Hyperproduction of poly(4-hydroxybutyrate) from glucose by recombinant *Escherichia coli*

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**Abstract**

**Background:** Poly(4-hydroxybutyrate) [poly(4HB)] is a strong thermoplastic biomaterial with remarkable mechanical properties, biocompatibility and biodegradability. However, it is generally synthesized when 4-hydroxybutyrate (4HB) structurally related substrates such as γ-butyrolactone, 4-hydroxybutyrate or 1,4-butanediol (1,4-BD) are provided as precursor which are much more expensive than glucose. At present, high production cost is a big obstacle for large scale production of poly(4HB).

**Results:** Recombinant *Escherichia coli* strain was constructed to achieve hyperproduction of poly(4-hydroxybutyrate) [poly(4HB)] using glucose as a sole carbon source. An engineering pathway was established in *E. coli* containing genes encoding succinate degradation of *Clostridium kluyveri* and PHB synthase of *Ralstonia eutropha*. Native succinate semialdehyde dehydrogenase genes *sad* and *gabD* in *E. coli* were both inactivated to enhance the carbon flux to poly(4HB) biosynthesis. Four PHA binding proteins (PhaP or phasins) including PhaP1, PhaP2, PhaP3 and PhaP4 from *R. eutropha* were heterologously expressed in the recombinant *E. coli*, respectively, leading to different levels of improvement in poly(4HB) production. Among them PhaP1 exhibited the highest capability for enhanced polymer synthesis. The recombinant *E. coli* produced 5.5 g L\(^{-1}\) cell dry weight containing 35.4% poly(4HB) using glucose as a sole carbon source in a 48 h shake flask growth. In a 6-L fermentor study, 11.5 g L\(^{-1}\) cell dry weight containing 68.2% poly(4HB) was obtained after 52 h of cultivation. This was the highest poly(4HB) yield using glucose as a sole carbon source reported so far. Poly(4HB) was structurally confirmed by gas chromatographic (GC) as well as 1H and 13C NMR studies.

**Conclusions:** Significant level of poly(4HB) biosynthesis from glucose can be achieved in *sad* and *gabD* genes deficient strain of *E. coli* JM109 harboring an engineering pathway encoding succinate degradation genes and PHB synthase gene, together with expression of four PHA binding proteins PhaP or phasins, respectively. Over 68% poly(4HB) was produced in a fed-batch fermentation process, demonstrating the feasibility for enhanced poly(4HB) production using the recombinant strain for future cost effective commercial development.

**Keywords:** Poly(4HB), PHB, Polyhydroxyalkanoates, PhaP, 4-hydroxybutyrate, *Escherichia coli*, Metabolic engineering, Synthetic biology

**Background**

A large variety of bacteria are able to accumulate diverse polyhydroxyalkanoates (PHA) as intracellular carbon and energy storage material under nutritional unbalanced conditions [1-4]. Due to their diverse structures, chirality, biodegradability and biocompatibility, PHA have attracted attentions from academic and industrial communities for their potential applications in areas of agriculture, medicine, and materials [2,5-7]. More than 150 types of hydroxyalkanoic acids have been known as monomers of PHA, leading to diverse polymer physical properties [8-11]. Some of the PHA monomers and oligomers were reported to stimulate cell proliferations [12,13].

Homopolymers of 4-hydroxybutyrate, or Poly(4HB), is a strong thermoplastic material with an elongation to break of 1000%, which means it can be stretched 10 times its original length before it is broken [14]. Due to
the remarkable mechanical properties, biocompatibility and biodegradability, poly(4HB) has been approved by the United States Food and Drug Administration (FDA) as the first PHA medical implant material among several PHA materials under investigation [5,15,16].

