

Cite this: *Energy Environ. Sci.*, 2012, **5**, 9857www.rsc.org/ees**PAPER**

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

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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,¹ renewable biofuels as an alternative energy resource to the current fossil fuels have attracted more and more attention.² 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.^{3,4} Currently,

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2ee22675h

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Broader context

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). Thus, there is a significant need to develop innovative technical routes for the production of bioethanols that are not biomass-based but direct 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. A theoretical calculation shows that the productivity of ethanol in a photosynthetic organism can reach ca. 5280 gal per acre per year. The previously reported production yield of ethanol in cyanobacteria is still far below the theoretical yield. Here we constructed a *Synechocystis* sp. PCC6803 mutant strain with significantly higher ethanol-producing efficiency (5.50 g L⁻¹, 212 mg L⁻¹ day⁻¹) and evaluated crucial culturing conditions, which displays great application potential for moving the production of solar-ethanol forward.

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.^{5,6} Another way of bioethanol production is to use inedible lignocellulose biomass as feedstock,^{7,8} the most abundant form of carbon on the earth. However, the cost of pretreatment and enzymatic hydrolysis^{9,10} and high energy consumption required¹¹ 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.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;¹³ from switchgrass, 330–810 gal per acre per year; and from corn stover, 290–580 gal per acre per year.¹⁴ Intrinsically cyanobacteria-based technology for bioethanol production is a consolidated bio-processing 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.^{15–17} For instance, cyanobacteria have been genetically engineered to produce ethylene (37 mg

L⁻¹),¹⁸ ethanol (200–500 mg L⁻¹)^{19,20} and isoprene (0.05 mg per g dry cell per day).²¹ More recently, Liao and his coworkers reported the production of isobutyraldehyde (1100 mg L⁻¹),²² isobutanol (450 mg L⁻¹)²² and 1-butanol (14.5 mg L⁻¹)²³ in genetically engineered *Synechococcus elongatus* PCC7942. Liu *et al.* described fatty acid production in genetically modified *Synechocystis* sp. PCC6803 with the yield of 197 mg L⁻¹.²⁴ Our group showed the production of fatty alcohols and alkanes in genetically engineered *Synechocystis* sp. PCC6803 previously.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.²⁶

In 1999, Deng and Coleman¹⁹ reported the first case of ethanol production by genetic engineering in *Synechococcus* sp. PCC7942, which expressed pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis* under control of the *rbcLS* promoter; the amount of ethanol accumulation reached approximately 5 mM (0.23 g L⁻¹). Ten years later, Dexter and Fu²⁰ demonstrated that bioethanol can be produced in *Synechocystis* sp. PCC6803 with the yield of *ca.* 10 mM (0.46 g L⁻¹). Algenol Biofuels Inc. recently constructed strains of *Synechocystis* sp. PCC6803 with the integration of pyruvate decarboxylase from *Z. mobilis* and endogenous alcohol dehydrogenase *slr1192* under control of different promoters, with a resulting ethanol accumulation of 3.6 g L⁻¹ for 38 days in the culture medium.²⁷

