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Production of 3-hydroxypropionate homopolymer and poly(3-hydroxypropionate-*co*-4-hydroxybutyrate) copolymer by recombinant *Escherichia coli*

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ABSTRACT

Conversion of 3-hydroxypropionate (3HP) from 1,3-propanediol (PDO) was improved by expressing dehydratase gene (*dhaT*) and aldehyde dehydrogenase gene (*aldD*) of *Pseudomonas putida* KT2442 under the promoter of *phaCAB* operon from *Ralstonia eutropha* H16. Expression of these genes in *Aeromonas hydrophila* 4AK4 produced up to 21 g/L 3HP in a fermentation process. To synthesize homopolymer poly(3-hydroxypropionate) (P3HP), and copolymer poly(3-hydroxypropionate-co-3-hydroxybutyrate) (P3HP4HB), *dhaT* and *aldD* were expressed in *E. coli* together with the *phaC1* gene encoding polyhydroxyalkanoate (PHA) synthase gene of *Ralstonia eutropha*, and *pcs*' gene encoding the ACS domain of the tri-functional propionyl-CoA ligase (PCS) of *Chloroflexus aurantiacus*. Up to 92 wt% P3HP and 42 wt% P3HP4HB were produced by the recombinant *Escherichia coli* grown on PDO and a mixture of PDO+1,4-butanediol (BD), respectively.

1. Introduction

Bacterial polyhydroxyalkanoates (PHA) are natural biodegradable thermoplastics produced by various microorganisms as intracellular energy and carbon storage compounds (Rehm, 2010; Steinbüchel and Fuchtenbusch, 1998; Steinbüchel and Hein, 2001; Verlinden et al., 2007). The potential application of PHA as alternatives to petroleum-based plastics elevated the importance of PHA in the field of bioengineering (Rincones et al., 2009; Keshavarz and Roy, 2010; Chen, 2009). PHA are biodegradable and biocompatible polyesters with piezoelectricity and flexible mechanical properties (Chen, 2009; Chen and Wu, 2005). They are used in packaging, medicine, pharmacy, agriculture, and food industry (Chen, 2009; Rehm, 2010; Cheng et al., 2006). Microbial enhanced production of various PHA have been realized by metabolic engineered recombinant microorganisms (Chen et al., 2004; Park and Lee, 2005; Park et al., 2005; Jung et al., 2010; Tyo et al., 2009a, b).

3-Hydroxypropionic acid (3HP) is considered as a very important bio-renewable platform chemical (Cho et al., 2010; Straathof et al., 2005; van Maris et al., 2004). For example, bulk chemical acrylic acid can be produced from 3HP by dehydration both chemically and biochemically (Straathof et al., 2005; Lee et al., 2009). Yamada et al. (2001) reported the microbial production of acrylic acid from 3HP. 3HP can be derived from agricultural products including sugar, starch, or even cellulose in an anaerobic stoichiometric way (van Maris et al., 2004). Over 10 synthetic pathways were designed for producing 3HP from bio-renewable sources such as glucose or glycerol (Cho et al., 2010; Jiang et al., 2009). Notably, 39 g/L 3HP was produced from glycerol by overexpressing aldehyde dehydrogenase (ALDH), α-ketoglutaric semialdehyde dehydrogenase in *Escherichia coli* (Rathnasingh et al., 2009).

Bacterial synthesis of homopolymer P3HP was first reported in 2010 (Andreessen et al., 2010). In that report, P3HP reached 12% of cell dry weight (CDW) when the *pduP* from *Salmonella enterica* serovar Typhimurium LT2 encoding propionaldehyde dehydrogenase was expressed to function as both aldehyde dehydrogenase and CoA ligase PHA copolymers containing 3HP monomer were reviewed by Andreessen and Steinbüchel (2010), including poly(3HB-3HP), poly(3HB-3HP-2HP), poly(3HB-3HP-4HB-2HP) and poly(3HP-3HB-3HH-3HO) (Fukui et al., 2009; Ichikawa et al., 1996; Nakamura et al., 1991; Shimamura et al., 1994; Valentin et al., 2000).

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[1] Both authors contributed equally to this paper. ZQ did most of the experiments and analyzed the data, and SZY designed the research.

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Two key enzymes, including malonyl-CoA reductase (MCR) and propionyl-CoA synthetase (PCS) from the 3-hydroxypropionate cycle of phototrophic green non-sulfur eubacterium *Chloroflexus aurantiacus*, were identified (Alber and Fuchs, 2002; Herter et al., 2002; Hugler et al., 2002). Since the over-expression of all the *accABCD* genes in *E. coli* can increase the intracellular malonyl-CoA level (Davis et al., 2000), pathways that can produce 3HP from sugar were proposed shortly after the discovery of the 3-hydroxypropanoate cycle genes. A simplified version of this idea was first demonstrated in 2009 where poly(3HB-3HP) were produced in *Ralstonia eutropha* (Fukui et al., 2009). However, there was only a small portion of P3HP in that study. Thus, the poor yield of all previous studies might imply that either the 3HP-CoA ligase or the PHA synthase might have low activity for 3HP or 3HP-CoA.

In order to find the reasons, 3HP-CoA ligase or PHA synthase have to be supplied with sufficient precursors. Therefore, an alternative pathway of producing 3HP and P3HP from PDO was proposed based on the fact that genes *dhaT* and *aldD* from *Pseudomonas putida* KT2442 are very likely to convert 1,3-propanediol to 3-hydroxypropionate because they can convert 1,4-butanediol to 4-hydroxybutyrate for P4HB synthesis (Zhang et al., 2009).

In this study, a P3HP synthesis pathway combining *dhaT*, *aldD*, *pcs*' and *phaC1* was constructed for the first time in recombinant *E. coli* (Fig. 1). We aimed to exploit possibilities of producing 3HP, P3HP and novel copolymers of 3HP and 4HB from 1,3-propanediol or/and 1,4-butanediol.

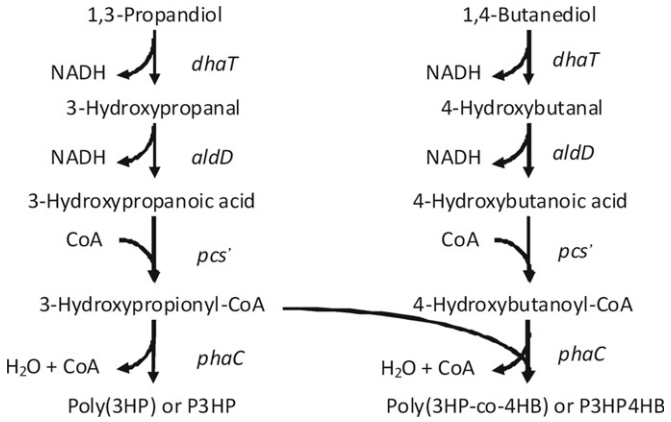

Fig. 1. Metabolically engineered pathways for production of P3HP and P3HP4HB in *E. coli*. Genes: *dhaT*, 1.3-propanediol dehydrogenase; *aldD*, aldehyde dehydrogenase; *pcs*', the gene encoding the ACS domain of propionyl-CoA synthase and functioning like 3-hydroxypropanoic acid CoA ligase; *phaC*, PHA synthase.