4-hydroxybutyrate (4HB) was first detected in a copolyester of 3HB and 4HB isolated from *R. eutropha* [17] and generally the incorporation of 4HB into PHA occurred only if 4-Hydroxybutyrate, 4-butyrolactone, 1,4-butanediol or 4-chlorobutyrate was provided as carbon source [18]. Poly(4HB) homopolyester was first reported to be synthesized by *R. eutropha* using 4-hydroxybutyrate as a precursor [19]. Wild-type bacteria like *Comamonas acidovorans* [20] and *Hydrogenophaga pseudovorans* [21] were also found to produce poly(4HB). Recombinant *E. coli* expressing *R. eutropha* PHA synthase gene *phaC* and *Clostridium kluyveri* 4HB-CoA:CoA transferase gene *orfZ* were found able to synthesize poly(4HB) homopolyester when both glucose and 4HB were provided as carbon sources [22,23]. As a general rule, structurally related substrates of 4HB such as γ-butyrolactone, 4-hydroxybutyrate or 1,4-butanediol (1,4-BD) are required as precursors for poly(4HB) synthesis [24]. However, these substrates are much more expensive than glucose, leading to the high cost of poly(4HB) production. Song et al. succeeded in producing poly(4HB) homopolyester using glucose as a sole carbon source in recombinant *E. coli* [25]. However, the reported yield (0.78 g L⁻¹) was low for mass cultivation. The high cost of raw material and the low yield of poly(4HB) prevents wide exploitation of poly(4HB) for more applications [26].

Studies on producing poly(3-hydroxybutyrate-co-4-hydroxybutyrate) or P3HB4HB from glucose in *E. coli* were reported [27,28]. The 4HB monomer was synthesized from anaerobic succinate degradation pathway of *C. kluyveri*. In this pathway, the intermediate of tricarboxylic acid (TCA) cycle succinyl-CoA was converted to succinate semialdehyde (SSA) by SSA dehydrogenase, and SSA was reduced to 4HB by 4HB dehydrogenase (Figure 1) [29,30]. Subsequently, 4HB was converted to 4HB-CoA via 4HB-CoA:CoA transferase. In *C. kluyveri*, these three enzymes were encoded by genes *sucD, 4hbD* and *orfZ*, respectively [28].

*E. coli* possesses two forms of SSA dehydrogenase (SSADH) encoded by *sad* and *gabD* first identified in *R. eutropha* [31], both SSADH catalyze degradation of SSA to succinate semialdehyde (SSA) by SSA dehydrogenase, and SSA was reduced to 4HB by 4HB dehydrogenase (Figure 1) [29,30]. Subsequently, 4HB was converted to 4HB-CoA via 4HB-CoA:CoA transferase. In *C. kluyveri*, these three enzymes were encoded by genes *sucD, 4hbD* and *orfZ*, respectively [28].

Phasins are small amphiphilic proteins localizing at the surface of PHA granules and there are interactions among various phasins [32-35]. They play important roles in PHA synthesis and granule formation [36]. The PhaP phasins were proven to promote PHB synthesis by regulating the surface/volume ratio of PHB granules or by interacting with PHA synthase yet without influencing PHA molecular weights [37]. Four genes encoding highly homologous phasins including *phaP1, phaP2, phaP3* and *phaP4* were found in *R. eutropha*, among which *phaP1* is the major phasin protein [38-40].

The aim of this study was to construct a recombinant *E. coli* for hyperproduction of poly(4HB) using glucose as a sole carbon source.
Results
Synthesis of poly(4HB) by recombinant E. coli grown in shake flasks
Biosynthesis pathway of poly(4HB) was constructed in sad and gabD deficient strain E. coli JM109SG by co-expressing sucD, 4hbD, orfZ and phaC heterologously using compatible plasmids pMCSH5 harboring sucD and 4hbD and pKSSE5.3 harboring orfZ and phaC (Figures 1 and 2). To study the function of PhaP on poly(4HB) production, four plasmids pKSSEP1, pKSSEP2, pKSSEP3 or pKSSEP4 were co-transformed with the plasmid pMCSH5 into E. coli JM109SG, respectively. In the pKSSEPx plasmid series, genes phaC and phaPx shared the same promoter PRe from R. eutropha while orfZ gene was initiated by its own promoter (Figure 2). In plasmid pMCSH5, sucD and 4hbD genes were controlled by promoter Ppdc. The function of poly(4HB) biosynthesis pathway with or without PhaP was tested in E. coli JM109 and its SSADH deficient strain was cultivated in shake flasks for 48 h in LB medium supplemented with 20 g L\(^{-1}\) glucose and PBS buffer.

