



# Construction of a “nutrition supply–detoxification” coculture consortium for medium-chain-length polyhydroxyalkanoate production with a glucose–xylose mixture

Yaru Liu<sup>1</sup> · Songyuan Yang<sup>1</sup> · Xiaoqiang Jia<sup>1,2,3</sup>

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## Abstract

In this study, we constructed a coculture consortium comprising engineered *Pseudomonas putida* KT2440 and *Escherichia coli* MG1655. Provision of “related” carbon sources and synthesis of medium-chain-length polyhydroxyalkanoates (mcl-PHAs) were separately assigned to these strains via a modular construction strategy. To avoid growth competition, a preference for the use of a carbon source was constructed. Further, the main intermediate metabolite acetate played an important role in constructing the expected “nutrition supply–detoxification” relationship between these strains. The coculture consortium showed a remarkable increase in the mcl-PHA titer (0.541 g/L) with a glucose–xylose mixture (1:1). Subsequently, the titer of mcl-PHA produced by the coculture consortium when tested with actual lignocellulosic hydrolysate (0.434 g/L) was similar to that achieved with laboratory sugars’ mixture (0.469 g/L). These results indicate a competitive potential of the engineered *E. coli*–*P. putida* coculture consortium for mcl-PHA production with lignocellulosic hydrolysate.

**Keywords** Lignocellulosic hydrolysate · Medium-chain-length polyhydroxyalkanoate · *Escherichia coli* · *Pseudomonas putida*

## Introduction

Environmental pollution caused by plastics and the consumption of fossil energy have accelerated the search for sustainable development materials to replace petroleum-based plastics. The bacterial polymers, polyhydroxyalkanoates (PHAs), are a class of high-molecular-weight polyesters

comprising hydroxy fatty acids as monomers in microorganisms. PHAs have attracted extensive interest as a new green alternative to petroleum-based plastics because they are biodegradable and can be produced from renewable resources [4, 5, 39, 47]. PHAs include short-chain-length (scl, 3–5 carbon atoms) and medium-chain-length (mcl, 6–14 carbon atoms) hydroxyalkanoic acid monomers depending on the strain, carbon substrate, and culture conditions provided [10]. Mcl-PHAs are polyesters of hydroxyalkanoates primarily produced by fluorescent pseudomonads under unbalanced growth conditions [34, 43, 44]. Mcl-PHAs have lower crystallinity but are more elastic than scl-PHAs. Furthermore, mcl-PHAs are more diverse than scl-PHAs in structure; therefore, they can be used in a wide range of applications [33]. However, high production costs make large-scale application of mcl-PHAs difficult, unlike the application of low-cost petroleum-based plastics. Several factors lead to the relatively high production cost of mcl-PHAs, such as high substrate cost, low polymer concentration, as well as low productivity [22, 48]. According to statistics, substrate costs account for approximately 50% of the total production cost.

Yaru Liu and Songyuan Yang contributed equally to this work.

✉ Xiaoqiang Jia  
xqjia@tju.edu.cn

<sup>1</sup> Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People’s Republic of China

<sup>2</sup> Frontier Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (MOE), School of Chemical Engineering and Technology, Tianjin University, Tianjin 300350, People’s Republic of China

<sup>3</sup> SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering, Tianjin 300072, People’s Republic of China

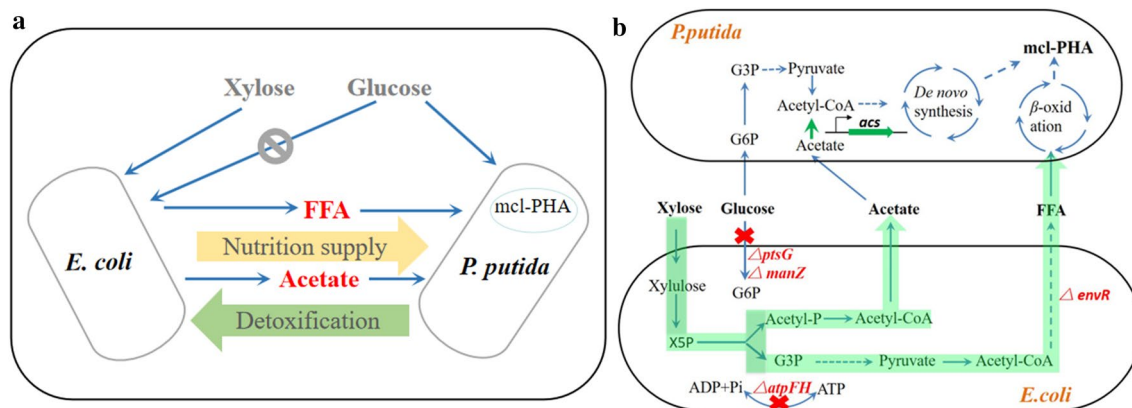
Therefore, mcl-PHA synthesis using inexpensive, renewable carbon sources is an effective strategy to reduce production cost [1, 30].

Lignocellulose comprising cellulose, hemicellulose, and lignin is inexpensive and the most abundant non-grain biomass in nature [27]. Cellulose is a water-insoluble polymer comprising glucose, hemicellulose is a polymer comprising pentose and hexose linked by glycoside bonds, and lignin is a three-dimensional polymer based on phenylpropane [18]. Currently, lignocellulose is converted into fermentable sugars (glucose and xylose) by various methods, and these sugars have been used to synthesize PHAs. However, most studies have focused on polyhydroxybutyrates (PHBs), whereas studies on PHAs are limited [8, 10, 13, 28, 29, 32, 35, 52]. Davis et al. [10] used several different *Pseudomonas* strains to synthesize mcl-PHAs from a wild ryegrass hydrolysate treated by different methods. Although the yield of mcl-PHAs was similar to that of laboratory-grade sugars, the highest yield was 0.3 g/L, which is insufficient to meet the needs of industrial production. Based on previous reports, we speculated that the reason for this finding is that sugars are structurally “unrelated” carbon sources and unbeneficial for PHA biosynthesis [47]. Different carbon source species considerably affect PHA biosynthesis. In general, carbon sources can be classified as “related” carbon sources (structures associated with PHA monomers, e.g., fatty acids) and “unrelated” carbon sources (structures unrelated to PHA monomers, e.g., xylose and glucose) [11, 21].

