time (days).

The *lldp* gene from *L. plantarum* was isolated using the primers listed in Table 1 and was cloned using the In-fusion Cloning Kit into pTSP2001 vector (a kind gift from Prof. Osanai) which has spectinomycin resistance and a psbA2 promoter. This plasmid was further inserted with 5 *ldh* genes (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL) to create 5 respective transformants. Positive clones were selected by PCR and were grown in BG 11 medium with 34 mg L^{-1} chloramphenicol and 100 mg L^{-1} spectinomycin. Other growth conditions were the same as those for the original 5 transformants. Growth of 2 transformants (PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp) was observed and OD_{750} was monitored at 0, 3, 6, 9, 12, 15, and 18 days. μ was calculated as described above.

Table 1 Primers and transformants constructed in this study using *Synechocystis* sp. strain PCC 6803

[1]*ldhB*, *ldhX*, *ldhL*, and *ldh* are lactate dehydrogenase genes, and *lldp* is a lactate transporter gene

Used Strain (*ldh*/ *lldp*)[1]	Primer	Sequence	Transformants constructed
Lactococcus lactis (ldhB)	F	GGAATTATAACCATATGTTACTTAATAGATTCTATTACC	PCC 6803/L.laldhB
	R	GGCATGGAGGACATATGATGAAAATTACAAGCAGAAAAG	
Lactococcus lactis (ldhX)	F	GGAATTATAACCATATGATGAAAATTAATAACAAAAAAG	PCC 6803/L.laldhX
	R	GGCATGGAGGACATATGTTACAAAGTACATTTTTCTTTA	
Lactobacillus plantarum (ldhL)	F	GGAATTATAACCATATGTGTCAAGCATGCCAAATCATCA	PCC 6803/L.plldhL
	R	GGCATGGAGGACATATGTTATTTATTTTCTAATTCAGCT	
Lactobacillus plantarum (ldh)	F	GGAATTATAACCATATGTTATTTATTTTCTAATTCAGCT	PCC 6803/L.plldh
	R	GGCATGGAGGACATATGTTGTCAAGCATGCCAAATCATC	
Lactobacillus rhamnosus (ldhL)	F	GGAATTATAACCATATGGTGGCAAGTATTACGGATAAGG	PCC 6803/L.rhldhL
	R	GGCATGGAGGACATATGTTACTGACGTGTTTCGATGTCG	
Lactobacillus plantarum (lldp)	F	GGAATTATAACCATATGTTAAGCTACTAGACGT	PCC 6803/L.laldhB/lldp, PCC 6803/L.laldhX/lldp, PCC 6803/L.plldhL/lldp, PCC 6803/L.plldh/lldp, and PCC 6803/L.rhldhL/lldp
	R	ATCCAATGTGAGGTTAACATGATTTTTATTGCAAG	

Transcriptional analysis of ldh genes

To test the expression of the *ldh* genes in the 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL), RNA from each sample was isolated using a Nucleospin RNAII kit (Macherry-Nagel GmbH& Co. KG, Düren, Germany). One microgram of RNA was used for cDNA synthesis using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Primers listed in Table 1 were used for reverse transcriptase PCR. For glucose tolerant *Synechocystis* sp. PCC 6803, all the primers were tested. Sequence analysis of *ldh* genes was conducted using Genetic analyzer 3130*xl* (Applied Biosystems, Foster, CA). The *rnpB* (RNA subunit of ribonuclease P) gene was used as a housekeeping gene. Primer sequences for *rnpB* are shown in Table 2.

Table 2 Primers used for amplification of housekeeping gene, *rnpB*, in reverse transcriptase PCR analysis

Amplified gene	Forward primer (5'-3')	Reverse primer (5'-3')
Housekeeping gene (*rnpB*)	AGCGGCCTATGGCTCTAATCA	CTGTGACAGGAATCCGCAACT

HPLC analysis

The concentrations of lactic acid within the first 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL) and in the supernatants of the 2 *lldp* transformants (PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp) were determined by HPLC (Shimadzu, Kyoto, Japan) with a reflective index detector (RID-10A; Shimadzu) equipped with a BioRad Aminex 87H column (Bio-Rad

Laboratories, Hercules, CA) using 5 mM H_2SO_4 as the mobile phase at a 0.6 ml min^{-1} flow rate. The oven temperature was maintained at 65°C.

To determine the lactic acid concentrations within the 5 transformants, 20 mg dry weight (DW) of cells were collected by centrifugation and washed with 20 mM ammonium carbonate buffer. Then, cells were disrupted by vortexing with glass beads (100 μm in diameter) in 30% ethanol and sonicated for 10 min. This was pelleted by centrifugation at 10,000 rpm for 5 min at 4°C, and the supernatant was collected for HPLC analysis.

Separation of D- and L-lactic acid using a chiral column.

To determine the chiral purity of lactic acid, HPLC (Shimadzu) with a UV detector (SPD-20A; Shimadzu) equipped with TSK gel Enantio L1 (Tosho, Tokyo, Japan) using 1 mM $CuSO_4$ as the mobile phase at 1 ml min^{-1} flow rate was utilized with the oven temperature maintained at 25°C. Samples were prepared in the same manner as for determining lactate concentration in the cell.

Lactate dehydrogenase assay.

For lactate dehydrogenase assay, the 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL) were grown for 12 days, and 50 mg of cells were collected by centrifugation. A lactate dehydrogenase kit (BioVision, San Francisco, CA) was used for analysis. Samples were prepared based on the manufacturer's protocol. Lactate dehydrogenase enzyme activity was determined using a plate reader (2104 EnVision; PerkinElmer, Waltham, MA) at 450 nm absorbance.

Results and Discussion

Growth analysis of the 5 transformants and wild-type

To check the effect of expressing the *ldh* genes on the growth of *Synechocystis* sp. PCC 6803, the 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL) were grown for 12 days. Figure 1 shows the growth of all transformants harboring *ldh* genes and the wild-type strain. Linear growth was found in all samples. Calculations of μ (0.03 ± 0.00, 0.04 ± 0.00, 0.04 ± 0.00, and 0.04 ± 0.00 h^{-1}, respectively) of 4 transformants, PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldh, and PCC 6803/L.rhldhL, were similar to that of the wild-type (0.03 ± 0.00 h^{-1}). However, the growth rate was slow in transformant PCC 6803/L.plldhL (μ = 0.01 ± 0.00 h^{-1}). From this data, we concluded that the insertion of 4 of the 5 *ldh* genes we studied here, with *ldhL* from *L. plantarum* as the exception, did not have much influence on the growth rate.

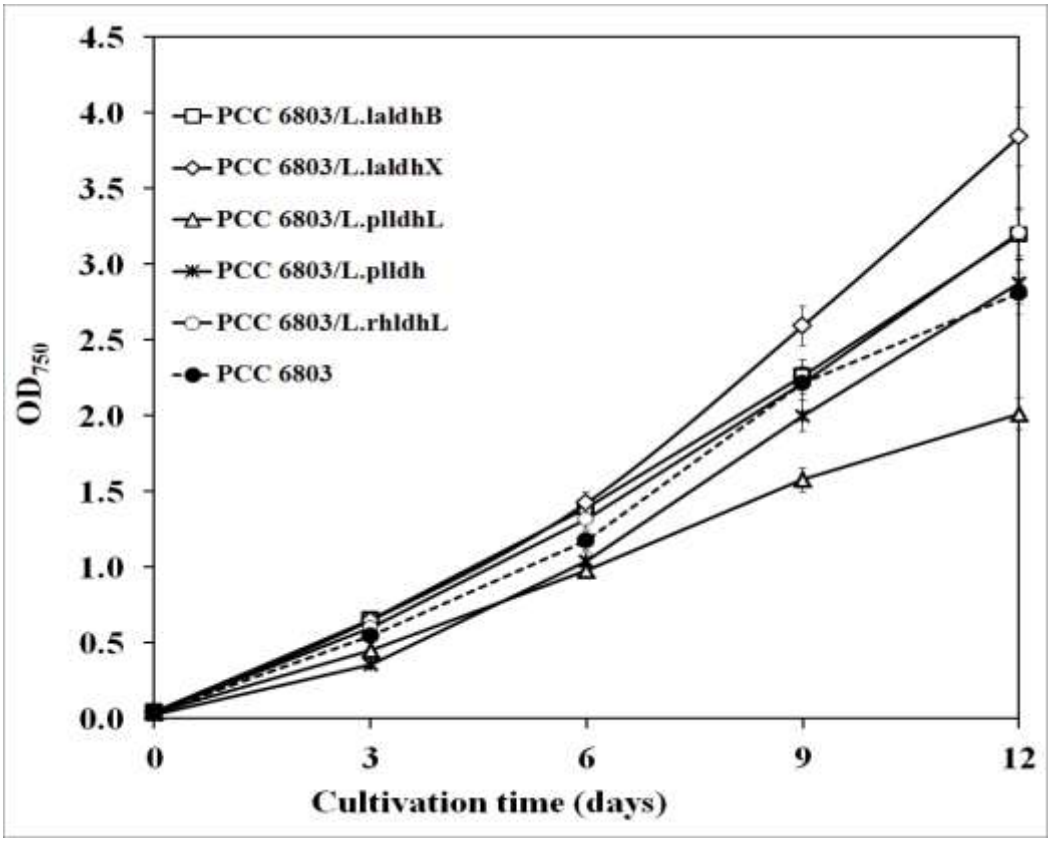

Fig. 1 Comparison of growth of 5 transformants with *Synechocystis* sp. PCC 6803 wild-type. Smooth lines indicate the growth of the 5 transformants and the dashed line indicates the growth of *Synechocystis* sp. PCC 6803 wild-type. The values are the means of three individual experiments. Bars indicate the standard deviation from triplicate determinations.

***ldh* expression and lactic acid production in the 5 transformants**

RT-PCR was employed to test whether the *ldh* genes, under the control of the psbA2 promoter, were transcribed in *Synechocystis* sp. PCC 6803. RT-PCR analysis showed that all *ldh* genes were indeed expressed (Fig. 2). *ldh* amplification products (size, around 950-1000 bp) were visualized on agarose gels, demonstrating the presence of *ldh* mRNA in these 5

transformants, whereas the wild-type strain did not produce any amplification as it has no *ldh* gene. Sequence analysis of reverse transcribed PCR products confirmed the transformation of *ldh* genes in proper orientation (data not shown). The *rnpB* gene (a housekeeping gene) was expressed in the 5 transformants and wild-type. After 12 days of cultivation for the 5 transformants (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL), higher lactate dehydrogenase activities than that detected in the wild-type were confirmed (Table 3).

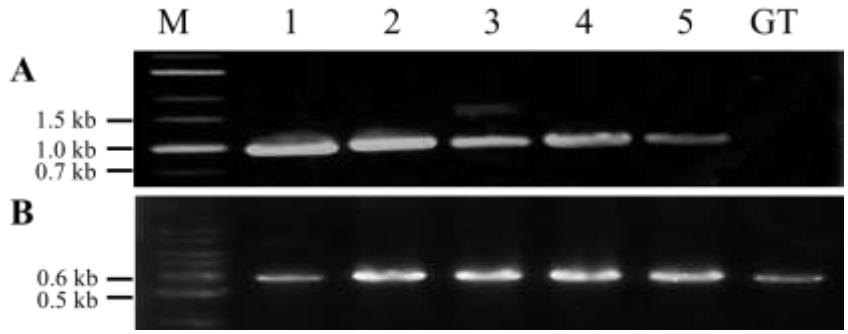

Fig. 2 Gene expression analysis on lactate dehydrogenase (*ldh*) genes in different transformants by reverse transcriptase PCR.

(A) Lanes 1-5, different transformants: 1. PCC 6803/L.laldhB, 2. PCC 6803/L.laldhX, 3. PCC 6803/L.plldhL, 4. PCC 6803/L.plldh, 5. PCC 6803/L.rhldhL. GT, Glucose tolerant *Synechocystis* sp. PCC 6803. (B) Control PCR with housekeeping gene *rnpB* to show that PCR worked for all samples. DNA ladder one (Nacalai Tesque; Kyoto, Japan) was used to determine size.

Table 3 Lactate dehydrogenase (*ldh*) activity in 5 transformants and *Synechocystis* sp. PCC 6803 wild-type (PCC6803).

Strains	*ldh* activity (mU/g dry weight)[1, 2]
PCC 6803/L.laldhB	1.12 ± 0.08
PCC 6803/L.laldhX	0.68 ± 0.06
PCC 6803/L.plldhL	0.76 ± 0.06
PCC 6803/L.plldh	1.00 ± 0.04
PCC 6803/L.rhldhL	0.82 ± 0.08
PCC 6803	0.10 ± 0.04

[1]Values are the means of three individual experiments plus/minus standard deviations from triplicate determinations.

[2]One unit is the amount of enzyme that generates 1.0 μmol NADH per min at 37°C (mU_ml^{-1} = nmol min^{-1} ml^{-1}).

To check whether the expressed *ldh* genes were metabolically competent to produce lactic acid within the 5 transformants, metabolites from cell pellets were measured using HPLC. Table 4 shows that *Synechocystis* sp. PCC 6803 engineered with various *ldh* genes produced lactic acid at around 0.03 to 0.04 mM. Detectable amounts of lactic acid were not found in the media using transformants with only the *ldh* genes.

Table 4 Concentration of lactic acid in the cells of the 5 transformants with *ldh* genes

Strains	Lactic acid production in cells[1] (mM)
PCC 6803/L.laldhB	0.04 ± 0.01
PCC 6803/L.laldhX	0.04 ± 0.02
PCC 6803/L.plldhL	0.03 ± 0.01
PCC 6803/L.plldh	0.03 ± 0.01
PCC 6803/L.rhldhL	0.03 ± 0.01
PCC 6803	0

[1]Lactic acid was not detected in the supernatant.

Separation of* D- *and* L-*lactic acid using a chiral column

Lactic acid exists in two optically active stereoisomers, the L(+) and D(-) forms. Figure 3 shows chromatograms of lactic acid produced in the transformant PCC 6803/L.laldhB. L-lactic acid production was confirmed by co-chromatography (Fig. 3B), in which a mixture of lactic acid from the transformant PCC 6803/L.laldhB and 10 mg L^{-1} of standard L-lactic acid produced a single peak. In addition, L-lactic acid production was ascertained from the remaining 4 transformants (data not shown).

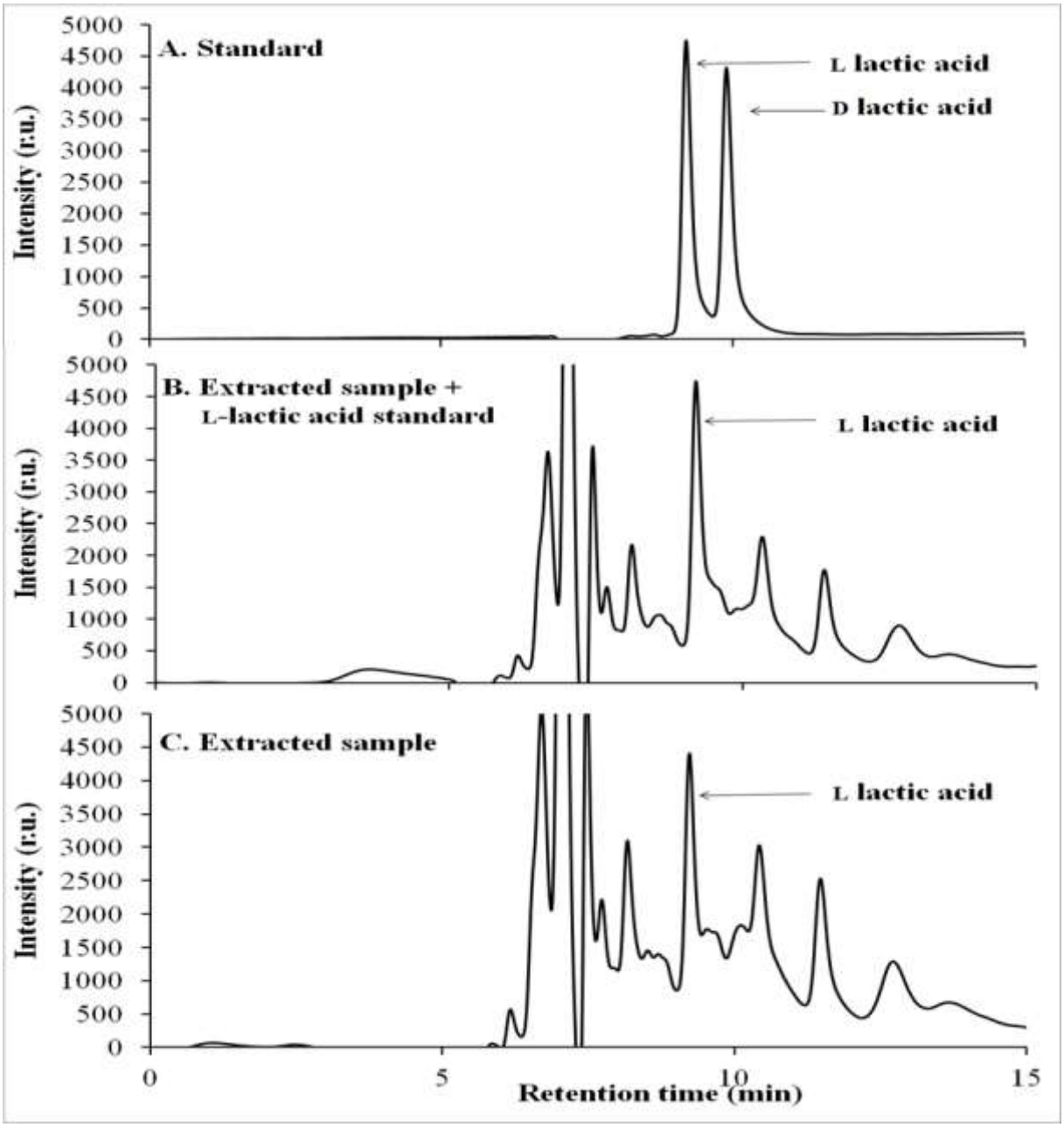

Fig. 3 Separation of D- and L-lactic acid using a chiral column.

