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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Biosynthetic pathway for acrylic acid from glycerol in recombinant *Escherichia coli*

Wenhua Tong[1,2] · **Ying Xu**[1,2] · **Mo Xian**[1] · **Wei Niu**[3] · **Jiantao Guo**[3] · **Huizhou Liu**[1] · **Guang Zhao**[1]

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Abstract Acrylic acid is an important industrial feedstock. In this study, a de novo acrylate biosynthetic pathway from inexpensive carbon source glycerol was constructed in *Escherichia coli*. The acrylic acid was produced from glycerol via 3-hydroxypropionaldehyde, 3-hydroxypropionyl-CoA, and acrylyl-CoA. The acrylate production was improved by screening and site-directed mutagenesis of key enzyme enoyl-CoA hydratase and chromosomal integration of some exogenous genes. Finally, our recombinant strain produced 37.7 mg/L acrylic acid under shaking flask conditions. Although the acrylate production is low, our study shows feasibility of engineering an acrylate biosynthetic pathway from inexpensive carbon source. Furthermore, the reasons for limited acrylate production and further strain optimization that should be performed in the future were also discussed.

Keywords Acrylic acid biosynthesis · Enoyl-CoA hydratase · Recombinant *Escherichia coli* · Inexpensive carbon source

✉ Huizhou Liu
liuhuizhou@qibebt.ac.cn

✉ Guang Zhao
zhaoguang@qibebt.ac.cn

[1] CAS Key Lab of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Introduction

Acrylic acid (CH_2 = CH-COOH) is an important unsaturated organic acid and industrial chemical feedstock and can be used in the fields of polymeric flocculants, dispersants, coatings, paints, adhesives, and binders for leather, paper, and textile. In addition, the polymeric products such as super absorbent polymer (SAP) further promote the value of acrylic acid. Currently, most of commercial acrylic acid is produced from oil via a two-step gas-phase oxidation of propene (Unverricht et al. 2003). With the depletion of fossil fuels, a lower-cost and bio-based route for relative chemicals production attracts great interest. As a consequence, researchers begin to focus on producing acrylic acid from renewable feedstock by biotechnological methods.

At present, the acrylic acid biosynthetic methods, including semi-biological method and entire biological method, are not commercially viable. Semi-biological method is based on chemical dehydration of lactic acid (Wang et al. 2013a), which is produced from biomass with high efficiency (Feng et al. 2014). However, high cost is a critical problem of this method. In the entire biological method, the process is dependent on the de novo synthesis in microorganisms. To our knowledge, acrylic acid is only a metabolic intermediate in some microorganisms, such as *Clostridium propionicum*, *Sulfolobus metallicus*, *Megasphaera elsdenii*, *Chloroflexus aurantiacus*, *Acidianus brierleyi*, *Metallosphaera sedula*, *Sulfolobus* sp. VE6, *Desulfovibrio acrylicus*, et al. (Hugler et al. 2003; Ishii et al. 2004; Marc J. E. C. van der Maarel 1996). Among these strains, *C. propionicum* is usually used for acrylic acid production in laboratory (Dalal et al. 1980; H. Danner 1998; O'Brien D J 1990). When grown on lactic acid, and provided methylene blue as an electron acceptor, *C. propionicum* produced 144 mg/L acrylic acid (Danner et al. 1998). However, *C. propionicum* grows slowly and lack available genetic

operation system, leading to great difficulty in enhancing acrylic acid production with metabolic engineering. So, it is necessary to design and construct an acrylic acid biosynthetic pathway in some model strains, like *Escherichia coli*, which has a high growth rate, clear genetic background, and simple genetic manipulation.

As a by-product of biodiesel production, glycerol has become an inexpensive and abundant carbon source for bioproduction of chemicals and fuels production (Duque 2011). In this study, we developed a recombinant *E. coli* strain to produce acrylic acid using glycerol as sole carbon source. The acrylic acid production was successfully enhanced by enzyme mutagenesis and chromosomal insertion of some exogenous function genes.

