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Production of Block Copolymer Poly(3-hydroxybutyrate)-*block*-poly(3-hydroxypropionate) with Adjustable Structure from an Inexpensive Carbon Source

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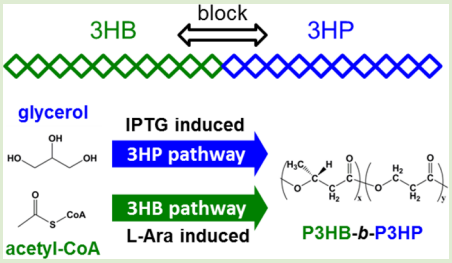
Supporting Information

ABSTRACT: The block copolymers poly(3-hydroxybutyrate)-*block*-poly(3-hydroxypropionate) (P3HB-*b*-P3HP) with a wide range of 3HP fractions from 7.4 mol % to 75 mol % were biosynthesized from inexpensive carbon sources for the first time, differing from previously reported approaches based on sequential addition of precursors. The engineered *Escherichia coli* strain carried two parallel synthetic pathways modulated by independent regulatory systems to produce the 3HB and 3HP monomers, respectively. Manipulating the expression levels of 3HB and 3HP pathways resulted in biosynthesis of block copolymers P3HB-*b*-P3HP with varied compositions. Nuclear magnetic resonance and differential scanning calorimetric studies demonstrated novel microstructure and thermal properties not available in related random copolymers and a blend of P3HB and P3HP homopolymers.



Poly(3-hydroxybutyrate) (P3HB), a well-characterized biodegradable and biocompatible thermoplastic, is brittle and difficult to process due to its poor thermal and mechanical properties.[1] Random incorporation of a second monomer into P3HB has been proved a feasible way to gain P3HB copolymers with remarkably enhanced properties.[2] However, these copolymers still suffer from property detrimental aging effects as native P3HB.[3] Block copolymer, containing two or more unique polymer regions covalently bonded together, could lead to local microphase separation different from the morphologies of random copolymer or polymer blending, making it able to endure aging effects.[4] The mechanical properties of block copolymers, including tensile strength and Young's modulus, are usually better than random copolymer and polymer blending.[5,6] The favorable properties make it more promising for block copolymers to acquire wider application.

The key point of forming block copolymer is the successive incorporation of each monomer in the actively elongating polymer chain sequentially. Previous reports on block copolymer production were all implemented by orderly exposing cells to different precursors.[3,5−9] Unfortunately, the precursors are costly, poorly miscible with water, and toxic to bacteria, which has become an obstacle for large-scale production of these copolymers.[10]

3-Hydroxypropionate (3HP) has been exploited as a monomer in P3HB copolymers for its high ductility and exceptional tensile strength.[2] In our previous study, an engineered strain HBP01 was constructed for synthesis of the poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) (P(3HP-*co*-3HB)) copolymer with controllable composition from glycerol. This strain contained polyhydroxyalkanoate (PHA) synthase PhaC1 of *Cupriavidus necator* and two parallel synthetic pathways to produce the 3HB and 3HP monomers, respectively. The genes involved in 3HP-CoA synthesis, glycerol dehydratase gene *dhaB* and its reactivatase genes and *gdrAB*, both from *Klebsiella pneumonia*, together with propionaldehyde dehydrogenase gene *pduP* from *Salmonella typhimurium*, were modulated by the T7 promoter, and their expression was induced by isopropyl *β*-D-1-thiogalactopyranoside (IPTG). The genes for 3HB-CoA production, *β*-ketothiolase gene *phaA*, and acetoacetyl-CoA reductase gene *phaB* from *C. necator* were controlled by the P_{BAD}-AraC regulatory system and inducer L-arabinose (Scheme 1). Through tuning the expression level of appropriate genes, P(3HP-*co*-3HB) copolyesters were synthesized with a wide range of 3HP fractions.[11]

In this study, the strain HBP01 was used to produce diblock copolymers poly(3-hydroxybutyrate)-*block*-poly(3-hydroxypropionate) (P3HB-*b*-P3HP) with varied composition. The expression of P_{BAD}-AraC controlled genes has been proved to be tightly regulated according to the L-arabinose concentration and inhibited by glucose,[12] so the formation of the P3HB segment becomes totally controllable. To synthesize P3HB-*b*-P3HP, the strain HBP01 was inoculated into a minimal