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

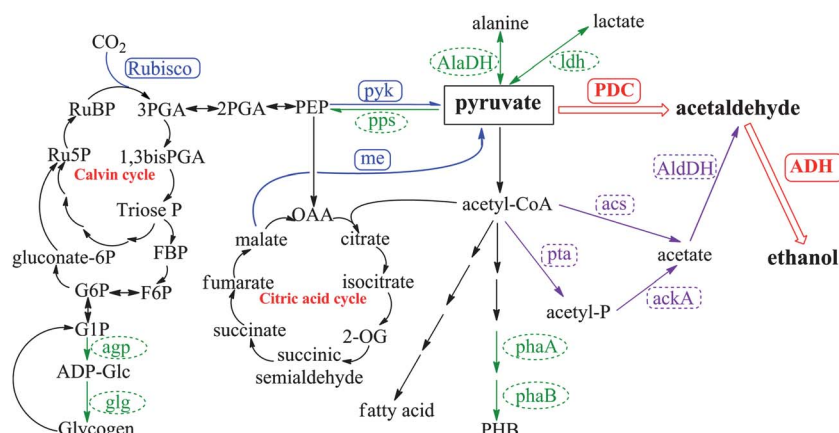


Fig. 1 Pyruvate relevant metabolic pathways in *Synechocystis* sp. PCC6803. PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; agp, ADP-glucose pyrophosphorylase; glg, glycogen synthase; pps, phosphoenolpyruvate synthase; AlaDH, alanine dehydrogenase; ldh, lactate dehydrogenase; phaA, PHA-specific β -ketothiolase; phaB, PHA-specific acetoacetyl-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.

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

Reference	Host strains	Ethanol production
Deng and Coleman ¹⁹	<i>Synechococcus</i> sp. PCC7942	0.23 g L ⁻¹
Dexter and Fu ²⁰	<i>Synechocystis</i> sp. PCC6803	0.46 g L ⁻¹
Duhring <i>et al.</i> ²⁷	<i>Synechocystis</i> sp. PCC6803	3.60 g L ⁻¹ (95 mg L ⁻¹ day ⁻¹)
This study	<i>Synechocystis</i> sp. PCC6803	5.50 g L ⁻¹ (212 mg L ⁻¹ day ⁻¹)

5.50 g L⁻¹ and 212 mg L⁻¹ day⁻¹ for 26 days of cultivation and remarkably exceeded previous titers reported (Table 1). At the same time, in order to reduce the culturing cost for scaling-up, the effects of different culturing conditions including tap water, metal ions and anoxic aeration on ethanol production were evaluated. Meanwhile, to select alcohol dehydrogenase with higher catalytic efficiency, nine alcohol dehydrogenases from different cyanobacteria strains were cloned and co-expressed with pyruvate decarboxylase from *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 DH5 α 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 *slr1993* and *slr1994* (*slr9394* for short) sites of wild type *Synechocystis* sp. PCC6803. Syn-HZ24 was constructed by recombination of plasmid pHZ23 into the *slr9394* 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 *P_{rbc}* promoter. The *pdc* ORF was amplified and fused in-frame with 6 \times 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 \times 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. Plasmid pXT43 was constructed by insertion of *pdc* and *adh II* into pFQ20,²⁵ under control of the *P_{rbc}* promoter. The *adh II* fragment was amplified and fused in-frame with 6 \times 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 pMSD15^{28,29} 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*, *sll0990*, *all2810* and *all5334* into pET-28b (Novagen, Germany), respectively. The gene Synpcc7942_0459 was amplified with primers 0459F and 0459R using the genomic DNA of *Synechococcus* sp. PCC7942 as the template. The genes *slr0942* and *sll0990* were separately amplified with primers 0942F and 0942R, 0990F and 0990R using the genomic DNA of *Synechocystis* sp. PCC6803 as the template. The genes *all0879*, *alr0895*, *alr0897*, *all2810* and *all5334* were amplified with primers 0879F and 0879R, 0895F and 0895R, 0897F and 0897R, 2810F and 2810R, 5334F and 5334R using the genomic DNA of *Anabaena* sp. PCC 7120 as the template, respectively.

Plasmid pZG63 was constructed by inserting *slr1192* and *alr0895* into the plasmid pET-28b. Plasmid pZG64 was constructed through inserting *adh II* and *slr1192* into the plasmid pET-28b. Plasmid pZG65 was constructed by inserting *alr0895* and *adh II* into the plasmid pET-28b. Plasmid pZG66 was constructed by inserting *alr0895*, *adh II* and *slr1192* into the plasmid pET-28b.

All 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†).