(A) Standard chromatogram obtained using a TSK chiral column (10 mg/L DL lactic acid used). (B) Co-chromatography using equal concentrations of L-lactic acid standard and an extracted sample from transformant PCC 6803/L.laldhB. (C) Chromatogram of transformant PCC 6803/L.laldhB.

Secretion of lactic acid from engineered Synechocystis

All 5 transforming plasmids (PCC 6803/L.laldhB, PCC 6803/L.laldhX, PCC 6803/L.plldhL, PCC 6803/L.plldh, and PCC 6803/L.rhldhL) were cloned with the *lldp* gene for the secretion of lactic acid. Insertion of the *lldp* gene into PCC 6803/L.laldhB and PCC 6803/L.rhldhL showed good growth (referred to as PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp, respectively) (Fig. 4). However, insertion of the *lldp* gene into PCC 6803/L.laldhX, PCC 6803/L.plldhL and PCC 6803/L.plldh showed no growth. The calculated μ of PCC6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp were 0.05 ± 0.00 and 0.06 ± 0.00 h^{-1}, respectively, which were higher than the μ of PCC 6803/L.laldhB and PCC 6803/L.rhldhL (0.03 ± 0.00 and 0.04 ± 0.00 h^{-1}, respectively). The reason for the faster growth of engineered *Synechocystis* sp. PCC 6803 with *lldp* is unclear; however, the secretion of lactic acid produced in the cell might encourage growth. The insertion of the *lldp* gene into these transformants led to an increase in lactic acid in the supernatant, corresponding with a previous study [12]. Lactic acid concentrations in the supernatant after 12 days cultivation were 0.05 ± 0.00 and 0.06 ± 0.01 mM, which was similar to lactic acid concentrations in cells lacking the *lldp* gene (0.03 - 0.04 mM). Prolonged cultivation (18 days) of PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp increased lactic acid concentrations in the supernatant to 0.17 ± 0.02 and 0.14 ± 0.02 mM, respectively. Lactic acid productivity was found to be 0.0034 mmol lactate/g DW/h. However, this value was lower than that from a previous report using coexpression of the *ldh* gene from *B. subtilis* and a transhydrogenase in which the productivity was found to be 0.0178 mmol lactate/g DW/h [11]. Final pH using PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp (18 days) were increased from 7.5 to 8.3 and 8.4 respectively. Therefore, *Synechocystis* PCC 6803 can be a valuable host for the

production of lactic acid or other acidic chemicals from CO_2 in future, because it increases pH in medium during cultivation.

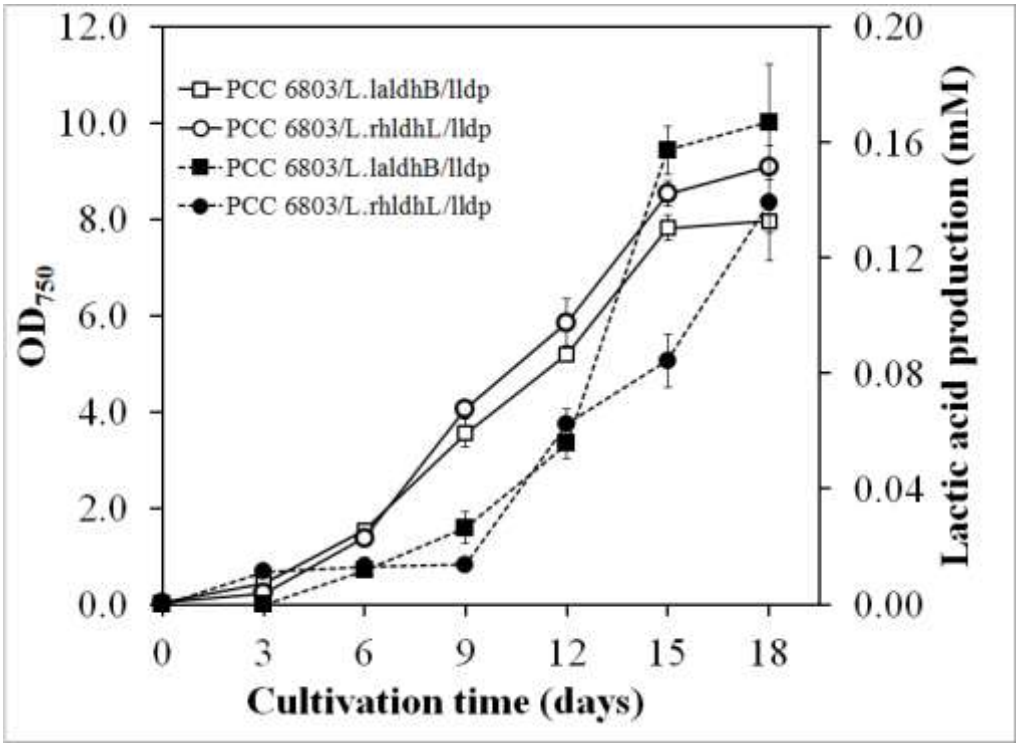

Fig. 4 Growth curve and lactic acid production in transformants with *ldh* and *lldp* genes. Smooth lines with open symbols indicate the growth of transformants (PCC 6803/L.laldhB/lldp and PCC 6803/L.rhldhL/lldp), and dashed lines with closed symbols indicate the lactic acid production in the supernatant. The values are the means of three individual experiments. Bars indicate the standard deviation from triplicate determinations.

These results confirmed that co-expression of genes from lactic acid bacteria with a transporter gene can be utilized in *Synechocystis* sp. PCC 6803 for the production of lactic acid without the addition of carbon source. In addition, coexpression of a *ldh* gene and the

transhydrogenase gene would be useful for increasing the cellular NADH/NAD^+ ratio so that NADH-dependent *ldh* benefits[11]. Such improvement is being studied in our laboratory. In conclusion, the current study increases our knowledge of how to produce L-lactic acid, and subsequently create polylactic acid polymers from CO_2, using *Synechocystis* sp. PCC 6803.

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Chapter 2

Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803

Introduction

Cyanobacteria, which perform oxygenic photosynthesis like eukaryotic algae and plants, inhabit almost all illuminated ecosystems and play key roles in the global carbon and nitrogen cycles [1,2]. In natural habitats, nitrogen, an essential macronutrient, is a limiting factor for cellular growth, and unicellular cyanobacteria have developed mechanisms to respond to nitrogen depletion [3]. Most cyanobacteria, including non-nitrogen fixing, unicellular cyanobacteria, like *Synechocystis* sp., store glycogen under nitrogen or sulfur deficiency [4]. Metabolic changes after nitrogen depletion have been demonstrated [5,6], showing increases in levels of tricarboxylic acid (TCA) cycle metabolites (such as malate, fumarate, succinate) and decreases in purine and pyrimidine nucleotides. Several amino acids including glutamine and glutamate decrease following nitrogen depletion [6], and particularly reflect limitation of available nitrogen sources. Similarly to other bacteria, nitrogen compounds in cyanobacteria are reduced to ammonium and incorporated into 2-oxoglutarate (2-OG) via the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle, known as the GS-GOGAT pathway [7]. The levels of 2-OG increase during nitrogen depletion, which initiates the nitrogen-starvation response in *Synechocystis* cells [8]. Thus, 2-OG is a key metabolite in *Synechocystis* for appropriate acclimation to nitrogen starvation.

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is one of the most widely studied cyanobacterial species due to its transformability and availability of its entire genome sequence [9,10]. Changes in gene expression under nitrogen depletion have been investigated by microarray analysis [11,12]. NtcA, which is a transcription factor belonging to the cAMP receptor protein family, regulates OmpR-type response regulator Rre37 and RNA polymerase sigma factor SigE, and binding of NtcA to the *rre37* promoter is enhanced in the presence of 2-OG *in vitro* [13]. PII signaling protein (encoded by *glnB*) in *Synechococcus* sp. PCC 7942

also binds 2-OG in a cooperative manner with ATP and is phosphorylated during nitrogen starvation [2]. PII bound to 2-OG releases PipX, a small protein conserved among several cyanobacteria, and PipX associates with NtcA, possibly controlling the transcriptional activity of NtcA [14,15]. The expression of various sugar catabolic genes and glycogen content are both increased by nitrogen depletion [16]. Rre37 and SigE are involved in nitrogen-induced expression of sugar catabolic genes [13,16,17]. Rre37 activates the expression of two glycogen catabolic genes [*glgX* (slr1857, encoding glycogen isoamylase) and *glgP* (slr1367, encoding glycogen phosphorylase)] and two glycolytic genes [*gap1* (slr0084, glyceraldehyde 3-phosphate dehydrogenase) and *pfkA* (sll1196, phosphofructokinase)] under nitrogen starvation [13]. SigE activates the expression of glycolytic genes (*gap1* and *pfkA*) independently of Rre37 [12,13]. In contrast to studies of the mechanism of transcriptional regulation after nitrogen depletion and the regulatory factors controlling primary metabolism during nitrogen starvation, few studies of the metabolome have been performed with mutants of nitrogen regulators.

In this study, an Rre37 mutant of *Synechocystis* was constructed and analysis of gene expression and metabolites were carried out to clarify which pathways or genes are regulated by Rre37 in carbon or nitrogen flow.

Materials and Methods

Strains and growth conditions

The glucose-tolerant (GT) strain of cyanobacterium *Synechocystis* sp. PCC 6803 isolated by Williams [18] and the cognate *rre37* knockout mutant (Δ*rre37*) were precultured for 5 days in 1.4 L modified BG-11 medium, which contained $NaNO_3$ 1.5 g/L, K_2HPO_4 0.04 g/L, $MgSO_4 \cdot 7H_2O$ 0.075 g/L, $CaCl_2 \cdot 2H_2O$ 0.036 g/L, citric acid 0.006 g/L, ferric ammonium

citrate 0.006 g/L, EDTA (disodium salt) 0.001 g/L, Na_2CO_3 0.02 g/L, H_3BO_3 2.86 mg/L, $MnCl_2 \cdot 4H_2O$ 1.81 mg/L, $ZnSO_4 \cdot 7H_2O$ 0.222 mg/L, $NaMoO_4 \cdot 2H_2O$ 0.39 mg/L, $CuSO_4 \cdot 5H_2O$ 0.079 mg/L, $Co(NO_3)_2 \cdot 6H_2O$ 49.4 μg/L [19] under continuous illumination at 70 μmol photons m^{-2} s^{-1} at 25 ± 2°C with 2% (w/w) CO_2 in air bubbled at 80 mL min^{-1}. 10.0 g/L agar was added in a solid medium. For culturing Δ*rre37*, 50 μg L^{-1} kanamycin was added to the medium. After cells reached the mid-exponential phase, they were collected by filtration using a 1 μm pore size polytetrafluoroethylene filter (Millipore, Billerica, MA), washed 3 times with BG-11 medium without a nitrogen source ($BG\text{-}11_0$), and then inoculated into fresh $BG\text{-}11_0$ medium at 0.2 g dry-cell weight L^{-1}.

Glycogen analysis

Glycogen was extracted from the dried cells as described previously [20]. Glycogen content was determined by high performance liquid chromatography (HPLC) (LC Prominence, Shimadzu, Kyoto, Japan) using a size exclusion HPLC column (OHpak SB-806 M HQ; Shodex, Tokyo, Japan) and a reflective index detector (RID-10A; Shimadzu) as previously described [21].

Quantitative gene expression analysis

Cells were harvested at different time points by centrifugation at 3500 × *g* for 5 min at 4°C. RNA was extracted using a Nucleospin RNAII kit (Macherey-Nagel GmbH & Co. KG Düren, Germany) and cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then loaded in a Agilent 2100 bioanalyzer (Agilent Technologies**,** La Jolla, CA) to check purity, concentration

and integrity. To investigate the expression of *rre37*, reverse transcription PCR was performed using 100 ng of cDNA and *rre37* primers (Table 1). Real time quantitative PCR was performed with the gene-specific oligonucleotides (Table 1) using Thunderbird SYBR qPCR mix (Toyobo). The reactions consisted of an initial heating step of 10 min at 95°C, then 40 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The transcript level of target genes was quantified using an Mx3000P qPCR system (Agilent Technologies**)**. The *rnpB* gene, which encodes RNase P subunit B, was used as a housekeeping gene. The amplification efficiency (E) of each oligonucleotide was calculated using the equation $E = 10^{(-1/slope)}$, and expression of the target gene relative to the reference gene was calculated using the Pfaffl method [22].

Table 1 Primers used for reverse-transcription PCR and quantitative reverse-transcription PCR. Forward primers are shown in first row of each pair, followed by reverse primers.

Gene	Gene function	Sequence
	Glycogen catabolism and anabolism	
glgX (*slr0237*) [a]	Glycogen isoamylase	GGTGGTGTTTGACCTGGATTTT
		TGGCCCTGCTGGAAGTTATT
glgP (*slr1367*) [a]	Glycogen phosphorylase	CGGCCTATTTGCTCAAGTATCG
		TCCGTTTGACCTGCACATCA
glgA	Glycogen synthase	GGCGTTTGTTACTGCGTAGCA
		ACGACCATATGCGCTTTGC
glgC	Glucose-1-phosphate	TTATTCTGTCCGGCGACCAT

	adenylyltransferase	
		CATTGGTTTCCCGGTGTCTT
	Glycolysis/pentose phosphate pathway/TCA cycle	
zwf	Glucose-6-phosphate dehydrogenase	GAAATTGGTGCCAGCCATCT
		CAAAACCGACCACCGTTAACTC
pfkA	Phosphofructokinase	TTAGTCCCTGGGTGGCTTCTT
		ACCATGCCATTGGCACCTA
gnd	6-Phosphogluconate dehydrogenase	GCAATTTCCGGCAGGTTAAC
		TGGCCCTAATTGCCAAAGC
gap1	Glyceraldehyde 3-phosphate dehydrogenase	TGGGCATCAATGACCTCGTT
		GAACCATGGGTGGAATCGTATT
pykF (*slr0587*) [a]	Pyruvate kinase	TGACCGTAGTAGGCGAACCAT
		TGGCCCGGCTGAATTTTT
ppc	Phosphoenolpyruvate carboxylase	TTCCTGCATTCGGTCTTTCC
		ACCGACTCTTCTTCAGAGTTGGA
gap 2	Glyceraldehyde 3-phosphate dehydrogenase (NADP+)	CCTGGGCAGAATGGAATGTAG
		CCCCTTCATGGGTAACAAAAAC
icd	Isocitrate dehydrogenase (NADP)	CCCCGATGAGTATTCCATTCTG
		GCGGCATCGGAAAGGTAAT
phaA.	Acetyl coenzyme A acetyltransferase (thiolase)	ACCCAGGTGTTAGCCAGTGATG
		AGCGCAATTTGCCTAGACTTTC
phaB	3-Oxoacyl-(acyl-carrier protein) reductase 2	GCCGGTATCACCAAAGACAAC

		CGTTCAACACTGCATCCCAAT
	GS-GOGAT pathway	
gltB	NADH-dependent glutamate synthase large subunit	TCGGGGTTACATCACCATTT
		GCGATCATACCGTCCAAAGT
gltD	NADH-dependent glutamate synthase small subunit	CCACTGCCACCAAAATTGAA
		ATCCTTTTCCCAACGGACATC
glnA	Glutamate-ammonia ligase (glutamine synthetase)	CGGCAAAACCGTTACCTTTA
		GGGTTAGTGAAGGCCAACAA
glsF	Ferredoxin-dependent glutamate synthase	CCAGGTTAAAGGCACCAAAA
		CCACTGCGACCAAAACCTAT
rnpB	RNase P subunit B	AGCGGCCTATGGCTCTAATCA
		CTGTGACAGGAATCCGCAACT
	Other functions	
ntcA	Global nitrogen regulator	GGGAAGAAAGCCCCTGTAACA
		TGTCTGCCCCCATTGAACA
ntcB	Nitrogen assimilation transcriptional activator	GTGTGGCAGCAATTCACTCAGT
		TGGCCCAGGCAAAATTTG
sigE	RNA polymerase sigma factor	ATCAGGTCGAGGAAGGGAACA
		ATCTCCGCCTAGTGGTTTCCA
gdhA	NADP-specific glutamate dehydrogenase	AATTACCCGCGACAATGCA
		GGGCCCATTAGCAACTTCAA
glnB	Nitrogen regulatory protein P-II	GCTGGTTAATGCAGGGATCGT
		GACCTTTTTGACGGCCAAAG

	Reverse transciptase PCR	
rre 37	Response regulator	CTAGGTAAGTACAGAGACTCCGGC
		GTGAATCCAGTCTACATATCAGTCG

[a]ORF no.

