Enhancing the light-driven production of D-lactate by engineering cyanobacterium using a combinational strategy

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It is increasingly attractive to engineer cyanobacteria for bulk production of chemicals from CO2. However, cofactor bias of cyanobacteria is different from bacteria that prefer NADH, which hampers cyanobacterial strain engineering. In this study, the key enzyme D-lactate dehydrogenase (LdhD) from Lactobacillus bulgaricus ATCC11842 was engineered to reverse its favored cofactor from NADH to NADPH. Then, the engineered enzyme was introduced into Synechococcus elongatus PCC7942 to construct an efficient light-driven system that produces D-lactic acid from CO2. Mutation of LdhD drove a fundamental shift in cofactor preference towards NADPH, and increased D-lactate productivity by over 3.6-fold. We further demonstrated that introduction of a lactic acid transporter and bubbling CO2-enriched air also enhanced D-lactate productivity. Using this combinational strategy, increased D-lactate concentration and productivity were achieved. The present strategy may also be used to engineer cyanobacteria for producing other useful chemicals.

Due to heavy use of fossil resources, atmospheric CO2 levels have increased approximately by 25% during the past 150 years1. The increased CO2 level has greenhouse effects and has altered the emissions of methane and nitrous oxide, which have a much higher global warming potential than CO22. Because of these concerns, biomass resources such as sugars are considered as the major substitutes for fossil resources3. However, using biomass as fossil substitutes leads to direct competition for resources between energy and food supplies. Therefore, it is necessary to develop biosynthetic processes which don’t need to use edible biomass as feedstocks. Direct conversion of CO2 to biofuels and carbohydrates using photoautotrophic organisms such as cyanobacteria can resolve the issues regarding both CO2 emission and resources shortage simultaneously4.

D-Lactate, an isomeric form of lactate, is used as basic feedstock of biodegradable polylactide, a well-known sustainable bioplastic material with lots of commercial applications4. D-Lactate is a chiral chemical, which is also used in the pharmaceutical industry and as a precursor for industrial chemicals such as cosmetics5. Moreover, its ester derivatives can be used to produce perfumes, coatings, adhesives, and printing ink, and have applications in the electronics industry6,7. Generally, D-lactate is produced by lactic acid bacteria from foods containing hexoses and pentoses or from sugar-containing raw materials8,9. To reduce utilization of food-related biomass resources, it is necessary to design cell factories that can directly use CO2 as the carbon source. More importantly, the cell factories consume CO2, thereby relieving material shortage and retarding climate change10,11. As lactate biosynthesis in cyanobacteria is attractive, several studies have investigated lactate production in cyanobacteria11–18. However, engineering of cyanobacteria for D-lactate production is limited by relatively low productivity, although efforts have been made to enhance its biosynthesis through both genetic engineering and optimization of culture conditions14,16,17. Low productivity may be attributed to cofactor imbalance (insufficient supply of NADH),
because cyanobacteria produce NADPH as the major carrier of reducing equivalents\(^{49}\). This hypothesis was confirmed by previous studies where soluble transhydrogenase (sth) was introduced into cyanobacteria during d-lactate synthesis\(^{11,16,17}\). Other studies using native NADPH-dependent enzymes were successful with high titers of target products\(^{20–23}\). However, considering that bacterial NADH-dependent oxidoreductases are more abundant than NADPH-dependent ones, it would be interesting to reverse coenzyme specificity using protein engineering\(^{24–29}\). Recently, Angermayr et al.\(^{30}\) reported that genetically engineered l-lactate dehydrogenase (coenzyme specificity changed from NADH to NADPH) resulted in increased l-lactate production in *Synechocystis* sp. PCC6803. Therefore, rational creation of efficient d-lactate dehydrogenase features a high preference for NADPH, and it is promising to utilize NADPH in cyanobacteria for d-lactate production.

In this study, a combinational strategy was used for construction of a cyanobacterial strain for d-lactate production. Firstly, the comparison of cyanobacterial genomes was performed. Then *Synechococcus elongatus* PCC7942 was selected as the host strain because it lacks l- and d-lactate dehydrogenases, ethanol dehydrogenase, and formate-lyase genes (GenBank ID, CP000100), and could grow to high density within enclosed bioreactors\(^{30}\). Secondly, the coenzyme specificity of the key d-lactate producing enzyme, LdhD, was switched from NADH to NADPH by protein engineering. Thirdly, codon usage of LdhD was optimized. Furthermore, as phototrophs, cyanobacteria generally lack transporters to move hydrophobic organic molecules across cell membranes\(^{31}\). Therefore, a lactate transporter was integrated into *S. elongatus* PCC7942. Finally, CO\(_2\) bubbling was used to enhance d-lactate production by the constructed *S. elongatus* strain.