Gas chromatographic analysis of derivatives obtained from lyophilized cells provided the single peak representing the methyl ester of 4HB, demonstrating that the resulting PHA was a poly(4HB) homopolyester. As expected, E. coli JM109 (pKSSE5.3, pMCSH5) did not produce any polyester. In comparison, its sad and gabD deficient mutant E. coli JM109SG (pKSSE5.3, pMCSH5) grew to 3.8 g L\(^{-1}\) CDW containing 12 wt% poly(4HB) (Table 1). The co-expression of PhaP1,4 in E. coli JM109SG (pKSSE5.3, pMCSH5) led to enhancements of poly(4HB) accumulation from 12 wt% without any PhaP to at least 22 wt% with PhaP4 to a maximum of 35 wt% with PhaP1. CDW also reached the highest of 5.5 g L\(^{-1}\) containing more than 35 wt% poly(4HB) when phaP1 was expressed in E. coli JM109SG (pKSSEP1, pMCSH5). Expression of phaP3 resulted in second highest poly(4HB) accumulation of 32 wt% CDW by E. coli JM109SG (pKSSEP3, pMCSH5). While PhaP2 and PhaP4 showed a similar lower ability on the improvement of poly(4HB) synthesis by the E. coli. The results were consistent with the different roles played by the four PhaP phasins for PHA synthesis [39,40].

Production and structure confirmation of poly(4HB) from fermentor study
As revealed by the shake flask results (Table 1), E. coli JM109SG (pKSSEP1, pMCSH5) showed the fastest growth rate and highest poly(4HB) accumulation level among all strains studied. It was therefore selected for further studies using well-controlled fermentor. E. coli JM109SG (pKSSEP1, pMCSH5) was grown in the modified LB medium containing tripled amount of yeast extract in a fed-batch fermentation process maintaining 10 g L\(^{-1}\) glucose during the entire period. After 52 h of fermentor cultivation, the cells grew to approximately 12 g L\(^{-1}\) CDW containing over 68 wt% poly(4HB) in the expense of a total of 90 g L\(^{-1}\) glucose consumed. This was by far, the highest poly(4HB) production using glucose as a sole carbon source. The poly(4HB) synthesis increased very fast during the exponential growth phase, and reached a relatively stable level at over 60 wt% poly(4HB) in CDW after 32 h (Figure 3).

To confirm homopolyester structure, gas chromatography and NMR were employed. As evidenced by spectra of \(^1\)H NMR and \(^13\)C NMR (Figure 4), three
well-characterized proton resonances, namely, 4HB (2): δ 2.39-2.37 ppm, 4HB (3): δ 1.97-1.93 ppm, 4HB (4): δ 4.12-4.09 ppm, appeared with identical intensities assigned to only 4HB units (Figure 4A). In the inserted dashed chart, the protons of 4HB (2) and 4HB (4) were found split into three peaks, while the peak of 4HB (3) proton was quadrupled based on the “N + 1 rule”: a proton with N neighbors appears as a cluster of N + 1 peaks. For example, the proton of 4HB (3) is neighbored to 4HB (2) and 4HB (4), and it has four protons around it as shown from the molecular structure of 4HB, thus, the proton of 4HB (3) is split into five peaks (Figure 4A).

Furthermore, the four 13C resonances at 20–180 ppm could be assigned to specific carbon species of 4HB units (Figure 4B). The carbon of carbonyl group 4HB (1) had the highest field in 13C NMR, the chemical shift was δ 172.74 ppm. From left to right, the chemical shifts of δ 63.61 ppm, δ 30.73 and δ 24.74 ppm belonged to 4HB

Figure 3 Growth and poly(4HB) production by recombinant E. coli harboring pKSSEP1 and pMCSH5 cultivated in a 6-l fermentor.

Figure 4 1H NMR (A) and 13C NMR spectra (B) of poly(4HB). Numbering scheme were the same as that of poly(4HB) molecular structure described in (A). The inserted dashed enveloped areas in (A) were the enlarged details of each proton split peaks. Chemical shifts were in ppm and tetramethylsilane (TMS) was employed as an internal chemical shift standard.
(4), 4HB (2) and 4HB (3), respectively. Based on these data, the NMR spectra confirmed the polyester sample be a homopolyester consisting of only 4-hydroxybutyrate.

Physical characterization of poly(4HB) produced by the recombinant *E. coli*

Poly(4HB) produced by *E. coli* JM109SG (pKSSEP1, pMCSH5) was extracted and purified before casting into films for mechanical property studies. The white and foggy poly(4HB) films exhibited a much higher elasticity than other known PHA. Its elongation at break ($\varepsilon_t$), tensile strength ($\sigma_t$), and Young’s modulus (E) was 1014%, 32.55 MPa and 12.8 MPa, respectively. The poly(4HB) had a molecular mass of weight average molecular weight (Mw) of $0.22 \times 10^6$ together with a polydispersity (Mw/Mn) of 2.11 as determined by gel-permeation chromatography.

