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# Coupled synthetic pathways improve the production of 3-hydroxypropionic acid in recombinant *Escherichia coli* strains

Dafeng Zhou [a,1], Diego Leandro Quiroga-Sánchez [a,1], Xuan Zhang [b], Yanhong Chang [b,c,**], Hui Luo [a,*]

[a] Department of Biological Science and Engineering, University of Science and Technology Beijing, Beijing, 100083, China
[b] Department of Environmental Engineering, University of Science and Technology Beijing, Beijing, 100083, China
[c] Beijing Key Laboratory of Resource-oriented Treatment of Industrial Pollutants, Beijing, 100083, China

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ABSTRACT

To improve the production of 3-HP with glucose as a substrate, the malonyl-CoA and propionyl-CoA pathways were coupled to regulate $NADP^+$/NADPH regeneration in the recombinant *E. coli*. The strain *Ec*-AM that overexpressed the key enzymes of the malonyl-CoA pathway, acetyl CoA carboxylase (ACC) from *Ustilago maydis* and malonyl CoA reductase (MCR) from *Chloroflexus aurantiacus*, produced 0.26 g/L of 3-HP in 25-h shake flask cultivation. The strain *Ec*-P overexpressing the key enzyme of the propionyl-CoA pathway, propionyl-CoA dehydrogenase (PACD) from *Candida rugosa*, produced 0.11 g/L of 3-HP. However, 3-HP titer of the strain *Ec*-PAM overexpressing PACD along with ACC and MCR, via two pathways cooperation, was 1.29 g/L. The addition of biotin and bicarbonate improved the 3-HP production of the strain *Ec*-PAM. 3-HP titer of strain *Ec*-ΔY-ΔP-PAM with double deletion of *ygfH* (encoding propionyl-CoA: succinate-CoA transferase) and *prpC* (encoding methylcitrate synthase) genes reached 1.94 g/L, which was 1.5-fold higher than that of the strain *Ec*-PAM cultured under the same conditions.

## 1. Introduction

In recent years, with the growing scarceness of non-renewable resources such as oil, researches around the world had turned their attention to production of renewable resources by biological methods. At the moment, 3-hydroxypropionic acid (3-HP) is one of the most promising chemical intermediates[1]; it has both, a hydroxyl and a carboxyl functional groups, and is the precursor of many important chemicals, such as 1,3-propanediol, special polyester and acrylic materials. 3-HP can also be used for production of water treatment chemicals, coatings, adhesives and personal care products.[2]

3-HP can be found in nature as a metabolic intermediate during the process of $CO_2$ fixation in some bacteria.[3] It can also be accumulated as an end product from glycerol, glucose, propionic acid, xylose, acrylic acid or acetate.[4–10] 3-HP exists as an intermediate metabolite in certain metabolic pathways, but in general, is not essential for microbial growth, so it cannot be highly accumulated by the cell. If there is excessive accumulation of 3-HP, it will destroy the metabolic balance of microbial cells.[11] In order to improve the strains for 3-HP accumulation it is required to develop specially designed recombinant strains.

In recent years, different routes for production of 3-HP had been proposed.[6,12] Among them, glycerol, malonyl-CoA and beta-alanine pathway are widely studied, and glycerol and glucose are the two main substrates for the synthesis of 3-HP.[13,14] Mutants of *Candida rugosa* could produce 40.2 g/l of 3-HP from propionate,[9] which makes it of great research value to produce 3-HP through the propionyl-CoA pathway in recombinant *E. coli*.[15] 3-HP production via the propionyl-CoA pathway requires few enzymes to produce 3-HP. Among them, propionyl-CoA dehydrogenase (PACD, EC:1.3.8.7)[9,16] is the essential enzyme for this pathway, which converts propionyl-CoA into acryloyl-CoA and simultaneously consumes one mol of $NADP^+$ to generate one mol of NADPH. Besides, propionate CoA-transferase (PCT, EC:2.8.3.1) and 3-hydroxypropionyl-CoA dehydratase (HPCD, EC:4.2.1.17) are necessary for the pathway, but *E. coli* possesses

* Corresponding author.
** Corresponding author. Department of Environmental Engineering, University of Science and Technology Beijing, Beijing 100083, China.
*E-mail addresses:* yhchang@ustb.edu.cn (Y. Chang), luohui@ustb.edu.cn (H. Luo).
[1] These authors contributed equally to this work.