**Results**

**Switching the coenzyme specificity of LdhD.** Bacteria are important gene sources for cyanobacterial engineering. For example, bacteria possess a large number of NADH-dependent oxidoreductases, which are crucial enzymes in metabolic pathways. Unfortunately, the concentration of NADPH in cyanobacteria is much higher than that of NADH\(^{15,17}\); this limits the application of NADPH-dependent oxidoreductases in cyanobacteria. A previous study has shown that it is possible to reverse the coenzyme specificity of xylitol dehydrogenase (XDH) from NADH to NADPH using site-directed mutagenesis\(^{25}\). To design NADPH-dependent enzymes for cyanobacterial engineering, several key enzymes in bacterial pathways were analyzed. As shown in Table S1, there are sequence gaps around the putative coenzyme binding regions of the first four enzymes, but Asp\(^{176}\)–Asn\(^{180}\) in LdhD and its corresponding regions in XDH and other enzymes are obviously homologous, indicating that aspartate, asparagine, and the hydrophobic residues are conserved. Therefore, these enzymes may be engineered to utilize NADPH as the preferred cofactor.

D-Lactate is a bio-based chemical that can be produced by fermentation. The key enzyme for d-lactate production in *Lactobacillus bulgaricus* ATCC11842\(^{32}\) has the same discriminatory sites between NADH and NADPH (Asp\(^{176}\), Ile\(^{177}\), Phe\(^{178}\), and Asn\(^{180}\)) as XDH (Table 1). Thus, this enzyme was selected to investigate the applications of LdhD in cyanobacterial engineering. To evaluate the effect of single substitution mutation on cofactor specificity, four single mutants, LdhD\(^\Delta\) (D176A), LdhD\(^\Delta\) (I177R), LdhD\(^\Delta\) (F178S), and LdhD\(^\Delta\) (N180R), were constructed and were expressed in recombinant *Escherichia coli* BL21(DE3) (Table S2). All four single mutations produced positive effects on NADPH kinetics, compared with wild-type LdhD, suggesting that these single substitutions might contribute independently to cofactor reversal in LdhD (Table 2). However, these single mutants still preferred NADH to NADPH.

To further increase the cofactor specificity of LdhD towards NADPH, a quadruple mutant LdhD\(^\Delta\) (D176A/I177R/F178S/N180R) was generated and was expressed in *E. coli* (Table S2). As shown in Table S2, the *k_{cat}/K_{m,NADH}* value of LdhD\(^\Delta\) dropped approximately 28.2-fold compared to LdhD; the *K_{m,*}* of the enzyme for NADH increased while the *k_{cat,*}* decreased. Interestingly, *k_{cat}/K_{m,NADPH}* was approximately 5.2-fold higher than *k_{cat}/K_{m,NADH}*, suggesting that there might be a synergistic effect in the quadruple mutant, leading to improved catalytic efficiency for NADPH. Although the *k_{cat}/K_{m,NADPH}* value of LdhD\(^\Delta\) did not reach the *k_{cat}/K_{m,NADH}* value of LdhD, the mutations drove a fundamental shift in cofactor preference toward NADPH. Furthermore, the kinetic constants for pyruvate were measured.

| Enzyme | Amino acid sequence |
|--------|----------------------|
| XDH\(^\Delta\) | VFGAGPVGLLAAAV AKTFGAKGVIVV |
| XDH\(^\Delta\) | VFGAGPVGLLAAAV AKTFGAKGVIVV |
| LdhD\(^\Delta\) | GVIGTHQGQYMFMQIMEGFGAKVIAV |
| LdhD\(^\Delta\) | GVIGTHQGQYMFMQIMEGFGAKVIAV |

Table 1. Partial amino acid sequences alignment of NAD(P)H-binding regions. *XDH*, xylitol dehydrogenase; LdhD, d-lactate dehydrogenase. *XDH\(^\Delta\)*, cofactor altered xylitol dehydrogenase; LdhD\(^\Delta\)*, cofactor altered d-lactate dehydrogenase. The coenzyme binding regions are underlined and in bold.
were cultured in the BG-11 medium 20 in the absence of IPTG. A small amount of d-lactate was detected transcription of all genes was detectable at this IPTG concentration. As controls, the five mutant strains

**ldhDc**

two genes, termed as

**Synechococcus**

control strain (Fig. 1). To enhance expression in

**S. elongates**

**S3**). Neutral site I of **PCC7942** chromosome was used to integrate the cassettes that contained

d-lactate production, **LldP**, a lactate transporter, was expressed under the same promoter (Fig. 2e; Table 2).

Table 2. Kinetic parameters of purified LdhDs for NADH, NADPH, and pyruvate. *Kinetic parameters for NADH or NADPH; **Kinetic parameters for pyruvate. Values represent mean ± S.D. (n = 3). *Under standard assay conditions as described under "Methods". ND, the kinetic parameters were not determined because the enzymes showed no activity towards the substrate.

(Table 2). $K_m$ for pyruvate with NADH was 1.1 ± 0.1 mM for wild type LdhD and 10.3 ± 0.5 mM for **LdhDnARSdR**; $K_m$ for NADPH with pyruvate was 2.25 ± 0.2 mM for wild type LdhD and 10.3 ± 0.5 mM for **LdhDnARSdR**. This result suggested that the $K_m$ for pyruvate had not changed significantly. Moreover, the catalytic activity of **LdhDnARSdR** did not decrease after incubation at 30°C for 24 h, suggesting that the enzyme is stable (data not shown).