Table 1
Strains and plasmids used in this study.

Strains/plasmids	Description	Reference/source
pBBR1MCS2	Cloning vector, Km^R	Kovach et al. (1995)
pET28-A	Expression vector, Km^R	Novagen
pBHR68	pkSE5.3 derived, containing *phaA phaB phaC* from *R. eutropha*, Amp^R	Spiekermann et al. (1999)
pZL-dhaT	pBBR1MCS2 derived, *dhaT* under the control of *lac* promoter, Km^R	Zhang et al. (2009)
pZL-aldD	pBBR1MCS2 derived, *aldD* under the control of *lac* promoter, Km^R	Zhang et al. (2009)
pZL-dhaT-aldD	pBBR1MCS2 derived, *dhaT* and *aldD* under the control of *lac* promoter, Km^R	Zhang et al. (2009)
pZQ01	pBBR1MCS2 derived, *dhaT* and *aldD* under the control of P_{Re} promoter, Km^R	This study
pZQ02	pET28A derived, *dhaT* and *aldD* under the control of T7 promoter, Km^R	This study
pZQ03	pBHR68 derived, *phaC* and *pcs*' under the control of *lac* promoter, Amp^R	This study
E.coli S17-1	*recA*, harbors the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi*-1	Simon et al. (1983)
E.coli JM109	Expression host	TransGen Biotech
E.coli Trans5α	Expression host	TransGen Biotech
E.coli Trans1-T1	The fastest growing chemically competent strain currently available	TransGen Biotech
E.coli BL21 (DE3)	Expression host for pET vectors, containing IPTG-inducible T7 RNA polymerase gene	TransGen Biotech
A. hydrophila 4AK4	Wild type. Host for producing 3-hydroxypropionic acid	Qiu et al. (2004)

3. Results

3.1. 3-Hydroxypropionate (3HP) production by A. hydrophila 4AK4, E. coli BL21 (DE3) and their respective recombinant strains

3-Hydroxypropionate (3HP) production pathway was constructed to obtain the P3HP monomer precursor (Fig. 1). *A. hydrophila* 4AK4, *E. coli BL21* (*DE3*) and their respective recombinant strains harboring plasmids pZL-dhaT, pZL-aldD, pZL-dhaT-aldD, pZQ01 or pZQ02 were cultured in LB medium supplemented with various concentrations of PDO. *A. hydrophila* 4AK4 harboring merely pBBR1MCS2 was also cultivated as a control strain. It was found that the cell dry weigh (CDW) of the strains decreased with the increase of PDO concentration, although more PDO led to more 3HP formation (data not shown). A PDO concentration of 10 g/L PDO was found to be the best for 3HP production. The yield of recombinant *A. hydrophila* 4AK4 harboring pZL-aldD or pZL-dhaT was 0.31–1.43 g/L 3HP, which was in great excess of the control strain (0.15 g/L) (Table 3). This result revealed that functional expression of *dhaT* or *aldD* gene effectively helped improve the conversion of PDO to 3HP.

When both genes *dhaT* and *aldD* were introduced into *A. hydrophila* 4AK4, 2.34 g/L 3HP was synthesized from 10 g/L PDO, representing a 15-fold increase compared with that of the control strain harboring pBBR1MC2 (Table 3).

P_{Re} promoter and T7 promoter had been shown to direct the gene expression more effectively than with a *lac* promoter (Chung et al., 2009; Zheng et al., 2006; Studier and Moffatt, 1986). Therefore, P_{Re} and T7 promoters were cloned and introduced into pZL-dhaT-aldD, generating pZQ01 and pZQ02, respectively. *A. hydrophila* 4AK4 harboring pZQ01 containing *dhaT* and *aldD* under P_{Re} promoter, produced 2.73 g/L 3HP; *E. coli* BL21 harboring pZQ01, produced 2.75 g/L 3HP from 10 g/L PDO. This yield was in excess of that achieved by strains using a *lac* promoter; *A. hydrophila* 4AK4 (pZL-dhaT-aldD) produced 2.34 g/L 3HP and *E. coli* BL21 (pZL-dhaT-aldD) produced 2.16 g/L. Thus, expression of *dhaT* and *aldD* was enhanced by P_{Re} promoter compared with the expression under *lac* promoter.

Similarly, induced by IPTG, expression of *dhaT* and *aldD* under T7 promoter was also enhanced in pZQ02. As a result, 3HP concentration in the culture of *E. coli* BL21 (pZQ02) reached 2.41 g/L in the presence of isopropyl-β- D-thiogalactopyranoside (IPTG), compared with only 0.98 g/L without IPTG (Table 3).

Table 3
3HP production from PDO by recombinant *Aeromonas hydrophila* 4AK4 *E. coli* BL21 (DEB).

Strain	Plasmids	IPTG (mM)	CDW (g/L)	3HP(g/L)
A. hydrophila 4AK4	pBBR1MCS2	–	1.18 ± 0.02	0.15 ± 0.04
A. hydrophila 4AK4	pZL-aldD	–	2.23 ± 0.00	0.31 ± 0.03
A. hydrophila 4AK4	pZL-dhaT	–	2.55 ± 0.04	1.43 ± 0.02
A. hydrophila 4AK4	pZL-dhaT-aldD	–	3.65 ± 0.08	2.34 ± 0.19
A. hydrophila 4AK4	pZQ01	–	4.59 ± 0.04	2.73 ± 0.04
A. hydrophila 4AK4	pZQ02	–	3.23 ± 0.05	2.23 ± 0.03
E. coli BL21(DE3)	pZL-dhaT-aldD	–	1.32 ± 0.03	2.16 ± 0.07
E. coli BL21(DE3)	pZQ01	–	1.44 ± 0.04	2.75 ± 0.09
E. coli BL21(DE3)	pZQ02	1	0.79 ± 0.05	2.41 ± 0.16
E. coli BL21(DE3)	pZQ02	–	1.26 ± 0.05	0.98 ± 0.23

Shake flask experiments was were carried out in 50 ml LB medium for 48 h, and 10 g/L PDO was added before sterilization. Each value represents the average of three samples. 3HP concentration was determined by HPLC. pZQ01: pBBR1MCS2 derived, *dhaT* and *aldD* under P_{Re} promoter, Km^R. pZQ02: pET28A derived, *dhaT* and *aldD* under T7 promoter, Km^R. *Abbreviations*: CDW, cell dry weight.

