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Engineering a synthetic pathway in cyanobacteria for isopropanol production directly from carbon dioxide and light

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ABSTRACT

Production of alternate fuels or chemicals directly from solar energy and carbon dioxide using engineered cyanobacteria is an attractive method to reduce petroleum dependency and minimize carbon emissions. Here, we constructed a synthetic pathway composed of acetyl-CoA acetyl transferase (encoded by *thl*), acetoacetyl-CoA transferase (encoded by *atoAD*), acetoacetate decarboxylase (encoded by *adc*) and secondary alcohol dehydrogenase (encoded by *adh*) in *Synechococcus elongatus* strain PCC 7942 to produce isopropanol. The enzyme-coding genes, heterogeneously originating from *Clostridium acetobutylicum* ATCC 824 (*thl* and *adc*), *Escherichia coli* K-12 MG1655 (*atoAD*) and *Clostridium beijerinckii* (*adh*), were integrated into the *S. elongatus* genome. Under the optimized production conditions, the engineered cyanobacteria produced 26.5 mg/L of isopropanol after 9 days.

1. Introduction

Solar energy is the most abundant and accessible renewable energy source available for sustainable production of chemicals and fuels. Therefore the development of effective systems for utilization of solar energy for the production of alternate fuels or chemicals is being investigated (Machado and Atsumi, 2012). One possible production scheme recycles carbon dioxide using phototrophic organisms such as cyanobacteria. The currently available genetic tools and genome sequences make cyanobacteria a user-friendly host for metabolic engineering. Recent literature has demonstrated that cyanobacteria produce exogenous chemicals such as isobutyraldehyde (1100 mg/L), isobutanol (450 mg/L) (Atsumi et al., 2009), 1-butanol (404 mg/L) (Lan et al., 2013; Lan and Liao, 2012, 2011), 2-methy-1-butanol (200 mg/L) (Shen and Liao, 2013), acetone (36 mg/L) (Zhou et al., 2012), ethylene (~171 mg/L·day) (Takahama et al., 2003; Ungerer et al., 2012), ethanol (5500 mg/L) (Gao et al., 2012), isoprene (0.05 mg/g dry cell·day) (Lindberg et al., 2010), fatty acids (197 mg/L) (Liu et al., 2011), 3-hydroxybutyrate (533.4 mg/L) (Wang et al., 2013), and 2,3-butanediol (2380 mg/L) (Oliver et al., 2013).

Isopropanol is a valuable secondary alcohol produced naturally by certain microbes (Osburn et al., 1937). Isopropanol can be dehydrated to yield propylene (Kibby and Hall, 1972), a petroleum-derived monomer for the production of polypropylene. Polypropylene is currently a popular industrial material with increasingly high global demand expected (Molenda, 2004).

Several species of *Clostridium* have been evaluated for isopropanol production with titers up to 1.8 g/L (Chen and Hiu, 1986). The pathway in *Clostridium beijerinckii* produces isopropanol from acetyl-coenzyme A (acetyl-CoA) via acetone (Fig. 1). First, an acetyl-CoA acetyltransferase (encoded by *thl*) condenses two molecules of acetyl-CoA into one molecule of acetoacetyl-CoA (Wiesenborn et al., 1988). Next, an acetoacetyl-CoA transferase (encoded by *ctfAB*) transfers the CoA to acetate or butyrate (Wiesenborn et al., 1989), forming acetoacetate. Acetoacetate is irreversibly converted to acetone and carbon dioxide by an acetoacetate decarboxylase (encoded by *adc*) (Petersen and Bennett, 1990). Finally, a primary-secondary alcohol dehydrogenase (hereafter referred to as SADH, encoded by *adh*) converts acetone to isopropanol in an NADPH-dependent reaction (Chen, 1995).

