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ORIGINAL RESEARCH PAPER

Metabolic engineering of *Escherichia coli* for poly(3-hydroxypropionate) production from glycerol and glucose

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Abstract A new poly(3-hydroxypropionate) (P3HP) biosynthetic pathway employing β-alanine as an intermediate from an inexpensive carbon source was developed in recombinant *Escherichia coli*. After a series of systematic optimization, the genes for L-aspartate decarboxylase and its maturation factor (*panD* and *panM*, from *E. coli*), β-alanine-pyruvate transaminase (*pp0596*, from *Pseudomonas putida*), 3-hydroxy acid dehydrogenase and 3-hydroxypropionyl-CoA synthase (*ydfG* and *prpE* respectively, from *E. coli*), and polyhydroxyalkanoate synthase (*phaC1*, from *Cupriavidus necator*) were cloned and expressed in *E. coli*. Under shake-flask conditions, the recombinant strain produced 0.5 g P3HP l^{-1} from glycerol and glucose, up to 10.2 % of CDW. Though the content of P3HP was low, this pathway has some advantages over other reported pathways, such as being redox neutral, does not require any coenzyme, and can use a wide range of carbon sources.

Keywords β-Alanine · Inexpensive carbon sources · Poly (3-hydroxypropionate) · Recombinant *Escherichia coli*

Introduction

Polyhydroxyalkanoates (PHA) are biodegradable and biocompatible thermoplastics. In bacteria, PHA is accumulated as a storage compound for energy and carbon under unbalanced growth conditions (Anderson and Dawes 1990). Nowadays, metabolic engineering approaches have been widely used to construct recombinant bacteria to obtain new types of polymers, one of which is poly (3-hydroxypropionate) (P3HP) (Andreessen and Steinbuchel 2010; Chen 2009).

With its high rigidity, ductility, and exceptional tensile strength in drawn films, P3HP has attracted growing interest. As 3-hydroxypropionate (3HP) is not a common compound in most metabolic pathways, synthesis of PHA copolymers containing 3HP monomer are generally dependent on structurally-related precursors, such as 3HP and acrylic acid (Andreessen and Steinbuchel 2010). However, the precursors are usually expensive and toxic to cells. For example, the

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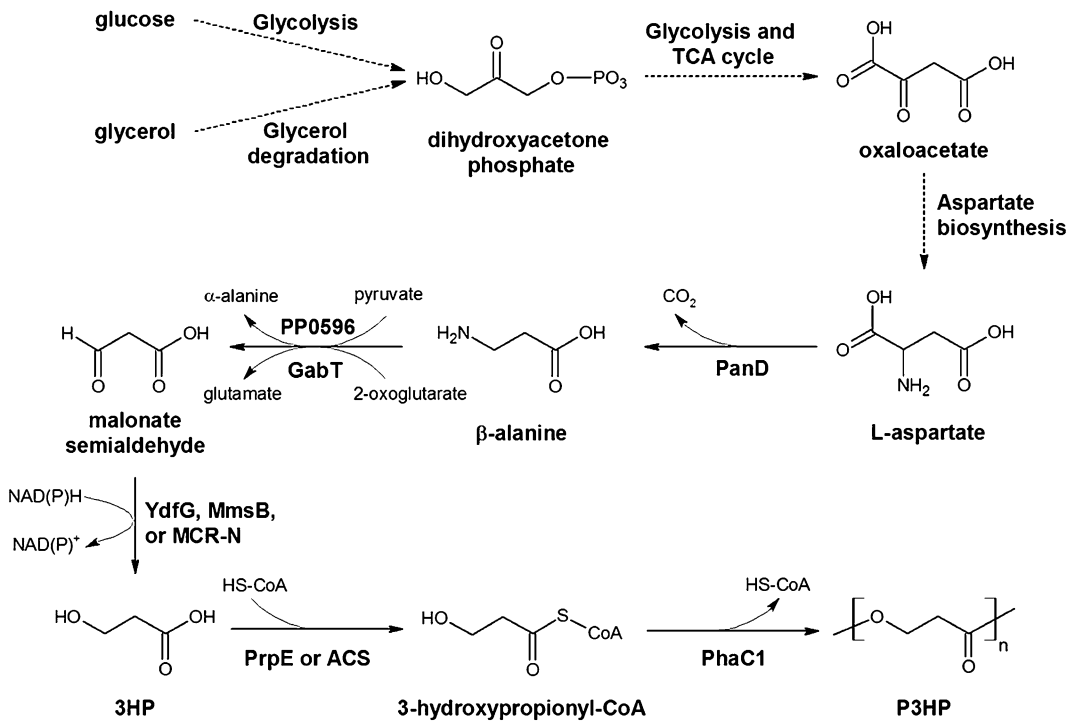


