**Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria†**

Zhengxu Gao,‡ab Hui Zhao,‡a Zhimin Li,a Xiaoming Tana and Xuefeng Lu*a

Received 29th April 2012, Accepted 26th September 2012
DOI: 10.1039/c2ee22675h

The rapidly growing demand for energy and the environmental concerns about carbon dioxide emissions make the development of renewable biofuels more and more attractive. Tremendous academic and industrial efforts have been made to produce bioethanol, which is one major type of biofuel. The current production of bioethanol is limited for commercialization because of issues with food competition (from food-based biomass) or cost effectiveness (from lignocellulose-based biomass). In this report we applied a consolidated bioprocessing strategy to integrate photosynthetic biomass production and microbial conversion producing ethanol together into the photosynthetic bacterium, *Synechocystis* sp. PCC6803, which can directly convert carbon dioxide to ethanol in one single biological system. A *Synechocystis* sp. PCC6803 mutant strain with significantly higher ethanol-producing efficiency (5.50 g L⁻¹, 212 mg L⁻¹ day⁻¹) compared to previous research was constructed by genetically introducing pyruvate decarboxylase from *Zymomonas mobilis* and overexpressing endogenous alcohol dehydrogenase through homologous recombination at two different sites of the chromosome, and disrupting the biosynthetic pathway of poly-β-hydroxybutyrate. In total, nine alcohol dehydrogenases from different cyanobacterial strains were cloned and expressed in *E. coli* to test ethanol-producing efficiency. The effects of different culturing conditions including tap water, metal ions, and anoxic aeration on ethanol production were evaluated.

**Introduction**

With the rapidly increasing consumption of energy and continuously growing concerns about climate change,™ renewable biofuels as an alternative energy resource to the current fossil fuels have attracted more and more attention.™ The production of bioethanol, which is one major type of biofuel and can be blended with gasoline in various ratios for use in the unmodified engines, has recently gained tremendous attention.™ Currently,
bioethanol is mainly produced from starch or sugar-rich agricultural biomass as the feedstock, such as sugarcane in Brazil and corn in US. Excessive exploitation of food-based feedstock to produce bioethanol would lead to competition with the world food supply, increase of the food prices, and problems with food security.\textsuperscript{5,6} Another way of bioethanol production is to use inedible lignocellulose biomass as feedstock,\textsuperscript{7,8} the most abundant form of carbon on the earth. However, the cost of pretreatment and enzymatic hydrolysis\textsuperscript{9,10} and high energy consumption required\textsuperscript{11} in the process of producing lignocellulose-based bioethanol make it less economically competitive.

Thus, there is a significant need to develop innovative technical routes for the production of bioethanol that are not biomass-based but directly photosynthesis-derived. Photosynthetic bacteria, e.g. cyanobacteria, are potential candidates as they harbor the photosynthetic capability to convert carbon dioxide to organic carbon metabolites by utilizing solar energy through the Calvin cycle and can be genetically modified to assemble an ethanol-producing pathway to metabolically convert organic carbon metabolites to ethanol products (Fig. 1). A theoretical calculation shows that the productivity of ethanol in a photosynthetic organism can reach ca. 5280 gal per acre per year.\textsuperscript{12} In contrast, the annual yield of ethanol from corn is 321 gal per acre per year; from sugar cane, 727 gal per acre per year; from switchgrass, 330–810 gal per acre per year; and from corn stover, 290–580 gal per acre per year.\textsuperscript{13} Intrinsically cyanobacteria-based technology for bioethanol production is a consolidated bioprocessing strategy to integrate photosynthetic biomass production and microbial conversion producing ethanol together into a bacterium that can directly convert carbon dioxide to ethanol in one single biological system, and can avoid using food-based biomass that causes food supply issues or lignocellulose-based biomass with low degradation efficiency and high process cost.

In the past couple of years, cyanobacteria have been modified to produce different types of biofuels and display huge potential for biotechnology applications.\textsuperscript{15–17} For instance, cyanobacteria have been genetically engineered to produce ethylene (37 mg L\textsuperscript{−1}),\textsuperscript{18} ethanol (200–500 mg L\textsuperscript{−1})\textsuperscript{19,20} and isoprene (0.05 mg per g dry cell per day).\textsuperscript{21} More recently, Liao and his coworkers reported the production of isobutyraldehyde (1100 mg L\textsuperscript{−1}),\textsuperscript{22} isobutanol (450 mg L\textsuperscript{−1})\textsuperscript{23} and 1-butanol (14.5 mg L\textsuperscript{−1})\textsuperscript{24} in genetically engineered \textit{Synechococcus elongatus} PCC7942. Liu et al. described fatty acid production in genetically modified \textit{Synechocystis} sp. PCC6803 with the yield of 197 mg L\textsuperscript{−1}.\textsuperscript{25} Our group showed the production of fatty alcohols and alkanes in genetically engineered \textit{Synechocystis} sp. PCC6803 previously.\textsuperscript{25} Furthermore, researchers at LS9 Inc. identified an alkane biosynthetic pathway in cyanobacteria, which opens the door for microbial production of hydrocarbons, major components of current fossil fuels.\textsuperscript{26}