Currently, synthetic microbial coculture strategy has become a novel strategy in metabolic engineering and synthetic biology and has been widely used to overcome the limitations of monoculture [16, 42, 53]. A major advantage of coculture consortia is that each expression system and pathway module can be constructed and optimized in parallel, which indicates substantially reduced product production time [54]. Moreover, coculture consortia can involve unique properties and functions of different

species and minimize problems arising from feedback inhibition by means of spatial pathway module segregation [54]. Researchers have applied coculture consortia to the PHA production. Shalin et al. [36] achieved an increase in the PHB yield by constructing a coculture consortium comprising *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII0925. Löwe et al. [25] used a coculture consortium comprising cyanobacteria and *Pseudomonas* to convert CO<sub>2</sub> to mcl-PHAs, and they achieved a yield of 156 mg/L. However, to the best of our knowledge, the use of a coculture consortium system to synthesize mcl-PHA with a glucose–xylose mixture as a carbon source and lignocellulosic hydrolysate as a substrate has not been reported to date.

In this study, we have reported on the construction of a coculture consortium comprising *Escherichia coli* and *Pseudomonas putida* using division of labor, i.e., separation of tasks, between these bacteria to achieve efficient biosynthesis of mcl-PHAs with only “unrelated” carbon sources (Fig. 1a). *E. coli* secreted acetate and “related” carbon sources such as extracellular free fatty acids (FFAs). As an excellent mcl-PHA synthetic strain, *P. putida* undertook the task of efficient biosynthesis of mcl-PHAs. As shown in Fig. 1b, *ptsG* and *manZ* were knocked out in *E. coli*, thereby prompting *E. coli* to preferably utilize xylose and slowly utilize glucose. Consequently, this reduced growth competition between *E. coli* and *P. putida*. In addition, the ability of *E. coli* to synthesize acetate and extracellular FFAs was promoted, which can promote mcl-PHA biosynthesis, by knocking out *atpFH* and *envR*. The main intermediate metabolite acetate played an important role in constructing the expected “nutrition supply–detoxification” relationship between these two strains. It was consumed by engineered *P. putida* to synthesize mcl-PHAs and produced by engineered *E. coli* from xylose, which was toxic to the latter in the meantime. The ultimate goal was to achieve efficient production of mcl-PHAs using inexpensive “unrelated” carbon sources.



**Fig. 1** Schematic diagram of double bacteria (a) and of metabolic engineering (b)

## Materials and methods

### Bacterial strains, plasmids, and reagents

The plasmids and bacterial strains used in this study are listed in Table 1. The parent strain *P. putida* KT2440 (American type culture collection; ATCC 47054) was obtained from ATCC (Manassas, VA, USA). The plasmid pBBR1MCS-2 was donated by Dr. Yingjin Yuan of Tianjin University, China. The plasmids pTKS/CS and pTKRED were donated by Dr. Tao Chen of Tianjin University. *E. coli* MG1655 was donated by Dr. Tao Chen of Tianjin University. Acetate [-performance liquid chromatography (HPLC) grade] was purchased from Concord Tech (China). Xylose (99% purity) was purchased from YuanYe (Shanghai, China). Glucose (AR) was purchased from Yuanli Chemical (Tianjin, China). DNA-manipulating agents, including restriction endonucleases and T4 DNA ligase, were purchased from Thermo Scientific (Beijing, China). Phanta Max Super-Fidelity DNA Polymerase and Taq for polymerase chain reaction (PCR) were purchased from Vazyme (Nanjing, China). PCR primers were synthesized by GENEWIZ (Suzhou, China) and are listed in Table 1.

### Plasmid and strain construction

Plasmids and strains were constructed according to standard protocols [9]. *P. putida* KT2440 was employed in mcl-PHA synthesis studies; 1689-bp *acs* (Gene ID 1045574), 1203-bp *ackA* (Gene ID 946775), and 2145-bp *pta* (Gene ID 946778) were amplified using PCR and the primer pairs *acs-F/acs-R*, *ackA-F/ackA-R*, and *pta-F/pta-R*. These amplified genes were cloned into pBBR1MCS-2 [19], which resulted in pBBR1-*acs*, pBBR1-*ackA*, and pBBR1-*ackA-pta*, respectively. Expression vectors were confirmed using enzymatic digestion and DNA sequencing, and then transformed into *P. putida* KT2440, thereby generating engineered strains. Transformation of *P. putida* strains was accomplished via electroporation using a voltage of 2.5 V, 200  $\Omega$ , and 25  $\mu$ F.

*Escherichia coli* MG1655 was used to synthesize “related” carbon sources using “unrelated” carbon sources and secrete them extracellularly. Gene knockout experiments based on  $\lambda$ -red and I-SceI described by Lin Zhenquan [23] were used to construct *ptsG*, *manZ*, *envR*, and *atpFH* knockout strains. *ptsG* knockout was performed as follows: using the genome of *E. coli* MG1655 as a template, the primers *ptsG-Af/ptsG-Ar* and *ptsG-Bf/ptsG-Br* were used to amplify the upstream and downstream homologous arm fragments A and B, respectively, of *ptsG*. Using

the plasmid pTKS/CS as a template, the fragment tet1 and downstream fragment tet2 of the tetracycline resistance gene tet were amplified with the primers *ptsG-tetf1/ptsG-tetr1* and *ptsG-tetf2/ptsG-tetr2*, respectively. Using the fragments A and tet1 as templates, the fragment A-tet1 was amplified by overlap extension PCR using the primer *ptsG-Af/ptsG-tetr1*. Similarly, using fragments tet2 and B as templates, the fragment tet2-B was amplified by overlap extension PCR using the primer *ptsG-tetf2/ptsG-Br*. Finally, overlap extension PCR was performed again using fragments A-tet1 and tet2-B as amplification templates and *ptsG-Af/ptsG-Br* as a primer to amplify the knockout fragment A-t-B. This knockout fragment A-t-B was transferred to *E. coli* MG1655 by electroporation. The *ptsG* knockout strain EC $\Delta$ *p* was obtained after two screenings. *manZ*, *envR*, and *atpFH* were sequentially knocked out by the same method to obtain the strains EC $\Delta$ *p-m*, EC $\Delta$ *p-m-a*, EC $\Delta$ *p-m-a*, and EC $\Delta$ *p-m-a-e*. PCR primers were synthesized by GENEWIZ (Suzhou, China) and are listed in Table 1.

### Culture media and conditions

Luria broth (LB) medium was used for strain maintenance and seed preparation. All batch fermentations, including pure culture and coculture, were performed in M9 medium comprising 12.8 g/L Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 3 g/L KH<sub>2</sub>PO<sub>4</sub>, 1 g/L NH<sub>4</sub>Cl, and 0.5 g/L NaCl supplemented with the desired amounts of glucose and/or xylose. This basic solution was autoclaved and supplemented with 0.24 g/L MgSO<sub>4</sub>, and a trace element solution containing 6.0 mg/L FeSO<sub>4</sub>·7H<sub>2</sub>O, 2.7 mg/L CaCO<sub>3</sub>, 2.0 mg/L ZnSO<sub>4</sub>·H<sub>2</sub>O, 1.16 mg/L MnSO<sub>4</sub>·H<sub>2</sub>O, 0.37 mg/L CoSO<sub>4</sub>·7H<sub>2</sub>O, 0.33 mg/L CuSO<sub>4</sub>·5H<sub>2</sub>O, and 0.08 mg/L H<sub>3</sub>BO<sub>3</sub> (all filter sterilized). Appropriate amounts of antibiotics (50  $\mu$ g/ml kanamycin and 100  $\mu$ g/ml streptomycin) were added to the medium when needed. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 2 mM when needed.