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Scheme 1. Biosynthetic Pathway for P3HB-*b*-P3HP

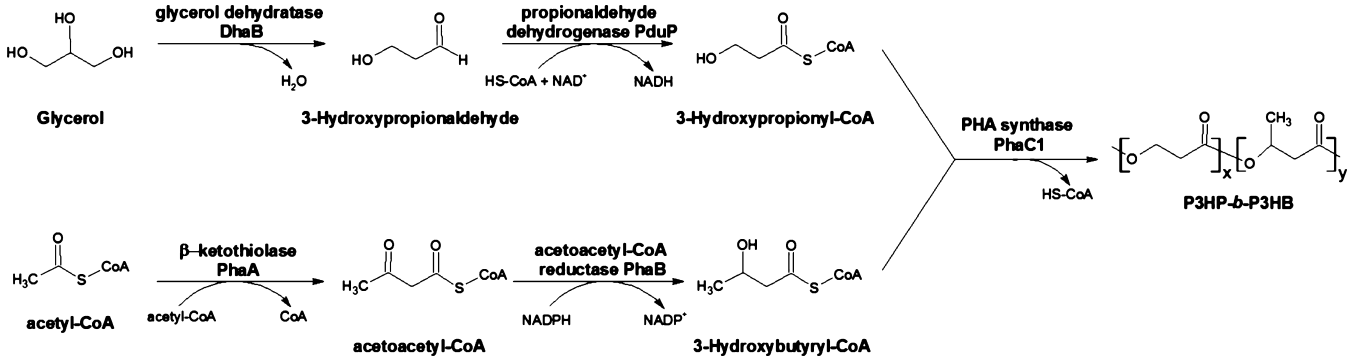

Table 1. Production of P3HB-*b*-P3HP from Inexpensive Carbon Sources by Recombinant *E. coli* in Shake Flasks[a]

L-arabinose (%)	IPTG (mM)	CDW (g/L)	P3HB-*b*-P3HP (g/L)	3HB (mol %)	3HP (mol %)
0	0.05	2.83	0.32	trace	>99
0.002	0	3.18	0.39	100	0
0.002	0.05	3.94	0.77	25	75
0.02	0.05	4.56	0.99	74.1	25.9
0.2	0.05	5.72	1.60	82	18
0.002	0.1	3.89	0.73	48.5	51.5
0.02	0.1	4.36	0.92	80.6	19.4
0.2	0.1	5.49	1.34	92.6	7.4

[a]The experiment was performed under shake flask conditions in triplicate.

medium with fructose as the sole carbon source. When L-arabinose was added into the culture, the expression of 3HB-CoA associated genes was turned on, and carbon flux was guided into synthesis of the first block segment P3HB. Several hours later, glucose and glycerol were supplied. Then the P3HB chain extension was shut off rapidly as glucose can efficiently inhibit the expression of genes controlled by the P_{BAD} promoter, and the metabolic flux was switched to the second block segment P3HP. Furthermore, block copolymers with desirable composition were achieved through changing the concentration of inducers (Table 1).

The suspected block copolymers P3HB-*b*-P3HP were characterized and compared with random copolymers P(3HB-*co*-3HP) and with blend PHAs containing homopolymers P3HB and P3HP. As shown in Figure 1, they have diverse chemical molecular structures. In each molecule of the block copolymer, there is only one chemical bond connecting the 3HB and 3HP units, while the 3HB units have random chemical bonds with 3HP in the random copolymer. There is no chemical conjugation between 3HB and 3HP monomers in a blend of P3HB and P3HP homopolymers.

The representative data from three classes of PHAs containing about 75 mol % 3HP fractions were shown in this paper. The structure differences among the block, random