Metabolic profile analysis using LC/QqQ-MS

Cell sampling was performed as described previously [5]. Dried extracts were dissolved in 50 μL Milli-Q water and applied to an LC/QqQ-MS system (HPLC system: Agilent 1200 series, MS system: Agilent 6460 with Jet Stream Technology, Agilent Technologies) controlled with MassHunter Workstation Data Acquisition software v. B.04.01 (Agilent Technologies). LC/QqQ-MS was performed with multiple reaction monitoring as described previously [5].

Results and discussion

This is the first metabolomics-based report showing changes in carbon and nitrogen metabolism of a GT mutant lacking a response regulator, Rre37.

Delay of glycogen accumulation in Δrre37 under nitrogen starvation

During nitrogen starvation for 72 h, *rre37* was expressed in GT, whereas Δ*rre37* lacked expression of the *rre37* gene, confirming the disruption of *rre37* in the mutant (Fig.1.a). Disruption of *rre37* did not influence cell growth in Δ*rre37* during nitrogen depletion (data not shown).

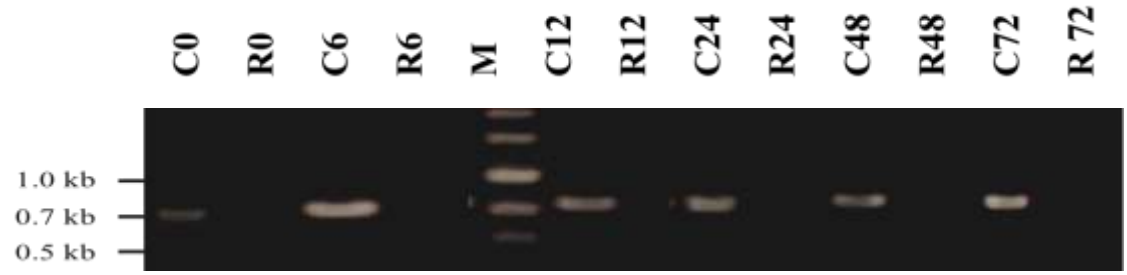

Fig. 1.a *rre37* expression in the glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (*Synechocystis*) and *rre37* knock out mutant. Expression in *Synechocystis* after 0, 6, 12, 24, 48, and 72 h was shown in lanes C0, C6, C12, C24, C48, and C72, respectively. Expression in *rre37* mutant after 0, 6, 12, 24, 48, and 72 h was shown in lanes R0, R6, R12, R24, R48, and R72, respectively. Lane M shows DNA ladder marker.

After nitrogen depletion, *Synechocystis* started to accumulate glycogen and the glycogen content reached 44% of dry-cell weight after 72 h [5]. Glycogen accumulation was delayed in Δ*rre37* for 24 h, although the glycogen content in both the GT and Δ*rre37* cells increased to 40% of dry-cell weight after 48 h (Fig. 1.b). Rre37 appears to have a functional role for glycogen accumulation up to 12 h after nitrogen depletion in GT.

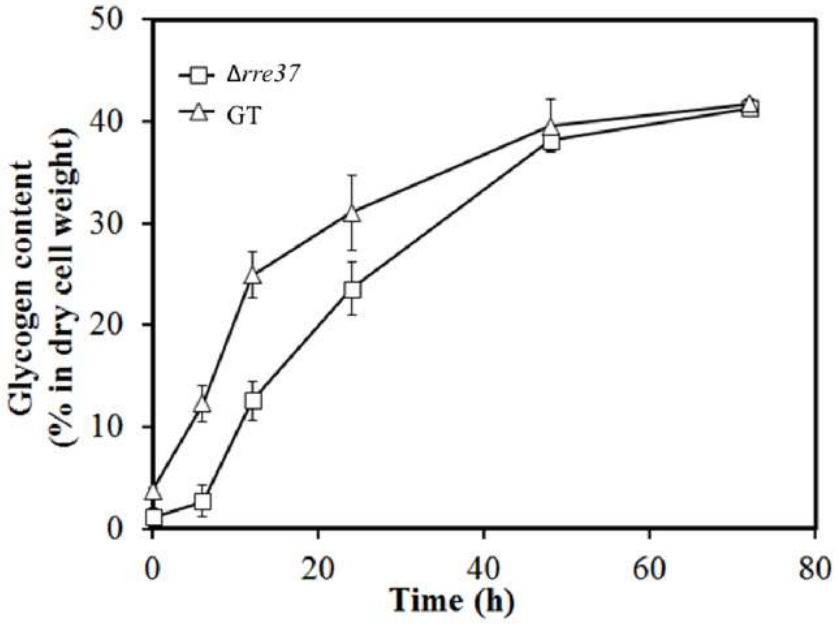

Fig. 1.b Glycogen content in glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (GT) (Δ) and *rre37* mutant (□) after nitrogen depletion.

Positive regulation of glycogen anabolic genes by rre37 under nitrogen starvation

To reveal the role of Rre37 during nitrogen depletion, changes in both metabolite content (related to glycogen catabolism and anabolism, glycolysis, pentose phosphate pathway, TCA cycle, and GS-GOGAT pathway) and gene expression (related to *glgA*, *glgC*, *glgP*, *glgX*, *gap1*, *gap2*, *zwf*, *gnd*, *ppc*, *pykF*, *icd*, *gdhA*, *gltB*, *gltD*, and *glnA*), which are involved in glycogen accumulation and the signaling pathway involved in nitrogen starvation, were compared for GT and Δ*rre37* after 6 h and 12 h (Fig. 2, Fig. 3 and table 2).

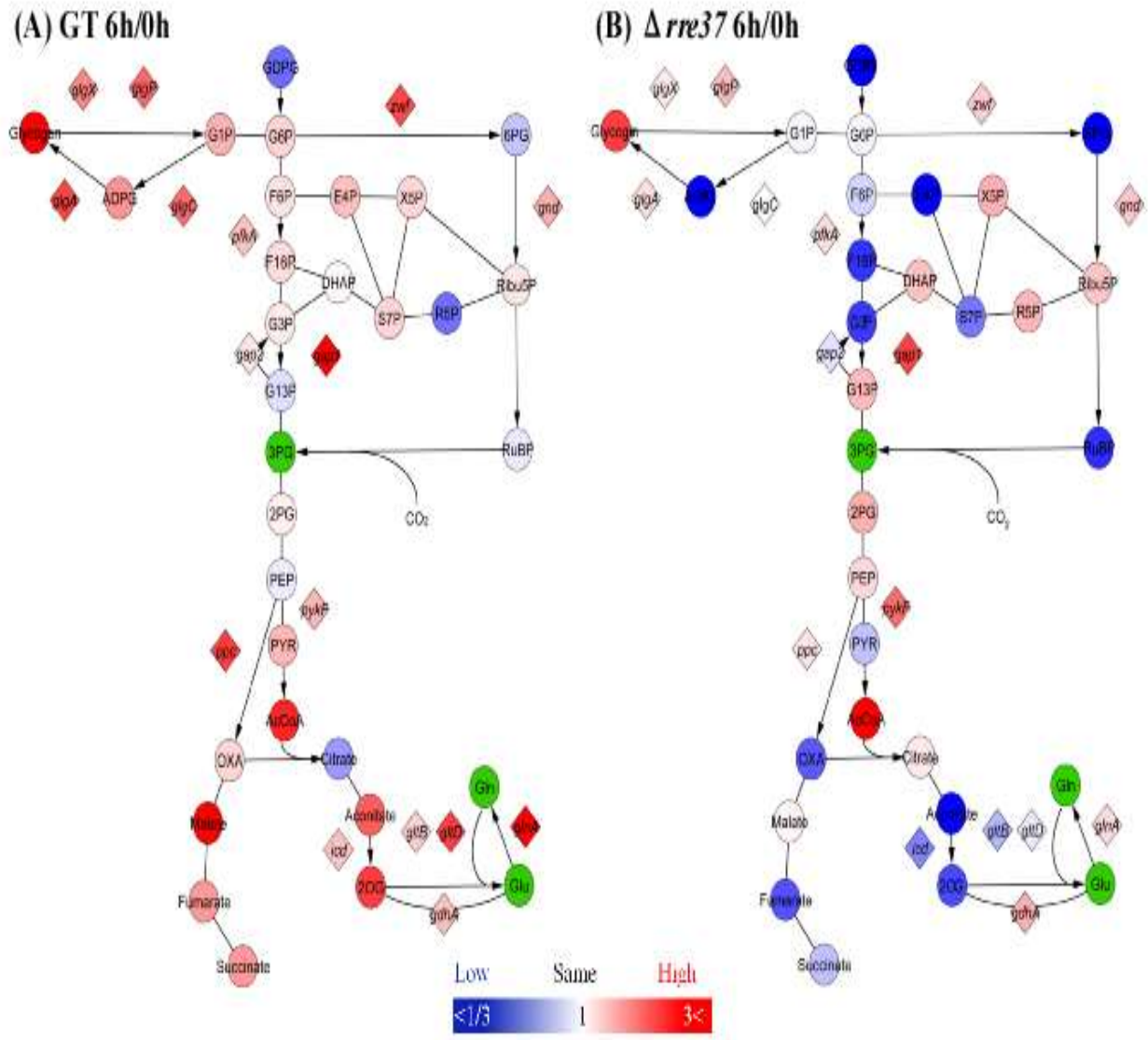

Fig. 2 Change in metabolite concentration (○) and gene expression (◊) in (A) *Synechocystis* sp. PCC 6803 (GT) and (B) *rre37* mutant (Δ*rre37*). Ratio of values after 6 h of nitrogen depletion to those at the starting point (0 h) were obtained. Fold change increases or decreases are indicated by shades of red and blue according to the scale bar.

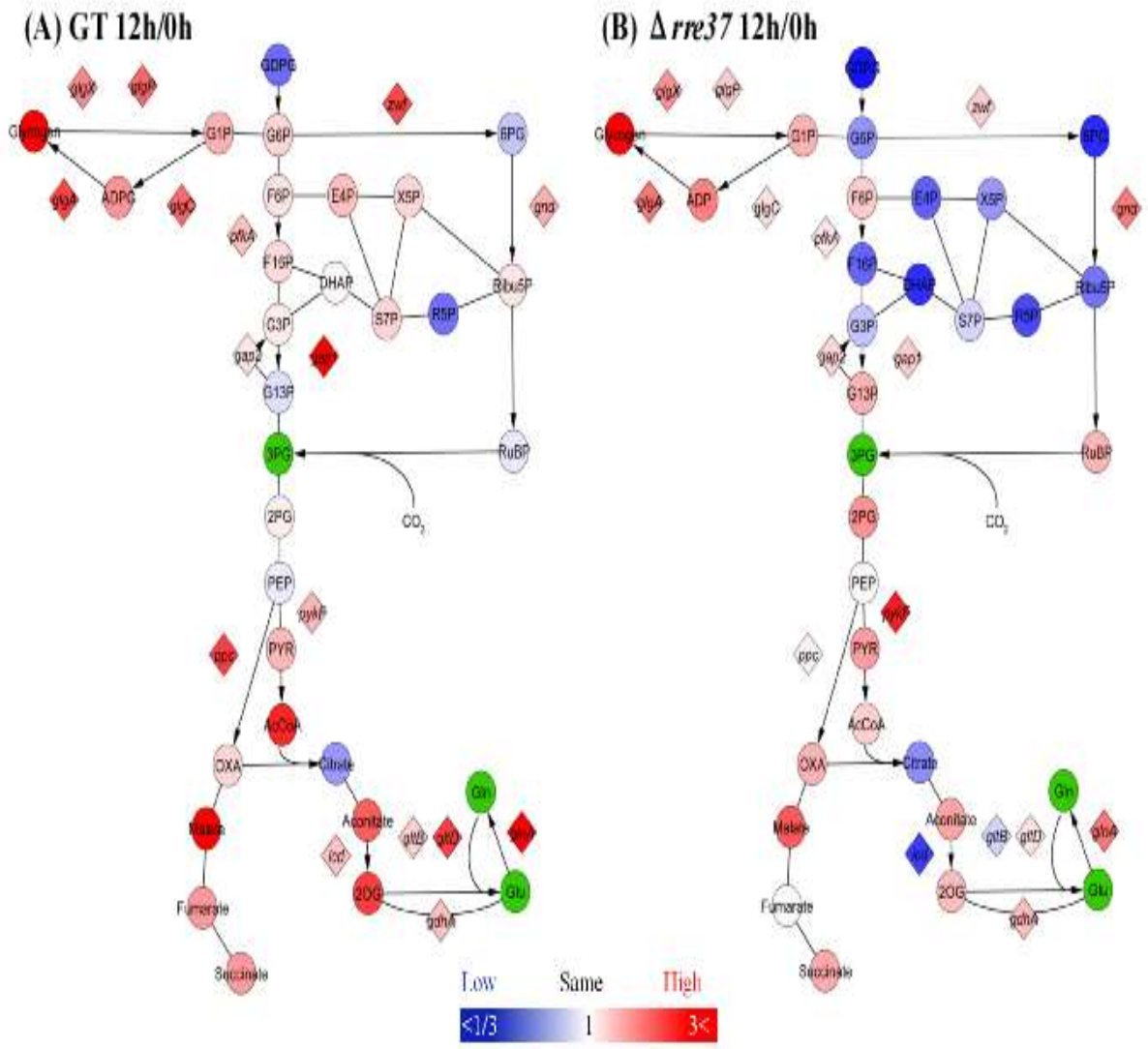

Fig. 3 Increase or decrease in metabolite concentration (◊) and gene expression (○) in (A) a glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (GT) and (B) the *rre37* mutant (Δ*rre37*). Values after 12 h of nitrogen depletion were compared with values at the starting point (0 h) to obtain the ratio of the values at 12 h to 0 h. Fold change increases or decreases are indicated by shades of red and blue according to the scale bar.

Abbreviations used for metabolites and genes in Fig 2 and 3

Metabolites: GDPG, GDP-glucose; ADPG, ADP-glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; G13P, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; 2-OG, 2-oxoglutarate; OXA, oxaloacetate; 6PG, 6-phosphogluconate; E4P, erythrose 4-phosphate; X5P, xylulose 5-phosphate; Ribu5P, ribulose-5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose-7-phosphate; RuBP, ribulose-1,5-bisphosphate; Gln, glutamine; Glu, glutamate. (Clear signals for 3PG, Gln, and Glu were not obtained and are shown in green.) Genes: *glgA* is glycogen (starch) synthase; *glgC*, glucose-1-phosphate adenylyltransferase; *glgX*, glycogen isoamylase; *glgP*, glycogen phosphorylase; *pfkA*, phosphofructokinase; *gap1*, glyceraldehyde 3-phosphate dehydrogenase (catabolic reaction); *gap1*, glyceraldehyde 3-phosphate dehydrogenase (NADP+); *ppc*, phosphoenolpyruvate carboxylase; *icd*, isocitrate dehydrogenase; *gdhA*, NADP-specific glutamate dehydrogenase; *gltB*, NADH-dependent glutamate synthase large subunit; *gltD*, NADH-dependent glutamate synthase small subunit; *glnA*, glutamate-ammonia ligase; *pykF*, pyruvate kinase 1; *zwf*, glucose-6-phosphate dehydrogenase; *gnd*, 6-phosphogluconate dehydrogenase.