Materials and methods

Strains and growth conditions

All strains and plasmids used in this study were listed in Table 1. The *E. coli* DH5α strain (Invitrogen) was used for plasmids preparation and BL21(DE3) was used for acrylic acid production and protein expression. The mutation of *yhdH* in Q1463 was constructed via P1vir-mediated transduction, and the procedure was performed as described previously (Moore 2011). Bacteria were grown at 37 °C in Luria-Bertani (LB) broth in construction of strains and plasmids. When necessary, antibiotics were added at final concentration of 100 μg/mL for ampicillin and 34 μg/mL for chloramphenicol.

Construction of recombinant plasmids

All PCRs were done using PrimeSTAR HS DNA Polymerase (TAKARA, Dalian, China). The plasmid pTW01 was constructed by cloning the *pduP* gene of *Salmonella typhimurium* (ATCC14028) into *Nde*I and *Kpn*I sites of vector pETDuet-1 with primers 929(5′-GGAATTCCATATGAATACTTCTGAACTCGAAAC-3′) and 930 (5′-CGGGGTACCTTAGCGAATAGAAAAGCCGTTG-3′). The plasmid pTW02 was constructed by cloning the *phaJ1* gene of *Pseudomonas aeruginosa* PAO1 (ATCC BAA-47) into *Bam*HI and *Sac*I sites of pTW01 with primers 1008 (5′-CATGGATCCTATGAGCCAGGTCCAGAACATTC-3′) and 1009 (5′-CAGAGCTCTCAGCCGATGCTGATCGGC-3′). The plasmid pTW03 was constructed by cloning the enoyl-CoA hydratase gene *ecH* of *P. aeruginosa* PAO1 into *Bam*HI and *Sac*I sites of pTW01 with primers 1010 (5′-CATGGATCCTATGAGCCAGGTCCAGAACATTC-3′) and 1011 (5′- CAGAGCTCTCAGCCGATGCTGATCGGC-3′). The plasmid pTW04 was constructed by cloning the 3-hydroxypropionyl-coenzyme A dehydratase gene *hcaD* of *Bacillus subtilis* (ATCC 6633) into *Bam*HI and *Sac*I sites of pTW01 with primers 1012 (5′- CATGGATCCTATGAATACCATATCATTAGCAGTCGATCAATTTG-3′) and 1013 (5′-CAGAGCTCTTATTCGCCTCTGAATTGAGGCTTTCTTTTTTC-3′). Then, *pcsII* from *Chloroflexus aurantiacus* J-10-fl (ATCC 29366) was synthesized and digested with enzymes *Bam*HI and *Sac*I, then ligated into pTW01 digested with the same enzymes, the resulting plasmid was named pTW05. Mutations were introduced into the *pcsII* gene in pTW05 using an overlap extension PCR method. All of the *pcsII* mutants were prepared using the same two outside primers on pTW05, T7 promoter-1 primer (5′-GATGCGTCCGGCGTAGAGG-3′) and DuetDOWN1 primer (5′-GATTATGCGGCCGTGTACAA-3′). The internal primers for R103K using 1065 (5′-GCTTATTCATCTTCTCAATCTTGCGGAAAGC-3′) and 1066 (5′-GATTGAGAAGATGAATAAGCCGTGTATC-3′), 1067 (5′-CAGCGCCTCAGCAGCCGGTACGCTACGCC-3′) and 1068 (5′-GTACCGGCTGCTGAGGCGCTGGAGCTGGG-3′) were used for D186A. To construct each mutant, two PCR reactions with the pTW05-*pcsII* as the template and consisting of one outside primer and the respective inside primer were performed, and the products were then pooled and used as a template for a second PCR using both outside primers. Then, the second PCR products containing the desired mutation were digested with the enzymes *Bam*HI and *Sac*I, and the appropriate fragments were isolated and ligated into reconstructed pTW05 to replace the wild *pcsII*; the resulting plasmids were named pTW06 and pTW07, respectively. Double mutants combined R103K with D186A in PcsII were performed by the method as described above; the resulting plasmid was named pTW08.

