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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Bio-based production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with modulated monomeric fraction in *Escherichia coli*

Dragan Miscevic[1] · Ju-Yi Mao[2] · Bradley Mozell[1] · Kajan Srirangan[3] · Daryoush Abedi[1,4] · Murray Moo-Young[1] · C. Perry Chou[1]

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Abstract

In this study, we applied metabolic engineering and bioprocessing strategies to enhance heterologous production of an important biodegradable copolymer, i.e., poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), with a modulated 3-hydroxyvalerate (3-HV) monomeric fraction from structurally unrelated carbon of glycerol in engineered *Escherichia coli* under different oxygenic conditions. We used our previously derived propanologenic (i.e., 1-propanol-producing) *E. coli* strain with an activated genomic Sleeping beauty mutase (Sbm) operon as a host for heterologous expression of the *phaCAB* operon. The 3-HV monomeric fraction was modulated by regulating dissimilated carbon flux channeling from the tricarboxylic acid (TCA) cycle into the Sbm pathway for biosynthesis of propionyl-CoA, which is a key precursor to (*R*)-3-hydroxyvaleryl-CoA (3-HV-CoA) monomer. The carbon flux channeling was regulated either by manipulating a selection of genes involved in the TCA cycle or varying oxygenic condition of the bacterial culture. With these consolidated strategies being implemented, we successfully achieved high-level PHBV biosynthesis with a wide range of 3-HV monomeric fraction from ~ 4 to 50 mol%, potentially enabling the fine-tuning of PHBV mechanical properties at the biosynthesis stage. We envision that similar strategies can be applied to enhance bio-based production of chemicals derived from succinyl-CoA.

Key points

- *TCA cycle engineering was applied to enhance 3-HV monomeric fraction in E. coli.*
- *Effects of oxygenic conditions on 3-HV incorporation into PHBV in E. coli were investigated.*
- *Bacterial cultivation for high-level PHBV production in engineered E. coli was performed.*

Keywords *Escherichia coli* · Glyoxylate shunt · Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) · Propionyl-CoA · Sleeping beauty mutase · TCA cycle

✉ C. Perry Chou
cpchou@uwaterloo.ca

[1] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, ON N2L 3G1, Canada

[2] Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 20224, Taiwan

[3] Biotechnology Research Institute, National Research Council of Canada, Montreal, QC H4P 2R2, Canada

[4] Department of Drug & Food Control, Tehran University of Medical Sciences, Tehran, Iran

Introduction

The global demand for renewable energy to meet human social and economic development has been increasing at an unprecedented pace. Rapidly emerging technologies surrounding bio-based production, which adopts biocatalysts to drive chemical conversions using clean and renewable carbon sources as feedstock, are gaining recognition as sustainable and environmentally friendly tools for manufacturing (Weiss et al. 2012). In particular, novel biotechnologies such as synthetic biology, genetic engineering, metabolic engineering, and systems biology have shown their potentials over

conventional organic chemistry for the synthesis of chemicals, specifically for those complex ones used in the pharmaceutical and fine chemical industries (Keasling 2010) and even bulk ones such as succinate, 1, 3-propanediol, ethanol, and polyhydroxyalkanoates (PHAs) (Hermann et al. 2007).