In a previous study (Hanai et al., 2007), we engineered a synthetic isopropanol pathway in *Escherichia coli*. In addition to the genes from *C. beijerinckii* mentioned above, we evaluated the native *E. coli* acetyl-CoA acetyltransferase (encoded by *atoB*) and acetoacetyl-CoA transferase (encoded by *atoA* and *atoD* [*atoAD*]) (Jenkins and Nunn, 1987). We also compared the activities of two SADH, the first from *C. beijerinckii* NRRL B593 (Ismaiel et al., 1993) and the second from *Thermoanaerobacter brockii* HTD4 (Lamed and Zeikus, 1981). Strain optimization led to an engineered *E. coli* strain with the synthetic gene combination of *Clostridium acetobutylicum*

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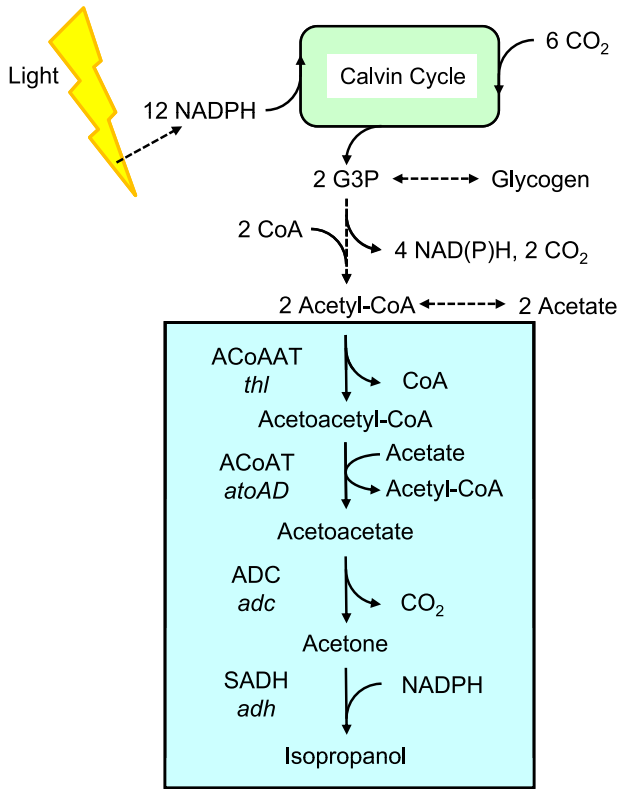

Fig. 1. The metabolic pathway for isopropanol production in engineered *S. elongatus* PCC7942. The box shows the synthetic pathway for isopropanol production from acetyl-CoA engineered in this study. The dashed line indicates omitted steps. ACoAAT: acetyl-CoA acetyltransferase (encoded by *thl*), ACoAT: acetoacetyl-CoA transferase (encoded by *atoAD*), ADC: acetoacetate decarboxylase (encoded by *adc*), SADH: secondary alcohol dehydrogenase (encoded by *adh*).

thl, *E. coli atoAD*, *C. acetobutylicum adc* and *C. beijerinckii adh*, that achieved the highest titer of 4.9 g/L (Hanai et al., 2007). Next, fermentation conditions were optimized for this bacterial isopropanol production. As the accumulation of isopropanol drastically reduced production yields, a gas stripping recovery method was incorporated into the fed-batch culture system to remove and recover the produced isopropanol from the culture medium. Using this approach, the strain produced 143 g/L of isopropanol after 10 days (Inokuma et al., 2010).

The high productivity in *E. coli* and the volatile property of isopropanol encourages us to transfer this pathway into cyanobacteria. However, isopropanol production in cyanobacteria has not been reported. The production of chemicals by using engineered cyanobacteria is more challenging than by model heterotrophic organisms such as *E. coli*, because of multiple copies of the genome per cell, poorly characterized metabolic physiology, and insufficiently developed genetic manipulation methods. Here, we engineered a synthetic pathway for the production of isopropanol in cyanobacteria.

2. Materials and methods

2.1. 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), ligase (rapid DNA ligation kit, Roche, Manheim, Germany), 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).