Culture conditions

The strain was inoculated into 500-mL baffled Erlenmeyer flasks containing 100 mL of minimal medium, which contains 20 g/L glycerol, 1.5 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 1 g/L citric acid, 1 g/L citrate sodium, 1.9 g/L KCl, 3 g/L $MgSO_4$, 0.138 g/L $FeSO_4{\cdot}7H_2O$, 4.5 mg/L vitamin B1, and 100 μL of trace element solution. The trace element solution contained (per liter) 3.7 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.47 g H_3BO_3, 1.58 g $MnCl_2{\cdot}4H_2O$, 0.29 g $ZnSO_4{\cdot}7H_2O$, and 0.25 g $CuSO_4{\cdot}5H_2O$. The culture broth was inoculated with the overnight culture and incubated in a gyratory shaker incubator at 37 °C and 200 rpm. The cells were induced at OD_{600} ~ 0.6 with 0.05 mM IPTG and further incubated at 30 °C. Five micrometer of vitamin B_{12} (VB_{12}) and appropriate antibiotic were added every 12 h. All shake flask experiments were carried out in triplicates.

Analytical methods

The OD at 600 nm was routinely used to monitor cell growth via ultraviolet spectrophotometer (Varian Cary 50 UV-Vis,

Table 1 Bacterial strains and plasmids used in this study

Stains or plasmids	Description	Source
Strains		
E. coli DH5α	F- *supE*44 Δ*lacU*169 (Φ80 *lacZ* Δ*M15*) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Invitrogen
E. coli BL21 (DE3)	F- *ompT hsdS*B (rB-mB-) *gal dcm* λ(DE3)	Invitrogen
Q1327	BL21(DE3) carrying pACYCDuet-1 and pETDuet-1	This study
Q1463	BL21(DE3), *prpR*::*lacI* P_{T7} *gdrAB* P_{T7} $dhaB_{123}$	(Gao et al. 2014)
Q2207	BL21(DE3), *prpR*::*lacI* P_{T7} *gdrAB* P_{T7} $dhaB_{123}$ Δ*yhdH*::*Kan*	This study
Q2117	BL21(DE3) carrying pWQ04 and pTW01	This study
Q2115	Q1463 carrying pTW05	This study
Q2178	BL21(DE3) carrying pWQ04 and pTW02	This study
Q2179	BL21(DE3) carrying pWQ04 and pTW03	This study
Q2180	BL21(DE3) carrying pWQ04 and pTW04	This study
Q2181	BL21(DE3) carrying pWQ04 and pTW05	This study
Q2251	Q1463 carrying pTW06	This study
Q2252	Q1463 carrying pTW07	This study
Q2288	Q1463 carrying pTW08	This study
Q2314	Q2207 carrying pTW08	This study
Plasmids		
pACYCDuet-1	rep_{p15A} Cm^R *lacI* P_{T7}	Novagen
pETDuet-1	rep_{pBR322} Amp^R *lacI* P_{T7}	Novagen
pWQ04	rep_{p15A} Cm^R *lacI* P_{T7} *dhaB123* P_{T7} *gdrAB*	(Wang et al. 2013b)
pTW01	rep_{pBR322} Amp^R *lacI* P_{T7} *pduP*	This study
pTW02	rep_{pBR322} Amp^R *lacI* P_{T7} *phaJ1 pduP*	This study
pTW03	rep_{pBR322} Amp^R *lacI* P_{T7} *ecH pduP*	This study
pTW04	rep_{pBR322} Amp^R *lacI* P_{T7} *hcaD pduP*	This study
pTW05	rep_{pBR322} Amp^R *lacI* P_{T7} *pcsII pduP*	This study
pTW06	rep_{pBR322} Amp^R *lacI* P_{T7} $pcsII^{R103K}$ *pduP*	This study
pTW07	rep_{pBR322} Amp^R *lacI* P_{T7} $pcsII^{D103A}$ *pduP*	This study
pTW08	rep_{pBR322} Amp^R *lacI* P_{T7} $pcsII^{R103K\text{-}D186A}$ *pduP*	This study

US). The concentrations of acrylic acid, glycerol, and other metabolites in the culture broth were determined by HPLC using the method described by previous reported with a slight modification (Larsson 2012; Shen and Jiang 2005). Briefly, the supernatants, obtained by centrifugation of the culture samples at 10,000×*g* for 10 min, were filtered through a Tuffryn membrane (China) and eluted through a 300 mm × 7.8 mm Aminex HPX-87 H (Bio-Rad, USA) column at 50 °C using 2.5 mM H_2SO4.

