Title

Optimization of isopropanol production by engineered cyanobacteria with a synthetic metabolic pathway

Running title

Optimization of cyanobacterial isopropanol production

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Abstract

Cyanobacterium is an attractive host for the production of various chemicals and alternative fuels using solar energy and carbon dioxide. In previous study, we succeeded to produce isopropanol using engineered *Synechococcus elongatus* PCC 7942 under dark and anaerobic conditions (0.43 mM, 26.5 mg/l). In the present study, we report the further optimization of this isopropanol producing condition. We then optimized growth conditions for production of isopropanol by the engineered cyanobacteria, including the use of cells in early stationary phase and buffering of the production medium to neutral pH. We observed that shifting of cultures from dark and anaerobic to light and aerobic conditions during the production phase dramatically increased isopropanol production by conversion to isopropanol from acetate, byproduct under dark and anaerobic condition. Under the optimized production conditions, the titer of isopropanol was elevated 6-fold, to 2.42 mM (146 mg/l).

36 **Introduction**

37 The bioconversion of cellulosic biomass using engineered microorganisms would be a key
38 technology for the production of alternatives to fossil fuel resources. *Escherichia coli* and yeast have
39 been widely used as hosts for these purposes because of these species' ideal properties, including fast
40 growth, the availability of well established genetic tools, and well characterized metabolic pathways.
41 The introduction of synthetic metabolic pathways composed of multiple genes derived from other
42 organisms has been used to enable these hosts to produce various chemicals that they are not able to
43 produce naturally (1, 2). Furthermore, methodologies in metabolic engineering (including knocking
44 out genes for byproduct producing pathways and overexpressing genes for target-producing
45 pathways) are effective for productivity improvements. Although there are many advantages to using
46 such microorganisms in bioconversion from cellulosic biomass to target chemicals and fuels, there
47 are also some difficulties. For example, there are some technological difficulties in the
48 saccharification process that is necessary to supply monosaccharides as substrate to microorganisms
49 from cellulosic biomass. Additionally, growth of the plants supplying cellulosic biomass is a very
50 time-consuming process. One of the expected solutions to these problems is the application of
51 photosynthetic microorganisms (cyanobacteria and algae) as host microorganisms for producing
52 chemicals and fuels. These photosynthetic microorganisms use photosystems to convert solar energy
53 to chemical energy (ATP and NADPH), which is used to fix carbon dioxide to organic compounds.
54 Thus, photosynthetic microorganisms introduced synthetic metabolic pathways would be able to
55 produce various chemicals directly from carbon dioxide using solar energy. Cyanobacteria have been
56 well studied in photosynthesis research, and are considered as an ancestor of chloroplast. Various
57 techniques and tools for gene manipulation in cyanobacteria have been developed (3, 4), and the

58 genomes of many species have been sequenced (5, 6).

59 Various chemicals already have been produced using engineered cyanobacteria, primarily in
60 strains derived from *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, and these
61 results have been summarized in several recent reviews (7-9). In a previous study, we constructed an
62 isopropanol producing strain derived from *S. elongatus* PCC 7942 (10). Isopropanol is an attractive
63 chemical, since the compound is readily converted to propylene via dehydration (11). Polypropylene
64 produced from propylene is currently a popular industrial material, with global demand expected to
65 increase (12). In the constructed synthetic metabolic pathway that we used (10, 13), isopropanol is
66 produced from cellular acetyl-CoA via a four step process, including [1] condensation of two
67 molecules of acetyl-CoA into one molecule of acetoacetyl-CoA, [2] CoA transfer to acetate and
68 formation of acetoacetate, [3] decarboxylation of acetoacetate to form acetone, and [4]
69 dehydrogenation of acetone to form isopropanol. For the construction of this synthetic metabolic
70 pathway, *thl* and *adc* were obtained from *Clostridium acetobutylicum* ATCC824, *atoAD* from *E. coli*
71 K-12 MG1655, and *sadh* from *C. beijerinckii* NRRL B593; the cloned genes were introduced into *E.*
72 *coli* (13) and cyanobacteria (10).

