1 **Photosynthetic production of itaconic acid in *Synechocystis* sp. PCC6803**

2
3 Taejun Chin^a, Mei Sano^a, Tetsuya Takahashi^b, Hitomi Ohara^a, Yuji Aso^{a,*}

4
5 ^a*Department of Biobased Materials Science, Kyoto Institute of Technology, 1 Hashigami-cho,*
6 *Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan*

7 ^b*Faculty of Education, Shimane University, 1060 Nishikawatsu-cho, Matsue, Shimane*
8 *690-8504, Japan*

9
10 **Abbreviations:** IA, itaconic acid; PCC6803, *Synechocystis* sp. PCC6803; CAD, *cis*-aconitate
11 decarboxylase; GFP, green fluorescence protein; CO₂, carbon dioxide; IPTG, isopropyl
12 β-D-1-thiogalactopyranoside; LED, light-emitting diode.

13
14 *Corresponding author: Department of Biobased Materials Science, Kyoto Institute of
15 Technology, 1 Hashigami-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan. Tel./Fax:
16 +81 75 724 7694; E-mail address: aso@kit.ac.jp (Y. Aso).

Abstract

Here, we report the photosynthetic production of itaconic acid (IA), a promising building block, from CO₂ by *Synechocystis* sp. PCC6803. The engineered PCC6803 strain expressing *cis*-aconitate decarboxylase, the key enzyme in IA biosynthesis, produced 0.9 mg/L and 14.5 mg/L of IA at production rates of 42.8 µg·L⁻¹·day⁻¹ and 919.0 µg·L⁻¹·day⁻¹, under conditions of constant bubbling with air and 5% CO₂, respectively. This is the first report on the possibility of IA production from CO₂ via the photosynthetic process in cyanobacteria.

Keywords: Itaconic acid, *Synechocystis* sp. PCC6803, Photosynthesis, *cis*-Aconitate decarboxylase

Cyanobacteria display high potential for use in bioindustrial processes. The production of valuable products such as biofuels and bulk chemicals from CO₂ has been achieved using engineered cyanobacteria (Chisti, 2013; Desai and Atsumi, 2013; Ducat et al., 2011; Quintana et al., 2011; Ruffing, 2011; Wang et al., 2012). Itaconic acid (IA) has attracted considerable attention as a promising building block for synthetic polymers and is industrially produced by the fungus *Aspergillus terreus* from sugars (Klement et al., 2013). The gene encoding *cis*-aconitate decarboxylase (CAD) has been identified and characterized; it encodes the key enzyme catalyzing the decarboxylation of *cis*-aconitate to IA in the tricarboxylic acid (TCA) cycle (Kanamasa et al., 2008). The heterologous production of IA has been demonstrated in the case of CAD expression in microorganisms that possess the TCA cycle (Okamoto et al., 2014a; Okamoto et al., 2014b). These studies suggest that cyanobacteria expressing CAD can produce IA from CO₂ through the TCA cycle.

Here, we report the photosynthetic production of IA via CAD expression in the cyanobacterium *Synechocystis* sp. PCC6803, using CO₂ as the carbon source (Fig. 1). The bacterial strains and plasmids used in this study are listed in Table S1. All the recombinant cyanobacterial strains were grown in BG11 medium supplemented with 50 mM sodium bicarbonate (BG11C) and 50 µg/mL of spectinomycin, with a light-emitting diode (LED) set at 30 µmol·m⁻²·s⁻¹. The CAD gene was synthesized by referring to the deposited CAD1 sequence from *A. terreus*, another IA producer, (GenBank database accession no. AB326105). The synthesized CAD gene was cloned into the expression vector pJAK12 containing an IPTG-inducible *tac* promoter, resulting in pJAK12-cad. There are three main reasons why pJAK12 was selected for this study. First, pJAK12 can be used as a conjugal plasmid in diverse gram-negative bacteria, including the PCC6803 strain (Marraccini et al. 1993). Second, the *tac* promoter has also been characterized in cyanobacteria for protein expression and production of high-value materials via IPTG induction (Ruffing, 2011). Lastly,