In 1999, Deng and Coleman\textsuperscript{27} reported the first case of ethanol production by genetic engineering in \textit{Synechococcus} sp. PCC7942, which expressed pyruvate decarboxylase and alcohol dehydrogenase II from \textit{Zymomonas mobilis} under control of the \textit{rbcL} promoter; the amount of ethanol accumulation reached approximately 5 mM (0.23 g L\textsuperscript{−1}). Ten years later, Dexter and Fu\textsuperscript{28} demonstrated that bioethanol can be produced in \textit{Synechocystis} sp. PCC6803 with the yield of ca. 10 mM (0.46 g L\textsuperscript{−1}). Algenol Biofuels Inc. recently constructed strains of \textit{Synechocystis} sp. PCC6803 with the integration of pyruvate decarboxylase from \textit{Z. mobilis} and endogenous alcohol dehydrogenase \textit{slr1192} under control of different promoters, with a resulting ethanol accumulation of 3.6 g L\textsuperscript{−1} for 38 days in the culture medium.\textsuperscript{29}

From previously published data, the production yield of ethanol in cyanobacteria is still far below the theoretical yield (Table 1).\textsuperscript{12} In an effort to increase the ethanol productivity in cyanobacteria, here an efficient ethanol-producing mutant strain of \textit{Synechocystis} sp. PCC6803 was constructed by genetically introducing exogenous pyruvate decarboxylase from \textit{Z. mobilis} and overexpressing endogenous alcohol dehydrogenase \textit{slr1192} from \textit{Synechocystis} sp. PCC6803 through homologous recombination at two different sites of the chromosome, and disrupting the biosynthetic pathway of poly-\textit{β}-hydroxybutyrate. The eventual ethanol concentration and productivity achieved were

---

**Fig. 1** Pyruvate relevant metabolic pathways in \textit{Synechocystis} sp. PCC6803. PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; cpg, ADP-glucose pyrophosphorylase; glg, glycogen synthase; pps, phosphoenolpyruvate synthase; AlaDH, alanine dehydrogenase; ldh, lactate dehydrogenase; phaA, PHA-specific β-ketothiolase; phaB, PHA-specific acetacetyl-CoA reductase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; pta, phosphotransacetylase; ackA, acetate kinase; me, malic enzyme; pyk, pyruvate kinase; acs, acetyl-coenzyme A synthetase; AldDH, acetaldehyde dehydrogenase.
This study used Synechocystis PCC6803 as the template. The sequences of primers used in this study were amplified with primers dc92F and dc92R using the genomic DNA of Synechocystis sp. PCC6803 as the template. The pdc and slr9394 expressed cassette was amplified with primers dc92F and dc92R using the plasmid pZG25 as the template. The pdc and adh II fragment was amplified and fused in-frame with 6× histidine tail by PCR with primers adhF and adhR using the genomic DNA of Z. mobilis in E. coli to test ethanol-producing efficiency.

Materials and methods

Chemicals and reagents

Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich (USA). Taq DNA polymerase and all restriction enzymes were purchased from Fermentas (Canada) or Takara (Japan). The kits used for molecular cloning were from Omega (USA) or Takara (Japan). Oligonucleotides were synthesized and DNA sequencing was performed by Sunnybio (Shanghai, China).

Strains and plasmids construction

Strains used and constructed in this study are shown in Table S1.† E. coli strain DH5α was used for molecular cloning and E. coli strain BL21 (DE3) was the host for protein expression. Strain Syn-LY225 was used for control strain. Strain Syn-XT43 was constructed by recombination of plasmid pXT43 into the slr0168 site of wild type Synechocystis sp. PCC6803. Strain Syn-ZG25 was constructed by recombination of plasmid pZG25 into the slr0168 site of wild type Synechocystis sp. PCC6803. Strain Syn-HZ23 was constructed by recombination of plasmid pHZ23 into the slr0934 site of Syn-ZG25.

Plasmids used and constructed in this study are shown in Table S1.† Plasmid pZG25 was constructed by insertion of pdc and slr1192 into pFQ20,25 under control of the Nac promoter. The pdc ORF was amplified and fused in-frame with 6× histidine tail by PCR with primers pdcF and pdcR using the genomic DNA of Z. mobilis as the template. The slr1192 fragment was amplified and fused in-frame with 6× histidine tail by PCR with primers 1192F and 1192R using the genomic DNA of Synechocystis sp. PCC6803 as the template. The sequences of primers used in this study are shown in Table S2.†

Plasmid pHZ23 was constructed by insertion of the fragment of pdc and slr1192 into pHZ22 which had the up and down homologous recombination arms of slr9394, encoding the key enzymes in the synthesis of PHB. The up and down homologous arms of slr9394 cloned into pMD18-T (Takara) were separately amplified with the primers 93F and 93R, 94F and 94R using the genomic DNA of Synechocystis sp. PCC6803 as the template. The pdc and slr1192 expressed cassette was amplified with primers dc92F and dc92R using the plasmid pZG25 as the template. The pdc and adh II fragment was amplified and fused in-frame with 6× histidine tail by PCR with primers adhF and adhR using the genomic DNA of Z. mobilis as the template.