Fig. 1 Pathway for P3HP biosynthesis from inexpensive carbon sources in the engineered strain of *E. coli*. PanD, L-aspartate decarboxylase of *E.coli*; PP0596, β-alanine-pyruvate transaminase of *P.putida*; GabT, 4-Aminobutyrate transaminase of *C.acetobutylicum*; YdfG, 3-hydroxyacid dehydrogenase of *E.coli*; MmsB, 3-hydroxyisobutyrate dehydrogenase of *P.putida*; MCR-N, 3-hydroxypropionate dehydrogenase of *C.aurantiacus*; PrpE, propionyl-CoA synthase of *E.coli*; ACS, 3-hydroxypropinyl-CoA synthase of *C.aurantiacus*; PhaC1, PHA synthase of *C.necator*

price of acrylic acid is about \$2,000 t^{-1}, and severe growth inhibition could be observed when it exceeds 5 M in minimal medium (Green et al. 2002). Only a few studies have been conducted on P3HP synthesis from inexpensive carbon sources, such as glucose and glycerol (Andreessen et al. 2010; Wang et al. 2012, 2013).

In our previous study, we constructed two pathways to produce P3HP. One pathway started from acetyl-CoA and the malonyl-CoA reductase (MCR) of *Chloroflexus aurantiacus* was the key enzyme in this pathway. Unfortunately, only 13 mg P3HP l^{-1} was accumulated by recombinant *Escherichia coli* probably due to the low enzyme activities of MCR and the instability of plasmids carrying exogenous genes (Wang et al. 2012). The other pathway employed glycerol dehydratase for conversion of glycerol into 3-hydroxypropioaldehyde, followed by further oxidation and polymerization. This pathway improved the production of P3HP to 10.1 g l^{-1} fermenter. However, the activity of glycerol dehydratase required exogenous supply of high-cost vitamin B_{12}, which severely impeded the commercial application of this pathway (Wang et al. 2013). Accordingly, it is indispensable to engineer new pathways to produce P3HP.

This study aimed to develop a new pathway to produce P3HP from glucose and glycerol without any addition of precursors and coenzymes (Fig. 1). Through enzyme activity screening, we finally employed L-aspartate decarboxylase and its maturation factor of *E. coli*, β-alanine-pyruvate transaminase of *Pseudomonas putida*, 3-hydroxyaciddehydrogenase and 3-hydroxypropionyl-CoA synthase of *E.coli*, and PHA synthase of *Cupriavidus necator*. Under shake-flask conditions, the recombinant *E.coli* produced 0.5 g P3HP l^{-1} from glycerol and glucose.

Materials and methods

Bacterial strains and plasmids construction

All bacterial strains, plasmids and primers used in this study were listed in Supplementary Table 1. The

pp0596 gene from *P. putida* and the *gabT* gene from *Clostridium acetobutylicum* were cloned in to pETDuet-1 to generate pHP501 and pHP505, respectively. The *pcs'* gene from *Chloroflexus aurantiacus* (pKS1 as template) was inserted into pHP301 to construct pHP514. The *ydfG* gene from *E.coli*, the *mmsB* gene from *P. putida* and the gene $mcr_{\text{-}N}$ from *C.aurantiacus* (pMCR as template) were inserted into pHP501 to generate the plasmids pHP502, pHP503 and pHP504, respectively, the gene $mcr_{\text{-}N}$ was inserted into pHP505 to generate pHP506. The plasmid pHP502 was digested by *Bam*HI and *Nde*I to generate a 2 kb fragment containing *ydfG* and *pp0596* genes, which was subsequently ligated between the same restriction sites of pACYCDuet-1 to construct pHP507. The plasmids pHP508 and pHP509 were constructed by inserting P_{Trc} and P_{Re} to substitute P_{T7} controlling *ydfG*, respectively. The P_{T7} controlling *pp0596* was substituted by P_{Trc} and P_{Re} to generate the plasmids pHP510 and pHP511. The *panD* fragment from *E.coli* was cloned in to pHP507 to generate pHP512, and then the *panM* fragments from *E.coli* were cloned in to pHP512 to generate pHP513. The constructed plasmids were transformed into *E.coli* JM109 (DE3) to obtain engineered strains.

