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Short Communication

Biosynthesis of poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) with fully controllable structures from glycerol

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HIGHLIGHTS

- P(3HP-*co*-3HB) copolymer with controllable monomer composition was biosynthesized.
- The inexpensive glycerol was used as sole carbon source.
- The highest P(3HP-*co*-3HB) production was achieved.
- The copolymer properties were dependent on its composition.

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ABSTRACT

As the most representative biodegradable thermoplastic, poly(3-hydroxybutyrate) (P3HB) has a limited range of applications because of its poor thermal and physical properties. To improve its properties, a novel biosynthetic system was designed to produce poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) (P(3HP-*co*-3HB)) with fully controllable structures from inexpensive carbon source. In this system, two parallel synthetic pathways controlled by independent regulatory systems were used to produce the 3HP and 3HB monomers, respectively. Through tuning the expression level of appropriate genes, P(3HP-*co*-3HB) copolyesters were synthesized with a wide range of 3HP fraction from 11.5 mol% to 94.6 mol%. Differential scanning calorimetry analysis demonstrated that the thermal properties of P(3HP-*co*-3HB) copolymer were totally dependent on its composition. The bioreactor cultivation was also performed and accumulated 9.8 g/L P(48.2 mol% 3HP-*co*-3HB) using glycerol as sole carbon source, which represented the highest production so far.

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1. Introduction

As a biodegradable and biocompatible thermoplastic, poly(3-hydroxybutyrate) (P3HB) has become the most representative homopolymer in PHA family since discovered by Lemoigne in 1926 (Chen, 2009). However, P3HB is a stiff and brittle material, and has high melting temperature, relative to the region of its thermal decomposition temperature, making P3HB difficult to be processed (Noda et al., 2005). To improve the characteristics of P3HB, random incorporation of a second monomer into P3HB was expected to be a feasible and effective approach. So far, various copolymers containing 3HB monomer have been biosynthesis and characterized. One of these copolymers, poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) (P(3HP-*co*-3HB)), has been proved exhibiting significant improved physical properties compared with P3HB, owing to the high rigidity, ductility, and exceptional tensile strength brought by poly(3-hydroxypropionate) (P3HP) (Andreessen and Steinbuchel, 2010; Cao et al., 1998; Feng et al., 2002). As 3HP is not a common compound in most organisms, the copolymer biosynthesis was dependent on addition of precursor molecules structurally related with 3HP, such as α, ω-alkanediols and acrylate. Through varying the feed ratio of precursors, copolymers with different 3HP content were obtained (Green et al., 2002; Hiramitsu and Doi, 1993; Valentin et al., 2000; Wang et al., 2002).

Considering the high cost of precursors, P(3HP-*co*-3HB) biosynthesis from structurally unrelated carbon sources has been investigated by Fukui et al. (2009). In that report, an engineered *Cupriavidus necator* strain harboring the genes encoding malonyl-CoA reductase and 3HP-CoA synthase from *Chloroflexus aurantiacus* under the control of P_{BAD} promoter was constructed to facilitate the production of P(3HP-*co*-3HB). Unfortunately, after several carbon sources and fermentation strategies have been attempted, only 2.1 mol% of 3HP monomer was incorporated into the

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copolymer. The incorporation of small 3HP fraction cannot effectively ameliorate the deficiency of P3HB to favor its practical applications. Therefore, it is worthwhile to develop a new strategy to produce P(3HP-*co*-3HB) with more 3HP composition, even fully controllable structures, from inexpensive carbon sources.