**Construction of d-lactate-producing S. elongates strains.** The biosynthetic pathway of d-lactate uses pyruvate, a central metabolic intermediate that can be reduced to lactate. To engineer **S. elongatus** PCC7942 for d-lactate production, **LdhDnARSdR** was then introduced into the strain to facilitate direct utilization of the NADPH pool (Fig. 1). The original enzyme, LdhD, was used to construct a control strain (Fig. 1). To enhance expression in **Synchococcus**, codon-optimized versions of the above two genes, termed as **ldhDc** and **ldhDARSdR**, were also synthesized (Table S2). These four genes were all expressed under the control of the IPTG-inducible promoter, **Ptrc**, to further enhance d-lactate production, **LldP**, a lactate transporter, was expressed under the same promoter (Fig. 2e; Table S3). Neutral site I of **S. elongates** PCC7942 chromosome was used to integrate the cassettes that contained in the constructed plasmids pYLW11, pYLW12, pYLW13, pYLW14, and pYLW24. The resulting strains were named as YLW01, YLW02, YLW03, YLW04, and YLW05, respectively (Table S3). Integration of the inserted genes into the chromosome was verified with PCR and DNA sequencing (Fig. 2f).

**d-Lactate production from CO2 by S. elongates.** To determine the optimal IPTG concentration required for d-lactate production, all the engineered **Synchococcus** strains were grown in the presence of 0.1, 0.5, 1, and 2 mM of IPTG. d-Lactate yields were highest at 1 mM IPTG for strains YLW01, YLW02, YLW03, YLW04, and YLW05 (titers of d-lactate were 101 ± 5.3, 104 ± 5.7, 362 ± 17.1, 452 ± 18.7, and 798 ± 30.3 mg/L, respectively; Figure S1a). d-Lactate synthesis was reduced when the concentration of IPTG was above 1 mM. Therefore, 1 mM IPTG was chosen as the optimal concentration for all subsequent experiments. In addition, reverse transcription (RT)-PCR was performed to investigate the expression of **ldhD, ldhDc, ldhDnARSdR, ldhDARSdR**, and **LldP** (Figure S1b). The result revealed that the transcription of all genes was detectable at this IPTG concentration. As controls, the five mutant strains were cultured in the BG-11 medium20 in the absence of IPTG. A small amount of d-lactate was detected in all the strains, indicating slight leaky expression of the LdhDs (data not shown). It is notable that the difficulty in obtaining the transformants (YLW03, YLW04, and YLW05) harboring **LdhDnARSdR** and **LdhDARSdR** proved to be quite difficult, suggesting that leaky expression of **LdhDnARSdR** might have resulted in low growth rate and transformation efficiency.
YLW01, YLW02, YLW03, YLW04, and YLW05 were cultured for d-lactate production under constant light exposure (100 μE·s−1·m−2); the wild type strain *S. elongates* 7942 was used as the control. After induction for 10 days, d-lactate was not detected in the wild type strain (Fig. 3a). Upon introduction of NADPH-utilizing LdhDn ARSdR, d-lactate production increased by 3.6-fold and 4.2-fold in YLW03 (37.9 mg/L per day) and YLW04 (46.1 mg/L per day), compared with YLW01 and YLW02 (which harbor native LdhD), respectively (Fig. 3a). The enzymatic activities of the LdhDs in crude *S. elongatus* cell lysate were estimated to confirm the expression of the introduced lactate dehydrogenase genes. As shown in Table 3, although high LdhD activity for NADH was detected in both YLW01 and YLW02, d-lactate production remained low (Fig. 3). This may be attributed to the insufficient concentration of intracellular NADH in cyanobacteria. Conversely, although the activity of LdhDn ARSdR in YLW03 and YLW04 was significantly low, d-lactate production was enhanced in these strains, attributable to the abundant NADPH pool for LdhDn ARSdR in these strains. These results are consistent with a recent report that l-lactate productivity was enhanced by introducing a mutated l-lactate dehydrogenase that could co-utilize NADPH13. In addition, to determine the effect of codon optimization on d-lactate production, the relative protein expression profiles of LdhDs were measured. Higher protein levels were observed in YLW02 and YLW04 (~0.80 and ~0.38 × 10⁻¹ mg/mg in YLW02 and YLW04, respectively; compared with ~0.56 and ~0.13 × 10⁻¹ mg/mg in YLW01 and YLW03, respectively). However, d-lactate production increased only by 1.04- and 1.21-fold in YLW02 and YLW04, compared with YLW01 and YLW03, respectively (Table 2; Table 3). This indicated that codon optimization of *ldhD* and *ldhDnARSdR* increased d-lactate production only marginally. Overall, our results reinforce the importance of using cofactor-altered LdhDnARSdR for d-lactate production in *S. elongatus* PCC7942.

It is notable that the growth rate of strains containing LdhDnARSdR was different from that of other strains. There was no significant difference in cell growth among the wild type, YLW01, and YLW02 strains, which had not reached stationary phase at the tenth day and seemed to be able to continue (Fig. 3b). On the other hand, the cell growth rate of strains YLW03, YLW04, and YLW05 was impaired (maximum OD₇₅₀ values of 0.95, 0.99, and 1.07, respectively). A similar phenomenon was also observed in previous reports in which a soluble transhydrogenase or NADPH co-utilizing l-lactate dehydrogenase was introduced11,13 (Fig. 3b).

