Increased Ethanol Production by Disrupting the Competitive Phosphoenolpyruvate Synthesis Pathway and Enhancing the Expression of Ethanol-producing Genes in Synechocystis Sp. PCC6803

E-Bin Gao
Jiangsu University
Ye Penglin
Jiangsu University https://orcid.org/0000-0002-6043-9141
Yunxiang Xu
Guangzhou University of Chinese Medicine
Zhu Yangjie
Jiangsu University
Kwasi Kyere-Yeboah
Jiangsu University
Guizhen Chen (gaofei@ujs.edu.cn)
Guangzhou University of Chinese Medicine https://orcid.org/0000-0002-3705-5510

Research

Keywords: metabolic engineering, ethanol, Synechocystis sp. PCC6803, microbial cell factories

Posted Date: October 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-75442/v1

Abstract

Background

Ethanol is a very important clean energy and it has many applications in medical and chemical fields. Large-scale production of ethanol has mainly been carried out through the fermentation of crops such as grain but its output and cost issues have attracted widespread attention.

Results

With the ability to fix carbon dioxide directly, cyanobacteria have been used as a photosynthetic microbial cell factory to generate biofuels and chemicals. Here, we constructed the biosynthetic pathway of ethanol in cyanobacterium *Synechocystis* sp. PCC 6803 through the following approaches. (1) We used homologous substitution to introduce pyruvate decarboxylase (*pdc*) gene from *Zymomonas mobilis* and NADPH-dependent aldehyde reductase (*yqhD*) gene from *Escherichia coli* into the neutral site of *Synechocystis* sp. PCC 6803. (2) The native superpromoter *Pcpc560*, consisting of two promoters from the cpcB gene and 14 predicted transcription factor binding sites (TFBSs) from *Synechocystis* sp. PCC6803 genome, was used to drive the over-expression of ethanol-producing genes. (3) To further increase ethanol production, we used molecular biotechnology to inhibit the metabolic pathway that direct the carbon flux of intermediate pyruvate metabolism to phosphoenolpyruvate (PEP) through disrupting the cyanobacterial endogenous PEP synthase. These approaches led to the production of 2.79g/g dry cell weight ethanol directly from light and greenhouse gas CO_2 in *Synechocystis* after cultivating for 9 days.

Conclusion

Our study provides insights into the biosynthetic pathway for ethanol production in *Synechocystis* indicating that knocking out the competitive pathway of the initial precursor and enhancing the expression of exogenous genes can effectively increase the amount of the targeted chemicals.

Background

With the consumption of fossil fuels such as coal and oil, a series of problems have been caused [1]. On one hand, the combustion of fossil fuels has caused serious environmental problems such as acid rain, greenhouse effect, and so on [2]. On the other hand, as a non-renewable resource, fossil fuels are facing a problem of energy depletion [3]. Therefore, the development of a renewable biofuel has attracted worldwide attention [4]. Bioethanol, as a renewable biofuel, has attracted the interest of researchers all over the world [4].

In the field of biotechnology, there is growing interest in using cyanobacteria as photosynthetic microbial cell factories for the production of a variety of high-value molecular products, such as ethylene [5] , acetone [6] , isoprene [7] , and isobutanol [8]. To obtain the effective production of the targeted biofuels,

cyanobacteria have been genetically modified by constructing many types of heterologous biosynthetic pathways. However, the cyanobacteria-based synthetic platform needs to be further studied for industrial production applications, in which the production yield is still not satisfactory. This is because the engineered metabolic pathways for the production of the selected chemicals have been constructed on the base of the utilization of endogenous metabolites as the initial materials, such as pyruvate and acetyl-CoA [9]. These metabolites are consumed in endogenous pathways for cell growth and physiological activities, including amino acid synthesis and the TCA cycle, which often limits the production yield of the synthesized chemicals by the biosynthetic pathway [10]. To get the most out of the yield of the synthesized chemicals, one way is to genetically modify the biosynthetic pathways by overexpressing codon-optimized exogenous genes in photosynthetic cyanobacteria under the drive of strong promoters [11]. Another alternative approach is to decrease the potential competition of endogenous metabolism for carbon resources and make more carbons and energy flux to the chemical production in cyanobacteria [9].

In this study, to further improve the application of cyanobacteria in ethanol synthesis, we used genetic engineering technology to introduce the pyruvate decarboxylase [12] from *Z. mobilis* and NADPH-dependent aldehyde reductase from *E. coli* [13] into the *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803) genome through homologous double exchange. To augment the expression of exogenous genes, two different promoters *PpetE* (Cu^{2+} inducible promoter) [14] and *Pcpc560* (super promoter) [15] were selected and integrated into the operon respectively. After obtaining two mutants with different promoters, a quantitative analysis of metabolites was carried out for comparison of the two promoters. To further increase ethanol production, we used molecular biotechnology to knock out the endogenous gene *slr0301* which encoding phosphoenolpyruvate synthase in *Synechocystis* 6803 [16] to increase the amount of intermediate pyruvate in the metabolism of cyanobacteria. The knockout of the gene *slr0301* in our experiment can verify whether the deleted gene affected cyanobacterial growth and ethanol production.

Results And Discussion

Construction of the ethanol-producing *Synechocystis*. To biosynthesize ethanol, the ethanol-producing pathway with acetyl-CoA or pyruvate from the Calvin cycle as the initial material was usually introduced into photosynthetic cyanobacterium *Synechocystis* 6803 [16,17]. Acetyl-CoA was directly reduced into ethanol via acetaldehyde using bifunctional aldehyde/alcohol dehydrogenase, CoA-dependent acetaldehyde dehydrogenase, and alcohol dehydrogenase, or indirectly converted to ethanol via acetate and aldehyde:ferredoxin oxidoreductase pathway [18]. The production of ethanol through acetyl-CoA was considered to be a less efficient route in cyanobacteria due to the low level of the reductant NADH (electron carrier), which limits the catalytic activities of NADH-dependent alcohol dehydrogenase [19, 20]. The ethanol-producing pathway was further optimized by expressing NADPH-dependent alcohol dehydrogenase, such as *E. coliyqhD*, which can exploit the reducing equivalent NADPH pool in *Synechocysitis* 6803 [20]. Compared with NADH-dependent alcohol dehydrogenase, NADPH-dependent

aldehyde reductase (as y*qhD*) has been confirmed to have high catalytic efficiency in the reduction of aldehydes to alcohols [21, 22, 23]. Therefore, in our study, codon-optimized gene coding NADPH-dependent aldehyde reductase from *E. coli* together with gene coding pyruvate decarboxylase from *Z. mobilis* was selected for the construction of the ethanol-producing pathway, which pyruvate was used as the initial precursor for this metabolic pathway in engineered cyanobacteria. As shown in Figure. 1, pyruvate decarboxylase catalyzes the conversion of pyruvate to acetaldehyde, which is then reduced to ethanol by NADPH-dependent aldehyde reductase (yqhD) and consumed NADPH. Compared with other NADPH-dependent alcohol dehydrogenases which have been used in previous studies like *slr1192* [16], *slr0942* [21], etc. *E. coli*'s NADPH-dependent aldehyde reductase (y*qhD*) has been confirmed to have high catalytic efficiency in the reduction of aldehydes to alcohols [21, 22, 23].

The first ethanol producer SynBE01 was constructed by introducing Z. *mobilispdc* gene and *E. coliyqhD* gene into *Synechocystis* 6803, which were co-expressed with the drive of the Copper ion inducible promoter *PpetE* (Figure.2A) [24]. To obtain cyanobacteria transformants with high copy transformation genes, we used a solid BG11 medium with corresponding antibiotics for repeated screening [25]. The high-copy transformed strains were cultivated on a large scale in plastic bottles filled with BG11 liquid medium without copper ions (Spectinomycin, 10μg/ml). To verify the stable integration of ethanol biosynthetic genes into the neutral *slr0168* site of the *Synechocystis* 6803 genome, the genomic DNA of the transformed strain was extracted and subjected to PCR analysis using primers binding to the coding regions of each transgene in the integrative locus (Figure.2B). PCR amplification products of the expected size (about 3.6kb, 2.9kb, and 2kb) for ethanol biosynthetic genes were present in the transformants but absent in the wild-type, indicating that ethanol biosynthetic genes were successfully integrated into the *Synechocystis* 6803 genome. These PCR analysis results provided evidence that all alien gene copies were completely segregated in the transformant and all original wild-type DNA copies were eliminated.