Regarding seed preparation, single colonies of *E. coli* and *P. putida* were inoculated into 5 mL LB medium in a 35-mL test tube and incubated overnight under aerobic conditions at 30 °C and 220 rpm. To produce mcl-PHAs, seed cultures of *P. putida* were inoculated into 500-mL baffled shake flasks comprising 50 mL defined M9 medium to an initial OD<sub>600</sub> of 0.05 and placed on a rotary shaker (220 rpm) under aerobic conditions at 30 °C. *E. coli* sugar utilization and acid production experiments were conducted as follows. A certain amount of glucose or xylose mother liquor was added to a 250-mL Erlenmeyer flask comprising 50 mL M9 medium, and a certain amount of activated seed solution was added. Then, this mixture was cultured under aerobic conditions at 30 °C and 220 rpm.

**Table 1** Bacterial strains and plasmids used in this study

Name	Description	Source references
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1</i>	TransGen biotech
DH5 $\alpha$ - <i>acs</i>	Carrying the <i>acs</i> gene cloned in pBBR1MCS-2	This study
DH5 $\alpha$ - <i>ackA</i>	Carrying the <i>ackA</i> gene cloned in pBBR1MCS-2	This study
DH5 $\alpha$ - <i>ackA-pta</i>	Carrying the <i>ackA-pta</i> genes cloned in pBBR1MCS-2	This study
MG1655	<i>E. coli</i> K-12 F <sup>-</sup> $\lambda$ - <i>ilvG<sup>-</sup> rfb-50 rph-1</i>	[3]
EC $\Delta$ p	<i>E. coli</i> MG1655, $\Delta$ <i>ptsG</i>	This study
EC $\Delta$ p- <i>m</i>	<i>E. coli</i> MG1655, $\Delta$ <i>ptsG</i> , $\Delta$ <i>manZ</i>	This study
EC $\Delta$ p- <i>m-a</i>	<i>E. coli</i> MG1655, $\Delta$ <i>ptsG</i> , $\Delta$ <i>manZ</i> , $\Delta$ <i>atpFH</i>	This study
EC $\Delta$ p- <i>m-a-e</i>	<i>E. coli</i> MG1655, $\Delta$ <i>ptsG</i> , $\Delta$ <i>manZ</i> , $\Delta$ <i>atpFH</i> , $\Delta$ <i>envR</i>	This study
Plasmids		
pBBR1MCS-2	Expression vector for <i>P. putida</i>	[19]
pBRR1- <i>acs</i>	<i>acs</i> inserted into pBBR1MCS-2	This study
pBRR1- <i>ackA-pta</i>	<i>ackA-pta</i> inserted into pBBR1MCS-2	This study
pBRR1- <i>ackA</i>	<i>ackA</i> inserted into pBBR1MCS-2	This study
pTKS/CS	p15A replication, Cm <sup>r</sup> , Tet <sup>r</sup> , I-Sce I restriction sites	Dr. Tao Chen of Tianjin University
pTKRED	pSC101 replication, temperature-sensitive replication origin, Spc <sup>r</sup> , Para BAD-driven I-Sce I gene, Red recombinase expression plasmid, lac-inducible expression	Dr. Tao Chen of Tianjin University
<i>Pseudomonas putida</i>		
KT2440	Wild-type	ATCC
KT2440-pBRR1	KT2440 harboring empty vector pBBR1MCS-2	This study
KT2440- <i>acs</i>	KT2440 harboring pBRR1- <i>acs</i>	This study
KT2440- <i>ackA</i>	KT2440 harboring pBRR1- <i>ackA</i>	This study
KT2440- <i>ackA-pta</i>	KT2440 harboring pBRR1- <i>ackA-pta</i>	This study
Primers <sup>a</sup>		
RT- <i>acs</i> -F	5'-ATGGACGAGCAGGGCTATGT-3'	
RT- <i>acs</i> -R	5'-CCCCTCACGCAACTCTACCT-3'	
RT-16S-F	5'-GAGACACGGTCCAGACTCCTAC-3'	
RT-16S-R	5'-CCAACTTGCTGAACCACTAC-3'	
<i>acs</i> -F	5'-CCGCTCGAGATGCCCCAGCCAAGCTACACC-3'	
<i>acs</i> -R	5'-CGCGGATCCTCAAATCTTGGGCAAGGCCG-3'	
<i>ackA</i> -F	5'-CCGGAATTCATGTTCGAGTAAGTTAGTACTG-3'	
<i>ackA</i> -R	5'-CGCGGATCCTCAGGCAGTCAGGCGGCTCGCG-3'	
<i>pta</i> -F	5'-CCGCTCGAGGTGTCCCGTATTATTATGCTG-3'	
<i>pta</i> -R	5'-CCGGAATTCCTACTGCTGCTGTGCAGACTG-3'	
<i>ptsG</i> -Af	5'-TGGCTGTGTTGAAAAGGTTGCGG-3'	
<i>ptsG</i> -Ar	5'-TATCCCTAAGCACAATGCCTCTATTTAGTTAACGTTCTG-3'	
<i>ptsG</i> -Bf	5'-AGGGTAATCAGAACGTTAACTAAATAGAGGCATTGTGCTAAAAACGAA CAAATGGCAGAG-3'	
<i>ptsG</i> -Br	5'-GTCTGCAAGGCGCTCAAGACG-3'	
<i>ptsG</i> -tetf1	5'-CAGAACGTTAACTAAATAGAGGCATTGTGCTTAGGGATAACAGGGTAA TATTTAC-3'	
<i>ptsG</i> -tetr1	5'-GGTAAAGCGATCCCACCACCAGCC-3'	
<i>ptsG</i> -tetf2	5'-GCGTGAAGTGGTTCGGTT-3'	
<i>ptsG</i> -tetr2	5'-TCGTTTTTAGCACAATGCCTCTATTTAGTTAACGTTCTGATTACCCTGTTA TCCCTAC-3'	
<i>manZ</i> -Af	5'-TGCAAGCCTTCATCTTCATGG-3'	
<i>manZ</i> -Ar	5'-TATCCCTAAGCACAATGCCTCTATTTAGTTAACGTTCTG-3'	
<i>manZ</i> -Bf	5'-AGGGTAATCAGAACGTTAACTAAATAGAGGCATTGTGCTAAAAACGAA CAAATGGCAGAG-3'	