Shake-flask cultures

The engineered strains listed in Supplementary Table 1 were inoculated into 500 ml baffled Erlenmeyer flasks containing 100 ml minimal medium (MM) with 20 g glycerol l^{-1} and 3 g glucose l^{-1} as described previously (Wang et al. 2013). 100 mg ampicillin l^{-1} and 34 mg chloramphenicol l^{-1} were supplemented when necessary. The cells were induced at OD_{600} ~0.6 with 0.05 mM IPTG and further incubated at 30 °C for 48 h. To screen enzymes more effectively, an amount of 5 g β-alanine l^{-1} was added to the medium as the precursor. All shake-flask experiments were performed in triplicate.

Analytical methods

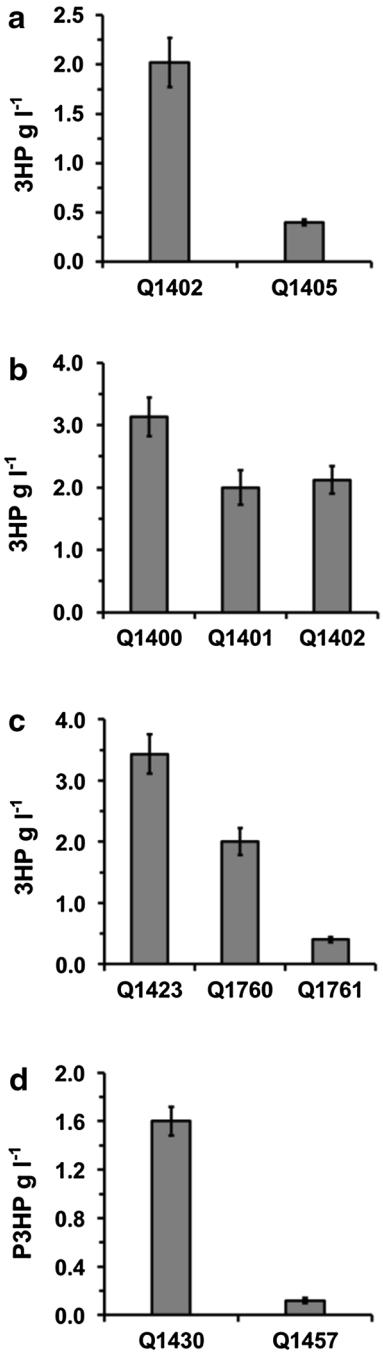
PHA extraction was performed by treating lyophilized cells with hot chloroform in a Soxhlet apparatus. NMR analysis was carried out on an Advance III 600 NMR Spectrometer (Bruker, Switzerland) to determine the polymer structure. The PHA samples were dissolved in deuterated chloroform ($CDCl_3$).Both the ^{1}H NMR and ^{13}C NMR spectra were measured at 25 °C. Tetramethylisilane (TMS) was used as an internal chemical shift standard. The molecular weight of P3HP was determined using gel permeation chromatography (Dawn Heleos II, Wyatt, USA). Chloroform was used as an eluent at 1 ml/min. The sample was at 2 mg/ml, and the injection volume was 50 µl. The calibration curve was generated with polystyrene standards (Liu et al. 2011). Differential scanning calorimetry (DSC) data were recorded using Diamond DSC (Perkin Elmer, USA).A sample of 2–4 mg in an aluminum-sealed pan was heated from −80 to 195 °C at 10 °C min^{-1}. After rapidly quenched, the sample was reheated from −100 to 195 °C at 20 °C min^{-1} (Cao et al. 1998).

3HP was quantified by HPL Cursing a refractive index and UV/vis detectors. Culture supernatants were eluted through an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, USA) at 55 °C. The mobile phase was 5 mM H_2SO_4 at 0.5 ml min^{-1}.

Results and discussion

Design of P3HP biosynthesis pathway

As 3HP is not a common compound in most organisms, precursors structurally related with 3HP are usually used in biosynthesis of 3HP-containing polymers. The supply of expensive precursors is a serious obstacle to an industrial process. Several attempts to produce polymers containing 3HP from inexpensive substrates have been reported (Fukui et al. 2009; Wang et al. 2012, 2013). However, all pathways exploiting malonyl-CoA reductase (MCR) from *C. aurantiacus* resulted in low 3HP or P3HP yields (Fukui et al. 2009; Wang et al. 2012). On the other hand, another pathway for P3HP production employing glycerol dehydratase has made great progress. Nevertheless, two main problems still remained unsolved. One problem is the requirement of vitamin B_{12} addition to maintain glycerol dehydratase activity, which led to a high cost. Another problem is redox unbalance. The conversion of glycerol to 3HP-CoA is accompanied by reduction of NAD^+ to NADH, but without regeneration of NAD^+ for continuous P3HP production. NAD^+ regeneration needs extra glycerol expenditure which inevitably decreases the amount of glycerol channeled into P3HP production.