3.2. Production of 3HP by recombinant A. hydrophila 4AK4 grown in a 6-L fermentor

Due to the rapid growth of *A. hydrophila* 4AK4 and its expression efficiency without induction under P_{Re} promoter, *A. hydrophila* 4AK4 harboring pZQ01 was considered as a suitable strain for 3HP production. In a fed-batch process, *A. hydrophila* 4AK4 (pZQ01) containing *dhaT* and *aldD* under P_{Re} promoter, was cultured in LB medium containing 10 g/L PDO; a total of 40 g PDO was supplemented during the growth process (the consumption rate of PDO monitored by HPLC).

After 59 h of cultivation, 21 g/L 3HP was produced, and continued to increase after fermentation. 85% PDO present in the culture was transformed into 3HP with 6 g/L PDO remaining (*ca*: 1:1conversion efficiency). Cells measured in CDW were grown to reach 9–10 g/L after 42 h of cultivation (Fig. 3).

3.3. Production of P3HP by recombinant Escherichia coli

The 3-hydroxypropionate synthesis pathway of *Chloroflexus aurantiacus*, together with *dhaT* and *aldD*, were combined and introduced into *E. coli*. Gene *pcs*' encoding 3-hydroxypropanoic acid CoA ligase was cloned and inserted into modified pBHR68 in which PHB synthesis genes *phaA* and *phaB* were deleted, generating plasmid pZQ03 (Fig. 2). Appropriate expression of the ACS domain encoded by *pcs*' in pZQ03 could be determined by the production of P3HP from 3HP. In addition, co-expression of pZQ01 containing *dhaT* and *aldD*, and pZQ03 containing *pcs*' and *phaC* of *R. eutropha*, should lead to the production of P3HP from PDO by the recombinant *E. coli* hosts.

First *E. coli* S17-1 (pZQ03) and *E. coli* JM109 (pZQ03) were cultured in LB medium supplemented with sterilized 3HP containing fermentation supernatant obtained from the above

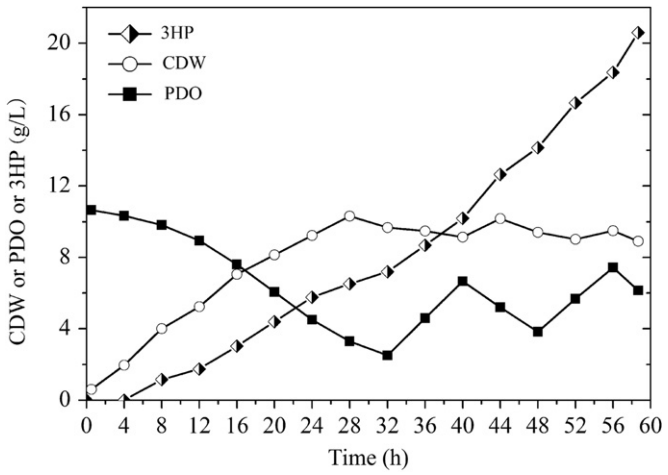

Fig. 3. Cell dry weight, 3HP accumulation and PDO consumption profile of *A. hydrophila* 4AK4 harboring pZQ01 grown in a 6-L fermentor. *A. hydrophila* 4AK4 harboring pZQ01 cultivated in LB medium produced up to 21 g/L 3HP in approximately 10.3 g/L CDW. The consumed PDO was converted to 3HP without by-product with 6 g/L PDO remaining at the end of fermentation.

cultures. GC and NMR revealed that both recombinants produced homopolymer P3HP from 3HP (Fig. 4). *E. coli* JM109 produced far less P3HP compared with *E. coli* S17-1 which synthesized 18 wt% P3HP in 0.98 g/L CDW in the presence of 3.3 g/L 3HP.

E. coli S17-1 harboring plasmids pZQ01 and pZQ03 increased P3HP production (Table 4); and it accumulated 50 wt% P3HP from LB medium containing 5 g/L PDO and 89 wt% P3HP from LB medium containing 10 g/L PDO, respectively. This is by far the highest P3HP accumulation reported. Obviously, the expression of pZQ01 containing *dhaT* and *aldD* genes significantly enhanced the conversion from PDO to 3HP and further to P3HP. An increase of PDO from 5 to 10 g/L not only led to more P3HP production but also resulted in more CDW (Table 4).

Two additional *E. coli* strains were investigated to further improve P3HP production; *E. coli* Trans5α and *E. coli* trans1-T1. Different *E. coli* strains have different expression capabilities, and are affected by the toxicity of PDO to different levels. Strains were cultured in nutrient-rich media, comprising LB' and TB media.

E. coli Trans5α (pZQ01, pZQ03) grown in 10 g/L PDO accumulated 41 wt% P3HP in LB medium, 67 wt% in LB' medium and 20 wt% in TB medium (Table 4). While *E. coli* Trans1-T1 (pZQ01, pZQ03) synthesized only 16 wt% in LB medium, 4.5 wt% in LB' medium and 7.7 wt% in TB medium, respectively, exhibiting the lowest P3HP yield among the three *E. coli* recombinants (Table 4). In all these shake flask studies, higher PDO concentration of 10 g/L led to more P3HP production compared with 5 g/L PDO. Nutrient rich medium including LB' and TB medium resulted in better cell growth but not necessarily more P3HP production. Comparison of the three strains cultured in identical conditions, demonstrated that recombinant *E. coli* S17-1 outperformed the other two *E. coli* strains, and demonstrated the highest P3HP yield and greatest CDW. Therefore, *E. coli* S17-1 (pZQ01, pZQ03) was selected for a further fermentor study in TB and LB' media.

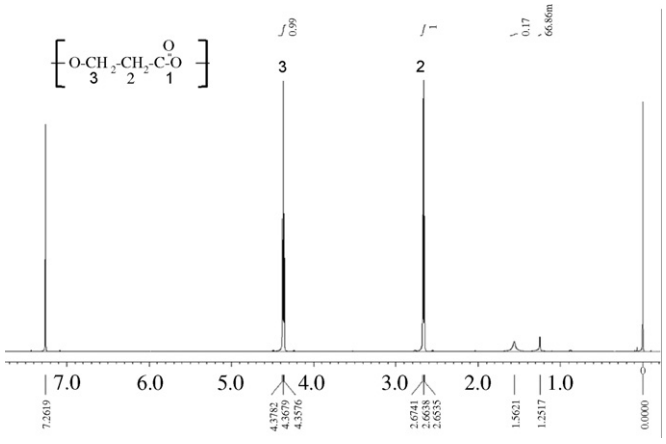

Fig. 4. ^{1}HNMR spectrum of P3HP synthesized by *E. coli* S17-1 (pZQ01, pZQ03).

3.4. P3HP production in 6-L fermentor containing LB' or TB medium

When grown in LB' medium, PDO was initially added into the fermentor at 16 h. 30 g PDO was used and only 1 g/L PDO remained after 48 h fermentation. *E. coli* S17-1 (pZQ01, pZQ03) produced 5.32 g/L CDW containing 85 wt% P3HP at the end of the 48 h fermentation (Fig. 5A). The CDW reached its peak at 5.52 g/L after 36 h growth, and P3HP reached its maximum content of 92 wt% after 44 h of growth (Fig. 5A), the maximum yield of P3HP was 22.2 g/L. During this process, PDO was consumed at a rate of 0.28 g/L/h, accompanying with a P3HP productivity of 0.14 g/L/h and a CDW formation rate of 0.11 g/L/h. Thus the conversion rate from PDO to P3HP was 0.50 g/g in our fermentor study.