73 It was reported that the carbon fixed by photosynthesis is fed into the Calvin cycle and
74 subsequently converted primarily to glycogen. It was also reported that the flux toward glycolysis
75 and the TCA cycle is considerably small during exposure to light (14, 15). The production of acetone
76 (36 mg/l) and 1-butanol (14.5 mg/l) from cellular acetyl-CoA in engineered cyanobacteria previously
77 was achieved under dark and anaerobic conditions (16, 17). Similarly, we achieved isopropanol
78 production (26.5 mg/l) in our engineered cyanobacteria under dark and anaerobic conditions. In our
79 work, the engineered cells were incubated under light and aerobic conditions (growth phase), and

then shifted to dark and anaerobic conditions (production phase) to enable the production of the target chemical. The production of isopropanol from acetyl-CoA was dependent on dark and anaerobic conditions under which the glycolytic pathway would be activated (18). Nevertheless, the obtained titers were lower than those obtained for 2,3-butanediol (2.38 g/l) and ethanol (552 mg/l) under photoautotrophic conditions (only under light and aerobic condition) derived from cellular pyruvate (19, 20). Based on these comparisons, production titer of chemicals by synthetic metabolic pathway from cellular acetyl-CoA in engineered cyanobacteria is much lower than that from pyruvate. The implication is that it would be challenging to produce high titer chemicals from acetyl-CoA using engineered cyanobacteria.

In a previous study, we constructed isopropanol producing cyanobacteria and proposed initial production conditions (10). However, optimization of production conditions was lacking, especially regarding the growth phase period and pH during production. In the present study, the engineered strain was regenerated by re-introducing the previously constructed synthetic metabolic pathway into a wild type *S. elongatus* PCC 7942 with confirmed genome sequence. Next, the isopropanol producing conditions (period in growth phase, medium pH, and medium composition in production phase) were optimized. During the isopropanol production phase, acetate was also produced as a byproduct. It is known that the addition of acetate enables isopropanol production under light and aerobic conditions (10). Hence, to further increase isopropanol titer, we examined whether the acetate produced under dark and anaerobic conditions could be utilized for isopropanol production under subsequent light and aerobic conditions.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) unless otherwise specified. Restriction enzymes, phosphatase (New England Biolabs, Ipswich, MA), and DNA polymerase (KOD Plus Neo DNA polymerase, TOYOBO Co., Ltd., Osaka, Japan) were used for cloning. Oligonucleotides were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan).

Culture medium

Unless otherwise specified, BG11 medium supplemented with 20 mM HEPES-NaOH (pH 7.5) (subsequently referred to simply as BG11 medium) was used as the standard medium for both growth and production phases. The composition of BG11 medium has been described previously (10). For selection of transformants, spectinomycin and kanamycin were added to 20 and 10 µg/ml.

To prepare BG11_{-N}, BG11_{-P}, and BG11_{-HEPES} media, medium components NaNO₃, K₂HPO₄, and HEPES-NaOH (pH 7.5) were removed from BG11 medium. BG11_{-N}, P, HEPES medium was prepared without any of these three components.

To prepare BG11_{T9.0, T8.5}, BG11_{H8.0, H7.5, H7.0}, BG11_{P7.5, P7.0, P6.5}, and BG11_{M6.0, M5.5} media, the HEPES-NaOH (pH 7.5) component of BG11 was replaced with Tris-HCl (pH 9.0, 8.5), HEPES-NaOH (pH 8.0, 7.5, 7.0), PIPES-NaOH (pH 7.5, 7.0, 6.5), and MES-NaOH (pH 6.0, 5.5). Thus, BG11 is synonymous with BG11_{H7.5}. For all BG11 derived media, buffers were supplemented to 20 mM.

Growth and production conditions

All cyanobacterial cultures were grown under fluorescent light ($150 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$) at 30 °C in a growth chamber (MLR-325H-PJ, Panasonic, Osaka, Japan). The cells grown under light and aerobic conditions were transferred into dark and anaerobic conditions. Thus, all isopropanol production cultures in this study consisted of two-phase incubations (growth and production phases).

