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Designing and creating a modularized synthetic pathway in cyanobacterium *Synechocystis* enables production of acetone from carbon dioxide

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ABSTRACT

Ketones are a class of important organic compounds. As the simplest ketone, acetone is widely used as solvents or precursors for industrial chemicals. Presently, million tonnes of acetone is produced worldwide annually, from petrochemical processes. Here we report a biotechnological process that can produce acetone from CO_2, by designing and creating a modularized synthetic pathway in engineered cyanobacterium *Synechocystis* sp. PCC 6803. The engineered *Synechocystis* cells are able to produce acetone (36.0 mg l^{-1} culture medium) using CO_2 as the sole carbon source, thus opens the gateway for biosynthesis of ketones from CO_2.

1. Introduction

Ketones are a class of compounds with a carbonyl group, which play important roles in synthetic chemistry and industry (Grogan, 2005). As the simplest ketone, acetone is not only widely used as a solvent, but also used as a precursor for other industrial chemicals such as isopropanol (Rahman, 2010). The consumption of acetone has grown rapidly in recent years. According to World Petrochemicals Report of January 2010, about 6.7 million tonnes acetone is produced worldwide annually.

Commercially, most acetone is produced from petrochemical processes (Sifniades and Levy, 2005). One petrochemical process is to produce acetone from propylene via Cumene process, in which acetone production is tied to phenol production. Other petrochemical processes involve direct oxidation of propylene (Wacker-Hoechst process), or hydration of propylene to produce 2-propanol, which is then oxidized to acetone (Sifniades and Levy, 2005). These processes are based on petrochemical raw materials, which are not renewable. Global climate change and energy shortage call for sustainable, renewable, carbon-neutral alternatives to replace fossil fuel resources; biosynthesis of acetone is therefore anticipated.

At the early 20th century, acetone was produced via bacterial fermentation of *Clostridia*, mostly using starch feedstocks (Jones and Woods, 1986; Lütke-Everslon and Bahl, 2011). In this process, acetate, butyrate, ethanol, and butanol are also produced along with acetone (Sillers et al., 2008). However, if hexose (such as glucose) were used as a sole carbon source for acetone production, only half of the carbon atom will be converted to acetone. The other half will be released as CO_2 during the decarboxylation of pyruvate to acetyl-CoA, and decarboxylation of acetoacetate to acetone. In addition, two molecules of NADH generated from glycolysis cannot be recycled, leading to redox imbalance. Therefore, production of acetone from glucose has to be associated with the production of other reductive metabolites. To date, a highly efficient process for biotechnological production of acetone has not yet been established.

Carbon dioxide (CO_2) is a cheap and abundant carbon source. The photoautotrophic prokaryotes cyanobacteria can convert CO_2 and water into carbohydrates by photosynthesis using sunlight as energy, and store them as glycogen, fatty acid or polyhydroxybutyrate (PHB) (Yoo et al., 2002; Radakovits et al., 2010; Schlebusch and Forchhammer, 2010). Therefore, cyanobacteria are ideal host for biosynthesis of acetone from CO_2. Based on the endogenous metabolic network of cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), a novel acetone biosynthetic pathway from CO_2 was designed, and the feasibility of using CO_2 to produce acetone was demonstrated in this study.

2. Materials and methods

2.1. Strains and growth conditions

The bacterial strains are listed in Table 1. *Escherichia coli* strain DH5α was used as a host for construction of vectors. *Synechocystis*

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6803 wild-type and transformants were grown in BG11 medium at 30 °C at an illumination intensity of 100 μmol photons $m^{-2} s^{-1}$ as described in the literature (Castenholz, 1988). Chloromycetin (10 mg l^{-1}) and/or kanamycin (10 mg l^{-1}) was added to the medium when necessary. For maintenance of strains on agar plates, the BG11 medium was supplemented with 1.5% (w/v) agar.

2.2. Construction of vectors

The plasmids used and constructed in this work are listed in Table 2. General strategy for constructing vectors used for deletion of *phaCE* and *pta* genes, or for expression of *ctfAB* and *adc* genes, in *Synechocystis* 6803, was shown in Supplementary Fig. 1. All primers used are listed in Table 3.

Plasmid pSM1 was constructed by inserting *phaCE* gene knockout cassette into the pMD18-T simple vector. The *phaCE* gene knockout cassette was constructed by ligating 600 bp sequence located immediately upstream *phaE* (*phaE* up), chloromycetin resistance cassette (Cm^r), and 600 bp sequence located immediately downstream *phaC* (*phaC* down), using fusion PCR (Wang et al., 2002). *phaE* up and *phaC* down were amplified from the genomic DNA of *Synechocystis* 6803.

Plasmid pSM2 was constructed by inserting *pta* gene knockout cassette into T-cloning site of pMD18-T simple vector. The *pta* gene knockout cassette was constructed by ligating 600 bp sequence located immediately upstream *pta* (*pta* up), kanamycin resistance cassette (Km^r), and 600 bp sequence located immediately downstream *pta* (*pta* down), using fusion PCR (Wang et al., 2002). *pta* up and *pta* down were amplified from the genomic DNA of *Synechocystis* 6803.

Plasmid pSM3 was constructed by inserting PrbcL-ctfAB-adc expression cassettes into the BamHI site of plasmid pSM1 (Supplementary Fig. 1). PrbcL-cftAB-adc expression cassettes were constructed by ligating promoter PrbcL (Onizuka et al., 2003) and genes *ctfAB-adc*, using fusion PCR (Wang et al., 2002). Promoter PrbcL and genes *ctfAB-adc* were amplified from the genomic DNA of *Synechocystis* 6803 and *C. acetobutylicum* DSM 1731 (Bao et al., 2011), respectively.

Table 1
Strains used and constructed in this study.