**Table 1** (continued)

Name	Description	Source references
<i>manZ-Br</i>	5'-GTCTGCAAGGCGCTCAAGACG-3'	
<i>manZ-tetf1</i>	5'-CAGAACGTAACTAAATAGAGGCATTGTGCTTAGGGATAACAGGGTAA TATTAC-3'	
<i>manZ-tetr1</i>	5'-GGTAAAGCGATCCCACCACCAGCC-3'	
<i>manZ-tetf2</i>	5'-GCGTGAAGTGGTTCGGTT-3'	
<i>manZ-tetr2</i>	5'-CAACAGTCTTCGCTCACCTGTTAGTCCAGTTCGATTACCCTGTTATCC CTAC-3'	
<i>atpFH-Af</i>	5'-TGCAAGCCTTCATCTTCATGG-3'	
<i>atpFH-Ar</i>	5'-TATCCCTAAGCACAATGCCTCTATTTAGTTAACGTTCTG-3'	
<i>atpFH-Bf</i>	5'-AGGGTAATCAGAACGTAACTAAATAGAGGCATTGTGCTAAAAACGAA CAAATGGCAGAG-3'	
<i>atpFH-Br</i>	5'-GTCTGCAAGGCGCTCAAGACG-3'	
<i>atpFH-Tetf1</i>	5'-CAGAACGTAACTAAATAGAGGCATTGTGCTTAGGGATAACAGGGTAA TATTAC-3'	
<i>atpFH-Tetr1</i>	5'-GGTAAAGCGATCCCACCAGCC-3'	
<i>atpFH-Tetf2</i>	5'-GCGTGAAGTGGTTCGGTT-3'	
<i>atpFH-Tetr2</i>	5'-TCGTTTTTAGCACAATGCCTCTATTTAGTTAACGTTCTGATTACCCTGTTA TCCCTAC-3'	
<i>envR-Af</i>	5'-TGCAAGCCTTCATCTTCATGG-3'	
<i>envR-Ar</i>	5'-TATCCCTAAGCACAATGCCTCTATTTAGTTAACGTTCTG-3'	
<i>envR-Bf</i>	5'-AGGGTAATCAGAACGTAACTAAATAGAGGCATTGTGCTAAAAACGAA CAAATGGCAGAG-3'	
<i>envR-Br</i>	5'-GTCTGCAAGGCGCTCAAGACG-3'	
<i>envR-tetf1</i>	5'-CAGAACGTAACTAAATAGAGGCATTGTGCTTAGGGATAACAGGGTAA TATTAC-3'	
<i>envR-tetr1</i>	5'-GGTAAAGCGATCCCACCACCAGCC-3'	
<i>envR-tetf2</i>	5'-GCGTGAAGTGGTTCGGTT-3'	
<i>envR-tetr2</i>	5'-TCGTTTTTAGCACAATGCCTCTATTTAGTTAACGTTCTGATTACCCTGTTA TCCCTAC-3'	

<sup>a</sup>Restriction sites are underlined

Regarding a reactor study, cells were cultured at 30 °C in a 5-L bioreactor with a working volume of 2 L. Sterile air was delivered at a flow rate of 2 vvm, and the dissolved oxygen level was kept above 25% of air saturation by controlling the agitation speed. pH was maintained at 7.0 by automatic addition of 0.5 M H<sub>2</sub>SO<sub>4</sub> and 1 M KOH.

### Enzymatic hydrolysis of corn stover

Corn stalk pretreated with ammonia water (water content 7.57%, glucan content 65.34%, xylan content 17.26%, and insoluble lignin 4.75%) was used as a substrate for enzymatic hydrolysis. The powder was placed in a 500-mL conical flask containing a citric acid buffer solution (50 mM, pH = 4.8) and reacted at a concentration ratio of 10% (w/v). Cellulase and hemicellulase were added at a ratio of 2:1 at a reaction temperature of 50 °C and rotational speed of 200 rpm; enzymatic hydrolysis was for 72 h in total. At the end of enzymatic hydrolysis, the reaction solution was placed in a boiling water bath for 10 min to kill the

enzyme and then subjected to suction filtration to obtain a hydrolyzate.

### Extraction and characterization of mcl-PHAs

The cultivated cells were harvested by centrifugation (9000 rpm, 4 °C, 10 min) and lyophilized for 24 h at –20 °C. The dried cells were placed in methanol (room temperature, treated at 160 rpm for 5 min, centrifuged at 9000 rpm for 10 min), and lyophilized again. The stem cells obtained in the previous step were placed in ten times (volume:mass) the acetone at 170 rpm and extracted at 22 °C for 5 h. The extracted PHAs were concentrated by rotary evaporation and precipitated with ten times the volume of ice-cold methanol [15]. The thermal properties of mcl-PHAs, including  $T_g$ ,  $T_m$ , and  $\Delta H$ , were analyzed by differential scanning calorimetry (DSC). The weight average molecular weight ( $M_w$ ), number average molecular weight ( $M_n$ ), and polydispersity index (PD,  $M_w/M_n$ ) of mcl-PHA were analyzed by gel permeation chromatography (GPC) [6, 40].

## Analysis

The growth of bacterial cells was determined by measuring optical density at a wavelength of 600 nm ( $OD_{600}$ ) using an UV-1200 spectrophotometer (Mapada, China). Based on the characteristics of tetracycline and chloramphenicol resistance in *E. coli* and *P. putida*, the culture solution was diluted to a certain multiple and then coated on a plate with tetracycline and chloramphenicol resistance. Colony counting was conducted to analyze the  $OD_{600}$  value of the two bacteria in the mixed culture process.

Acetate, glucose, and xylose were quantified in the culture supernatant using an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H ion-exchange column (Bio-Rad, USA) operating at 65 °C with a flow rate of 0.6 mL min of 5 mM  $H_2SO_4$  using a differential refraction detector.

Extraction and detection of fatty acids were performed as described previously [37]. Fermentation supernatant was harvested by centrifugation at 12,000 rpm for 10 min. Subsequently, 200  $\mu$ L acetate was added to 2 mL supernatant, 150 mg undecanoic acid was added as an internal standard, and then, it was extracted with 2 mL *n*-hexane:chloroform (4:1, v/v). After the organic layer was evaporated to dryness, it was dissolved in 1 mL chloroform methanolic sulfuric acid (10:8.5:1.5) and then subjected to methanolysis at 100 °C for 1 h. Gas chromatography (Agilent, USA) was used for detecting FFAs. The resulting 1  $\mu$ L FFA methyl esters was injected for gas chromatography. The column temperature was initially maintained at 60 °C for 3 min, increased to 250 °C at the rate of 10 °C/min, and finally maintained at 250 °C for 10 min.