In growth phase, cells inoculated to 20 ml BG11 medium in 50-ml flask were incubated under fluorescent light with rotary shaking at 150 rpm as preculture (NR-30 shaker; TAITEC, Saitama, Japan). When OD_{730} of the preculture reached 1.0-2.0, cells were grown by inoculation of 500 ml BG11 medium in a turtle-shaped flask (#62040; Vidrex, Fukuoka, Japan) to an initial cell density (OD_{730}) of 0.025. To increase the growth rate, the cultures were grown by continuous aeration with air containing 5% carbon dioxide at 1.0 vvm (volume per volume per minute) for two weeks. To transfer into production phase, the cells were harvested by centrifugation at $3,000 \times g$ and 25 °C. The harvested cells were resuspended in an appropriate volume of fresh BG11 medium supplemented to 1.0 mM IPTG. The cell density after resuspension was adjusted to an OD_{730} of 5.0. Anaerobic conditions were obtained by using evacuated blood collection tubes (Vacutainer, BD, NJ, USA) and sterilized ultra-pure nitrogen gas, as mentioned in the previous study (10). Dark conditions were obtained by wrapping the tubes with aluminum foil. In the production phase, the tubes for isopropanol production were shaken at 100 rpm in a BR-23FP shaker (TAITEC, Saitama, Japan) for 15 days. To change dark and anaerobic conditions to light and aerobic conditions in the production phase, cultures incubated under dark and anaerobic conditions for 1, 3, 5, and 10 days were transferred to screw-cap test tubes (71-063-010, Asahi Glass, Chiba, Japan) and incubated for the subsequent 10 days with rotary shaking at 150 rpm with fluorescent light.

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146 ***Product analysis***

147 The filtered supernatant obtained after centrifugation ($20,000 \times g$, 10 min, 4 °C) was applied
148 to quantification analysis. Isopropanol and acetone were quantified using a GC-2014 gas
149 chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an AOC-20
150 automatic injector and sampler (Shimadzu). Acetate was quantified using a high-performance liquid
151 chromatograph (LC-20AD, Shimadzu) equipped with an autosampler and electric conductivity
152 detector (CDD-10A, Shimadzu). The detailed conditions for analysis were described previously (10).

153

154 ***Cloning of synthetic metabolic pathway containing mutation (G66D in AtoA) from TA1317***

155 The genome of TA1317, the isopropanol producing strain constructed in our previous study,
156 was extracted with a DNeasy Blood and Tissue kit (Cat. 69504, Qiagen, Venlo, the Netherlands) and
157 used as a PCR template. The synthetic metabolic pathway containing a missense mutation (encoding
158 the G66D substitution in AtoA) was amplified by PCR using primers T548 (5'- GCCAT CCCTA
159 GGAAT TGTGA GCGGA TAACA ATTGA CATTG-3') and T550 (5'- GCCAT CGGAT CCTTA
160 CTTAA GATAA TCATA TATAA CTTCA GCTC-3'). The PCR product was digested with AvrII
161 and BamHI, and inserted into an AvrII, BamHI site of pTA371. The resulting plasmid was designated
162 pTA821 [*P_{LacO1}:: thl-atoAD(mutation)-adc*]. All amplified genes were sequenced.

163 The cloned synthetic metabolic pathway was integrated into the *S. elongatus* PCC 7942
164 (Life Technologies Corporation, CA, USA) genome by sequential homologous recombination using
165 two plasmids (pTA821 and pTA634). First, *P_{LacO1}:: sadh* and the kanamycin resistance gene were
166 integrated into the NS II (21) site of TA1297, creating strain TA1684. Second, *P_{LacO1}::*

thl-atoAD(mutation)-*adc*, *lacI*^q and the spectinomycin resistance gene were integrated into the NS I (22) site of TA1684, creating strain TA1741. All integrated genes were sequenced and confirmed.

Results and Discussion

Cloning of synthetic metabolic pathway from TA1317 and construction of a new isopropanol producing strain

In this study, a wild type of *S. elongatus* PCC 7942 with confirmed genome sequence (<http://genome.kazusa.or.jp/cyanobase/SYNPCC7942>) was purchased from Life Technologies Corporation. This strain, described as TA1297 in our laboratory, was used as a host strain. The synthetic metabolic pathway for isopropanol production was constructed by integration of four genes (*thl*, *atoAD*, *adc*, and *sadh*) into two neutral sites (NS I and II) on the cyanobacterial chromosome. The synthetic metabolic pathway with a missense mutation (encoding AtoA with a G66D substitution) was cloned from TA1317, the isopropanol producing strain established in our previous study (10), and a plasmid (designated as pTA821) containing the *thl*, mutated *atoAD*, and *adc* was newly constructed. Sequential transformation (starting from TA1297) with the two plasmids, pTA821 and pTA634, resulted in an isopropanol producing strain without further mutations in the genes of the synthetic metabolic pathway (as confirmed by amplification and sequencing of the integrated genes). This strain, referred to as TA1741, showed isopropanol productivity equivalent to that of TA1317 under conditions reported in the previous study (data not shown). Therefore, TA1741, the newly established isopropanol producing strain, was used for further experiments aimed at optimizing the conditions for isopropanol production. Because of no obtaining of isopropanol producing strain by transformation using pTA372 (*thl*, *atoAD*, and *adc* in NS I) as well as in the

previous study, pTA821 was newly constructed and used instead of pTA372.

