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

Jie Zhou, Haifeng Zhang, Yanping Zhang, Yin Li*, Yanhe Ma

Institute of Microbiology, Chinese Academy of Sciences, No.1 West Beichen Road, Chaoyang District, Beijing 100101, China

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 CO₂ 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 CO₂ as the sole carbon source, thus opens the gateway for biosynthesis of ketones from CO₂.

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 (Sillers 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 (Sillers 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-Eversløn 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 CO₂ 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 (CO₂) is a cheap and abundant carbon source. The photoautotrophic prokaryotes cyanobacteria can convert CO₂ 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 CO₂. Based on the endogenous metabolic network of cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis 6803), a novel acetone biosynthetic pathway from CO₂ was designed, and the feasibility of using CO₂ to produce acetone was demonstrated in this study.

2. Materials and methods

2.1. Strains and methods

The bacterial strains are listed in Table 1. Escherichia coli strain DH5α was used as a host for construction of vectors. Synechocystis...
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–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 OD730 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 OD730 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 N2 gas and sealing the tube with rubber plug.
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 Aminex 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 (Carriére et al., 2010) with modification. For total carbohydrate determination, 20 μl of culture (OD730 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 (Carriére et al., 2010) with modification. 20 μl of culture (OD730 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 CO2

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 CO2. 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 CO2 was designed based on the fatty-acid β-oxidation and glycogenolysis in Synechocystis 6803 (Fig. 1).

To achieve biosynthesis of acetone from CO2, 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; Cohen-Bazire, 1977), which can convert acetoacetate to acetone, and subsequently decarboxylate acetoacetate to acetone. In SM3, the phosphotransacetylase (PtA) and phosphotransacetylase (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 (Juntarajumphong 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 CO2 in Synechocystis 6803 mutant (SM) strains

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![Fig. 2. The genetic modifications in SM strains for acetone synthesis.](image-url)

(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 chloromycin resistance cassette to block PHB synthesis from acetyl-CoA. In SM2, both phaCE and phosphotransacetylase encoding gene pta were replaced with chloromycin 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 Ppcp. 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.
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 CO₂ 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 PhaE of the phaE gene, which is a weak promoter. Expression level of exogenous gene in cyanobacteria is a limiting factor for production of target metabolite (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 Ppcp (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 Ppcp, the acetone production from CO₂ 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
before and after fermentation, were determined (Table 4). It is known that acetone produced by strains SM4 and SM5 (Fig. 3d), the production of acetate and acetone significantly increased acetone production. Increasing the activities of enzymes involved in acetone biosynthesis will contribute to improving the production of acetone. This study demonstrated the feasibility of constructing a modularized synthetic pathway for acetone biosynthesis from CO₂. In general, this synthetic pathway consists of two main modules (Fig. 1): acetyl-CoA is synthesized from CO₂ in the first module, and then converted to acetone in the second module. In the designed pathway, CO₂ 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 acetate. 6803.}

Table 3
Primer name | Sequence (5'-3') | Used for plasmid
---|---|---
phuCupF | CAGATTCGCTTCCTCCACACG | pSM1, pSM3, pSM4
phuCupR | ATCCAGAGCCACGATCGATGAACG | pSM1, pSM3, pSM4
CmR | GGATCCCATTTCGGAGATGGTCG | pSM1, pSM3, pSM4
phEdownF | GATGACCTGCACTGAGGACGCA | pSM1, pSM3, pSM4
ptadownR | GCTGTGTCAGGAGCCAGTCAGG | pSM3, pSM4
ptaupR | ATATCGAAGCGGACAACGGCAT | pSM1, pSM3, pSM4
ptaupF | ATCGAGCCATGTTGCATCTA | pSM2
ptauupR | CTAAACTACCGGCGGTAG | pSM2
KmF | CCATGAAGCGGTGAGTTAGGATA | pSM2
KmR | GGATCCGGTCAAAATCCACCTTACTA | pSM1, pSM3, pSM4
PrbcR | ACAAATCTAATTATTTTAGAGTTCATCTAGGTCAGTCCTCCATAAAC | pSM3, pSM4
PrbcF | ACAGTGAGT GCATTGAAGGATGATAATTTAACAC | pSM3, pSM4
ctfAB-adcF | ATGACCTTAAATTTTACTCGTCATCACGGTCAGTCCCTCCATACAAC | pSM3, pSM4
ctfAB-adcR | ACAGTGAGT GATGGTAACAGGGATGATAATTTAACAC | pSM3, pSM4
phaEdownF | GATGAATGGCAGAAATTCGATTTGCTGGAATACATTAGGGCAA | pSM1, pSM3, pSM4
phaEdownR | ATATCGAAGCGGACAACGGCAT | pSM1, pSM3, pSM4
CmR | ATCGAATTTCTGCCATTCATC | pSM1, pSM3, pSM4
CmF | GTTGATAATGAACTGTGCTGAT | pSM1, pSM3, pSM4
ptaupR | ATATCGAAGCGGACAACGGCAT | pSM1, pSM3, pSM4
ptaupF | ATCGAGCCATGTTGCATCTA | pSM2

BamHI 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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Total carbohydrate (mg l⁻¹)</th>
<th>Acetone conversion rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before fermentation</td>
<td>After fermentation</td>
</tr>
<tr>
<td>(a) Total carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM2</td>
<td>1330.5 ± 4.1</td>
<td>1160.3 ± 3.2</td>
</tr>
<tr>
<td>SM5</td>
<td>1390.0 ± 6.3</td>
<td>1180.0 ± 8.1</td>
</tr>
<tr>
<td>Strains</td>
<td>Insoluble glycogen (mg l⁻¹)</td>
<td>Acetone conversion rate (%)</td>
</tr>
<tr>
<td>---------</td>
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<td>-----------------------------</td>
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<tr>
<td></td>
<td>Before fermentation</td>
<td>After fermentation</td>
</tr>
<tr>
<td>(b) Insoluble glycogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM2</td>
<td>1060.0 ± 7.1</td>
<td>1010.2 ± 3.0</td>
</tr>
<tr>
<td>SM5</td>
<td>1090 ± 5.0</td>
<td>1000.0 ± 5.2</td>
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(40.0 ± 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 concentration of acetate (about 0.2 g l⁻¹) at day 4, which was more than 6-fold higher than that in SM4 culture (5.0 ± 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 CO₂. In general, this synthetic pathway consists of two main modules (Fig. 1): acetyl-CoA is synthesized from CO₂ in the first module, and then converted to acetone in the second module. In the designed pathway, CO₂ 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 acetate. After that, the synthesis 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. 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 directed 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
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 CO₂ 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