When grown in TB medium, PDO was initially added at 14 h. 105 g PDO was added during the process and only 9.5 g/L PDO was remained at the end of the fermentation. 17.2 g/L CDW containing 51.8 wt% P3HP was obtained at the end of the 48 h fermentation (Fig. 5B). The CDW reached its peak at 20 g/L after 28 h growth, P3HP reached its maximum content of 66.8 wt% at 44 h, and the maximum yield of P3HP was 42.9 g/L. During the fermentation process, PDO was consumed at 0.75 g/L/h, P3HP was produced at 0.26 g/L/h and the CDW formation rate is 0.36 g/L/h. Therefore, the conversion rate (P3HP/PDO) from PDO to P3HP was 0.35 g/g.

According to the above fermentation studies, it was found that almost all of the consumed PDO was converted to 3HP with some of the 3HP utilized for P3HP synthesis. P3HP formation seemed to be non-growth associated as observed from Fig. 5C and D. The residual CDW (rCDW: CDW minus PHA) decreased with increasing P3HP formation (Fig. 5C), and the rCDW fluctuated between 6

Table 4
P3HP production from PDO by recombinant *Escherichia coli* strains harboring pZQ01 and pZQ03 grown in different media.

Medium and *E.coli* strains	PDO (g/L)	CDW (g/L)	P3HP (wt%)	P3HP (g/L)
LB medium				
S17-1 (pZQ01, pZQ03)	0	1.27 ± 0.12	NT	NT
S17-1 (pZQ01, pZQ03)	5	1.88 ± 0.08	50.03 ± 1.09	0.94 ± 0.05
S17-1 (pZQ01, pZQ03)	10	2.17 ± 0.16	88.71 ± 1.79	1.82 ± 0.45
Trans5α (pZQ01, pZQ03)	0	0.83 ± 0.08	NT	NT
Trans5α (pZQ01, pZQ03)	5	1.11 ± 0.03	32.74 ± 1.10	0.38 ± 0.02
Trans5α (pZQ01, pZQ03)	10	1.62 ± 0.09	41.46 ± 6.24	0.67 ± 0.06
Trans1-T1 (pZQ01, pZQ03)	0	0.96 ± 0.03	NT	NT
Trans1-T1 (pZQ01, pZQ03)	5	1.05 ± 0.02	11.35 ± 2.40	0.12 ± 0.03
Trans1-T1 (pZQ01, pZQ03)	10	1.15 ± 0.04	16.11 ± 3.45	0.19 ± 0.04
LB' medium				
S17-1 (pZQ01, pZQ03)	10	3.65 ± 0.08	80.57 ± 1.00	2.98 ± 0.02
Trans5α (pZQ01, pZQ03)	10	1.77 ± 0.09	67.15 ± 8.00	1.20 ± 0.19
Trans1-T1 (pZQ01, pZQ03)	10	2.12 ± 0.03	4.45 ± 0.30	0.09 ± 0.00
TB medium				
S17-1 (pZQ01, pZQ03)	10	7.76 ± 0.28	43.47 ± 1.45	3.38 ± 0.22
Trans5α (pZQ01, pZQ03)	10	4.88 ± 0.96	20.13 ± 0.62	1.09 ± 0.08
Trans1-T1 (pZQ01, pZQ03)	10	4.09 ± 0.06	7.70 ± 2.93	0.31 ± 0.12

Shake flask experiments were carried out in 50 ml LB medium for 48 h as described in Section 2. PDO was added to the medium prior to the sterilization. pZQ01: pBBR1MCS2 derived, *dhaT* and *aldD* under the control of P_{Re} promoter, Km^R. pZQ03: pBHR68 derived, *phaC* and *pcs*' under the control of *lac* promoter, Amp^R. *Abbreviations*: CDW, cell dry weight; wt, weight percentage; NT, not detected.