Protein expression and purification

E. coli BL21 (DE3) (Takara) was transformed with plasmid pXT113A, pXT5, pZG35, pZG36, pZG37, pZG38, pZG39, pZG40, pZG41 and pZG42 were constructed by insertion of pdc and adh II into pFQ20,25 under control of the P_{nac} promoter. The adh II fragment was amplified and fused in-frame with 6× histidine tail by PCR with primers adhF and adhR using the genomic DNA of Z. mobilis as the template.

Plasmid pZG62 was constructed by insertion of pdc into pMSD1529,29 under control of the T7 promoter. Plasmid pXT113A, pZG35, pZG36, pZG37, pZG38, pZG39, pZG40, pZG41 and pZG42 were constructed by insertion of slr1192, Synpcc7942_0459, all0879, alr0895, alr0897, slr0942, slr1192, all2810 and all5334 into pET-28b (Novagen, Germany), respectively. The gene Synpcc7942_0459 was amplified with primers O459F and O459R using the genomic DNA of Synechococcus sp. PCC7942 as the template. The genes slr0942 and slr0990 were separately amplified with primers O942F and O942R, O990F and O990R using the genomic DNA of Synechocystis sp. PCC6803 as the template.

The transformants have been molecularly characterized and identified by the PCR experiments for proving DNA integration and segregation as shown in the ESI (Fig. S1†).

Table 1: The comparison of ethanol production in cyanobacteria from literature and this study

<table>
<thead>
<tr>
<th>Reference</th>
<th>Host strains</th>
<th>Ethanol production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deng and Coleman19</td>
<td>Synechococcus sp. PCC7942</td>
<td>0.23 g L⁻¹</td>
</tr>
<tr>
<td>Dexter and Fu20</td>
<td>Synechocystis sp. PCC6803</td>
<td>0.46 g L⁻¹</td>
</tr>
<tr>
<td>Duhring et al.27</td>
<td>Synechocystis sp. PCC6803</td>
<td>3.60 g L⁻¹ (95 mg L⁻¹ day⁻¹)</td>
</tr>
<tr>
<td>This study</td>
<td>Synechocystis sp. PCC6803</td>
<td>5.50 g L⁻¹ (212 mg L⁻¹ day⁻¹)</td>
</tr>
</tbody>
</table>
pelleted by centrifugation at 10 000g, 4 °C for 30 min. The supernatant was immediately added to the Ni-NTA resin (Novagen), pre-equilibrated with the binding buffer, which was gently agitated at 4 °C for 1 h. The resin was transferred into a 5 mL column that was washed sequentially with 5 column volumes of the binding buffer, five column volumes of the washing buffer containing 20 mM, 60 mM, and 100 mM imidazole to remove nonspecifically bound proteins, and then 25 mL of the washing buffer containing 250 mM imidazole to elute the target protein. The eluted proteins were examined by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were subsequently desalinated with 30 mM Tris–HCl (pH 7.9) and concentrated in 30% PEG20000 before quantitation using the Bradford method. 

**Ethanol production from genetically engineered *E. coli***

The transformed cells *E. coli* BL21 (DE3) were grown in 10 mL LB medium at 37 °C overnight. Then 50 μL seed culture was re-inoculated in 50 mL fresh LB medium without other carbon sources. The cultures were incubated at 37 °C with shaking at 80 rpm and induced by 0.4 mM IPTG. The OD_{600} and ethanol production was detected every two hours during cell growth.

**Transformation of *Synechocystis* sp. PCC6803**

Transformation of plasmids into *Synechocystis* sp. PCC6803 was carried out with the published method. The *Synechocystis* sp. PCC6803 cultures were grown to the exponential phase. The cells were collected by centrifugation, washed with fresh liquid BG11 twice, and resuspended to a density of about 1 × 10^{10} cells mL⁻¹. Then the cell suspension was mixed with plasmid DNA to a final concentration of 10 μg mL⁻¹. The cell and plasmid mixture was incubated for 5 h at 30 °C under luminous intensity of approximately 50 μE m⁻² s⁻¹ before spreading on nitrocellulose filters, which were rested on BG11 agar plates without antibiotic for 24 h. Finally, the filters were moved to selective BG11 agar plates containing antibiotics with an appropriate concentration. After 1–2 weeks incubation, a single colony was separated and grown in liquid BG11 medium for examination.

**Extracting crude enzyme of cells**

Cyanobacteria culture (300 mL) was centrifuged at OD_{730} of 2.0. Then the harvested cells were resuspended in 10 mL Tris buffer (30 mM Tris–HCl, pH 8.0) and lysed by sonication in ice–water mixture. The cellular extracts were centrifuged at 10 000g, 4 °C for 30 min. The supernatant was collected for western blot analysis or enzymatic assay.