Strains	Relevant genotype	Reference
Escherichia coli		
DH5α	Commercial transformation host for cloning	Takara Co., Ltd.
Cyanobacteria		
Synechocystis 6803	*Synechocystis* sp. PCC 6803 wild-type	Prof. D.J. Shi
SM1	phaCE::Cm^r	This study
SM2	phaCE::Cm^r, pta::Km^r	This study
SM3	phaCE::PrbcL-cftAB, PphaE-adc, Cm^r	This study
SM4	phaCE::Prbc-cftAB, Pcpc-adc, Cm^r	This study
SM5	phaCE::Prbc-cftAB, Pcpc-adc, Cm^r; pta::Km^r	This study

Plasmid pSM4 was constructed by inserting PrbcL-ctfAB-adc-Pcpc expression cassettes into the BamHI site of plasmid pSM1 (Supplementary Fig. 1). PrbcL–ctfAB–adc–Pcpc expression cassettes were constructed by ligating promoter PrbcL (Onizuka et al., 2003), genes *ctfAB-adc*, and promoter Pcpc (Imashimizu et al., 2003), using fusion PCR (Wang et al., 2002). Promoter PrbcL and Pcpc were amplified from the genomic DNA of *Synechocystis* 6803, and genes *ctfAB-adc* were amplified from the genomic DNA of *C. acetobutylicum* DSM 1731 (Bao et al., 2011).

2.3. Construction of SM strains

Mutants of *Synechocystis* 6803, designated as SM strains, were constructed by transforming *Synechocystis* 6803 with plasmids listed in Table 2. Transformations were performed as previously described (Lindberg et al., 2010; Zhou et al., 2008). All constructed SM strains were listed in Table 1. Briefly, strain SM1, SM3 and SM4 were constructed by integration of plasmids pSM1, pSM3 and pSM4, respectively, at the *phaCE* locus of wild-type *Synechocystis* 6803 via double crossover homologous recombination. Strain SM2 was constructed by integration of plasmid pSM2 at the *pta* locus of strain SM1. Strain SM5 was constructed by integration of plasmid pSM2 at the *pta* locus of strain SM4.

2.4. Rt-PCR

RT-PCR was performed as previously described (Zhou et al., 2008). Total RNA of *Synechocystis* 6803 wild-type (WT) and acetone producing strain SM5 cells were isolated using Redzol reagent and SiMaxTM membrane spin columns (SBS Genetech, Beijing, China). Residual DNA in RNA preparations was eliminated by digestion with RNAse-free DNAse (Qiagen, Beijing, China). Reverse transcription reactions using random primers were performed with M-MLV RT (Takara, Dalian, China). To detect possible DNA contamination, control reactions were performed without M-MLV RT but with Taq DNA polymerase. Expression of *rnpB* was used as a positive control. Reverse transcription products were amplified by PCR and analyzed by electrophoresis on 1.2% (w/v) agarose gels.

2.5. Production of acetone

Cells were grown in 150 ml flask in BG11 medium under normal growth conditions (Castenholz, 1988) until OD_{730} reached 2.0. The cells were then collected by centrifugation and washed twice with nitrogen-free and phosphate-free BG11 medium, and resuspended in the same medium to reach an OD_{730} of 15 (about 4 g l^{-1} dry weight). The culture was separated into different tubes to test acetone accumulation under light or fermentative conditions. To induce fermentation, dark condition was created by wrapping the test tubes with aluminum foil, while anoxic condition was accomplished by purging the head-space of the test tube with N_2 gas and sealing the tube with rubber plug.

Table 2
Plasmids used and constructed in this study.

Plasmids	Relevant characteristics	Reference
pMD18-T	Amp^r, *E. coli* cloning vector	Takara Co.,Ltd.
pSM1	pMD18-T derivate, Amp^r Cm^r, containing *phaCE* knockout cassette	This study
pSM2	pMD18-T derivate, Amp^r Km^r, containing *pta* knockout cassette	This study
pSM3	pSM1 derivate, $Amp^r Cm^r$, containing PrbcL-cftAB and PphaE-adc expression cassettes	This study
pSM4	pSM1 derivate, $Amp^r Cm^r$, containing PrbcL-cftAB and Pcpc-adc expression cassettes	This study