The hydrophobic cell membrane is the main barrier for the production and secretion of hydrophilic products such as lactate by genetically engineered cyanobacteria33. An l-lactate transporter, LldP, has been described as a nonspecific d-lactate transporter that efficiently transports d-lactate by using proton motive force in *E. coli* and cyanobacteria16,34. With the expression of the additional *lldP* gene, YLW05 secreted 829 mg/L of d-lactate in 10 days with an average productivity of 82.9 mg/L per day (Fig. 3a). Although the activity of LdhDnARSdR was lower in YLW05 (compared with that in YLW04), probably because of downregulation of the two genes upon co-expression (Table 3), d-lactate titer in YLW05 was approximately 1.8-fold higher than that in YLW04. This indicates that the transporter efficiently translocated lactate in YLW05, thereby improving d-lactate productivity.
Effect of aerating CO₂ on d-lactate production. In order to test if lactate production by the Synechococcus mutant strain could be enhanced, strain YLW05 was grown in a bubble column photobioreactor by continuously aerating CO₂-enriched air (5%, v/v; Fig. 4). As expected, aeration with CO₂ increased d-lactate production in YLW05 (~1.6-fold), reaching a titer of 1.31 g/L in 10 days with a maximum productivity of 221 mg/L per day. Moreover, d-lactate production did not cease after the ninth day, although productivity decreased slightly. As for cell growth, the YLW05 culture with CO₂-enriched air exhibited slight increase in cell density (Fig. 3; Fig. 4). To further simulate natural production conditions, S. elongatus mutant YLW05 was maintained at alternating dark and light periods (at an interval of 12 h) instead of constant light exposure. Under the conditions employed, cell growth was limited to the light period, and cell density decreased slightly in the dark period (Fig. 4b). This result suggests that d-lactate production in Synechococcus strains might be promoted by light and inhibited in the dark. This hypothesis is consistent with a previous report stating that cyanobacterial cultures accumulate polysaccharides when they are exposed to light, and they mobilize these intracellular reserve materials in the dark. Finally, strain YLW05 produced a mere 563 mg/L of d-lactate (maximum production rate is 75.1 mg/L per day) after 10 days (Fig. 4b). Nevertheless, these results (productivity of 56.3 mg/L per day) under the day-night cycle conditions suggest that Synechococcus strains may be applied to d-lactate production.

Discussion

Cofactor preference of enzymes is important for microbial organisms to produce metabolites. In this study, to directly use the abundant NADPH pool in cyanobacteria for d-lactate production, a cyanobacterial cell factory was designed by introducing an NADPH-utilizing enzyme, LdhDnARSdR. Significant changes in the kinetic constants of LdhDnARSdR suggested that the increased d-lactate productivity in YLW03 and YLW04 might stem from the increased activity with NADPH (Table 2). Multiple strategies were tested to optimize lactate production. Altering the cofactor preference of LdhD resulted in over 3.6-fold increase while introducing the transporter, LldP, resulted in approximately 1.8-fold increase, and bubbling CO₂ resulted in approximately 1.6-fold increase, in d-lactate production. These results show that altering cofactor specificity contributes mostly in enhancing the d-lactate production, which...
Figure 3. d-Lactate production by engineered cyanobacteria. (a) Cumulative production of d-lactate by the wild-type 7942 and mutants YLW01, YLW02, YLW03, YLW04, and YLW05 under constant light exposure. (b) Time courses for the growth of wild-type PCC7942 and mutants YLW01, YLW02, YLW03, YLW04, and YLW05. Values are the averages of biological replicates; error bars indicate the standard deviations (n = 3); if errors bar are not visible, they are smaller than the respective data point symbol. OD_{730}, optical density at 730 nm.

| Strain   | Activity* | Enzyme    |
|----------|-----------|-----------|
|          | NADH      | NADPH     |
| PCC7942  | ND± 0.02  | ND± 0.02  | —c       |
| YLW01    | 0.21± 0.02| 1.96± 0.08| LdhDnARSdR |
| YLW02    | 0.68± 0.03| 5.76± 0.25| LdhDnARSdR |
| YLW03    | 0.53± 0.03| 4.34± 0.21| LdhDnARSdR |

Table 3. Activities of LdhD and LdhDnARSdR of crude extracts of S. elongates. Values represent mean ± S.D. (n = 3). *Under standard assay conditions as described under "Methods": bND, the enzymes showed no activity towards the substrate. cNot exist.
indicates the feasibility of altering the cofactor specificity. In addition, altering the cofactor preference of an existing enzyme has the following possible advantages. First, the cofactor-altered enzyme could directly utilize NADPH, which might be more efficient for product synthesis. Second, compared to the use of a transhydrogenase, it simplifies the metabolic pathways using just one enzyme. Third, altering cofactor specificity might be faster than the process of identifying NADPH-dependent enzymes.