Expression of two glycogen anabolic genes, *glgA* (encoding glycogen synthase) and *glgC* (encoding glucose-1-phosphate adenyltransferase) were about 2-fold upregulated after 6 h in GT, and ADP-glucose, a precursor of glycogen, accumulated; however, expression in Δ*rre37* was unaltered and the ADP-glucose level decreased (Fig. 2 and fig 3). Also, the expression of *glgX* and *glgP* increased after 6 h in GT and the glucose-1-phosphate level increased, whereas

in Δ*rre37*, the expression of these genes was lower than in GT and the amount of glucose-1-phosphate did not change. After 12 h, glycogen started to be accumulated in Δ*rre37* in accordance with the increase in expression of *glgA* and *glgC*, which reached a level similar to their expression in GT (Fig 4). However, the expression of *glgP* and *glgX* in Δ*rre37* were lower than in GT after 12 h. These results suggest that a delay in upregulation of expression of genes, *glgA* and *glgC*, caused a delay in glycogen accumulation in Δ*rre37*. Glycogen accumulation was delayed by low expression of glycogen anabolic genes, such as *glgA* and *glgC* in particular, after nitrogen depletion in Δ*rre37*. Reportedly, expression of *rre37* is correlated with an increase in expression of glycogen catabolic genes (*glgX* and *glgP*) under nitrogen depletion [13]. Our research showed that expression of *rre37* is also correlated with an increase in expression of glycogen anabolic genes, resulting in glycogen accumulation at the initial stage of nitrogen depletion.

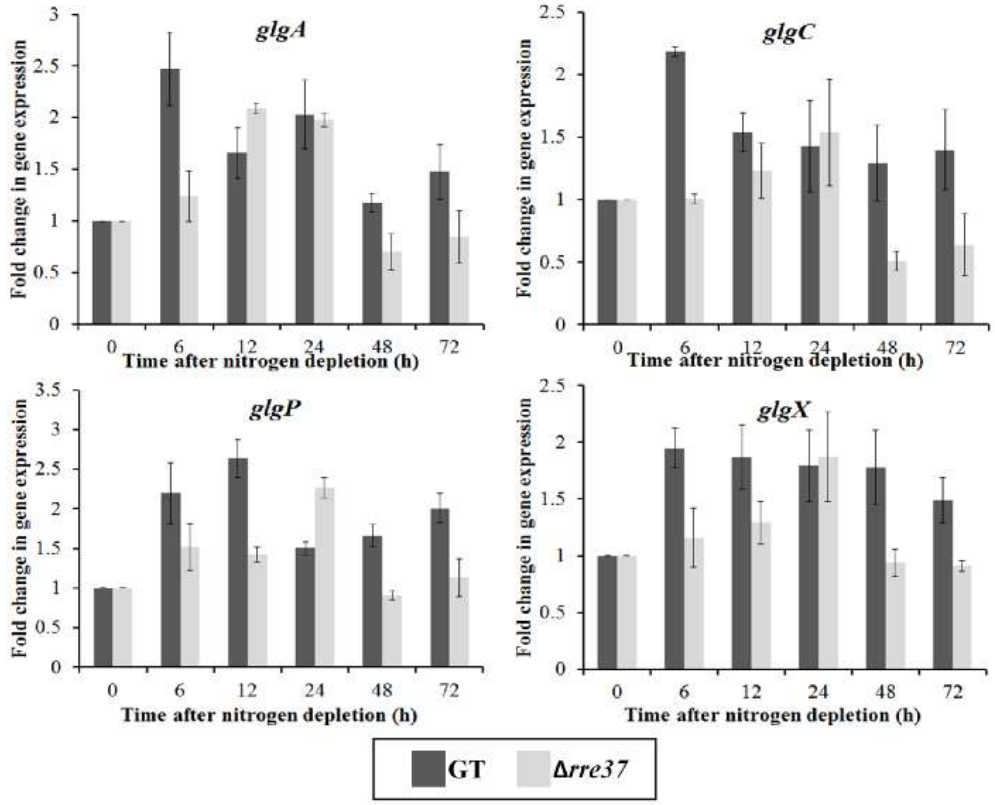

Fig. 4 Expression of catabolic and anabolic genes under nitrogen depletion in a glucose tolerant strain of *Synechocystis* sp. PCC 6803 (GT) and the *rre37* mutant (Δ*rre37*). *glgA* (glycogen synthase), *glgC* (glucose-1-phosphate adenylyl transferase), *glgP* (glycogen phosphorylase), *glgX* (glycogen isoamylase). Values are the means of 3 individual experiments. Error bars indicate standard deviation of triplicates.

Table 2 Change in Metabolite concentration after nitrogen depletion in GT and *Δrre37.* Values of metabolites are the ratio of respective hour to 0 hour after nitrogen depletion. Data represent means ± standard deviation (SD) of values from independent experiments (n = 3).

Metabolites	GT 6/0h	*Δrre37 6*/0h	GT12/0h	Δ*rre37* 12/0h
2-phosphoglycerate (2PG)	1.13 ± 0.34	1.62 ± 0.52	1.04 ± 0.47	1.86 ± 0.31
6-phosphogluconate (6PG)	0.84 ± 0.22	0.18 ± 0.04	0.63 ± 0.09	0.42 ± 0.05
Acetyl-CoA (AcCoA)	4.28 ± 0.34	4.74 ± 0.51	2.55 ± 0.11	1.80 ± 0.41
Aconitate	2.02 ± 0.02	0.40 ± 0.09	2.93 ± 0.28	1.68 ± 0.10
ADP-glucose (ADPG)	1.85 ± 0.49	0.39 ± 0.08	1.92 ± 0.56	2.00 ± 0.29
2-oxoglutarate (2-OG)	2.69 ± 0.02	0.50 ± 0.20	3.00 ± 0.17	1.52 ± 0.06
Citrate	0.72 ± 0.21	1.16 ± 0.03	0.53 ± 0.07	0.68 ± 0.12
Dihydroxyacetone phosphate (DHAP)	1.07 ± 0.01	1.48 ± 0.19	1.09 ± 0.11	0.43 ± 0.07
Erythrose 4-phosphate (E4P)	1.52 ± 0.03	0.22 ± 0.16	1.50 ± 0.13	0.43 ± 0.06
Fructose-1,6-bisphosphate (F16P)	1.27 ± 0.75	0.44 ± 0.33	1.52 ± 0.39	0.58 ± 0.19
Fructose-6-phosphate (F6P)	1.22 ± 0.71	0.88 ± 0.63	1.30 ± 0.12	1.40 ± 0.12
Fumarate	1.81 ± 0.34	0.52 ± 0.00	1.87 ± 0.22	1.04 ± 0.18
1,3-bisphosphoglycerate (G13P)	0.91 ± 0.30	1.54 ± 0.41	0.76 ± 0.47	1.61 ± 0.93
Glucose-1-phosphate (G1P)	1.61 ± 0.42	0.98 ± 0.11	1.52 ± 0.10	1.61 ± 0.05
Glyceraldehyde-3-phosphate (G3P)	1.25 ± 0.10	0.46 ± 0.12	1.20 ± 0.02	0.84 ± 0.09
Glucose-6-phosphate (G6P)	1.44 ± 0.21	0.97 ± 0.06	1.52 ± 0.27	0.62 ± 0.01
GDP-glucose (GDPG)	0.60 ± 0.06	0.20 ± 0.04	0.72 ± 0.24	0.36 ± 0.14
Malate	4.78 ± 0.35	1.07 ± 0.16	5.01 ± 0.58	2.29 ± 0.55
Oxaloacetate (OXA)	1.32 ± 0.28	0.54 ± 0.12	1.54 ± 0.05	1.60 ± 0.29
Phosphoenolpyruvate (PEP)	0.91 ± 0.12	1.51 ± 0.32	0.95 ± 0.01	1.12 ± 0.03
Pyruvate (PYR)	1.55 ± 0.32	0.65 ± 0.19	1.51 ± 0.39	1.38 ± 0.19
Ribose 5-phosphate (R5P)	0.61 ± 0.05	1.57 ± 0.35	0.58 ± 0.04	0.50 ± 0.04
Ribulose-5-phosphate (Ribu5P)	1.20 ± 0.07	1.45 ± 0.26	1.22 ± 0.21	0.62 ± 0.13
Ribulose-1,5-bisphosphate (RuBP)	0.94 ± 0.23	0.43 ± 0.08	0.85 ± 0.17	1.58 ± 0.25
Sedoheptulose-7-phosphate (S7P)	1.33 ± 0.04	0.62 ± 0.02	1.33 ± 0.15	0.78 ± 0.52
Succinate	1.58 ± 0.09	0.83 ± 0.17	1.67 ± 0.58	1.66 ± 0.18
Xylulose 5-phosphate (X5P)	1.33 ± 0.10	1.62 ± 0.30	1.23 ± 0.02	0.71 ± 0.14

Effect of Rre37 on glycolysis, pentose phosphate pathway, and TCA cycle

For metabolites related to glycolysis pathway, fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate were increased and 1,3-bisphosphoglycerate was decreased in GT after 6 h of nitrogen depletion; however, contradictory tendencies of these metabolites were observed in Δ*rre37*, showing that drastic change in glycolytic pathway by disruption of Rre37 (Fig. 2 and table 2). For metabolites related to pentose phosphate pathway, erythrose 4-phosohate and sedoheptulose-7-phosphate were increased and ribose 5-phosphate was decreased in GT after 6 h of nitrogen depletion; however, contradictory tendencies of these metabolites were observed in Δ*rre37*, showing that metabolites nearly associated with glycolytic pathway were changed in pentose phosphate pathway. For genes related to glycolysis pathway, expression of *gap1* was 5.2-fold increased in GT, however this was 2.5-fold increased in Δ*rre37* (Fig. 2 and table 2). Expression of *gap2* was slightly increased (1.2-fold) in GT, but was slightly decreased (0.9-fold) in Δ*rre37*, correlating with increase of glyceraldehyde-3-phosphate level in GT. For genes related to pentose phosphate pathway, expressions of glucose-6-phosphate dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) were not so changed between GT (2.4, and 1.6-fold, respectively) and Δ*rre37* (1.4, and 1.5-fold, respectively) after 6 h of nitrogen starvation.

All the metabolites of the TCA cycle except for citrate increased during nitrogen depletion in the *Synechocystis* cells, as previously described [6], but oxaloacetate, fumarate, succinate, aconitate, and 2-OG decreased in Δ*rre37* (Fig. 2). Also under photomixotrophic conditions, *Synechocystis* increased the levels of malate, fumarate, and isocitrate [23]. The expression of *icd*, encoding isocitrate dehydrogenase, which catalyzes the production of 2-OG, was examined in both GT and Δ*rre37* cells. As previously reported [11, 24], *icd* gene expression was upregulated in GT (1.5-fold) after 6 h of nitrogen depletion but it was downregulated (0.7-fold) in Δ*rre37* (Fig. 2). *icd* gene expression was upregulated 1.4-fold in

GT after 12 h of nitrogen depletion, but in Δ*rre37*, it was downregulated except at 24 h (Fig 5). These data suggest that downregulation of *icd* gene expression is one reason for the delayed accumulation of 2-OG in Δ*rre37*. In *Synechocystis*, the gene product of *ppc* (phosphoenolpyruvate carboxylase) converts phosphoenolpyruvate to oxaloactetae [25]. Expression of *ppc* was upregulated in GT (2.5 fold) and unchanged in Δ*rre37* (1.2 fold), which showed decreased accumulation of oxaloacetate in Δ*rre37* after 6 h of nitrogen depletion (Fig. 2).

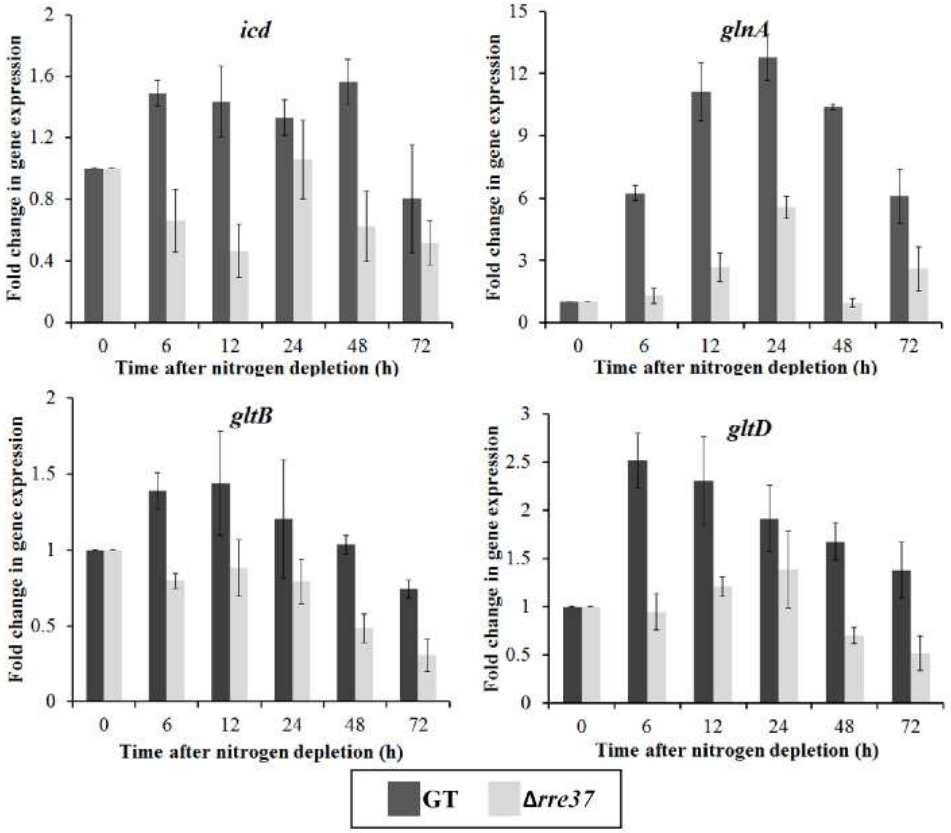

Fig. 5 Expression of genes involved in carbon-nitrogen balance under nitrogen depletion in a glucose tolerant strain of *Synechocystis* sp. PCC 6803 (GT) and the *rre37* mutant (Δ*rre37*). *icd* (isocitrate dehydrogenase), *glnA* (glutamate-ammonia ligase), *gltB* (NADH-dependent glutamate synthase large subunit), and *gltD* (NADH-dependent glutamate synthase small

subunit). Values are the means of 3 individual experiments. Error bars indicate standard deviation of triplicates.

The observed differences in TCA cycle metabolites between GT and Δ*rre37* are likely partially due to differential expression of *ppc*. Expression level of *ppc*, was 1.4-fold in wild type after 12 h of nitrogen starvation [11], correlating with our research (1.9-fold after 12 h of nitrogen starvation) (Fig 3). Similar expression level of *ppc* in Δ*rre37* after nitrogen starvation was one reason of decreases in oxaloacetate, fumarate, and succinate. Decreased sugar catabolism in Δ*rre37* would also affect low accumulation of TCA cycle metabolites.

Decrease in downstream glycolytic metabolites by *rre37* knockout correlates with previous research that increase in metabolites such as glucose-6-phosphate, fructose-6-phosphate, and dihydroxyacetone phosphate, was observed after 4 or 6 h of nitrogen depletion in GT [6,26]. Decreased glycolytic metabolites caused decrease of erythrose 4-phosphate and sedoheptulose-7-phosphate in pentose phosphate pathway, which are connected with glycolysis pathway. Rre37 activates *gap1* in glycolysis under nitrogen starvation [13] and higher expression of *gap1* was observed in GT, compared with Δ*rre37*. Similar expression levels of *zwf* and *gnd* in pentose phosphate pathway between GT and Δ*rre37* correlate with the previous research that SigE activates *zwf* and *gnd* [13].