Effect of period in growth phase on isopropanol productivity

The engineered *S. elongatus* PCC 7942 strain, TA1741, produced only trace amounts of isopropanol under conditions of light and aerobic condition. Therefore, two-phase incubation was applied to isopropanol production. At first, TA1741 was grown under light and aerobic condition (growth phase). Secondary, the grown culture was corrected and incubated under dark and anaerobic condition (production phase).

The effect of growth phase period on isopropanol productivity was examined. Figure 1 shows a growth profile of TA1741 during two weeks under light and aerobic condition, starting from an initial cell density (OD₇₃₀) of 0.025. Isopropanol productivity was compared among cultures in four different periods of growth phase prior to the shift to production phase, specifically, growth to: early logarithmic phase (2 days); late logarithmic phase (4 days); early stationary phase (8 days); and late stationary phase (12 days) (Fig. 2A). For all cultures, the cell density at the time of the shift to production phase was adjusted to an OD₇₃₀ of 5.0. The cultures transferred at early logarithmic phase showed the lowest titer, with a value lower than 0.1 mM. A moderate titer (0.83 mM) was obtained from cultures transferred at late stationary period. The highest titer (> 1.0 mM) was achieved by using the cultures transferred at late logarithmic or early stationary phase. Notably, the cell density at early stationary phase was higher than that at late logarithmic phase. In order to obtain highest isopropanol titer from same volume of culture in growth phase, the transferring cells at early stationary phase was optimal. Therefore, cultures at early stationary phase were used for further experiments. Under dark and anaerobic conditions (production phase), cells could not grow and

uptake carbon dioxide. During production phase, the values of OD₇₃₀ and pH gradually decreased. The produced isopropanol under these conditions was probably derived from cellular carbon storage, for example glycogen. In the report of acetone production by engineered *Synechocystis* sp. PCC 6803 (16), the relation between glycogen consumption and acetone production was discussed. In cyanobacteria, the cellular content of glycogen gradually increases during growth (23, 24). This phenomenon is one of the reasons for our results that cultures in later growth phase produced isopropanol with higher titers. The almost same titer (about 1.0 mM) was also achieved by the early stationary culture of TA1317, previously constructed isopropanol strain.

We observed increased acetone levels with decreased isopropanol levels after 10 days into the production phase (Fig. 2A, B). In constructed metabolic pathway, a reaction catalyzed by secondary alcohol dehydrogenase is reversible dependently on NADPH/NADP⁺ balance. One molecule of NADPH is necessary for one molecule conversion of acetone to isopropanol. We hypothesized that the conversion of isopropanol to acetone reflected a reverse reaction catalyzed by a secondary alcohol dehydrogenase, which would be driven by depleted reducing power (NADPH). The capacity of the NADPH supply in production phase was evaluated by isopropanol production following supplementation with acetone (10 g/l). When excess acetone was added, the titer of isopropanol reached 25 mM (Fig. S1), indicating that a reducing power corresponding to 25 mM NADPH was provided under these conditions. This result suggested that the bottleneck in isopropanol production was the metabolic flux to acetone.

During isopropanol production, acetate accumulation was observed (Fig. 2C). The amount of acetate correlated to length of the growth period and did not decrease during 15 days in production phase. The same accumulation of acetate was observed in the host strain, TA1297 (data not shown).

In a metabolic pathway of *S. elongatus* PCC 7942, acetate is expected to be produced solely from acetyl-CoA, in a reaction catalyzed by acetyl-CoA synthase. Acetyl-CoA is a shared substrate for production of acetate (by an endogenous pathway) and isopropanol (by the synthetic metabolic pathway), thus explaining the correspondence between acetate producing period and isopropanol one (Fig. 2A, C). Additionally, the second reaction in the synthetic metabolic pathway (which is catalyzed by acetoacetyl-CoA transferase) uses acetate as a CoA receptor. These facts indicated that the appropriate acetate supply was important for isopropanol production via the synthetic metabolic pathway. For cultures shifted in early stationary phase, acetate levels reached 8.77 mM after 15 days in production phase. Since one molecule of isopropanol is produced from two molecules of acetyl-CoA, we expected that the conversion of acetate to isopropanol could elevate isopropanol titer by up to 4 mM.