To further confirm acrylic acid was synthesized in our cultures, the same sample was analyzed by gas chromatography-mass spectrometry (GC-MS) after HPLC determined. The GC–MS analysis was performed with an Aglient GC quadrupole instrument. The analysis conditions were as follows: a 30-m HP-5 column (internal diameter 0.25 mm, film thickness 0.25 μm); the column temperature program was composed of an initial hold at 50 °C for 2 min, ramping at 10 °C per minute to 240 °C, followed by heating until 240 °C for 10 min. The injector and transfer line temperature were 240 and 250 °C, respectively. The mass spectrometry full scan from 30 to 400; ion source and quadrupole temperature were 230 and 150 °C.

Results

Design of acrylic acid biosynthesis pathway

In our previous study, a poly (3-hydroxypropionate) (P3HP) biosynthetic pathway from glycerol was constructed in recombinant *E. coli* strain, in which glycerol was converted into 3HP-CoA, then polymerized to form P3HP (Wang et al. 2013b). In addition, acrylyl-coenzyme A is an intermediate derived from 3HP-CoA in the 3-hydroxypropionate cycle of phototrophic bacterium *Chloroflexus aurantiacus* (Ishii et al. 2004). These facts inspire us to construct an acrylic acid pathway from glycerol. As shown in Fig. 1, glycerol was

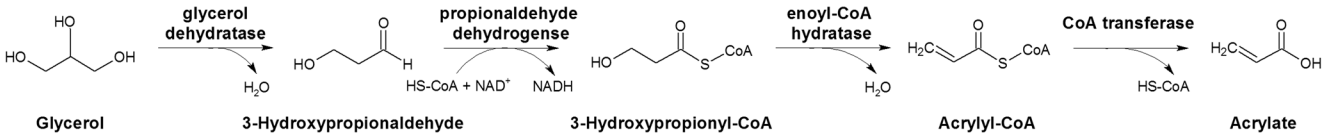

Fig. 1 Pathway for acrylic acid biosynthesis from glycerol in the engineered *E. coli* strain

converted into 3-hydroxypropionaldehyde and 3HP-CoA by glycerol dehydratase and propionaldehyde dehydrogenase. Then, enoyl-CoA hydratase catalyzes the dehydration of 3HP-CoA to acrylyl-CoA, which is transformed into acrylate by CoA transferase. As *E. coli* encodes some CoA transferase with broad substrate spectrum (Cho and Cronan 1994; Elssner et al. 2001; Heider 2001; Naggert et al. 1991; Zhuang et al. 2008), and we have cloned the *dhaB-gdrAB* (encoding glycerol dehydratase and its reactivatase, from *Klebsiella pneumoniae*) and *pduP* (encoding propionaldehyde dehydrogenase, from *Salmonella typhimurium*) genes, the focus of this study is finding a suitable enoyl-CoA hydratase.

Screening of enoyl-CoA hydratase for acrylic acid production

We cloned the following enoyl-CoA hydratase genes to compare their efficiencies on acrylic acid production. The propionyl-CoA synthase PCS from *C. aurantiacus* (accession No. YP_001634242) is a trifunctional enzyme with CoA ligase, enoyl-CoA hydratase, and enoyl-CoA reductase activities and can convert 3HP into propionyl-CoA (Alber and Fuchs 2002). The enoyl-CoA hydratase domain of PCS (amino acids 856–1184) was cloned and defined as PcsII thereafter. PhaJ1 from *Pseudomonas aeruginosa* (accession No. BAA92740) is a short chain-length enoyl-CoA hydratase (Tsuge et al. 2000). In addition, EcH from *P. aeruginosa* (accession No. WP_003085498) and HcaD from *Bacillus subtilis* (accession No. WP_003325180) are two hypothetical enoyl-CoA hydratases based on conserved domain analysis.