◀**Fig. 2** Optimization of the pathway for P3HP production in recombinant *E.coli*. **a** 3HP precursor production by employing PP0596 and GabT as transaminase, MCR-N as 3-hydroxypropionate dehydrogenase. **b** 3HP precursor production by employing YdfG, MmsB, and MCR-N as 3-hydroxypropionate dehydrogenase, PP0596 as transaminase. **c** Optimization of the expression of PP0596 and YdfG. **d** P3HP production by employing PrpE and ACS as 3-hydroxypropionyl-CoA synthase, PP0596 as transaminase and YdfG as 3-hydroxypropionate dehydrogenase. All the experiments were conducted based on addition of 5 g β-alanine l^{-1}

In order to overcome these problems, we explored a new pathway for P3HP production with β-alanine as intermediate (Fig. 1). Compared with the P3HP synthesis pathway using glycerol dehydratase, vitamin B_{12} is not required, resulting in decreased cost. Furthermore, the production of P3HP in this pathway is redox neutral. The conversion of glucose or glycerol into 1 mol aspartate generates 1 mol NADH, while subsequent reduction of malonate semialdehyde to 3HP consumes 1 mol NADPH, which comes from $NADP^+$ reduction by NADH and pyridine nucleotide transhydrogenase, PntAB. Additionally, β-alanine is a common intermediate of sugar metabolism, so that various sugars can be used as raw material for P3HP production.

Production of 3HP precursor in engineered strains employing transaminase PP0596 or GabT

β-Alanine-pyruvate transaminase catalyzes the transamination between β-alanine and pyruvate, resulting in the formation of malonate semialdehyde and α-alanine(Hayaishi et al. 1961). 4-Aminobutyrate transaminase catalyzes the transamination of 4-aminobutyrate with 2-oxoglutarate, the products of which are succinate semialdehyde and glutamate (Scott and Jakoby 1959). Considering the structure similarity between β-alanine and 4-aminobutyrate, 4-aminobutyrate transaminase encoded by *gabT* gene from *C. acetobutylicum* was overexpressed and compared with β-alanine-pyruvate transaminase of *P. putida* (PP0596). When co-expressed with 3-hydroxypropionate dehydrogenase from *C. aurantiacus* (MCR-N), the strain with PP0596 (Q1402) produced 2 g 3HP l^{-1}, higher than the strain harboring *gabT* (Q1405) (Fig. 2a). Therefore, PP0596 was used for the following study.

Comparison of the efficiencies of three 3-hydroxyacid dehydrogenase

3-Hydroxyisobutyrate dehydrogenase of *Pseudomonas putida* (MmsB) catalyzes the reversible oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde. Lysine and asparagine residues, which are essential in catalysis of the 3-hydroxyacid dehydrogenase family, were also conserved in this enzyme, suggesting the potential catalysis for 3-hydroxyacids (Chowdhury et al.

2003). Another 3-hydroxyacid dehydrogenase of *E.coli* (YdfG) also catalyzes the oxidation of 3-hydroxy acids with three or four carbon atoms (Fujisawa et al. 2003). In addition, MCR *N*-terminal region (MCR-N; amino acids 1–549) can function as 3-hydroxypropionate dehydrogenase, catalyzing the conversion of malonate semialdehyde to 3HP (Liu et al. 2013). Based on the overexpression of PP0596, we introduced MmsB, YdfG and MCR-N separately into recombinant *E. coli* strains to compare their catalysis efficiency. Figure 2b shows that the strain with YdfG overproduction (Q1400) produced 3.1 g 3HP l^{-1}, in contrast to 2 and 2.1 g 3HP l^{-1} produced by the other two strains (Q1401 and Q1402).

Optimization of the expression level of transaminase and 3-hydroxyacid dehydrogenase

Bacterial promoters play an important role in metabolic regulation, determining the transcription level of genes and subsequently influencing the expression level of proteins. Either the insufficient enzyme synthesis or metabolic burden to the cells caused by improper inducing intension would limit the synthesis of product. It is therefore worthwhile to optimize the expression level of heterologous genes. P_{T7} promoter always directs high-level of protein expression, probably giving rise to the formation of inclusion bodies. On the other hand, the promoter of the *phaCAB* operon from *R.eurtopha* (P_{Re}) has been proved to be effective in regulating gene expression and leads to high production of the desired product (Zhou et al. 2011). Furthermore, as a medium expression level promoter, P_{Trc}, might be more effective than the P_{T7} promoter. Accordingly, P_{Re} and P_{Trc} promoters were used to replace P_{T7} promoter.