Effect of medium composition on isopropanol productivity

The effect of medium composition on isopropanol production was examined (Fig. 3). As in our previous study, production was tested using media removed nitrogen, phosphate, or both components. As noted in the Methods, BG11 medium was typically supplemented with 20 mM HEPES-NaOH (pH 7.5) in this study. In BG11_{-N} medium, which created nitrogen limited conditions, isopropanol titer was increased to 1.43 mM from 1.06 mM in BG11 medium. In contrast, no significant difference was detected between BG11 medium and phosphate limited or nitrogen and phosphate limited medium. The difference in titers between BG11_{-N} and BG11_{-N,P} medium indicated that there was a negative effect of phosphate limitation in addition to the nitrogen limited condition. Phosphate limitation has been reported to cause increased accumulation of intracellular acetyl-CoA,

and an increased acetyl-CoA pool has been reported to boost titers of acetyl-CoA derived products (25, 26). However, our results indicated that phosphate limitation did not contribute to improved isopropanol productivity. This distinction from previous literature may reflect differences in metabolic pathway(s) subsequent to acetyl-CoA.

We additionally noted that removing buffer from BG11 medium drastically decreased isopropanol production by our strain (Fig. 3), suggesting that maintenance of neutral pH is important for isopropanol production. In our previous study, HEPES buffer was not included in the medium used for the production phase, and the highest titer of isopropanol was obtained in medium removed both nitrogen and phosphate (referred to as BG11-N,P medium in the previous study, and as BG11-N,P,HEPES medium in the present study). The enhanced titer seen here with buffer containing medium revealed that BG11-N,P,HEPES medium was not an optimal medium for isopropanol production.

Notably, BG11 medium without buffer also resulted in decreased acetate accumulation (Fig. 3B). The Rapid decrease in pH during production phase in this medium was observed (data not shown). The decreased pH was expected to inhibit cellular metabolism, including glycolysis, which would be sufficient to explain the reductions in titer of both acetate and isopropanol. We additionally noted decreased acetate production using BG11-N and BG11-N,P media compared to BG11 and BG11-P. This result suggested that nitrogen limitation caused decreased acetate production. Although a correlative increase in isopropanol production was observed for BG11-N compared to BG11, a similar increase was not observed for BG11-N,P compared to BG11-N. The details of the synergistic effects of nitrogen and phosphate limitation remain unclear, and will require further investigation.

The decrease in isopropanol titer observed with unbuffered BG11 medium indicated the

importance of maintaining neutral pH for isopropanol production. To further characterize the buffer effect, a series of media with different initial pHs was tested in production phase (Fig. 4). The range of pH from 5.5 to 9.0 was covered using four kinds of Good buffers (MES, PIPES, HEPES, Tris; see Materials and Methods). Figure 4A shows isopropanol production by production phase cultures grown for 10 days in medium at each pH. The culture in BG11_{P7.0} showed higher titer than that observed in the standard medium. Isopropanol titer was maximized in medium with an initial pH 7.0-7.5. We confirmed that pH of the medium decreased during incubation under dark and anaerobic conditions (data not shown). We hypothesized that the buffering capacities of BG11_{H7.0} and BG11_{P6.5} were comparatively low because the target pHs (7.0 and 6.5) were lower than the proper buffering range of HEPES and PIPES, respectively. Presumably, the differences in isopropanol titers between cultures in BG11_{H7.0} and BG11_{P7.0}, or in BG11_{P6.5} and BG11_{M6.5}, primarily reflected buffering capacity.

The pH and buffer dependence in acetate production were different from those in isopropanol production (Fig. 4B). With the exception of the culture in BG11_{M5.5}, cultures in the various media produced similar amounts of acetate. Hence, the range of pH for proper isopropanol production was narrower than that for acetate production, indicating that the synthetic metabolic pathway is more sensitive to pH change than natural cellular metabolism.

Enhanced isopropanol production by utilization of accumulated acetate

The isopropanol productivity was improved by examination of the period in growth phase and the medium conditions in production phase. For further production improvement, we focused on the utilization of acetate accumulated under dark and anaerobic conditions. As mentioned above, the

isopropanol producing cyanobacteria produced only trace amounts of isopropanol under light and aerobic conditions, but the addition of acetate enabled this strain to produce isopropanol even under light and aerobic conditions (10). Other laboratories have reported, in cyanobacteria, that supplementation with acetate increases accumulation of poly- β -hydroxybutyrate (PHB) synthesized from cellular acetyl-CoA (27, 28). We employed a shift from dark and anaerobic condition to light and aerobic one during the production phase to permit the conversion of the accumulated acetate into isopropanol (Fig. 5).