To date, plastics have become popular products of choice because of their high stability, durability, low cost, and ease to use. However, the benefits of plastics can be outweighed by their environmental impacts, such as non-biodegradability (Zheng et al. 2005) and leaching (Mileva et al. 2014), resulting in adverse health effects to humans (Rustagi et al. 2011). Hence, there is a growing interest in manufacturing biodegradable plastics. The most common alternative is PHAs, which are linear aliphatic polyesters with high thermostability, versatility, biocompatibility, and biodegradability (Tian et al. 2012). PHAs have a range of justified applications in manufacturing (Lam et al. 2017) and medical/healthcare (Ali and Jamil 2016; Patel 2015). PHAs are naturally synthesized in many Gram-negative and Gram-positive bacteria, such as those from the genera *Cupriavidus* (Berezina 2013; Passanha et al. 2014), *Pseudomonas* (Song et al. 2008), and *Bacillus* (Halami 2008; Valappil et al. 2007), and accumulate as nutrient-storing granules inside the cell (Pötter and Steinbüchel 2006). Note that, in addition to bacteria, PHA production in archaea has also been reported in the past (Hermann-Krauss et al. 2013). The most common and extensively studied PHA is poly(3-hydroxybutyrate) (PHB), which is a short-chain homopolymer with (*R*)-3-hydroxybutyryl-CoA (3-HB-CoA) as the substrate (Ali and Jamil 2016; Hyakutake et al. 2011). Nevertheless, PHB has limited applications due to its high melting point and crystallinity, making it rather brittle and stiff for mechanical processing (Anbukarasu et al. 2015). The structural disadvantages of PHB can be overcome by incorporating a longer-chain (*R*)-3-hydroxyvaleryl-CoA (3-HV-CoA) monomer, resulting in a copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) which is more flexible and less crystalline than PHB. Despite such structural advantages for PHBV, its bio-based production has faced a major technical issue associated with co-feeding of structurally related carbons (e.g., propionate and valerate) as the precursors to 3-HV-CoA (Fig. 1), significantly limiting its commercial and practical applicability (Aldor et al. 2002).

Over the past decades, considerable progress has been made for microbial production of PHBV, in particular from genetically tractable bacterium *E. coli* (Chen et al. 2011; Srirangan et al. 2016; Wong et al. 2008). PHBV biosynthesis in *E. coli* requires heterologous expression of the *phaCAB* operon, encoding enzymes for three distinct conversions, from natural producers (Fig. 1). The first reaction involves the Claisen condensation catalyzed by PhaA (encoded by *phaA*), a thiolase that simultaneously (i) homo-fuses two acetyl-CoA moieties to form acetoacetyl-CoA and (ii) hetero-fuses acetyl-CoA and propionyl-CoA moieties to form 3-ketovaleryl-CoA. The second reaction is a reduction catalyzed by PhaB (encoded by *phaB*), an NADPH-dependent reductase that simultaneously reduces (i) acetoacetyl-CoA to 3-HB-CoA and (ii) 3-ketovaleryl-CoA to 3-HV-CoA. The last reaction is a polymerization catalyzed by PhaC (encoded by *phaC*), a polymerase that incorporates both monomers of 3-HB-CoA and 3-HV-CoA into the polymer chain to form PHBV.

Natural *E. coli* strains do not produce propionyl-CoA, making it impossible for their PHBV biosynthesis. Hence, we previously derived a propanologenic (i.e., 1-propanol-producing) *E. coli* strain by activating the genomic Sleeping beauty mutase (Sbm) operon (Srirangan et al. 2013, 2014), and use it as the host for heterologous expression of the *phaCAB* operon (Srirangan et al. 2016). While successfully demonstrating PHBV biosynthesis in this engineered *E. coli* from structurally unrelated carbons of glucose and glycerol, the 3-HV monomeric fraction of the copolymer remained low and could not be modulated due to generally low intracellular levels of propionyl-CoA. Since the 3-HV monomeric fraction typically correlates with PHBV biopolymer properties, it is technically desirable to modulate this parameter at the biosynthesis stage. In this study, we tackled major challenges surrounding propionyl-CoA availability by manipulating selected oxygen-sensitive pathways in the tricarboxylic acid (TCA) cycle for effective dissimilated carbon flux channeling into the Sbm pathway, enabling modulation of the 3-HV monomeric fraction. Note that succinate is a plausible target for such metabolic manipulation as its biosynthesis under various oxygenic conditions in *E. coli* has been well characterized and succinyl-CoA is a key metabolic node for such carbon flux channeling (Cheng et al. 2013; Thakker et al. 2012; Vuoristo et al. 2016). Using a selection of engineered *E. coli* strains for cultivation under different oxygenic conditions, we successfully demonstrated high-level PHBV biosynthesis with a wide range of 3-HV monomeric fraction from ~ 4 to 50 mol%.

