

Comparison of glucose, acetate and ethanol as carbon resource for production of acetyl-CoA derived chemicals in engineered *Escherichia coli*

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Abstract

Background: Acetyl-CoA is a fundamental metabolite in *Escherichia coli*, and also a precursor for biosynthesis of chemicals and materials suitable for multiple applications. The acetyl-CoA synthesis route from glucose presents low atomic economy due to the release of CO₂ in pyruvate decarboxylation reaction. Because ethanol and acetate, both ordinary and inexpensive chemicals, can be converted into acetyl-CoA directly, they could be alternative substrates for production of acetyl-CoA derivatives.

Results: In this study, the bifunctional reductase AdhE mutant (A267T/E568K), which converts ethanol into acetyl-CoA, was used to enable *E. coli* to grow on ethanol, and AMP-forming acetyl-CoA synthetase ACS was employed to enhance the ability of *E. coli* to utilize acetate. Several products derived from acetyl-CoA, including polyhydroxybutyrate, 3-hydroxypropionate, and phloroglucinol, were produced from glucose, ethanol, and acetate, respectively, by engineered *E. coli* strains. Compared with glucose and acetate, the strains grown on ethanol presented the highest production and yield of carbon source, and metabolome analysis revealed the reasons of high yield from ethanol.

Conclusions: The conversion of ethanol into acetyl-CoA presents high atomic economy along with generation of reducing power, and the yield of target chemical from ethanol is much higher than those from glucose and acetate. All these results suggested that ethanol could be a suitable carbon source for production of acetyl-CoA derived bioproducts.

Background

Production of value-added chemicals and materials by recombinant microorganism has attracted wide attention in recent years. *Escherichia coli*, a common inhabitant of the mammalian intestines, undoubtedly is one of the most important chassis hosts because of its fast growth, clear genetic background, facility in genetic manipulation, and low cultivation cost [1, 2]. Acetyl-CoA is a fundamental metabolite in central metabolic pathways of *E. coli*, and also a precursor for biosynthesis of large number of industrial chemicals [3, 4] and natural products [5]. A series of acetyl-CoA derived chemical and material have been successfully produced by engineered *E. coli* strains, such as phloroglucinol (PG) [6], 3-hydroxypropionate (3HP) [4, 7], fatty acids [8], polyketides [9], terpenoids [5], and polyhydroxyalkanoates [3].

The most common metabolic route for acetyl-CoA synthesis in *E. coli* is the glycolysis pathway coupled with decarboxylation of pyruvate by pyruvate dehydrogenase [10]. Through this pathway, each mol of glucose is converted into 2 mol of acetyl-CoA with generation of 4 mol of NADH, 2 mol of ATP and 2 mol of CO₂ (Fig. 1). The release of CO₂ lowers the atomic economy of targeted chemical biosynthetic pathway, leading to decrease of theoretical production yield, titer and productivity [11]. To improve the atomic economy of biosynthesis process, fatty acids were tested as an alternative carbon source [7]. Catabolized through β -oxidation pathways, fatty acids are transformed into acetyl-CoA with 100% carbon recovery in addition to production of reducing power. Although the production and yield of target

chemical were increased significantly [7], the water-insoluble nature of fatty acids makes it difficult to handle and monitor the fermentation process. Several synthetic pathways for acetyl-CoA from one-carbon substrates have been constructed in *E. coli* [12, 13], but their efficiency remains far below what is required for effective production of chemicals and materials.

Acetate and ethanol are both ordinary and inexpensive commodity chemicals, which can be directly converted into acetyl-CoA by microorganism, and could be considered as alternative substrate for production of acetyl-CoA with high atomic economy, and then production of acetyl-CoA derived chemicals. In *E. coli*, acetyl-CoA is produced from acetate via two different pathways, AMP-forming acetyl-CoA synthetase (ACS) catalyzing the higher-efficient pathway and phosphotransacetylase/acetate kinase (Pta-AckA) catalyzing the lower-efficient pathway [14]. In both pathways, ATP is consumed for the production of acetyl-CoA from acetate, and no reducing power is produced during this process (Fig. 1), indicating that extra acetate is demanded to generate ATP and reducing power when acetate is used as sole carbon source.

E. coli possesses a bifunctional reductase AdhE, which catalyzes the two-step reduction of acetyl-CoA to acetaldehyde and then to ethanol during anaerobic growth [15]. Although both reactions catalyzed by AdhE are reversible, *E. coli* cannot grow aerobically on ethanol as the sole carbon source due to the insufficiency of adhE expression [16] and the susceptibility of AdhE protein to metal-catalyzed oxidation [17]. Further study revealed an AdhE A267T/E568K mutant with acquired and improved aerobic growth ability on ethanol when overexpressed in *E. coli* [18], suggesting that this AdhE mutant can convert ethanol into acetyl-CoA efficiently and aerobically. *Pseudomonas aeruginosa* ATCC 17933 grows aerobically with ethanol as sole carbon source, and encodes an unusual quinoprotein-dependent ethanol oxidizing system ExaABC, which catalyzes the oxidation of ethanol to acetate (Fig. 1) [19, 20]. Although the conversion of ethanol to acetyl-CoA through both routes present a carbon recovery of 100%, the pathway employing AdhE A267T/E568K mutant produces more NADH, and does not require energy supply.

Previously, we have accomplished the production of PG and mevalonate from acetate in recombinant *E. coli* with ACS overexpression [21, 22]. In this study, we constructed an engineered *E. coli* strain growing with ethanol as sole carbon source by introduction of AdhE A267T/E568K mutant, and achieved the conversion of ethanol into various bioproducts including polyhydroxybutyrate (PHB), PG and 3HP. Furthermore, the fermentation process using glucose, ethanol or acetate as sole carbon source were compared.

Results And Discussion

Tolerance of *E. coli* to ethanol and acetate

Ethanol usually shows antimicrobial activity at high concentration, and acetate represses the growth rate and maximum cell density of *E. coli* [23]. To figure out appropriate concentrations of ethanol and acetate as carbon source, they were added into minimal medium containing 10 g/L glucose at a series of

concentrations from 1 g/L to 20 g/L, respectively. As shown in Fig. 2, addition of ethanol slightly slowed the growth, but did not affect maximum cell density of *E. coli* W3110 wild-type strain, as well as acetate at concentrations of 1 g/L and 2 g/L. However, acetate at concentrations of 5–15 g/L severely retarded *E. coli* growth, and 20 g/L acetate almost inhibited bacterial growth. As *E. coli* can catabolize acetate naturally, the maximum cell density with addition of 5–15 g/L acetate was higher than that at control conditions. Therefore, the concentration of ethanol was fixed at 10 g/L, and 2 g/L acetate was supplied at the beginning of cultivation with follow-up addition when exhausted.