plasmid-based gene expression is likely to be preferred in cases where high gene expression may require a high copy number, or when the desired host may have low recombination efficiency (Ruffing, 2011). In order to evaluate protein expression by using pJAK12, a green fluorescence protein (GFP) encoded by pGreenTIR was used. The CAD and GFP genes were introduced into the PCC6803 strain by conjugation using *E. coli* DH5 α harboring a mating helper plasmid pRK2013 and pJAK12-cad and pJAK12-gfp, respectively. The specific fluorescence intensity was measured when the PCC6803 harboring pJAK12-gfp was grown in 1 mL of BG11C at 30°C in the presence of different IPTG concentrations; the optimal IPTG concentration was determined to be 5 mM (Fig. S1). In addition, the fluorescence intensity was dramatically increased by adding 5 mM IPTG, and GFP fluorescence was observed while cultivating PCC6803 cells (pJAK12-gfp) with 5 mM IPTG induction (Fig. S2).

To investigate the effect of CAD expression and CO₂ supplementation on IA production, the PCC6803 strain harboring pJAK12-cad was cultivated in 50 mL of BG11C at 25°C by constantly bubbling with air or with 5% CO₂ at 1 vvm. After the growth rate reached the stationary phase, 50 mM sodium bicarbonate was added to the cultures. The specific growth rate (μ) was calculated as the slope of the regression line. The IA produced in the culture supernatant was quantified using a Prominence HPLC system (Shimadzu; Kyoto, Japan) equipped with a SCR-102H column (Shimadzu). As a result, the cultivation of PCC6803 (pJAK12-cad) after air bubbling resulted in a μ of $0.010 \pm 0.000 \text{ h}^{-1}$ and IA production of $0.9 \pm 0.1 \text{ mg/L}$ after 527 h (Fig. 2B). On the other hand, 5% CO₂ supplementation led to an increase in each productive property (μ , $0.018 \pm 0.000 \text{ h}^{-1}$; IA production, $14.5 \pm 1.0 \text{ mg/L}$ after 379 h) (Fig. 2D). The production rates of IA were calculated to be $42.8 \text{ }\mu\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ and $919.0 \text{ }\mu\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ for cells grown in the presence of constant air bubbling and 5% CO₂, respectively. In addition, the CAD activity in the

PCC6803 transformants was assayed using a previously described method (Okamoto et al., 2014a). It is remarkable that the CAD activity at the log growth phase of PCC6803 (pJAK12-cad) in the presence of air with 5% CO₂ was similar to that observed in the presence of air without CO₂ (Fig. 3). However, PCC6803 (pJAK12) produced no significant amount of IA (Figs. 2A and C) and did not exhibit any CAD activity (data not shown). These results indicate that the expression of CAD in the PCC6803 strain led to IA production from CO₂, and that the IA synthesis was improved with increasing CO₂ concentration (of the bubbled air) in the medium. The photosynthetically fixed carbon is used primarily for biomass production in the PCC6803 strain during cell growth in the log growth phase. As the cells enter the stationary phase, an increasing portion of the fixed carbon is used from the storages, resulting in improved objective compound productivity (Ungerer et al., 2012). IA production could act as another carbon sink, in addition to the native storage compounds. Moreover, it is evident that <5% of the CO₂ is fixed into the TCA cycle in the PCC6803 strain (Knoop et al., 2013). The problem caused by a limited carbon flux could be overcome by bubbling more CO₂ during cultivation. It is possible to improve IA productivity in PCC6803 expressing CAD. For instance, carbon fixation could be enhanced by overexpression of the enzyme, which is implicated as the limiting step in CO₂ fixation. It is also beneficial for IA production if cultivation conditions such as light intensity and medium components are improved. Additionally, sustained production of IA can be achieved through continuous cell growth. Our bioprocess is more advantageous and attractive for IA production than the bioprocesses previously used in non-photosynthetic microorganisms.

Acknowledgements

We thank the National BioResource Project (National Institute of Genetics, Japan)

for providing us with pGreenTIR. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (24580110), the Adaptable and Seamless Technology Transfer Program Through Target-Driven R&D of the Japan Science and Technology Agency (AS232Z01368E), and the Sasakawa Scientific Research Grant from The Japan Science Society.