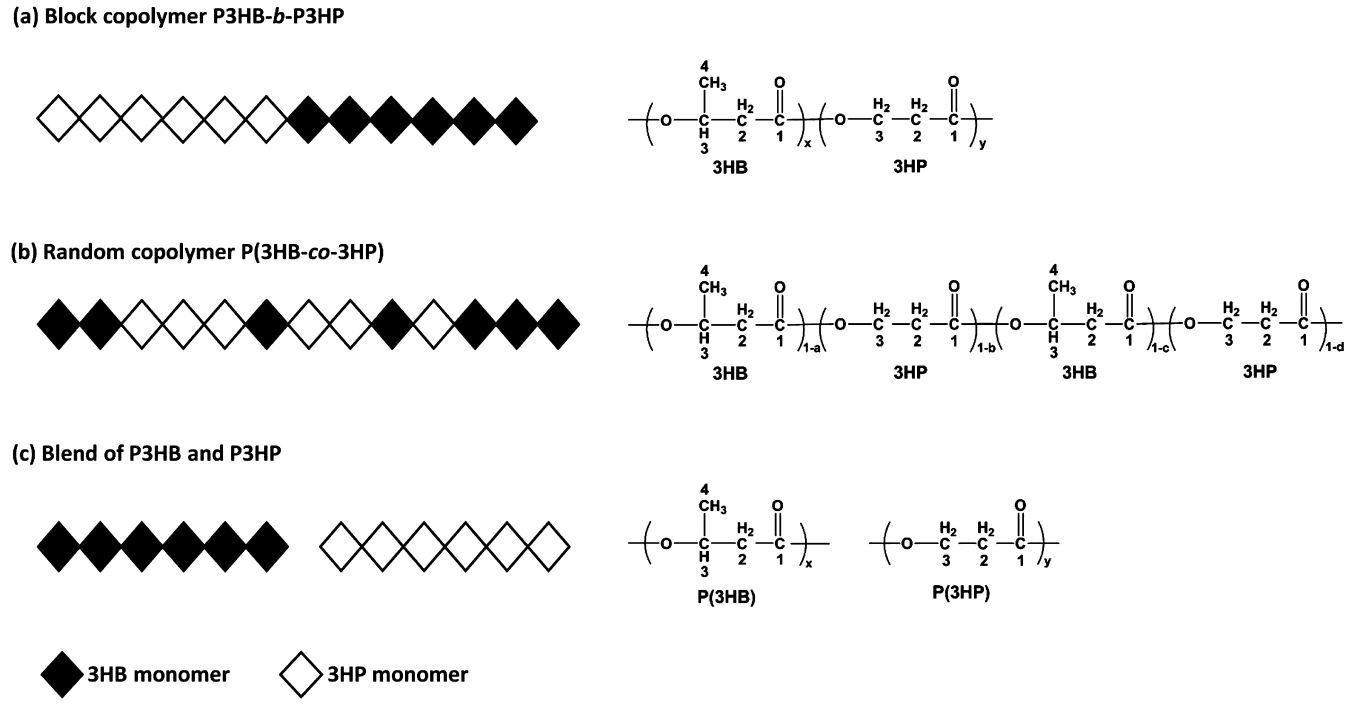

Figure 1. Graphical representation of the chemical molecular structures of block copolymer P3HB-*b*-P3HP (a), random copolymer P(3HB-*co*-3HP) (b), and a blend of P3HB and P3HP (c).