Expression of nitrogen assimilatory genes is reduced by rre37 knockout

Since 2-OG accumulation differed between GT and Δ*rre37*, the transcript levels of genes related to nitrogen assimilation were examined. Expression of *glnA* (encoding glutamate-ammonia ligase) was upregulated 6-fold under nitrogen starvation in GT, consistent

with previous studies [11,16] whereas *glnA* was upregulated only 1.2-fold in Δ*rre37* after 6 h of nitrogen depletion (Fig. 2 and 3). Expression of *gdhA*, which encodes glutamate dehydrogenase, catalyzing the reversible reaction between 2-OG and glutamate [27], was upregulated in GT and in Δ*rre37* (1.4 and 1.6-fold, respectively) after 6 h of nitrogen depletion. *Synechocystis* possess two types of GOGATs; one using ferredoxin and a second type using NADH as electron donors [7]. Both *gltD* (encoding NADH dependent glutamate synthase small subunit) and *gltB* (NADH-dependent glutamate synthase large subunit) were upregulated in GT (2.4 and 1.7-fold, respectively) but in Δ*rre37*, these genes were downregulated (0.8-fold) after 6 h of nitrogen depletion. On the other hand, expression of *glsF* (encoding ferredoxin-dependent glutamate synthase) was upregulated in both GT and Δ*rre37* (3.0 and 3.4-fold, respectively). These results suggested that expression of genes in the GS-GOGAT pathway is partly inhibited by *rre37* knockout.

Expression analysis of genes related to nitrogen regulators

Since *rre37* knockout widely affected primary carbon and nitrogen metabolism, the transcript levels of four nitrogen regulators, *ntcA, ntcB, sigE* and *glnB* were measured for GT and Δ*rre37* cells. In the presence of excess 2-OG under nitrogen limited conditions, the PII protein is phosphorylated on binding 2-OG and ATP, a phenomenon that has been well investigated in *Synechococcus* sp. PCC 7942, and 2-OG also activates NtcA [28- 30]. Under nitrogen starvation in GT, *ntcA* was upregulated 2.3-fold and *glnB* 4.6-fold after 6 h of nitrogen depletion, whereas *ntcA* and *glnB* were slightly increased in Δ*rre37* (1.4 and 2.2-fold, respectively), (Fig. 6). In Δ*rre37*, the level of *ntcA* and *glnB* transcripts increased gradually, a similar pattern to that of glycogen catabolic and anabolic genes (*glgA, glgC, glgP*, and *glgX*). Our results showed that *ntcA* and *glnB* transcription correlates 2-OG accumulation, which agrees with the 2-OG dependence of NtcA mediated gene expression. Similar expression

pattern of *sigE* in GT and Δ*rre37* was consistent with *sigE* and *rre37* (Fig. 6) working independently [13]. In *Synechocystis*, expression of *ntcB*, a nitrate assimilation transcriptional activator, is enhanced even in the absence of nitrite [31]. In GT, *ntcB* was not upregulated at 6 h, corresponding with previous results [11], but in Δ*rre37*, *ntcB* expression decreased in response to nitrogen depletion, suggesting that *rre37* affects nitrate assimilation.

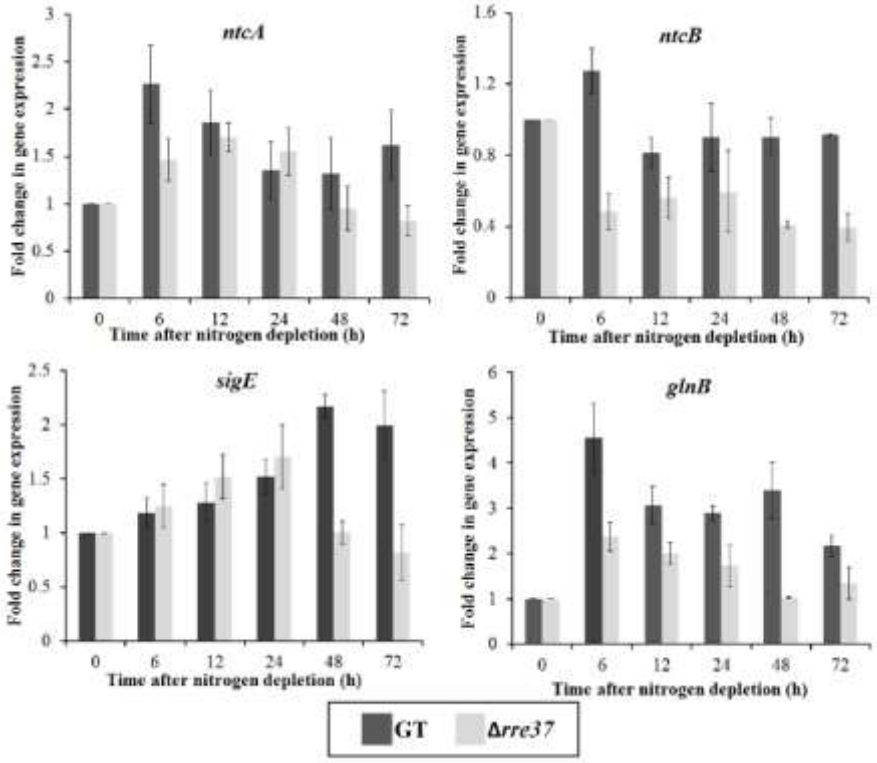

Fig. 6 Expression analysis of genes involved in nitrogen regulation during nitrogen depletion in *Synechocystis* sp. PCC 6803 (GT) and *rre37* mutant (Δ*rre37*). *ntcA* (global nitrogen regulator), *ntcB* (nitrate assimilation transcription factor), *sigE* (RNA polymerase group 2 sigma factor), *glnB* (nitrogen regulatory protein P II).

Our studies showed that nitrogen depletion led to 2-OG accumulation and expression of genes related to *icd* and the GS-GOGAT pathway, such as *glnA*, *gltB*, and *gltD*, were upregulated in GT, whereas in Δ*rre37*, there was delayed accumulation of 2-OG and relatively

low expression of these genes (Fig.7). *icd* and *glnA* are reported to have NtcA-activated promoters [7, 32], and low upregulation of *icd* and *glnA* gene expression in Δ*rre37* is partially due to low upregulation of *ntcA* gene expression. However, NtcA promoter for *gltB* and *gltD* genes, related to the GOGAT system, has not been found [7, 32] Therefore, regulatory mechanism on expression of *gltB* and *gltD* during nitrogen starvation is interesting and should be investigated.

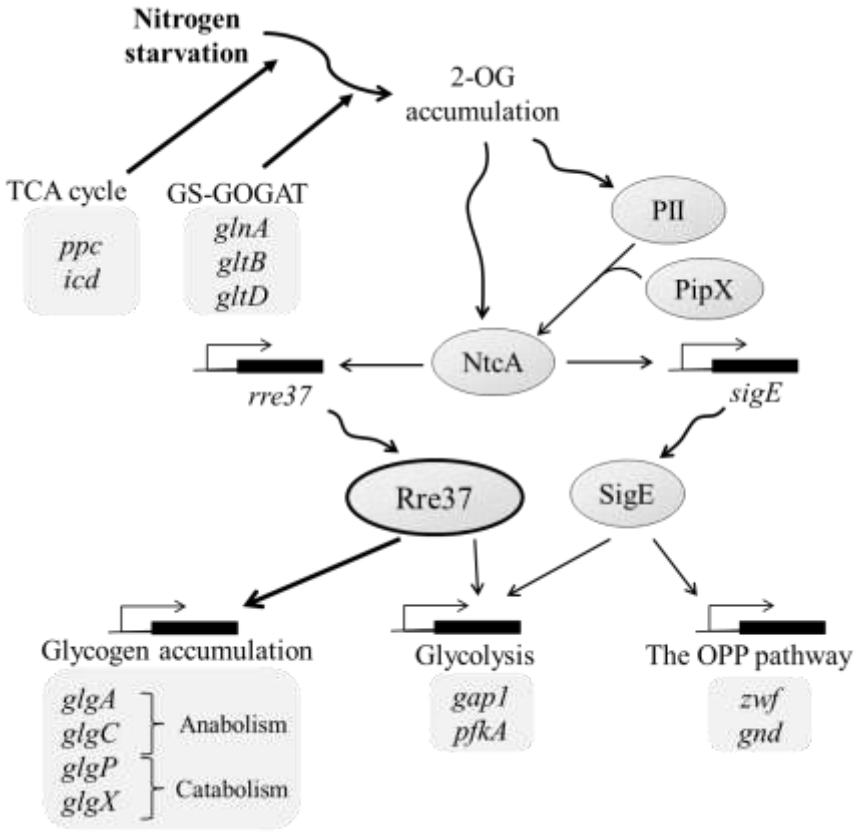

Fig. 7 Schematic model for Rre37 function under nitrogen starvation in *Synechocystis* sp. PCC 6803 [Partially revised from Azuma et al. [13]. In this research, Rre37 positively regulates glycogen anabolic genes (*glgA* and *glgC*). Expression of *icd* gene and activation of GS-GOGAT system urges 2-OG accumulation.]. OPP, oxidative pentose phosphate.

Metabolomics and quantitative gene expression analysis using the *rre37* mutant showed the role of Rre37 under nitrogen starvation. 1) Rre37 positively regulated glycogen anabolic genes, thereby accumulating glycogen. 2) Rre37 positively regulated sugar catabolism, and was involved in 2-OG accumulation. 3) Rre37 urged accumulation of TCA cycle metabolites such as malate, fumarate, and succinate. In addition to sugar metabolism, previous study showed that expressions of *phaA* and *phaB* genes (encoding β-ketothiolase and acetoacetyl-CoA reductase, respectively), related to polyhydroxybutyrate accumulation, were decreased (about 0.3-fold) in Δ*rre37*, compared with GT after 4 h of nitrogen depletion [13]. Here we showed that Rre37 is also involved in the regulation of nitrogen metabolism (Supplementary Fig. S3), and therefore, future studies could extend the roles of Rre37 into areas other than sugar metabolism in this cyanobacterium.

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Chapter 3

Increased biomass production and glycogen accumulation in *apcE* gene deleted *Synechocystis* sp. PCC 6803

Introduction

Biorefinery processes that convert sustainable biomass into commercially valuable chemical and energy sources have received increasing attention because of depleting fossil fuel reserves and concerns about the accumulation of greenhouse gases [1]. Recently, microalgae and cyanobacteria have been utilized as feedstocks for biorefinery processes due to their high photosynthetic efficiency compared with sugar or starch crops such as sugar cane and corn, and CO_2-neutral fuel production [2-4]. For example, the freshwater microalgae *Hydrodictyon reticulum* has been was utilized as a carbon source for the efficient production of lactic acid through a simultaneous enzymatic hydrolysis and fermentation process involving lactic acid bacteria [3]. In addition, bioethanol was produced directly from the cyanobacterium *Arthrospira platensis*, which contains high levels of storage glycogen, by a recombinant yeast strain diplaying an amylolytic enzyme [4]. To further increase the production of energy and valuable chemical products using these biorefinery processes, it is necessary to increase the mass productivity and carbohydrate content of oxygenic photosynthetic microorganisms.

At high light intensities, the rate of photon absorption by the chlorophyll antenna molecules in the surface layer of microalgal and cyanobacterial cells in cultures or pond environments exceeds the rate of photosynthetic reactions, resulting in the dissipation or loss of excess photons and/or photoinhibition of photosynthesis [5]. In addition, as a result of this excess photon absorption by surface-layer cells, cells at greater distances from the surface are deprived of sufficient light to support photosynthesis [5]. However, it was shown that a

truncated chlorophyll antenna mutant of the green alga *Chlamydomonas reinhardtii* exhibited reduced light absorbance of light by the first layers of cells and alleviated photoinhibition, resulting in high biomass production [6].

The cyanobacterium *Synechocystis* sp. PCC 6803 has been widely studied as a model species for photosynthetic processes because of its transformability and full sequenced genome [7]. *Synechocystis* sp. PCC 6803 have a large membrane-extrinsic phycobilisome antenna, which is composed of rods of the pigment phycocyanin, an allophycocyanin core, and several linker proteins connecting the rods to the core and the core to the thylakoid membrane [8]. However, truncation of phycobilisome antenna by deleting phycocyanin rods or the entire phycobilisome assembly decreases biomass productivity [9]. Previous studies have shown that deletion of *apcE* gene, whose product is involved in the interection between the thylakoids and phycobilisome [10], leads to the loss of phycobilins from the thylakoid fraction and decrease in antenna size [10, 11]. Degradation of phylicobisome and induction of glycogen synthesis has been observed in *Synechocystis* sp. PCC 6803 under nitrogen starvation conditions [12]. To date, however, the effect of *apcE* deletion on biomass production has not been investigated.

In this study, the effect of *apcE* gene deletion on the growth of *Synechocystis* sp. PCC 6803 was investigated under photoautotrophic and atmospheric air conditions. In addition, the effect of *apcE* gene deletion on glycogen accumulation was also investigated under these conditions.

Materials and Methods

Microorganisms and growth condition

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (here after referred to as GT) was obtained from Prof. Masahiko Ikeuchi of the University of Tokyo. *Synechocystis* sp. PCC 6803 was routinely cultured in BG11 medium, which contained 1.5 g L^{-1} $NaNO_3$, 0.04 g L^{-1} K_2HPO_4, 0.075 g L^{-1} $MgSO_4 \cdot 7H_2O$, 36 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 6 mg L^{-1} citric acid, 6 mg L^{-1} ferric ammonium citrate, 1 mg L^{-1} EDTA (disodium salt), 20 mg L^{-1} $NaCO_3$, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.222 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.39 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 0.079 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 49.4 μg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$ [13], under continuous illumination at 50 or 200 μmol photons m^{-2} s^{-1} using white fluorescence bulbs (Life Look HGX and NHG; NEC, Tokyo, Japan) at 28±2°C under atmospheric air conditions. Light intensity was measured in the middle of the culture using an LI-250A light meter (LI-COR, Lincoln, NE) equipped with an LI-190SA quantum sensor (LI-COR). *Escherichia coli* strain DH5α was used to propagate pBluescriptSK- and the *apcE* inactivation plasmids.

Cloning and transformation

For the construction of an *apcE* mutant, the 500-bp upstream and downstream regions of the *apcE* (slr0335) coding region of GT were isolated by PCR using the primers listed in Table 1. The obtained DNA fragments were joined through standard PCR-driven overlap extension to form a single DNA segment harbouring a AatII restriction site in place of the

coding region. After cloning the fused DNA fragment into pBluescriptSK, the resulting plasmids were digested with AatII, and an AatII-digested Km^r cassette, which was amplified from the pCR® II-Blunt-TOPO® vector (Toyobo Life science, Japan) was ligated into the plasmid in the same orientation as the coding region. The resulting deletion cassette was verified by PCR and nucleotide sequencing (Big Dye kit, ABI Perking Elmer). For the selection and maintenance of plasmids, the culture medium was supplemented with 50 μg L^{-1} of kanamycin. The vector containing the deletion casette was transformed into GT by homologous recombination and positive colonies were identified by PCR analysis. Selected clones were restreaked on plates supplemented with appropriate antibiotics to obtain complete deletion of the *apcE* gene. The *apcE* gene-disrupted GT mutant (Δ*apcE* mutant) was routinely cultivated in BG11 medium supplemented with 50 μg L^{-1} of kanamycin under the same conditions used for GT, unless otherwise mentioned.

Table 1 Primers used in this study.

Primer Name	Sequence (5′-3′)
ApcE_up-F	CGGGCCCCCCCTCGAGGGGCATTTCCCACCCCGTTTCA
ApcE_up-R	ATGAATTGTTTTAGGACGTCTTGGATTTCATTATCTCCCATTAACA
ApcE_down-F	CTCATTTTAGCCATGACGTCGGTTGCGGTTGTTTAAAGCTTAGC
ApcE_down-R	GTGGATCCCCCGGGCTGCAGTGGGCATAATCCTCCAATTGGGCTAGC
kanamycin-F	ATAATGAAATCCAAGACGTCCTAAAACAATTCATCCAGTAA
kanamycin-R	AAACAACCGCAACCGACGTCATGGCTAAAATGAGAATATCACCG
ApcE gene-F	ATGAGTGTTAAGGCAAGTGG TGGC
ApcE gene-R	CTAACCGCCCACTTTTACTACTGGGGTA

Spectral analysis

After cultivation of GT for 9 days under the conditions described above, the culture was diluted with fresh medium to adjust the OD_{750} to 0.1. Steady-state absorption spectra were collected at room temperature using a spectrometer equipped with an integrating sphere (JASCO V-650/ISV-722), as described previously [11]. All absorption spectra were normalized to the chlorophyll Qy band (~676 nm).

Analytical Methods

Cell growth was monitored tubidimetrically by measuring OD_{750}. Cell concentration in culture media is reported as dry cell weight, as a linear correlation was obtained between dry cell weight and optical density. It was determined that 1.0 OD_{750} equals approximately 0.26 g dry-cell weight L^{-1}.