Protein expression and purification

E. coli BL21 (DE3) (Takara) was transformed with plasmid pXT113A, pXT5, pZG35 and pZG37, respectively. One liter of LB medium was inoculated with 10 mL of the overnight cultured transformed *E. coli* BL21 (DE3) from a single colony and grown at 37 °C. Cell cultures were induced by adding 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) at OD₆₀₀ = 0.6 and the cultures were incubated at 16 °C overnight with shaking at 180 rpm. Thereafter, cells were harvested by centrifugation, resuspended in binding buffer (20 mM Tris-HCl, 50 mM NaCl and 5 mM imidazole, pH 7.9) and lysed by sonication. The cell lysate was

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.³⁰

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 μ L 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 OD₆₀₀ 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.³¹ *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 1×10^9 cells mL⁻¹. Then the cell suspension was mixed with plasmid DNA to a final concentration of 10 μ g mL⁻¹. The cell and plasmid mixture was incubated for 5 h at 30 °C under luminous intensity of approximately 50 μ E 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 OD₇₃₀ 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 \times 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.^{19,20} One milliliter sample from the column bioreactor and 500 μ L 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;³² 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 μ E m⁻² s⁻¹ white light at the initial OD₇₃₀ 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 \times 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 OD₇₃₀ with constant 100 μ E 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 μ M 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 study³³ 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 μ M 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 study³⁴ 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 μ M NADPH, 0.1 mM MgCl₂, 0.1 mM thiamine pyrophosphate, 400 μ M purified alcohol dehydrogenase (*slr1192*) 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 *pdh* gene driven by P_{T7} promoter), respectively. All resulting *pdh* and *adh* co-expressing strains, as well as *pdh* expressing strain of *E. coli*, were cultured in 250 mL flasks containing 100 mL LB medium at the initial OD₇₃₀ 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.¹⁹ Here, the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) promoter *P_{rbc}* was used instead of the light-driven *psbAII* promoter in Dexter and Fu's report²⁰ 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.²⁵ The genes encoding pyruvate decarboxylase (*pdh*), alcohol dehydrogenase II (*adh II*) and the promoter *P_{rbc}* were integrated into the neutral *slr0168* site³¹ of the genome of *Synechocystis* sp. PCC6803 (Fig. S1A†).

The *pdh* and *adh II* genes were successfully expressed in Syn-XT43, which was confirmed by western blot analysis (Fig. 2B). Syn-LY2 without the introduced *pdh* 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 Dexter²⁰ 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

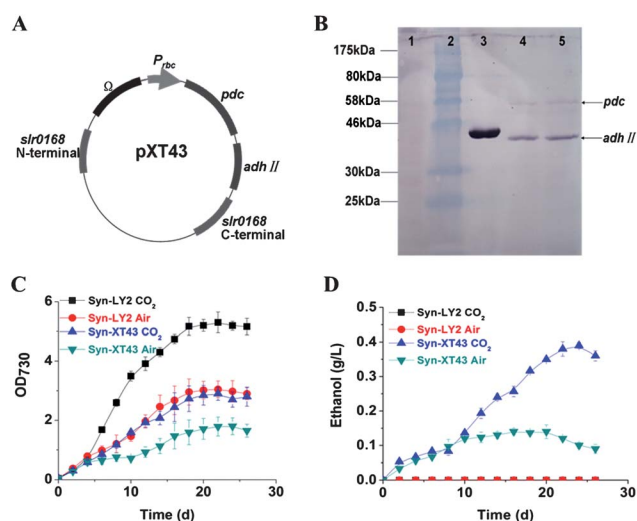


Fig. 2 First generation ethanol producer. (A) Plasmid map of pXT43 used to transform *Synechocystis* sp. PCC6803. (B) Western blot analysis of protein expression. Lane 1: the soluble proteins of wild type strain of *Synechocystis* sp. PCC6803 was used as negative control. Lane 2: protein marker. Lane 3: the purified glycerol-3-phosphate dehydrogenase from *Thermotoga maritima* MSB8 with a C-terminal 6 × histidine tag as positive control (molecular weight: 37 kDa). Lane 4 and 5: soluble proteins with histidine tag of Syn-XT43 (two paralleled samples). Syn-XT43 and Syn-LY2 (control) strains were cultured in flasks pumped with air (air) or 5% CO₂ (CO₂), and growth (C) and ethanol production (D) were measured every two days.