Extraction and detection of PHAs were performed as described previously [30]. Bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min, washed with distilled water, frozen at –80 °C, and lyophilized in a freeze dryer. The obtained lyophilizate was subjected to methanolysis at 100 °C for 4 h in the presence of 15% (v/v)  $H_2SO_4$ , and benzoic acid was used as an internal standard. Gas chromatography (Agilent, USA) was used for this study. The resulting 1  $\mu$ L PHA methyl esters was injected for gas chromatography. Column temperature was initially maintained at 80 °C for 1 min, then increased to 250 °C at a rate of 20 °C/min, and finally maintained at 250 °C for 1.5 min. To identify the mcl-PHA monomer composition, the sample was first analyzed by gas chromatography and mass spectrometry (GC–MS), the interface temperature of mass spectrometry was 250 °C, the ion source voltage was 70 eV, ion current was 40  $\mu$ A, and the mass scanning range was from 50 to 650 m/z. The inlet temperature was set to 290 °C. And then the standards of different monomers were used as internal standards in gas chromatography, and the monomer composition of mcl-PHA was

determined according to the different peak positions of different monomers.

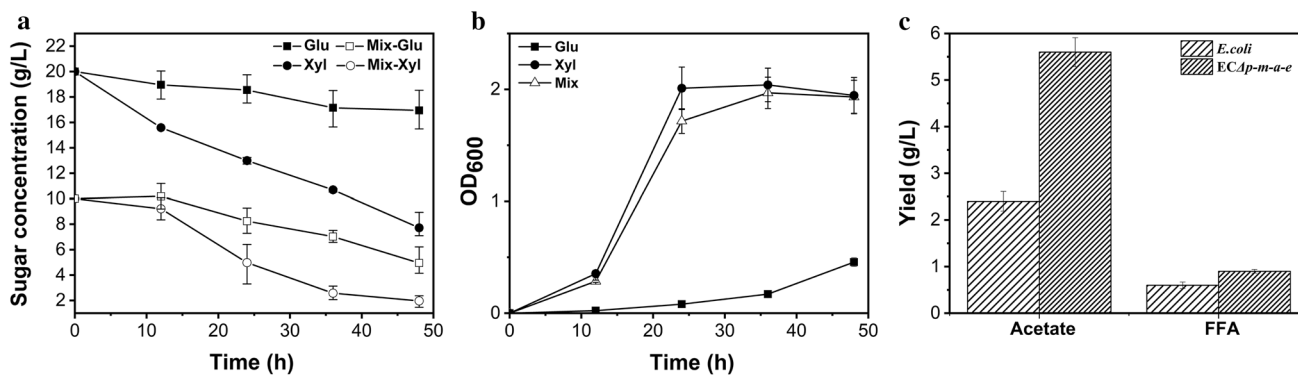
## Results and discussion

### Engineering *P. putida* KT2440 and *E. coli* MG1655 to increase mcl-PHA production from “unrelated” carbon sources

Results of previous studies conducted at our laboratory have reported on the application of engineered *P. putida* KT2440 to synthesize mcl-PHAs using acetate by overexpressing *acs* and *ackA-pta* pathways; *acs* overexpression can increase mcl-PHA production. The final yield of KT2440-*acs* was 0.674 g/L, which was 92% higher than that of wild-type bacteria; further, the intracellular accumulation of mcl-PHAs increased by approximately 43%, and the yield of mcl-PHAs from acetate increased by approximately 50%. Real-time quantitative PCR has demonstrated that the expression level of *acs* of KT2440-*acs* was 12 times higher than that of wild-type bacteria [51].

To resolve the limitation of low mcl-PHA production of *P. putida* monocultures with glucose and xylose, we introduced *E. coli* into the culture system to provide relevant carbon sources for *P. putida* [10]. When glucose is used as a single carbon source, these two bacteria have a competitive relationship, resulting in instability and inconsistency in the system. Engineered and cocultivated strains using different carbon sources serve as an effective strategy to avoid competitive growth and enhance the coordination of the growth of two bacteria. However, in the presence of glucose, *E. coli* preferentially consumes glucose instead of xylose owing to carbon catabolite repression (CCR). To promote the ability of *E. coli* to prefer xylose and relieve the effect of glucose, Gosset et al. [12] demonstrated that *ptsG* inactivation abolishes the CCR of *manZ* involved in glucose uptake and secretion. Liu et al. [24] knocked out *ptsG* and *manZ* simultaneously in *E. coli* BMT 23 to determine its slow metabolism of glucose and preferential utilization of xylose.

We knocked out *ptsG* and *manZ* in *E. coli* MG1655. The engineered strain  $EC\Delta p-m$  was cultured, and the results are presented in Fig. 2a, b. When the strain was cultured with 20 g/L of glucose as the sole carbon source, its concentration decreased slowly, and only a small amount (3.06 g/L) of glucose was consumed after 48 h. However,  $OD_{600}$  increased to only 0.457, which indicated that the glucose metabolism ability of the engineered strain was greatly inhibited. When the strain was cultured with 20 g/L xylose as the sole carbon source, the strain could consume xylose rapidly (12.28 g/L xylose was consumed 48 h later), and  $OD_{600}$  increased rapidly to 1.946 at 48 h, which indicated that the strain had a strong xylose consumption capacity. When the strain was



**Fig. 2** Sugar consumption (a) and biomass (b) of the strain *ECΔp-m*, (filled square) Glu: glucose was the single carbon source; (filled circle) Xyl: xylose was the single carbon source; (open triangle) Mix: carbon source was mixture of glucose and xylose; (open square) Mix-

Glu: glucose concentration under mixed carbon source; (open circle) Mix-Xyl: xylose concentration under mixed carbon source. Production of acetate and free fatty acids in *E. coli* (c), *E. coli*: wild-type *E. coli* and *ECΔp-m-a-e*: engineered strain *ECΔp-m-a-e*

cultured with a mixture of 10 g/L glucose and 10 g/L xylose, xylose was rapidly consumed during cultivation, whereas glucose was almost unconsumed. Glucose was only slowly consumed when the xylose concentration was low. These results suggest that knocking out *ptsG* and *manZ* in *E. coli* MG1655 can promote its preferential use of xylose and slow down the metabolism of glucose in the presence of both glucose and xylose.