Construction of *E. coli* strain growing on ethanol

As mentioned above, two systems, *E. coli* AdhE mutant and *P. aeruginosa* ExaABC, can support bacterial growth on ethanol as sole carbon source (Fig. 1). As the route employing AdhE mutant has some advantages like shorter pathway, more NADH production and no energy requirement, it was chosen to construct engineered *E. coli* strain which can grow using ethanol as sole carbon source. The gene encoding AdhE A267T/E568K mutant was cloned into the vector pACYCDuet-1 under the IPTG-inducible promoter P_{lac1-6} [24], and transformed into *E. coli* W3110 strain, along with empty vector, to generate strains Q3092 and Q3352, respectively. When grown in LB broth and induced by IPTG, AdhE mutant protein was observed as distinct band with the expected molecular weight on SDS-PAGE (Fig. 3A). Then, these two strains were inoculated into minimal medium supplemented with 10 g/L ethanol, but neither could grow under this condition, implying that overexpression of heterologous gene at beginning of cultivation might impair the cell growth seriously. So, 1 g/L glucose was added into the medium, and transcription of $adhE_{mut}$ gene was initiated at an OD_{600} of 0.6. After 57 h cultivation in shaking flask, expression of AdhE mutant significantly increased the cell density, up to 6.7-time higher than the strain carrying empty vector (Fig. 3B), suggesting that appropriate expression of AdhE double mutant is efficient to support *E. coli* growth on ethanol.

Production Of PHB From Ethanol

PHB is the most representative biodegradable and biocompatible thermoplastic, and suitable for applications in packaging, medicine, pharmacy, and food industries [25]. It is synthesized from its precursor acetyl-CoA by three enzymes encoded by *phaCAB* operon of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Fig. 4A) [26].

In this study, PHB was used as example to evaluate the feasibility of ethanol as carbon source for production of acetyl-CoA derived bioproducts, and the plasmid pBAD-Ae-*pha* carrying *phaCAB* operon [27] was transformed into strains Q3352 and Q3092 to generate Q3095 and Q3094, respectively. Then, these two strains were grown in minimal medium containing 1 g/L glucose and 10 g/L ethanol, and expression of $adhE_{mut}$ and *phaCAB* was initiated by addition of IPTG and l-arabinose, respectively. During the cultivation process, the OD_{600} and residual ethanol concentration in medium were measured.

As shown in Fig. 4B, the final OD₆₀₀ of Q3094 strain was 19.77 ± 0.36 , while the control strain Q3095 only reached an OD₆₀₀ of 3.65 ± 0.07 . Growth of Q3094 strain depleted all ethanol in the medium, however the ethanol concentration in Q3095 culture decreased slightly, similar with that of medium without inoculation but incubated at 37 °C (Fig. 4C). After 54 h cultivation, the strain Q3094 accumulated 3.12 ± 0.15 g/L PHB representing 48.7% of cell dry weight (CDW), while the control strain Q3095 produced 0.40 ± 0.11 g/L CDW and trace amount of PHB (Fig. 4D). All these results demonstrated that *E. coli* with overexpression of AdhE_{mut} acquired the ability to produce value-added bioproducts using ethanol as carbon source.

Comparison of glucose, ethanol and acetate as carbon source for PHB production

Combining the reactions in Fig. 1A and Fig. 4A, the overall theoretical stoichiometry of PHB synthesis from various carbon source is deduced (Fig. 5A). It shows that the PHB production from both glucose and ethanol is coupled with generation of energy and reducing power, but extra acetate is oxidized to meet the demand of energy and reducing power in pathway from acetate to PHB. The theoretical yield of PHB from ethanol is 0.935 g/g, much higher than its theoretical yields from the other two feedstocks. So, it is hypothesized that ethanol could be an ideal carbon source for PHB production in engineered bacteria.

To verify this hypothesis, comparison of glucose, ethanol and acetate as carbon source for PHB production was conducted. The gene *acs* was cloned into the vector pACYCDuet-1 under the IPTG-inducible promoter P_{lac1-6}, and co-transformed with pBAD-Ae-*pha* into *E. coli* W3110 to generate strain Q3140. Then Q3095, Q3094 and Q3140 were grown on glucose, ethanol and acetate, respectively, and the cell growth, consumption of carbon source, PHB production, and intracellular metabolites were monitored during the cultivation process.

After 54 h cultivation, the strain Q3094 presented the highest cell density, followed by strain Q3095 and Q3140 (Fig. 5B). At D1 and D2 stages shown in Fig. 5B, the consumption of carbon sources was measured. The strain Q3095 almost exhausted 10 g/L glucose in 24 h, while 4.81 ± 0.17 g/L ethanol and 1.29 ± 0.01 g/L acetate were consumed by strain Q3094 and Q3140, respectively. At the end of cultivation, the strain Q3094 consumed 9.68 ± 0.02 g/L ethanol, and 4.85 ± 0.13 g/L acetate was catabolized by strain Q3140 (Fig. 5C). The PHB production of Q3094 is 3.12 ± 0.18 g/L, and the yield of PHB from ethanol is 0.32 ± 0.02 g/g ethanol. Both PHB production and yield were much higher than those from glucose and acetate (Fig. 5D). These results suggested that ethanol have some advantages as carbon source for PHB production than glucose and acetate, such as high cell density, high production and yield.

Differences Between The Metabolism Of Glucose, Ethanol And Acetate

To figure out the differences between the metabolism of glucose, ethanol and acetate, the targeted metabolome analysis was performed. Relative amount of acetyl-CoA, by-products, and main metabolites in central carbon metabolism pathways was determined at D1 and D2 stages (Fig. 5B) by LC-MS/MS, and shown in Fig. 6.

At D1 stage, Q3095 strain grown on glucose produced the most acetyl-CoA, which was 8.73 times and 6.91 times higher than strains Q3094 and Q3140, respectively, and the similar results were observed in supply of energy and reducing power (Fig. 6). It is probably because that glucose is the favorite carbon source of *E. coli* [28], and was rapidly metabolized. This was also confirmed by the highest consumption rate of glucose (Fig. 5C). At D2 stage, acetyl-CoA and NADH were undetectable in Q3095 strain as glucose had been depleted.

For metabolites in glycolysis pathway, Q3095 strain also showed the highest abundance at D1 stage. In particular, the pyruvate amount in Q3095 strain was more than 20 times higher than those in the other two strains (Fig. 6). It was very reasonable because that glucose was metabolized mainly through glycolysis pathway, and in Q3094 and Q3140 strains these metabolites have to be produced from ethanol and acetate through gluconeogenesis as building blocks for synthesis of components for construction of cell architecture. At D2 stage, there was not significant difference between three strains.

In contrast, the relative amount of TCA cycle intermediates in Q3140 strain was significantly higher than those in the other two strains at both D1 and D2 stages (Fig. 6). This phenomenon proved our hypothesis that extra acetate was oxidized through TCA cycle to produce ATP and NADH.

Remarkably, two by-products, lactate and acetate, were produced in Q3095 strain in large quantities at D1 stage, and the accumulation of acidic products could inhibit further growth of Q3095 strain. The lactate production of Q3095 strain was 229.22 times and 48.59 times as much as those of Q3094 strain and Q3140 strain, respectively. The accumulation of lactate was consistent with the high contents of pyruvate and NADH in Q3095 strain. As is known, glucose is catabolized through glycolysis pathway to pyruvate, which is converted into acetyl-CoA under catalysis of pyruvate dehydrogenase, with reduction of NAD^+ to NADH in both glycolysis pathway and pyruvate dehydrogenation. When NADH could not be oxidized through respiratory chain sufficiently, the build-up of NADH rapidly inactivated the pyruvate dehydrogenase [29], leading to accumulation of pyruvate in cells, and the recycling of NAD^+ must be achieved through the reduction of some metabolites. Given the circumstances, a large quantity of lactate was produced by lactate dehydrogenase LdhA via reduction of pyruvate with consumption of NADH molecule [30].