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 acetaldehyde³⁵ 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.²⁷ 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.^{19,20}

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 (*slr1192*) of *Synechocystis* sp. PCC6803. Western blot analysis showed that the two genes of *pdh* and *slr1192* 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 *slr1192* was overexpressed in Syn-ZG25 instead of the exogenous *adh II* from *Z. mobilis* in Syn-XT43. Obviously, this alternation increased the

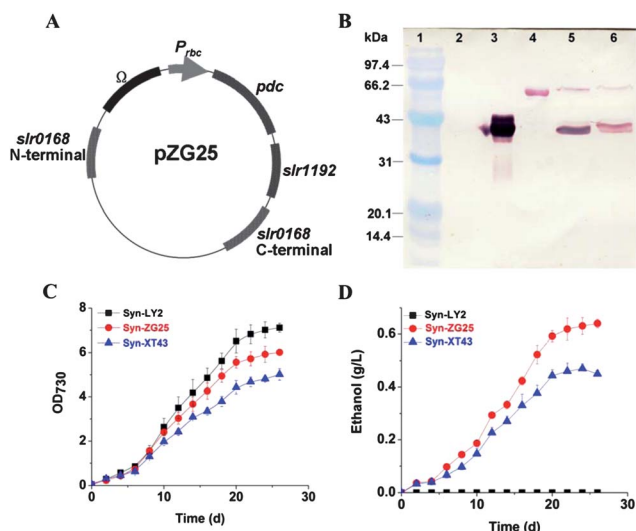


Fig. 3 Second generation ethanol producer. (A) Plasmid map of pZG25 used to transform *Synechocystis* sp. PCC6803. (B) Western blot analysis of protein expression. Lane 1: protein marker, Lane 2: the soluble proteins of wild type strain of *Synechocystis* sp. PCC6803 was used as negative control, Lane 3: purified *slr1192* with 6 \times histidine tag, Lane 4: purified PDC with 6 \times histidine tag, Lane 5: soluble proteins with histidine tag of Syn-HZ24, Lane 6: soluble proteins with histidine tag of Syn-ZG25. Syn-ZG25, Syn-XT43 and Syn-LY2 (control) strains were cultured in flasks, and growth (C) and ethanol production (D) were measured every two days.

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.*³⁶ 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*,³⁷ *Synechocystis* sp. PCC6803,³⁸ 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 *slr1993* encoding) and polyhydroxyalkanoate-specific acetoacetyl-CoA reductase (*phaB* or *slr1994* encoding) in the biosynthetic pathway of PHB (Fig. S1C \dagger).

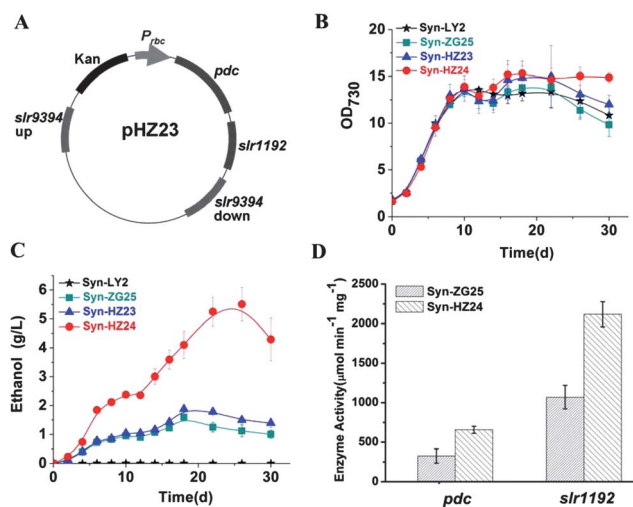


Fig. 4 Third generation ethanol producer. (A) Plasmid map of pHZ23 used to transform *Synechocystis* sp. PCC6803 and Syn-ZG25. Syn-ZG24, Syn-ZG23, Syn-ZG25 and Syn-LY2 (control) strains were cultured on column photobioreactors with a condensation device, and growth (B) and ethanol production (C) were measured every two days. (D) The enzymatic activities of pyruvate decarboxylase and alcohol dehydrogenase in Syn-ZG25 and Syn-HZ24. The activities were measured with crude cell culture extract at 12 days of cell growth.