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endogenous genes encoding PrpE (propionyl-CoA synthetase) and enoyl-CoA hydratase, which are functionally similar to PCT and HPCD, respectively (Fig. 1).[15] Therefore, introducing PACD alone is enough to establish the propionyl-CoA pathway in *E. coli*. In addition, the deletion of *ygfH* gene (encoding propionyl-CoA: succinate CoA-transferase) and *prpC* gene (encoding methylcitrate synthase) in the competitive pathways led in the increase of 3-HP titer.[15]

The malonyl-CoA pathway is one of the most promising methods to produce 3-HP from glucose[17–22] (Fig. 1). Carboxylation of acetyl-CoA into malonyl-CoA occurs in the presence of acetyl-CoA carboxylase (ACC), and then malonyl-CoA reductase (MCR) uses malonyl-CoA as a substrate to form 3-HP.[22] In malonyl-CoA pathway, the cofactor NADPH is necessary for the reduction of malonyl-CoA and its regeneration may be a limiting factor of this pathway.[23] Interestingly, for the propionyl-CoA pathway, NADPH is a byproduct in the oxidation of propionyl-CoA to produce acryloyl-CoA.[15] Thus, the combination of these two pathways into a recombinant strain would be promising for the increment of 3-HP titer due to the *in vivo* regeneration of NADPH (Fig. 1).

In this work, the genes PACD along with ACC and MCR were obtained from different bacterial species, and the recombinant strains containing the single or combining pathways of the propionyl-CoA pathway and the malonyl CoA pathway for 3-HP production were constructed. The comparisons of these recombinant strains in the production of 3-HP were investigated. The effects of biotin and sodium bicarbonate on 3-HP accumulation were also discussed. In order to further improve 3-HP titer of the recombinant strains, the genes of competitive pathways were deleted to increase the propionyl-CoA pool in the strain, and 3-HP accumulation of the resultant strains was investigated.

## 2. Materials and methods

### 2.1. Materials

The genomic DNA isolation kit was purchased from Tiangen Co. Ltd. (Beijing, China). The DNA gel purification kit was purchased from Promega (USA). The restriction endonucleases and DNA-modifying enzymes were obtained from Takara Co. Ltd. (Dalian, China). Primers were synthesized by Sunbiotech Co., Ltd. (Beijing, China). 3-HP was purchased from Tokyo Chemical Industry Co., Ltd. Propionic acid and all other chemicals and enzymes, unless otherwise indicated, were obtained from Biodee Co., Ltd. (Beijing, China).

### 2.2. Cloning of acc, mcr and pacd genes

Bacterial strains, along with the plasmids used in this study are summarized in Table 1. Genetic engineering, protein expression and harvesting of the strains were performed using Lysogeny Broth (LB) medium containing a final concentration of 50 mg/L kanamycin (Kan), 50 mg/L ampicillin (Amp) and 25 mg/L chloramphenicol (Cm). Gene manipulations were carried out using standard methods.[24] To express the *pacd* gene from *C. rugosa*, the pACYCDuet-1 vector with the T7 promoter was adopted.[15] A *acc* gene was cloned from the genomic DNA

**Table 1**
Bacterial strains and plasmids used in the study.

| Strains and plasmids | Description | Source |
|---|---|---|
| Strains | | |
| *C. aurantiacus* | Source for the *mcr* gene | CAS, China |
| *E. coli* BL21(DE3) | Expression host | Novagen |
| *E. coli* JM109 (DE3) | Expression host | Tiangen |
| *Ec-ΔY* | *E. coli* JM109(DE3) *ΔygfH* | Reference[15] |
| *Ec-ΔP* | *E. coli* JM109(DE3) *ΔprpC* | Reference[15] |
| *Ec-ΔY-ΔP* | *E. coli* JM109(DE3) *ΔygfH, ΔprpC* | Reference[15] |
| *Ec*-P | Recombinant *E. coli* BL21(DE3) harboring plasmid pACYCDuet-1-PACD | Reference[15] |
| *Ec*- AM | Recombinant *E. coli* BL21(DE3) harboring plasmids pCS11 and pBAD18-MCR | This study |
| *Ec*- PAM | Recombinant *E. coli* BL21(DE3) harboring plasmids pACYCDuet-PACD, pCS11 and pBAD18-MCR | This study |
| Plasmids | | |
| pET-28a | *lacI*, expression vector, T7 promoter, PBR322-ori, kanamycin (Kan)$^r$ | Novagen |
| pBAD18 | expression vector, arabinose PBAD promoter, Amp$^r$ | Reference[26] |
| pCS11 | *acc* in pET28a vector, Kan$^r$ | Reference[25] |
| pACYCDuet-PACD | *pacd* in pACYCDuet-1 vector, Cm$^r$ | Reference[15] |
| pBAD18-MCR | *mcr* in pBAD18 vector, Amp$^r$ | This study |