**SDS-PAGE and western blot analysis**

The purified or extracted proteins were separated on 12% SDS-PAGE according to a standard procedure and blotted to PVDF membranes, sealed in 5% nonfat milk–TBST (0.05% Tween-20 in TBS) at 4 °C overnight. First, the membranes were incubated with the anti-6×His-tag monoclonal antibodies (from mouse, Tiangen, China) for 2 h and washed three times with TBST (15 min each). Second, the membranes were incubated with an alkaline phosphatase-linked secondary antibody (goat anti-mouse, Tiangen, China) for 1 h and washed three times with TBST (15 min each). Finally, the membranes were colored using BCIP/NBT in an Alkaline Phosphatase Color Development Kit (Amresco, USA) following the manufacturer’s instructions.

**Ethanol production assay**

To measure the ethanol production more accurately, a condenser and a recovery bottle were connected with the column bioreactor due to the volatility of ethanol (Fig. S4†). This method was not used in the previous study. One milliliter sample from the column bioreactor and 500 μL sample from the recovery bottle were collected every two days separately. Each sample of cell culture was centrifuged at 10 000g for 2 minutes, and the supernatant was used for ethanol assay by a SBA-40c biosensor analyzer (Shandong Academy of Sciences, China) equipped with the ethanol oxidase immobilized membrane, the recovered ethanol was also measured using this method. Finally, we calculated the total ethanol production by adding the ethanol in both the bioreactor and the recovery bottle.

**Various cultivation conditions of *Synechocystis* sp. PCC6803**

**Cultivation of cyanobacteria with a flask.** The *Synechocystis* sp. PCC6803 cells were inoculated in a 500 mL flask containing 300 mL BG11 medium with constant 50 μE m⁻² s⁻¹ white light at the initial OD_{730} of 0.05, and pumped with air or 5% CO₂–air (v/v) at 30 °C.

**Cultivation of cyanobacteria with a column photo-bioreactor.** The column bioreactor was a 580 mm × 30 mm glass column with a rubber plug. The cells were grown in a flask to the exponential phase and harvested by centrifugation. The harvested cells were re-suspended in fresh BG11 medium using tap water or distilled water, and transferred to the column photo-bioreactor at an appropriate OD_{730} with constant 100 μE m⁻² s⁻¹ white light at 30 °C. For normal cultivation, the cultures were sparged with 5% CO₂–air (normal conditions). For cultivation under anoxic conditions, 5% CO₂–N₂ was pumped into the cultures with (anoxic condition-2) or without (anoxic condition-1) the addition of 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the medium after 10 days culturing. The rate of gas addition is about 200 mL min⁻¹. And, for evaluating the ethanol yield of the third generation ethanol producer, a condensation device (Fig. S4†) was specially assembled to the outlet of the column photo-bioreactor in order to recover the evaporated ethanol.

**Alcohol dehydrogenase activity assay in vitro.** The measurement of alcohol dehydrogenase activity was carried out by monitoring the decrease in absorbance at 340 nm according to the previous study with utilization of different cofactors such as NAD(H) or NADP(H), and a Beckman-coulter DU-800 spectrophotometer was used in this progress. The reaction buffer contained 30 mM Tris (pH 8.0), 200 μM NADP(H), and different concentrations of acetaldehyde or ethanol.

**Pyruvate decarboxylase activity assay in vitro.** The measurement of pyruvate decarboxylase activity was carried out by
monitoring the decrease in absorbance at 340 nm according to the previous study by utilizing the cofactor of NADPH, and a Beckman-coulter DU-800 spectrophotometer was used in this progress. The reaction buffer contained 100 mM Tris (pH 7.5), 200 μM NADPH, 0.1 mM MgCl2, 0.1 mM thiamine pyrophosphate, 400 μM purified alcohol dehydrogenase (sr1192) and 10 mM pyruvate.

**Evaluation of ethanol-producing capability of different alcohol dehydrogenases in *E. coli*** The different alcohol dehydrogenases expressing plasmids (pXT113A, pZG35, pZG36, pZG37, pZG38, pZG39, pZG40, pZG41 and pZG42) were introduced into *E. coli* BL21 (DE3) together with pZG62 (harbouring pdc gene driven by P77 promoter), respectively. All resulting pdc and adh co-expressing strains, as well as pdc expressing strain of *E. coli*, were cultured in 250 mL flasks containing 100 mL LB medium at the initial OD570 of 0.4–0.5, and incubated on a rotary shaker with a shaking speed of 200 rpm at 37 °C. Expression of the target proteins was induced by adding 0.4 mM isopropylthiogalactoside (IPTG) before monitoring the ethanol production.

**Results and discussions**

**First generation ethanol producing strain**

Based on the previous research, the first generation ethanol producer Syn-XT43 was constructed by introducing the ethanol-producing pathway of *Z. mobilis* consisting of pyruvate decarboxylase and alcohol dehydrogenase II into *Synechocystis* sp. PCC6803, which was used as the host strain instead of *Synechococcus* sp. PCC7942 in Deng and Coleman’s work. Here, the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) promoter Pspe was used instead of the light-driven pshbAll promoter in Dexter and Fu’s report to control the expression of pyruvate decarboxylase and alcohol dehydrogenase in *Synechocystis* sp. PCC6803. Both the construction of plasmid pXT43 (Fig. 2A) and the transformation of *Synechocystis* sp. PCC6803 host cells with plasmid were carried out according to the published procedures. The genes encoding pyruvate decarboxylase (pdc), alcohol dehydrogenase II (adh II) and the promoter Pspe were integrated into the neutral site of the genome of *Synechocystis* sp. PCC6803 (Fig. S1A).