The isopropanol production and acetate accumulation in dark and anaerobic conditions continued for 10 days (Fig. 2). Therefore, cultures incubated for 1, 3, 5, and 10 days under dark and anaerobic conditions were subsequently transferred to light and aerobic conditions. The concentrations of isopropanol, acetone, and acetate were measured until 10 days under light and aerobic conditions. The culturing conditions are abbreviated as D1L10 [dark and anaerobic conditions for 1 day, light and aerobic conditions for 10 days; Fig. 5 (A), (E)], D3L10 [(B), (F)], D5L10 [(C), (G)], and D10L10 [(D), (H)]. Following the shift to light and aerobic conditions, levels of accumulated acetate decreased, while levels of isopropanol increased. These observations indicated that isopropanol was produced from the acetate accumulated under dark and anaerobic conditions. Two conditions, D3L10 and D5L10, showed higher titers (Fig. 5B, C) than continuous 10 days dark and anaerobic conditions (Fig. 2A). The highest titer of isopropanol (2.42 mM, 146 mg/l) was observed in the D5L10 culture. Although the culture incubated for 10 days under dark and anaerobic conditions produced the highest amount of acetate (8.61 mM), the cells in D10L10 did not consume the accumulated acetate, and exhibited photobleaching (whitening) after shifting to light and aerobic conditions. In contrast to cultures under other conditions, the reverse reaction (from

isopropanol to acetone) was observed in D10L10. The similar reverse reaction was also observed in the case of longer incubation under dark and anaerobic condition (Fig. 2). In another cyanobacterium, *Arthrospira maxima*, long incubation under dark and anaerobic conditions has been reported to cause a significant decrease in PSII activity due to degradation of the reaction center (29). We hypothesize that the longer incubation under dark and anaerobic conditions may make our strain difficult to grow after shifting to light and aerobic conditions.

With the exception of the D10L10 culture, isopropanol production rate after the shift from dark and anaerobic to light and aerobic conditions was higher than that before this shift. These results suggest that the production of isopropanol by conversion from acetate exceeds that from isopropanol production under dark and anaerobic conditions. The titers of isopropanol after shifting conditions in D1L10, D3L10, and D5L10 were 0.38 mM, 1.18 mM, and 1.90 mM, respectively. Assuming that all of the increase in isopropanol was produced by consumption of acetate, the conversion ratio was calculated to be 50-67% of the theoretical yield. This conversion ratio was quite similar to that observed for added acetate under light and aerobic conditions (data not shown). In the D5L10 culture, which showed the highest isopropanol titer, accumulated acetate under dark and anaerobic conditions was detected even after 10 days of incubation under light and aerobic conditions.

In this study, we succeeded in improving isopropanol productivity by optimizing the period in growth phase and the production phase conditions. The maximized titer of isopropanol reached 2.42 mM (146 mg/L), about 6-fold higher than that previously reported (0.43 mM, 26.5 mg/l) (10). Shifting of conditions from dark and anaerobic to light and aerobic during production phase was an effective method for increasing isopropanol production. Cultures under this subsequent light and

343 aerobic conditions are expected to fix carbon dioxide again, so it may be possible to achieve
344 continuous production by subsequent dark-light cycling. To achieve practically applicable level,
345 however, a lot of further challenges in improvement of strain and engineering are necessary