As shown in Fig. 1a, glucose was preferentially used for growth and reproduction by *P. putida*, whereas acetate and FFAs secreted by *E. coli* were mainly used for mcl-PHA biosynthesis or other product biosynthesis. Therefore, increasing the production of acetate and FFAs in *E. coli* could promote mcl-PHA production. Causey et al. [7] used various metabolic modification strategies to improve the acetate production of *E. coli* W3110. Under aerobic conditions, *E. coli* converted approximately 50% of the substrate into biomass [41]. If carbon flux can be regulated to acetate synthesis, synthesis of acetate can be effectively increased, and oxidative phosphorylation is a key process related to this. This process can drive the main synthesis of ATP under aerobic conditions. Disruption of this pathway will force cells to synthesize ATP via the acetate synthesis pathway, thereby achieving the goal of metabolic flux regulation. Therefore, we attempted to promote acetate synthesis by knocking out the key gene *atpFH* in *ECΔp-m* oxidized phosphoric acid.

FFAs are synthesized by microorganisms using acyl-ACP as a substrate catalyzed by thioesterases. In the past decade, rational metabolic engineering was widely used to improve the synthesis of FFAs and their precursors [49, 50]. EnvR was known to inhibit the expression of the forward transporter AcrAB. AcrAB excretes intracellular antibiotics, surfactants, and organic complexes [14, 31]. AcrAB is the major transporter of fatty acids. Shin et al. [37] demonstrated that knocking out *envR* can eliminate AcrAB inhibition, thereby promoting the secretion of extracellular FFA

by *E. coli*. Promoting the secretion of extracellular FFA by *E. coli* also contributed to mcl-PHA synthesis. Therefore, we knocked out *envR* in *ECΔp-m-a*.

The final engineered strain *ECΔp-m-a-e*, which was knocked out of *ptsG*, *manZ*, *atpFH*, and *envR*, was cultured and tested. The main components of extracellular FFAs were *n*-hexanoic acid, *n*-heptanoic acid, *n*-octanoic acid, and *n*-dodecanoic acid. These FFAs can be used by *P. putida* to synthesize mcl-PHAs. As shown in Fig. 2c, when 20 g/L xylose was a single carbon source, cultured for 60 h, wild-type *E. coli* MG1655 could synthesize 2.4 g/L acetate and 0.62 g/L FFA, whereas *ECΔp-m-a-e* could synthesize 5.5 g/L acetate and 0.91 g/L FFAs. Therefore, the ability of *E. coli* MG1655 to use xylose for synthesizing acetate and extracellular FFAs can be promoted by knocking out *atpFH* and *envR*.

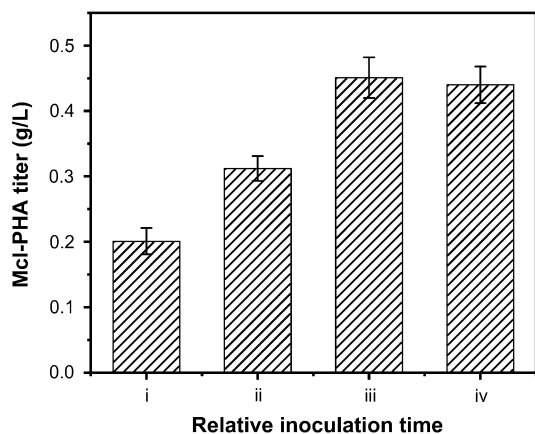
## Developing a coculture consortium for efficient production of mcl-PHA

### Construction and optimization of culture conditions for the coculture consortium

Although *E. coli* and *P. putida* have been engineered and optimized, the question of how to coculture for PHA production remains unsolved. The relative inoculation time, inoculation ratio, and corresponding culture conditions (e.g., temperature and pH) have important effects on the coculture consortium. In addition, the nitrogen source also affects the production of mcl-PHA.

First, the relative inoculation time of the two bacteria mainly involves whether the double bacteria are simultaneously or sequentially inoculated to the medium. Using *E. coli* as a reference, *P. putida* was inoculated 12 h before *E. coli* inoculation, simultaneously with *E. coli*, 12 h after *E. coli* inoculation, and 24 h after *E. coli* inoculation. As

shown in Fig. 3, the yield of mcl-PHAs by *P. putida* inoculated 12 h before *E. coli* inoculation and simultaneously with *E. coli* was 0.201 and 0.312 g/L, respectively. Then, 0.451 and 0.440 g/L of mcl-PHAs were synthesized when *P. putida* was inoculated 12 and 24 h after *E. coli* inoculation, respectively. The results showed that the latter two relative inoculation times were significantly better than the former two relative inoculation times for mcl-PHA production, and PHA production was the highest when *P. putida* was inoculated 12 h after *E. coli* inoculation. Consequently,



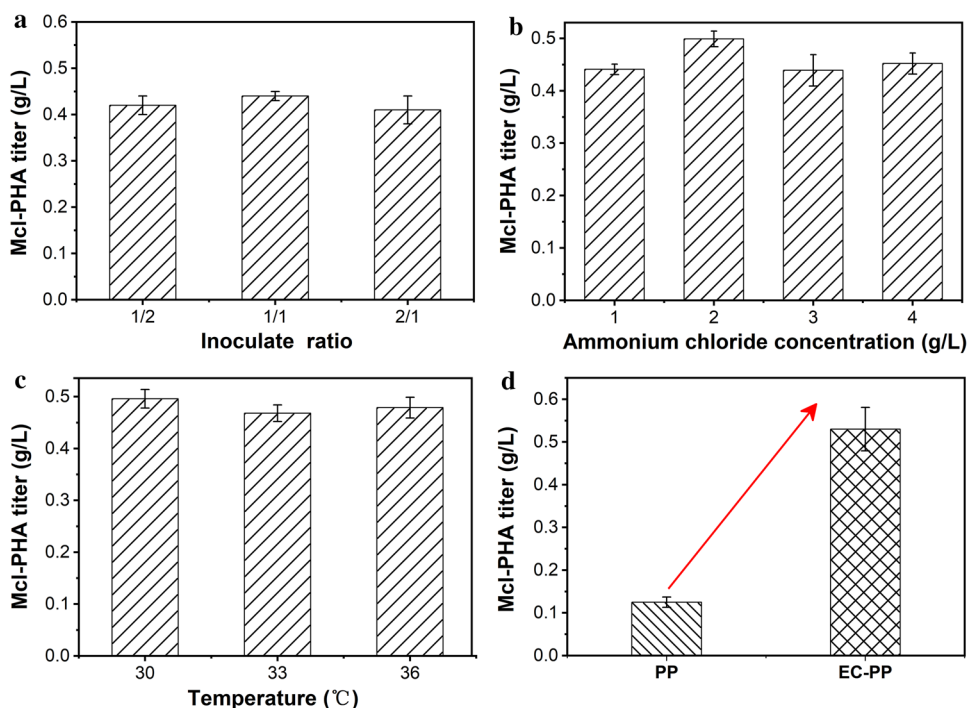
**Fig. 3** Mcl-PHA production by the coculture consortium under different relative inoculation times. *P. putida* inoculated 12 h before *E. coli* inoculation (i), simultaneously with *E. coli* (ii), 12 h after *E. coli* inoculation (iii), and 24 h after *E. coli* inoculation (iv)

the subsequent culture was inoculated with *P. putida* 12 h after *E. coli* inoculation.