Use of the cofactor-altered LdhDn ARSdR resulted in impaired cell growth. This might be attributed to the high rate of d-lactate production, resulting in decreased NADPH level and activation of the oxidative pentose phosphate cycle. This cycle is the major route of carbon metabolism in cyanobacteria. To confirm this hypothesis, the intracellular levels of NADPH/NADH in both wild type S. elongatus PCC7942 and mutant strains were determined during the cultivation process. The ratio of NADPH/NADH slightly decreased in strain YLW04, compared with that in S. elongatus PCC7942 (Figure S2). This suggested that the NADPH/NADH ratio altered in the mutant strains, which might have affected the cell growth of strains YLW03, YLW04, and YLW05 (Fig. 3b), respectively. Another possible reason is that redirection of carbon flux from cellular biomass toward synthesis of d-lactate disrupts cell growth. Therefore, the intracellular pyruvate concentration in the wild type and mutant strains was measured. Pyruvate concentration was slightly higher in the wild type strain than that in YLW04 (Figure S3). To overcome this problem of attenuated cell growth, it is necessary to maintain the balance between growth and lactate production by precisely controlling the expression of mutated ldhD. This result is also consistent with the above IPTG concentration optimization.

As photoautotrophs, cyanobacteria lack many of the transporters found in E. coli or yeast. In two previous studies in which a transporter was introduced into a cyanobacterium for the secretion of lactate, significant improvement in lactate production was observed. Here, strain YLW05 expressing the ldhD ARSdR and lldP genes secreted relatively high levels of d-lactate into the medium, suggesting that
integration of the lactate transporter aids lactate secretion. Moreover, the substrate transport process was mediated by proton translocation, resulting in the accumulation of H⁺ — a necessary material for the synthesis of NADPH. Therefore, introduction of LdhP might contribute to the high yield of d-lactate in strain YLW05 by promoting NADPH production, which can then be used by LdhDnARSdR.

Biosynthesis of d-lactate from CO₂ has been achieved and is characterized in cyanobacteria, such as Synechocystis sp. PCC6803, through genetic engineering and recombinant DNA technologies. As shown in Table S4, both Hollinshead et al. and Varman et al. have reported the enhanced d-lactate production using Synechocystis sp. PCC6803 by adding acetate as an organic carbon source. Herein, the concentration and average productivity of d-lactate increased by approximately 90% using S. elongatus strain YLW05, compared with strain AV10, within 10 days. It should be noted that the production in this case was purely photosynthetic. Apparently, the titer values and average productivity of YLW05 were considerably higher than those of other reported strains (10 days), without the addition of an additional carbon source. Based on the above results, it is reasonable to conclude that our combinational strategy for the production of d-lactate might be more effective. Furthermore, to examine whether S. elongatus PCC7942 was superior, the actual partitioning of carbon between cellular biomass and d-lactate production was evaluated at the late log phase of growth (6 to 8 days for YLW05; 18 to 21 days for AV10). The results revealed that the values for strains YLW05 and AV10 were approximately 80.7 mg/L/OD₇₃₀ and 5.9 mg/L/OD₇₃₀ per day, respectively. This result suggests that strain YLW05 might be more efficient than strain AV10.

In summary, LdhD, a key enzyme in the d-lactate production pathway, was successfully engineered for cofactor reversal, and was used in engineered cyanobacteria for efficient production of d-lactate. Other methods, including introducing a lactate transporter and optimizing codon usage were also adopted in the construction. Under conditions of constant light exposure and bubbling CO₂-enriched air, the resulting strain (YLW05) achieved the highest lactate concentration and productivity reported for engineered cyanobacteria within 10 days (Table S4). This indicates that the systematic combination of different methods is promising in cyanobacteria engineering. Thus, the method of cyanobacterial engineering might have applications in the efficient biosynthesis of other chemicals as well.

Methods

Chemicals and reagents. The d-lactate standard, NADH, NADPH, and isopropyl-β-d-thiogalactoside (IPTG) were obtained from Sigma-Aldrich (St. Louis, MO). Oligonucleotides and gene synthesis were carried out by Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and reagents were of at least analytical grade and were available commercially.

Strains and growth conditions. Lactobacillus bulgaricus ATCC11842 and Escherichia coli K-12 strain MG1655 were used as the sources of ldhD (GenBank no. 103422405) and the l-lactate transporter gene (lldP) (GenBank no. 1790031), respectively. E. coli strain DH5α was used as the host for vector construction. S. elongatus PCC7942 (ATCC33912) was from ATCC (American Type Culture Collection). The S. elongatus strain was cultured in the BG-11 medium (20) unless otherwise stated, and cells were incubated statically, at 30°C and at an illumination intensity of 100 μE·s⁻¹·m⁻², as described elsewhere. Cell growth was monitored by measuring the optical density at 730 nm (OD₇₃₀).

For d-lactate production, S. elongatus cells in the exponential phase were diluted to 0.05 (OD₇₃₀) in 100 mL BG-11 medium containing 20 mg/L spectinomycin in 300 mL flasks. Cultures were induced with a suitable concentration of IPTG after growing to an OD₇₃₀ of 0.4–0.6. Daily, 1 mL of each sample was collected for analysis.