To construct an acrylate-producing strain, two plasmids were introduced into *E. coli* BL21(DE3): one is pACYCDuet-1 carrying *gdrAB* and *dhaB123* (Wang et al. 2013b), the other is pETDuet-1 carrying *pduP* and one of enoyl-CoA hydratase gene described above. After 48-h cultivation in shaking flask, the strain with PcsII overexpression (Q2181) produced 3.51 mg/L acrylic acid, 11.7-fold, 1.9-fold, and 1.9-fold higher than the strains carrying PhaJ1 (Q2178), EcH (Q2179) and HcaD (Q2180), respectively (Fig. 2). Meanwhile, the control strain Q1327 did not produce acrylic acid. As a result, PcsII was selected for acrylic acid production. Furthermore, no acrylic acid was detected when glycerol was replaced with glucose, demonstrating that glycerol is an indispensable substrate to produce acrylic acid.

The production of acrylic acid was confirmed by GC-MS analysis. GC analyses of Q2181 culture showed a specific peak with a retention time of R_T = 11.7 min, which is identical to the retention time of acrylic acid standard (Fig. 3A). This particular peak was extracted for MS analysis. Once again, the extracted peak showed a mass of 72 Da, and dissociation of this ion led to other masses such as 45 and 55 Da, exactly the same with acrylic acid standard (Fig. 3B).

Chromosomal gene integration to improve acrylic acid production

In previous study, we found that the loss of recombinant plasmids carrying exogenous genes severely suppressed the production of P3HP and the plasmids derived from pACYCDuet-1 showed higher segregational instability than those from pETDuet-1 (Gao et al. 2014). To improve the acrylic acid production, the plasmid pTW05 was introduced into a strain with chromosomal *dhaB-gdrAB* genes (Gao et al. 2014) to generate strain Q2115. Under shaking flask conditions, Q2115 strain produced 6.92 mg/L acrylic acid after 48 h cultivation, 2-fold higher than that of strain Q2181. This result indicated that plasmid instability really limited the production of acrylic acid.

Site-directed mutagenesis of PcsII

As DhaB and PduP can produce adequate 3HP-CoA precursor for acrylic acid production, we speculated that low activity of PcsII might be the reason of low acrylic acid yield. To improve PscII catalytic activity, we carried out the protein sequence alignment of PcsII with several enoyl-CoA hydratase with known structure. As shown in Fig. 4, six amino acid residues were found conserved in all other proteins but not in PcsII. So, site-directed mutagenesis was performed to construct PcsII

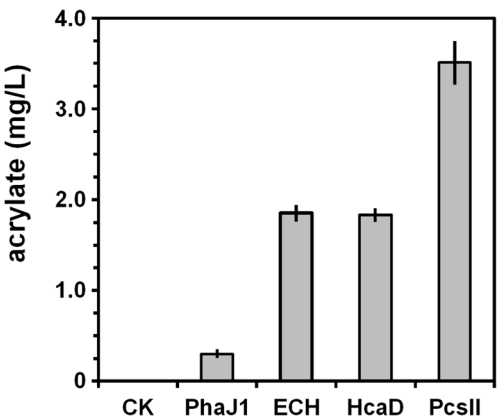

Fig. 2 Screening of enoyl-CoA hydratase for acrylic acid production. The experiment was carried out in shaking flask in triplicate

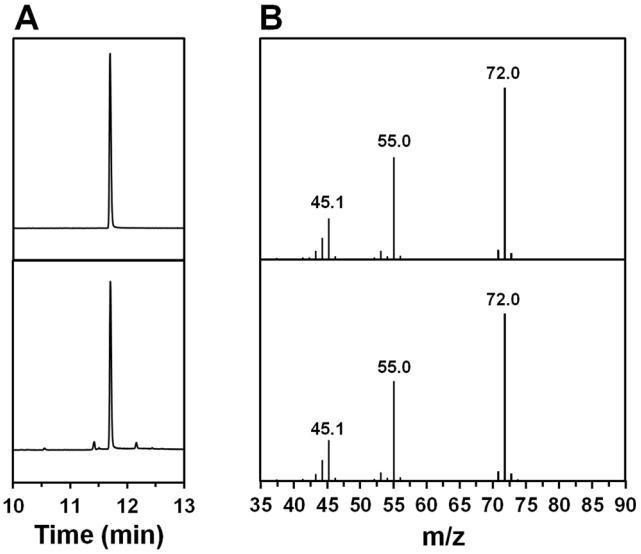

Fig. 3 Conformation of the production of acrylic acid from the Q2181 strain. Gas chromatography (**a**) and mass spectrometry (**b**) results are shown for acrylic acid standard (*up panel*) and Q2181 culture (*bottom panel*)