Furthermore, Q3095 strain accumulated acetate 10.33-fold higher than Q3094 strain at D1 stage. In addition to be oxidized through TCA cycle, acetyl-CoA could be converted into acetate by PTA and AckA, in coupling with generation of one ATP molecule [31]. It was demonstrated that the majority of the acetyl-CoA is converted into acetate through the PTA-AckA pathway, and only a minority is metabolized via TCA cycle to generate NADH and CO_2 when *E. coli* grows on glucose aerobically [32]. Therefore, acetate accumulated in medium in large amount. Acetate overflow is caused by an imbalance between the pathways of glycolysis and TCA cycle in rapidly growing cells, and severely decreases the yield of target chemicals from glucose [33, 34].

When the glucose supply is depleted, lactate and acetate can be taken back into the cells and respired. So, the amount of lactate and acetate dramatically decreased at D2 stage. Based on the above, the growth of *E. coli* on glucose was classically a diauxie, consuming glucose in the first half and consuming lactate and acetate in the second half. It was reported that *E. coli* cells grown on glucose produce acetate and consume it after glucose exhaustion, but do not grow on acetate due to the decoupling of acetate anabolism and acetate catabolism, and the growth restores only after prolonged exposure to acetate [35]. Here, it is believed that this glucose-lactate/acetate transition would delay the growth of *E. coli*, and further affect the production of target product.

Another thing that deserves special attention was that the levels of cyclic AMP (cAMP) in Q3095 and Q3094 strains were essentially the same, much lower than that in Q3140 strain grown on acetate at D1 stage. The secondary messenger cAMP is synthesized from ATP, catalyzed by the adenyl cyclase Cya whose activity is controlled by glucose availability [36]. When the preferred carbon source glucose is absent, the intracellular cAMP concentration increases ~ 10-fold, converting the cAMP receptor protein CRP into an active form to activate a number of genes for utilization of carbon sources other than glucose [37]. The low cAMP level in Q3094 strain grown on ethanol indicated that ethanol could be one of preferred carbon sources of *E. coli*.

Production Of Other Acetyl-CoA Derivatives From Ethanol

To test whether ethanol is suitable for production of other acetyl-CoA derived chemicals, the biosynthetic pathways for PG and 3HP from glucose, ethanol and acetate was constructed in *E. coli*, respectively, and comparison of those three carbon sources was carried out. 3HP and PG are both derived from the intermediate malonyl-CoA, which is produced from acetyl-CoA under catalysis of acetyl-CoA carboxylase ACC. Then malonyl-CoA is reduced to 3HP by malonyl-CoA reductase MCR, or catalyzed by polyketide synthase PhID to form PG (Fig. 7A). The theoretical yields of 3HP and PG from various carbon sources were calculated, and those from ethanol were the highest (Fig. 7A).

Then, the 3HP- and PG-producing *E. coli* strains from glucose, ethanol and acetate were constructed, respectively, and were inoculated into minimal medium with corresponding carbon sources. After cultivation in shaking flasks, Q3398 strain grown on ethanol produced 0.50 ± 0.01 g/L 3HP, and Q3433 strain grown on ethanol produced 0.38 ± 0.02 g/L PG, much higher than strains grown on the other two carbon sources. These results demonstrate that ethanol can be used as carbon source for production of acetyl-CoA derived chemicals besides PHB.

Ethanol is a suitable carbon source for production of acetyl-CoA derivatives

In this study, *E. coli* strain growing on ethanol was constructed, and the bioconversion of ethanol into a series of bioproducts including PHB, 3HP and PG was achieved in recombinant *E. coli* strains. Furthermore, metabolome analysis was carried out to discover the differences between metabolism of glucose, ethanol and acetate in engineered *E. coli* strains. All these results suggested that ethanol may be a suitable carbon source for production of acetyl-CoA derived bioproducts.

Firstly, ethanol is an ordinary and inexpensive commodity chemical. Besides fermentation of lignocellulosic biomass [38], ethanol can also be produced from coal by chemical methods. There are three ethanol-producing routes from coal with syngas [39], acetate [40], and dimethyl ether [41] as intermediate, respectively, which were all applied in industrial scale. The capacity of ethanol production from coal has exceeded 2 million tons per year in China. Secondly, the conversion of ethanol into acetyl-CoA presents high atomic economy, in addition to the generation of NADH. However, CO₂ is released in glucose catabolism and ATP is consumed to synthesis acetyl-CoA from acetate (Fig. 1). Therefore, the theoretical yield of target chemical from ethanol is much higher than those from glucose and acetate (Fig. 5A, Fig. 7A and 7B). Furthermore, ethanol has a higher energy density. If oxidized completely to CO₂ and H₂O in bacteria, 0.326 mol of ATP is generated from 1 g ethanol, while 0.178 and 0.133 mol of ATP can be produced from 1 g glucose and acetate, respectively. Moreover, *E. coli* is easy to reach a balance between growth and production when grown on ethanol. As glucose is the favorite carbon source of *E. coli*, it was rapidly metabolized in the first half of cultivation (Fig. 5C) to produce a large amount of lactate and acetate (Fig. 6), leading to a bi-phasic growth which may delay the further bacterial growth and lower the production of target chemical. The assimilation of ethanol was neither as fast as glucose to accumulate by-products, nor as slow as acetate to retard the bacterial growth, helping bacteria reach a balance between growth and production. Additionally, the nature of ethanol, such as low toxicity and high water-solubility, makes it friendly to the bacterial cultivation process.

Although our study showed the feasibility of ethanol to support *E. coli* growth and bioproduction, there are still some problems remaining. The ethanol utilizing gene *adhE_{mut}* was carried by a plasmid vector, leading to addition of antibiotic into medium and increasing of production cost. Besides that, the strain performance may be affected by the strain instability due to plasmid loss. This problem can be dissolved by the integration of *adhE_{mut}* gene into bacterial chromosome. Furthermore, the PHB yield was 0.32 ± 0.02 g/g ethanol in our engineered strain, representing only 34% of the theoretical limit (Fig. 4), and the productions of 3HP and PG from ethanol were both much lower than previous reports from other carbon sources probably due to the lack of overexpression of acetyl-CoA carboxylase [6, 7]. It is necessary to carry out further development to achieve a higher production and yield from ethanol.

Conclusion

In summary, the bifunctional dehydrogenase AdhE A267T/E568K mutant, which converts ethanol into acetyl-CoA, was introduced into *E. coli* strain, conferring *E. coli* the growth capability on ethanol. Several products derived from acetyl-CoA, such as PHB, 3HP and PG, were produced from ethanol by recombinant *E. coli* strains. Compared with glucose and acetate, the strains grown on ethanol presented the highest production and yield, and metabolome analysis revealed the reasons of high yield from ethanol. All these results demonstrate that ethanol is a putative carbon source for production of acetyl-CoA derived bioproducts.