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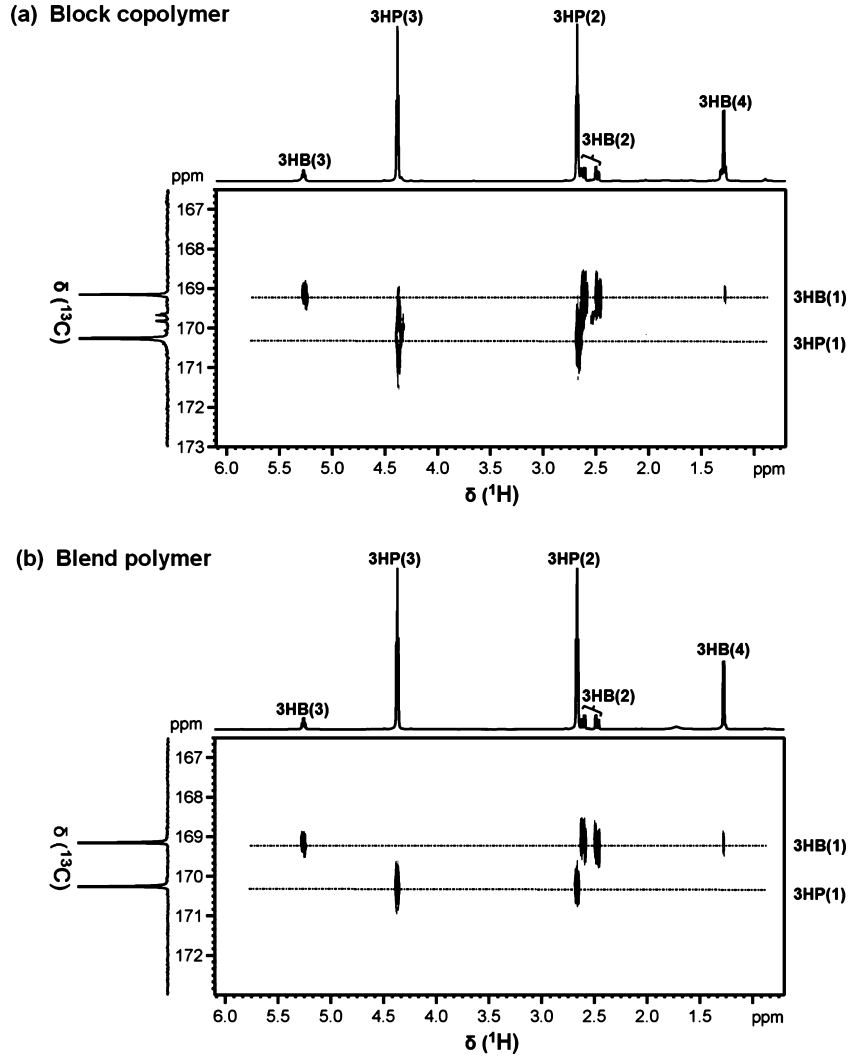

Figure 2. 2D NMR $^{1}H-^{13}C$ HMBC spectra of block copolymer (a) and blend of P3HB and P3HP (b). The numbers in the parentheses represent the numbering scheme of carbon atoms, and the detailed labelscan be referred to in Figure 1. Chemical shifts are in parts per million, and tetramethylsilane was employed as an internal chemical shift standard.

Table 2. Molecular Weights and Thermal Properties of Microbial 3HB and 3HP Related Polymers

	molecular weights		thermal properties		
sample	$M_w(10^5)$	M_w/M_n	T_g (°C)	T_m (°C)	T_c (°C)
P3HB	5.89	1.8	4	176	50
P3HP	1.83	1.6	−22	76	8
P3HB-*b*-75 mol % P3HP	8.92	3.2	−20,−1	73,164	18
P(3HB-*co*-77.5 mol % 3HP)	3.31	2.1	−20	72	15
25 mol % P3HB + 75 mol % P3HP	6.42	2.5	−21,3	76,173	14,51

copolymers, and blend of P3HB and P3HP were elucidated by nuclear magnetic resonance (NMR) analysis (Figure S1, Supporting Information). The ^{1}H NMR spectrum of the block copolymer showed new hydrogen resonances besides 3HP(3), 3HB (3), 3HP (2), and 3HB (2) derived signals, revealing the neighboring effect of the 3HB and 3HP units (Figure S1, Supporting Information, left panel), while in the case of a random copolymer the peak from 3HB (3) and 3HB (2) appears curving rather than sharp in shape because of the strong coeffect among random chemically bonded monomers. In contrast, no new peak was detected in a blend of P3HB and P3HP homopolymers. The similar phenomenon was also observed in ^{13}C NMR spectra (Figure S1, Supporting Information, right panel). The carbonyl carbon resonances of both block copolymer and random copolymer were resolved into four peaks, and the new signals in the random copolymer were more intensive than the block. The carbon resonance of 3HB(3) split into quadrupled peaks in the random copolymer, while for the block copolymer only one small new peak appeared, indicating the slight interrelation of 3HB and 3HP at the changing point. Especially when the monomer sequence distribution was calculated, the *D* value for the block copolymer is in accord with Bernoullian statistics.[13]