The pdc and adh II genes were successfully expressed in Syn-XT43, which was confirmed by western blot analysis (Fig. 2B). Syn-LY2 without the introduced pdc and adh II genes was used as a control strain. The time course for cell growth and ethanol production of Syn-XT43 under different conditions including sparged with air or 5% mixed CO2–air (v/v) were examined (Fig. 2C and D). Fig. 2C shows that 5% CO2 increased the rate of cell growth by 2-fold for both Syn-LY2 and Syn-XT43. Furthermore, under the same culturing conditions, Syn-XT43 strain grows to about half of the cell density level of the control strain Syn-LY2. Fu and Dexter showed that *Synechocystis* can tolerate ethanol up to 10.6 g L⁻¹ in the medium with negligible impacts on cell growth; hence, the ethanol accumulation in the culture of Syn-XT43 (ca. 0.4 g L⁻¹, Fig. 2D) should have no direct effect on the cell growth. One possible reason for the lower cell density of Syn-XT43 is that the carbon resource is utilized to produce ethanol rather than biomass in the case of the control strain without ethanol production. The other reason might be that the acetaldehyde accumulated in the medium caused by reverse catalysis of alcohol dehydrogenase II with conversion of ethanol to acetaldehyde is toxic to cells. Algenol biofuels Inc. reported that a certain amount of acetaldehyde could be detected in *Synechocystis* sp. PCC6803 mutant strain harboring the adh II gene from *Zymomonas mobilis*, which also has a lower growth rate compared to the control strain. The yield of ethanol production in Syn-XT43 can reach up to 0.4 g L⁻¹ in flasks with continuously sparged 5% CO2 (four-fold higher than with air, Fig. 2D) and is comparable with the previous results.

**Second generation ethanol producing strain**

Syn-ZG25 was generated as the second generation ethanol producer by transformation of *Synechocystis* sp. PCC6803 with plasmid pZG25 (Fig. 3A and S1B†), which substituted the adh II gene from *Z. mobilis* in pXT43 (Fig. 2A) with the endogenous alcohol dehydrogenase gene (sr1192) of *Synechocystis* sp. PCC6803. Western blot analysis showed that the two genes of pdc and sr1192 in Syn-ZG25 were expressed successfully (Fig. 3B). Again, the final cell density level of control strain Syn-LY2 is slightly higher than that of Syn-XT43 and Syn-ZG25 (Fig. 3C). Compared with that of Syn-XT43, the ethanol production capacity of Syn-ZG25 increased by 50% (Fig. 3D) to 0.6 g L⁻¹. The only difference between Syn-XT43 and Syn-ZG25 is that the endogenous alcohol dehydrogenase sr1192 was overexpressed in Syn-ZG25 instead of the exogenous adh II from *Z. mobilis* in Syn-XT43. Obviously, this alternation increased the
ethanol producing capability. As a result, Syn-ZG25 was used as the host strain for further genetic modification.

Third generation ethanol producing strain

To further increase the ethanol productivity by cyanobacteria, more genetic engineering was carried out. Previously, Wu et al. constructed a *Synechocystis* sp. PCC6803 mutant strain in which an erythromycin resistant cassette substituted the ADP-glucose pyrophosphorylase gene in the wild-type strain. Experiments showed that the poly-β-hydroxybutyrate (PHB) content accumulated up to 14% of the dry cell weight, much higher than that of the wild-type strain (ca. 3.5%) under photoautotrophic growth conditions. This result indicated that the carbon partitioning in cyanobacteria for PHB production was enhanced by blocking the glycogen biosynthetic pathway. Therefore, we hypothesize that the flux to pyruvate, the precursor of ethanol production, may be increased by knocking out related competitive pathways. PHB was synthesized by many cyanobacteria, including *Spirulina maxima*. *Synechocystis* sp. PCC6803, which diverts the carbon flux fixed by Calvin cycle from the ethanol-producing pathway (Fig. 1). So, another strain Syn-HZ23 was constructed by transformation of *Synechocystis* sp. PCC6803 wild type with plasmid pHZ23 (Fig. 4A), in which the *pdc* gene from *Z. mobilis* and endogenous *slr1192* gene were incorporated into the position of genes coding the enzymes polyhydroxyalkanoate-specific β-ketothiolase (*phaA* or *shr1993* encoding) and polyhydroxyalkanoate-specific acetooacetyl-CoA reductase (*phaB* or *shr1994* encoding) in the biosynthetic pathway of PHB (Fig. S1C†).

Unexpectedly, the result shows that there is no significant difference in the production of biomass and ethanol between Syn-HZ23 and Syn-ZG25 (Fig. 4B and C). Thus, only blocking the biosynthetic pathway of PHB did not increase ethanol production. Pyruvate is a central metabolite and its physiological concentration is controlled by many diversified metabolic pathways. The concentration of pyruvate in *Synechocystis* cannot be significantly enhanced by only disrupting the synthesis pathway of PHB.