The total carbohydrate content was analyzed by a colorimetric method using anthrone reagent [14]. Briefly, algal powder was reacted in 75% sulfuric acid containing 2 g L^{-1} anthrone reagent for 15 min at 100°C. The absorbance of the resulting solution and glucose as standard was measured at 620 nm. Total protein content was extracted from cells as described previously [15]. Protein concentrations were determined using a Sigma QuantiPro BCA Assay Kit (Sigma-Aldrich, St Louis, MO, USA) as directed by the manufacturer's protocol and using bovine serum albumin as a standard. Glycogen content was determined by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a size exclusion HPLC

column (OHpak SB-806 M HQ; Shodex, Tokyo, Japan) and a reflective index detector (RID-10A; Shimadzu) as described previously [16]. Glycogen was extracted from the dried cells using a previously reported method with slight modifications [17]. The cells used for the assay were collected by centrifugation at 6,300 × *g* for 2 min at 25 ± 2°C and washed once with 0.3 M ammonium carbonate, which was volatilized during the subsequent lyophilisation process.

Photosynthetic oxygen evolution and dark respiration by cells were determined in the exponential growth phase with a Clark-type oxygen electrode (DW2/2, Hansatech Instruments Ltd., King's Lynn, UK) controlled by a computerized oxygen monitoring system (OMS; Hansatech Instruments Ltd.). After system calibration, dark respiratory O_2 uptake was recorded for 8 min, followed by photosynthetic O_2 evolution, which was monitored for 8 min at 30°C during irradiation at 50, 100, 300, 600 μmol photons m^{-2} s^{-1} from a halogen light source. Cell concentration in the reaction mixture was adjusted to an OD_{750} of 1.0 with BG11 medium.

Results and Discussion

Construction of an Δapc E mutant

Complete deletion of the *apcE* gene was confirmed by PCR analysis using the primers designed to bind 500 bp upstream and downstream of the *apcE* gene, as listed in Table 1. The Δ*apcE* mutant gave a single amplicon of 2.0 kb, confirming the deletion of the *apcE* gene and

insertion of the kanamycin cassette, whereas GT produced an amplicon of 3.6 kb corresponding the *apcE* gene (Fig 1a).

Absorption spectra of GT and ΔapcE mutant

For a quantitative analysis of the spectral features of GT and the Δ*apcE* mutant, absorption spectra of intact cells were measured (Fig. 1b). All absorption spectra were normalized at the chlorophyll Qy band. The absorption spectra of GT showed four peaks, which were assigned to the chlorophyll Soret (~435 nm), carotenoid (~500 nm), phycobilisome (~620 nm), and chlorophyll Qy (~676 nm) bands based on a previous report [18]. The Δ*apcE* mutant showed a large reduction in the 620-nm absorption maximum of GT, as was previously observed for a phycobiliprotein-less mutant of *Synechocystis* sp. PCC 6803 [10, 19]. The relative amount of chlorophyll (chlorophyll Soret and chlorophyll Qy) in the Δ*apcE* mutant and wild-type GT were similar. These results suggested that deletion of the *apcE* gene, which would have impaired phycobilisome assembly and attachment, does not affect the amount of chlorophyll in cells.

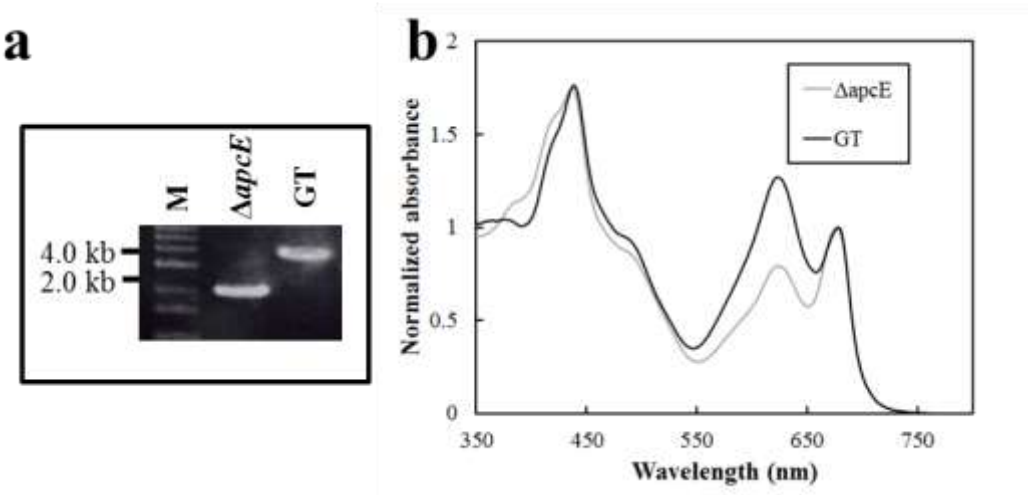

Fig. 1 (a) Confirmation of complete deletion of the *apcE* gene in glucose-tolerant *Synechocystis* sp. PCC 6803 (GT) by PCR with primers flanking the *apcE* gene. Lane 1, DNA size markers (M); lane 2, deletion of the *apcE* gene in GT; and lane 3, full-length *apcE* gene in GT. (b) Absorption spectral analysis of GT (black line) and Δ*apcE* (grey line).

Effect of apcE gene deletion on growth of GT

To determine the impact of minimizing phycobilisome antenna size through deletion of the *apcE* gene on photoautotrophic growth, GT and the Δ*apcE* mutant were grown under photoautotrophic conditions in atmospheric air conditions. Under these conditions, biomass production of the Δ*apcE* mutant, as determined from OD_{750}, was approximately 1.7-fold higher than that of GT after 12 days of cultivation with 50 μmol photons m^{-2} s^{-1} illumination (0.75 ± 0.05 and 1.24 ± 0.03 g-cell-dry weight L^{-1} for GT and the Δ*apcE* mutant, respectively) (Fig. 2). When the light intensity was increased to 200 μmol photons m^{-2} s^{-1}, biomass production of the Δ*apcE* mutant was higher than that of GT (data not shown). Deletion of the

apcE gene caused a colour change in GT. The Δ*apcE* mutant appeared olive green in colour due to a reduction in the amount of phycocyanin, as reported previously [11], whereas GT cell appeared blue-green in colour

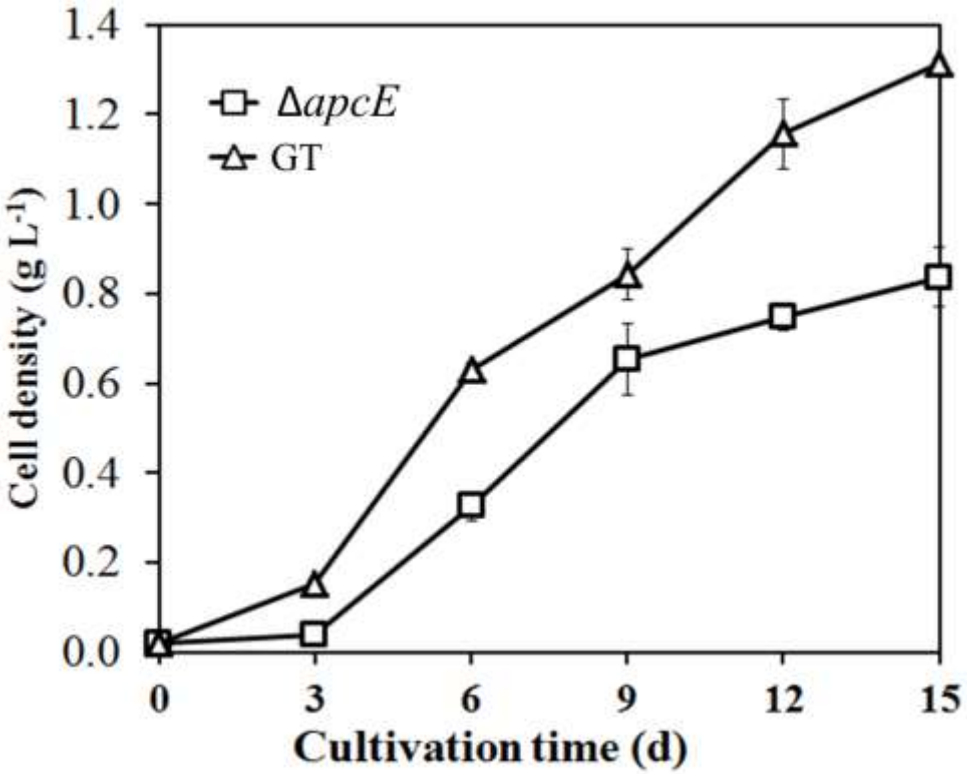

Fig. 2 Growth curves of glucose-tolerant *Synechocystis* sp. PCC 6803 (GT) (open triangles) and the Δ*apcE* mutant (open squares) under photoautotrophic and air conditions with illumination at 50 μmol photons m^{-2} s^{-1}. Error bars represent the mean of three triplicate experiments.

Here, reducing the chlorophyll antenna size of the photosystems through mutation of the *apcE* gene in *Synechocystis* sp. PCC 6803 led to incresed productivity, as determined by the increase in cell density under photoautotrophic and low CO_2 conditions (0.04% in air). The Δ*apcE* mutant has a similar doubling time as the glucose-tolerant strain of *Synechocystis*

sp. PCC 6803 (21.25 ± 0.55 h and 24.88 ± 0.81 h for GT and Δ*apcE* mutant, respectively), between 3 to 6 days of culture. These doubling times are similar to that (25.2 h) reported previously for wild-type *Synechocystis* sp. cells [9]. Deletion of the Δ*apcE* gene increased the maximum cell density of cultures due to the decreased efficiency of light use by cells at the surface layer. Previous investigations [20, 21] showed that the growth rate of a *Synechocystis* Δ*apcE* mutant was slower than that of the wild-type strain under photoautotrophic conditions in air enriched with 1% CO_2. Together, these findings suggest that the presence of high concentrations of CO_2 might reduce the growth of Δ*apcE* mutants. Although the reason behind this phenomenon is presently unclear, metabolic and gene expression analysis may provide insight into this responses.

Effect of apcE deletion on cell compositions of GT and ΔapcE mutant

To investigate the effect of *apcE* deletion on cell composition under photoautotrophic and air conditions, carbohydrate and protein contents were analysed in GT and the Δ*apcE* mutant after 15 days of cultivation with 50 μmol photons m^{-2} s^{-1} illumination (Table 2). The protein and carbohydrate contents in the Δ*apcE* mutant were similar with those in GT, showing that *apcE* deletion did not affect protein and carbohydrate levels. However, in GT, only an average of 42.0% ± 0.3% of available carbohydrate was converted to glycogen, whereas the Δ*apcE* mutant converted 85.0% ± 4.8% of carbohydrate to glycogen. The accumulation of glycogen in cyanobacterial cells, which are attractive feedstocks for biorefinery processes [4]. Glycogen accumulation in the Δ*apcE* mutant corresponded to approximately 24% of total dry cell weight after 15 days of cultivation, whereas the accumulated glycogen in GT cells

corresponded to only 11% of dry cell weight (Table 2). At 200 μmol photons m^{-2} s^{-1} illumination, the glycogen content as a fraction of total dry cell weight was higher in the Δ*apcE* mutant than that of GT (data not shown). These results showed that the restriction of photoantennae size directed carbon flux towards glycogen accumulation under photoautotrophic and air conditions.

Table 2 Biochemical composition of glucose-tolerant *Synechocystis* sp. PCC 6803 (GT) and the Δ*apcE* mutant under photoautotrophic and air conditions with illumination at 50 μmol photons m^{-2} s^{-1}.

Strain	Protein (% in dry-cell weight)	Carbohydrate (% in dry-cell weight)		Others (% in dry-cell weight)
		Glycogen	Others	
GT	47.3 ± 2.5	11.1 ± 0.3	15.4 ± 0.5	26.1 ± 2.3
Δ*apcE*	48.4 ± 0.2	24.2 ± 0.7	4.3 ± 1.5	23.1 ± 1.0

Data are presented as the mean and standard deviation of three individual experiments.

In light conditions, cyanobacteria fix CO_2 via the Calvin-Benson-Bassham (CBB) cycle, and CBB cycle intermediates enter central metabolic pathways, such as glycolysis and the tricarboxylic acid (TCA) cycle, and that leading to glycogen synthesis [22]. Glycogen synthesis is the major carbon and energy storage pathway, whereas glycolysis and the TCA cycle produce building blocks for cell growth. During prolonged nitrogen starvation, *Synechocystic* sp. PCC 6803 stores glycogen and degrades nitrogen-rich phycobilisomes,

resulting in loss of the pigment phycocyanin, a condition which is referred to as bleaching or chlorosis [12]. Here, deletion of the *apcE* gene caused a decrease in phycobilisomes, similar to observed in response to nitrogen starvation, and hence resulted in glycogen accumulation.

Effect of apcE gene deletion on photosynthesis

The rate of oxygen evolution under *in-vivo* conditions with photoautotrophically grown cells of GT and the Δ*apcE* mutant under different light intensities was measured to generate light-photosynthesis curve (Fig. 3). The rate of oxygen evolution by GT and Δ*apcE* mutant cells at 50, 100, and 300 μmol photons m^{-2} s^{-1} were similar, suggesting that the efficiency of photon use was similar by the two strains under these light conditions, , although biomass production was higher in the Δ*apcE* mutant, which was shown to markedly decreased levels of phycobilisomes. However, the rate of oxygen evolution at 600 μmol photons m^{-2} s^{-1} was higher in the Δ*apcE* mutant than that in GT, suggesting that the light-saturated evolution (P_{max}) was higher in Δ*apcE* mutant. This result was consistent with the previous finding that the green alga *Chlamydomonas reinhardtii* with a truncated chlorophyll antenna has an increased P_{max} [6]. The cellular respiration rate of the Δ*apcE* mutant was 1.4-fold higher than that of GT. Thus, under photoautotrophic and low CO_2 conditions, absorbed CO_2 would be utilized by the Δ*apcE* mutant for cell growth, rather than the production of phycobilisomes.

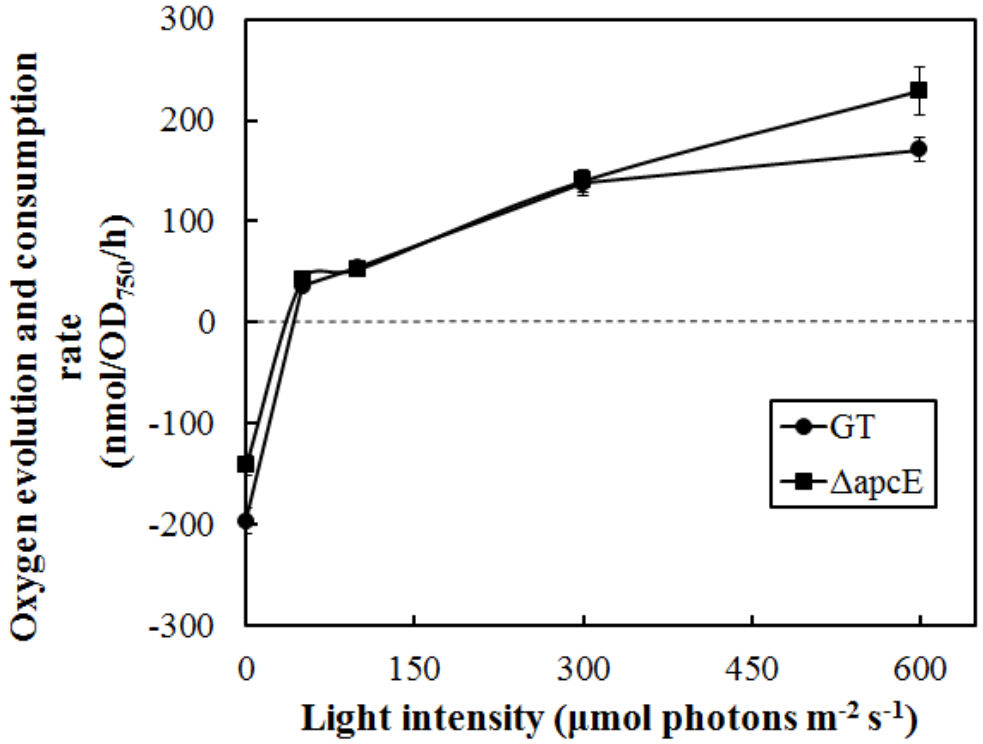

Fig. 3 Light-saturation curves for photosynthesis obtained for the Δ*apcE* mutant (closed squares) and glucose-tolerant *Synechocystis* sp. PCC 6803 (GT) (closed circles) under air conditions. Data are presented as the mean and standard deviation of three individual experiments.