Site-directed mutagenesis and plasmid construction. All primers used for plasmid construction are listed in Table S5. The constructed plasmids are listed in Table S3. A neutral site I (NSI) in Escherichia coli ATCC11842. These two DNA fragments were then ligated by SOE-PCR using primers O4_F and O4_R (Table S5). The resulting gene was cloned into pMD18-T for sequencing. The primers, O4_F and O4_R, AB_F and AB_R, ARSdR_F and ARSdR_R, were then designed to clone ldhDnARSdR, ldhDc, and ldhDARSdR, respectively (Table S5).

Two primers, mcs12_F and mcs12_R, were designed for cloning MCS1 and MCS2 fragments (MCS12) from pETDuet-1. For plasmid construction, the PCR product of MCS12 was firstly cloned into the EcoRI/BamHI site of plasmid pAM2991 to introduce cloning sites AflIII, BglII, and XhoI, creating
plasmid pAM-MCS12. The PCR product of ldhD was then cloned into the EcoRI/XhoI site of plasmid pAM-MCS12, creating plasmid pYLW11. Similarly, ldhDc was cloned into the BamHI/AflII site of plasmid pAM-MCS12, resulting in plasmid pYLW12. Then, ldhD\textsuperscript{ARSdR} and ldhD\textsuperscript{ARSdR} were cloned into the AflII/XhoI site of plasmid pAM-MCS12, resulting in plasmid pYLW13 and pYLW14, respectively. The Shine-Dalgarno (SD) sequence of ldhD, ldhDc, ldhD\textsuperscript{ARSdR} and ldhD\textsuperscript{ARSdR} was obtained from pET28a (+).

**Transformation of Synechococcus.** Transformation of Synechococcus host cells was carried out by using a double homologous-recombination procedure as previously described	extsuperscript{16}. Integration of vectors into neutral site I was verified by PCR using gene-specific primers (Table S5) to demonstrate the presence of appropriate novel chromosome-transgene junctions and the absence of uninserted sites. The genetic stability of the mutant strains was evaluated by the serial subcultivation. Table S3 lists the strains that were constructed and used in this study. Briefly, mutant strains were obtained via integrating the aimed DNA fragments harbored by plasmids pYLW11, pYLW12, pYLW13, pYLW14, and pYLW24 to Synechococcus chromosome, respectively.

**Culture conditions of Synechococcus cells.** To investigate the effect of the initial concentration of IPTG on d-lactate production, IPTG concentration was adjusted in the culture media to 0.1, 0.5, 1, and 2 mM. To determine the effect of aeration on d-lactate production, a bubble column photobioreactor equipped with a glass column was used. S. elongatus strains were separately suspended in BG11 medium by aerating CO\textsubscript{2}-enriched air under constant light exposure as described in a previous study	extsuperscript{10}. To study the effect of the day-night cycle on d-lactate production, S. elongatus strain was grown under the aeration condition with day and night periods that alternated every 12 h.

**Enzyme assays.** S. elongatus cells were harvested via centrifugation (6,000 × g, 5 min) 72 h after induction, washed twice with 50 mM Tris-HCl buffer (pH 7.0), and resuspended in the same buffer containing 2 mM dithiothreitol. Crude extracts were prepared via bead beating	extsuperscript{22}. Total protein concentration was determined according to the method of Bradford	extsuperscript{10} using bovine serum albumin as the standard. The standard reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.0), 0.2 mM NAD(P)H, and 0.05 mM pyruvate. One unit of protein activity was calculated as micromoles of pyruvate consumed per minute per milligram of the total protein at 30°C.

To characterize the kinetic constants of LdhD\textsuperscript{ARSdR} after the reversal of coenzyme specificity, the enzyme was expressed in E. coli BL21(DE3), with the wild-type LdhD as a control. The purification of the two enzymes were performed using the method of Wang et al.	extsuperscript{40} The reduction activity of purified LdhD and LdhD\textsuperscript{ARSdR} on pyruvate were assayed at 30°C. Oxidation of NADPH/NADH (ε\textsubscript{340} = 6220 M\textsuperscript{−1} cm\textsuperscript{−1}) was monitored by the decrease in absorbance at 340 nm	extsuperscript{41}. One unit of protein activity was defined as the amount enzyme that catalyzed the consumption of 1 μmol pyruvate per minute. The reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.0), 0.2 mM NAD(P)H, and different concentrations of substrate. The Michaelis-Menten equation was used for determination of the kinetic parameters. To determine the stability of LdhD\textsuperscript{ARSdR}, the purified LdhD\textsuperscript{ARSdR} were incubated at 30°C for 24 h.

**Reverse transcription PCR (RT-PCR).** RT-PCR was performed as previously described	extsuperscript{21}. Total RNA of the various cyanobacteria strains was extracted using an RNAprep Pure Cell/Bacteria Kit (TIANGEN Biotech Co., Ltd, Beijing, China). RNA was quantified using a NanoVue (GE Healthcare Bio-Sciences AB, Sweden). Residual DNA in RNA preparations was treated with RNase-free DNase I (Thermo Scientific, Shanghai, China). Reverse transcription using random primers was performed with SuperScript\textsuperscript{TM} III Reverse Transcriptase (Invitrogen, Shanghai, China). Reverse transcription products were amplified using the specific primers listed in Table S5. The expression of rnpB was used as a positive control, and the wild-type S. elongates PCC7942 was used as the negative control. PCR products were analyzed with electrophoresis on 2% (w/v) agarose gels.