To determine whether the transformants expressed the integrated ethanol biosynthetic genes, we analyzed the transcription of each transgene by reverse transcriptase (RT)-PCR. The concentration of 500nM copper ion was added into the transformant *Synechocystis* SynBE01 cultures to drive the expression of exogenous gene *pdc* and *yqhD*. Total RNA was extracted from the transformant or wild-type cultures and digested with RNA free-DNase. The expected size fragments for ethanol biosynthetic genes were amplified from cDNA isolated from the transformant by using the specific primers. However, these fragments were not amplified in the wild-type cDNA (Figure.2C). The *16S* gene was used as a positive control and was present in the transformant and wild-type cDNA which means the correctness of RNA extraction. These results indicated that the introduced ethanol biosynthetic genes were all expressed successfully in the mutant SynBE01.

To verify the impact of the introduced ethanol biosynthesis genes on the growth of *Synechocystis.* During the cultivation, the growth state of SynBE01 was observed and the growth curve within 14 days was drawn (Figure.2D). It was found that SynBE01 grow normally, and its growth was not inhibited by other conditions.

Strong expression of the ethanol-producing genes. To enhance the expression of ethanol-producing genes, SynBE02 was constructed by transformation of *Synechocystis* 6803 with plasmid pBE02 (Figure.3A), which substituted the promoter *PpetE* with the super promoter *Pcpc560*. The promoter *Pcpc560* has been reported to make the expression level of functional proteins reach 15% of the total soluble protein in *Synechocystis* 6803, which is comparable to the protein production in *E. coli* [11].

Similar to SynBE01, DNA and RNA of SynBE02 were extracted to verify the construction of mutant strain and the successful expression of exogenous gene *pdc* and *yqhD*. The extracted DNA of SynBE02 was also subjected to PCR analysis with primers binding to the coding regions of each transgene in the integrative locus (Figure.3B). PCR products (about 3.9kb, 2.9kb, and 2kb) were amplified successfully with the extracted DNA of SynBE02 as the template. At the same time, when using the extracted DNA of wild-type 6803 as a template to amplify the target product, there are no bands in the gel electrophoresis pattern (Figure.3B). This evidence indicates that SynBE02 successfully integrated the target fragment into the corresponding site. The total RNA of SynBE02 was also extracted to determine the expression level of ethanol-producing genes in SynBE02. The expected fragments for ethanol biosynthetic genes were also amplified successfully with the cDNA which transformed from RNA in SynBE02 as the templet (Figure.3C). Total RNA extracted from SynBE01 and SynBE02 were also used for a quantitative (RT)-PCR analysis. The Table.2 results showed that *pdc* and *yqhD* gene expression was increased by 1.27-fold and 1.70-fold in the SynBE02 strain when compared with the SynBE01 strain. These results indicate that the exogenous ethanol-producing genes in SynBE02 were successfully expressed, and compared with SynBE01, the super-strong promoter *Pcpc560* effectively increased the expression of ethanol-producing genes.

Under the same culture conditions as SynBE01, SynBE02 was also cultured in a BG11 liquid medium containing 10μg/ml spectinomycin antibiotic. Without optimizing any culture conditions, the growth curve of SynBE02 is shown in Figure.3D. The ethanol producer SynBE01 and SynBE02 were constructed on the expression platform that knocks out the gene *slr0168*. It has been confirmed that the knockout of *slr0168* has no effect on the growth of *Synechocystis* 6803, and our observations are consistent with this conclusion.

HPLC detection of metabolites. During the observation of SynBE01 and SynBE02, we collected the expressed cyanobacterial samples accurately and prepared for the detection of ethanol production. To measure ethanol production, the SynBE01 medium was collected regularly after adding copper ions to drive expression. SynBE02 samples were regularly collected during the observation of growth status. The ethanol content in the sample was determined by high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 Series) system equipped with a refractive index detector (RID) using Biorad Aminex HPX-87H column (300×7.8 mm). The column was eluted with 0.5mM of H_2SO_4 at a flow rate of 0.6 mL/min at 50°C [26]. The ethanol production graph shows that on the 14th day when the OD_{730} reached 1.12, the maximum ethanol production of SynBE01 was 389 mg/L. On the 9th day, when OD_{730} reached 0.75, the ethanol production of SynBE02 reached 591.7 mg/L. At the highest yield, the dry cell weights of SynBE01 and SynBE02 were 0.3g and 0.315g, respectively. By weighing and corresponding

calculations, the maximum ethanol output of SynBE02 is 1.97g/dry cell weight (1g) and the maximum output of SynBE01 is 0.9g/dry cell weight (1g). So, the ethanol production of SynBE02 is much higher than SynBE01. The only difference between the SynBE02 and SynBE01 is the promoter. It has been found that the super-strong promoter can effectively enhance the expression of ethanol-producing genes. Therefore, enhancing the expression of ethanol-producing genes in *Synechocystis* 6803 can improve ethanol production.

Inhibition of competing phosphoenolpyruvate synthase activity. It has been suggested that the deletion of the related competitive pathways may contribute to increasing the production yield of ethanol in the engineered cyanobacteria. A previous experiment carried out by Wu et al. indicated that disrupting the ADP-glucose pyrophosphorylase gene leads to the accumulation of the poly-β-hydroxybutyrate (PHB) in *Synechocystis* 6803 through blocking the glycogen biosynthetic pathway. To divert the PHB carbon flux to the ethanol biosynthetic pathway, Gao et al. constructed a *Synechocystis* 6803 mutant in which the position of genes *phaAB* coding the enzymes polyhydroxyalkanoate-specific bketothiolase and polyhydroxyalkanoate-specific acetoacetyl-CoA reductase was substituted by the *pdc* gene from *Z. mobilis* and endogenous *slr1192* gene [10, 26, 27]. No significant increase in the production of ethanol was observed in the *Synechocystis* 6803 mutant cultures. This result may be the suggestion that the physiological level of the precursor metabolite is regulated by many different metabolic pathways. However, the genes *phaAB* is used by acetyl-CoA as the precursor metabolite to produce PHB, and thus the disruption of the PHB metabolic pathway may not directly influence the concentration of pyruvate. To investigate if the ethanol productivity is influenced by the related competitive pathways, regulating the concentration of the precursor pyruvate, the *Synechocystis* SynBE03 strain was constructed by knocking out the gene *slr0301*.

In *Synechosytis*, the precursor pyruvate is produced through the EMP pathway. Phosphoenolpyruvate is catalyzed by pyruvate kinase to generate pyruvate and ATP. The gene *slr0301* in *Synechocystis* 6803 also encodes phosphoenolpyruvate synthase, which controls the conversion of pyruvate to phosphoenolpyruvate reversal pathway [28]. To increase the carbon flux to pyruvate, the initial precursor of ethanol production, SynBE03 was generated as the last ethanol producer by knocking out phosphoenolpyruvate competitive pathways. The ethanol-producing genes were incorporated into the site of the *slr0301* gene encoding phosphoenolpyruvate synthase and expressed in *Synechocystis* 6803 under the drive of the promoter *Pcpc560*. The PCR results of genome DNA extracted indicated that the gene *pdc* and *yqhD* were integrated into *Synechocystis* 6803 genome, suggesting the successful construction of the engineered cyanobacterium SynBE03 lacking phosphoenolpyruvate competitive pathways (Figure.4A-B). Furthermore, the RT-PCR results indicated that the exogenous gene *pdc* and *yqhD* in SynBE03 were successfully expressed, as shown in Figure.4C. For quantitative RT-PCR analysis, the total RNA of SynBE03 was extracted and converted to cDNA. As shown in Table2, in multiple parallel experiments, the average △CT values of *pdc* and *yqhD* in SynBE03 were -0.93, -0.96. The relative abundance of different mRNA molecules was estimated using 2-△△CT. The difference of the RNA abundance of the *pdc* gene between strain SynBE03 and SynBE02 is 2^-(-0.93-(-0.25)). The difference of the RNA abundance of the *yqhD* gene between strain SynBE03 and SynBE02 is 2^-(-0.96-0.22). The data demonstrated that *pdc* and

yqhD gene expression was increased by 1.6-fold and 2.26-fold in the SynBE03 strain when compared with the SynBE02 strain.

In terms of ethanol production, in our experiment, we found that the modified strain SynBE03 which knocked out *slr0301* improved significantly when compared with SynBE02. SynBE03 reached the highest output of 878mg/L on the 9th day, its OD_{730} was 0.81. We weighed the dry weight of the algae cells (DCW) at the highest yield, the dry cell weight of SynBE03 was 0.43g. By weighing and corresponding calculations, the maximum ethanol output of SynBE03 is 2.79g/dry cell weight (1g). The output of SynBE02 is much lower than SynBE03. SynBE03 has the highest ethanol production which is consistent with the analysis of RT-qPCR results. The knockout of the gene *slr0301* promotes the expression of exogenous ethanol-producing genes and effectively increases the yield of ethanol synthesis. Knocking out the competitive phosphoenolpyruvate synthesis pathway can effectively increase the expression level of ethanol-producing genes and ethanol production in *Synechocystis* 6803.