In conclusion, this is the first report that antenna truncation by *apcE* gene deletion in *Synechocystis* sp. PCC 6803 increased both biomass production and glycogen content under photoautotrophic and air conditions. Due to these properties, this mutant is a potentially useful candidate for use in various biorefinery processes.

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Chapter 4

Direct conversion of *Spirulina* to ethanol without pretreatment or enzymatic hydrolysis processes

Introduction

Currently, bioethanol is typically produced from agricultural crops such as corn and sugarcane. However, because these crops are major contributors to human food supplies, bioethanol production using these starting materials has contributed to increased food prices. As an alternative feedstock for bioethanol production, oxygenic photosynthetic microorganisms, including cyanobacteria and microalgae, have considerable potential because they convert solar energy to biomass more efficiently (0.5%-2.0% efficiency) than energy crops such as switch grass (0.2% efficiency)[1-4]. Oxygenic photosynthetic microorganisms capable of growing in aquatic environments provide the additional benefit of year-round cultivation without the requirement for arable land. In particular, the use of salt-tolerant species as a feedstock for bioethanol eliminates the impact on freshwater resources[5]. Starch accumulated by green algae has been used as carbon source for ethanol production in yeast fermentation[8-10]. However, green algae generally have low salt tolerance,[6,7] and carbohydrate content[6-8,11]. Additionally, complicated pretreatment steps, such as alkaline and acid treatment at high temperature, are required for the extraction of starch from algal cells [6-8,12]. In contrast, cyanobacteria, such as *Arthrospira* (*Spirulina*) *platensis*, can grow in high-salinity environments,[13] and produce large amounts of α-polyglucan in the form of glycogen [11]. Moreover, it is expected that glycogen can be extracted from *A. platensis* cells without complicated pretreatment because of their brittle cell membrane architecture[14]. Thus, *A. platensis* is an attractive candidate as a carbohydrate feedstock for biorefinery use, particularly bioethanol production.

Starch can be converted to ethanol through hydrolysis with amylolytic enzymes and fermentation by microorganisms, such as yeast. However, as yeast lack the ability to hydrolyze starch, enzymatic treatment processes are indispensable for the production of

ethanol from starchy feedstock. To eliminate the need for complicated pre-processing steps, we developed recombinant yeast *Saccharomyces cerevisiae* strains that display amylolytic enzyme on the cell surface and are capable of utilizing starch as a carbon source to produce ethanol[15].

In the present study, a recombinant *S. cerevisiae* strain secreting α-amylase from *Streptococcus bovis* and displaying glucoamylase from *Rhizopus oryzae* on the cell surface was used for the direct production of ethanol from *A. platensis* glycogen. This is the first report of direct ethanol production from cyanobacterial glycogen. Additionally, the effect of treating the *A. platensis* cell membrane with lysozyme on enhancing ethanol production was examined. Ethanol was successfully produced from *A. platensis* at high yield without any pretreatment or extraction steps by the combined use of amylase-expressing yeast and lysozyme addition.

Materials and methods

Microorganism and growth conditions

The cyanobacterium *Arthrospira platensis* NIES-39 was obtained from the Global Environmental Forum (Tsukuba, Japan). Cells were pre-cultured in 500-mL Erlenmeyer flasks containing 250 mL SOT medium (16.8 g L^{-1} $NaHCO_3$, 0.5 g L^{-1} K_2HPO_4, 1.0 g L^{-1} K_2SO_4, 1.0 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.08 g L^{-1} Na_2 EDTA, and 0.1% (v/v) A5 solution [2.86 g L^{-1} H_3BO_3, 2.5 g L^{-1} $MnSO_4 \cdot 7H_2O$, 0.222 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.079 g L^{-1} $CuSO_4 \cdot 5H_2O$, and 0.021 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$]) with 100 rpm agitation under continuous illumination at 50 μmol photons m^{-2} s^{-1} for 6 days at 30 ± 2°C in an NC350-HC plant chamber (Nippon Medical and Chemical Instruments, Osaka, Japan). Pre-

cultivated cells were inoculated into 1.4 L SOT medium containing 3 mM $NaNO_3$ for increasing the glycogen content in *A. platensis* at a dry-based biomass concentration of 0.03 g L^{-1}. The cells were grown for 4 days in 2-L flattened flasks (Vidrex, Tokyo, Japan) containing 1.4 L SOT medium supplemented with 3 or 30 mM $NaNO_3$ under continuous illumination at 500 μmol photons m^{-2} s^{-1} using white fluorescent bulbs (Life Look HGX and NHG; NEC, Tokyo, Japan) at 29 ± 1°C with bubbling air at 350 mL min^{-1}. Light intensity was measured in the middle of the medium using an LI-250A light meter (LI-COR, Lincoln, NE) equipped with an LI-190SA quantum sensor (LI-COR). After 4 days of cultivation, cells were collected by filtration with a nylon net filter (30 × 40 cm, 20-μm pore size; Millipore, Billerica, MA), and then washed once with distilled water on the filter.

Ethanol fermentation from Arthrospira platensis

A. platensis glycogen for ethanol fermentation was extracted by treating lyophilized cells in 30% KOH (w/v) at 90°C for 30 min, and glycogen extracts were then adjusted to pH 7.0 using 98% H_2SO_4 (w/w). To examine direct ethanol production from non-pretreated *A. platensis* cells, cells collected by filtration were added directly to the fermentation medium. The water content of filtered *A. platensis* cells was 88−90% (w/w), which was calculated from a decrease in cell weight after lyophilization for 36 h.

Saccharomyces cerevisiae MT8-1 and MT8-1δGS cells were grown aerobically in 1-L Erlenmeyer flasks containing 500 mL YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} glucose) at 30°C with 150 rpm agitation for 96 h. Yeast cells were then collected by centrifugation at 5,000 × *g* for 3 min at 25°C, washed twice with distilled water, and then inoculated into YPG (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 0.1 M phosphate buffer adjusted to pH 6.0, 10 mM disodium EDTA, and 10 g L^{-1} extracted *A. platensis* glycogen, pH 7.0) or

YPA fermentation medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 0.1 M phosphate buffer adjusted to pH 6.0, 10 mM disodium EDTA, and 200 g wet cell weight L^{-1} of *A. platensis*). The initial yeast cell concentration was adjusted to 50 g wet cells weight L^{-1}. Furthermore, 1 g L^{-1} chicken egg white lysozyme (Sigma-Aldrich, St. Louis, MO) was added to YPA fermentation medium for the extraction of intracellular glycogen from *A. platensis*.

Ethanol production was performed at 30°C and an agitation speed of 500 rpm in 100-mL closed bottles equipped with a bubbling CO_2 outlet and a stir bar as described previously [16]. Agitation speed was maintained with a magnetic stirrer (VARIOMAG Telesystem; Thermo Fisher Scientific, Waltham, MA).

Analytical methods

Bioethanol concentration was analyzed using a GC2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a GC-FID flame ionization detector (Shimadzu) and a DB-FFAP column (60 m × 0.250 mm i.d., 0.5-µm film thickness; Agilent, Palo Alto, CA) with helium as the carrier gas. The column temperature was held at 40°C for 1 min before being raised to 170°C with a linear gradient of 10°C min^{-1}. The injector and detector temperature was maintained at 230°C. The injection volume was 1 µL and the split ratio was adjusted to 1:50.

Glucose was determined by a high-performance liquid chromatography (HPLC) (LC20A; Shimadzu) using an ion-exchange HPLC column (Unison UK-Amino UKA06; Imtakt, Kyoto, Japan) and an evaporative light scattering detector (ELSD-LTII; Shimadzu), as previously described [17].

The total carbohydrate content of *A. platensis* cells was analyzed by a colorimetric method using an anthrone reagent. Briefly, algal powder was reacted in 75% sulfuric acid

containing 2 g L^{-1} anthrone reagent for 15 min at 100°C. The absorbance of the resulting solution and glucose as standard was measured at 620 nm.

The total protein content of *A. platensis* cells was measured using a Quantipro BCA assay kit (Sigma-Aldrich) as directed by the manufacturer.

The glycogen content of *A. platensis* cells was determined by HPLC after the extraction of glycogen from cells using a modified method of Ernst *et al.*[18].

All experimental data are presented as the means of triplicate samples and error bars indicate the standard deviation.

Results and Discussion

Biochemical composition of Arthrospira platensis cells

The biochemical composition of *A. platensis* cells cultivated for 4 days in SOT medium containing 3 and 30 mM nitrate is shown in Table 1. The total carbohydrate content of *A. platensis* cells cultured in 3 mM nitrate was 1.5-fold higher than that of cells in 30 mM nitrate. Similarly, the glycogen content of *A. platensis* in 3 mM nitrate was more than 60% of the dry cell weight, whereas that of cells cultured in 30 mM nitrate was only 22.4%. In addition, the total protein accumulated in *A. platensis* cells was decreased by half when the initial nitrate concentration was reduced from 30 to 3 mM. The following ethanol fermentation experiments were performed using 200 g wet-cell weight L^{-1} *A. platensis* cells (20 g dry-cell weight L^{-1}) containing 12.2 g L^{-1}glycogen.

Table 1. Biochemical composition of *Arthrospira platensis* cells in media with different nitrate concentrations

Component	Composition based on dry cell weight (% w/w)	
	30 mM $NaNO_3$*	3 mM $NaNO_3$†
Carbohydrates	48.3 ± 1.9	70.9 ± 1.6
Glycogen	22.4 ± 0.7	60.9 ± 2.2
Proteins	32.9 ± 4.9	16.4 ±2.1
Other	18.8 ± 5.9	12.6 ±1.1

**A. platensis* cultivated under 500 μmol photons m^{-2} s^{-1} in SOT medium containing 30 mM $NaNO_3$ for 4 days

†*A. platensis* cultivated under 500 μmol photons m^{-2} s^{-1} in SOT medium containing 3 mM $NaNO_3$ for 4 days

Ethanol production from A. platensis by yeast and amylases

As there are no reports of bioethanol production from cyanobacterial glycogen by microbial fermentation, here, ethanol production from glycogen extracted from *A. platensis* cells was examined with a wild-type strain of *S. cerevisiae*, MT8-1, in the presence of 0.3 U mL^{-1} α-amylase and 0.1 U mL^{-1} glucoamylase (Figure 1). The *A. platensis* glycogen added as a carbon source was prepared by first lyophilizing *A. platensis* cells, which were then treated with 30% KOH at 90°C for 30 min, and the extract solution containing glycogen was adjusted to pH 7 with H_2SO_4. Although strain MT8-1 produced 6 g L^{-1} ethanol from 11.5 g L^{-1} *A. platensis* glycogen, multipartite and time-consuming steps, including cell drying, glycogen

extraction, and extract neutralization, should be required for using *A. platensis* glycogen as a carbon source for yeast.

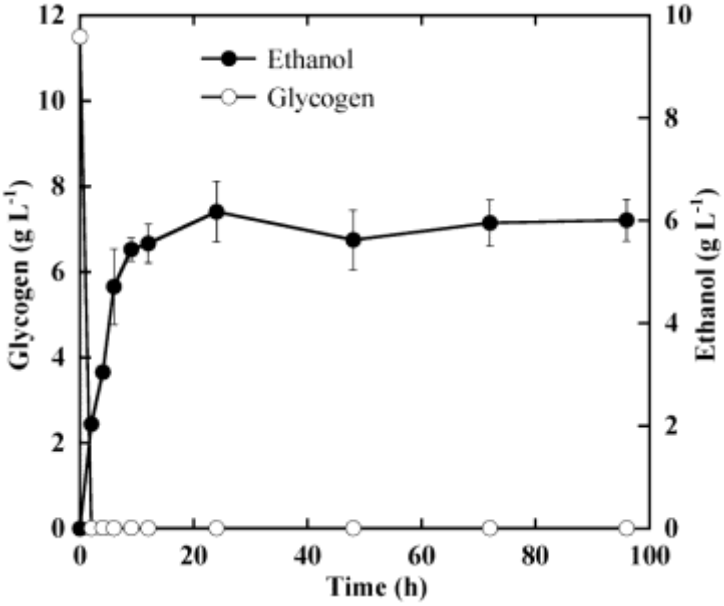

Fig. 1 Ethanol production from extracted *A. platensis* glycogen in a SSF process. Closed circle indicate ethanol concentration (g L^{-1}), and open circle indicated glycogen concentration (g L^{-1}). Error bars indicate standard deviations (SD) of three replicated experiments.

To evaluate the potential for ethanol production without glycogen extraction steps, *A. platensis* cells were directly added to the fermentation medium. Ethanol production, glucose concentration, and glycogen consumption in the presence and absence of amylases were then measured (Figure 2). Although no ethanol was detected in the absence of added amylase, 2.5 g L^{-1} ethanol was produced from 200 g wet-cell weight L^{-1} *A. platensis* after 96 h fermentation in the presence of 0.3 U mL^{-1} α-amylase and 0.1 U mL^{-1} glucoamylase. Although a total of 12.2 g L^{-1} glycogen was contained in 200 g wet-cell weight L^{-1} *A. platensis*, the ethanol yield based on the complete consumption of glycogen was only 36% of the theoretical yield. The low yield was likely due to the low glycogen hydrolysis activity, because glucose was not

detected during the fermentation, which proceeded through a simultaneous saccharification and fermentation (SSF) process (Figure 2). In addition, the poor ethanol yield may have been a result of partial glycogen consumption by *A. platensis* because the total glycogen in the fermentation medium decreased to 5 g L^{-1} despite the absence of added amylases.

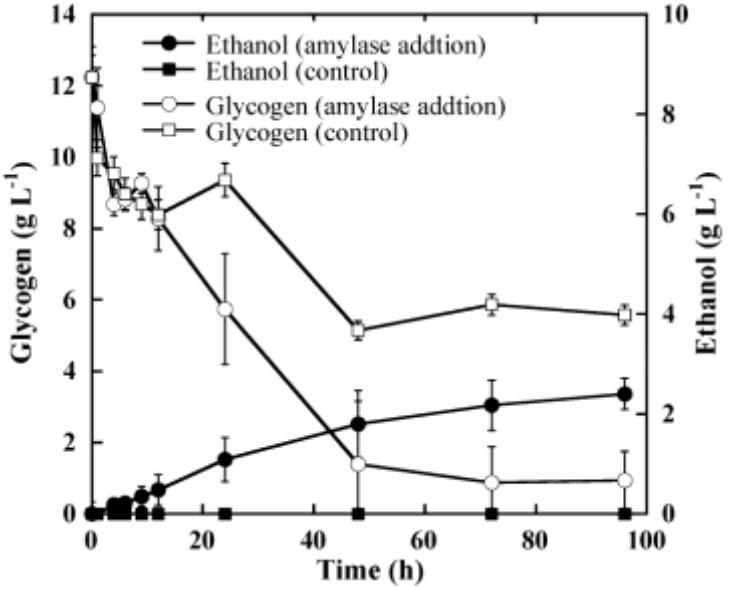

Fig. 2 Ethanol production from non-treated *A. platensis* in a SSF process in the presence and absence of amylase. Closed symbols indicate ethanol concentration (g L^{-1}), and open symbols indicated glycogen concentration (g L^{-1}). Circles indicate in the presence of amylases (amylase addition) and squires indicate in the absence of amylases (control). Data points are mean values from three separate cultures with SD of triplicates.

Based on the above findings, ethanol was successfully produced from cyanobacterial glycogen by SSF without any pretreatment processes. However, to further simplify this ethanol production process and improve the economic viability of that, it is necessary to eliminate the need for added amylases.

Ethanol production from A. platensis by amylase-expressing yeast

In our previous report, ethanol was directly produced from raw starch using a recombinant *S. cerevisiae* strain, MT8-1δGS, which expresses α-amylase from *S. bovis* and glucoamylase from *R.* oryzae [15]. Here, MT8-1δGS was used for the fermentation of glycogen produced by *A. platensis* in the absence of added amylases (Figure 3).