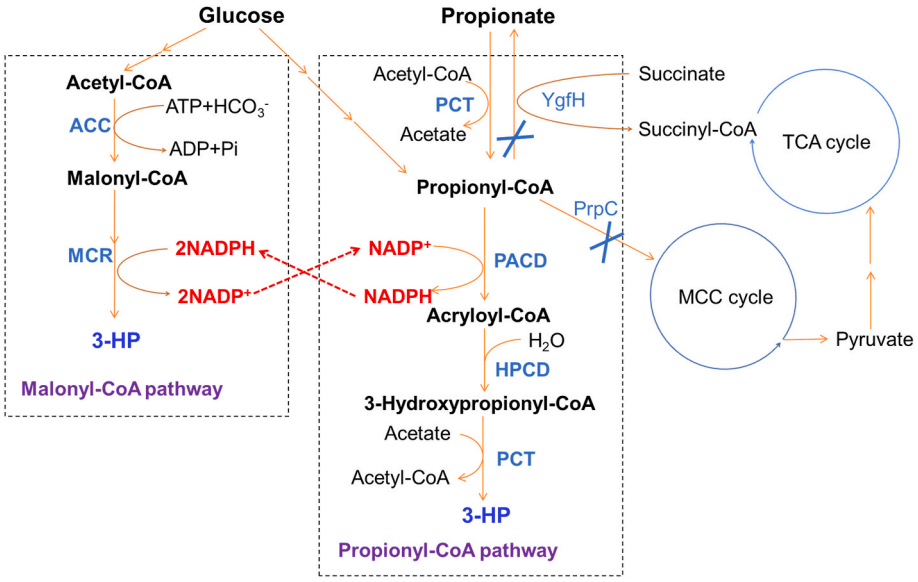


**Fig. 1.** The production of 3-HP involving malonyl-CoA pathway and propionyl-CoA pathway. ACC: acetyl-CoA carboxylase; MCR: malonyl-CoA reductase; PCT: propionate CoA-transferase; PACD: propionyl-CoA dehydrogenase; HPCD: 3-hydroxypropionyl-CoA dehydratase; YgfH: propionyl-CoA: succinate-CoA transferase; PrpC: methylcitrate synthase; MC cycle: methylcitrate cycle; TCA cycle: tricarboxylic acid cycle.

of a fungus *U. maydis*, and then ligated in pET30a vector to construct a recombinant plasmid pCS11.[25]

The *mcr* gene was obtained by PCR amplification form the genomic DNA of *C. aurantiacus* using the primers 5′-CTCGGATCCATGAGCGGAACAGGACGACT-3′ and 5′-CCTAAGCTTACACGGTAATCGCCCGTCC-3’. The PCR products were digested with *Bam*H1 and *Hin*d III and then ligated to the pET-28a vector. The obtained plasmid pET-MCR was further digested by *Xba* I and *Hin*d III, and the *mcr* gene was then subcloned into pBAD18 vector to construct the recombinant plasmid pBAD18-MCR. The gene sequences of *pacd, acc* and *mcr* could be found in Supplementary Information.

The plasmids pCS11, pBAD18-MCR and pACYCDuet-PACD were transformed into *E. coli* BL21(DE3) individually or in combination to create the 3 strains used in this study: *Ec*-AM (pCS11, pBAD18-MCR), *Ec*-P (pACYCDuet-PACD) and *Ec*-PAM (pACYCDuet-PACD, pCS11, pBAD18-MCR), for protein expression and 3-HP production assessment. The plasmids pACYCDuet-PACD, pCS11 and pBAD18-MCR were also the transformed into three gene-deleted host cells (*Ec-ΔY*, *Ec-ΔP* and *Ec-ΔY-ΔP*, which were constructed in our previous work[15]) to construct the recombinant strains *Ec-ΔY*-PAM, *Ec-ΔP*-PAM and *Ec-ΔY-ΔP*-PAM.

### 2.3. Protein expression and gel electrophoresis

To verify that the process of modification of the strain was successful, the strain *Ec*-PAM was cultivated in LB medium and induced at 25 °C, 30 °C and 37 °C with 100 μM IPTG at 0.6 $OD_{600}$. The cells were harvested after 12 h induction, and centrifuged at 10,000 g at 4 °C for 15 min. The cell pellets were washed twice and then re-suspended in 100 mM potassium phosphate buffer (pH 7.0). The cells were lysed and then centrifuged at 10,000 g for 20 min. The supernatants were used for SDS-PAGE which examined protein expression.[24]

### 2.4. Cultivation of recombinant E. coli strains

Unless indicated otherwise, shake flask cultivation was carried out with a 20 mL working volume in 100 ml Erlenmeyer flasks at 37 °C in an incubator shaker at 200 rpm. The following medium was used in this study: peptone 1%, yeast extract 0.5%, NaCl 1%, pH 7.0. And Cm at 25 mg/L was added to the strain *Ec*-P. For *Ec*-AM, Kan and Amp at 50 mg/L, were added in the medium. And for *Ec*-PAM, Kan, Amp and Cm at 50 mg/L, 50 mg/L, and 25 mg/L, respectively, were added to the medium. The recombinant strains were cultured in the above mediums and induced at 0.6 $OD_{600}$ with 100 μM IPTG. For *Ec*-AM and *Ec*-PAM, in addition to 100 μM IPTG, 6.7 mM arabinose was added. After 12 h induction, 0.1% propionate and 0.4% glucose were added to the culture broth of *Ec*-P and *Ec*-PAM and the culture temperature was raised to 37 °C. Since the cultivation was carried out in shake flask and the pH was not controlled, thus low-concentration carbon sources were added to the medium to prevent a drastic drop of pH value during the cultivation. Each sample was withdrawn periodically to determine the concentrations of 3-HP during a period of 25 h. For the gene deletion strains *Ec-ΔY*-PAM, *Ec-ΔP*-PAM and *Ec-ΔY-ΔP*-PAM, the same culture condition as *Ec*-PAM were adopted. The yield (g/g) was calculated by dividing the produced 3-HP by the total carbon source (propionic acid and glucose).