Materials and methods

Bacterial strains and plasmids

Bacterial strains, plasmids, and deoxynucleic acid (DNA) primers used in this study are listed in Table 1. Genomic DNA from bacterial cells was isolated using the Blood & Tissue DNA Isolation Kit (Qiagen, Hilden, Germany). Standard recombinant DNA technologies were applied for molecular cloning (Miller 1992). All plasmids were constructed by Gibson enzymatic assembly (Gibson et al. 2009). Phusion HF and *Taq* DNA polymerases were obtained from New England Biolabs (Ipswich, MA, USA). All synthesized oligonucleotides were obtained from Integrated DNA

Technologies (Coralville, IA, USA). DNA sequencing was conducted by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada). *E. coli* BW25113 was the parental strain for derivation of all mutant strains in this study and *E. coli* DH5α was used as a host for molecular cloning. It should be emphasized that the *ldhA* gene (encoding lactate dehydrogenase) was previously inactivated in BW25113, generating BWΔ*ldhA* (Srirangan et al. 2014), a strain with higher metabolic potential as it limits side production of lactate. Activation of the genomic Sbm operon in BWΔ*ldhA* to generate propanologenic *E. coli* CPC-Sbm was described previously (Srirangan et al. 2014).

Implementation of PHBV-biosynthetic pathway in propanologenic *E. coli* was previously reported (Srirangan et al. 2016). Briefly, the *phaCAB* operon was PCR-amplified using the primer set g-phaCAB