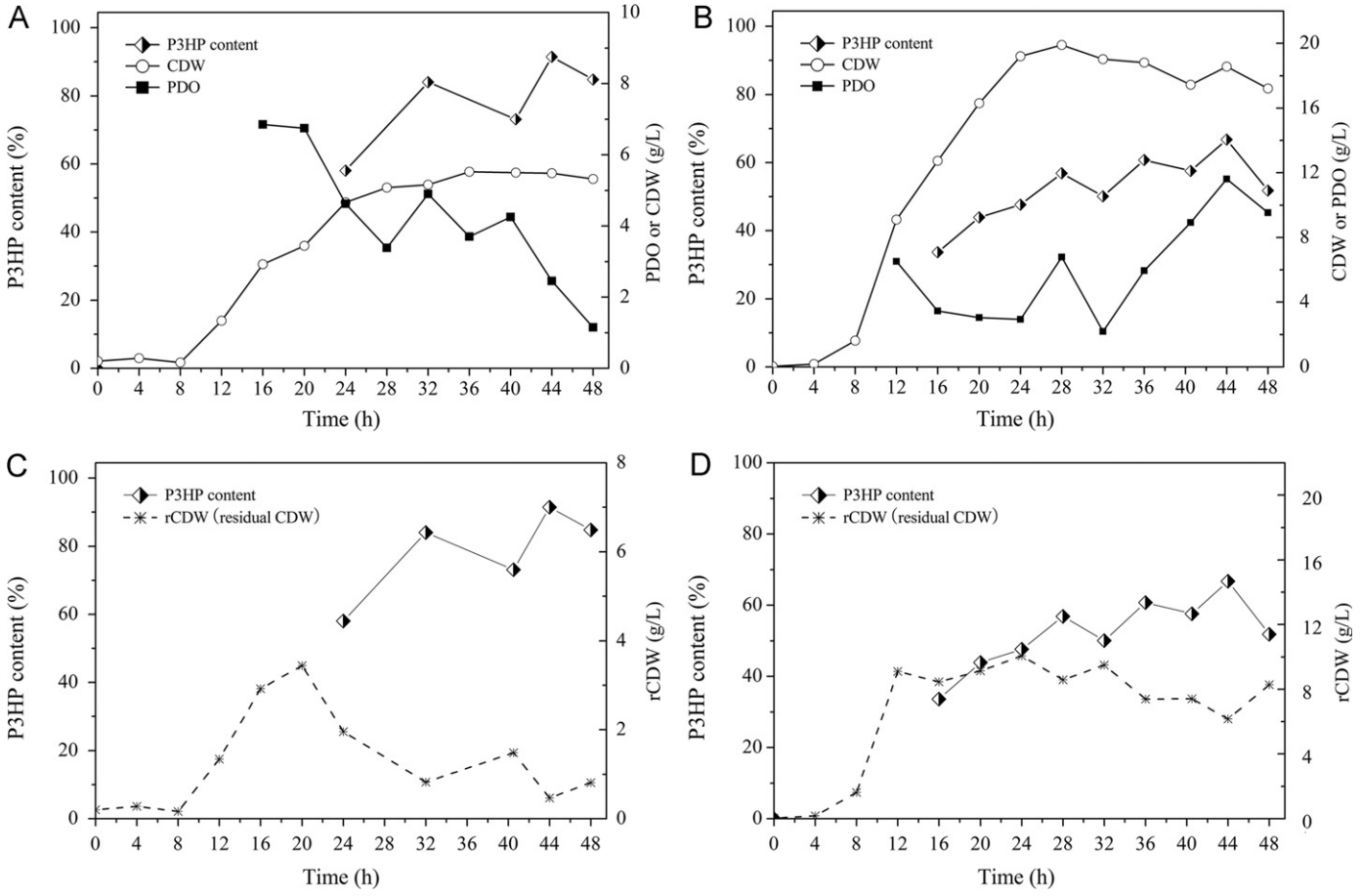

Fig. 5. Cell dry weight, PHA content, residual cell dry weight (rCDW=CDW-PHA) and PDO consumption profile of *E. coli* S17-1 harboring pZQ01 and pZQ03 grown in a 6-L fermentor. (A) *E. coli* S17-1 (pZQ01, pZQ03) cultivated in LB' medium produced maximally 92 wt% P3HP in approximately 5.5 g/L CDW, yielding 22.2 g/L P3HP. (B) *E. coli* S17-1 (pZQ01, pZQ03) cultivated in TB medium grew to maximally 20 g/L CDW containing 57 wt% P3HP, yielding 42.9 g/L P3HP. (C) Residual CDW (rCDW) in relation to P3HP production by recombinant *E. coli* cultured in LB' medium. (D) Residual CDW (rCDW) in relation to P3HP production by recombinant *E. coli* cultured in TB medium.

and 10 g/L while P3HP continued to increase (Fig. 5D). Therefore, P3HP formation was not affected by the fluctuation of cell growth when the bacteria were in their steady state. However, better cell growth is still important for P3HP formation since it leads to a higher expression level.

3.5. P4HB or P3HP4HB production by E. coli S17-1 (pZQ01, pZQ03) grown in LB, LB' or TB medium

The PCS' enzyme can catalyze the conversion of 3-hydroxypropionate to 3-hydroxypropionyl-CoA. Therefore it was likely that it could also catalyze 4-hydroxybutyrate (4HB) to 4-hydroxybutanoyl-CoA, due to the structural similarity between 3HP and 4HB, and thus lead to the formation of novel PHA copolymer P3HP4HB in the presence of PHA synthase PhaC. To investigate this, *E. coli* S17-1 (pZQ01, pZQ03) containing *dhaT*, *aldD*, *pcs*', and *phaC* was cultivated in different media supplemented with various concentrations of PDO or/and BD (Table 5).

In the presence of 5 g/L BD, supplemented with LB, LB' or TB medium, P4HB synthesis was low, merely 2–5 wt% in CDW. This suggested that the PCS' enzyme had a much lower activity on 4HB than on 3HP. Cell growth was greatest in TB medium and the poorest in LB medium (Table 5). When both PDO and BD were added to the medium, P3HP4HB was produced, ranging from 23 to 42 wt% in CDW. This was dependent on both the BD concentration and the species of the host strain (Table 5). However, 4HB monomer content was all less than 2 mol% in P3HP4HB.

3.6. Physical characterization of bacterial P3HP

P3HP produced by *E. coli* S17-1 (pZQ01, pZQ03) was extracted, purified and analyzed. ^{1}H NMR spectrum confirmed the PHA as a 3HP homopolymer (Fig. 4). The number-average molecular weight (M_n) of the P3HP was 3.0×10^5 Da for the strain cultured in TB medium, and 1.8×10^5 Da in LB' medium. These M_n results were similar to that of P3HB3HP (Shimamura et al., 1994), and lower than that of PHB homopolymer, which reached $5–10\times10^5$ Da (Fukui et al., 2009; Wang et al., 2010).

The thermal properties of P3HP synthesized in TB medium and LB' medium proved to be similar, by DSC analysis (Table 6). P3HP showed a significantly lower melting temperature T_m of 78 °C compared with its copolymer P(3HB-*co*-11 mol%3HP) with a T_m of 152 °C, and PHB with a T_m of 177 °C (Fukui et al., 2009). The microbial P3HP had a glass transition temperature (T_g) of about –21 °C and an enthalpy of fusion (ΔH_m) of about 54 J/g. This was similar to result achieved from P3HP synthesized by ring-opening polymerization of β-propiolactone (Shimamura et al., 1994).

The tensile strength (σ_t) of P3HP synthesized in TB and LB' medium were 28.28 and 26.47 MPa, respectively. The elongation at break (ε_b) was 683.5% and 583.9% for P3HP produced in TB and LB' media, respectively. These results were much higher than that of traditional plastics polypropylene (PP) and poly(lactic acid) (PLA) (Jem et al., 2010) (Table 6). The Young's modulus (E) was 333.3 MPa for P3HP in TB and 327.9 MPa in LB' medium (Table 6). The tensile strength (σ_t) and elongation at break (ε_b) of the

Table 5
P3HP4HB and P4HB production from PDO or/and BD by recombinant *E. coli* harboring pZQ01 and pZQ03.

Medium and strain	Carbon sources	CDW (g/L)	P4HB (wt%)	P3HP4HB (wt%)	4HB monomer (mol%)
LB medium					
S17-1 (pZQ01, pZQ03)	BD (5)[a]	1.72 ± 0.03	4.61 ± 0.35	NT	100
S17-1 (pZQ01, pZQ03)	BD(5)+PDO(5)	1.95 ± 0.02	NT	26.79 ± 2.85	1.98 ± 0.05
S17-1 (pZQ01, pZQ03)	BD(5)+PDO(10)	2.22 ± 0.03	NT	41.70 ± 0.54	0.49 ± 0.01
LB' medium					
S17-1 (pZQ01, pZQ03)	BD(5)	2.71 ± 0.03	4.09 ± 0.15	NT	100
S17-1 (pZQ01, pZQ03)	BD(5)+ PDO(5)	3.25 ± 0.04	NT	24.70 ± 0.39	1.45 ± 0.01
S17-1 (pZQ01, pZQ03)	BD(5)+PDO(10)	3.46 ± 0.05	NT	38.74 ± 3.18	/
TB medium					
S17-1(pZQ01, pZQ03)	BD(5)	6.28 ± 0.20	1.99 ± 0.20	NT	100
S17-1(pZQ01, pZQ03)	BD(5)+PDO(5)	7.80 ± 0.10	NT	22.54 ± 2.65	/
S17-1(pZQ01, pZQ03)	BD(5)+PDO(10)	8.35 ± 0.07	NT	25.95 ± 3.90	/

Shake flask experiments was were carried out in 50 ml LB medium for 48 h as described in Section 2. PDO and BD were added before sterilization. pZQ01: pBBR1MCS2 derived, *dhaT* and *aldD* under the control of P_{Re} promoter, Km^R. pZQ03: pBHR68 derived, *phaC* and *pcs*' under the control of *lac* promoter, Amp^R. *Abbreviations*: CDW, cell dry weight; wt, weight percentage.