To verify the impact of *slr0301* disruption on cell growth, we transferred the plasmid pBE409 into *Synechocystis* 6803 and observed the growth status. During the period of cultivation under constant light condition, we observed that the mutant strain lacking the gene *slr0301* showed no obvious difference in the cell growth compared with wild-type *Synechocystis* 6803 (Figure.4D, Figure.5). Therefore, the knockout of the *slr0301* gene had no obvious effect on the growth of *Synechocystis* 6803.

In our experiment, as shown in Figure.6, our highest output is 878mg/L/9days. In terms of output, our results are still far from Gao and Rajendran Velmurugan [10, 16]. However, the cyanobacteria were cultivated with a column photo-bioreactor and optimized for cultivation in their experiment. In their experiment, they still need a relatively long cultivation period and a high concentration of cultivation to achieve high yield. For example, as shown in Table.3, in Gao's experiment, the ethanol production reached $5.50gL^{-1}$ with the OD_{730} reached 12. That means its average output just reached $0.458g/OD_{730.}$ Our experiment provides a modified strain that can achieve relatively high yields through simple cultivation and has the advantages of simple production and short production cycle in industrial production. It has great application prospects in industrial production. In our experiment, we also found that ethanol production increased significantly by knocking out the competitive phosphoenolpyruvate synthesis pathway and enhancing the expression of ethanol-producing genes in *Synechocystis* 6803. It will serve as the basis for optimizing ethanol synthesis in the future.

Future study directions. All the cyanobacteria described in this article are cultivated in plastic air bottles. Ethanol is easy to volatilize. Under normal culture conditions, ethanol volatilization will cause experimental deviation. So, we plan to design a bioreactor to cultivate cyanobacteria. Blowing a certain amount of CO_2 into the cyanobacteria will effectively promote the growth of cyanobacteria and increase the metabolic yield of ethanol. Under normal conditions, our cyanobacteria were only cultivated to OD_{730} =1.3. Compared with other experiments, we need to further optimize the experimental conditions to optimize the growth status of cyanobacteria.

As documented in previous studies, *Synechocystis* can grow in the maximum concentration of 10.6g/L ethanol without significant impacts on cells [16, 27]. Nowadays, the maximum yield of ethanol has not yet been obtained in engineered cyanobacteria due to the physiological impacts of ethanol tolerance and metabolic pathways on the cyanobacterial cells. Thus, to further improve ethanol production, the next work will be performed to increase the expression of ethanol tolerance genes, such as *slr0982* [29], which may contribute to reducing the effect of high-yield ethanol on the cyanobacteria cell growth. Another alternative approach is to modify the ethanol-producing metabolic pathways in *Synechocystis* 6803 through introducing exogenous genes such as *maeB* and *Ycf21,* which may increase the carbon flux to the initial precursor pyruvate, or deleting other related competitive pathways such as the lactic acid metabolism pathway. Also, enhancing the expression of ethanol-producing genes will become the focus of the future. Transferring the plasmid pBE02 into the modified strain SynBE03 potentially double the expression of ethanol-producing genes.

Conclusions

In this study, three genetically engineered *Synechocystis* 6803 strains, SynBE01, SynBE02, and SynBE03 with gradually improved ethanol productivity were constructed. A final ethanol concentration of 2.79g/dry cell weight (1g cyanobacteria cells) was achieved over 9 days of cultivation in SynBE03 with the genetic introduction of the exogenous pyruvate decarboxylase from *Z. mobilis* and NADPH-dependent aldehyde reductase from *E. coli* into the site of the chromosome of *Synechocystis* 6803 and disruption of the biosynthetic pathway of phosphoenolpyruvate. By comparing the ethanol production of SynBE01 with SynBE02, we found that the promoter *Pcpc560* increased the expression of exogenous genes compared with the *PpetE*. By observing the growth of Syn409, we found that knocking out the pyruvate reverse metabolic pathway encoded by the gene *slr0301* did not affect the growth of *Synechocystis* 6803. By comparing SynBE02 and SynBE03, we found that knocking out the gene *slr0301* encoding phosphoenolpyruvate synthase can significantly increase ethanol productivity.

Methods

Chemicals and reagents. All chemicals were purchased from Sangon Biotech. (Shanghai, China) unless noted otherwise in the text. T4 DNA polymerase and all restriction enzymes were purchased from NEW ENGLAND BioLabs. (Beijing, China). The molecular biology kits used were from Sangon Biotech (Shanghai, China). Synthesis of DNA oligonucleotides and DNA sequencing were provided by Sangon Biotech. (Shanghai, China).

Culture conditions. *E. coli* Trans5α used as a host to construct all recombinant plasmids were grown using a standard LB medium. *Synechocystis* 6803 and its derivatives were grown in a BG11 medium under a light intensity of approximately 50 μmol photons m^{-2} s^{-1}unless otherwise specified [30]. For solid agar plates, 1.5% agar was supplemented to the liquid BG11 medium before autoclaving.

Plasmids and strains constructed. All strains used and constructed in this study are listed in Table 1. Strain SynBE01 was constructed by recombination of plasmid pBE01 into the *slr0168* site of wild type *Synechocystis* 6803. Strain SynBE02 was constructed by recombination of plasmid pBE02 into the *slr0168* site of wild type *Synechocystis* 6803. Strain SynBE03 was constructed by recombination of plasmid pBE03 into the *slr0301* site of wild type *Synechocystis* 6803. Strain Syn409 was constructed by recombination of plasmid pBE409 into the *slr0301* site of wild type *Synechocystis* 6803.

All plasmids used and constructed in this study are also listed in Table 1. The vector pMD18-T was purchased from Sangon Biotech. (Shanghai, China), and used as a backbone. All the fragments were amplified by PCR, the vector and the fragment are double-digested and ligated by T4 ligase to obtain a new vector.

Plasmid pMD0168 was constructed which had the up and down homologous recombination arms of *slr0168* on pMD18-T. The up and down homologous arms of *slr0168* cloned into pMD18-T were separately amplified with the primers *s*lr0168Up-F and slr0168Up-R, slr0168Dw-F and slr0168Dw-R using the genomic DNA of *Synechocystis* 6803 as the template.

Plasmid pBE406 was constructed which the Spectinomycin gene was cloned into the plasmid pMD0168. The Spectinomycin gene cloned into the plasmid pMD0168 was amplified with the primers SP-F and SP-R using the optimized spectinomycin gene as the template. Plasmid pBE407 was constructed which the *PpetE* promoter gene and *TrbcL* terminator gene were cloned into the plasmid pBE406. The *PpetE* and *TrbcL* gene cloned into pBE406 were separately amplified with the primers PetE-F and PetE-R, TrbcL-F and TrbcL-R using the genomic DNA of *Synechocystis* 6803 as the template. Plasmid pBE408 was constructed which substituted the *PpetE* promoter in pBE407 with the *Pcpc560* promoter. The *Pcpc560* promoter gene and *TrbcL* gene cloned into pBE406 were separately amplified with the primers Pcpc-F and Pcpc-R, TrbcL-F and TrbcL-R using the genomic DNA of Synechocystis 6803 as the template.

Through bioinformatics analysis and comparison of Metacyc and NCBI databases, pyruvate carboxylase gene (*pdc*) derived from *Z. mobilis* and NADPH-dependent aldehyde reductase gene (*yqhD*) obtained from *E. coli* were selected as exogenous gene combination for the synthesis of ethanol in the present invention. Specific method: We obtain the nucleotide sequence of the pyruvate carboxylase gene (*pdc*) of *Zymomonas mobilis* and NADPH-dependent aldehyde reductase gene (*yqhD*) of *E. coli* through NCBI database. Then according to the codon preference of the *Synechocystis* PCC6803 biological system, the online software (http://www.jcat.de/) was used to optimize the nucleotide sequence of pyruvate carboxylase gene (*pdc*) of *Z. mobilis* and NADPH-dependent aldehyde reductase gene (*yqhD*) of *E. coli*. The optimized sequence was synthesized by Sangon Biotech (Shanghai, China).

Plasmid pBE01 was constructed by insertion of *pdc* and *yqhD* into pBE407 under control of *PpetE* promoter. The exogenous gene *pdc* was amplified by PCR with primers Pdc-F and Pdc-R using the optimized *pdc* gene as the template. The *yqhD* fragment was amplified with primers YqhD-F and YqhD-R using the optimized *yqhD* gene as the template. Plasmid pBE02 was constructed by insertion of *pdc* and

yqhD into pBE408 under control of *Pcpc560* promoter. The sequences of primers used in this study are shown in Table S1.