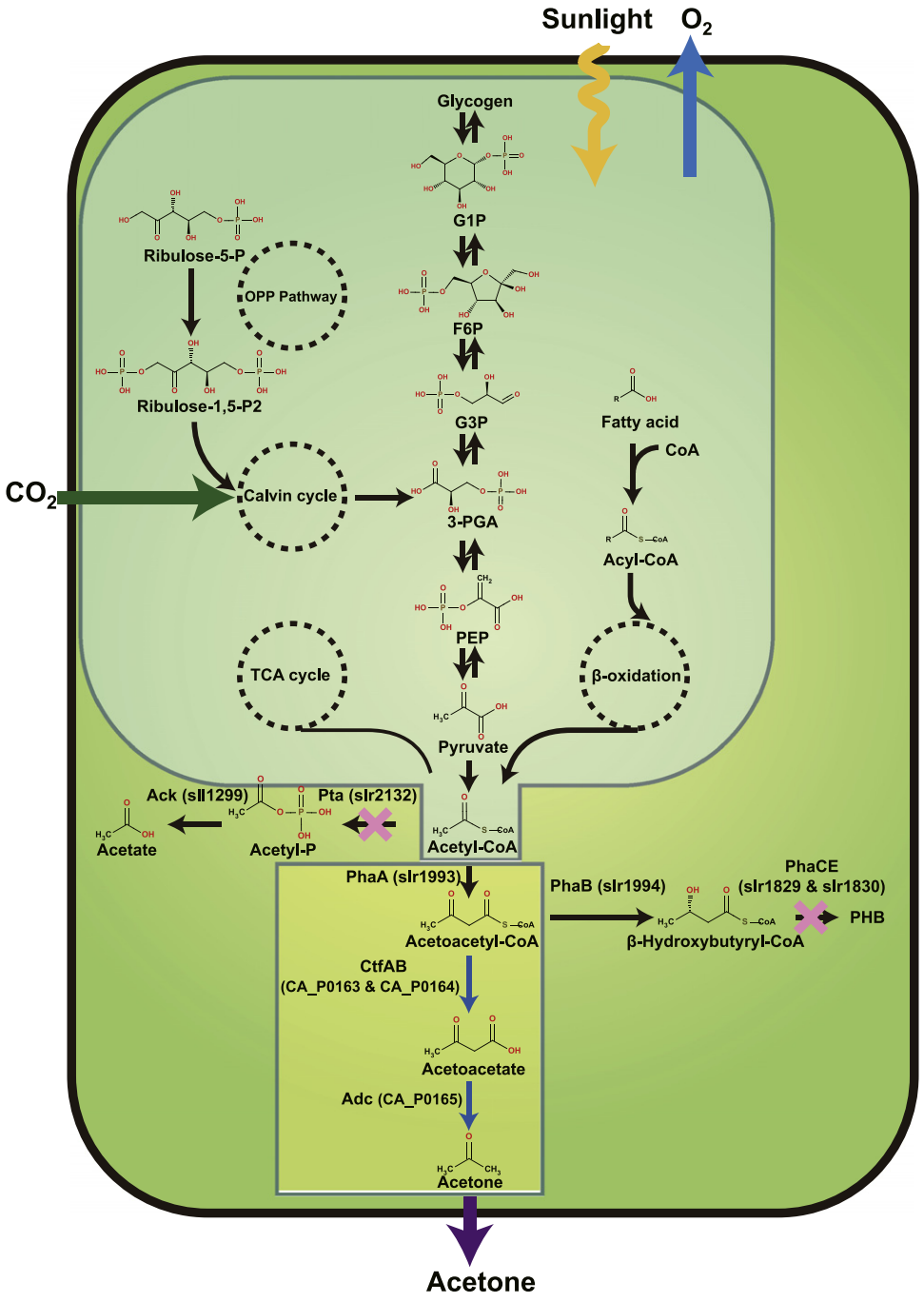

Fig. 1. Modularized biosynthetic pathway of acetone from carbon dioxide constructed in *Synechocystis* 6803. The acetone synthetic pathway was an acetyl-CoA dependent pathway. Acetyl-CoA was from fatty-acid β-oxidation and glycogenolysis under dark and anaerobic conditions, and fatty-acid and glycogen were from CO_2 fixation during photosynthesis under nitrogen and phosphate deprived conditions (the first module in light green box). From acetyl-CoA to acetone (the second module in yellow-green box), three enzymes were required: 3-ketothiolase (PhaA, UniProt accession number P73825) is the native enzyme for conversion of acetyl-CoA to acetoacetyl-CoA; Coenzyme A transferase (CtfAB, UniProt accession number P33752 and P23673) and acetoacetate decarboxylase (Adc, UniProt accession number P23670) were enzymes from *Clostridium acetobutylicum* DSM 1731 for conversion of acetoacetyl-CoA to acetone. To block PHB and acetate syntheses from acetyl-CoA, PHB synthase (PhaCE, UniProt accession number P73390 and P73389) and phosphotransacetylase (Pta, UniProt accession number P73662) were inactivated. Abbreviations: OPP, oxidative pentose phosphate; TCA, tricarboxylic acid; G1P, glucose-1-phosphate; F6P, fructose-6- phosphate; G3P, glyceraldehyde-3-phosphate; 3-PGA, 3-phospho-glycerate; PEP, phosphoenolpyruvic acid.

2.6. GC–MS analysis for acetone

Qualitative and quantitative analysis of acetone was performed by gas chromatography–mass spectrometry (GC–MS) as described (Zhang et al., 2007) with modification. 0.5 ml of culture samples were centrifuged at 14,000 rpm for 2 min. After filtrating, the supernatant was subjected to GC–MS analysis on Agilent Technologies 6890N GC-5973N MSD (Agilent Technologies, Palo Alto, CA, USA) with an Agilent DB-WAX column (30 m × 0.15 mm × 0.25 μm).

2.7. HPLC analysis for acetate

The concentration of acetate was analyzed by HPLC (Agilent 1200; Agilent Technologies, Beijing, China) as described

(Zhu et al., 2011). An Aminexs HPX-87H ion exchange column (7.8 mm × 300 mm; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used with a mobile phase of 0.05 mM sulfuric acid flowing at 0.50 ml/min at 15 °C. A refractive index (RI) detector (Agilent) was used for signal detection.

2.8. Carbohydrate determination

Total carbohydrate and insoluble glycogen concentration in strain SM2 and SM5 before and after fermentation were determined by anthrone method (Carrieri et al., 2010) with modification. For total carbohydrate determination, 20 μl of culture (OD_{730} of about 15) and 180 μl water were added to 1.8 ml anthrone reagent solution (consisting of 0.2 g anthrone per 100 ml of 71% sulfuric acid in water) and heated at 100 °C for 10 min. The absorbance of the resulting solution was measured in a spectrophotometer at 620 nm. Calibration curve was prepared with glucose standards ranging from 5 μg to 50 μg.

Isolation of insoluble glycogen from cells was performed as described (Carrieri et al., 2010) with modification. 20 μl of culture (OD_{730} of about 15) and 80 μl water were added to 200 μl of 48% KOH and incubated at 100 °C for 1 h. Subsequently, 600 μl of cold (0 °C) absolute ethanol was added, and this suspension was centrifuged at 14,000 rpm. The supernatant was discarded, and the pellet was washed twice with cold ethanol. 200 μl water and 1.8 ml anthrone reagent solution were added to the isolated insoluble glycogen. Then this solution was treated as described above.