Methods

Bacterial strains and plasmids construction

All bacterial strains, plasmids and primers used in this study were listed in Table 1. The plasmids pACYCDuet-adhE and pACYCDuet-acs were constructed by inserting the adhE gene (accession No. AP_001865.1) and the acs gene (accession No. AP_004570.1) from *E. coli* into pACYCDuet1, respectively. The plasmid pACYCDuet-adhE_{mut} was constructed using Fast Site-Directed Mutagenesis Kit (TIANGEN).

Table 1
Bacterial strains, plasmids, and primers used in this study

Plasmids, strains and primers	Description	Source
Plasmids		
pACYCDuet1	T7 promoter, lacI ^q , p15A ori, Cm ^r	Novagen
pETDuet1	T7 promoter, lacI ^q , pBR322 ori, Amp ^r	Novagen
pET-28a	T7 promoter, lacI ^q , pBR322 ori, Kan ^r	Novagen
pACYCDuet-adhE	lac1-6 promoter, adhE, lacI ^q , p15A ori, Cm ^r	This study
pACYCDuet-adhE _{mut}	lac1-6 promoter, adhE A267T/E568K, lacI ^q , p15A ori, Cm ^r	This study
pACYCDuet-acs	lac1-6 promoter, acs, lacI ^q , p15A ori, Cm ^r	This study
pBAD-Ae-pha	araBAD promoter, phaCAB, Amp ^r , araC, pBR322 ori	[27]
pET-MCRCN	pBR322 ori, lacI ^q , T7 promoter, mcr ₅₅₀₋₁₂₁₉ N940V/K1106W/S1114R, lac ₂₋₅₁ promoter, mcr ₁₋₅₄₉ , Amp ^r	Lab collection
pET28a-phID	trc promoter, phID, lacI ^q , pBR322 ori, Kan ^r	Lab collection
Strains		
E. coli DH5α	F ⁻ , supE44, ΔlacU169, (φ80 lacZ ΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1	Lab collection
E. coli W3110	F ⁻ , λ ⁻ , IN(rrnD-rrnE)1, rph-1	Lab collection
E. coli MG1655 (DE3)	F ⁻ , λ(DE3), ilvG ⁻ , rfb-50, rph-1	Lab collection
Q3352	E. coli W3110/pACYCDuet1	This study
Q3092	E. coli W3110/pACYCDuet-adhE _{mut}	This study
Q3094	E. coli W3110/pACYCDuet-adhE _{mut} /pBAD-Ae-pha	This study
Q3095	E. coli W3110/pACYCDuet1/pBAD-Ae-pha	This study
Q3140	E. coli W3110/pACYCDuet-acs/pBAD-Ae-pha	This study
Q3397	E. coli MG1655 (DE3)/pACYCDuet1/pET-MCRCN	This study
Q3398	E. coli MG1655 (DE3)/pACYCDuet1-adhE _{mut} /pET-MCRCN	This study

Plasmids, strains and primers	Description	Source
Q3399	E. coli MG1655 (DE3)/pACYCDuet1-acs/pET-MCRCN	This study
Q3432	E. coli W3110/ pACYCDuet1/pET28a-phID	This study
Q3433	E. coli W3110/pACYCDuet1-adhE _{mut} /pET28a-phID	This study
Q3434	E. coli W3110/pACYCDuet1-acs/pET28a-phID	This study
Primers	Sequence(5'-3')	Restriction site
adhE-F	CGCGGATCCGATGGCTGTTACTAATGTCGC	BamHI
adhE-R	CCGGAGCTCTTAAGCGGATTTTTTCGCTT	SacI
adhE(A267T)-F	CGCTGTACGTGAACGTTTTACCACCCACGGCGGCTATCTGTT	/
adhE(A267T)-R	AACAGATAGCCGCGTGGGTGGTAAAACGTTACGTACAGCG	/
adhE(E568K)-F	ATCCGGAAACTCACTTCGAAAAACTGGCGCTGCGCTTTATGGA	/
adhE(E568K)-R	TCCATAAAGCGCAGCGCCAGTTTTTCGAAGTGAGTTTCCGGAT	/
acs-F	CGCGGATCCGATGAGCCAAATTCACAAACAC	BamHI
acs-R	CCGGAGCTCTTACGATGGCATCGCGATAGCC	SacI

Protein Expression And Gel Electrophoresis Analysis

For the expression of recombinant proteins, single colonies of E. coli W3110 harboring recombinant plasmids were used to inoculate Luria–Bertani (LB) medium containing appropriate antibiotics and grown at 37 °C overnight. The culture was diluted 1:100 into fresh LB medium and incubated under the same conditions. When the OD₆₀₀ reached about 0.6, the protein expression was induced by 100 μM IPTG and growth was continued for 12 h at 30 °C. Cells were collected by centrifugation and suspended in 100 μL 1 × SDS sample buffer, heated at 100 °C for 10 min and then analyzed by SDS-PAGE.

Shaking Flask Cultivation

Shaking flask experiments were carried out in triplicate in 250 mL flasks containing 50 mL medium with appropriate antibiotics. For the production of PG and PHB, the medium contains 9.8 g/L K₂HPO₄·3H₂O, 2.1 g/L C₆H₈O₇·H₂O, 0.3 g/L ammonium ferric citrate, 3 g/L (NH₄)₂SO₄. For the production of 3HP, the medium contains 14 g/L K₂HPO₄·3H₂O, 5.2 g/L KH₂PO₄, 1 g/L NaCl, 1 g/L NH₄Cl, 0.25 g/L MgSO₄·7H₂O, 0.2 g/L yeast extract. E. coli strains were grown overnight at 37 °C with shaking in LB broth, and then 1:50 diluted into 50 mL minimal medium supplemented with corresponding carbon sources. When the OD₆₀₀ reached about 0.6, 100 μM IPTG and 1 g/L L-arabinose was added and the strains are further incubated

48 h at 30 °C. Samples were taken out to determine the OD₆₀₀, the concentrations of PHB, 3HP, PG, and residual carbon sources.

CDW And PHB Content Analysis

E. coli cells were harvested with centrifugation at 5000 × g for 15 min, and washed with distilled water twice. To determine CDW, cell pellets were lyophilized, and the CDW was gravimetrically determined. P3HP was extracted from lyophilized cells with hot chloroform in a Soxhlet apparatus, and precipitated by icecold ethanol as described [42].

Quantification Of 3HP, PG, Ethanol, Acetate And Glucose

Concentrations of 3HP, ethanol, acetate and glucose in medium were determined using an Agilent 1200 Infinity HPLC system equipped with an Aminex HPX-87H (Bio-Rad, Hercules, CA) column (300 × 7.8 mm). The concentration of PG was determined using the colorimetric reaction between cinnamaldehyde and PG as previously described [21].