The copolymer P(3HP-*co*-3HB) is synthesized directly from two substrates: 3HP-CoA and 3HB-CoA. In this study, two parallel synthetic pathways for both substrates were introduced into *Escherichia coli*, and controlled by two independent regulatory systems. The genes involved in 3HP-CoA formation were under the control of T7 promoter, and could be induced by IPTG. The pathway responsible for 3HB-CoA synthesis was modulated by the P_{BAD}-AraC regulatory system. By varying the concentration level of arabinose, a broad range of P(3HP-*co*-3HB) copolymers with different 3HP content were synthesized by the engineered *E. coli* strain using glycerol as sole carbon source. In a fed-batch fermentation, P(48.2 mol% 3HP-*co*-3HB) was accumulated up to 9.8 g/L. To our best knowledge, this is the first report on microbial production of P(3HP-*co*-3HB) with fully controllable structures without addition of any precursor, and the highest production of P(3HP-*co*-3HB) was also achieved.

2. Methods

2.1. Bacterial strains and plasmids construction

The *E. coli* DH5α (Invitrogen) strain was used for plasmids preparation and *E. coli* JM109(DE3) was used for the copolymers production. The plasmids pWQ02 and pWQ04 were constructed previously (Wang et al., 2013). The plasmid pBAD18-*phaAB* was constructed by cloning the *phaAB* genes of *Cupriavidus necator* H16 into *Eco*RI and *Pst*I sites of vector pBAD18-kan with primers 227 (5′-GAGAATTCGTTTCCATTGAAAGGACTACAC-3′) and 228 (5′-CAGCTGCAGCTGCCGACTGGTTGAACCAGG-3′).

2.2. Culture conditions

Shake flask experiments were performed using minimal medium (MM) with 20 g/L glycerol as described previously (Wang et al., 2013). 100 mg/L ampicillin, 34 mg/L chloramphenicol and 50 mg/L kanamycin were supplemented when necessary. The strain *E. coli* JM109(DE3)/pWQ02/pWQ04/pBAD18-*phaAB* was inoculated into 500 mL baffled Erlenmeyer flasks containing 100 mL MM at 37 °C. The cells were induced at $OD_{600} \sim 0.6$ with 0.05 mM IPTG and various L-arabinose concentrations, and further cultured at 30 °C. Five micromole per liter of vitamin B_{12} (VB_{12}) was added every 12 h during 60 h culture. All shake flask experiments were performed in triplicates.

Fed-batch cultures were carried out in a Biostat B plus MO5L fermentor (Sartorius Stedim Biotech GmbH, Germany) containing 2 L MM. The pH and dissolved oxygen (DO) were kept at 7.0 and 5%, respectively. Concentrated glycerol (10 M) was used as feeding solution. When OD_{600} reached 0.6, 0.05 mM IPTG and different concentration L-arabinose were used to induce gene expression. IPTG, L-arabinose and 5 μM of VB_{12} were added every 12 h during 84 h fermentation.

2.3. Analytical methods

PHA was extracted from lyophilized cells with hot chloroform in a Soxhlet apparatus, and precipitated by ice-cold ethanol (Brandl et al., 1988). PHA structure was determined by NMR analysis using an Avance III 600 NMR spectrometer (Bruker, Switzerland), and the molar fraction was determined by the relative intensity of the resonance of C3 methylene protons in 3HP and C3 methine proton in 3HB (Wang et al., 2009). Molecular weight data was obtained via gel permeation chromatography (DAWN HELEOS II, Wyatt, USA) (Wang et al., 2009). The thermal properties of PHA were determined using differential scanning calorimetry (DSC, Perkins Elmer, USA) (Arai et al., 1999).

3. Results and discussion

3.1. Design of P(3HP-co-3HB) biosynthesis pathway

As 3HP is not a natural compound in common metabolic pathways, the 3HP skeletons in copolymers mostly come from external precursors which are usually detrimental to the cells and result in high industrial cost. Therefore, the engineered pathway has to be equipped with 3HP-CoA formation genes from renewable carbon sources to supply 3HP monomer without addition of precursors. In our previous study, an engineered *E. coli* strain for P3HP production from glycerol was constructed by employing the glycerol dehydratase DhaB of *Klebsiella pneumonia*, propionaldehyde dehydrogenase PduP of *Salmonella typhimurium*, and PHA synthase PhaC1 of *C. necator* (Wang et al., 2013). In this study, *C. necator* genes coding β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) were also introduced into this strain to provide 3HB-CoA (Fig. 1). To dial the composition of the copolymer, two independent control systems, P_{BAD}-AraC and P_{T7}-LacI, were employed. As