When Syn-ZG25 was transformed with the plasmid pHZ23 to generate Syn-HZ24, in which two copies of exogenous *pdc* gene from *Z. mobilis* and endogenous *slr1192* gene were integrated into *Synechocystis* sp. PCC6803 at two different sites of the chromosome, including the neutral *slr0168* site and the position of genes *phaAB* (Fig. S1D†), the ethanol productivity reached 5.50 g L⁻¹ over 26 days (Fig. 4C). In comparison to the ethanol yield of Syn-ZG25 and Syn-HZ23 over 26 days, this is 4.9-fold higher than that of Syn-ZG25 (1.12 g L⁻¹) or 3.7-fold higher than that of Syn-HZ23 (1.49 g L⁻¹). We designated Syn-HZ24 as the third generation ethanol producer, which surpasses the previous highest concentration of 3.6 g L⁻¹ for 38 days claimed by Algenol Biofuels. In order to investigate why ethanol production improved greatly, the pyruvate decarboxylase and alcohol dehydrogenase activities in crude cell culture extract were measured spectrophotometrically. As shown in Fig. 4D, enzymatic activities of both pyruvate decarboxylase and alcohol dehydrogenase in Syn-HZ24 were approximately two-folds higher than those in Syn-ZG25. This result is consistent with the protein expression level as shown in Fig. 3B by western blot analysis, in which the protein expression level of Syn-ZG25 is higher than those in Syn-ZG25. This result is consistent with the protein expression level as shown in Fig. 3B by western blot analysis, in which the protein expression level of Syn-ZG25 is higher than those in Syn-ZG25. Therefore it indicates that higher enzymatic activities of pyruvate decarboxylase and alcohol dehydrogenase will increase the ethanol productivity. The
direction of our next study will be to explore pyruvate decarboxylase and alcohol dehydrogenase with higher catalytic efficiency or higher expression level.

Enzymatic characterization and evaluation of ethanol-producing capability of different alcohol dehydrogenases in *E. coli*

The only difference between strain Syn-XT43 and Syn-ZG25 is the alcohol dehydrogenase overexpressed. Specifically, exogenous alcohol dehydrogenase II (*adh II*) from *Z. mobilis* is used in Syn-XT43 and endogenous alcohol dehydrogenase *slr1192* is used in Syn-ZG25. Fig. 3D shows that the ethanol yield of Syn-ZG25 is 50% higher than that of Syn-XT43, so the activity of alcohol dehydrogenase plays an important role in the production of ethanol. Alcohol dehydrogenases exist in many organisms including cyanobacteria. Based on the annotation in CyanoBase (http://genome.kazusa.or.jp/cyanobase), nine different alcohol dehydrogenases from three different cyanobacteria strains were cloned and co-expressed with pyruvate decarboxylase from *Z. mobilis* in *E. coli* (Table S3†).

The *slr1192* gene in *Synechocystis* sp. PCC6803 has been proven to code a protein functioning as alcohol dehydrogenase by Vidal *et al.* The cell growth curves (Fig. 5A) showed that the cell cannot grow to high density when only pyruvate decarboxylase was expressed in *E. coli* due to the toxicity of acetaldehyde generated by pyruvate decarboxylase. When pyruvate decarboxylase and alcohol dehydrogenase were co-expressed in *E. coli* (Fig. S3†), the cell grows well and there is no significant difference in both the final cell density and doubling time between strains with different alcohol dehydrogenases (Fig. 5A and C and Table S4†). The ethanol production capability of these alcohol dehydrogenases in *E. coli* was examined (Fig. 5B and D). Fig. 5B shows that all the *E. coli* strains with alcohol dehydrogenase genes can produce ethanol (the one without alcohol dehydrogenase gene does not generate ethanol). This means that all the selected nine genes possess the function of alcohol dehydrogenase. The ethanol production ability of *slr1192* and *alr0895* is among the top of the selected genes. Furthermore, we examined the ethanol productivity of *E. coli* when *pdc* was co-expressed with different combinations of *adh II*, *slr1192* and *alr0895* genes (Fig. 5D). Unlike the results shown in Fig. 4C, more expressed copies of alcohol dehydrogenase in *E. coli* did not increase the ethanol productivity. The strain with only *pdc* and one copy of *slr1192* has the highest yield.

To further investigate why the ethanol productivity is different when using different alcohol dehydrogenases, four alcohol dehydrogenase enzymes were much higher than that of others (up to 74 000-fold difference) when acetaldehyde and NADPH were used as substrates. Although the activity of *adh II* was 94-fold higher than that of *slr1192* when acetaldehyde and NADH were used as substrates, the cellular concentration of NADP(H) is about 10-fold that of NAD(H) in *Synechocystis* sp. PCC6803. At the same time, all the tested alcohol dehydrogenases prefer acetaldehyde as a substrate rather than alcohol (40–270 fold higher activity towards acetaldehyde than alcohol, Table 2).