[a] Compound concentration, indicated as g/L; NT, not detected.

Table 6
Physical characterization of the microbial P3HP compared with other polymers.

PHA samples	Thermal properties			Mechanical properties		
	T_m (°C)	ΔH_m (J/g)	T_g (°C)	E (MPa)	σ_t (MPa)	ε_b (%)
P3HP[a]	78.0	54.0	−21.46	333.3	28.3	683.5
P3HP[b]	77.7	53.6	−20.96	327.9	26.5	583.9
P3HP[c]	77	74	−19	–	–	–
P3HB[d]	177.3	83.0	0.8	–	20.4	5.9
PP[e]	175	–	−20	–	31	200
PLA[e]	140–175	–	57–58	–	53	6

Abbreviations: P3HB, poly(3-hydroxybutyrate); PP, polypropylene; PLA, poly(lactic acid); T_m, melting temperature; ΔH_m, apparent heat of fusion; T_g, glass transition temperature; E, Young's modulus; σ_t, tensile strength; ε_b, elongation at break.

[a] P3HP samples extracted from the strain cultured in TB medium.
[b] P3HP samples extracted from the strain cultured in LB' medium.
[c] Physical properties of P3HP synthesized by ring-opening polymerization of β-propiolactone (Shimamura et al., 1994).
[d] P3HB synthesized by *E. coli* strains (Li et al., 2010).
[e] Physical properties of traditional plastics including PP and PLA (Jem et al., 2010).

microbial P3HP were both higher than those of PHB (Li et al., 2010), indicating that P3HP was elastic and quite ductile.

4. Discussion

3-hydroxypropionic acid (3HP) is an important industrial intermediate, with a diverse range of applications (Cho et al., 2010; Straathof et al., 2005; van Maris et al., 2004). Biological production of 3HP has been developed in recent years, mainly utilizing glycerol or glucose as the substrate (Jiang et al., 2009). P3HP homopolymer could only be synthesized using chemical methods until 2010 when bacterial P3HP was accumulated up to 12 wt% from glycerol (Andreessen et al., 2010). This low P3HP content is insufficient for industrial-scale production. In this study, a different 3HP synthesis pathway was constructed to provide sufficient precursor for P3HP, and thus enhancing production of P3HP from 3HP or further from PDO, which could also be obtained from glucose or glycerol (Liu et al. 2010).

Genes *dhaT* and *aldD* have been proven functional in the conversion of BD to 4HB, since 4HB and 3HP are structurally similar, a plasmid containing these two genes was transformed into *E. coli* strains to realize 3HP production, the first step for synthesizing P3HP homopolymer.

Recombinant *A. hydrophila* exhibited a fast growth rate, which is essential for rapid conversion of PDO to 3HP. This strain was also selected as a host strain for higher 3HP productivity. To avoid the toxicity of PDO, its concentration was maintained to less than 10 g/L in shake flasks and fermentor studies (Table 3). Less production of 3HP in the wild strain might be due to a low expression level of *dhaT* and *aldD*. Additionally, *A. hydrophila* 4AK4 harboring pZL-dhaT produced 1.43 g/L 3HP, which was much higher than the strain harboring pZL-aldD with a yield of only 0.31 g/L 3HP (Table 3). Thus, for biotransformation of PDO to 3HP by *A. hydrophila* or *E. coli*, *dhaT* expression may be the limiting step, since over expression of *dhaT* produced a more significant effect than *aldD*. Simultaneous expression of both *dhaT* and *aldD* led to an enhanced 3HP production of 2.34 g/L compared with the control strain harboring pBBR1MCS2, which produced only 0.15 g/L 3HP (Table 3).

This result further suggested that 3HP yield was positively associated with higher expression level of these two genes and higher cell density. The promoter of the *phaCAB* operon from *Ralstonia eutropha* (P_{Re}) was shown to be more active than the *lac* promoter transcriptionally (Chung et al., 2009; Zheng et al., 2006). Similarly, T7 promoter was also reported to direct high-level protein expression in *E. coli* (Studier and Moffatt, 1986). Therefore, pZL-dhaT-aldD with *dhaT* and *aldD* with *lac* promoter was modified by replacing the *lac* promoter with P_{Re} promoter to generate pZQ01, and by replacing the *lac* promoter with T7 promoter to generate pZQ02 (Table 1). Consequently, both higher CDW and 3HP yield were obtained by strains *A. hydrophila* 4AK4 and *E. coli* BL21 (DE3) harboring pZQ01, compared with *A. hydrophila* containing pZL-dhaT-aldD under *lac* promoter (Table 3). However, expression of pZQ02 containing *dhaT* and *aldD* under the T7 promoter in *E. coli* BL21(DE3) resulted in very low CDW (Table 3). This could possibly be attributed to the strong T7 promoter which led to over production of the proteins and formation of inactive inclusion bodies with low enzyme activities (Baneyx, 1999). Thus, pZQ01 containing P_{Re} promoter was found to be the optimum for 3HP production. In fact, over 80% PDO added in the culture was transformed into 3HP (without obvious by-products), providing sufficient precursors for P3HP synthesis.

P3HP was recently reported to be synthesized biotechnologically by Andreessen et al. (2010) in a two step fed-batch fermentation process. The genes *pduP* encoding propionaldehyde dehydrogenase and *phaC* from *Ralstonia eutropha*, were co-expressed in recombinant *E. coli*. Cultivation of the resultant strain with 3HP containing supernatant led to an accumulation of

12 wt% P3HP. However, none of the previous studies had reached more than 15% P3HP in CDW. It was estimated that only higher activity of PhaC or the CoA ligase is not enough for producing higher P3HP content in the cells, continuous supply of sufficient intracellular 3HP should also be very important to synthesize more P3HP.

The propionyl-CoA synthetase (PCS) from the 3HP cycle is a tri-functional natural fusion protein. According to Herter et al. (2002), residues 18–850 of the PCS, which is termed ACS domain, showed high sequence similarity to acetyl-CoA synthase, and this domain is very likely to function as 3-hydroxypropanoic acid CoA ligase. Considering the domain distribution determination is the result of sequence analysis, the cloned segment of the fusion *pcs* may affect the activity of assumed 3-hydroxypropanoic acid CoA ligase.