Plasmid pMD0301 was constructed which had the up and down homologous recombination arms of *slr0301* on pMD18-T. The up and down homologous arms of *slr0301* cloned into pMD18-T were separately amplified with the primers slr0301Up-F and slr0301Up-R, slr0301Dw-F and slr0301Dw-R using the genomic DNA of Synechocystis sp. PCC6803 as the template. Similar to the construction of the plasmid pBE408, the plasmid pBE409 was constructed by cloning the amplified spectinomycin gene, *Pcpc560* promoter gene, *TrbcL* terminator gene into pMD0301. Plasmid pBE03 was constructed by insertion of the fragment of *pdc* and *yqhD* into pBE409.

Transformation of *Synechocystis.* *Synechocystis* 6803 was cultivated to the exponential phase (OD_{600} = 1.1-1.2, OD_{730} = 0.6-0.8). Then take 30ml of *Synechocystis* liquid and centrifuge in a centrifuge (4500r/min, 15min) to remove the supernatant and retain the precipitate. The precipitate was washed two times with an equal volume of fresh antibiotic-free BG11, discard the supernatant by centrifugation (operate in a sterile bench). The remaining pellet was resuspended in a 1ml BG11 solution before transferred to a 1.5mL EP tube. The plasmid (total concentration is 10μg) was added into the cell and was mixed by shaking [31]. The cell and plasmid mixture was incubated at 30 ℃ for 5 h under the luminous intensity of approximately 50 μmol photons m^{-2} S^{-1}. Then prepare some non-resistant BG11 agar plates, covered with a 0.45-micron film. Add 200μL of the mixture to each plate film. After 24 hours, change the membrane to a plate of the corresponding resistant solid medium（spectinomycin. antibiotic 10ng/μL）.After about one month of culture, transformants were obtained.

The transformants SynBE02, SynBE03 contained Spectinomycin resistance gene, promoter *Pcpc560*, *pdc*, *yqhD* gene, and *TrbcL* terminator. All these genes were obtained and passed several times on fresh BG11 plates supplemented with 10μg/mL Spectinomycin to achieve complete chromosome segregation (confirmed by PCR). The transformants SynBE01 contained Spectinomycin resistance gene, promoter *PpetE*, *pdc*, *yqhD* gene, and *TrbcL* terminator as shown in Figure.6. All these genes were obtained and passed several times on fresh BG11 plates supplemented with 10μg/mL Spectinomycin to achieve complete chromosome segregation (confirmed by PCR).

Ethanol quantifification. The obtained transformants need to be further purified to get positive colonies. After obtaining positive colonies, it was transferred into liquid BG11 medium for growth. 10μg/ml spectinomycin was added into liquid BG11 medium. Engineered cyanobacteria SynBE01 liquid medium needs to be added with 500nmol/L Cu^{2+} to induce reaction to produce ethanol.

Purified mutant is obtained until the growth is stable (confirmed by PCR), the growth status is observed, the growth curve is drawn and the ethanol concentration is tested. 1 ml of cyanobacterial cultures was regularly taken within two weeks and centrifuged at 10000×g for 2min. The supernatant was filtered through a 0.22-micron filter membrane and used for qualitative and quantitative analysis by high-performance liquid chromatography (refractive index detector). The cyanobacterial pellet was washed

twice with deionized water and crushed for 5min by a cell disrupter. After filtered with a 0.22-micron filter, the lysed samples were also used for ethanol detection by high-performance liquid chromatography (refractive index detector). The final product is the sum of the amount of ethanol detected in the supernatant due to the absence of ethanol in the precipitate. Based on the final result, we plotted the ethanol production curve.

Dry weight determination. The transformants were grown in BG11 liquid medium at 30°C under constant illumination for about 6 days. 20 ml of samples were collected daily and the optical density (730 nm) was measured using a Shanghai Linchylab 723(N) spectrophotometer. Each of the samples collected was filtered through a 0.45 um hydrophilic PVDF filter (Durapore Membrane Filter, Sigma-Aldrich). The biomass obtained was washed twice with 20ml distilled water (milli-Q) and afterward dried at 70°C for 24 hours in the oven (Sheldon Manufacturing Inc). The dry filtered biomass was allowed to cool and weighed using a balance.

RT-qPCR. After *Synechocystis* cultures were grown to the exponential phase (OD_{600} = 1.1-1.2, OD_{730} = 0.6-0.8). 30 ml of *Synechocystis* liquid was centrifuged at 4500×r/min for 15min to remove the supernatant and retain the precipitate. The precipitate was used for RNA extraction and RT-qPCR analysis using methods described previously. The relative abundance of different mRNA molecules could be estimated using $2^{-\triangle\triangle CT}$; the higher the $\triangle C_T$ value is, the less abundant the corresponding mRNA [32].

Declarations

Acknowledgements

E-Bin Gao and Guizhen Chen are acknowledged for their technical advice and expertise.

Authors' contributions

E-BG and GC designed the experiment. PY, KKY, YZ and YX performed the experiments and PY, KKY, YX collected the data. PY, YZ analyzed the data and PY, KKY drafted the article. E-BG, GC, PY critically revised the article. All authors approved the final draft for submission.

Funding

This work was supported by the National Natural Science Foundation of China (no. 31200019, no.81473755, no.81574064), Shenzhen Science and Technology Planning Project (grant to : JCYJ20170306152650625) and the Open Fund of Key Laboratory of Tropical Marine Bio-resources and Ecology, Chinese Academy of Sciences (LMB131001).

Corresponding author

Correspondence to Guizhen Chen.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional fle.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Clauss R, Mayes J, Hilton P, et al. The influence of weather and environment on pulmonary embolism: pollutants and fossil fuels[J]. Medical Hypotheses, 2005, 64(6):1198-1201.
2. Németh. Environment and energy: Problems, resolutions, solutions[J]. International Journal of Hydrogen Energy, 1990, 15(7):457-462.
3. Afgan N H, Gobaisi D A, Carvalho M G, et al. Sustainable energy development[J]. Renewable & Sustainable Energy Reviews, 2011, 2(3):235-286.
4. Dolgykh L, Stolyarchuk I, Deynega I, et al. The use of industrial dehydrogenation catalyst for hydrogen production from bioethanol. Int J Hydrogen Energy[J]. International Journal of Hydrogen Energy, 2006, 31(11):1607-1610.
5. Wang, Jinsheng, et al. "Simulation of Ethylene Production by Recombinant Cyanobacteria." Ifac Proceedings Volumes 31.8(1998):117-119.
6. Lee H J, Son J, Sim S J, et al. Metabolic rewiring of synthetic pyruvate dehydrogenase bypasses for acetone production in cyanobacteria[J]. Plant Biotechnology Journal, 2020.
7. Lindberg P, Park S, Melis A. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism[J]. Metabolic Engineering, 2009, 12(1):70-79.
8. Miao R, Liu X, Englund E, et al. Isobutanol production in Synechocystis PCC 6803 using heterologous and endogenous alcohol dehydrogenases[J]. Metabolic Engineering Communications, 2017, 5:45-53.
9. Hirokawa Y, Kubo T, Soma Y, et al. Enhancement of acetyl-CoA flux for photosynthetic chemical production by pyruvate dehydrogenase complex overexpression in Synechococcus elongatus PCC 7942[J]. Metabolic Engineering, 2020: 23-30.