single mutants harboring each substitution as its homologs, and the effect of point mutations was verified by shake flask fermentation. Strains with PcsII R103K (Q2251) and PcsII D186A (Q2252) produced 16.1 and 20.8 mg/L acrylic acid, respectively (Fig. 5), and all other mutations have no effect on yield (data not shown). When the double point mutations R103K/D186A were constructed in PcsII (Q2288), the titer of acrylic acid was increased to 37.7 mg/L.

Toxic effects of acrylic acid on *E. coli*

Previous study has shown that *E. coli* YhdH protein exhibits NADPH-dependent acrylyl-CoA reductases activity, catalyzing acrylyl-CoA to propionyl-CoA (Marie Asao and Alber 2013). To block the by-product propionate, Δ*yhdH* mutation was introduced into strain Q2288 by phage P1 transduction to generate strain Q2314. However, the acrylate titer was significantly decreased and the growth of stain Q2314 was severely repressed.

As *E. coli* BW25113 strain with *yhdH* knockout was hypersensitive to acrylic acid (Todd et al. 2012), we proposed that this was caused by the toxicity of acrylate and tested growth of *E. coli* BL21(DE3) strain with presence of acrylic acid at different concentrations. Similar to BW25113, *E. coli* BL21(DE3) strain carrying *yhdH* mutation was incapable of growing even with 3.6 mg/L of exogenous acrylic acid, whereas the wild-type strain could grow normally with presence of 36 mg/L acrylic acid (Fig. 6). Unfortunately, the absence of *yhdH* gene makes *E. coli* strain hyper-sensitive to acrylic acid and functional YhdH will decrease the acrylate production by converting it into propionate.

Discussion

In this study, we constructed a recombinant *E. coli* strain to produce acrylic acid from glycerol by introducing glycerol dehydratase, propionaldehyde dehydrogenase, and enoyl-CoA hydratase. It is a more environmental-friendly substitute for the traditional production of acrylate from petroleum. Although the acrylate production is low, our study shows feasibility of engineering an acrylate biosynthetic pathway from inexpensive carbon source glycerol.

As acrylyl-CoA is a very active cytotoxic electrophile attacking sulfhydryl group (Clayden et al. 2001), accumulation of acrylate and acrylyl-CoA in culture severely repressed *E. coli* growth and is responsible for the low acrylic acid production. Previous study showed that *E. coli* growth showed 50 % reduction under aerobic conditions with ~1.4 g/L acrylic acid or under anaerobic conditions with ~28 mg/L acrylic acid (Arya et al. 2013). In this study, the excessive glycerol concentration and the use of shake flasks, which may resulted in the cells under micro-aerobic conditions, had a negative impact on acrylic acid accumulation. So far, no efficient means could overcome acrylate toxicity