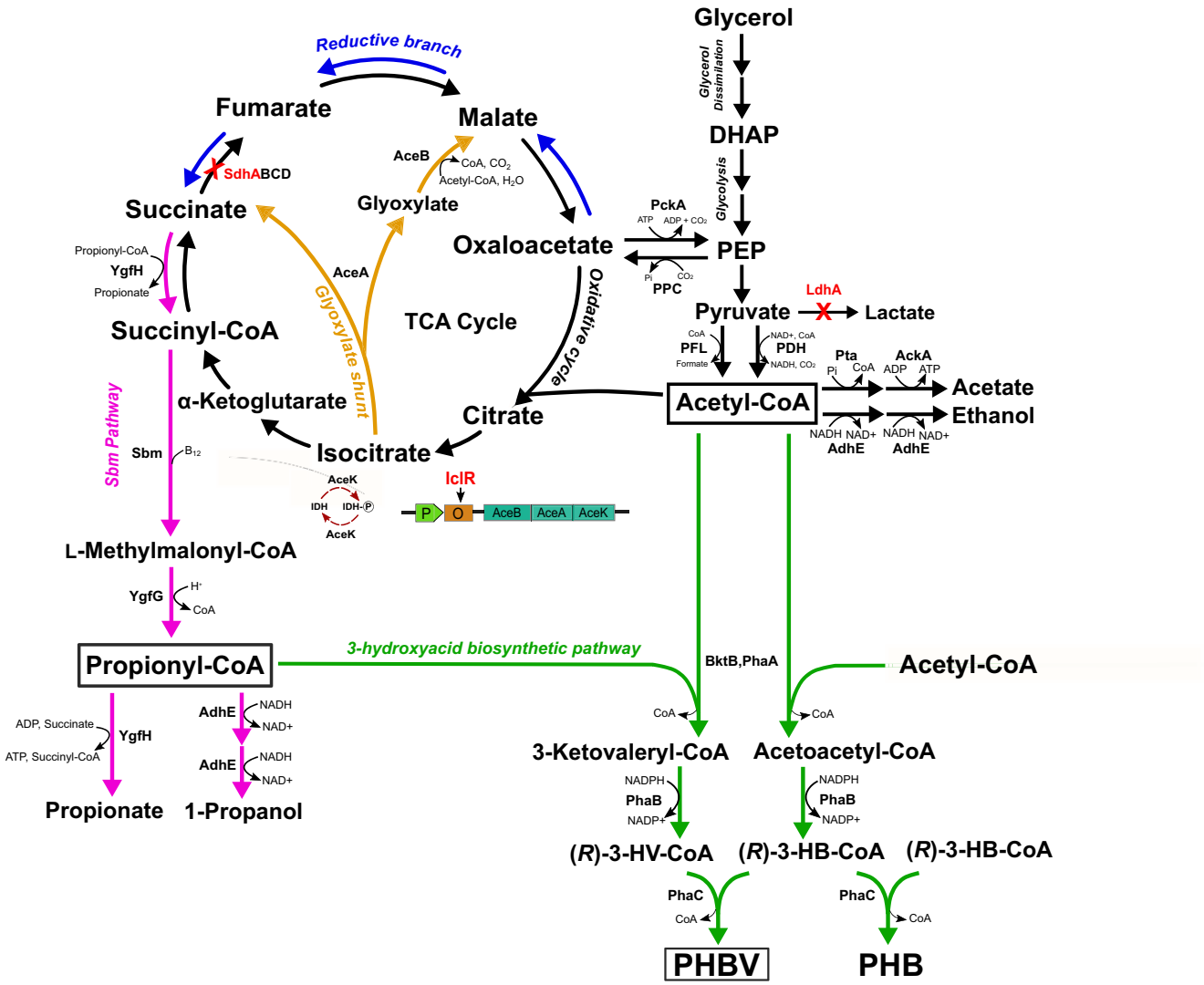

Fig. 1 Schematic representation of engineered PHBV pathway in *E. coli* using glycerol as the carbon source. Metabolic pathways outlined: glycerol dissimilation, central metabolism, and oxidative TCA cycle (in black); glyoxylate shunt of TCA cycle (in dark yellow); reductive branch of TCA cycle (in blue); Sbm pathway (in purple); 3-hydroxyacid biosynthetic pathway (in green). Metabolite abbreviations: 3-HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; (*R*)-3-HB-CoA, (*R*)-3-hydroxybutyryl-CoA; (*R*)-3-HV-CoA, (*R*)-3-hydroxyvaleryl-CoA. Protein abbreviations: AceA, isocitrate lyase; AceB, malate synthase A; AceK, isocitrate dehydrogenase kinase/phosphatase; AckA, acetate kinase; AdhE, aldehyde-alcohol dehydrogenase; BktB, beta-ketothiolase; IclR, AceBAK operon repressor; IDH, isocitrate dehydrogenase; IDH-P, isocitrate dehydrogenase-phosphate; LdhA, lactate dehydrogenase A; PckA, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase; PhaA, acetoacetyl-CoA thiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; PK, pyruvate kinase; PPC, phosphoenolpyruvate carboxylase; Pta, phosphotransacetylase; Sbm, methylmalonyl-CoA mutase; SdhABCD, succinate dehydrogenase complex; YgfG, (*R*)-methyl-malonyl-CoA carboxylase; YgfH, propionyl-CoA: succinate CoA-transferase

and the genomic DNA of wild-type *Cupriavidus necator* ATCC 43291 as the template. The amplified operon was Gibson–assembled (Gibson et al. 2009) (note that "assembled" is used for subsequent appearance) with the PCR-linearized pTrc99a using the primer set g-pTrc-phaCAB to generate pTrc-PhaCAB. All genes inserted into pTrc99a vector were under the control of the P_{trc} promoter. Plasmid pK-BktB was previously constructed in our lab by PCR-amplifying *bktB* from *C. necator* ATCC 43291 using the corresponding g-bktb primer set, followed by assembling all three PCR-amplified fragments with the PCR-linearized pK184 using the primer set g-pK-bktB. All genes inserted into pK184 vector were under the control of the P_{lac} promoter.

Knockouts of the genes *sdhA* and *iclR* were introduced into *E. coli* recipient strains by P1 phage transduction (Miller 1992) using the appropriate Keio Collection strains (The Coli Genetic Stock Center, Yale University, New Haven, CT, USA) as donors (Baba et al. 2006). To eliminate the co-transduced FRT-Kn^R-FRT cassette, the transductants were transformed with pCP20 (Cherepanov and Wackernagel 1995), a temperature-sensitive plasmid expressing a flippase (Flp) recombinase. Upon Flp-mediated excision of the Kn^R cassette, a single Flp recognition site (FRT "scar site") was generated. Plasmid pCP20 was then cured by growing cells at 42 °C. The genotypes of derived knockout strains were confirmed by colony polymerase chain reaction (PCR) using the appropriate verification primer sets listed in Table 1.