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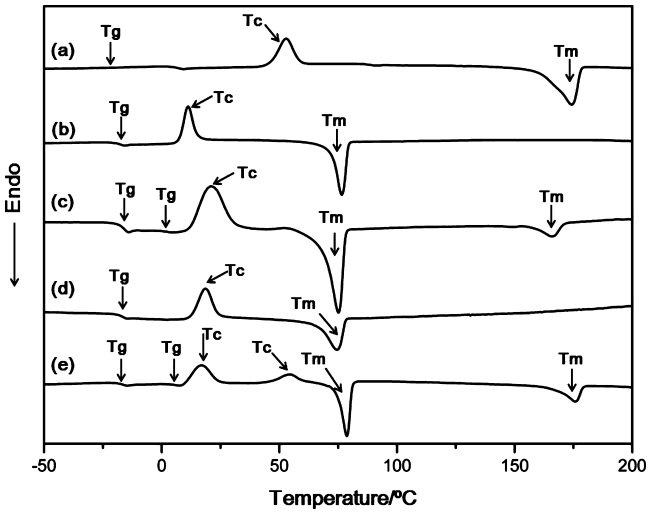

Figure 3. Typical DSC thermographs of microbial 3HB and 3HP related polymers: (a) P3HB; (b) P3HP; (c) block copolymer of P3HB-*b*-75 mol % P3HP; (d) random copolymer of P(3HB-*co*-77.5 mol % 3HP); (e) blend polymer P3HB (25 mol %) + P3HP (75 mol %). The curves were collected during the second heating run.

To further provide solid evidence for the existence of the block copolymer, the $^{1}H-^{13}C$ HMBC spectrum on the block and blend samples was recorded (Figure 2). The HMBC spectrum exhibited clear coherences between the proton of 3HP (3) and both carbonyl groups of 3HB and 3HP units, indicating the covalent link of each block. At the same time, no cross correlation between the proton of 3HB (3) and the carbonyl group of the 3HP monomer suggested that the microstructure sequence of the block copolymer was P3HB-*b*-P3HP. On terms of the blend polymer, there is only correlation inside the monomer itself.

The diversity of PHA microstructures led to the difference of thermal properties among three classes of PHA (Table 2 and Figure 3). The blend polymer, physically mixed by two homopolymers, had two glass transition temperatures (T_g) of −21 and 3 °C, two melting temperatures (T_m) of 76 and 173 °C, and also two cooling crystallization temperatures (T_c) of 14 and 51 °C, almost the same as typical T_g, T_m, and T_c in homopolymers P3HB and P3HP, suggesting the immiscibility of the 3HB and 3HP monomer. Compared with the blend polymer, the block copolymer also displayed two T_g and two T_m corresponding to P3HB and P3HP polymer chains, respectively. However, the T_g of the P3HB segment in the block copolymer was shifted from 4 °C to a low T_g at −1 °C, while the T_m lowered from 176 to 164 °C, indicating chemical conjugation between the P3HB segment and P3HP segment. On the differential scanning calorimetry (DSC) curve of both the blend polymer and block copolymer, the T_g peaks of the P3HB fraction were very weak as a result of the interference of cooling crystallization temperature (T_c) and low content of P3HB. In the case of a random copolymer, only one T_g and T_m were observed as a result of random assembly of monomers in the whole chain. Only one value for T_c corresponding to P3HP was observed because the rearrangement of the P3HB chain was severely restrained by its covalent connection with the P3HP segment, which has formed crystals at lower temperature. Coupled with the low percentage of P3HB segments, the cooling crystallization peak for P3HB was not detectable. In contrast, the blend polymer has two clear cooling crystallization peaks on account of no chemical bonds of two independent phases.

The block copolymer P3HB-*b*-P3HP is composed of two different microphases P3HP and P3HB with diverse thermodynamic properties and exhibits the conserved T_g and T_m of each block. This combination brings not only enlargement of temperature range for elastomeric state but also improved tensile strength and stiffness for the block copolymer. As evaluated in a previous study, with the increase of microphase in polymers, the elastomeric properties of the polymer raised accordingly.[4]

In summary, diblock copolymers P3HB-*b*-P3HP were produced by a recombinant *E. coli* strain from renewable inexpensive carbon sources. The copolymer composition was totally controllable by manipulating the expression levels of independent P3HB and P3HP synthetic pathways, instead of feeding cells various precursors orderly. NMR and DSC analysis strongly confirmed the existence of the block copolymer and showed a difference in microstructure and thermal properties among relevant block, random copolymers, and blend polymers. To our best knowledge, this is the first report on block copolymer biosynthesis with controllable structures from an inexpensive carbon source.