3. Results

3.1. Design of acetone biosynthetic pathway from CO_2

Analysis of six enzyme families (oxido-reductase, transferase, hydrolase, lyase, isomerase, and ligase) and their subfamilies brought up two possible acetone biosynthetic pathways: oxidation of secondary alcohol or decarboxylation of β-ketoacid. In the first pathway, additional NADH is required for biosynthesis of secondary alcohol from CO_2. In the latter process, β-ketoacid can be derived from β-oxidation of fatty acid, or from glycogenolysis. Both pathways are present in cyanobacterium *Synechocystis* 6803 (Liu et al., 2011), leading to the generation of acetyl-CoA. Under regular conditions, acetyl-CoA is further oxidized to generate NADH, FADH, or GTP in the citric acid cycle (TCA cycle). Since the TCA cycle in most cyanobacteria is incomplete (Stanier and Cohen-Bazire, 1977), the acetyl-CoA from fatty-acid β-oxidation and glycogenolysis can be directed to synthesize other useful chemicals. Therefore, a novel pathway for producing acetone from CO_2 was designed based on the fatty-acid β-oxidation and glycogenolysis in *Synechocystis* 6803 (Fig. 1).

To achieve biosynthesis of acetone from CO_2 in *Synechocystis* 6803, several exogenous and endogenous genes are required (Fig. 1). As acetoacetyl-CoA is the intermediate metabolite for PHB synthesis in *Synechocystis* 6803 (Asada et al., 1999), native enzymes for conversion of CO_2 to acetoacetyl-CoA can be used. Consequently, genes involved in the conversion of acetoacetyl-CoA to acetone are required to be introduced into *Synechocystis* 6803. The ketone bodies synthetic pathway via β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) in mammals is a well characterized pathway to convert acetoacetyl-CoA into acetone. This pathway consists of the formation of HMG-CoA by HMG-CoA synthase, formation of acetoacetate by HMG-CoA lyase, and non-enzymatic decarboxylation of acetoacetate to acetone (Hegardt, 1999). Concerning the low feasibility of introducing this mammalian pathway into prokaryotes, coenzyme A transferase (CtfAB) (Cary et al., 1990) and acetoacetate decarboxylase (Adc) (Petersen and Bennett, 1990) from *C. acetobutylicum*, which can convert acetoacetyl-CoA to acetoacetate, and subsequently decarboxylate acetoacetate to acetone, were selected by searching protein database. To direct the carbon flux towards the biosynthesis of acetone from acetyl-CoA, the PHB synthase encoding gene *phaCE* (Han et al., 2007; Xie et al., 2011) and the phosphotransacetylase encoding gene *pta* (Juntarajumnong et al., 2007) were deleted in order to block the carbon flux towards biosynthesis of PHB and acetate (Fig. 1).

3.2. Synthesis of acetone from CO_2 in Synechocystis 6803 mutant (SM) strains

To achieve biosynthesis of acetone from CO_2, the designed synthetic pathway was constructed in *Synechocystis* 6803. As shown in Fig. 2, five *Synechocystis* 6803 mutant strains SM1, SM2, SM3, SM4, and SM5 were constructed (Fig. 2a). The gene insertions were verified by PCR and sequencing (Fig. 2b). The expression of *ctfAB* and *adc* was confirmed by RT-PCR (Fig. 2c) and the synthesis of acetone was analyzed (Fig. 2a).

Under normal conditions, no acetone was detected in all of the 5 strains. Previous studies showed that nitrogen and phosphate deprivation can contribute to the accumulation of carbon storages such as glycogen, fatty acid and PHB (Radakovits et al., 2010;

a

Strain	Insertion	Acetone production (mg l^{-1})
SM1	*phaE* up — Cm^r — *phaC* down	0
SM2	*phaE* up — Cm^r — *phaC* down; *pta* up — Km^r — *pta* down	0
SM3	PphaE, PrbcL; *phaE* up — *adc* — *ctfAB* — Cm^r — *phaC* down	3.0 ± 0.4
SM4	Pcpc, PrbcL; *phaE* up — *adc* — *ctfAB* — Cm^r — *phaC* down	5.0 ± 1.0
SM5	*pta* up — Km^r — *pta* down; Pcpc, PrbcL; *phaE* up — *adc* — *ctfAB* — Cm^r — *phaC* down	36.0 ± 1.5

b c

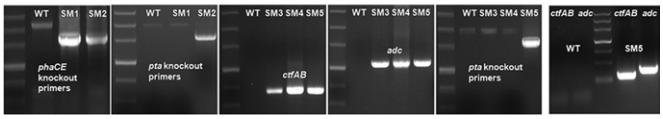

Fig. 2. The genetic modifications in SM strains for acetone synthesis. (a) Schematics of gene arrangements in all SM strains. SM1 and SM2 were control mutant strains for SM4 and SM5, respectively. In SM1, PHB synthase encoding gene *phaCE* was replaced with chloromycetin resistance cassette to block PHB synthesis from acetyl-CoA. In SM2, both *phaCE* and phosphotransacetylase encoding gene *pta* were replaced with chloromycetin and kanamycin resistance cassette, respectively, to block PHB and acetate syntheses from acetyl-CoA. SM3, SM4 and SM5 were acetone producing strains, in which *phaCE* gene was inactivated by inserting exogenous gene expression cassettes of *ctfAB* and *adc*, which encode enzymes for conversion of acetoacetyl-CoA to acetone. In SM3, the promoter for *adc* expression was promoter of *phaE*; while in SM4 strain, promoter PphaE was replaced with strong promoter Pcpc. The difference between SM4 and SM5 was that acetate synthetic pathway was blocked by replacing *pta* gene with kanamycin resistance cassette in SM5. (b) Whole cell PCR with specific primers demonstrated the integration of each gene into the chromosome of individual mutants of *Synechocystis* 6803. (C) RT-PCR confirmation of expression of *ctfAB* and *adc* gene in acetone production strain.