To investigate the impact of biotin or sodium bicarbonate on 3-HP production, the recombinant strain *Ec*-PAM was cultured and induced in LB medium containing antibiotics Kan, Amp and Cm at 25 °C for 12 h. Then, 0.1% propionate and 0.4% glucose were added to the culture broth. At the same time biotin was added at different final concentrations 0 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L, 30 mg/L and cultured at 37 °C. While for sodium bicarbonate optimization, after the induction of *Ec*-PAM, sodium bicarbonate was added at the concentration of 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L, 2 g/L, 2.5 g/L. Each sample was periodically taken to analyze the final concentration of 3-HP by HPLC.

### 2.5. Analytical methods

The concentrations of 3-HP and the by-products were assayed by HPLC. Supernatants, obtained by centrifugation of culture samples at 10,000 g for 10 min, were filtered through 0.22 μm filter and isocratically eluted through a Venusil MP C18 column (4.6 mm × 250 mm, 5 μm particle size, Agela Technologies Inc.) with methanol-10 mM $H_3PO_4$ (5:95, v/v) as the mobile phase. The flow rate and the detection wavelength were selected at 1 ml/min and UV 208 nm, respectively. The samples were assayed for three times and the standard deviation of the measurements was less than 5% for 3-HP.

## 3. Results and discussion

### 3.1. Expression of ACC, MCR and PACD in recombinant E. coli

In order to enhance the production of 3-hydroxypropionic acid, three genes (ACC, MCR and PACD) were overexpressed in *E. coli*. For this purpose, three strains with different 3-HP synthetic pathways were studied. (i) Strain *Ec*-P produced 3-HP via the propionyl-CoA pathway by overexpressing PACD from *C. rugose*. (ii) Strain *Ec*-AM produced 3-HP via the malonyl-CoA pathway involving two enzymes: ACC from *U. maydis* and MCR from *C. aurantiacus*. (iii) To investigate the effect of the overexpression of these three genes (PACD, ACC, MCR) on the production of 3-HP, a strain *Ec*-PAM coupling the propionyl-CoA pathway and the malonyl-CoA pathway was engineered.

The expression of the strain *Ec*-PAM with IPTG induction was carried out at 25 °C, 30 °C and 37 °C respectively, and the enzyme expression was assessed by SDS-PAGE. Fig. 2 shows the expression profile for the recombinant enzymes. The extract of *Ec*-PAM showed the presence of the ACC enzyme with a molecular weight of 225 kDa, MCR with a molecular weight of 145 kDa and PACD with a molecular weight of 49 kDa. The soluble expression level of the enzymes was higher at 25 °C under shake flask fermentation conditions, suggesting that culture temperature of 25 °C was beneficial to the production of 3-HP.

### 3.2. Shake flask fermentation of the strains for production of 3-HP

With the intention to evaluate the production of 3-HP via the propionyl-CoA pathway and the malonyl-CoA pathway, the recombinant strains *Ec*-AM, *Ec*-P and *Ec*-PAM were cultured and induced as described above. When ACC and MCR were expressed together, after 25 h of fermentation, the strain *Ec*-AM produced a 3-HP titer of 0.26 g/L (Fig. 3A). When PACD was expressed alone, the strain *Ec*-P produced

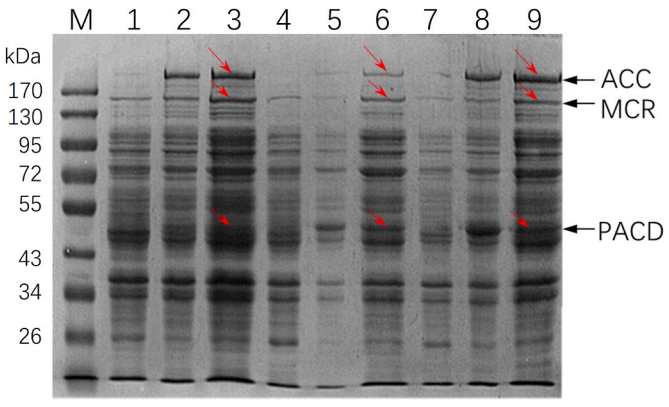


**Fig. 2.** SDS-PAGE analysis of the Ec-PAM. (Lane M) protein standard, (Lane 1, 4, 7) whole cell samples of the cell cultured without inducer at 25 °C, 30 °C and 37 °C, (Lane 2, 5, 8) whole cell samples of cell cultured with inducer at 25 °C, 30 °C and 37 °C, (Lane 3, 6, 9) supernatant samples of cell cultured with inducer at 25 °C, 30 °C and 37 °C. The arrow marks indicate the corresponding target proteins.