References

- Chisti, Y., 2013. Constraints to commercialization of algal fuels. *J. Biotechnol.* 167, 201–214.
- Desai, S. H., Atsumi S., 2013. Photosynthetic approaches to chemical biotechnology. *Curr. Opin. Biotech.* 24, 1031–1036.
- Ducat, D.C., Way, J.C., Silver, P.A., 2011. Engineering cyanobacteria to generate high-value products. *Trends Biotechnol* 29, 95–103.
- Kanamasa, S., Dwiarti, L., Okabe, M., Park, E.Y., 2008. Cloning and functional characterization of the *cis*-aconitic acid decarboxylase (CAD) gene from *Aspergillus terreus*. *Appl. Microbiol. Biot.* 80, 223–229.
- Klement, T., Buchs, J., 2013. Itaconic acid - a biotechnological process in change. *Bioresource Technol.* 135, 422–431.
- Knoop, H., Grundel, M., Zilliges, Y., Lehmann, R., Hoffmann, S., Lockau, W., Steuer, R., 2013. Flux balance analysis of cyanobacterial metabolism: the metabolic network of

130 *Synechocystis* sp. PCC6803. PLoS Comput. Biol. 9, e1003081.

131 Marraccini, P., Bulteau, S., Cassier-Chauvat, C., Mermet-Bouvier, P., Chauvat, F., 1993. A
132 conjugative plasmid vector for promoter analysis in several cyanobacteria of the genera
133 *Synechococcus* and *Synechocystis*. Plant Mol. Biol. 23, 905–909.

134 Okamoto, S., Chin, T., Hiratsuka, K., Aso, Y., Tanaka, Y., Takahashi, T., Ohara, H., 2014a.
135 Production of itaconic acid using metabolically engineered *Escherichia coli*. J. Gen.
136 Appl. Microbiol, in press.

137 Okamoto, S., Chin, T., Nagata, K., Takahashi, T., Ohara, H., Aso, Y., 2014b. Production of
138 itaconic acid in *Escherichia coli* expressing recombinant α -amylase using starch as
139 substrate. J. Biosci. Bioeng., in press.

140 Quintana, N., Van der Kooy, F., Van de Rhee, M.D., Voshol, G.P., Verpoorte, R., 2011.
141 Renewable energy from cyanobacteria: energy production optimization by metabolic
142 pathway engineering. Appl. Microbiol. Biot. 91, 471–490.

143 Ruffing, A.M., 2011. Engineered cyanobacteria: Teaching an old bug new tricks. Bioeng.
144 Bugs 2, 136–149.

145 Ungerer, J., Tao, L., Davis, M., Ghirardi, M., Maness, P.-C., Yu, J., 2012. Sustained
146 photosynthetic conversion of CO₂ to ethylene in recombinant cyanobacterium
147 *Synechocystis* 6803. Energ. Environ. Sci. 5, 8998.

148 Wang, B., Wang, J., Zhang, W., Meldrum, D.R., 2012. Application of synthetic biology in
149 cyanobacteria and algae. Front. Microbiol. 3, 344.

Figure legends

Fig. 1. Biosynthetic pathway of itaconic acid from CO₂ in *Synechocystis* sp. PCC6803 expressing CAD.

Fig. 2. Itaconic acid production in PCC6803 transformants after 5 mM IPTG induction and constant air bubbling. The arrow indicates 5 mM IPTG addition. Error bars indicate $\pm$ SD ($n = 2$). PCC6803 (pJAK12) bubbled with (A) air and with (C) 5% CO₂. PCC6803 (pJAK12-cad) bubbled with (B) air and with (D) 5% CO₂.

Fig. 3. Specific CAD activities in PCC6803 (pJAK12-cad) at the early-log and mid-log growth phases, in the presence of air and 5% CO₂. Error bars indicate SD ($n = 2$). The CAD activity was assayed according to the methodology detailed in a previous study (Okamoto et al., 2014a).

Figure

Fig. 1 Chin *et al.*

Fig. 2 Chin *et al.*

Fig. 3 Chin *et al.*