Metabolite Measurements

Metabolites were extracted from seven individual Bacteria samples. Bacteria samples were taken 200 µL, then added 800 µL pre-cooled methanol/acetonitrile (1:1, v/v), and mixed in vortex. After sonication for 20 min on ice, the mix was stored at -20 °C for 1 h to precipitate proteins. The mix was centrifuged for 15 min (13000 rpm, 4 °C) and supernatant was dried by a vacuum drying system. A targeted metabolic analysis was performed using an LC-MS/MS system. The dried metabolites were dissolved in 100 µL of acetonitrile/ H₂O (1:1, v/v) and centrifuged (14000 × g) for 20 min. Electrospray ionization was conducted with an Agilent 1290 Infinity chromatography system and AB Sciex QTRAP 5500 mass spectrometer. NH₄COOH₄ (10 mM) and acetonitrile were used as mobile phases A and B, respectively. The sample was placed in a 4°C automatic sampler with a column temperature of 45°C, a flow rate of 300 µL/min and an injection volume of 2 µL. A binary solvent gradient was used as follows: A, NH₄COOH; B, 0–18 min at 90–40% acetonitrile; 18-18.1 min at 40–90% acetonitrile; and 18.1–23 min at 90% acetonitrile. The LC-MS/MS was operated in the negative mode under the following conditions: source temperature, 450 °C; ion source gas 1, 45; ion source gas 2, 45; curtain gas, 30; and ion spray voltage floating, -4500 V.

List Of Abbreviations

PG, phloroglucinol; 3HP, 3-hydroxypropionate; ACS, AMP-forming acetyl-CoA synthetase; PHB, polyhydroxybutyrate; CDW, cell dry weight; cAMP, cyclic AMP; TCA cycle, tricarboxylic acid cycle.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated during the present study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GZ and MX conceived and designed the study. SS, YD, and ML performed the strain construction, fermentation, and metabolome analysis. SS, LM, MX, and GZ analyzed the data. SS and GZ wrote the manuscript. All authors read and approved the final manuscript.

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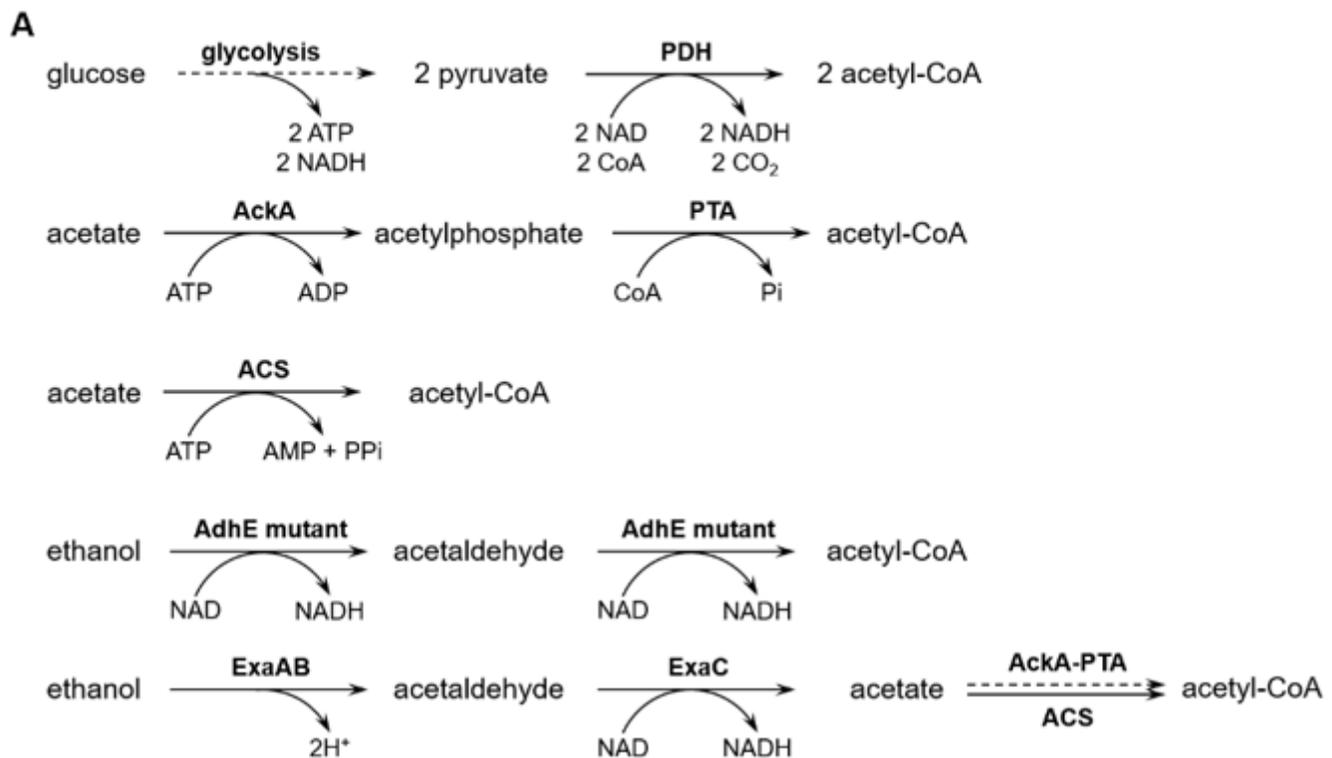
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Figures



B

substrate	enzymes	NADH yield (mol/mol acetyl-CoA)	ATP yield (mol/mol acetyl-CoA)	CO ₂ release (mol/mol acetyl-CoA)
glucose	glycolysis pathway and pyruvate dehydrogenase	2	1	1
acetate	AckA and PTA	0	-1	0
acetate	ACS	0	-2	0
ethanol	AdhE A267T/E568K	2	0	0
ethanol	ExaABC and ACS ExaABC and AckA-PTA	1	-1 (AckA-PTA) -2 (ACS)	0

Figure 1

Production of acetyl-CoA from various carbon resource. (A) Different metabolic routes for acetyl-CoA production from glucose, acetate or ethanol. The solid lines represent single step, and dotted line represent multiple steps. (B) Comparison of these pathways. The enzymes involved are as follows: PDH, pyruvate dehydrogenase; AckA, acetate kinase; PTA, phosphotransacetylase; ACS, AMP-forming acetyl-CoA synthetase; AdhE, bifunctional acetyl-CoA reductase; ExaA, quinoprotein ethanol dehydrogenase; ExaB, cytochrome c550; ExaC, NAD⁺-dependent acetaldehyde dehydrogenase.

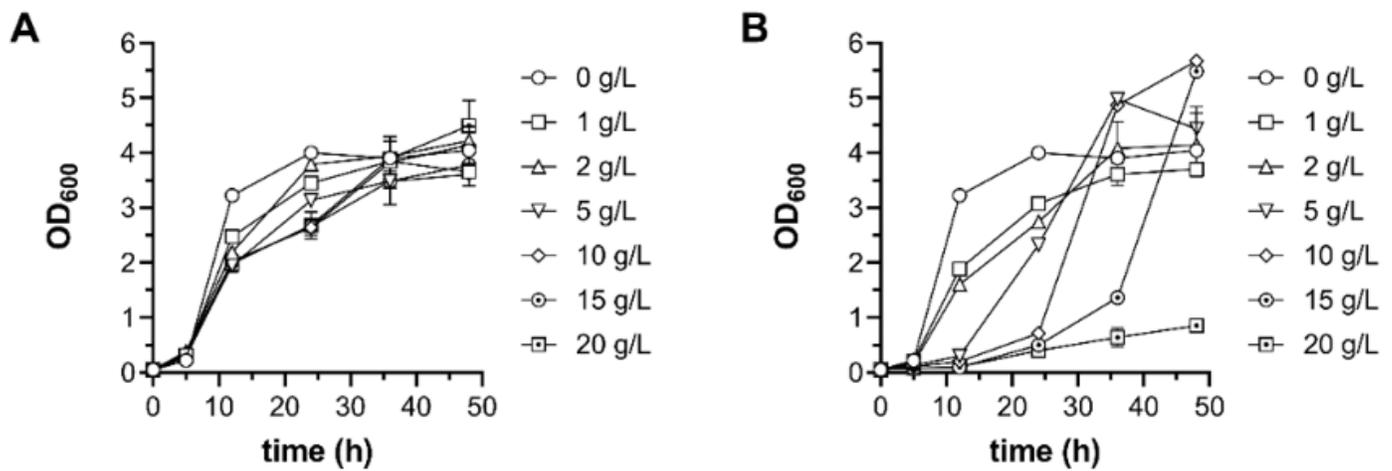


Figure 2

Growth of *E. coli* W3110 strain in minimal medium supplemented with 10 g/L glucose and different concentration of ethanol (A) and acetate (B). When acetate was added, pH was adjusted to 7.0 with sodium hydroxide. This experiment was carried out in duplicate, and data represent mean \pm SEM.