346

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References

1. **McEwen, J.T. and Atsumi, S.:** Alternative biofuel production in non-natural hosts. *Current Opinion in Biotechnol.*, **23**, 744-750 (2012).
2. **Zhang F., Rodriguez, S. and Keasling, J.D.:** Metabolic engineering of microbial pathways for advanced biofuels production. *Current Opinion in Biotechnol.*, **22**, 775-783 (2011).
3. **Koksharova, O.A. and Wolk, C.P.:** Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol.*, **58**, 123-137 (2002).
4. **Clerico, E.M., Ditty, J.L. and Golden, S.S.:** Specialized techniques for site-directed mutagenesis in cyanobacteria. p. 155-171. In Rosato, E. (ed.), *Circadian Rhythms*. Humana Press Inc., New Jersey (2007).
5. **Kotani, H., Kaneko, T., Matsubayashi, T., Sato, S., Sugiura, M. and Tabata, S.:** A physical map of the genome of a unicellular cyanobacterium *Synechocystis* sp. Strain PCC6803. *DNA Res.*, **1**, 303-307 (1994).
6. **Holtman, C.K., Chen, Y., Sandoval, P., Gonzales, A., Nalty, M.S., Thomas, T.L., Youderian, P. and Golden, S.S.:** High-throughput functional analysis of the *Synechococcus elongatus* PCC 7942 genome. *DNA Res.*, **12**, 103-115 (2005).
7. **Rosegaard, L., Porcellinis, A.J., Jacobsen, J.H., Frigaard, N.U. and Sakuragi, Y.:** Bioengineering of carbon fixation, biofuels, and biochemical in cyanobacteria and plants. *J. Biotechnol.*, **162**, 134-147 (2012).
8. **Wang, B., Wang, J., Zhang, W. and Meldrum, D.R.:** Application of synthetic biology in cyanobacteria and algae. *Front. Microbiol.*, **3**, Article344 (2012).
9. **Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y.J. and Zhang, F.:** Development of

- 373 *Synechocystis* sp. PCC 6803 as a phototropic cell factory. Mar. Drugs, **11**, 2894-2916 (2013).
- 374 10. **Kusakabe, T., Tatsuke, T., Tsuruno, K., Hirokawa, T., Atsumi, S., Liao, J.C. and Hanai, T.:**
- 375 Engineering a synthetic pathway in cyanobacteria for isopropanol production directly from
- 376 carbon dioxide and light. Metab. Eng., **20**, 101-108 (2013).
- 377 11. **Kibby, C.L., and Hall, W.K.:** Studies of acid catalyzed reactions. XII. Alcohol decomposition
- 378 over hydroxyapatite catalysts. J. Catal., **29**, 144-159 (1972).
- 379 12. **Molenda, J.:** The oil and petrochemical industries are facing process changes consequent upon
- 380 the expected propylene demand rise. Przem. Chem., **83**, 320-324 (2004).
- 381 13. **Hanai, T., Atsumi, S. and Liao, J.C.:** Engineered synthetic pathway for isopropanol production
- 382 in *Escherichia coli*. Appl. Environ. Microbiol., **73**, 7814-7818 (2007).
- 383 14. **Alagesan, S., Gaudana, S.B., Sinha, A. and Wangikar, P.P.:** Metabolic flux analysis of
- 384 *Cyanothece* sp. ATCC 51142 under mixotrophic conditions. Photosynth. Res., **118**, 191-198
- 385 (2013).
- 386 15. **Young, J.D., Shastri, A.A., Stephanopoulos, G. and Morgan, J.A.:** Mapping photoautotrophic
- 387 metabolism with isotopically nonstationary ¹³C flux analysis. Metab. Eng., **13**, 656-665 (2011).
- 388 16. **Zhou, J., Zhand, H., Zhang, Y., Li, Y. and Ma, Y.:** Designing and creating a modularized
- 389 synthetic pathway in cyanobacterium *Synechocystis* enables production of acetone from carbon
- 390 dioxide. Metab. Eng., **14**, 394-400 (2012).
- 391 17. **Lan, E.I. and Liao, J.C.:** Metabolic engineering of cyanobacteria for 1-butanol production from
- 392 carbon dioxide. Metab. Eng., **13**, 353-363 (2011).
- 393 18. **Stal, L.J. and Moezelaar, R.:** Fermentation in cyanobacteria. FEMS Microbiol. Rev., **21**,
- 394 179-211 (1997).