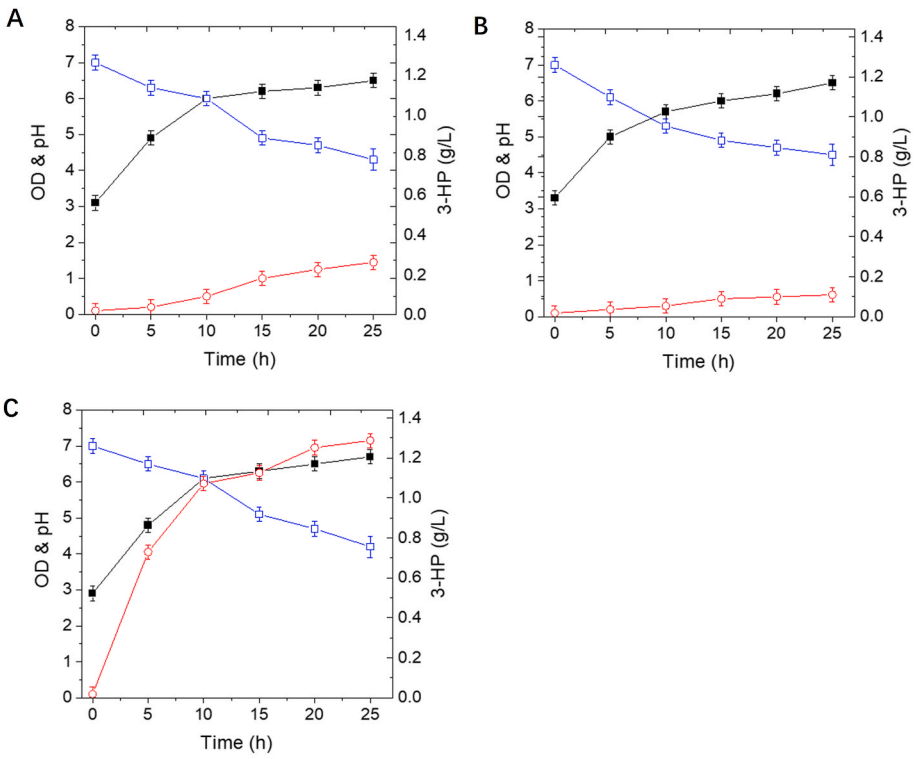


**Fig. 3.** Time-course profile of production of 3-HP for the strains *Ec*-P, *Ec*-AM and *Ec*-PAM. (A) *Ec*-P (expressing PACD) (B) *Ec*-AM (expressing ACC and MCR) and (C) *Ec*-PAM (expressing PACD, ACC and MCR). Symbols: 3-HP (circle), cell density (square) and pH (triangle). When optical density of the broth was around 0.6, the cell was induced at 25 °C for 12 h, and then the substrates of propionate and glucose were added to the culture. The experiment was done in triplicate. The error bars show standard errors.

0.11 g/L 3-HP (Fig. 3B). When the 3 genes were expressed together, 3-HP titer of *Ec*-PAM was 1.29 g/L (Fig. 3C), which was 4.9-fold and 11.7-fold higher than that of *Ec*-AM and *Ec*-P, respectively. This shows that the introduction of the ACC and MCR genes, along with PACD expression significantly improved the accumulation of 3-HP in the engineered strain.

When the malonyl-CoA pathway was introduced alone in *E. coli*, the expression of ACC and MCR did not result in a high production of 3-HP (0.26 g/L). However, 3-HP titer of 0.26 g/L was still higher than that of the recombinant *E.coli* expressing MCR of *C. aurantiacus* and ACC of *E. coli* (1.6 mM) in literature,[23] which might be due to the higher activity of ACC from fungus *U. maydis* than that of the prokaryotic ACC from *E. coli*.[26] Since most of the acetyl-CoA is metabolized via the tricarboxylic acid (TCA) cycle,[23] the malonyl-CoA pathway is competing with the TCA cycle for acetyl-CoA. Thus, a higher activity of ACC is crucial to improve the efficiency of the malonyl-CoA pathway. In addition, the reaction requires 2 mol of the reduced cofactor NADPH to convert 1 mol malonyl-CoA into 3-HP,[6,27] the low production of 3-HP by this strain can also be explained as a result of lack of NADPH.

Another potential pathway for production of 3-HP is the propionyl-CoA pathway.[15] One of the key enzymes of this pathway is propionyl-CoA dehydrogenase (PACD), a heterogeneous protein of *E. coli*, which interacts with the endogenous enzymes, PrpE (propionyl-CoA synthetase)[15,28,29] and enoyl-CoA hydratase,[6] to produce 3-HP as an end product. In order to reduce the metabolic stress of the recombinant strain, we decided to overexpress PACD to interact with the native metabolic fluxes for the metabolism of propionyl-CoA. But as it was reported previously,[15] due to the low specific activity of other enzymes regarding the propionyl-CoA pathway, *Ec*-P had a low titer of 3-HP (0.11 g/L).