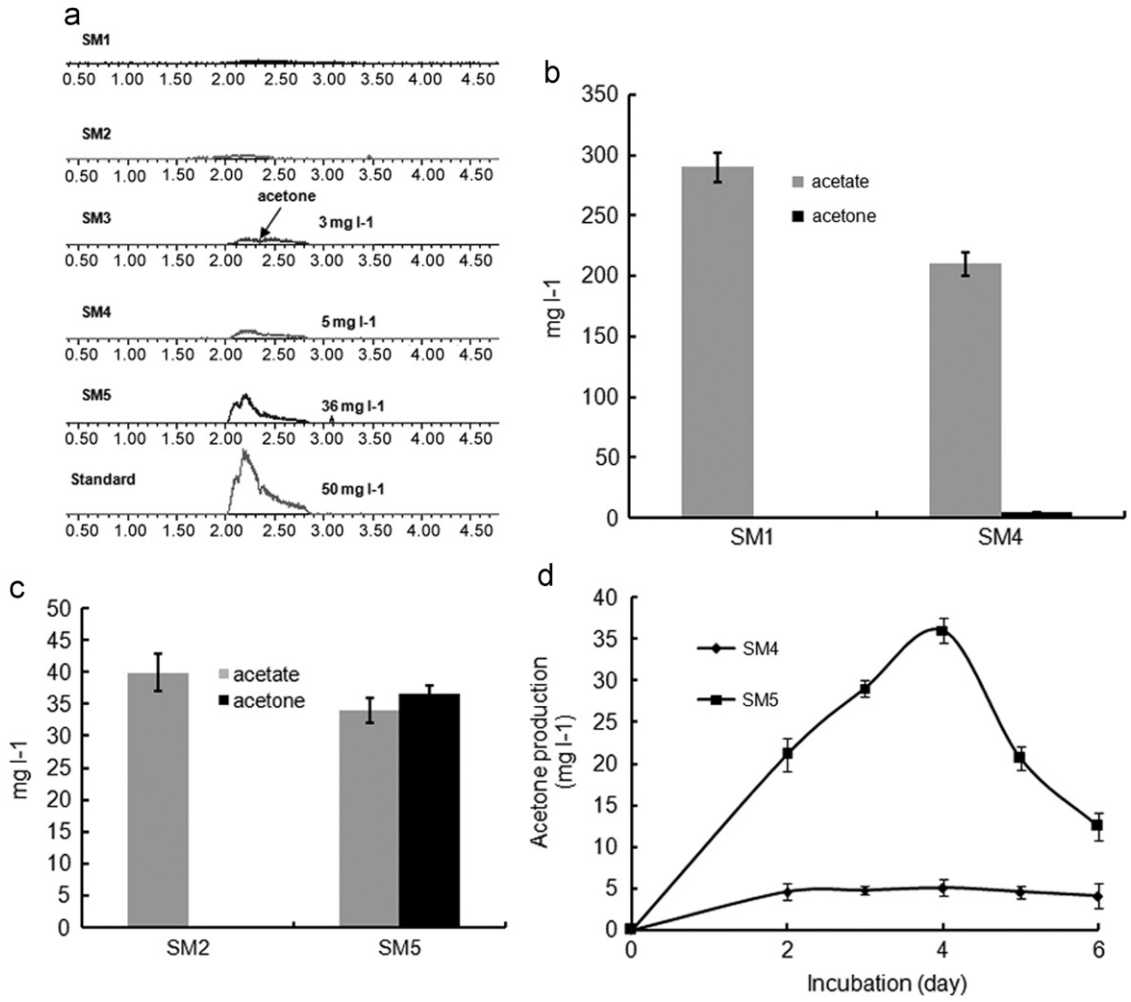

Fig. 3. Acetone and acetate production from SM strains under fermentative conditions in nitrogen and phosphorous deprived medium. (a) GC–MS analysis of acetone production in all SM cultures. Selected-ion monitoring (SIM) parameter: $m/z=58$ (acetone). Comparison of acetate and acetone accumulation in SM1 and SM4 cultures (b) and in SM2 and SM5 cultures (c) on the fourth day of incubation. (d) Time-course of acetone production from SM4 and SM5 cultures. Error bars indicate standard deviation of the data collected at different times from three independent experiments. For each experiment, two technical replicates were performed.

Schlebusch and Forchhammer, 2010; Ernst and Boger, 1985), while dark and anaerobic culture conditions can induce degradation of these carbon storages (such as glycogenolysis (Carrieri et al., 2010)) in *Synechocystis* 6803. Therefore, nitrogen and phosphate deprived medium and dark and anaerobic culture conditions were used as acetone producing conditions in this study. 3.0 ± 0.4 mg l^{-1} of acetone was produced in the culture of strain SM3, in which *C. acetobutylicum* DSM 1731 *ctfAB* gene and *adc* gene were constitutively expressed by replacing PHB synthase encoding gene *phaCE*, whereas no acetone was detectable in the control strain SM1 in which the *phaCE* was replaced by chloromycetin resistance cassette (Fig. 2a; Fig. 3a).