2.2. Culture medium

A BG-11 medium (1.5 g/L $NaNO_3$, 0.027 g/L $CaCl_2$, 0.006 g/L ferric ammonium citrate, 0.001 g/L $Na_2EDTA \cdot 2H_2O$, 0.039 g/L K_2HPO_4, 0.075 g/L $MgSO_4 \cdot 7H_2O$, 0.020 g/L Na_2CO_3, 0.006 g/L citrate, 2.860 mg/L H_3BO_3, 1.810 mg/L $MnCl_2 \cdot 4H_2O$, 0.220 mg/L $ZnSO_4 \cdot 7H_2O$, 0.390 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.080 mg/L $CuSO_4 \cdot 5H_2O$, and 0.050 mg/L $Co(NO_3)_2 \cdot 6H_2O$) was used as a basic medium for *Synechococcus elongatus*. BG-11$_{+HEPES}$, BG-11$_{-N}$, BG-11$_{-P}$, and BG-11$_{-N,\ P}$ media were modified from BG-11 medium for the cell growth or isopropanol production with the following modifications: 20 mM HEPES was added for BG-11$_{+HEPES}$, $NaNO_3$ was removed for BG-11$_{-N}$, K_2HPO_4 was removed for BG-11$_{-P}$, and both $NaNO_3$ and K_2HPO_4 were removed for BG-11$_{-N,\ P}$. Antibiotics were added as appropriate; spectinomycin 20 μg/ml and kanamycin 10 μg/ml.

2.3. Growth and production conditions

For isopropanol production under standard aerobic incubation in constant light exposure, cells were grown at 30 °C under fluorescent light (50 μmol/m^2·s) using 50 ml BG-11$_{+HEPES}$ medium in a baffled 300 mL Erlenmeyer flask, shaken at 100 rpm with a rotary shaker Shake LR (TAITEC, Koshigaya, Japan). When OD_{730} reached 0.4, 1.0 mM IPTG was added. The cells were harvested at 0, 3, 5 and 7 days after addition of IPTG.

For isopropanol production under further examined conditions, 5% carbon dioxide at 250 mL/min was supplied to cells grown in 500 mL turtle shape flasks 62040 (Vidrex, Fukuoka, Japan). Cells were grown using 500 ml BG-11 medium at 30 °C under fluorescent light (100 μmol/m^2·s) until OD_{730} reached 1.0. The grown cells were then collected by centrifugation at 1500 × *g*. The cells were resuspended in one-fifth the original volume of the appropriate medium (BG-11, BG-11$_{-N}$, BG-11$_{-P}$, and BG-11$_{-N,\ P}$) with 1.0 mM IPTG. Anaerobic conditions were accomplished by purging the headspace of evacuated blood collection tubes Vacutainer (BD, Franlin Lakes, USA) with sterilized ultra-pure nitrogen gas several times. Aerobic conditions were maintained by incubating cultures in a test tube with a plastic cap. All culture tubes grown under light conditions were maintained with fluorescent light (50 μmol/m^2·s). Dark growth was accomplished by wrapping the tubes with aluminum foil. The tubes for isopropanol production were shaken at 100 rpm with a rotary shaker INNOVA 44R (New Brunswick, Edison, USA), and cells were harvested at 0, 3, 5, 7, 9 and 15 days.

2.4. Plasmid and strain construction

Table 1 shows the strains and plasmids used in this study. pTA216 and pTA418 were used to examine the effect of G66D missense mutation in *atoD* gene in *E. coli*, while pTA372 and pTA634 were used for the integration of genes into *S. elongatus* genome. The *S. elongatus* strain was kindly provided from Dr. Kondo (Nagoya University). For the construction of plasmids, *E. coli* XL1-Blue or XL10-Gold (Agilent technologies, Santa Clara, USA) was used.

2.4.1. pTA216 (*lacI^q*)

lacI^q was amplified from the F plasmid in XL1-Blue by PCR using primers: T329 (5′-GCCAT CCTCG AGCGT TGACA CCATC GAATG GTGCA AAACC-3′) and T330 (5′-GCCAT CGGAT CCTCA CTGCC CGCTT TCCAG TCG-3′). The PCR product was digested by XhoI and BamHI, and inserted into an XhoI and BamHI site of