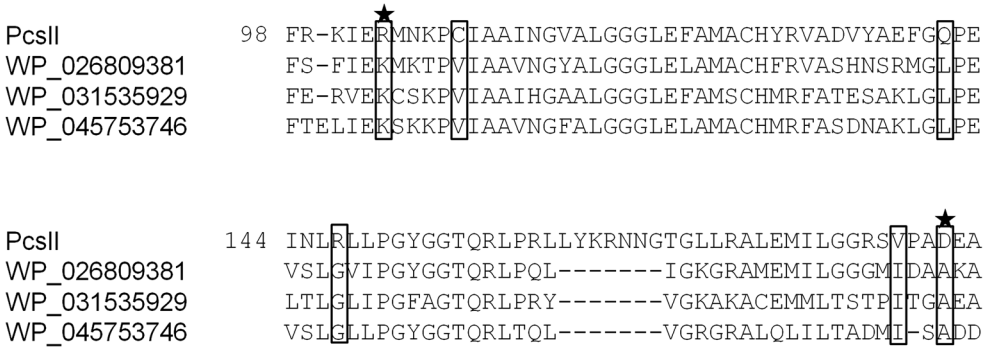

Fig. 4 Alignment of PcsII and the enoyl-CoA hydratases with known structure from *Arenibacter latercius* (accession No. WP_026809381), *Bacillus anthracis* (accession No. WP_031535929), and *Sphingobacterium* sp. (accession No. WP_045753746). *Boxes* correspond to amino acid residues conserved in all other enoyl-CoA hydratases but not in PcsII, and mutations enhancing acrylate production are *highlighted* by *asterisks*

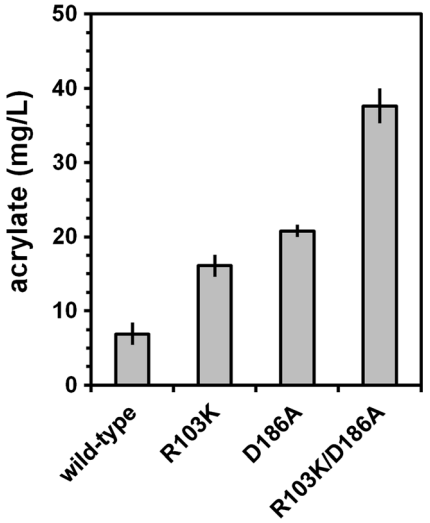

Fig. 5 Effect of PcsII mutagenesis on acrylate production. The experiment was carried out in shaking flask in triplicate

to growing cells, and we can bypass this puzzle via forming resting cells. *C. propionicum* resting cells were able to rapidly metabolize acrylate in a 35 g/L solution, although they could not grow in this solution (Sinskey et al. 1981), and the acrylate productivity from propionate had been successfully improved by using *C. propionicum* resting cells in previous report (O'Brien et al. 1990). Meanwhile, cell-free system may also contribute to accumulation of more acrylic acid.

We proposed that low activity of enoyl-CoA hydratase PcsII is also related to the low yield of acrylic acid, based on the following facts: (i) the precursor 3HP-CoA should be adequate as the P3HP-producing strain with chromosomal *dhaB* and *gdrAB* genes accumulated 25.7 g/L P3HP; (ii) site-directed mutagenesis of PcsII increased the acrylate production. As a powerful vehicle for the development of proteins exhibiting desirable properties, directed evolution should be an ideal way to further improve PcsII activity. To establish high-throughput PcsII activity assay, PcsII mutants

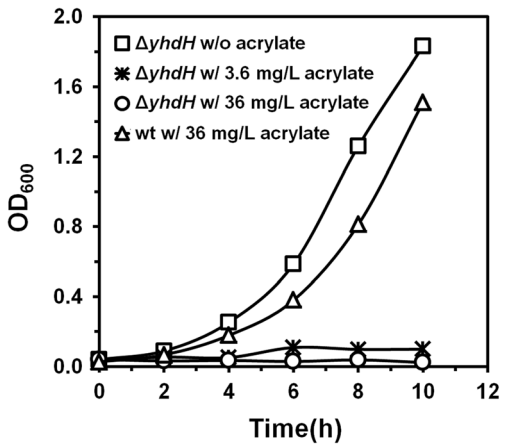

Fig. 6 Growth of *E. coli* BL21(DE3) wild-type strain and Δ*yhdH* mutant with acrylate at different concentrations

can be co-expressed with a NAD(P)H-dependent acrylyl-CoA reductase, so the acrylyl-CoA produced by PcsII will be converted into propionyl-CoA along with NAD(P)H oxidation, which is easily monitored by spectrometry. Additionally, the final step from acrylyl-CoA to acrylic acid is currently catalyzed by some undefined CoA transferase of *E. coli*, and discovery of a specific acrylate CoA transferase will not only improve the acrylic acid production but also benefit survival of *E. coli* cells.

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Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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