**Quantification of d-lactate.** For d-lactate measurement, 1 mL of the sample was centrifuged (13,000 × g, 2 min), cell debris was removed, and the supernatant was boiled for 10 min and centrifuged at 13,000 × g for 5 min. The final supernatant was used to determine d-lactate content. Thereafter, d-lactate assay kit (Megazyme) was used to determine d-lactate concentration according to the manufacturer's instructions	extsuperscript{16}. As a control, l-lactate was also assayed in the cell-free supernatant of the wild type strain. Assays were performed in triplicate, and standard deviations were determined.

**References**

1. Atsumi, S., Higashide, W. & Liao, J. C. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* 27, 1177–1180 (2009).
2. Groenigen, K. J., Osenberg, C. W. & Hungate, B. A. Increased soil emissions of potent greenhouse gases under increased atmospheric CO\textsubscript{2}. *Nature* 475, 214–216 (2011).
3. Lan, E. I. & Liao, J. C. ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 109, 6018–6023 (2011).
29. Opgenorth, P. H., Korman, T. P. & Bowie, J. U. A synthetic biochemistry molecular purge valve module that maintains redox

22. Oliver, J. W., Machado, I. M., Yoneda, H. & Atsumi, S. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol.

25. Watanabe, S., Kodaki, T. & Makino, K. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of

24. Katzberg, M., Skorupa-Parachin, N., Gorwa-Grauslund, M. F. & Bertau, M. Engineering cofactor preference of ketone reducing

32. Zhou, J., Zhang, H., Meng, H., Zhang, Y. & Li, Yin. Production of optically pure d-lactate from CO₂ by blocking the PHB and

31. Ducat, D. C., Way, J. C. & Silver, P. A. Engineering cyanobacteria to generate high-value products.

26. Hou, J., Shen, Y., Li, X. P. & Bao, X. M. Effect of the reversal of coenzyme specificity by expression of mutated

33. Wang, B.

36. Olavarria, K., Valdes, D. & Cabrera, R. The cofactor preference of glucose-6-phosphate dehydrogenase from

35. Smith, A. J. Modes of cyanobacterial carbon metabolism.

11. Angermayr, S. A., Paszota, M. & Hellingwerf, K. J. Engineering a cyanobacterial cell factory for production of lactic acid.

14. Hollinshead, W. D.

15. Joseph, A.

40. Wang, X.

41. Zheng, Z. J.

5. Abdel-Rahman, M. A., Tashiro, Y. & Sonomoto, K. Lactate production from lignocellulose-derived sugars using lactate bacteria: modeling the physiological production of reduced cofactors. J. Bacteriol. 179, 61–68 (1989).

6. Okano, K. et al. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. J. Biotechnol. 100–102 (2014).

7. Datta, R. & Michael, Henry. Lactic acid: recent advances in products, processes and technologies—a review. Trends Biotechnol. 1249–1254 (2013).

8. Okano, K.

9. Yun, J. S., Wee, Y. J., Kim, J. N. & Ryu, H. W. Fermentative production of α-lactate from amylosis-treated rice and wheat brans hydrolyzate by a novel lactate bacterium, Lactobacillus sp. Biotechnol. Lett. 26, 1613–1616 (2004).

10. Tan, X. M. et al. Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. Metab. Eng. 13, 169–176 (2011).

11. Angermayr, S. A., Paszota, M. & Hellingwerf, K. J. Engineering a cyanobacterial cell factory for production of lactic acid. Appl. Environ. Microbiol. 78, 7098–7106 (2012).

12. Angermayr, S. A. & Hellingwerf, K. J. On the use of metabolic control analysis in the optimization of cyanobacterial biosol lar cell factories. Phys. Chem. B 117, 11619–11715 (2013).

13. Angermayr, S. A. et al. Exploring metabolic engineering design principles for the photosynthetic production of lactic acid by Synechocystis sp. PCC6803. Biotechnol. Biofuels 7, 99 (2014).

14. Hollinshead, W. D. et al. Boosting n-lactate production in engineered cyanobacteria using sterilized anaerobic digestion effluents. Bioreour. Technol. 169, 462–467 (2014).

15. Joseph, A. et al. Utilization of lactate bacterial genes in Synechocystis sp. PCC6803 in the production of lactate. Bioosci. Biotechnol. Biochem. 77, 966–970 (2013).

16. Niederholtmeyer, H. et al. Engineering cyanobacteria to synthesize and export hydrophilic products. Appl. Environ. Microbiol. 76, 3462–3466 (2010).

17. Varman, A. M., Yu, Y., You, L. & Tang, Y. J. Photoautotrophic production of n-lactate in an engineered cyanobacterium. Microb. Cell Fact. 12, 117 (2013).