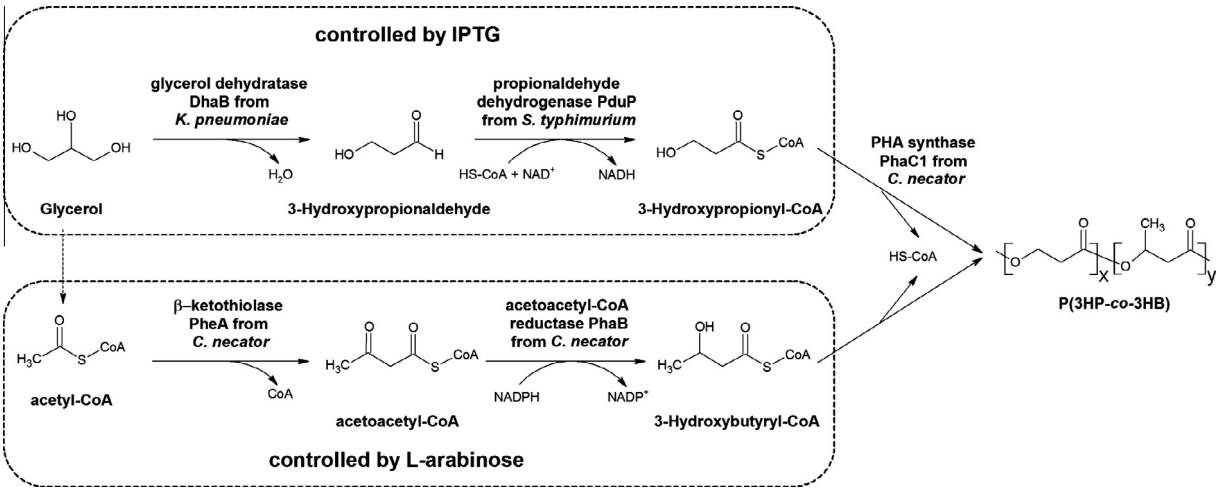

Fig. 1. Pathway for P(3HP-*co*-3HB) biosynthesis from glycerol in the engineered *E. coli* strain.

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Table 1
Production and characterization of P(3HP-*co*-3HB) produced from glycerol by recombinant *E. coli* in shake flasks.[a]

L-arabinose (%)	CDW (g/L)	PHA/CDW (wt%)	PHA (g/L)	3HB (mol%)	3HP (mol%)	Molecular weights		Thermal properties		
						M_n ($\times 10^5$ Da)	M_w/M_n	T_g (°C)	T_m (°C)	ΔH_m (J/g)
0.002	3.63	30.3	1.10	5.4	94.6	1.32	1.9	−22	76	68
0.02	3.68	31.9	1.17	8.3	91.7	1.36	2.0	−21	76	62
0.05	3.2	34.8	1.11	22.5	77.5	1.57	2.1	−20	72	48
0.1	3.59	31.2	1.12	37.5	62.5	1.96	3.0	−20	72	19
0.2	3.4	32.6	1.11	43.8	56.2	2.55	2.8	−19	68	13
0.3	3.54	37.3	1.32	68.7	31.3	3.24	2.4	−15	73	8
0.4	3.9	38.3	1.49	74.4	25.6	2.15	2.1	−4	163	11
0.5	3.6	40.5	1.46	75.6	24.4	2.89	2.1	−3	162	12
0.6	4.4	37.2	1.64	78.3	21.7	2.64	2.3	−3	167	17
0.7	4.25	41.2	1.75	80.4	19.6	2.61	2.3	−1	162	32
0.8	5.46	41.3	2.25	85.7	14.3	3.37	2.6	0	168	41
1	5.86	44	2.58	88.5	11.5	3.12	2.5	3	162	67
							P3HB[b]	4	177	97
							P3HP[b]	−19	77	74

[a] The experiment was performed under shake flask condition in triplicates.
[b] The thermal properties of P3HB and P3HP were derived from review by Andreessen and Steinbuchel (2010).