Discussion

As a strong pliable thermoplastic material with good flexibility, poly(4HB) has been approved by FDA as a suture material (www.tepha.com). Biomedical applications are usually not sensitive to high cost. However, a reduction on poly(4HB) production cost should allow for more application exploitation. High production cost for poly(4HB) comes from expensive 4HB precursors including 4-hydroxybutyric acid, y-butyrolacton or 1,4-butanediol [41,42], and from very low yield of poly(4HB) by recombinant bacteria. Therefore, simple and low cost substrates as well as a highly productive strain can help reduce poly(4HB) production cost.

The anaerobic succinate degradation pathway employed in this study conferred on the recombinant *E. coli* the ability to utilize glucose as a sole carbon source for poly(4HB) production. The additional expression of PHA granule associate protein PhaP provided a further enhancement on poly(4HB) yield, allowing for further fermentor exploitation. While in wild *E. coli* strain, succinate semialdehyde can be degraded to succinate by SSA dehydrogenase (SSADH) encoded by *sad* and *gabD* [30], leading to a decreased metabolic flux to 4HB production (Figure 1). To channel more flux to 4HB, the native SSADH genes of *E. coli* were inactivated in the poly(4HB) producing recombinant. Shake flask studies of *E. coli* JM109 and its SSADH deficient mutant JM109SG harboring pMCSH5 and pKSSE5.3 showed that inactivation of SSADH genes significantly improved poly(4HB) synthesis compared with the wild strain JM109 which had no poly(4HB) production at all (Table 1). On the other hand, the highest 4HB molar fraction in P3HB4HB synthesized from glucose in *E. coli* was 11% reported so far [28]. Our result indicated that the recombinant enzymes in this pathway were active enough to provide sufficient 4HB from glucose for polymerization.

Expression of all four PhaPs (phasin) cloned from *R. eutropha* provided additional improvement on poly(4HB) accumulation in the order of PhaP1 > PhaP3 > PhaP2 > PhaP4 (Table 1). The differences of their different influences are not clear but probably due to the different roles of PhaP played on PHA granules formation. PhaP1 was the major phasin with the highest expression amount in *R. eutropha* while PhaP2, PhaP3 and PhaP4 were small in quantity [39,40], indicating its dominating function for PHA granule formation, and PhaP3 was expressed at a significantly high level in PhaP1 deficient strains, other PhaPs were in much lower levels. Our results therefore suggested that the poly(4HB) yields were positively related to the expression levels of PhaP.

The recombinant *E. coli* JM109SG (pKSSEP1, pMCSH5) grown to 12 g L$^{-1}$ CDW under a well-controlled fermentor run in a fed-batch process accumulated over 68% poly (4HB) using glucose as the only carbon source over a 52 h period (Figure 3). This is by far the highest yield for poly (4HB). In its exponential growth period of 8–24 h after inoculation, poly(4HB) content increased most rapidly and reached a relatively stable level when cells entered the stationary phase. As in the exponential phase, TCA cycle is most active, supplying the most succinyl-CoA for the poly(4HB) synthesis, leading to a rapid poly(4HB) accumulation rate. A continuous fermentation process that maintains the cells in their exponential growth phase may further improve poly(4HB) accumulation level.

Conclusion

In summary, *Escherichia coli* strain JM109 harboring an engineering pathway encoding succinate degradation genes of *Clostridium kluyveri* and PHB synthase gene of *Ralstonia eutropha* together with its native succinate semialdehyde dehydrogenase genes *sad* and *gabD* [30], leading to a decreased metabolic flux to 4HB production, was able to achieve significant level of poly(4HB) biosynthesis from glucose. Additional expression of four PHA binding proteins PhaP or phasins in the recombinant strain, respectively, led to a further improvement of poly(4HB) accumulation. PhaP1 was found most useful among the four PhaPs used. Over 68 wt% poly (4HB) was produced in a fed-batch fermentation process, demonstrating the feasibility for enhanced poly(4HB) production using the recombinant strain for future cost effective commercial development.

Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study were listed in Table 2. *E. coli* Trans1-T1 from TransGen Biotech (Beijing, China) was used for plasmids construction. *Ralstonia eutropha* was used as a template for gene cloning [43]. *E. coli* JM109 (TaKaRa, Dalian, China) and
its sad and gabD deficient strain E. coli JM109SG were used for gene expression and poly(4HB) accumulation.

Plasmid pKSSE5.3 was constructed by Hein et al. containing genes phaC and orfZ [22]. Plasmids pKSSEPx, with “x” referring to 1, 2, 3 and 4 in this study, were constructed by subcloning genes phaP1, phaP2, phaP3 and phaP4 from genome of R. eutropha, followed by inserting them into the plasmid pKSSE5.3, respectively. Primers phaPxF and phaPxR were used for PCR amplification. The PCR products of phaP1, phaP2 and phaP3 fragments were digested by StuI while phaP4 fragment digested by pFII. The gel electrophoresis-purified fragments were then ligated with pKSSE5.3 digested by the respective endonuclease (Figure 2). Plasmid pMCSH5 was constructed by Li et al. containing sucD and 4hbD genes [28].

Cultivation conditions and culture medium
Plasmids pKSSE5.3 or pKSSEPx were co-transformed with pMCSH5 into E. coli JM109 and its SSADH deficient mutant by electroporation.

For shake flask cultivations, the growth process was carried out on a rotary shaker at 200 rpm in 500 mL conical flasks containing 50 mL LB medium supplemented with 20 g L⁻¹ glucose at an inoculation volume of 4% for 48 h. For fermentation studies, seed culture was inoculated into a 6-L fermentor (NBS3000, New Brunswick, USA) at 10% inoculation volume with an operating volume of 3 L. The fermentation process was carried out at 37°C, pH 7.0 under a dissolved oxygen concentration (DO) of 30% of saturation. For poly(4HB) accumulation, concentrated glucose was complemented when OD600 began to increase exponentially to maintain the concentration of glucose above 10 g L⁻¹ during the fermentation process.

For shake flask studies, Luria-Bertani (LB) medium supplemented with 20 g L⁻¹ glucose in phosphate buffered saline (PBS) solution with a working concentration of 2.31 g L⁻¹ KH₂PO₄ and 16.42 g L⁻¹ K₂HPO₃·3H₂O to maintain a pH around 7.0. Glucose and PBS were added to the medium after heat sterilization. During the fermentation process, LB medium with 15 g L⁻¹ yeast

| Table 2 Bacterial strains and plasmids used in this study |
|---------------------------------|----------------------------------|---------------------------------|
| **Strains**                      | **Plasmids**                     | **Primers (5‘→3’)**             |
| **E. coli JM109**                | pKSSE5.3                         | phaP1F                          |
| recA1, endA1, gyrA96, thi, hsdR17, supF44, relA1, Δ(lac proAB) F [traD36, proAB, lacZΔM15] | phaP1 gene inserted into pKSSE5.3, AmpR | AGTCTAGGCTCTAAAGAATGCCTTGACCCACCC |
| **E. coli JM109SG**              | pKSSEP1                          | phaP1R                          |
| JM109 Δsad ΔgabD                 | phaP1 gene inserted into pKSSE5.3, AmpR | AGTCTAGGCTCGAAACACACCCGAAACGCAG |
| **E. coli Trans1-T1**            | pKSSEP2                          | phaP2F                          |
| The fastest growing chemically   | phaP2 gene inserted into pKSSE5.3, AmpR | CAGCGAGGCGCTGTCGCAATGCTGCAATCTTTAT |
| available                      | pKSSEP3                          | phaP2R                          |
|                                   | phaP3 gene inserted into pKSSE5.3, AmpR | ACTATAGGCGCTATACCCACCGTGACAACGGCAAG |
|                                   | pKSSEP4                          | phaP3F                          |
|                                   | phaP4 gene inserted into pKSSE5.3, AmpR | ACTATAGGCGCTAACACCTCGGATGCTGCCGCT |
|                                   | pMCSH5                           | phaP3R                          |
| sucD-4hbD inserted into pBBR1MCS-2, KmR | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| **Primers (5‘→3’)**              |                                   | phaP4F                          |
| phaP1F                           | AGTCTAGGCTCTAAAGAATGCCTTGACCCACCC | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP1R                           | AGTCTAGGCTCGAAACACACCCGAAACGCAG  | AGTCTAGGCGCTTGTACCCACCGTGACAACGGCAAG |
| phaP2F                           | CAGCGAGGCGCTGTCGCAATGCTGCAATCTTTAT | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP2R                           | ACTATAGGCGCTATACCCACCGTGACAACGGCAAG | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP3F                           | ACTATAGGCGCTAACACCTCGGATGCTGCCGCT | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP3R                           | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP4F                           | CAGCGAGGCGCTTGTACCCACCGTGACAACGGCAAG | CAGCGAGGCGCTTGTAGCATCCGAGCGGAAGATT |
| phaP4R                           | AGTCTAGGCGCTTGTACCCACCGTGACAACGGCAAG | AGTCTAGGCGCTTGTACCCACCGTGACAACGGCAAG |