Second, we researched three initial inoculation ratios (*E. coli*:*P. putida*, 1/2, 1/1, 2/1) of the two bacteria. The results are shown in Fig. 4a. Different inoculation ratios had no significant effect on the yield of mcl-PHAs. The initial inoculum can accelerate the entry of the strain into the log phase. Under the study conditions, both *P. putida* and *E. coli* grew rapidly. The change in inoculum had no significant effect on their growth rate. In addition, *P. putida* was inoculated after *E. coli*, further weakening the effect of inoculation on double bacteria exchange. Therefore, changing the inoculation ratio did not significantly affect mcl-PHA production. Next, we designed four groups of experiments on the concentration of the nitrogen source (1, 2, 3, and 4 g/L  $\text{NH}_4\text{Cl}$ ). As shown in Fig. 4b, we found that 2 g/L of  $\text{H}_4\text{Cl}$  showed the highest yield of mcl-PHAs. Last, we explored the culture temperature and pH. The optimum culture pH of *P. putida* and *E. coli* was approximately 7.0; therefore, the culture pH was selected to be 7.0. However, there were differences between the two cultures in terms of optimum temperature (*P. putida*: 30 °C and *E. coli*: 37 °C). Therefore, we designed culture experiments at different temperatures for the two bacteria. The results presented in Fig. 4c show that the yield of mcl-PHAs was the highest at 30 °C, and the change in culture temperature did not significantly affect this yield.

Experiments related to coculture consortium were conducted under the abovementioned optimum conditions to produce mcl-PHAs (Fig. 4d). In M9 medium, with a total sugar concentration of 20 g/L (glucose:xylose, 1:1), the

**Fig. 4** Mcl-PHA production by the coculture consortium under different inoculum ratios (a); production of mcl-PHA under different concentrations of ammonium chloride (b) and different temperatures (c); mcl-PHA production of coculture and monoculture (d)





yield of mcl-PHA produced by the coculture consortium was 0.541 g/L, which was 3.9 times higher than that synthesized by *P. putida* monoculture.

#### “Nutrition supply–detoxification” relationship between *E. coli* and *P. putida*

As shown in Fig. 5, the sugar concentration changed and biomass were analyzed during culture. A comparison of the maximum OD<sub>600</sub> revealed that the biomass yields of the coculture consortium were higher than those of the respective monoculture (Fig. 5a). The increase in biomass yield of *E. coli* may be mainly owing to the release of acetate inhibition by *P. putida* through the metabolic consumption of acetate. As can be seen in Fig. 5b, acetate hardly accumulates in the coculture consortium compared with how it accumulates in monocultures. It can also be deduced that *E. coli* grows better based on the observation that xylose consumption was faster in the coculture consortium, and there was no residue (Fig. 5d). The increase in biomass yield of *P. putida* is due to the increase in available carbon sources in the coculture consortium. Regarding monocultures, the biomass yield of *P. putida* cannot continue to increase because the only carbon source glucose is consumed within 24 h of culture (Fig. 5c). In the coculture consortium, *P. putida* can also use acetate and FFAs secreted by *E. coli* to grow and synthesize mcl-PHAs (Fig. 5b).

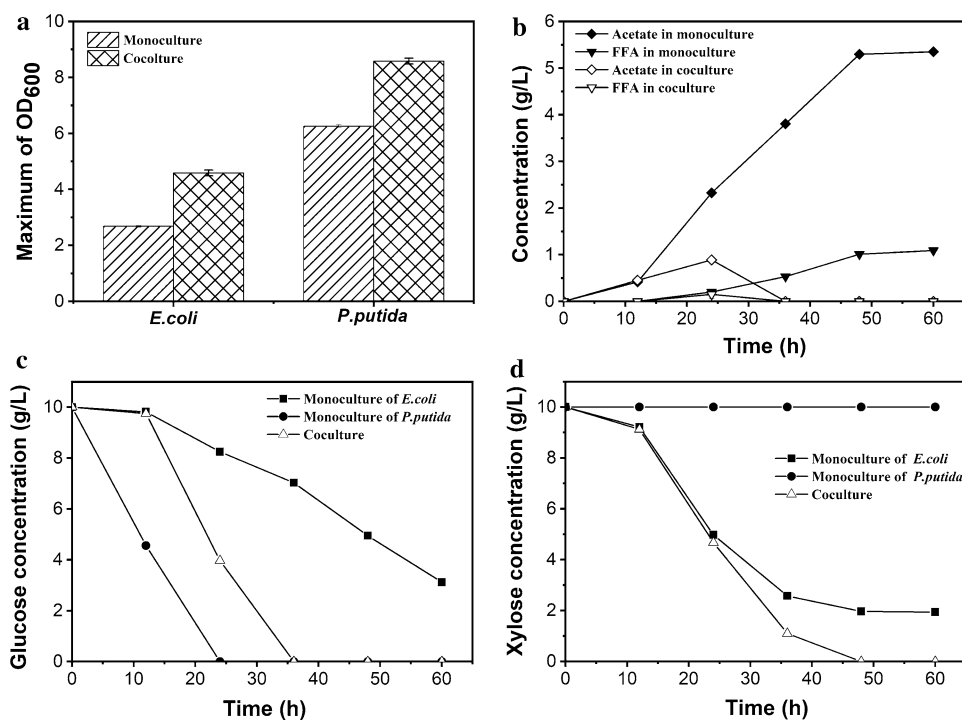
Acetate, the intermediate metabolite, was responsible for the symbiotic “nutrition supply–detoxification” relationship between two bacteria, i.e., *P. putida* used acetate and FFAs

secreted by *E. coli* as carbon sources. Simultaneously, it relieved the inhibition of acetate on *E. coli*. Compared with the monoculture of *P. putida*, the coculture consortium can efficiently synthesize mcl-PHAs using a sugar-based carbon source.