Although the recombinant *A. hydrophila* 4AK4 is a good 3HP monomer producer, it is also a native PHA producer. When cultivated in LB medium, *A. hydrophila* 4AK4 produces a small amount of polyhydroxybutyrate (PHB). In order to produce P3HP homopolymer without PHB, *E. coli* was chosen as a host for P3HP production.

E. coli S17-1 harboring both plasmids pZQ01 and pZQ03 accumulated P3HP up to 89% of the CDW from PDO in shake flask cultivation, depending on the amount of PDO added to the cultures (Table 4). The P3HP content of maximally 92 wt% in CDW in a fermentation process was the highest P3HP content reported so far. Firstly, P3HP formation seemed to be non-growth associated since P3HP formation was not affected by the fluctuation of cell growth when the bacteria were in their steady state. Secondly, these results indicated that the ACS domain is an active 3HP-CoA ligase and the *R. eutropha* PhaC has sufficient activity in polymerizing 3HP-CoA, although the host strain of the *pcs* gene, *C. aurantiacusm*, grows optimally at 55 °C.

However, 18.9 g/L and 4.5 g/L 3HP monomer were detected in fermentor culture broth of TB medium and LB' medium, respectively, for producing P3HP by *E. coli* S17-1 harboring both pZQ01 and pZQ03, while little PDO was left. If the recombinant *E. coli* S17-1 harboring only pZQ03 (*pcs*' and *phaC*) was incubated in LB medium supported with 2–4 g/L 3HP monomer, no more than 18 wt% CDW of P3HP could be produced. This implies that *pcs*' gene may actually require a relative high intracellular concentration of 3HP monomer. Therefore, increasing the PCS activity and reducing the 3HP leakages are both possible ways to improve the yield of P3HP.

Furthermore, since 1,3-PDO can be produced from glucose (Celinska, 2010; Sauer et al., 2008), it is possible to produce P3HP from glucose with a two-step fermentation.

On the other hand, a small amount of 4HB could be incorporated into P3HP to form P3HB4HB (Table 5), demonstrating that 3-hydroxypropanoic acid CoA ligase also has the 4-hydroxybutyrate:CoA ligase activity. But this activity on 4HB was much lower than that on 3-hydroxypropanoate (Table 5). Since minor amounts of 4HB could be incorporated into P3HP to form P3HP4HB, it could be expected that P3HP4HB with high 4HB content could be synthesized if 4HB specific 4-hydroxybutyrate CoA transferase (*orfZ*) (Li et al., 2010; Valentin and Dennis, 1997; Zhang et al., 2009) or 4-hydroxybutyrate CoA ligase (Msed_1422) from *Metallosphaera sedula* (Berg et al., 2007) was functionally expressed in the recombinants. This is a subject for future investigation.

In this study, metabolic engineering approaches were successfully employed to produce 3HP and P3HP homopolymers. The new pathway effectively converted PDO to 3HP, and further to P3HP homopolymer. When BD was added, 4HB was incorporated into P3HP to form P3HP4HB although 4HB content was minor due to the low enzyme specificity on 4HB. Most importantly, P3HP could be produced to approximately 90% of cell dry weight, demonstrating the mass-production potential of this unique homopolymer. Furthermore, P3HP synthesized using this pathway exhibited improved properties for future applications, such as high ductility and tensile strength.

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References

Alber, B.E., Fuchs, G., 2002. Propionyl-coenzyme A synthase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO_2 fixation. J. Biol. Chem. 277, 12137–12143.

Andreessen, B., Lange, A.B., Robenek, H., Steinbüchel, A., 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. Appl. Environ. Microbiol. 76, 622–626.

Andreessen, B., Steinbüchel, A., 2010. Biosynthesis and biodegradation of 3-hydroxypropionate-containing polyesters. Appl. Environ. Microbiol. 76, 4919–4925.

Baneyx, F., 1999. Recombinant protein expression in *Escherichia coli*. Curr. Opin. Biotechnol. 10, 411–421.

Berg, I.A., Kockelkorn, D., Buckel, W., Fuchs, G., 2007. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Science 318, 1782–1786.

Celinska, E., 2010. Debottlenecking the 1.3-propanediol pathway by metabolic engineering. Biotechnol. Adv. 28, 519–530.

Chen, G.Q., 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem. Soc. Rev. 38, 2434–2446.

Chen, G.Q., Wu, Q., 2005. Microbial production and applications of chiral hydroxyalkanoates. Appl. Microbiol. Biotechnol. 67, 592–599.

Chen, J.Y., Liu, T., Zheng, Z., Chen, J.C., Chen, G.Q., 2004. Polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas stutzeri* 1317 had different substrate specificities. FEMS Microbiol. Lett. 234, 231–237.

Cheng, S., Chen, G.Q., Leski, M., Zou, B., Wang, Y., Wu, Q., 2006. The effect of D,L-β-hydroxybutyric acid on cell death and proliferation in L929 cells. Biomaterials 27, 3758–3765.

Cho, A., Yun, H., Park, J.H., Lee, S.Y., Park, S., 2010. Prediction of novel synthetic pathways for the production of desired chemicals. BMC Syst. Biol. 4, 35.

Chung, A., Liu, Q., Ouyang, S.P., Wu, Q., Chen, G.Q., 2009. Microbial production of 3-hydroxydodecanoic acid by *pha* operon and *fadBA* knockout mutant of *Pseudomonas putida* KT2442 harboring *tesB* gene. Appl. Microbiol. Biotechnol. 83, 513–519.

Davis, M.S., Solbiati, J., Cronan, J.E., 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J. Biol. Chem. 275, 28593–28598.

Fukui, T., Suzuki, M., Tsuge, T., Nakamura, S., 2009. Microbial synthesis of poly((R)-3-hydroxybutyrate-*co*-3-hydroxypropionate) from unrelated carbon sources by engineered *Cupriavidus necator*. Biomacromolecules 10, 700–706.

Herter, S., Fuchs, G., Bacher, A., Eisenreich, W., 2002. A bicyclic autotrophic CO_2 fixation pathway in *Chloroflexus aurantiacus*. J. Biol. Chem. 277, 20277–20283.

Hugler, M., Menendez, C., Schagger, H., Fuchs, G., 2002. Malonyl-coenzyme A reductase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO_2 fixation. J. Bacteriol. 184, 2404–2410.

Ichikawa, M., Nakamura, K., Yoshie, N., Asakawa, N., Inoue, Y., Doi, Y., 1996. Morphological study of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). Macromol. Chem. Phys. 197, 2467–2480.

Jem, K.J., van der Pol, J.F., de Vos, S., 2010. Microbial lactic acid, its polymer poly(lactic acid), and their industrial applications. In: Chen, G.Q. (Ed.), Plastics from Bacteria: Natural Functions and Applications, Springer, Heidelberg, pp. 246–323.

Jiang, X., Meng, X., Xian, M., 2009. Biosynthetic pathways for 3-hydroxypropionic acid production. Appl. Microbiol. Biotechnol. 82, 995–1003.