Beyond our expectation (Fig. 2c), the strain harboring PP0596 and YdfG under the control of P_{Trc} or P_{Re} promoter (Q1760 or Q1761) resulted in low production of 3HP, other than promoting the production, in contrast to that under the P_{T7} promoter (Q1423), demonstrating that in this pathway, the high-lever expression of P_{T7} promoter promote the production of 3HP. Hence, the P_{T7} promoter was the optimal choice for this pathway.

Screening of 3-hydroxypropionyl-CoA synthase used in P3HP production

Propionyl-CoA synthase (PCS) from *C. aurantiacus*is a trifunctional enzyme. Its *N*-terminal region (amino acids 18–850) showed high homologies with acetyl-CoA synthase, indicating the ability to link 3-hydroxypropionate and coenzyme A, and was defined as ACS domain (Herter et al. 2002). In addition, (Zhou et al. 2011) has also shown the feasibility of this likely 3-hydroxypropinyl-CoA synthase in the 3HP production. Propionyl-CoA synthase from *E.coli* (PrpE) was used as a 3-hydroxypropionyl-CoA synthase in our previous study (Wang et al. 2012). For P3HP production, pHP509 was co-transferred with pHP302 and pHP516 into *E. coli* JM109 (DE3) to construct the engineered strains Q1430 and Q1457, respectively. The strain Q1430 accumulated 1.6 g P3HP l^{-1}, while only 0.12 g P3HP l^{-1} was detected in Q1457 culture (Fig. 2d), demonstrating the higher efficiency of PrpE than that of ACS domain.

P3HP synthesis from inexpensive carbon sources

We have already obtained three enzymes with high activities in the pathway through supplying β-alanine as precursor. To produce P3HP without addition of any precursors, on enzyme for β-alanine synthesis from inexpensive carbon sources is required to increase the intracellular β-alanine level. $_L$-Aspartate decarboxylase (PanD) catalyzes the decarboxylation of $_L$-aspartate to form β-alanine. However, this enzyme is translated as inactive proprotein (pro-PanD) but can be activated by self-catalyzed cleavage. Notably, PanM, as an acetyl-CoA sensor, could trigger the maturation of pro-PanD. To verify the effect of PanM on PanD maturation, we constructed two strains Q1598 and Q1749. In contrast to 0.87 g 3HP l^{-1} produced by Q1749 harboring PanMand PanD, Q1598 with only PanD produced 0.41 g 3HP l^{-1}, suggesting the important role of PanM.

Thus far, all the enzymes for synthesis of P3HP from inexpensive carbon sources have been successfully obtained, and the corresponding strain, harboring both pHP302 and pHP513, was named as Q1911. Under shake-flask conditions, strain Q1911 produced 0.5 g P3HP l^{-1}, representing 10.2 % of CDW, with glycerol and glucose as carbon sources. ^{1}H- and ^{13}C-NMR analyses confirmed the structure of P3HP (Supplementary Fig. 1). The number-average molecular (M_n) and weight-average molecular weight (M_w) of the P3HP were 1.28×10^5 and 2.32×10^5 Da, respectively. The melting temperature (T_m), glass transition temperature (T_g), and enthalpy of fusion

(ΔH_m) of P3HP were 76 °C, −21 °C, and 78 J/g, respectively.

As shown above, the strain Q1430 (harboring PP0596, YdfG, PrpE and PhaC1) produced 1.6 g P3HP l^{-1} with β-alanine addition, while the strain Q1911 (harboring PanD, PanM, PP0596, YdfG, PrpE and PhaC1) only produced 0.5 g P3HP l^{-1} without the addition of β-alanine. It is assumed that the poor yield of the strain Q1911 was caused for two reasons: low activity of PanD and low intracellular $_L$-aspartate concentration. Directed evolution on PanD and introduction of this pathway into an $_L$-aspartate-producing strain will be done during further strain optimization.

Conclusion

A new pathway to produce P3HP from glycerol and glucose, without any addition of precursors or coenzymes, was developed. The final engineered strain, with *panM*, *panD*, *pp0596*, *ydfG*, *prpE* and *phaC1* coexpression, accumulated 0.5 g P3HP l^{-1} under shake-flask conditions. Though the P3HP content was relatively low, this pathway still has some advantages over others including redox neutral, no addition of coenzyme and wide range of carbon sources. So it is worthwhile to further improve the production of this new pathway.

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