Therefore, we decided to construct a strain that couples the malonyl-CoA pathway and the propionyl-CoA pathway. The coupling expression of PACD, ACC and MCR positively affected the production of 3-HP. As it was previously explained, the intracellular NADPH pool can be a limiting factor for the production of 3-HP via the malonyl-CoA pathway. The presence of a membrane-bound transhydrogenase catalyzing the reversible redox reaction ($NADH + NADP^+ + H^+_{out} \leftrightarrow NAD^+ + NADPH + H^+_{in}$) in *E. coli* has been verified long before.[30,31] In a previously reported work, a transhydrogenase was introduced into malonyl-CoA pathway to converts NADH to NADPH and the titer of 3-HP increased from 1.6 mM to 2.14 mM.[23] On the other hand, the upregulated metabolic flux of propionyl-CoA pathway and tricarboxylic acid (TCA) cycle, which might result in generation of NADPH in an excess of cellular needs. The excess NADPH could be balanced by the conversion of NADPH to NADH by the transhydrogenase. These transhydrogenases can catalyze the transfer of hydride from NADPH to $NAD^+$, and in the case of NADPH overproduction, are important to balance the NADPH/NADH pool.[32–34] Obviously, the excess NADPH in the propionyl-CoA pathway is an indispensable substrate for the malonyl-CoA pathway. Thus, the high production of 3-HP by *Ec*-PAM can be the result of a $NADP^+$/NADPH redox balance regulated by the combination of malonyl-CoA pathway and propionyl-CoA pathway.

In a previous work, a recombinant strain with a dual synthetic pathway (ALDH pathway and Pdu pathway) was constructed for 3-HP production with glycerol as carbon source, and the resultant strain exhibited a 70% increase in 3-HP titer compared to one harboring the ALDH pathway alone. Though two pathways were involved in that work, there was no benefit of redox balance for these two pathways.[35]

### 3.3. Analysis of by-products in the production of 3-HP

Production of 3-HP by bacterial fermentation can produce some kinds of carboxylic acids, for example, lactic acid and acetic acid. In order to analyze the effect of these by-products on the production of 3-HP, shake-flask fermentation of the strains was conducted and the production of some carboxylic acids was evaluated.

As shown in Table 2, the relationship between 3-HP and the other by-products is negatively correlated, meaning that the lower is the production of by-products, the higher will be the production of 3-HP. Acetic acid and lactic acid were two main by-products that have attracted much attention, and in some literature the gene deletion of *pta-ackA* (encoding phosphotransacetylase and acetate kinase) and *ldhA* (encoding lactate

**Table 2**
By-products produced during bacterial fermentation.

| Metabolite ( mM ) | *EC*-P | *EC*-AM | *EC*-PAM |
|---|---|---|---|
| Acetic acid | 3.37 | 2.36 | 1.67 |
| Lactic acid | 5.55 | 3.45 | 3.10 |
| Succinic acid | 14.87 | 8.71 | 8.23 |
| Formic acid | 11.93 | 6.94 | 4.89 |
| 3-HP | 1.22 | 2.89 | 14.29 |

dehydrogenase) was performed to investigate the effect on 3-HP accumulation.[23,36,37] As expected, when their concentrations in the broth were high, the production of 3-HP was low. *Ec*-P presented the highest levels of by-products. On the other hand, when combined with the malonyl-CoA pathway, the production of these by-products decreased significantly in the culture broth of *Ec*-PAM. The highest titer of 3-HP with the lowest by-products concentration for *Ec*-PAM suggested that this two-pathway coupling strain had a higher substrate transformation efficiency than the single-pathway strains *Ec*-P and *Ec*-AM, exhibiting a good prospect for the 3-HP fermentation with glucose as carbon source.

### 3.4. Optimization of the cultivation conditions

In the production of 3-HP by malonyl-CoA pathway, there are some factors that may have impact on the ACC-catalyzed carboxylation of acetyl-CoA to form malonyl-CoA.[21] Considering ACC is a biotin-dependent enzyme, and bicarbonate is necessary for the synthesis of malonyl-CoA, we decided to identify the optimal concentration of biotin and bicarbonate to improve the 3-HP production of the engineered strain.

The strain *Ec*-PAM was cultured with biotin at different concentrations, and the final concentration of 3-HP of each sample was shown in Fig. 4A. In different concentrations of biotin, the 3-HP production had a small increasement. When biotin was added at a concentration of 25 mg/L, 3-HP increased to a titer of 1.41 g/L, indicating that the optimal concentration of biotin is 25 mg/L.

ACC requires a covalently bound biotin prosthetic group for activity, and in a previous study,[25] addition of biotin to the bacterial culture resulted in a 65% increase of the specific activity of ACC. Based on this, we can infer that the addition of biotin to the medium could enhance the intracellular ACC activity and it is possible to some extent improve the 3-HP production of the recombinant strain.