*American Type Culture Collection.*
extract without PBS was used to promote cell growth. 50 mg L⁻¹ kanamycin and 100 mg L⁻¹ ampicillin were added to the medium to maintain stability of the plasmids during the growth processes.

Analytical methods
Bacterial cultures were harvested by centrifugation at 3000 g for 10 min and then washed with distilled water. The cell dry weight (CDW) was measured after vacuum lyophilization. PHA content and composition were analyzed by gas chromatography (SHIMADZU GC-2014 C, Kyoto, Japan) after methanolysis of lyophilized cells in chloroform with γ-butyrolactone (Sigma-Aldrich) used as standard [44,45].

PHA extraction and physical characterization
PHA were extracted from the lyophilized cells [42]. In details: 10 mL chloroform was added to 1 g of dry cells in screw-capped tubes. The tubes were maintained at 100°C for 4 h. Subsequently, equal volume of water was added to the tube and the chloroform at the bottom was sucked out and precipitated with an excess of 10 volumes of ice-cold ethanol [46]. The molecular structure of poly(4HB) was studied using nuclear magnetic resonance (NMR). The sample was dissolve in deuterated-chloroform (CDCl3) and recorded the 1H NMR and 13C NMR spectra. The proton (1H) NMR was performed on JOEL JNM- ECA 300 NMR spectrophotometer in deuterated chloroform as a solvent, tetrathylethylsilane (TMS) was used as an internal chemical shift standard. Carbon (13C) NMR spectra was measured on 600 MHz spectrophotometer.

To study its mechanical properties, PHA samples were spread into films by the conventional solvent-casting method [47]. The resulting PHA films were cut into rectangle-shaped specimens with a width of 10 mm and a thickness of approximately 120 μm. The stress–strain measurements of films were carried out using an AL-7000 S testing machine (Gotech Testing Machine, China) at room temperature. The speed of the cross-head was 50 mm min⁻¹ [48]. Molecular weights were obtained via gel permeation chromatography (GPC Spectra System P2000) equipped with a Shimadzu RID-10A detector.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
XYZ designed the experiments, constructed the plasmids, performed fermentation studies and prepared the manuscript. XXY performed shake flask experiments. ZYS provided suggestions. DCM and WJJ participated in the fermentation process. LPW analyzed the NMR data. ICC and GQC supervised the study. All authors read and approved the final manuscript.