10. Velmurugan R, Incharoensakdi A. Co-cultivation of two engineered strains of Synechocystis sp. PCC 6803 results in improved bioethanol production[J]. Renewable Energy, 2020: 1124-1133.
11. Zhou J, Zhang H, Meng H, et al. Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria[J]. Scientific Reports, 2015, 4(1): 4500-4500.
12. Wang X, He Q, Yang Y, et al. Advances and Prospects in Metabolic Engineering of Zymomonas mobilis[J]. Metabolic Engineering, 2018, 50: 57-73.
13. Lan E I, Liao J C. Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide[J]. Metabolic Engineering, 2011, 13(4):353-363.
14. Ghassemian M, Wong B, Ferreira F, et al. Cloning, sequencing and transcriptional studies of the genes for cytochrome c-553 and plastocyanin from Anabaena sp. PCC 7120[J]. Microbiology, 1994, 140(5): 1151-1159.
15. Rippka R, Deruelles J, Waterbury J B, et al. Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria[J]. Microbiology, 1979, 111(1): 1-61.
16. Gao Z, Zhao H, Li Z, et al. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria[J]. Energy and Environmental Science, 2012, 5(12): 9857-9865.
17. Woo J E, Jang Y S. Metabolic engineering of microorganisms for the production of ethanol and butanol from oxides of carbon[J]. Applied Microbiology and Biotechnology, 2019, 103(4):1-10.
18. Kopke M, Held C, Hujer S, et al. Clostridium ljungdahlii represents a microbial production platform based on syngas[J]. Proceedings of the National Academy of Sciences of the United States of America, 2010, 107(29): 13087-13092.
19. Ma W, Mi H. Effect of exogenous glucose on the expression and activity of NADPH dehydrogenase complexes in the cyanobacterium *Synechocystis* strain PCC 6803[J]. Plant Physiology & Biochemistry Ppb, 2008, 46(8-9):775-779.
20. Choi, Yun-Nam, Park, et al. Enhancing biomass and ethanol production by increasing NADPH production in Synechocystis sp. PCC 6803. [J]. Bioresource Technology, 2016.
21. Miao R, Liu X, Englund E, et al. Isobutanol production in Synechocystis PCC 6803 using heterologous and endogenous alcohol dehydrogenases[J]. Metabolic Engineering Communications, 2017, 5:45-53.
22. Atsumi S, Hanai T, Liao J C. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels[J]. Nature, 2008, 451(7174):86-89.
23. Atsumi S, Wu T Y, Eckl E M, et al. Engineering the isobutanol biosynthetic pathway in Escherichia coli by comparison of three aldehyde reductase/alcohol dehydrogenase genes[J]. Appl Microbiol Biotechnol, 2010, 85(3):651-657.
24. Duran R V, Hervas M, De La Rosa M A, et al. The efficient func2 tioning of photosynthesis and respiration in synechocystis sp. PCC 6803 strictly requires the presence of either cytochrome c6 or plastocyanin [J]. Journal of Biological Chemistry, 2004, 279 :7229 —7233
25. Tan X, Yao L, Gao Q, et al. Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. [J]. Metabolic Engineering, 2011, 13(2):169-176.

26. Seo S O, Wang Y, Lu T, et al. Characterization of a Clostridium beijerinckii spo0A mutant and its application for butyl butyrate production[J]. Biotechnology & Bioengineering, 2016, 114(1):106.
27. Dexter J, Fu P. Metabolic engineering of cyanobacteria for ethanol production[J]. Energy and Environmental Science, 2009, 2(8): 857-864.
28. Machado I M, Atsumi S. Cyanobacterial biofuel production. [J]. Journal of Biotechnology, 2012, 162(1): 50-56.
29. Silva T L, Passarinho P C, Galrica R, et al. Evaluation of the ethanol tolerance for wild and mutant Synechocystis strains by flow cytometry[J]. Biotechnology Reports, 2018: 137-147.
30. Liu Y, Cui Y, Chen J, et al. Metabolic engineering of Synechocystis sp. PCC6803 to produce astaxanthin[J]. Algal Research, 2019, 44:101679.
31. Williams J G K. [85] Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in Synechocystis 6803[J]. Methods Enzymol, 1988, 167.
32. Livak, K.J. and Schmittgen, T.D, “Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method,” Methods, 25. 402-408. 2001.

Tables

Table 1

Strains and plasmids used in this study		Reference
Strains		
E. coli Trans5α		Sangon Biotech
Synechocystis sp. PCC6803	Wild-type *Synechocystis*	ATCC 27184
SynBE01	*PpetE-pdc-yqhD-TrbcL* integrated and *slr0168* deleted by pBE01 in *Synechocystis* sp. PCC6803	This study
SynBE02	*Pcpc560-pdc-yqhD-TrbcL* integrated and *slr0168* deleted by pBE02 in *Synechocystis* sp. PCC6803	This study
SynBE03	*Pcpc560-pdc-yqhD-TrbcL* integrated and *slr0301* deleted by pBE03 in *Synechocystis* sp. PCC6803	This study
Syn409	Plasmid pBE409 was transformed into PCC6803	This study
Plasmid		
pMD18-T	Amp^R	Sangon Biotech
pMD0168	Amp^R Upstream gene and downstream gene of *slr0168* were cloned it into the site of pMD18-T	This study
pBE406	Spe^R spectinomycin gene was cloned into the site of pMD0168	This study
pBE407	Spe^R The promoter *PpetE* and terminator *TrbcL* were cloned into the site of pBE406	This study
pBE407-pdc	Spe^R Exogenous gene(*pdc*) was cloned into the site of pBE407	This study
pBE01	Spe^R Exogenous gene(*yqhD*) was cloned into the XhoI/PstI site of pBE407-pdc	This study

pBE408	Spe^R The promoter *Pcpc560* and terminator *TrbcL* were cloned into the site of pBE406	This study
pBE408-pdc	Spe^R Exogenous gene(*pdc*) was cloned into the site of pBE408	This study
pBE02	Spe^R Exogenous gene(*yqhD*) was cloned into the site of pBE408-pdc	This study
pMD0301	Amp^R Upstream gene and downstream gene of *slr0301* were cloned it into the site of pMD18-T	This study
pBE409	Spe^R The spectinomycin gene, the promoter *Pcpc560* and terminator *TrbcL* were cloned into the site of pMD0301	This study
pBE409-pdc	Spe^R Exogenous gene(*pdc*) was cloned into the site of pBE409	This study
pBE03	Spe^R Exogenous gene(*yqhD*) was cloned into the site of pBE409-pdc	This study

Table2. RT-qPCR analysis of gene expression of *pdc and yqhD*

Genes	△CT		
	SynBE01	SynBE02	SynBE03
pdc	0.1±0.005	-0.25±0.01	-0.93±0.05
yqhD	0.99±0.05	0.22±0.01	-0.96±0.05

Table 3 Ethanol production in Synechocystis PCC6803

Name	culture time	OD_{730}	Site/Genotype	Yield (mg/L)	references
WT	6 days	0.32	*slr0168/PrbcL-pdc-adhII*	460	[28]
Syn-ZG25	20 days	6	*slr0168/PrbcL-pdc-slr1192*	600	[16]
Syn-HZ23	20days	15	*slr9394/ PrbcL-pdc-slr1192*	About 600	[16]
Syn-HZ24	26days	15	*slr0168/PrbcL-pdc-slr1192;* *slr1993/ PrbcL-pdc-slr1192*	5500	[16]
GBK-APK	25days	-----	*glgC-phaA* was knocked out Overexpression/ *PpsbA-pdc-slr0942*	4103	[10]
SynBE01	14	1.136	*slr0168/PpetE-pdc-yqhD*	389	This study
SynBE02	9	0.75	*slr0168/Pcpc560–pdc-yqhD*	591.7	This study
SynBE03	9	0.805	*slr0301/Pcpc560–pdc-yqhD*	878	This study