Considering bicarbonate is necessary for the synthesis of malonyl-CoA from acetyl-CoA by ACC, the influence of sodium bicarbonate on the 3-HP production was also evaluated (Fig. 4B). The titer of 3-HP was 1.29 g/L without adding sodium bicarbonate, and 3-HP production was increased to 1.44 g/L when the sodium bicarbonate was added at a concentration of 2 g/L.

Finally, based on these results, 25 mg/L of biotin and 2 g/L sodium bicarbonate were added to the medium after induction, the production of 3-HP by *Ec*-PAM increased from 1.29 g/L to 1.69 g/L. The results of increasing 3-HP titer by adding biotin and bicarbonate in this work are similar to those published by Cheng et al.[21]

### 3.5. Deletion of the ygfH and prpC genes

Propionyl-CoA is an important precursor for 3-HP formation via propionyl-CoA pathway, however, it can be utilized in different pathways, for example the TCA and the MCC cycles.[29,38] In our previous study on propionyl-CoA pathway, an increase on the production of 3-HP was obtained by deleting two genes, *ygfH* and *prpC*.[15] In this work, the recombinant strains with *ygfH* and *prpC* genes knocked out were constructed, and the resultant strains *Ec*-ΔY-PAM, *Ec*-ΔP-PAM and *Ec*-ΔY-ΔP-PAM were cultured in shake flask to study the improvement of 3-HP production.

3-HP titers of the single-gene deletion strains *Ec*-ΔY-PAM and *Ec*-ΔP-PAM were 1.55 g/L and 1.74 g/L, respectively, while in the case of the double-gene deletion strain *Ec*-ΔY-ΔP-PAM, 3-HP titer was 1.94 g/L, which was 1.5-fold higher than that of *Ec*-PAM cultured under the same conditions (Fig. 5). The increased accumulation of 3-HP could be attributed to the increased propionyl-CoA pool caused by the deletion of *ygfH* and *prpC* genes.[39–41]

As shown in Table 3, some pathways for the synthesis of 3-HP in *E. coli* were recently reported. Compared with beta-alanine pathway and glycerol pathway, propionyl-CoA pathway and malonyl-CoA pathway showed lower yields of 3-HP. While in this work, the coupled-pathway strategy could markedly improve the yield of 3-HP to 0.39, a relatively high value. Though the primary result of 3-HP titer and yield in shake flask was still low in this work, there are some methods that will further improve the production of 3-HP. First, in this work, only one key enzyme PACD of propionyl-CoA pathway was overexpressed in the engineered strains. Thus, propionyl-CoA pathway could be further strengthened by co-expressing other key enzymes, HPCD and PCT,[15] and more NADPH could be provided for the coupled malonyl-CoA pathway. The two pathways of propionyl-CoA and malonyl-CoA could be elaborately regulated to generate a redox balance conducive to the synthesis of 3-HP. Second, the culture conditions (for example, the substrates concentration, pH, temperature, etc.) could be optimized, and the fed-batch fermentation will effectively promote 3-HP titer.[38]

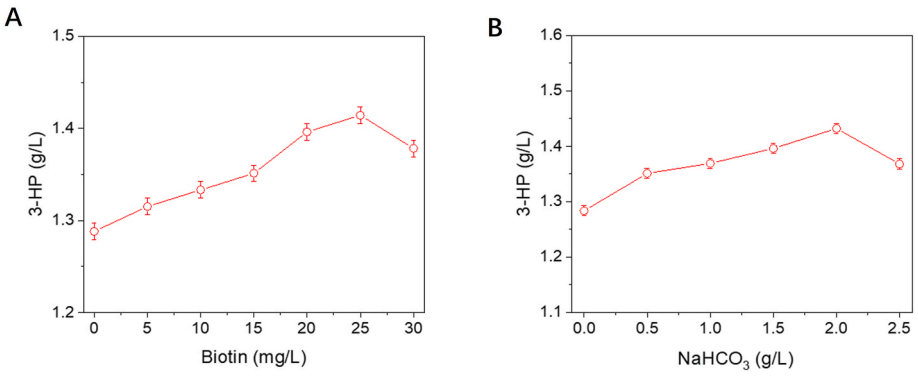


**Fig. 4.** Optimization of the cultivation of strain *Ec*-PAM. The strains were cultured in the media containing different concentrations of biotin (A) and sodium bicarbonate (B) to determine the optimal concentration at which enhances the 3-HP production of the strain. The experiment was done in triplicate. The error bars show standard errors.