Although the biosynthesis of acetone from CO_2 was achieved in strain SM3, the titer of acetone produced was quite low. Further analysis of the genetic structure of strain SM3 showed that only the expression of *ctfAB* gene was driven by endogenous strong promoter PrbcL (Onizuka et al., 2003), whereas the promoter for *adc* gene was PphaE of the *phaE* gene, which is a weak promoter. Expression level of exogenous gene in cyanobacteria is a limiting factor for production of target metabolites (Lan and Liao, 2011). In order to increase the expression efficiency of the introduced genes in *Synechocystis* 6803, weak promoter PphaE for *adc* gene in SM3 strain was replaced with endogenous strong promoter Pcpc (Imashimizu et al., 2003) to generate strain SM4 (Fig. 2a). The titer of the acetone produced in SM4 culture increased to 5.0 ± 1.0 mg l^{-1} (Fig. 2a; Fig. 3a), which was 1.6-fold of the acetone produced by strain SM3 (3.0 ± 0.4 mg l^{-1}).

Although the acetone produced by strain SM4 was enhanced by replacing the weak promoter PphaE with strong promoter Pcpc, the acetone production from CO_2 was still lower than expected. Redirecting carbon flux is a useful approach to increase the production of target metabolite. We found that the control strain SM1 and acetone producing strain SM4 all produced unusually high concentration of acetate (290 ± 12 mg l^{-1} in control strain SM1 culture; 210 ± 10 mg l^{-1} in acetone producing strain SM4 culture) at day 4 under the conditions used for acetone production (Fig. 3b). This suggests that acetone production might be increased by blocking the acetate synthetic pathway in *Synechocystis* 6803. The acetate synthetic pathway of strain SM4 was then disrupted by replacing the *pta* gene (encoding phosphotransacetylase, the first enzyme of the acetate pathway) with kanamycin resistance cassette, resulting in strain SM5. Meanwhile, a control strain SM2 for acetone producing strain SM5 was derived from control strain SM1. The acetate synthetic pathway of strain SM2 was also disrupted by replacing the *pta* gene with kanamycin resistance cassette.

As shown in Fig. 3b, c, compared to acetone producing strain SM4 (210 ± 10 mg l^{-1}) and its control strain SM1(290 ± 12 mg l^{-1}), acetate production in acetone producing strain SM5 culture (34.0 ± 2.0 mg l^{-1}) and its control strain SM2 culture

Table 3
Primers used in this study.

Primer name	Sequence (5′-3′)	Used for plasmid
phaCupF	CAGATTCGGTCTTCCCCCAG	pSM1, pSM3, pSM4
phaCupR	ATCAGCACAGTTCATTATCAAC**GGATCC**GGTCAAAATCCACCTTACTA	pSM1, pSM3, pSM4
CmF	GTTGATAATGAACTGTGCTGAT	pSM1, pSM3, pSM4
CmR	ATCGAATTTCTGCCATTCATC	pSM1, pSM3, pSM4
phaEdownF	GATGAATGGCAGAAATTCGATTTGCTGGAATACATTAGGGCAA	pSM1, pSM3, pSM4
phaEdownR	ATATCGAAGCGGACAACGGCAT	pSM1, pSM3, pSM4
ptaupF	ATCGAGCCATGTTGCATCTA	pSM2
ptaupR	CTAAACTCACCGCTTCATGG	pSM2
KmF	CCATGAAGCGGTGAGTTTAGGATCAAGAGACAGGATGAGGATC	pSM2
KmR	GCCTTGTAAGTATTGTTACCGGAACGATTCCGAAGCCCAACC	pSM2
ptadownF	CCGGTAACAATACTTACAAGGC	pSM2
ptadownR	GCTGTGGTGGGACTGTTTCA	pSM2
PrbcF	ACAGT**GGATCC**ATGTTAAAGGATGAAGTAATTAAAC	pSM3, pSM4
PrbcR	CAAATCTAATTATTTTAGAGTTCATCTAGGTCAGTCCTCCATAAAC	pSM3, pSM4
ctfAB-adcF	ATGAACTCTAAAATAATTAGATTG	pSM3, pSM4
ctfAB-adcR	ACAGT**GGATCC**ATGTTAAAGGATGAAGTAATTAAAC	pSM3
PcpcF	GTTTAATTACTTCATCCTTTAACATTGAATTAATCTCCTACTTGACTTT	pSM4
PcpcR	GTCAGT**GGATCC**ACCTGTAGAGAAGAGTCCCTG	pSM4

Bam HI site is in bold.

Table 4
Total carbohydrate and insoluble glycogen contents in strain SM5 with highest acetone production and its control strain SM2 before and after fermentation. Standard deviation was calculated from the data collected at different times from three independent experiments. For each experiment, two technical replicates were performed.

Strains	Total carbohydrate ($mg\,l^{-1}$)		Carbon consumption ($mg\,l^{-1}$)	Acetone conversion rate (%)
	Before fermentation	After fermentation		
(a) Total carbohydrate				
SM2	1330.5 ± 4.1	1160.3 ± 3.2	170.2	0
SM5	1390.0 ± 6.3	1180.0 ± 8.1	210.0	17.1
Strains	Insoluble glycogen ($mg\,l^{-1}$)		Carbon consumption ($mg\,l^{-1}$)	Acetone conversion rate (%)
	Before fermentation	After fermentation		
(b) Insoluble glycogen				
SM2	1060.0 ± 7.1	1010.2 ± 3.0	49.8	0
SM5	1090 ± 5.0	1000.0 ± 5.2	90.0	40

($40.0 \pm 3.0\ mg\,l^{-1}$) dramatically decreased, demonstrating that disruption of the *pta* gene inhibited acetate production. As a result, disruption of the acetate synthetic pathway led to a significantly increased acetone production in strain SM5 as compared to strain SM4 (Fig. 3c). According to the time-course of acetone produced by strains SM4 and SM5 (Fig. 3d), the acetone produced in SM5 culture reached the highest ($36.0 \pm 1.5\ mg\,l^{-1}$) at day 4, which was more than 6-fold higher than that in SM4 culture ($5.0 \pm 1.0\ mg\,l^{-1}$). This indicated that disruption of acetate production directed the carbon flux from acetyl-CoA towards the synthesis of acetone.

