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

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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).

[**Key words:** Cyanobacteria; Synthetic metabolic pathway; Isopropanol production; Dark and anaerobic condition; Acetate utilization]

The bioconversion of cellulosic biomass using engineered microorganisms would be a key technology for the production of alternatives to fossil fuel resources. *Escherichia coli* and yeast have been widely used as hosts for these purposes because of these species' ideal properties, including fast growth, the availability of well established genetic tools, and well characterized metabolic pathways. The introduction of synthetic metabolic pathways composed of multiple genes derived from other organisms has been used to enable these hosts to produce various chemicals that they are not able to produce naturally (1,2). Furthermore, methodologies in metabolic engineering (including knocking out genes for byproduct producing pathways and overexpressing genes for target-producing pathways) are effective for productivity improvements. Although there are many advantages to using such microorganisms in bioconversion from cellulosic biomass to target chemicals and fuels, there are also some difficulties. For example, there are some technological difficulties in the saccharification process that is necessary to supply monosaccharides as substrate to microorganisms from cellulosic biomass. Additionally, growth of the plants supplying cellulosic biomass is a very time-consuming process. One of the expected solutions to these problems is the application of photosynthetic microorganisms (cyanobacteria and algae) as host microorganisms for producing chemicals and fuels. These photosynthetic microorganisms use photosystems to convert solar energy to chemical energy (ATP and NADPH), which is used to fix carbon dioxide to organic compounds. Thus, photosynthetic microorganisms introduced synthetic metabolic pathways would be able to produce various chemicals directly from carbon dioxide using solar energy. Cyanobacteria have been well studied in photosynthesis research, and are considered as an ancestor of chloroplast. Various techniques and tools for gene manipulation in cyanobacteria have been developed (3,4), and the genomes of many species have been sequenced (5,6).

Various chemicals already have been produced using engineered cyanobacteria, primarily in strains derived from *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942, and these results have been summarized in several recent reviews (7,8,9). In a previous study, we constructed an isopropanol producing strain derived from *S. elongatus* PCC 7942 (10). Isopropanol is an attractive chemical, since the compound is readily converted to propylene via dehydration (11). Polypropylene produced from propylene is currently a popular industrial material, with global demand expected to increase (12). In the constructed synthetic metabolic pathway that we used (10,13), isopropanol is produced from cellular acetyl-CoA via a four step process, including (i) condensation of two molecules of acetyl-CoA into one molecule of acetoacetyl-CoA, (ii) CoA transfer to acetate and formation of acetoacetate, (iii) decarboxylation of acetoacetate to form acetone, and (iv) dehydrogenation of acetone to form isopropanol. For the construction of this synthetic metabolic pathway, *thl* and *adc* were obtained from *Clostridium acetobutylicum* ATCC824, *atoAD* from *E. coli* K-12 MG1655, and *sadh* from *C. beijerinckii* NRRL B593; the cloned genes were introduced into *E. coli* (13) and cyanobacteria (10).

It was reported that the carbon fixed by photosynthesis is fed into the Calvin cycle and subsequently converted primarily to glycogen. It was also reported that the flux toward glycolysis and the TCA cycle is considerably small during exposure to light (14,15).

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The production of acetone (36 mg/l) and 1-butanol (14.5 mg/l) from cellular acetyl-CoA in engineered cyanobacteria previously was achieved under dark and anaerobic conditions (16,17). Similarly, we achieved isopropanol production (26.5 mg/l) in our engineered cyanobacteria under dark and anaerobic conditions. In our 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, USA), 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 $NaNO_3$, K_2HPO_4, 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 same as $BG11_{H7.5}$. For all BG11 derived media, buffers were supplemented to 20 mM.

Growth and production conditions All cyanobacterial cultures were grown under fluorescent light (150 μmol photon m^{-2} 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 (no. 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 ×*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 ultrapure 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.

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

Cloning of synthetic metabolic pathway containing mutation (G66D in AtoA) from TA1317 The genome of TA1317, the isopropanol producing strain constructed in our previous study, was extracted with a DNeasy Blood and Tissue kit (cat. 69504, Qiagen, Venlo, the Netherlands) and used as a PCR template. The synthetic metabolic pathway containing a missense mutation (encoding the G66D substitution in AtoA) was amplified by PCR using primers T548 (5′-GCCAT CCCTA GGAAT TGTGA GCGGA TAACA ATTGA CATTG-3′) and T550 (5′-GCCAT CGGAT CCTTA CTTAA GATAA TCATA TATAA CTTCA GCTC-3′). The PCR product was digested with AvrII and BamHI, and inserted into an AvrII, BamHI site of pTA371. The resulting plasmid was designated pTA821 [$P_L$lacO1:: *thl-atoAD* (G66D AtoA)-*adc*, NS I site]. All amplified genes were sequenced.

The cloned synthetic metabolic pathway was integrated into the *S. elongatus* PCC 7942 (Life Technologies Corporation, CA, USA) genome by sequential homologous recombination using two plasmids (pTA821 and pTA634) (Table 1). First, $P_L$lacO1:: *sadh* and the kanamycin resistance gene were integrated into the NS II (21) site of TA1297, creating strain TA1684. Second, $P_L$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 (Table 1).