Interestingly, the ethanol productivity of Syn-ZG25 is only about 50% higher than that of Syn-XT43 (Fig. 3D), although the activity of *slr1192* in Syn-ZG25 is about 74 000-fold higher than that of *adh II* in Syn-XT43. This observation indicates that the pyruvate decarboxylation is probably the rate-limiting step of ethanol production. It may be promising to explore more pyruvate decarboxylases to test this hypothesis.

Effects of growing *Synechocystis* mutant in tap water or with addition of different metal ions on ethanol production

Alcohol dehydrogenase is a metal-dependent enzyme. Metal ions can either inhibit or increase the enzyme activity. The above experiments show that the activity of alcohol dehydrogenase will affect the ethanol production in *Synechocystis* sp. PCC6803. To determine the effect of metal ions on the ethanol production, the effect of various metal ions on the activity of purified enzyme *slr1192* was assayed first (Fig. 6A). The activity of *slr1192* without metal ion was arbitrarily defined as 100%. The enzyme activity was 85% inhibited by 10 μM Zn2+ or Co2+, and only 50% and 75% activity remained when 50 μM Mn2+ and Cu2+ were used as the metal factors, respectively. There was no significant effect on the enzyme activity when Mg2+ and Ca2+ were added to the assay solution.

The concentration of Zn2+ in BG11 medium, the typical culture medium for cyanobacteria, is 0.77 μM, which can still inhibit 50% activity of *slr1192*. The concentrations of Co2+, Mn2+, Cu2+, Mg2+ and Ca2+ in BG11 medium are 0.042 μM, 9.15 μM, 0.32 μM, 0.30 mM and 0.245 mM, respectively. Therefore, the effect of different metal ions on the production of ethanol was tested. Five-fold, 1-fold, 1/2-fold and 1/4-fold of commonly used concentrations of metal ions in the BG11 medium were added to the culture. And 1/4-fold concentration of metal ions without Zn2+ was also examined. Although the activity of the purified *slr1192* protein was significantly inhibited by Zn2+ at even very low concentration, there was no significant difference in growth and ethanol production of strain Syn-ZG25 with various concentrations of metal ions (Fig. 6B and C). Meanwhile we
tested the activity of crude slr1192 protein from the above cultures after 14 days of cultivation and the results suggested that the crude activity was close to each other even with different concentrations of metal ions in the cell culture (Fig. 6D).

Since there is no significant effect of various concentrations of different metal ions on the ethanol production in Synechocystis, we also tested the ethanol productivity of Synechocystis sp. PCC6803 cultured with tap water instead of distilled water used in the aforementioned experiments. Obviously, it is impossible to use distilled water for future industrialization of ethanol production in cyanobacteria. So it is important to know if the yield of ethanol production can be affected by using tap water. The results show that there is no difference for both cell growth (data not shown here) and the ethanol productivity when the Syn-ZG25 strain was cultivated in tap water and distilled water (Fig. 6E).

### Effects of growing Synechocystis mutant under anoxic conditions on ethanol production

Liao and Lan\(^{23}\) demonstrated that oxygen would inhibit the production of 1-butanol in genetically engineered Synechococcus elongatus PCC7942. To test the effect of oxygen on ethanol production in Synechocystis sp. PCC6803, the Syn-ZG25 strain was cultivated under normal and anoxic conditions respectively (Fig. 7). Unlike the results reported by Liao,\(^ {23}\) comparable ethanol accumulation was obtained under normal (5% CO\(_2\) and 95% air) and anoxic (5% CO\(_2\) and 95% N\(_2\)) conditions during the first 15 days of cultivation (Fig. 7B). Interestingly, when pumped with 5% CO\(_2\) and 95% N\(_2\), the culture can maintain the ethanol production constantly until 30 days, whereas the ethanol production drops sharply from 15 days.

### Table 2  The kinetic characterization of alcohol dehydrogenases with NADP(H) or NAD(H) as cofactor

<table>
<thead>
<tr>
<th>Enzyme Origin</th>
<th>Acetaldehyde + NADPH</th>
<th>Ethanol + NADP(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mM) (k_{cat}) (min(^{-1})) (k_{cat}/K_m) (min(^{-1}) mM(^{-1}))</td>
<td>(K_m) (mM) (k_{cat}) (min(^{-1})) (k_{cat}/K_m) (min(^{-1}) mM(^{-1}))</td>
</tr>
<tr>
<td>slr1192 Synechocystis sp. PCC6803</td>
<td>1.56 1271 814.7</td>
<td>19.4 268.2 13.81</td>
</tr>
<tr>
<td>adh II Zymomonas mobilis</td>
<td>380 4.22 0.011</td>
<td>146 0.037 2.53 (\times 10^{-4})</td>
</tr>
<tr>
<td>all0879 Anabaena sp. PCC7120</td>
<td>0.13 2.70 20.8</td>
<td>0.65 0.32 0.49</td>
</tr>
<tr>
<td>Synpc7942_0459 Synechococcus sp. PCC7942</td>
<td>0.64 1.44 2.25</td>
<td>128 1.08 8.4 (\times 10^{-3})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme Origin</th>
<th>Acetaldehyde + NADH</th>
<th>Ethanol + NAD(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mM) (k_{cat}) (min(^{-1})) (k_{cat}/K_m) (min(^{-1}) mM(^{-1}))</td>
<td>(K_m) (mM) (k_{cat}) (min(^{-1})) (k_{cat}/K_m) (min(^{-1}) mM(^{-1}))</td>
</tr>
<tr>
<td>slr1192 Synechocystis sp. PCC6803</td>
<td>9.56 67.7 7.08</td>
<td>730.6 89.3 0.122</td>
</tr>
<tr>
<td>adh II Zymomonas mobilis</td>
<td>2.73 1816 665.2</td>
<td>63.3 581.6 9.19</td>
</tr>
</tbody>
</table>