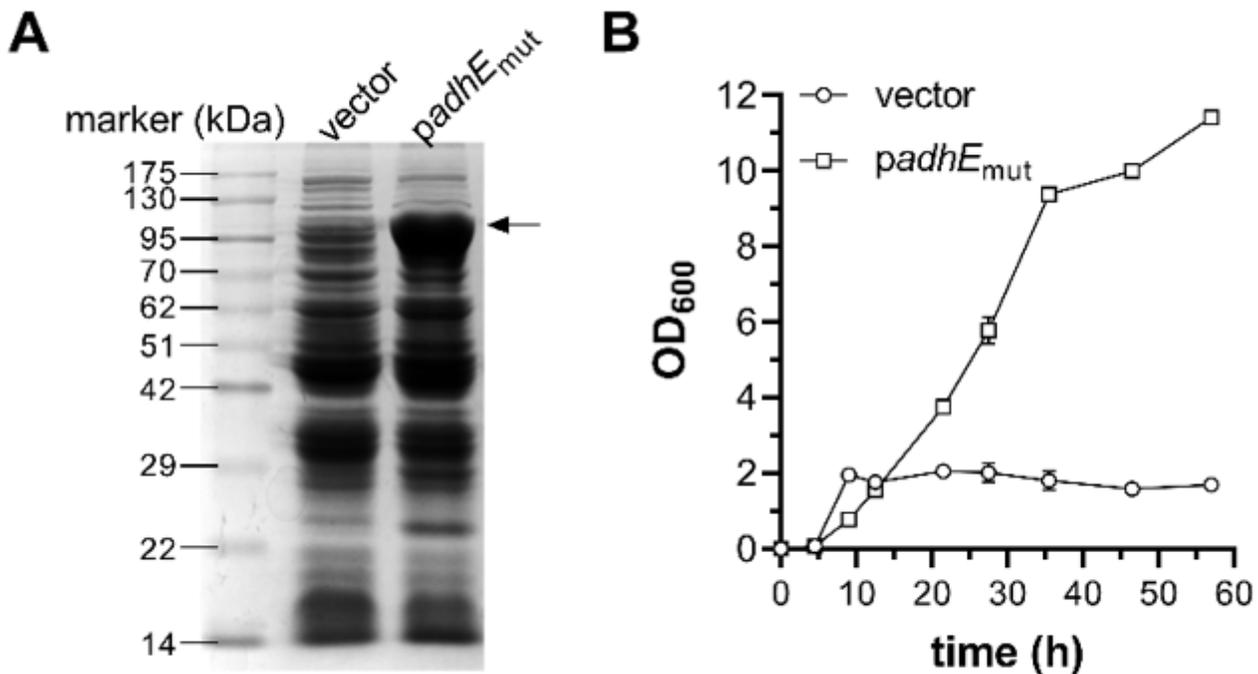


Figure 3

Construction of *E. coli* strain growing on ethanol as sole carbon source. (A) Expression of acetyl-CoA reductase AdhE mutant in *E. coli* cells. Crude cell extracts of *E. coli* W3110 strains carrying empty vector

(Q3352) or recombinant plasmid for overexpression of AdhE A267T/E568K mutant (Q3092) were used. The position of overexpressed AdhE mutant (98 kDa) is indicated by arrow. (B) Growth profile of these strains in minimal medium supplemented with 1 g/L glucose and 10 g/L ethanol. This experiment was carried out in triplicate, and data represent mean \pm SEM.

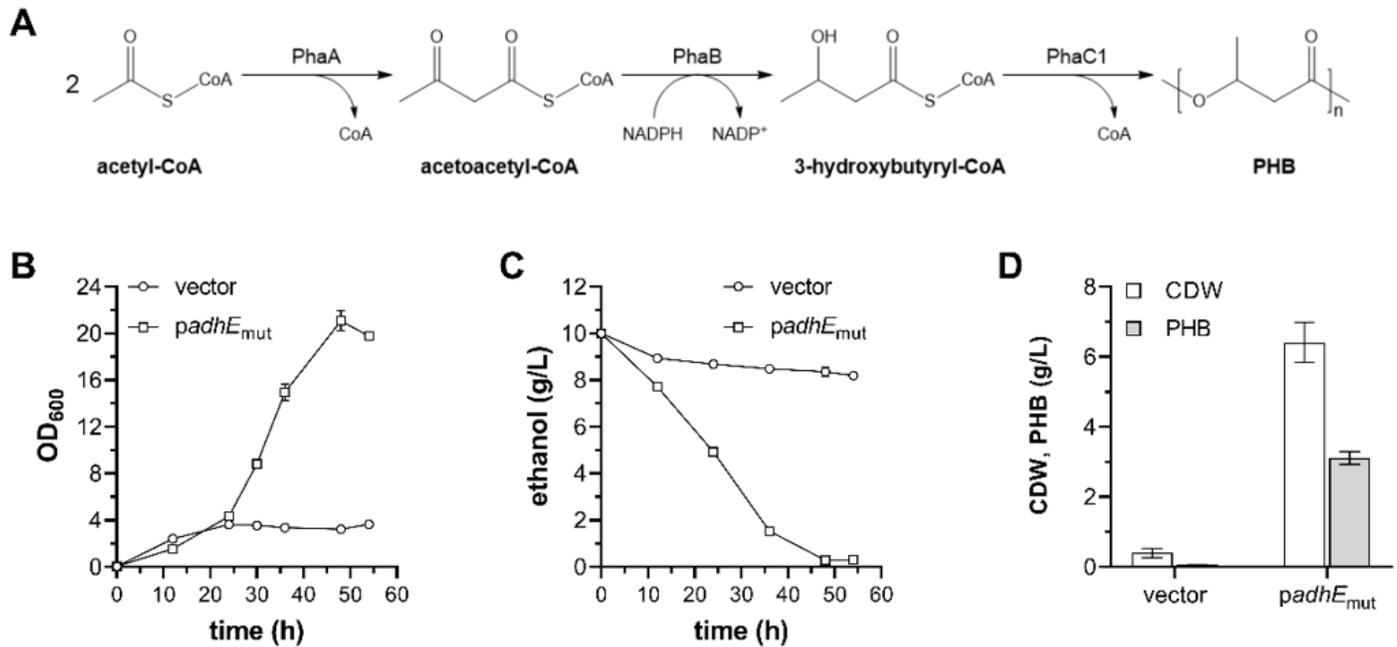


Figure 4

PHB production of engineered *E. coli* W3110 strain with ethanol as sole carbon source. (A) PHB biosynthetic pathway from acetyl-CoA. PhaA, 3-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC1, PHA synthase. (B) The growth profile, (C) residual ethanol in medium, and (D) cell dry weight (CDW) and PHB production of *E. coli* W3110 strains carrying PHB-producing plasmid pBAD-Ae-pha in addition to the empty vector (Q3095) or AdhEmut plasmid (Q3094). Minimal medium used in these experiments is supplemented with 1 g/L glucose and 10 g/L ethanol. These experiments were carried out in triplicate, and data represent mean \pm SEM.