EXPERIMENTAL SECTION

The strain HBP01 constructed previously was utilized to allow the production of P3HB-*b*-P3HP.[11] The plasmids pBAD18-*phaAB* and pHP301[11] were transformed into *E. coli* JM109 (DE3) to produce P3HB homopolymer. The strain *E. coli* JM109 (DE3)/pWQ02/pWQ04 and the culture conditions described previously were employed to produce P3HP.[14] For block copolymer production, shake flask inoculations were conducted in minimal medium (MM) containing 10 g of fructose as described previously.[11] Amounts of 100 mg/L of ampicillin, 50 mg/L of kanamycin, and 34 mg/L of chloramphenicol were used to maintain the plasmids. The cells were induced at OD_{600} ∼ 0.6 with various IPTG and L-arabinose concentrations and further cultured at 30 °C. An amount of 10 g/L of glucose was supplemented into the medium at approximately 20 h of cell growth. Then, one hour later, 10 g/L of glycerol and 5 μM vitamin B_{12} (VB_{12}) were provided. During 60 h culture, antibiotics and VB_{12} were added every 12 h. All shake flask experiments were performed in triplicate. Control experiments were carried out with addition of only one inducer, IPTG or L-arabinose. For P3HB production, the minimal medium (MM) as above was used. When OD_{600} reached about 0.6, 0.2% L-arabinose and 0.05 mM IPTG were added. The culture was carried out for 60 h.

PHA extraction was carried out with hot chloroform in a Soxhlet apparatus on lyophilized cells.[15] NMR analysis was employed to determine the microstructure and the molar fraction of the product using an Avance III 600 NMR spectrometer.[3] DSC data were recorded on Pyris Diamond DSC system (Perkins Elmer, USA).[11] The molecular weight was determined using gel permeation chromatography (DAWN HELEOS II, Wyatt, USA) as described.[16]

ASSOCIATED CONTENT

Supporting Information

Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Noda, I.; Green, P. R.; Satkowski, M. M.; Schechtman, L. A. *Biomacromolecules* **2005**, *6*, 580−586.
(2) Andreeβen, B.; Steinbuchel, A. *Appl. Environ. Microbiol.* **2010**, *76*, 4919−4925.
(3) Hu, D.; Chung, A. L.; Wu, L. P.; Zhang, X.; Wu, Q.; Chen, J. C.; Chen, G. Q. *Biomacromolecules* **2011**, *12*, 3166−3173.
(4) McChalicher, C. W. J.; Srienc, F. *J. Biotechnol.* **2007**, *132*, 296−302.
(5) Li, S. Y.; Dong, C. L.; Wang, S. Y.; Ye, H. M.; Chen, G. Q. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 659−669.
(6) Tripathi, L.; Wu, L. P.; Meng, D. C.; Chen, J. C.; Wu, Q.; Chen, G. Q. *Bioresour. Technol.* **2013**, *142*, 225−231.
(7) Pederson, E. N.; McChalicher, C. W.; Srienc, F. *Biomacromolecules* **2006**, *7*, 1904−1911.
(8) Tripathi, L.; Wu, L. P.; Meng, D. C.; Chen, J. C.; Chen, G. Q. *Biomacromolecules* **2013**, *14*, 862−870.
(9) Tripathi, L.; Wu, L. P.; Chen, J.; Chen, G. Q. *Microb. Cell Fact.* **2012**, *11*, 44.
(10) Wang, Q.; Zhuang, Q. Q.; Liang, Q. F.; Qi, Q. S. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 3301−3307.
(11) Wang, Q.; Yang, P.; Xian, M.; Yang, Y.; Liu, C.; Xue, Y.; Zhao, G. *Bioresour. Technol.* **2013**, *142*, 741−744.
(12) Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J. *J. Bacteriol.* **1995**, *177*, 4121−4130.
(13) Kamiya, N.; Yamamoto, Y.; Inoue, Y.; Chujo, R.; Doi, Y. *Macromolecules* **1989**, *22*, 1676−1682.
(14) Wang, Q.; Yang, P.; Liu, C.; Xue, Y.; Xian, M.; Zhao, G. *Bioresour. Technol.* **2013**, *131*, 548−551.
(15) Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller, R. C. *Appl. Environ. Microbiol.* **1988**, *54*, 1977−1982.
(16) Liu, Q.; Luo, G.; Zhou, X. R.; Chen, G. Q. *Metab. Eng.* **2011**, *13*, 11−17.