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 \dagger), 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.²⁷ 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 about half that of Syn-HZ24. 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.*³³ 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 purified (Fig. S2†) and characterized (Table 2). The results showed that the activity of *slr1192* was 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. PCC6803.³⁹ 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 Zn^{2+} or Co^{2+} , and only 50% and 75% activity remained when 50 μM Mn^{2+} and Cu^{2+} were used as the metal factors, respectively. There was no significant effect on the enzyme activity when Mg^{2+} and Ca^{2+} were added to the assay solution.

The concentration of Zn^{2+} in BG11 medium, the typical culture medium for cyanobacteria, is 0.77 μM , which can still inhibit 50% activity of *slr1192*. The concentrations of Co^{2+} , Mn^{2+} , Cu^{2+} , Mg^{2+} and Ca^{2+} 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 Zn^{2+} was also examined. Although the activity of the purified *slr1192* protein was significantly inhibited by Zn^{2+} 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

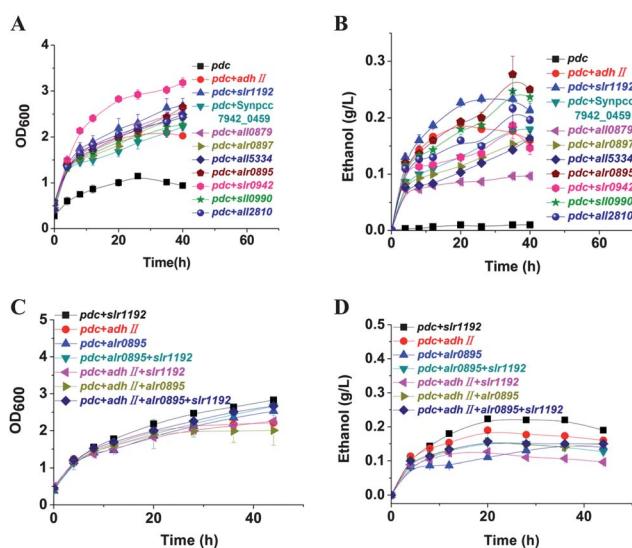


Fig. 5 Growth curves (A and C) and ethanol production curves (B and D) of *E. coli* mutants with heterologous overexpression of pyruvate decarboxylase from *Zymomonas mobilis* and different alcohol dehydrogenases.

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

Enzyme	Origin	Acetaldehyde + NADPH			Ethanol + NADP ⁺		
		K _m (mM)	k _{cat} (min ⁻¹)	k _{cat} /K _m (min ⁻¹ mM ⁻¹)	K _m (mM)	k _{cat} (min ⁻¹)	k _{cat} /K _m (min ⁻¹ mM ⁻¹)
<i>slr1192</i>	<i>Synechocystis</i> sp. PCC6803	1.56	1271	814.7	19.4	268.2	13.81
<i>adh II</i>	<i>Zymomonas mobilis</i>	380	4.22	0.011	146	0.037	2.53 × 10 ⁻⁴
<i>all0879</i>	<i>Anabaena</i> sp. PCC7120	0.13	2.70	20.8	0.65	0.32	0.49
Synpcc7942_0459	<i>Synechococcus</i> sp. PCC7942	0.64	1.44	2.25	128	1.08	8.4 × 10 ⁻³