A

Glucose \rightarrow PHB + 2 ATP + 3 NADH + 2 CO₂

2 Ethanol \rightarrow PHB + 3 NADH

2.8125 Acetate \rightarrow PHB + 1.625 CO₂

theoretical yield (g/g)

0.478

0.935

0.510

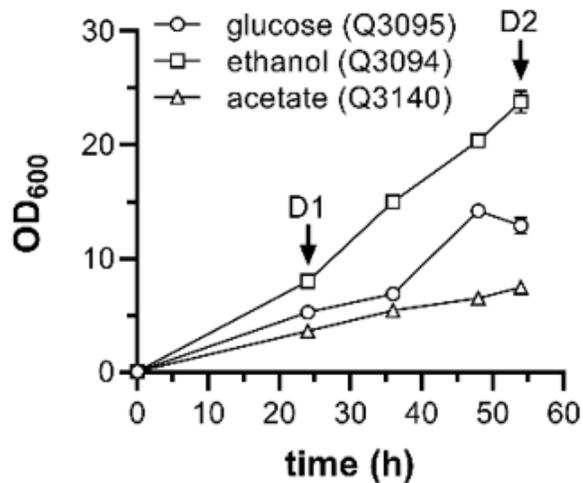
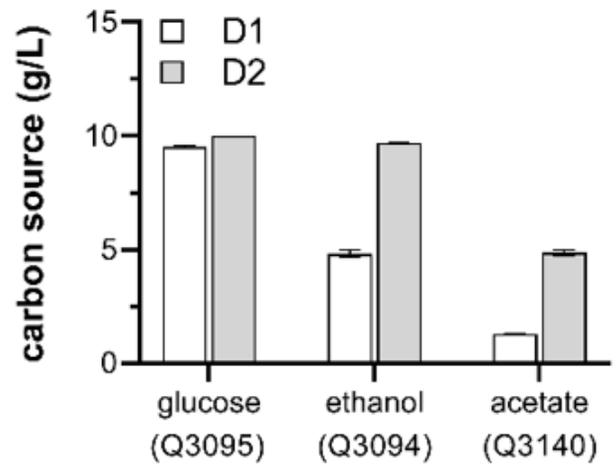
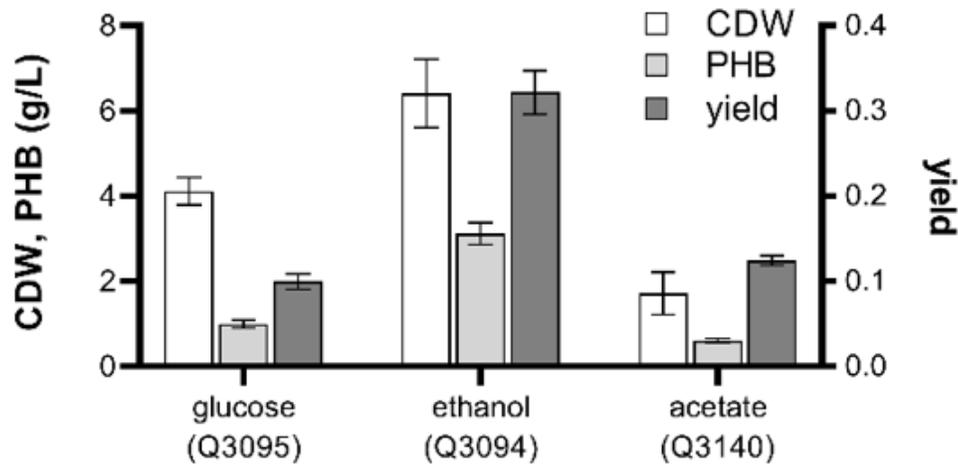
B**C****D**

Figure 5

PHB production from glucose, ethanol and acetate by engineered *E. coli*. (A) Theoretical yield of PHB from different carbon sources. (B) Growth curve. (C) Carbon source consumed by various strains at D1 and D2 stage in Fig 5B. (D) Cell dry weight, PHB production and yield by different *E. coli* strains. Three strains carrying PHB-producing plasmid pBAD-Ae-pha in addition to the empty vector (Q3095), AdhE plasmid (Q3094), and ACS plasmid (Q3140) were grown with glucose, ethanol, and acetate as carbon source, respectively. For Q3095 and Q3094, 10 g/L glucose and 10 g/L ethanol are supplemented in

addition of 1 g/L glucose, respectively. For Q3140, 1 g/L glucose and 2 g/L acetate were added into the medium at 0 h, and 3 g/L acetate was added at D1 stage. These experiments were carried out in triplicate, and data represent mean \pm SEM.