#### Mcl-PHA production by the coculture consortium on cellulose hydrolysate

Currently, renewable resources such as lignocellulose are mainly used for scl-PHA production; for example, Narayanan [26] used *Bacillus mycoides* to synthesize 0.39 g/L P (3HB-co-3HV) with rice hull hydrolysate as a substrate. Johanna et al. [38] synthesized 0.23 g/L P (3HB-co-3HV) using acid-hydrolyzed wood chip hydrolysate. Kulkarni [20] synthesized 0.365 g/L P (3HB-co-3HV) using bagasse extract as a substrate along with *Halomonas campisalis* MCM B-1027 isolated from an extreme environment. However, there are a few reports on synthetic mcl-PHA studies. Davis [10] used wild ryegrass hydrolysate as a substrate to synthesize mcl-PHAs using several different *Pseudomonas* strains, producing a maximum yield of 0.3 g/L. Löwe et al. [25] constructed a coculture consortium comprising *Synechococcus elongatus* cscB and *P. putida* cscAB. The two bacteria synthesized mcl-PHAs using CO<sub>2</sub> as a substrate through material exchange, but the production efficiency was only 23.8 mg/L/day. There remains a large gap between the mcl-PHAs produced by renewable resources and scl-PHAs in terms of yield and production efficiency.

**Fig. 5** Maximum biomass of *E. coli* and *P. putida* in monocultures and coculture (a); Concentrations of acetate and FFA during monoculture of *E. coli* and coculture (b); Concentrations of glucose during monoculture and coculture (c); Concentrations of xylose during monoculture and coculture (d)



We obtained the hydrolysate by enzymatic hydrolysis of corn straw (water content 7.57%, glucan content 65.34%, xylan content 17.26%, insoluble lignin 4.75%) pretreated with ammonia (181 °C, 30 min). HPLC was used to analyze the sugar composition in the hydrolysate. As a result, the main sugar components in the hydrolysate were glucose and xylose (ratio 4.3:1). Sugar solution obtained by enzymatic hydrolysis was used for fermentation. The total sugar concentration was approximately 20 g/L, and the glucose–xylose mixture (4.3:1) was used as a control. The result showed that the titer of mcl-PHAs produced by the coculture with the hydrolysate (0.434 g/L) was slightly lower than that of mcl-PHAs produced with a glucose–xylose mixture (0.469 g/L). This shows that the coculture consortium had the ability to utilize lignocellulosic resources. The mcl-PHA yield and production efficiency of the coculture consortium were improved compared with those previously reported (Table 2).

### Property characterization of mcl-PHAs

Property characterization of mcl-PHAs showed that mcl-PHA monomer synthesized by the coculture consortium was composed of 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, and 3-hydroxytetradecanoate, with 3-hydroxydecanoate (accounting for 60% of the total mcl-PHA monomer) being the main component followed by 3-hydroxyoctanoate (accounting for 26.8% of the total mcl-PHA monomer). The monomer with the highest carbon atom number was hydroxytetradecanoate, whereas that with the lowest carbon atom number was hydroxyhexanoate.

As shown in Table 3,  $T_g$  of mcl-PHAs synthesized by the coculture consortium was  $-46.78$  °C, and  $T_m$  was  $40.81$  °C. It has typical thermal properties of mcl-PHAs mainly comprising a hydroxydecanoate monomer [2, 17].

**Table 3** Molecular weight and thermal performance data of mcl-PHAs

$M_n$	$M_w$	PD	$T_g$ (°C)	$T_m$ (°C)	$\Delta H$ (J/g)
40222	94146	2.3	$-46.78$	40.81	5.6

The experimental data measured by GPC showed that  $M_w$  of mcl-PHA was 94146,  $M_n$  was 40222, and PD was 2.3. The molecular weight and thermal properties of mcl-PHAs synthesized by coculture are similar to those of mcl-PHAs synthesized by Davis et al. [10] using ryegrass hydrolysate.

### Conclusions

In this study, we constructed a coculture consortium comprising engineered *P. putida* KT2440 and *E. coli* MG1655 for mcl-PHA production using “unrelated” carbon sources. *E. coli* used xylose to secrete acetate and “related” carbon sources such as extracellular FFAs, whereas *P. putida* undertook the task of efficient biosynthesis of mcl-PHAs. We knocked out *ptsG*, *manZ*, *atpFH* and *envR* in *E. coli* MG1655, and the yield of acetate and FFA in  $EC\Delta p\text{-}m\text{-}a\text{-}e$  was 2.3 and 1.4 times higher than the parent strain, respectively.

Consequently, *E. coli* used xylose to secrete acetate and extracellular FFAs, whereas *P. putida* used glucose as the main carbon source for growth. The main intermediate metabolite acetate played an important role in constructing the expected “nutrition supply–detoxification” relationship between these two strains. Next, the coculture consortium was applied to produce mcl-PHAs with renewable carbon resources; the glucose–xylose mixture first and then, the actual lignocellulosic hydrolysate. In M9 medium, with a total sugar concentration of 20 g/L (glucose:xylose, 1:1),

**Table 2** PHA production from cost-effective sources in different studies

Strain	Substrate	Type of PHA	Yield of PHA (g/L)	Production efficiency	References
<i>P. putida</i> KT2440 + <i>E. coli</i> MG1655	Glucose + xylose	mcl-PHA	0.541	0.01 (g/L/h)	This study
<i>P. putida</i> KT2440 + <i>E. coli</i> MG1655	Corn straw hydrolysate	mcl-PHA	0.434	0.01 (g/L/h)	This study
<i>Pseudomonas</i> strains	Ryegrass hydrolysate	mcl-PHA	0.3	–	[10]
<i>P. putida</i> KT2440	Glucose	mcl-PHA	1.8	0.006 (g/L/h)	[30]
<i>Bacillus mycoides</i>	Rice husk hydrolysate	P(3HB-co-3HV)	0.39	–	[26]
<i>S. Macrogoltabida</i>	Sawdust	P(3HB-co-3HV)	0.23	–	[38]
<i>E. coli</i>	Glucose	mcl-PHA	0.4	–	[46]
<i>S. elongatus</i> cscB + <i>P. putida</i> cscAB	CO <sub>2</sub>	mcl-PHA	0.156	23.8 (mg/L/day)	[25]
<i>P. putida</i> KT2440	Xylose + octanoic acid	mcl-PHA	0.53	–	[21]
<i>H. scampisalis</i> MCM B-1027	Bagasse extract	P(3HB-co-3HV)	0.365	–	[20]
<i>Alcaligenes latus</i>	Sugarbeet juice	PHB	4.01	0.22	[45]

the yield of mcl-PHAs produced by the coculture consortium was 0.541 g/L, which was 3.9 times higher than that observed for the engineered *P. putida* monoculture. The titer of mcl-PHA produced by the coculture consortium with lignocellulosic hydrolysate was 0.434 g/L, which was slightly lower than that of mcl-PHAs (0.469 g/L) produced with the glucose–xylose mixture (glucose:xylose, 4.3:1). These results indicate that the coculture consortium has the potential to apply lignocellulose resources. Simultaneously, it has a competitive advantage over other studies that use renewable resources to synthesize PHAs. Research on increasing FFA production is underway at our laboratory, which could be used to further improve the yield of mcl-PHAs.

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