Figures

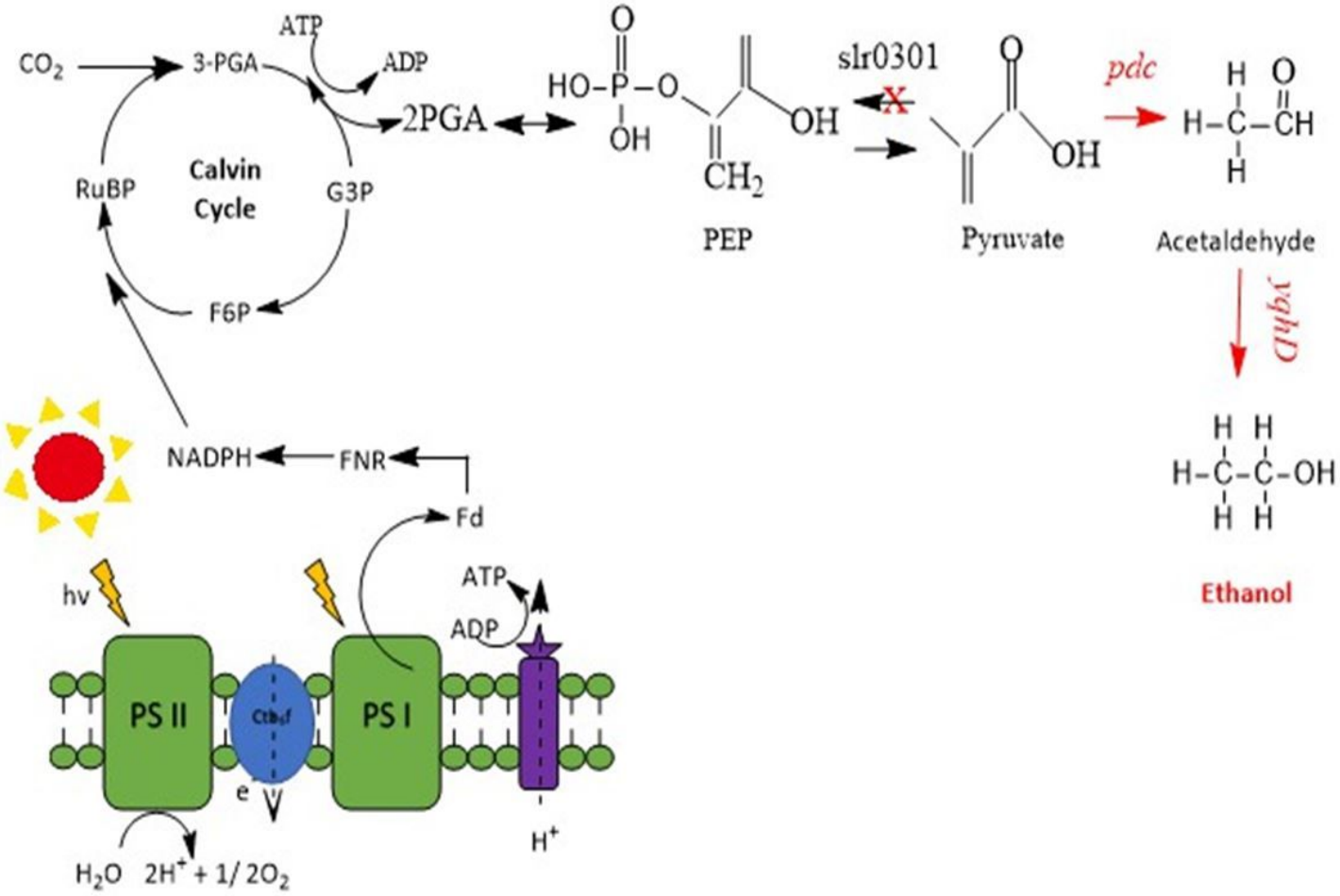

Figure 1

Scheme of the biosynthetic pathway of ethanol from CO2 in Synechocystis. The genetic modifications made in this study were highlighted in red. (pdc, pyruvate decarboxylase; yqhD, NADPH-dependent aldehyde reductase; PEP, phosphoenolpyruvate; 3-PGA, 3-Phosphoglyceric acid; 2-PGA, 2-Phosphoglyceric acid; G3P, Glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate; RuBP, Ribulose-1,5-bisphosphate).

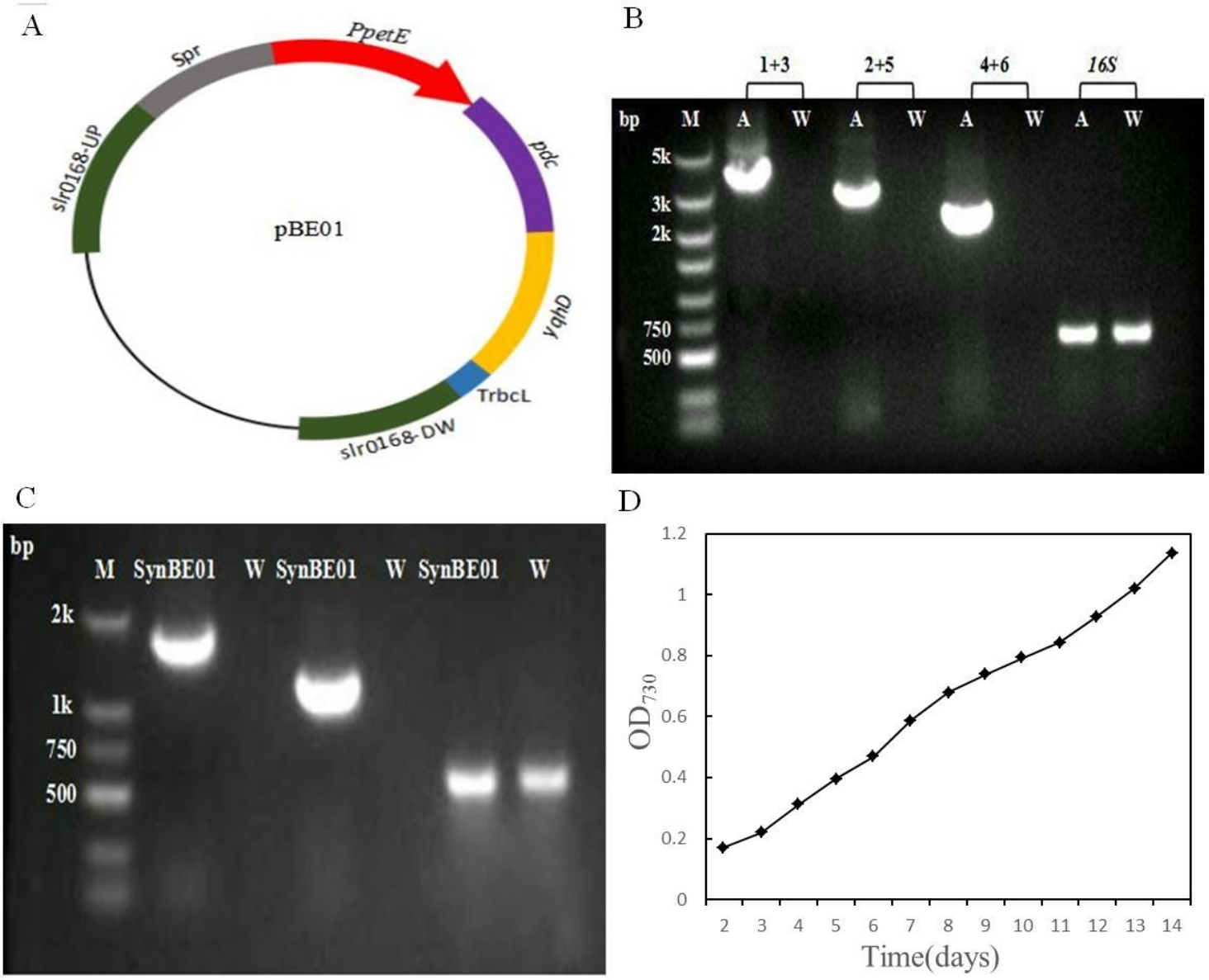

Figure 2

A: Plasmid map of the vector pBE01 B: The PCR verification of SynBE01 (The M in the left column is the marker; A represents the mutant strain SynBE01; W represents wild-type Synechocystis PCC6803; 1+3 means using primer 1 and primer 3 to verify the corresponding fragment,3.6kb in SynBE01 and cannot be detected in wild type. 2+5 means using primer 2 and primer 5 to verify the corresponding fragment, 2.9kb in SynBE01 and cannot be detected in wild type. 4+6 means using primer 4 and primer 6 to verify the corresponding fragment, 2kb in SynBE01 and cannot be detected in wild type. The endogenous gene 16s 521bp can be detected in SynBE01 and wild-type PCC6803 genome, which means the correctness of DNA extraction.) C: The PCR identification diagram performed by converting the extracted RNA from SynBE01 into cDNA(The M in the left column is the marker; The pdc gene 1.7kb was successfully expressed in SynBE01, but it could not be detected in the wild-type Synechocystis PCC6803; The yqhD gene 1.2kb was successfully expressed in SynBE01, but it could not be detected in the wild-type Synechocystis PCC6803, The endogenous gene 16S 521bp was expressed successfully in SynBE01 and wild-type Synechocystis

PCC6803, which means the correctness of RNA extraction.) (Primer: 1. slr0168Up-F, 2. Pdc-F, 3. Pdc-R, 4. Yqhd-F, 5. YqhD-R, 6. slr0168Dw-R) D: Growth curve of SynBE01 in 14 days