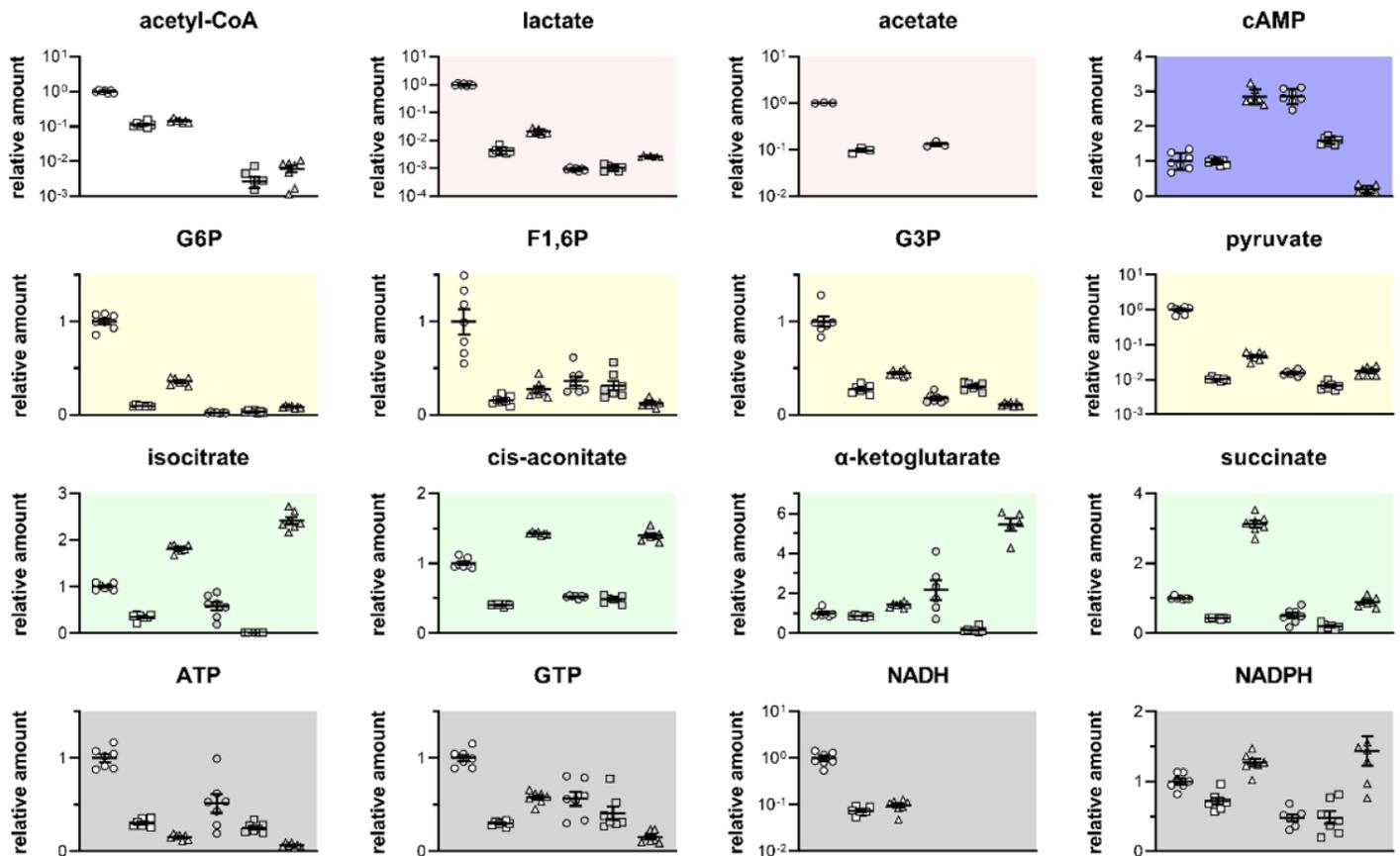


Figure 6

Relative quantification of metabolites in different strains at D1 and D2 stages in Fig 5B. Circle, Q3095 grown on glucose; square, Q3094 grown on ethanol; triangle, Q3140 grown on acetate; open symbol, D1 stage; filled symbol, D2 stage. Background colors represent the categories of corresponding metabolite. Yellow, glycolysis intermediate; green, TCA cycle intermediate; grey, energy and reducing power; pink, by-product; purple, secondary messenger. G6P, glucose-6-phosphate; F1,6P, fructose 1,6-biphosphate; G3P, 3-phospho-glycerate. These experiments were carried out in septuplicate, and data represent mean \pm SEM. The acetate production of Q3140 strain cannot be determined as acetate was added as carbon source.

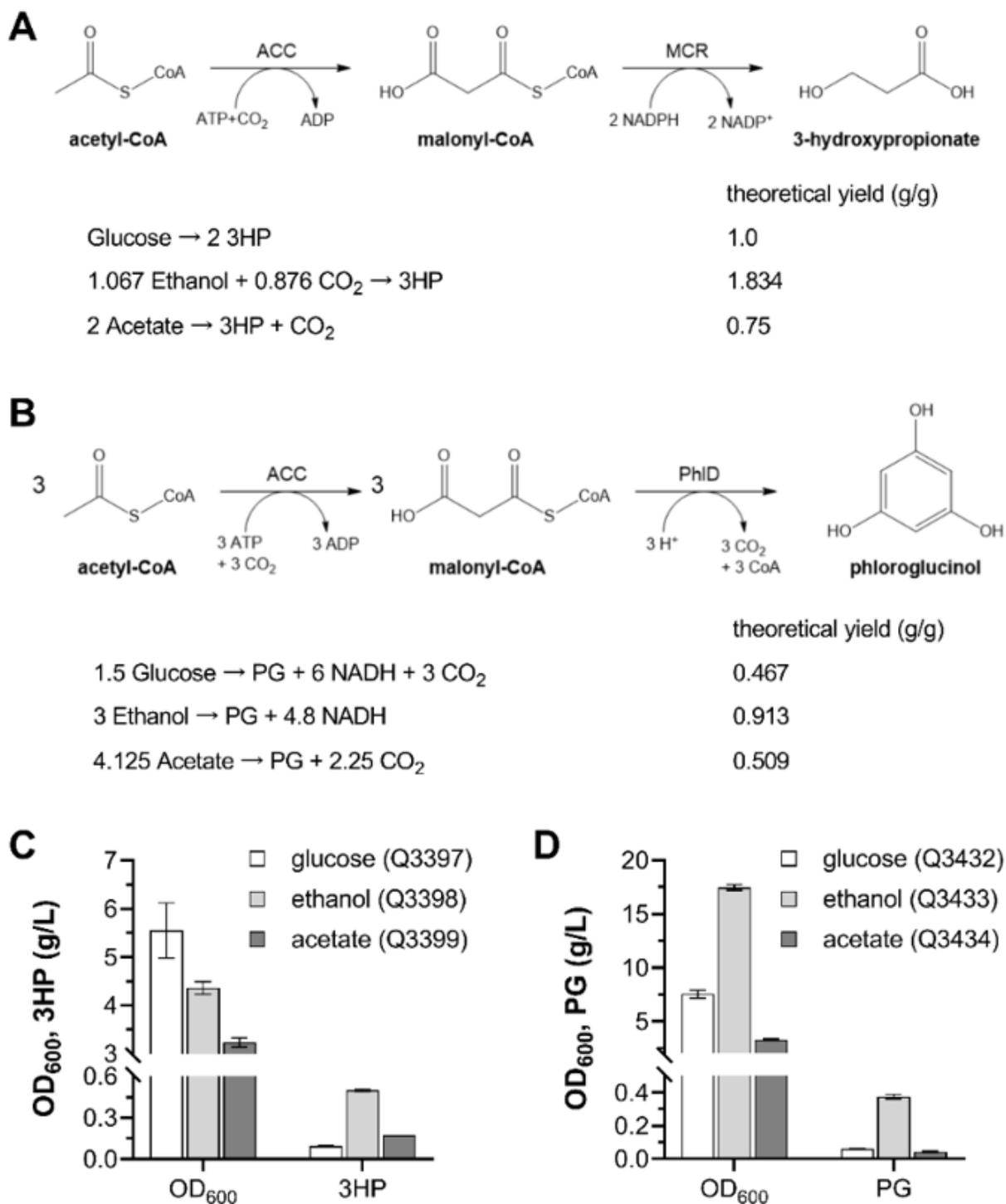


Figure 7

Production of 3HP and PG from various carbon sources by engineered *E. coli* strains. (A) 3HP biosynthetic pathway and theoretical yield from different carbon sources. (B) PG biosynthetic pathway and theoretical yield from different carbon sources. (C) Cell density and 3HP production when grown with glucose, ethanol and acetate as sole carbon source. (D) Cell density and PG production when grown with glucose, ethanol and acetate as sole carbon source. ACC, acetyl-CoA reductase; MCR, malonyl-CoA

reductase; PhID, polyketide synthase. These experiments were carried out in triplicate, and data represent mean \pm SEM.