Fig. 6 Effects of growing Synechocystis mutant in tap water or with addition of different metal ions on ethanol production. (A) The effect of different metal ions on the activity of pure alcohol dehydrogenase encoded by the slr1192 gene of Synechocystis sp. PCC6803. (B) Growth curves and (C) ethanol production curves of the Syn-ZG25 strain cultivated under column photobioreactor conditions in medium containing various metal ions with different concentrations. (D) Analysis of crude enzymatic activity of the slr1192 enzyme from the culture of Syn-ZG25 strain cultivated in BG11 medium containing different ion concentrations. (E) Ethanol production curves of the Syn-ZG25 strain cultivated in tap water and distilled water.

Fig. 7 Effects of growing Synechocystis mutant under anoxic conditions on ethanol production. Syn-ZG25 strain was cultivated on column photobioreactors: (A) Growth curves. (B) Ethanol production curves. Normal conditions: continuously sparged with 5% CO\(_2\) and 95% air. Anoxic condition 1: continuously sparged with 5% CO\(_2\) and 95% N\(_2\). Anoxic condition 2: continuously sparged with 5% CO\(_2\) and 95% N\(_2\) with addition of 20 \(\mu\)M DCMU after 10 days’ culturing. The blue arrow symbol indicates the time points when the DCMU was added into the cultures.
days pumped with 5% CO₂ and 95% air. It is consistent with the cell densities of corresponding conditions (Fig. 7A). At the 10th day, 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which can inhibit photosystem II’s function was added to the culture medium under the above anoxic conditions. As a result, the cell growth and the ethanol production were stopped after the addition of DCMU and dramatically lower than that without DCMU (Fig. 7B).

Future study directions

A robust ethanol-producing cyanobacteria strain with significantly improved titer of ethanol production has to be genetically constructed for future industrialization of a solar-ethanol production system. Based on the analysis of a metabolic network of an ethanol-producing pathway in Synechocystis sp. PCC6803 (Fig. 1), it is reasonable to foresee that the efficiency of ethanol production can be further increased through genetic engineering. First, metabolic pathways competing for a carbon source could be knocked out or knocked down. These pathways include the biosynthetic pathway of glycogen, citric acid cycle and acetate metabolic pathway. Second, the enzymes involved in strengthening pyruvate production could be overexpressed. For example, overexpressing the Rubisco enzyme can enhance the carbon fixation efficiency and overexpression of malate enzyme and pyruvate kinase might increase the metabolic flux of pyruvate biosynthesis in the Synechocystis cell. And third, the determining factors for catalytic conversion of pyruvate to ethanol need to be identified, and pyruvate dehydrogenase with higher catalytic efficiency can be screened and applied to increase biosynthetic flux from the pyruvate to ethanol product. Aside from developing excellent ethanol-producing cyanobacteria strains, other challenges for developing competitively economical large-scale cultivation and separation systems specifically used for photosynthetic production of the evaporated ethanol product need to be overcome.

Conclusions

In this study, three generations of genetically engineered Synechocystis sp. PCC6803 strains, Syn-XT43, Syn-ZG25 and Syn-HZ24, with gradually improved ethanol productivity were constructed. A final ethanol concentration of 5.50 g L⁻¹ was achieved over 26 days of cultivation in Syn-HZ24 with genetic introduction of the exogenous pyruvate decarboxylase from Zymomonas mobilis and endogenous alcohol dehydrogenase slr1192 into two different sites of the chromosome of Synechocystis sp. PCC6803 and disruption of the biosynthetic pathway of poly-β-hydroxybutyrate. Although the enzymatic activity of the purified alcohol dehydrogenase slr1192 protein can be significantly inhibited by some metal ions (e.g., Zn²⁺ at 0.77 μM can inhibit the enzyme activity by 50%), there is no effect on ethanol production in cyanobacteria and tap water can be used for cultivation of ethanol-producing Synechocystis sp. PCC6803 mutant strains. Anoxic cultivation conditions can maintain the ethanol production for a much longer time without a decrease, which is important for saving the cost of large-scale industrialization.

Acknowledgements

The research was supported by the National High-Tech Research and Development Program of China (2012AA052103), Qicheng Carbon Energy Inc. and 100-Talent Program of the Chinese Academy of Sciences (Grant O91001110A). We would like to thank Dr Kenneth Reardon for valuable discussion.

References

21. P. Lindberg, S. Park and A. Melis, Metab. Eng., 2010, 12, 70–79.