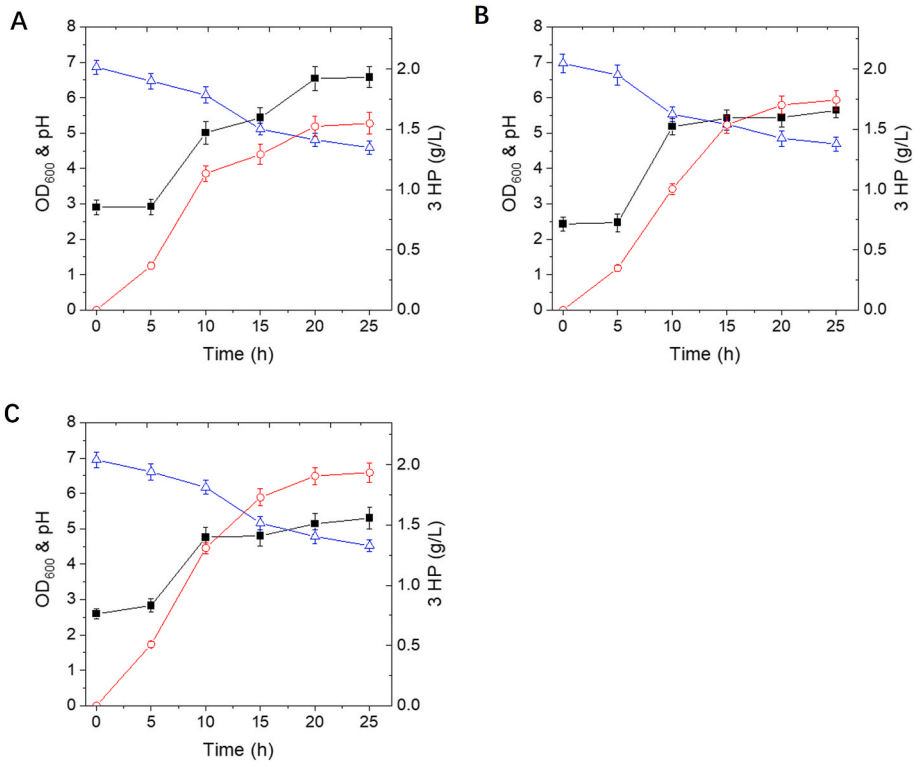


**Fig. 5.** Production of 3-HP by recombinant *E. coli* deletion mutants expressing PACD, ACC and MCR (A) EC-ΔY -PAM, (B) EC-ΔP -PAM, and (C) EC-ΔY-ΔP -PAM. Symbols: 3-HP (circle), cell density (square), pH (triangle). When optical density of the broth was around 0.6, the cell was induced at 25 °C for 12 h, and then the substrates of propionate and glucose were added to the culture. The experiment was done in triplicate. The error bars show standard errors.

**Table 3**
Production of 3-HP through different pathways by engineered *E. coli* strains.

| Pathway | Fermentation mode | Substrate | Titer (g/L) | Yield (g/g) | Reference |
|---|---|---|---|---|---|
| Malonyl-CoA | Shake flask | 100 mM glucose | 0.19 | 0.02 | Reference [23] |
| Malonyl-CoA | Fed-batch culture | glucose | 40.6 | 0.19 | Reference [42] |
| Beta-alanine | Fed-batch culture | glucose | 31.3 | 0.42 | Reference [43] |
| Propionyl-CoA | Shake flask | 10 g/L glucose, 5 g/L propionate | 2.17 | 0.14 [a] | Reference [15] |
| Glycerol | Fed-batch culture | 41.79 g/L glycerol, 34.27 g/L glucose | 40.51 | 0.53 [a] | Reference [14] |
| Malonyl-CoA | Fed-batch culture | glucose, acetate | 7.3 | 0.26 [b] | Reference [37] |
| Malonyl-CoA & Propionyl-CoA | Shake flask | 4 g/L glucose, 1 g/L propionate | 1.94 | 0.39 [a] | This study |

[a] The yield was calculated based on the total carbon source (g/g).
[b] The yield was calculated based on acetate (mol/mol).

## 4. Conclusion

In order to synthesize 3-HP from biobased material, some recombinant strains harboring the key enzymes of the malonyl-CoA and propionyl-CoA pathways were constructed. By coupling the malonyl-CoA and propionyl-CoA pathways we successfully enhanced the production of 3-HP in recombinant *E. coli*, and also managed to improve its production by adding biotin and sodium bicarbonate to the medium. On the other hand, increasing the propionyl-CoA pool *in vivo* by deleting the competitive pathways also resulted in an improvement of 3-HP production. The titer and yield of 3-HP for the dual-pathway strain *Ec*-ΔY-ΔP-PAM were 1.94 g/L and 0.39, respectively, which were much higher than those of single-pathway strains. The production of 3-HP is strongly correlated with the regeneration of NADPH *in vivo*, suggesting that it is necessary to precisely regulate the $NADP^+$/NADPH redox balance for the pathways to work more efficiently. The titer and yield of 3-HP are still low, and the challenge of current work is to improve 3-HP productivity by optimizing the strain and cultivation conditions.

## Declaration of competing interest

The authors declare no conflict of interest.

## CRediT authorship contribution statement

**Dafeng Zhou:** Methodology, Validation, Formal analysis, Data curation. **Diego Leandro Quiroga-Sánchez:** Methodology, Validation, Writing – original draft. **Xuan Zhang:** Methodology, Validation. **Yanhong Chang:** Supervision, Project administration. **Hui Luo:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2022.02.002.

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