Jung, Y.K., Lee, S.Y., Tam, T.T., 2010. Towards systems metabolic engineering of PHA producers. In: Chen, G.Q. (Ed.), Plastics from Bacteria: Natural Functions and Applications, Springer, Heidelberg, pp. 63–84.

Kato, M., Bao, H.J., Kang, C.K., Fukui, T., Doi, Y., 1996. Production of a novel copolyester of 3-hydroxybutyric acid and medium chain length

3-hydroxyalkanaic acids by *Pseudomonas sp*. 61-3 from sugars. Appl. Microbiol. Biotechol. 45, 363–370.
Keshavarz, T., Roy, I., 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr. Opin. Microbiol. 13, 321–326.
Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., Peterson, K.M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.
Lee, S.H., Park, S.J., Park, O.J., Cho, J., Rhee, J.W., 2009. Production of 3-hydroxypropionic acid from acrylic acid by newly isolated *Rhodococcus erythropolis* LG12. J. Microbiol. Biotechnol. 19, 474–481.
Li, Z.J., Shi, Z.Y., Jian, J., Guo, Y.Y., Wu, Q., Chen, G.Q., 2010. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) from unrelated carbon sources by metabolically engineered *Escherichia coli*. Metab. Eng. 12, 352–359.
Liu, H.J., Xu, Y.Z., Zheng, Z.M., Liu, D.H., 2010. 1.3-Propanediol and its copolymers: research, development and industrialization. Biotechnol. J. 5, 1137–1148.
Nakamura, S., Kunioka, M., Doi, Y., 1991. Biosynthesis and characterization of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). J. Macromol. Sci. Chem. 28, 15–24.
Ouyang, S.P., Luo, R.C., Chen, S.S., Liu, Q., Chung, A., Wu, Q., Chen, G.Q., 2007. Production of polyhydroxyalkanoates with high 3-hydrooxydodecanoate monomer content by *fadB* and *fadA* knockout mutant of *Pseudomonas putida* KT2442. Biomacromolecules 8, 2504–2511.
Park, S.J., Choi, J.I., Lee, S.Y., 2005. Engineering of *Escherichia coli* fatty acid metabolism for the production of polyhydroxyalkanoates. Enzyme Microb. Technol. 36, 579–588.
Park, S.J., Lee, S.Y., 2005. Systems biological approach for the production of various polyhydroxyalkanoates by metabolically engineered *Escherichia coli*. Maromol. Symp. 224, 1–9.
Qiu, Y.Z., Ouyang, S.P., Shi, Z.Y., Wu, Q., Chen, G.Q., 2004. Metabolic engineering for the production of copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyhexanoate by *Aeromonas hydrophila*. Macromol. Biosci. 4, 255–261.
Raj, S.M., Rathnasingh, C., Jo, J.E., Park, S., 2008. Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia coli* BL21 strain. Process Biochem. 43, 1440–1446.
Rathnasingh, C., Raj, S.M., Jo, J.E., Park., S., 2009. Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. Biotechnol. Bioeng. 104, 729–739.
Rehm, B.H.A., 2010. Bacterial polymers: biosynthesis, modifications and applications. Nat. Rev. Microbiol. 8, 578–592.
Rincones, J., Zeidler, A.F., Grassi, M.C.B., Carazzolle, M.F., Pereira, G.A.G., 2009. The golden bridge for nature: the new biology applied to bioplastics. Polym. Rev. 49, 85–106.
Sauer, M., Marx, H., Mattanovich, D., 2008. Microbial production of 1.3-propanediol. Recent Pat. Biotechnol. 2, 191–197.
Shen, X.W., Yang, Y., Jian, J., Wu, Q., Chen, G.Q., 2009. Production and characterization of homopolymer poly(3-hydroxyvalerate) (PHV) accumulated by wild type and recombinant *Aeromonas hydrophila* strain 4AK4. Bioresour. Technol. 100, 4296–4299.
Shimamura, E., Scandola, M., Doi, Y., 1994. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). Macromolecules 27, 4429–4435.
Simon, R., Priefer, U., Pühler, A., 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat. Biotech. 1, 784–791.
Spiekermann, P., Rehm, B.H., Kalscheuer, R., Baumeister, D., Steinbüchel, A., 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. 171, 73–80.
Steinbüchel, A., Fuchtenbusch, B., 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol. 16, 419–427.
Steinbüchel, A., Hein, S., 2001. Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. Adv. Biochem. Eng. Biotechnol. 71, 81–123.
Straathof, A.J., Sie, S., Franco, T.T., van der Wielen, L.A., 2005. Feasibility of acrylic acid production by fermentation. Appl. Microbiol. Biotechnol. 67, 727–734.
Studier, F.W., Moffatt, B.A., 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189, 113–130.
Tartof, K.D., Hobbs, C.A., 1987. Improved media for growing plasmid and cosmid clones. Bethesda Res. Lab. Focus 9, 12–14.
Tyo, K., Ajikumar, P.K., Stephanopoulos, G., 2009a. Stabilized gene duplication enables long-term selection-free heterologous pathway expression. Nat. Biotechnol. 27 760-U115.
Tyo, K.E.J., Espinoza, F.A., Stephanopoulos, G., Jin, Y.S., 2009b. Identification of gene disruptions for increased poly-3-hydroxybutyrate accumulation in *Synechocystis* PCC 6803. Biotechnol. Progr. 25, 1236–1243.
Valentin, H.E., Dennis, D., 1997. Production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. J. Biotechnol 58, 33–38.
Valentin, H.E., Mitsky, T.A., Mahadeo, D.A., Tran, M., Gruys, K.J., 2000. Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) accumulation in recombinant *Escherichia coli*. Appl. Environ. Microbiol. 66, 5253–5258.
van Maris, A.J., Konings, W.N., van Dijken, J.P., Pronk, J.T., 2004. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab. Eng. 6, 245–255.
Verlinden, R.A.J., Hill, D.J., Kenward, M.A., Williams, C.D., Radecka, I., 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. J. Appl. Microbiol. 102, 1437–1449.
Wang, H.H., Zhou, X.R., Liu, Q., Chen., G.Q., 2010. Biosynthesis of polyhydroxyalkanoate homopolymers by *Pseudomonas putida*. Appl. Microbiol. Biotechnol. 89, 1497–1507.
Yamada, H., Nagasawa, T., Nakamura, T., 2001. Process for Biological Production of Organic Acids. U.S. Patent No. 5135858.
Zhang, L., Shi, Z.Y., Wu, Q., Chen, G.Q., 2009. Microbial production of 4-hydroxybutyrate, poly-4-hydroxybutyrate, and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) by recombinant microorganisms. Appl. Microbiol. Biotechnol. 84, 909–916.
Zheng, Z., Li, M., Xue, X.J., Tian, H.L., Li, Z., Chen, G.Q., 2006. Mutation on N-terminus of polyhydroxybutyrate synthase of *Ralstonia eutropha* enhanced PHB accumulation. Appl. Microbiol. Biotechnol. 72, 896–905.