To investigate how much glycogen was converted to acetone, total carbohydrate and insoluble glycogen concentrations in strain SM5 with highest acetone production and its control strain SM2, before and after fermentation, were determined (Table 4). It is calculated that about 40% of the consumed glycogen in strain SM5 was converted to acetone, and about 17.1% of the consumed total carbohydrate in strain SM5 was converted to acetone. Since the acetone produced only accounts for 2.5% of the total carbohydrate, there is great potential to further improve the acetone production from the storage carbohydrate by engineered *Synechocystis* 6803.

4. Discussion

This study demonstrated the feasibility of constructing a modularized synthetic pathway for acetone biosynthesis from CO_2. In general, this synthetic pathway consists of two main modules (Fig. 1): acetyl-CoA is synthesized from CO_2 in the first module, and then converted to acetone in the second module. In the designed pathway, CO_2 was first converted to glycogen and fatty acids via photosynthesis, which were then converted to acetyl-CoA through glycogenolysis or β-oxidation, respectively. The first step of PHB synthetic pathway was used to convert acetyl-CoA to acetoacetyl-CoA. Subsequently, enzymes from *C. acetobutylicum* DSM1731were used to convert acetoacetyl-CoA to acetone.

Recently, several fuels and chemicals, such as ethanol (Dexter and Fu, 2009), isobutanol (Atsumi et al., 2009), butanol (Lan and Liao, 2011), fatty alcohol (Tan et al., 2011, fatty acid (Liu et al., 2011), isoprene (Lindberg et al., 2010), and H_2 (Baebprasert et al., 2011), have been successfully produced by engineered cyanobacteria. In these studies, several exogenous synthetic pathways have been successfully constructed in cyanobacteria. However, very few attempts have been made to redirect native carbon flux for production of useful chemicals. In this study, the combination of introducing an exogenous synthetic pathway and redirecting the native carbon flux led to the production of acetone in cyanobacterium. The low yield of acetone relative to the storage carbohydrates suggests that future efforts should be put on improving the efficiency of utilizing the storage carbohydrates. Increasing the driving force towards acetone synthesis and increasing the activities of enzymes involved in acetone biosynthesis will contribute to improving the acetone production. Increasing the activities of enzymes involves two approaches. One is to improve the efficiency for the expression of genes *ctfAB* and *adc* in *Synechocystis* 6803 via optimization of codon usage, the other is to screen enzymes with higher activity from other microbes, or to evolve the present enzymes through direction evolution.

Acetate is one of the fermentative products from acetyl-CoA in cyanobacteria (Carrieri et al., 2010; McNeely et al., 2010). We found high concentration of acetate (about $0.2\ g\,l^{-1}$; Supplementary Fig. 2) can be produced in strains SM1 and SM4 (acetate synthetic pathway unblocked) grown in nitrogen and phosphate deprived medium under dark and anaerobic culture conditions. Since the production of acetate and acetone

competitively uses the acetyl-CoA (Fig. 1), blocking acetate pathway could theoretically divert more acetyl-CoA into acetone biosynthesis, which was demonstrated in our study. Furthermore, from the designed pathway, acetyl-CoA, acetoacetyl-CoA and β-hydroxybutyryl-CoA can be produced (Fig. 1). These acyl compounds are important units for synthesis of many diverse ketones such as diketone and polyketides, which are widely used in industry and medicine production (Grogan, 2005; Dalby and Paterson, 2010). Therefore, other valuable ketones might also be produced from CO_2 by designing a modularized synthetic pathway.

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Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2012.03.005.

References

Asada, Y., Miyake, M., Miyake, J., Kurane, R., Tokiwa, Y., 1999. Photosynthetic accumulation of poly-(hydroxybutyrate) by cyanobacteria—the metabolism and potential for CO_2 recycling. Int. J. Biol. Macromol. 25, 37–42.

Atsumi, S., Higashide, W., Liao, J.C., 2009. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat. Biotechnol. 27, 1177–1180.

Baebprasert, W., Jantaro, S., Khetkorn, W., Lindblad, P., Incharoensakdi, A., 2011. Increased H_2 production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. Metab. Eng. 13, 610–616.

Bao, G., Wang, R., Zhu, Y., Dong, H., Mao, S., Zhang, Y., Chen, Z., Li, Y., Ma, Y., 2011. Complete genome sequence of *Clostridium acetobutylicum* DSM 1731, a solvent producing strain with multi-replicon genome architecture. J. Bacteriol. 191, 5007–5008.

Carrieri, D., Momot, D., Brasg, I.A., Ananyev, G., Lenz, O., Bryant, D.A., Dismukes, C.G., 2010. Boosting autofermentation rates and production yields with sodium stress cycling: application to production of renewable fuels by cyanobacteria. Appl. Environ. Microbiol. 76, 6455–6462.

Cary, J.W., Petersen, D.J., Papoutsakis, E.T., Bennett, G.N., 1990. Cloning and expression of *Clostridium acetobutylicum* ATCC 824 acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase in *Escherichia coli*. Appl. Environ. Microbiol. 56, 1576–1583.

Castenholz, R.W., 1988. Culturing methods for cyanobacteria. Methods Enzymol. 167, 68–93.

Dalby, S.M., Paterson, I., 2010. Synthesis of polyketide natural products and analogs as promising anticancer agents. Curr. Opin. Drug. Discov. Devel. 13, 777–794.