AraC-P_{BAD} system can be effectively modulated over a wide range of inducer concentrations and the inducer is non-toxic to the cells (Guzman et al., 1995), we changed the concentration of L-arabinose to obtain desirable composition of copolymer. Compared with natural PHA producing bacterial, *E. coli* has numerous advantages, such as not tied to culture conditions like nutrient limitation, and no intracellular depolymerization system of PHA. So *E.coli* was employed as a host cell in our study.

3.2. P(3HP-co-3HB) production

The P(3HP-*co*-3HB)-producing strain HBP01 was constructed by transferring plasmids pBAD18-*phaAB*, pWQ02 and pWQ04 to *E. coli* JM109 (DE3).

In shake flask cultivation, 0.002%–1% L-arabinose was added to regulate the level of 3HB-CoA associated genes' transcription, while the inducer for genes involved in 3HP-CoA formation remained at 0.05 mM IPTG. Table 1 showed that the percentage of 3HB fraction in the copolyesters increased from 9.5 mol% to 89.9 mol% along with the increase of L-arabinose concentration from 0.002% to 1%. In this experiment, a noteworthy phenomenon was observed that the weight of 3HP fraction in copolymers varied while the inducer IPTG concentration was constant. The possible explanation is that the pool of available carbon source within the bacterial cells is certain, and when L-arabinose concentration increased, a significant amount of carbon flux was guided into 3HB-CoA synthetic pathway, which seriously reduced the 3HP-CoA production even in the presence of sufficient 3HP-CoA associated enzymes.

Bioreactor cultivation was performed to further investigate the results obtained by flask cultures. Two different concentrations of L-arabinose, 0.02% and 0.2%, were used to induce the production of PHB, respectively. When 0.02% L-arabinose was added, the engineered strain accumulated 20.2 g/L cell dry weight (CDW) containing 45.5% (wt/wt CDW) P(85.4 mol% 3HP-*co*-3HB). While in the case of 0.2% L-arabinose, 9.8 g/L P(48.2 mol% 3HP-*co*-3HB) representing 46.2% of CDW was produced.

3.3. Analysis of P(3HP-co-3HB) with various monomer composition

The copolymers' structures were confirmed by NMR analysis, and a representative spectrum of P(77.5 mol% 3HP-*co*-3HB) is shown in Fig. S1. ^{1}H NMR exhibited a special resonance at 4.33 ppm for the C_3 methylene group of 3HP unit because of the neighboring effect between two monomers, indicating the copolymerization of 3HP and 3HB. In addition, hydrogen resonances for C_2 methylene of 3HB unit was strongly affected by 3HP unit, and split into multiple peaks instead of four sharp peaks for every hydrogen atom in non-interaction blend molecules. Furthermore, in the spectrum of ^{13}C NMR, the carbonyl carbon resonances were resolved into four peaks, in a similar manner of those in previous report (Shimamura et al., 1994).

The molecular weights and thermal properties of P(3HP-*co*-3HB) samples are listed in Table 1. Among all the polymers, P(94.6 mol% 3HP-*co*-3HB) had the lowest number-average molecular weight (M_n) and polydispersity (M_w/M_n), while P(14.3 mol% 3HP-*co*-3HB) had the highest M_n. The scope of these copolymers' M_n value is $1.32 \sim 3.37 \times 10^5$ Da and the M_w/M_n value ranged from 1.9 to 3.0. As 3HP fractions increased, the molecular weight decreased probably because PHA synthase PhaC could not catalyze the polymerization of 3HP-CoA as sufficiently as 3HB-CoA, its natural substrate.