Enzyme	Origin	Acetaldehyde + NADH			Ethanol + NAD ⁺		
		K _m (mM)	k _{cat} (min ⁻¹)	k _{cat} /K _m (min ⁻¹ mM ⁻¹)	K _m (mM)	k _{cat} (min ⁻¹)	k _{cat} /K _m (min ⁻¹ mM ⁻¹)
<i>slr1192</i>	<i>Synechocystis</i> sp. PCC6803	9.56	67.7	7.08	730.6	89.3	0.122
<i>adh II</i>	<i>Zymomonas mobilis</i>	2.73	1816	665.2	63.3	581.6	9.19

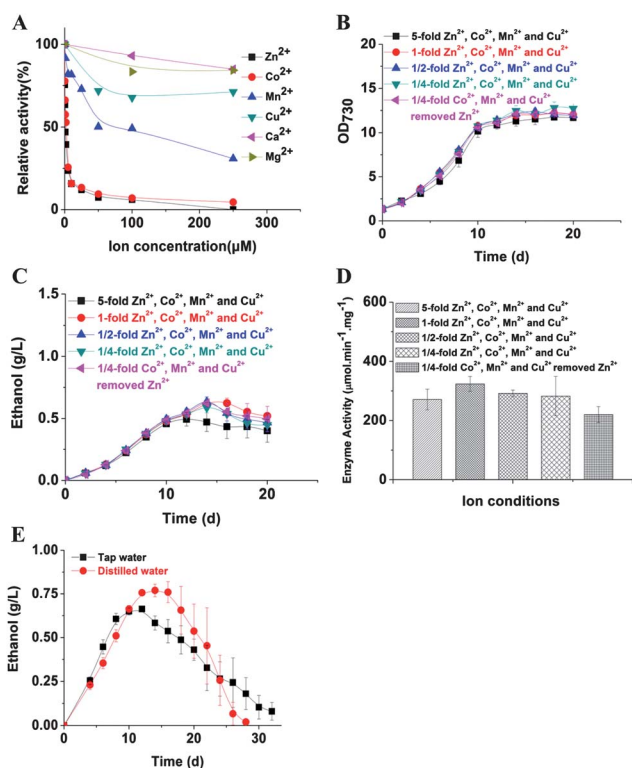


Fig. 6 Effects of growing *Synechocystis* mutant in tap water or with addition of different metal ions on ethanol production. (A) The effect of different metal ions on the activity of pure alcohol dehydrogenase encoded by the *slr1192* gene of *Synechocystis* sp. PCC6803. (B) Growth curves and (C) ethanol 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.

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 Lan²³ 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% CO₂ and 95% air) and anoxic (5% CO₂ and 95% N₂) conditions during the first 15 days of cultivation (Fig. 7B). Interestingly, when pumped with 5% CO₂ and 95% N₂, the culture can maintain the ethanol production constantly until 30 days, whereas the ethanol production drops sharply from 15

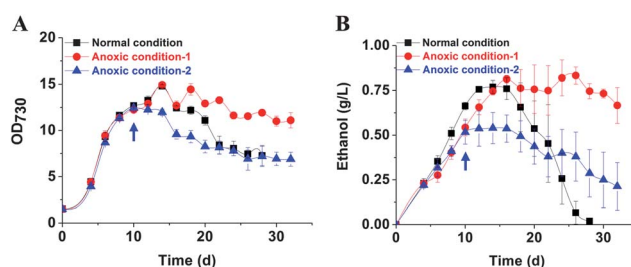


Fig. 7 Effects of growing *Synechocystis* mutant under anoxic conditions on ethanol production. Syn-ZG25 strain was cultured on column photobioreactors: (A) Growth curves. (B) Ethanol production curves. Normal conditions: continuously sparged with 5% CO₂ and 95% air. Anoxic condition 1: continuously sparged with 5% CO₂ and 95% N₂. Anoxic condition 2: continuously sparged with 5% CO₂ and 95% N₂ with addition of 20 µM 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 decarboxylase and alcohol 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.

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