Dexter, J., Fu, P., 2009. Metabolic engineering of cyanobacteria for ethanol production. Energy Environ. Sci. 2, 857–864.

Ernst, A., Boger, P., 1985. Glycogen accumulation and the induction of nitrogenase activity in the heterocyst-forming cyanobacterium *Anabaena variabilis*. J. Gen. Microbiol. 131, 3147–3153.

Grogan, G., 2005. Emergent mechanistic diversity of enzyme-catalysed β-diketone cleavage. Biochem. J. 388, 721–730.

Han, J., Lu, Q., Zhou, L., Zhou, J., Xiang, H., 2007. Molecular characterization of the $phaEC_{Hm}$ genes, required for biosynthesis of poly(3-Hydroxybutyrate) in the extremely halophilic archaeom *Haloarcula marismortui*. Appl. Environ. Microbiol. 73, 6058–6065.

Hegardt, F.G., 1999. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. Biochem. J. 338, 569–582.

Imashimizu, M., Fujiwara, S., Tanigawa, R., Tanaka, K., Hirokawa, T., Nakajima, Y., Higo, J., Tsuzuki, M., 2003. Thymine at—5 is crucial for *cpc* promoter activity of *Synechocystis* sp. strain PCC 6714. J. Bacteriol. 185, 6477–6480.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50, 484–524.

Juntarajumnong, W., Eaton-Rye, J.J., Incharoensakdi, A., 2007. Two-component signal transduction in *Synechocystis* sp. PCC 6803 under phosphate limitation: role of acetyl phosphate. J. Biochem. Mol. Biol. 40, 708–714.

Lan, E.I., Liao, J.C., 2011. Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. Metab. Eng. 13, 353–363.

Lindberg, P., Park, S., Melis, A., 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. Metab. Eng. 12, 70–79.

Liu, X., Sheng, J., Curtiss ^{3rd}, R., 2011. Fatty acid production in genetically modified cyanobacteria. Proc. Natl. Acad. Sci. USA 108, 6899–6904.

Lütke-Everslon, T., Bahl, H., 2011. Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr. Opin. Biotechnol. 22, 1–14.

McNeely, K., Xu, Y., Bennette, N., Bryant, D.A., Dismukes, G.C., 2010. Redirecting reductant flux into hydrogen production via metabolic engineering of fermentative carbon metabolism in a cyanobacterium. Appl. Environ. Microbiol. 76, 5032–5038.

Onizuka, T., Akiyama, H., Endo, S., Kanai, S., Hirano, M., Tanaka, S., Miyasaka, H., 2003. CO_2 response for expression of ribulose-1,5-bisphosphate carboxylase/oxygenase genes is inhibited by AT-rich decoy in the cyanobacterium. FEBS Lett. 542, 42–46.

Petersen, D.J., Bennett, G.N., 1990. Purification of acetoacetate decarboxylase from *Clostridium acetobutylicum* ATCC 824 and cloning of the acetoacetate decarboxylase gene in *Escherichia coli*. Appl. Environ. Microbiol. 56, 3491–3498.

Radakovits, R., Jinkerson, R.E., Darzins, A., Posewits, M.C., 2010. Genetic engineering of algae for enhanced biofuel production. Eukaryot. Cell. 9, 486–501.

Rahman, A., 2010. Catalytic hydrogenation of acetone to isopropanol: an environmentally benign approach. Bull. Chem. React. Eng. Catal. 5, 113–126.

Schlebusch, M., Forchhammer, K., 2010. Requirement of the nitrogen starvation-induced protein Sll0783 for polyhydroxybutyrate accumulation in *Synechocystis* sp. strain PCC 6803. Appl. Environ. Microbiol. 76, 6101–6107.

Sifniades, S., Levy, A.B., 2005. "Acetone" in Ullmann's Encyclopedia of Industrial Chemistry. Wiley-VCH, Weinheim.

Sillers, R., Chow, A., Tracy, B., Papoutsakis, E.T., 2008. Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab. Eng. 10, 321–332.

Stanier, R.Y., Cohen-Bazire, G., 1977. Phototrophic prokaryotes: the cyanobacteria. Annu. Rev. Microbiol. 31, 225–274.

Tan, X., Yao, L., Gao, Q., Wang, W., Qi, F., Lu, X., 2011. Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. Metab. Eng. 13, 169–176.

Wang, H.L., Postier, B.L., Burnap, R.L., 2002. Polymerase chain reaction-based mutageneses identify key transporters belonging to multigene families involved in Na^+ and pH homeostasis of *Synechocystis* sp. PCC 6803. Mol. Microbiol. 44, 1493–1506.

Xie, J., Zhou, J., Zhang, H., Li, Y., 2011. Increasing reductant NADPH content via metabolic engineering of PHB synthesis pathway in *Synechocystis* sp. PCC 6803. Chin. J. Biotechnol. 27, 998–1004.

Yoo, S.H., Spalding, M.H., Jane, J.L., 2002. Characterization of cyanobacterial glycogen isolated from the wild type and from a mutant lacking of branching enzyme. Carbonhydr. Res. 337, 2195–2203.

Zhang, J.X., Rao, X.P., Sun, L., Zhao, C.H., Qin, X.W., 2007. Putative chemical signals about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). Chem. Senses 32, 293–303.

Zhou, J., Zhou, J.X., Yang, H.M., Yan, C.S., Huang, F., 2008. Characterization of a sodium-regulated glutaminase from cyanobacterium *Synechocystis* sp. PCC 6803. Sci. Chin. Ser. C-Life Sci. 51, 1066–1075.

Zhu, L., Dong, H., Zhang, Y., Li, Y., 2011. Engineering the robustness of *Clostridium acetobutylicum* by introducing glutathione biosynthetic capability. Metab. Eng. 13, 426–434.