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References
1. Anderson AJ, Dawes EA: Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 1990, 54:450–472.
2. Steinbüchel A, Valentin HE: Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 1995, 128:219–228.
3. Chen GQ, Patel MK: Plastics derived from biological sources: present and future: a technical and environmental review. Chem Rev 2012, 112:2082–2099.
4. Cai L, Tan D, Aibaidula G, Dong XR, Chen JC, Tian WD, Chen GQ: Comparative genomics study of PHA and ektoine relevant genes from Halomonas sp. TD01 revealed extensive horizontal gene transfer events and co-evolutionary relationships, Microb Cell Fact 2011, 10:88.
5. Martin DP, Williams SF: Medical applications of poly-4-hydroxybutyrate: a strong flexible absorbable biomaterial. Biochim Biophys Acta 2003, 1697–1705.
6. Hazer B, Steinbüchel A: Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. Appl Microbiol Biotechnol 2007, 74:1–12.
7. Chen GQ: A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 2009, 38:2434–2446.
8. Madison LL, Huisman GW: Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microb Mol Biol Rev 1999, 63:21–53.
9. Gao X, Chen JC, Wu Q, Chen GQ: Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels. Curr Opin Biotechnol 2011, 22:768–774.
10. Chen GQ, Wu Q: Microbial production and applications of chiral hydroxyalkanoates. Appl Microbiol Biotechnol 2005, 67:592–599.
11. Tripathi L, Wu LP, Chen GQ: Microbial Synthesis of Diblock Copolymer Poly-3-Hydroxybutyrate-block-Poly-3-Hydroxyhexanoate [P(3HB)-b-P(3HHx)] by a Genome Reduced Pseudomonas putida KT2442. Microb Cell Fact 2012, 11:44.
12. Cheng S, Chen GQ, Leski M, Zou B, Wang Y, Wu Q: The effect of D, L-β-hydroxybutyric acid on cell death and proliferation in L929 cells. Biomaterials 2006, 27:358–365.
13. Sun J, Dai ZW, Chen GQ: Oligomers of polyhydroxyalkanoates stimulated calcium ion channels in mammalian cells. Biomaterials 2007, 28:3896–3903.
14. Saito Y, Nakamura S, Hiratsumi M, Doi Y: Microbial Synthesis and Properties of Poly(3-hydroxybutyrate-co-4-hydroxybutyrate). Polym Int 1996, 39:169–174.
15. Mergaert J, Webb A, Anderson C, Wouters A, Swings J: Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. Appl Environ Microbiol 1995, 61:3233–3238.
16. Chen GQ, Wu Q: The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 2005, 26:6565–6578.
17. Kunicka M, Nakamura Y, Doi Y: New bacterial copolyesters produced in Alcaligenes eutrophus from organic-acids. Polymer Commun 1988, 29:174–176.
18. Steinbüchel A, Valentin HE, Schönbaeum A: Application of recombinant gene technology for production of polyhydroxyalkanoic acids.
biosynthesis of poly(4-hydroxybutyric acid) homopolyester. J. Polymer Environ 1994, 2(6):7–14.
19. Nakamura S, Doi Y, Scandola M: Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate). Macromolecules 1992, 25:4237–4241.
20. Sudesh K, Fukui T, Taguchi K, Iwata T, Doi Y: Improved production of poly (4-hydroxybutyrate) by Comamonas acidovorans and its freeze-fracture morphology. Int J Biol Macromol 1999, 25:79–85.
21. Choi MH, Yoon SC, Lenz RW: Production of poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) and poly(4-hydroxybutyric acid) without subsequent degradation by Hydrogenophaga pseudoflava. Appl Environ Microbiol 1999, 65:1570–1577.
22. Hein S, Söhling B, Gottschalk G, Steinbüchel A: Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of Escherichia coli. FEMS Microb Lett 1997, 153:411–418.
23. Song SS, Hein S, Steinbüchel A: Production of poly(3-hydroxybutyric acid) by fed-batch cultures of recombinant strains of Escherichia coli. Biotechnol Lett 1999, 21:193–197.
24. Lee WH, Azizan MNM, Sudesh K: Effects of culture conditions on the composition of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) synthesized by Comamonas acidovorans. Polymer Degrad Stabil 2004, 84:129–134.
25. Song SS, Ma H, Gao ZX, Ja ZH, Zhang X: Construction of recombinant Escherichia coli strains producing poly(3-hydroxybutyric acid) homopolymer from glucose. Wei Sheng Wu Xue Tong Bao 2005, 45:382–386.
26. Singh M, Patel SKS, Kala VC: Bacillus subtilis as potential producer for polyhydroxyalkanates. Microb Cell Fact 2009, 8:38.
27. Valentin HE, Dennis D: Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant Escherichia coli grown on glucose. J Biotechnol 1997, 58:233–28.