The glass transition temperature (T_g) decreased from 3 °C to −22 °C as the 3HP content increased from 11.5 mol% to 94.6 mol%. The alteration of melting temperature (T_m) has a different manner. During the 3HP fraction increased from 0 mol% to 56.2 mol%, the T_m value decreased from 173 °C to 68 °C. However, it increased to 76 °C with further increasing of 3HP fraction. The trend of enthalpy of fusion (ΔH_m) was similar to that of T_m, first decreased from 97 J/g (P3HB) to 8 J/g (P(31.3 mol% 3HP-*co*-3HB)) and then increased to 74 J/g (P3HP).

4. Conclusion

For the first time, P(3HP-*co*-3HB) with adjustable monomer content was successfully synthesized from glycerol when pathways for P3HP and P3HB production were coexpressed under independent promoters in recombinant *E. coli*. Also, the bioreactor cultivation reached the highest production of P(3HP-*co*-3HB). NMR and DSC analysis confirmed the existence of the copolymers and the dramatically changed thermal properties depending on their monomer ratios, which should widen their range of applications compared with the 3HP or 3HB homopolymer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.05.121.

References

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

Arai, Y., Cao, A., Yoshie, N., Inoue, Y., 1999. Studies on comonomer compositional distribution and its effect on some physical properties of bacterial poly(3-hydroxybutyric acid-*co*-3-hydroxypropionic acid). Polym. Int. 48, 1219–1228.

Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., 1988. *Pseudomonas oleovorans* as a source of poly(β-Hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54, 1977–1982.

Cao, A., Kasuya, K., Abe, H., Doi, Y., Inoue, Y., 1998. Studies on comonomer compositional distribution of the bacterial poly(3-hydroxybutyric acid-*co*-3-hydroxypropionic acid)s and crystal and thermal characteristics of their fractionated component copolyesters. Polymer 39, 4801–4816.

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

Feng, L., Watanabe, T., Wang, Y., Kichise, T., Fukuchi, T., Chen, G.Q., Doi, Y., Inoue, Y., 2002. Studies on comonomer compositional distribution of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)s and thermal characteristics of their factions. Biomacromolecules 3, 1071–1077.

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.

Green, P.R., Kemper, J., Schechtman, L., Guo, L., Satkowski, M., Fiedler, S., Steinbuchel, A., Rehm, B.H., 2002. Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid β-oxidation inhibited *Ralstonia eutropha*. Biomacromolecules 3, 208–213.

Guzman, L.M., Belin, D., Carson, M.J., Beckwith, J., 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. 177, 4121–4130.

Hiramitsu, M., Doi, Y., 1993. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). Polymer 34, 4782–4786.

Noda, I., Green, P.R., Satkowski, M.M., Schechtman, L.A., 2005. Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. Biomacromolecules 6, 580–586.

Shimamura, E., Scandola, M., Doi, Y., 1994. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). Macromolecules 27, 4429–4435.

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.

Wang, Y., Inagawa, Y., Saito, T., Kasuya, K., Doi, Y., Inoue, Y., 2002. Enzymatic hydrolysis of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxypropionate)s by poly(3-hydroxyalkanoate) depolymerase from *Acidovorax* Sp. TP4. Biomacromolecules 3, 828–834.

Wang, H.H., Li, X.T., Chen, G.Q., 2009. Production and characterization of homopolymer polyhydroxyheptanoate (P3HHp) by a *fadBA* knockout mutant *Pseudomonas putida* KTOY06 derived from *P. putida* KT2442. Process Biochem. 44, 106–111.

Wang, Q., Yang, P., Liu, C., Xue, Y., Xian, M., Zhao, G., 2013. Biosynthesis of poly(3-hydroxypropionate) from glycerol by recombinant *Escherichia coli*. Bioresour. Technol. 131, 548–551.