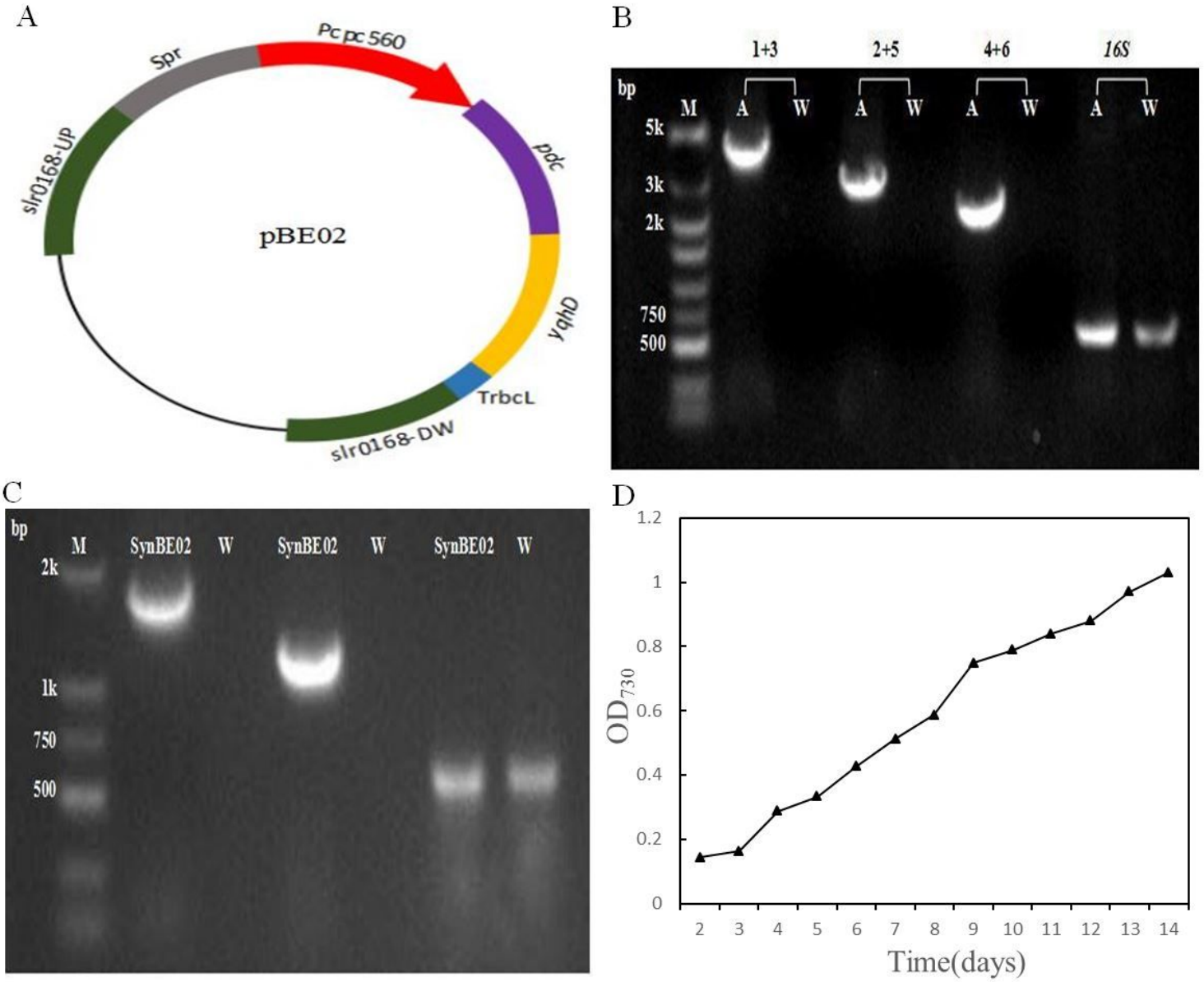

Figure 3

A: Plasmid map of the vector pBE02 B: The PCR verification of SynBE02 (The M in the left column is the marker; A represents the mutant strain SynBE02; W represents wild-type Synechocystis PCC6803; 1+3 means using primer 1 and primer 3 to verify the corresponding fragment,3.9kb in SynBE02 and cannot be detected in wild type. 2+5 means using primer 2 and primer 5 to verify the corresponding fragment, 2.9kb in SynBE02 and cannot be detected in wild type. 4+6 means using primer 4 and primer 6 to verify the corresponding fragment, 2kb in SynBE02 and cannot be detected in wild type. The endogenous gene 16s 521bp can be detected in SynBE02 and wild-type PCC6803 genome, which means the correctness of DNA extraction.) C: The PCR identification diagram performed by converting the extracted RNA from SynBE02 into cDNA (The M in the left column is the marker; The pdc gene 1.7kb was successfully expressed in SynBE02, but it could not be detected in the wild-type Synechocystis PCC6803; The yqhD gene 1.2kb was

successfully expressed in SynBE02, but it could not be detected in the wild-type Synechocystis PCC6803. he endogenous gene 16S 521bp was expressed successfully in SynBE02 and wild-type Synechocystis PCC6803, which means the correctness of RNA extraction.) (Primer: 1. slr0168Up-F, 2. Pdc-F, 3. Pdc-R, 4. Yqhd-F, 5. YqhD-R, 6. slr0168Dw-R) D: Growth curve of SynBE02 in 14 days.

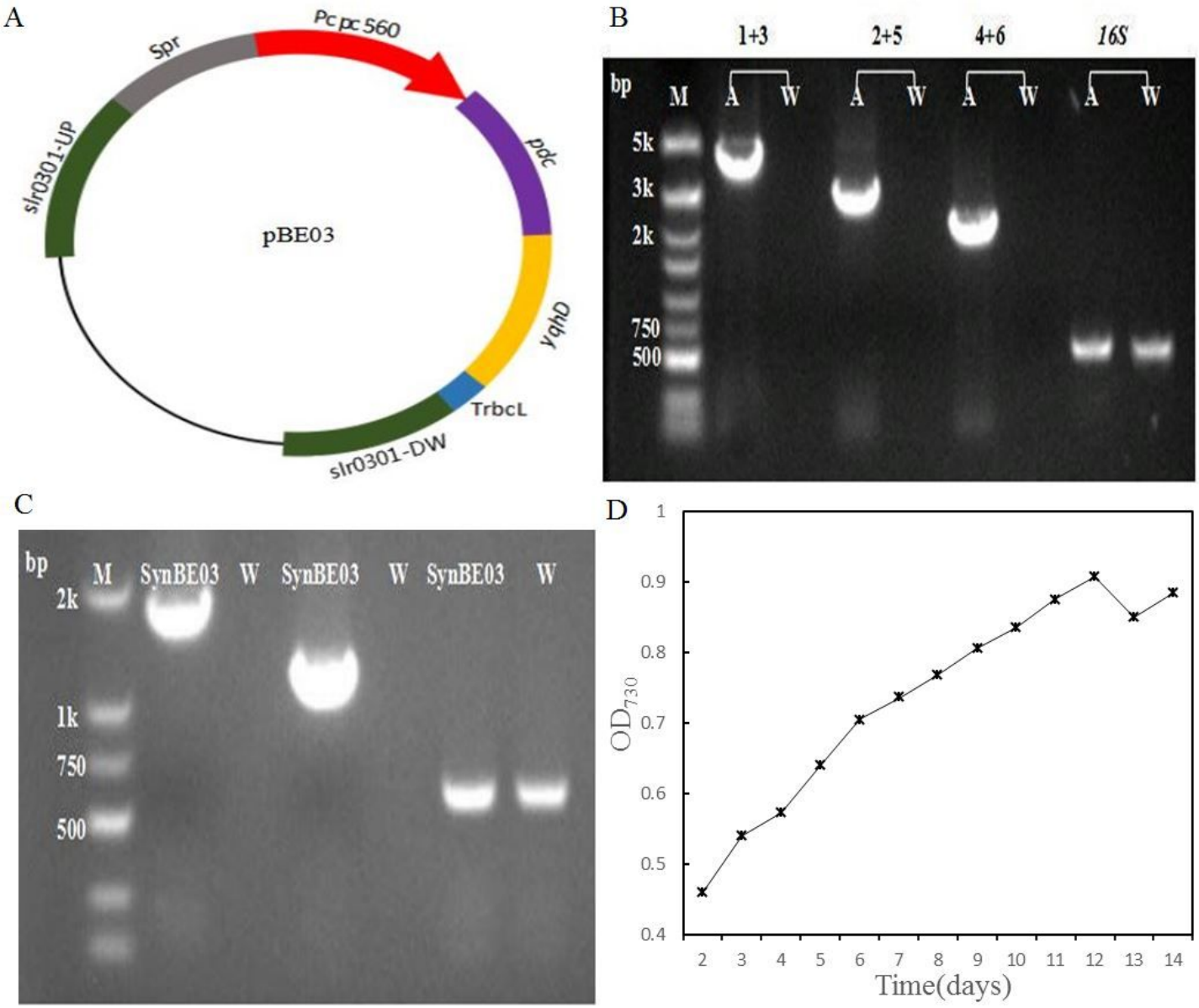

Figure 4

A: Plasmid map of the vector pBE03 B: The PCR verification of SynBE03 (The M in the left column is the marker; A represents the mutant strain SynBE03; W represents wild-type Synechocystis PCC6803; 1+3 means using primer 1 and primer 3 to verify the corresponding fragment,3.9kb in SynBE03 and cannot be detected in wild type. 2+5 means using primer 2 and primer 5 to verify the corresponding fragment, 2.9kb in SynBE03 and cannot be detected in wild type. 4+6 means using primer 4 and primer 6 to verify the corresponding fragment, 2kb in SynBE03 and cannot be detected in wild type. The endogenous gene 16s 521bp can be detected in SynBE03 and wild-type PCC6803 genome, which means the correctness of DNA

extraction.) C: The PCR identification diagram performed by converting the extracted RNA from SynBE03 into cDNA(The M in the left column is the marker; The pdc gene 1.7kb was successfully expressed in SynBE03, but it could not be detected in the wild-type Synechocystis PCC6803; The yqhD gene 1.2kb was successfully expressed in SynBE03, but it could not be detected in the wild-type Synechocystis PCC6803, The endogenous gene 16S 521bp was expressed successfully in SynBE03 and wild-type Synechocystis PCC6803, which means the correctness of RNA extraction.) (Primer: 1. slr0168Up-F, 2. Pdc-F, 3. Pdc-R, 4. Yqhd-F, 5. YqhD-R, 6. slr0168Dw-R) D: Growth curve of SynBE03 in 14 days