28. Li ZJ, Shi ZY, Jian J, Gao YY, Wu Q, Chen GQ: Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) from unrelated carbon sources by metabolically engineered Escherichia coli. Metab Eng 2010, 12:352–359.
29. Söhling B, Gottschalk G: Molecular analysis of the anaerobic succinate degradation pathway in Clostridium kluyveri. J Bacteriol 1996, 178:871–880.
30. Spyros A, Marchesauft W: Nuclear magnetic-relaxation measurements of poly(4-hydroxybutrate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in the bulk. Macromolecules 1995, 28:6108–6111.
31. Valentin HE, Zwingmann G, Schonebaum A, Steinbüchel A: Metabolic pathway for biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) from 4-hydroxybutyrate by Alcaligenes eutrophus. Eur J Biochem 1995, 227:49–60.
32. Zhang S, Wang ZH, Chen GQ: Microbial polyhydroxyalkanate synthesis repression protein PhaR as an affinity tag for recombinant protein purification. Microb Cell Fact 2010, 9:28.
33. Wang ZH, Ma P, Chen J, Zhang J, Yao YC, Zhang HF, Chen GQ: A heterogeneous two-system hybrid in Escherichia coli based on polyhydroxyalkanates synthesis regulatory proteins PhaR. Microb Cell Fact 2011, 10:221.
34. Dennis D, Liebig C, Holley T, Thomas KS, Khosla A, Wilson D, Augustine B: Preliminary analysis of polyhydroxyalkanate inclusions using atomic force microscopy. FEMS Microb Lett 2003, 226:113–119.
35. Wieczorek R, Pries A, Steinbüchel A, Mayer F: Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanonic acid granules in Alcaligenes eutrophus. J Bacteriol 1995, 177:2425–2435.
36. Potter M, Muller H, Steinbüchel A: Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in Ralstonia eutrophus H16. Microbiology 2005, 151:825–833.
37. Luz G, Stabenow-Lohbauer U, Langer M, Boskurt T: Gastroenterology in Germany determination of current status and perspectives. Results of a survey among members of the German Society of Digestive and Metabolic Diseases. Z Gastroenterol 1996, 34:542–548.
38. Potter M, Muller H, Reinecke F, Wieczorek R, Frick F, Bowien B, Friedrich D, Steinbüchel A: The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in Ralstonia eutrophus. Microbiol 2004, 150:2301–2311.
39. Potter M, Steinbüchel A: Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. Biomacromolecules 2005, 6:552–560.
40. Neumann L, Spinozzi F, Sinibaldi R, Rustichelli F, Potter M, Steinbüchel A: Binding of the major phasin, PhaP1, from Ralstonia eutrophus H16 to poly (3-hydroxybutyrate) granules. J Bacteriol 2004, 186:2911–2919.
41. Kunioka M, Kawaguchi Y, Doi Y: Production of biodegradable copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate by Alcaligenes eutrophus. Appl Microbiol Biotechnol 1989, 30:569–573.
42. Mitomo H, Hishe W, Nishiyama K, Kasuga K, Doi Y: Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by Comamonas acidovorans. Polymer 2001, 42:3455–3461.
43. Pohlmann A, Frick W, Reinecke F, Kusian B, Liesegang H, Cramm R, Ettinger E, Ewering C, Potter M, Schwartz E, Stettmüller V, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B: Genome sequence of the bioplastic-producing “Knollgas” bacterium Ralstonia eutrophus H16. Nat Biotechnol 2006, 24:1257–1262.
44. Kato M, Bao HJ, Kang CK, Fukui T, Doi Y: Production of a novel copolyester of 3-hydroxybutyric acid and medium chain length 3-hydroxyalkanolic acids by Pseudomonas sp 61–3 from sugars. Appl Microbiol Biotechnol 1996, 45:363–370.
45. Chen JY, Liu T, Zheng Z, Chen JC, Chen GQ: Polyhydroxyalkanolate synthases PhaC1 and PhaC2 from Pseudomonas stutzeri 1317 had different substrate specificities. FEMS Microb Lett 2004, 234:231–237.
46. Ouyang SP, Luo RC, Chen SS, Liu Q, Chung A, Wu Q, Chen GQ: Production of polyhydroxyalkanoates with high 3-hydroxydodecanoate monomer content by fadB and fadD knockout mutant of Pseudomonas putida KT2442. Biomacromolecules 2007, 8:2504–2511.
47. Gao Y, Kong L, Zhang L, Gong YD, Chen GQ, Zhao NM, Zhang XF: Improvement of mechanical properties of poly(DL-lactide) films by blending of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Eur Polymer J 2005, 42:764–775.
48. Luo R, Chen J, Zhang L, Chen GQ: Polyhydroxyalkanolate copolymers produced by Ralstonia eutrophus PHB – 4 harboring a low-substrate-specificity PHA synthase PhaC2P from Pseudomonas stutzeri 1317. Biochim Biophys Acta 2006, 1721:218–225.

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