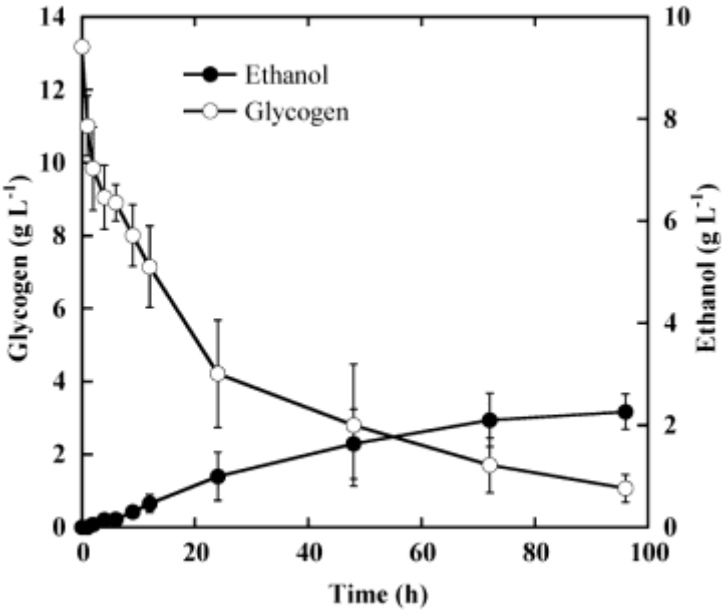

Fig. 3 Ethanol production from *A. platensis* by a recombinant amylase-expressing yeast strain. Closed circle indicate ethanol concentration (g L^{-1}), and open circle indicated glycogen concentration (g L^{-1}). Data are the means of three independent replicates with SD of triplicates.

After 96 hours of fermentation under these conditions, ethanol production reached 2.2 g L^{-1}, which was similar to the amount of ethanol produced by MT8-1 in the presence of

added amylases. Although glycogen and starch are both glucose-based polymers consisting of linear chains joined by α-1-4 glycosidic bonds and branched chains linked with α-1-6 bonds, the level and distribution of branching are not identical in starch and glycogen. The result presented in Figure 3 indicates that glycogen, as well as starch, is a suitable substrate for ethanol fermentation by the recombinant amylase-expressing yeast strain. Notably, although glycogen was not extracted from the *A. platensis* cells before fermentation, strain MT8-1δGS was able to utilize glycogen as a carbon source. Evidence suggests that *A. platensis* cells are susceptible to disruption by physical agitation because of their brittle cell wall, and are therefore likely to release glycogen into the medium upon stirring [14]. However, as the ethanol yield remained low in the fermentation with the amylase-expressing yeast, glycogen might be utilized in *A. platensis* cells before cell disruption, which is required for yeast fermentation, as described above.

Effect of lysozyme addition on glycogen extraction from A. platensis in fermentative conditions

To promote the disruption of *A. platensis* cells, the effects of lysozyme addition on the extraction of glycogen from *A. platensis* cells was examined in YPA medium at an agitation speed of 500 rpm in the presence of 0.3 U mL^{-1} α-amylase and 0.1 U mL^{-1} glucoamylase. Glucose and glycogen concentrations in the presence and absence of lysozyme are shown in Figure 4. Glycogen concentration decreased faster in the presence of lysozyme. After 24-h reaction, glucose released in the medium was 6 and 4 g L^{-1} in the presence and absence of lysozyme, respectively. These results revealed that the addition of lysozyme to the fermentation medium led to glycogen being released more rapidly from *A. platensis* cells than it could be consumed.

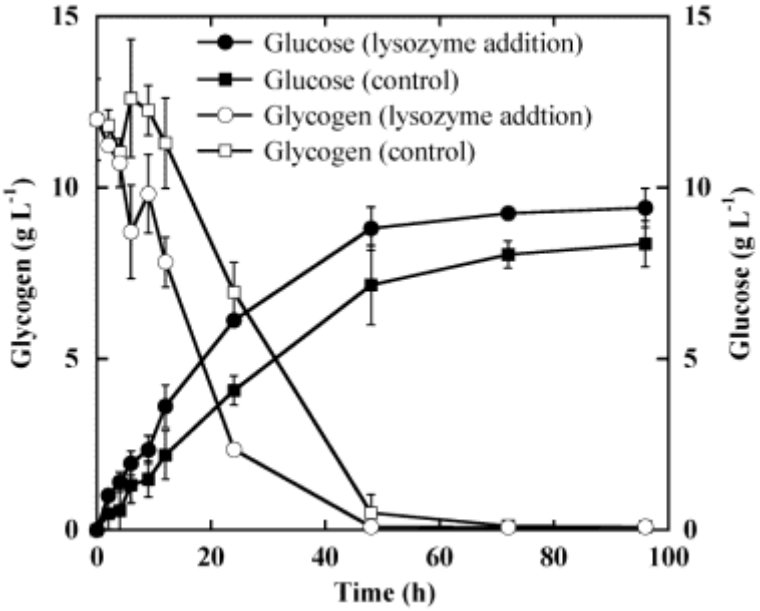

Fig. 4 Effect of lysozyme addition on glycogen extraction from *A. platensis* in the presence of added amylases. Closed symbols indicate glucose concentration (g L^{-1}), and open symbols indicated glycogen concentration (g L^{-1}). Circles indicate in the presence of lysozyme (lysozyme addtion) and squires indicate in the absence of lysozyme (control). Error bars indicate SD from triplicate determinations.

Direct ethanol production from A. platensis by amylase-expressing yeast in the presence of lysozyme

Ethanol production from *A. platensis* by strain MT8-1δGS was evaluated after adding lysozyme to the fermentation medium (Figure 5). In the presence of lysozyme, the maximum yield of ethanol production was 6.5 g L^{-1}, which represented an improvement from 36% (no lysozyme) to 86% based on glycogen consumption.

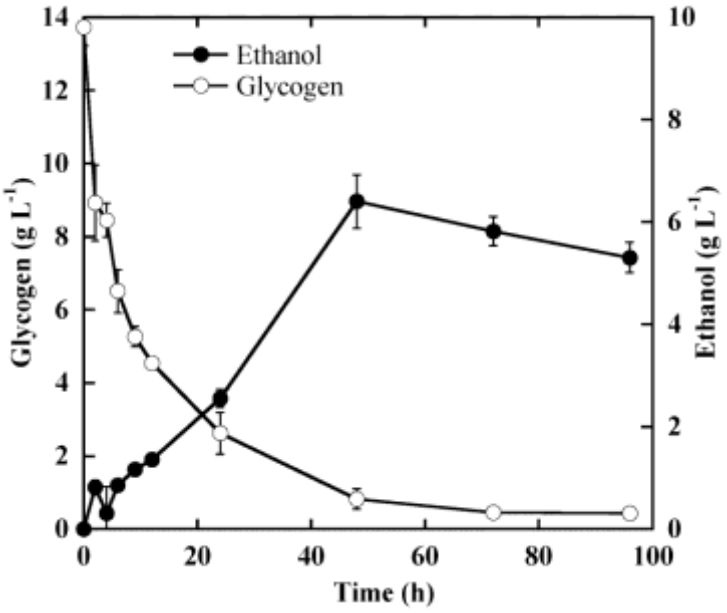

Fig. 5 Ethanol production from *A. platensis* by a recombinant amylase-expressing yeast strain in the presence of lysozyme. Closed circle indicate ethanol concentration (g L^{-1}), and open circle indicated glycogen concentration (g L^{-1}). Error bars indicate standard deviations (SD) of three replicated experiments.

Ethanol production increased with the addition of lysozyme due to the enhancement of glycogen extraction and suppression of glycogen consumption by *A. platensis*. Together, these results demonstrate that ethanol was successfully produced at high yield from non-pretreated cyanobacterial cells without added amylases using an amylase-expressing strain of *S. cerevisiae*. To improve direct ethanol production from *A. platensis*, the removal of lysozyme addition is future issue which might be resolved with recombinant yeast producing lysozyme.

Prior to the present study, bioethanol has been produced by yeast using starch extracted from green algae [6,7,12]. The production and yield of ethanol from glycogen extracted from the cyanobacterium *A. platensis* determined here was compared with the values previously reported for various species of green algae (Table 2). As shown in this comparison, we obtained the highest yield of ethanol from a photosynthetic microorganism without

pretreatment steps or amylase addition reported to date. The utilization of a cyanobacterial carbon source without pre-treatment for direct bioethanol production is the novelty of this work.

Bioethanol production from oxygenic photosynthetic microorganisms has primarily been investigated using three techniques: yeast fermentation using oxygenic photosynthetic microorganisms as carbon source, autofermentation of cyanobacteria or green algae under dark and anaerobic conditions,[19,20]and genetic engineering of cyanobacteria[21-24]. The highest ethanol yields by autofermentation and transgenic cyanobacteria are shown in Table 2. Ethanol production by the autofermentation of *C. reinhardtii* reached 7.5 g L^{-1} for 48 hours, but pH have to be regulated between 6.5 to 8.0 during ethanol production period and the ethanol yield is lower than that achieved by the yeast fermentation of treated *C. reinhardtii* cells. *Synechocystis* sp. PCC6803 introducing pyruvate decarboxylase from *Zymomonas mobilis* and overexpressing endogenous alcohol dehydrogenase from *Synechocystis* sp. PCC6803 directly produced 5.5 g L^{-1} of ethanol.[24] Although transgenic cyanobacteria directly produce ethanol from CO_2, volumetric ethanol productivity is significantly low.

Table 2 Ethanol production and yield from cyanobacteria and green algae and pretreatment steps

Ethanol production technique	Species name of cyanobacteria or green algae	Ethanol yield (mg g dry-cell weight^{-1})	Ethanol conc. (g L^{-1})	Substrate pretreatment steps	Reference
Fermentation by yeast	*A. platensis*	350	6.5	None	This study
	Chlorococcum sp.	n.d.	3.6	Drying at 60°C, 60°C heat treatment, 400 mL min^{-1} CO_2 bubbling, and drying at 60°C	12
	C. infusionum	260	n.d.	Freeze-thawed, dried at 60°C, extraction at 120°C with 0.75% (v/v) NaOH for 30 min	6
	Chlamydomonas reinhardtii	235	11.7	Liquefaction by 0.005% (w/v) α-amylase at 90°C for 30 min, and saccharification by 0.2% (w/v) glucoamylase at 55°C for 30 min	7
Auto-fermentation	*C. reinhardtii*	197	7.5	None	19
Genetic engineering	*Synechocystis* sp. PCC6803	n.d.	5.5	None	24

n.d., not determined

Table 3 Ethanol yield from various bioethanol sources

Bioethanol source	Ethanol yield (L kg dry-biomass weight^{-1})	Reference
Cyanobacteria (*A. platensis*)	0.44	This study
Barley	0.41	23
Corn	0.46	23
Oat	0.41	23
Rice	0.48	23
Wheat	0.40	23
Sweet sorghum	0.08	24
Sugarcane	0.07	24
Cassava	0.15	24
Switch grass	0.38	25

The ethanol yields from various carbohydrate feedstocks are presented in Table 3. The ethanol yield from *A. platensis* was comparable to the highest levels achieved using carbohydrate feedstocks [25-27]. Additionally, the annual biomass production of *A. platensis* is reported to range from 30 to 70 t dry weight ha^{-1} year^{-1},[5] a level that is higher than that of agricultural feedstocks such as barley, corn, oat, rice, and wheat, which range from 1 to 10 t

dry weight ha^{-1} $year^{-1}$,[25] and it is similar to that of sweet sorghum, cassava, and sugarcane, which is 35, 40, 73 t dry weight ha^{-1} $year^{-1}$, respectively [27,29]. Moreover, annual *A. platensis* biomass is also higher than that of switch grass cultivated as a potential bioethanol crop, which ranges from 5 to 11 t dry weight ha^{-1} $year^{-1}$[28].Therefore, the use of *A. platensis* as a substrate for bioethanol production would be more sustainable than other carbohydrate feedstocks. In addition, *A. platensis* can be utilized as carbon source without pretreatment, unlike most agricultural crops. Therefore, *A. platensis* is a remarkable carbohydrate feedstock in the form of glycogen, which is expected to become a useful material for the production of multiple industrially important chemicals.

Currently, several companies such as Earthrise Farms and Siam Algae Company have a large scale commercial production of food-grade *A. platensis* for nutritional supplement in open outdoor raceway ponds [30]. In case of bioethanol production, it would be important to reduce further cost related to *A. platensis* cultivation and harvest. *A. platensis* as a carbon source would be supplied at low cost than food-grade *A. platensis*, because *A. platensis* used as feedstock does not need cell-dry process as indicated in this study.

We succeeded in direct ethanol production without pretreatment steps or amylase addition from the cyanobacterium *A. platensis*, which is high glycogen producer, using the amylase-expressing *S. cerevisiae* strain MT8-1δGS. Additionally, by the addition of lysozyme to the fermentation, the ethanol production and yield, as calculated from glycogen consumption, improved to 6.5 g L^{-1} and 86%, respectively. Our findings demonstrate that *A. platensis* enables to consolidate ethanol production processes consisting of carbohydrate extraction, saccharification, and yeast fermentation without any physicochemical pretreatments.

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General Conclusion

This study was carried out to produce lactic acid and glycogen from cyanobacteria and to develop a consolidated methodology for production of ethanol from cyanobacterial Glycogen.

In Chapter 1, Lactic acid production in five transformants of *Synechocystis* PCC 6803 in which *ldh* genes that are specific for L-lactic acid production from various lactic acid bacteria (*L. lactis*, *L. plantarum*, and *L. rhamnosus*) were used. The results confirmed that co-expression of genes from lactic acid bacteria with a transporter gene can be utilized in *Synechocystis* sp. PCC 6803 for the production of lactic acid without the addition of carbon source. In addition, coexpression of a *ldh* gene and the transhydrogenase gene would be useful for increasing the cellular NADH/NAD^+ ratio so that NADH-dependent *ldh* benefits. The current study increases our knowledge of how to produce L-lactic acid, and subsequently create polylactic acid polymers from CO_2, using *Synechocystis* sp. PCC 6803.

In Chapter 2, Metabolomics and quantitative gene expression analysis using the *rre37* mutant showed the role of Rre37 under nitrogen starvation. 1) Rre37 positively regulated glycogen anabolic genes, thereby accumulating glycogen. 2) Rre37 positively regulated sugar catabolism, and was involved in 2-OG accumulation. 3) Rre37 urged accumulation of TCA cycle metabolites such as malate, fumarate, and succinate. The knowledge of regulation of genes and metabolomics provides better understanding of cyanobacterilal response to nitrogen starvation. Gene expression analysis elucidates new pathway for genetic engineering and overexpression of *rre37* may increase the glycogen production.

In Chapter 3, we constructed phycobilisome linker protein-deficient *Synechocystis* sp. PCC 6803 by deleting the *apcE* gene. This is the first report that *apcE* gene deleted mutant of *Synechocystis* sp. PCC 6803 increased both biomass production and glycogen content under photoautotrophic condition and thus we proved that minimizing, or truncating, the antenna size of the photosystems can improve photosynthetic solar energy conversion efficiency and productivity. This mutant would be one of useful candidates for biorefinery use.

In Chapter 4, we constructed an efficient methodology for the utilization of cyanobacterial glycogen for the production of bioethanol by yeast fermentation. This was achieved by using lysozyme and a recombinant amylase expressing yeast strain. This technology has overcome the multiple pretreatment and enzymatic hydrolysis steps which are labourious and costly. In the direct conversion process from *A. platensis* to ethanol, 6.5 g L^{-1} (ethanol productivity of 1.08 g L^{-1} d^{-1}) ethanol was produced. The total ethanol yield based on glycogen consumption was 86% of theoretical yield, which to our knowledge, is the highest yield of bioethanol from an oxygenic photosynthetic microorganism. The present findings indicate that *A. platensis* is a remarkable carbohydrate feedstock in the form of glycogen, which is a promising material for the production of bioethanol and various other commercially valuable chemicals.

Publication list

Chapter 1

Joseph, A., Aikawa, S., Sasaki, K., Matsuda, F., Tanaka, T., Kondo, A. (2013) Utilization of lactic acid bacrterial genes in *synechocystis* sp. PCC 6803 for the production of lactic acid. *Bioscience, Biotechnology, and Biochemistry*, 77(5): 966-70.

Chapter 2

Joseph, A., Aikawa, S., Sasaki, K., Teramura, H., Hasunuma, T., F Matsuda, F., Osanai, T., Hirai, M.Y., Kondo, A. (2013) Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803. (FEBS letters, accepted.,in press)

Chapter 3

Joseph, A., Aikawa, S., Sasaki, K., Hasunuma, T., F Matsuda, F., Kondo, A. (2013) Increased biomass production and glycogen accumulation in *apcE* gene deleted *Synechocystis* sp. PCC 6803 (submitted)

Chapter 4

Aikawa, S., **Joseph, A**., Yamada, R., Izumi, Y., Yamagishi, T., Matsuda, F., Kawai, H., Chang, JS., Hasunuma, T., Kondo, A. (2013) Direct conversion of *Spirulina* to ethanol without pretreatment or enzymatic hydrolysis processes *Energy & Environmental Science*, 6:1844-1849.

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