18. Van der Woude, A. D. et al. Carbon sink removal: increased photosynthetic production of lactic acid by Synechocystis sp. PCC6803 in a glycogen storage mutant. J. Biotechnol. 184, 100–102 (2014).

19. Tamoi, M., Miyazaki, T., Fukamizo, T. & Shigeoka, S. The Calvin cycle in cyanobacteria is regulated by CP12 via the NAD(H)/NADP(H) ratio under light/dark conditions. Plant J. 42, 504–513 (2005).

20. Lan, E. I., Ro, S. Y. & Liao, J. C. Oxygen-tolerant coenzyme A-acylating aldehyde dehydrogenase facilitates efficient photosynthetic n-butanol biosynthesis in cyanobacteria. Energy Environ. Sci. 6, 2672–2681 (2013).

21. Li, H. & Liao, J. C. Engineering a cyanobacterium as the catalyst for the photosynthetic conversion of CO₂ to 1,2-propanediol. Microb. Cell Fact. 12, 4 (2013).

22. Oliver, J. W., Machado, I. M., Yoneda, H. & Atsumi, S. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. Proc. Natl. Acad. Sci. USA 110, 1249–1254 (2013).

23. Savakis, P. E., Angermayr, S. A. & Hellingwerf, K. J. Synthesis of 2,3-butanediol by Synechocystis sp. PCC6803 via heterologous expression of a catabolic pathway from lactate- and enterobacteria. Metab. Eng. 20, 121–130 (2013).

24. Hagen, K. D. & Meeks, J. C. The unique cyanobacterial protein Opca is an allosteric effector of glucose-6-phosphate dehydrogenase from Escherichia coli—modeling the physiological production of reduced cofactors. FEBS J. 279, 2296–2309 (2012).

25. Horton, R. M. et al. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61–68 (1989).

26. Hoover, D. M. & Lubkowski, J. DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. Nucleic Acids Res. 30, e43 (2002).

27. Wang, X. et al. Cloning, expression, purification, and activity assay of proteins relatd to d-lactic acid formation in Lactobacillus sp. PCC6803 via heterologous expression of a catabolic pathway from lactate- and enterobacteria. J. Biotechnol. 184, 184–189 (2007).

28. Brinkmann-Chen, S. et al. General approach to reversing ketol-acid reductoisomerase cofactor dependence from NADPH to NADH. Proc. Natl. Acad. Sci. USA 110, 10946–10951 (2013).

29. Brinkmann-Chen, S., Cahn, J. K. B. & Arnold, F. H. Uncovering rare NADH-prefering ketol-acid reductoisomerases. Metab. Eng. 26, 17–22 (2014).

30. Li, H. & Liao, J. C. Engineering a cyanobacterium as the catalyst for the photosynthetic conversion of CO₂ to 1,2-propanediol. Microb. Cell Fact. 12, 4 (2013).

31. Ducat, D. C., Way, J. C. & Silver, P. A. Engineering cyanobacteria to generate high-value products. Trends Biotechnol. 29, 95–103 (2011).

32. Zhou, J., Zhang, H., Meng, H., Zhang, Y. & Li, Yin. Production of optically pure n-lactate from CO₂ by blocking the PHB and acetate pathways and expressing α-lactate dehydrogenase in cyanobacterium Synechocystis sp. PCC 6803. Process Biochem. 49, 2071–2077 (2014).

33. Wang, B. et al. Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO₂. Metab. Eng. 16, 68–77 (2013).

34. Nunez, M. F. et al. Transport of α-lactate, β-lactate, and glycolate by the LldP and GlcA membrane carriers of Escherichia coli. Biochem. Biophys. Res. Commun. 309, 824–829 (2003).

35. Smith, A. J. Modes of cyanobacterial carbon metabolism. Ann. Microbiol. (Paris) 134B, 93–113 (1983).

36. Olavarria, K., Valdes, D. & Cabrera, R. The cofactor preference of glucose-6-phosphate dehydrogenase from Escherichia coli—modeling the physiological production of reduced cofactors. FEBS J. 279, 2296–2309 (2012).

37. Hagen, K. D. & Meeks, J. C. The unique cyanobacterial protein Opca is an allosteric effector of glucose-6-phosphate dehydrogenase in Nostoc punctiforme ATCC 29133. J. Bacteriol. 176, 11477–11486 (2001).

38. Horton, R. M. et al. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61–68 (1989).

39. Wang, X. et al. Cloning, expression, purification, and activity assay of proteins relatd to n-lactic acid formation in Lactobacillus rhamnosus. Appl. Microbiol. Biotechnol. 87, 2117–2123 (2010).

40. Wang, X. et al. Relative catalytic efficiency of ldhL- and ldhD-encoded proteins is crucial for optical purity of lactate produced by Lactobacillus strains. Appl. Environ. Microbiol. 78, 3480–3483 (2012).
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Author Contributions
F. T. and P. X. conceived and designed the project and experiments. C. L., F. T., J. N., Y. W., and F. Y. performed the experiments. F. T., C. L., and P. X. analyzed the data. C. L., F. T., and P. X. wrote the paper.

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