- 395 19. **Oliver, J.W.K., Machado, I.M.P., Yoneda, H. and Atsumi, S.:** Combinatorial optimization of
396 cyanobacterial 2,3-butanediol production. *Metab. Eng.*, **22**, 76-82 (2014).
- 397 20. **Dexter, J. and Fu, P.:** Metabolic engineering of cyanobacteria for ethanol production. *Energy*
398 *Environ. Sci.*, **2**, 857-864 (2009).
- 399 21. **Andersson, C.R., Tsinoremas, N.F., Shelton, J., Lebedeva, N.V., Yarrow, J., Min, H.T. and**
400 **Goldem, S.S.:** Application of bioluminescence to the study of circadian rhythms in
401 cyanobacteria. *Methods Enzymol.*, **305**, 527-542 (2000).
- 402 22. **Bustos, S.A. and Golden, S.S.:** Expression of the *psbDII* gene in *Synechococcus* sp. Strain-PCC
403 7942 requires sequences downstream of the transcription start site. *J. Bacteriol.*, **173**, 7525-7533
404 (1991).
- 405 23. **Suzuki, E., Ohkawa, H., Moriya, K., Matsubara, T., Nagaike, Y., Iwasaki, I., Fujiwara, S.,**
406 **Tsuzuki, M. and Nakamura, Y.:** Carbohydrate metabolism in mutants of the cyanobacterium
407 *Synechococcus elongatus* PCC 7942 defective in glycogen synthesis. *Appl. Environ. Microbiol.*,
408 **76**, 3153-3159 (2010).
- 409 24. **Monshupanee, T. and Incharoensakdi, A.:** Enhanced accumulation of glycogen, lipids and
410 polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium
411 *Synechocystis* sp. PCC 6803. *J. Appl. Microbiol.*, **116**, 830-838 (2013).
- 412 25. **Panda, B. and Mallick, N.:** Enhanced poly- β -hydroxybutyrate accumulation in a unicellular
413 cyanobacterium, *Synechocystis* sp. PCC 6803. *Lett. Appl. Microbiol.*, **44**, 194-198 (2006).
- 414 26. **Wang, B., Pugh, S., Nielsen, D.R., Zhang, W. and Meldrum, D.R.:** Engineering cyanobacteria
415 for photosynthetic production of 3-hydroxybutyrate directly from CO₂. *Metab. Eng.*, **16**, 68-77
416 (2013).

- 417 27. **Takahashi, H., Miyake, M., Tokiwa, Y. and Asada, Y.:** Improved accumulation of
418 poly-3-hydroxybutyrate by a recombinant cyanobacterium. *Biotechnol. Lett.*, **20**, 183-186
419 (1998).
- 420 28. **Sharma, L. and Mallick, N.:** Accumulation of poly- β -hydroxybutyrate in *Nostoc muscorum*:
421 regulation by pH, light-dark cycles, N and P status and carbon source. *Bioresour. Technol.*, **96**,
422 1304-1310 (2005).
- 423 29. **Ananyev, G.M., Skizim, N.J. and Dismukes, G.C.:** Enhancing biological hydrogen production
424 from cyanobacteria by removal of excreted products. *J. Biotechnol.*, **162**, 97-104 (2012).
425

Figure legends

Fig. 1. Growth profile of isopropanol producing strain under light and aerobic conditions. Data are represented as means $\pm$ S.D. of three individual experiments.

Fig. 2. Isopropanol (A), acetone (B), and acetate (C) production by cultures at different periods of growth phase. Circles and squares represent cells at early and late logarithmic phase, respectively. Triangles and diamonds represent cells at early and late stationary phase, respectively. Data are represented as means $\pm$ S.D. of four individual experiments.

Fig. 3. Isopropanol (A) and acetate (B) production in media of different compositions. Data are represented as means $\pm$ S.D. of three individual experiments.

Fig. 4. Isopropanol (A) and acetate (B) production in media of differing initial pH. Circles, squares, triangles, and diamonds represent Tris, HEPES, PIPES, and MES, respectively (the kinds of buffer used in each medium). Data are represented as means $\pm$ S.D. of three individual experiments.

Fig. 5. Isopropanol, acetone (A-D), and acetate (E-H) concentrations during production phase with shifting of conditions from dark and anaerobic to light and aerobic. Open, closed, and gray symbols represent isopropanol, acetone, and acetate, respectively. Shadowed area in each graph represents period under dark and anaerobic conditions. Data are represented as means $\pm$ S.D. of four individual experiments.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
<i>Strain</i>		
TA1297	<i>S. elongatus</i> PCC 7942, wild type strain	Life technologies
TA1684	<i>sadh</i> integrated at NS II in TA1297 genome	This study
TA1741	<i>thl-atoAD-adc</i> integrated at NS I in TA1684 genome	This study
<i>Plasmid</i>		
pTA371	ColE1, <i>lacIq</i> , NS I targeting, <i>spec^r</i>	(10)
pTA372	<i>P_{LacO1}:: thl, atoAD, adc</i> , ColE1, <i>lacIq</i> , NS I targeting, <i>spec^r</i>	(10)
pTA634	<i>P_{LacO1}:: sadh</i> , ColE1, NS II targeting, <i>kan^r</i>	(10)
pTA821	<i>P_{LacO1}:: thl, atoAD (G66D AtoA), adc</i> , ColE1, <i>lacIq</i> , NS I targeting, <i>spec^r</i>	This study

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.