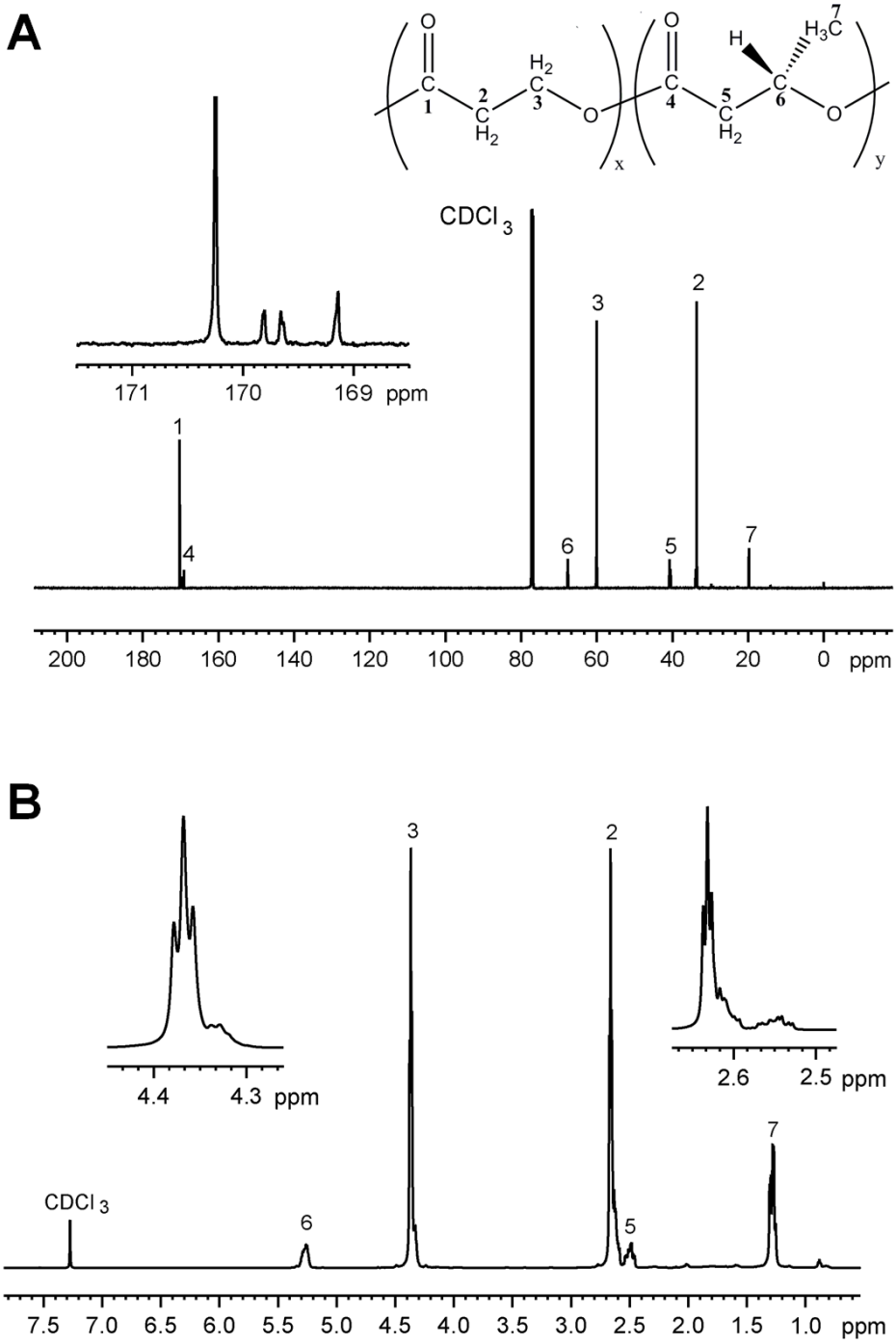

Fig. S1 The ^{13}C (A) and ^{1}H (B) NMR spectra of P(77.5 mol% 3HP-*co*-3HB) synthesized by *E. coli* HBP01 in $CDCl_3$ solution. The chemical shift assignment for each proton and carbon resonance was showed.