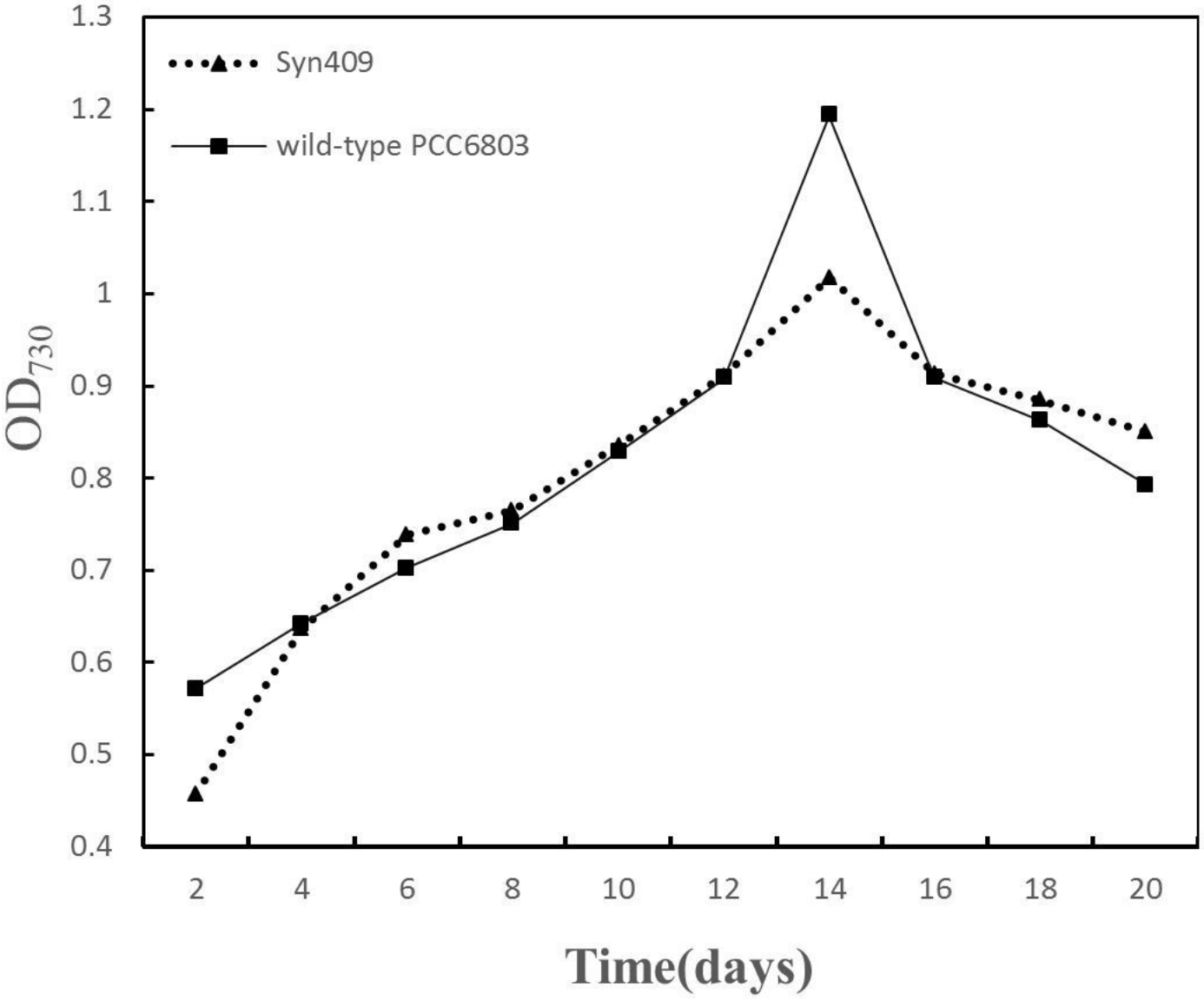

Figure 5

Comparison of growth status between Syn409 and wild-type Synechocystis PCC6803

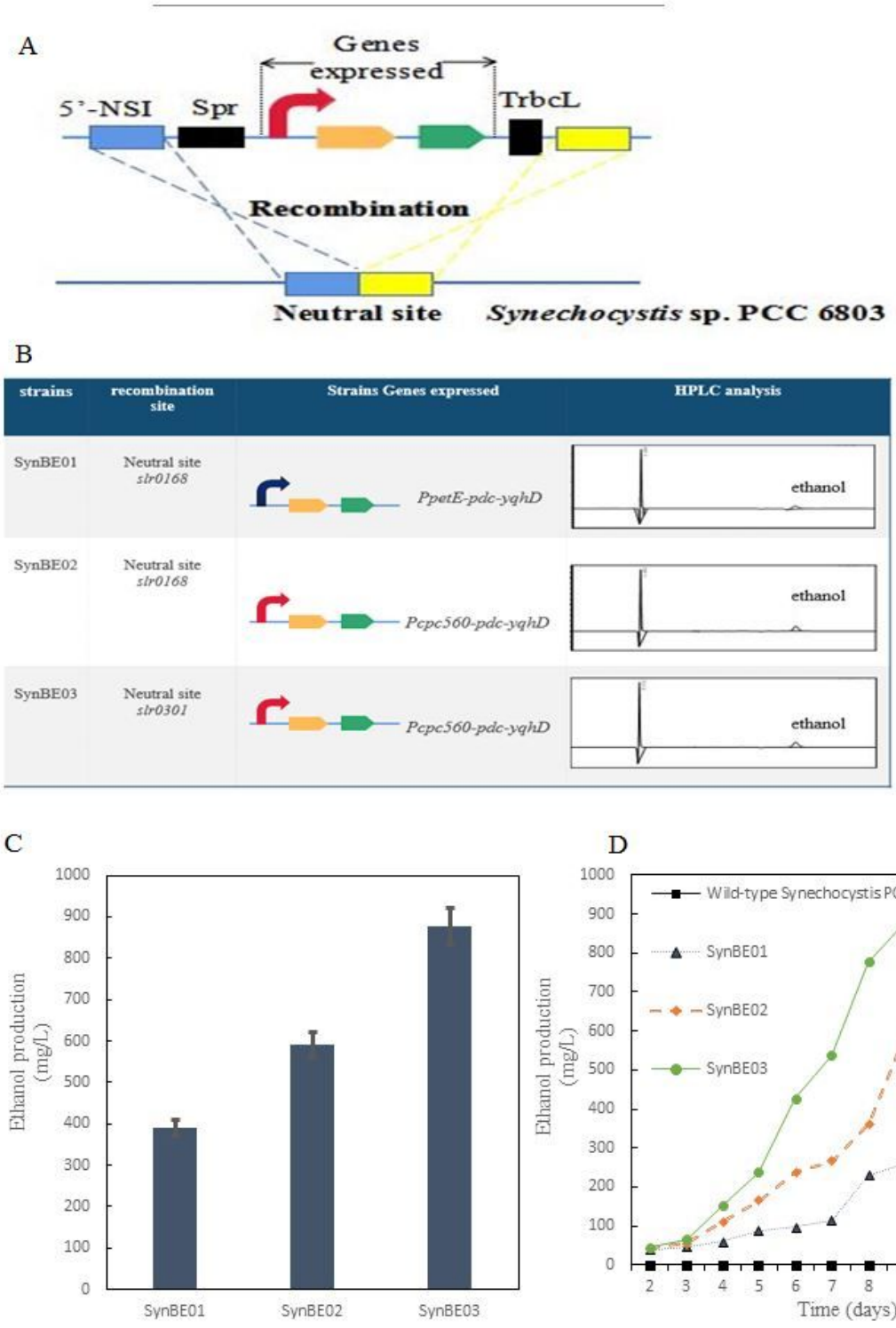

Figure 6

A: Schematic diagrams of the construction of strains that produce ethanol. B: The concrete recombination site, expressed gens, and HPLC analysis of the modified strains. C: Comparison of the highest ethanol yields of the modified strains SynBE01, SynBE02 and SynBE03 D: SynBE01, SynBE02, SynBE03, and wild-type Synechocystis PCC6803 ethanol production in 14 days

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx
- TableS1.docx