Table 1 *E. coli* strains, plasmids, and oligonucleotides used in this study

Name	Description, relevant genotype or primer sequence (5′ ➔ 3′)	Source
E. coli host strains		
DH5α	F−, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG φ80d lacZΔacZd ladlacZYA—argF*, *U169*, *hsdR17(rK-mK +)*, λ-	Lab stock
BW25113	F-, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, λ-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	(Datsenko and Wanner 2000)
BWΔ*ldhA*	BW25113 *ldhA* null mutant	(Srirangan et al. 2014)
CPC-Sbm	BWΔ*ldhA*, P*trc*::*sbm* (i.e., with the FRT-P*trc* cassette replacing the 204-bp upstream of the Sbm operon)	(Akawi et al. 2015)
PHBV-C	CPC-Sbm/pTrc-PhaCAB and pK- BktB	(Srirangan et al. 2016)
PHBVΔ*sdhA*	*sdhA* null mutant of PHBV	This study
PHBVΔ*iclR*	*iclR* null mutant of PHBV	This study
PHBVΔ*sdhA*Δ*iclR*	*sdhA* and *iclR* null mutant of PHBV	This study
Plasmids		
pCP20	FLP+ , λ cI857+ , λ pR Rep(pSC101 ori)ts, ApR ,CmR	(Cherepanov and Wackernagel 1995)
pKD46	RepA101ts ori, ApR , araC-P*araB:gam-bet-exo*	(Datsenko and Wanner 2000)
pTrc99a	ColE1 ori ApR P*trc*	(Amann et al. 1988)
pK184	p15A ori, KmR, P*lac::lacZ′*	(Jobling and Holmes 1990)
pTrc-PhaCAB	Derived from pTrc99a, P_{trc}::*phaCAB*	Lab stock
pK-BktB	Derived from pK184, P_{lac}::*bktb*	Lab stock
Primers		
v-ldhA	GATAACGGAGATCGGGAATGATTAA; GGTTTAAAAGCGTCGATGTCCAGTA	(Akawi et al. 2015)
v-sdhA	CTCTGCGTTCACCAAAGTGT; ACACACCTTCACGGCAGGAG	(Miscevic et al. 2019)
v-iclR	GGTGGAATGAGATCTTGCGA; CCGACACGCTCAACCCAGAT	(Miscevic et al. 2019)
c-frt	AGATTGCAGCATTACACGTCTTGAG; CCAGCTGCATTAAT GAATCGGGCCATGGTCCATATGAATATCCTCC	(Srirangan et al. 2014)
c-ptrc	CCGATTCATTAATGCAGCTGG; GGTCTGTTTCCTGTGTGAAATTGTTA	(Srirangan et al. 2016)
g-phaCAB	**CACACAGGAAACAGAC**ATGGCGACCGGCAAAGGC; **CGAGCTCGAATTCC** **AT**TCAGCCCATATGCAGGCC	Lab stock
g-bktb	**CACAGGAAACAGCTATGACC**ATGACGCGTGAAGT GGTAGT; **TAAACAGACCTCCCTTAAATTTAAT**TCAG ATACGCTCGAAGATGG	Lab stock
g-pTrc-phaCAB	GCGGCCTGCATATGGGCTGA**TGGAATTCGAGCTCGGTACC**; ACGATGACAACGTCAGTCAT **GTCTGTTTCCTGTGTGAAATTGTTATCCG**	Lab stock
g-pK-bktB	**GGTGATGCGTAATCACAATTAA**TGATTACGAATTCGAGCTCGG; **TACCACTTCACGCGTCAT**GGTCATAGCTGTTTCCTGTGTGAA	Lab stock

Media and bacterial cell cultivation

All medium components were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) except glucose, yeast extract, and tryptone which were obtained from BD Diagnostic Systems (Franklin Lakes, NJ, USA). The media were supplemented with antibiotics as required: 25 μg mL^{-1} kanamycin and 50 μg mL^{-1} ampicillin. *E. coli* strains, stored as glycerol stocks at − 80 °C, were streaked on lysogeny broth (LB) agar plates with appropriate antibiotics and incubated at 37 °C for 14–16 h. Single colonies were picked from LB plates to inoculate 30 mL super broth (SB) medium (32 g L^{-1} tryptone, 20 g L^{-1} yeast extract, and 5 g L^{-1} NaCl) with appropriate antibiotics in 125 mL conical flasks. Overnight cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ, USA) and used as seed cultures to inoculate 220 mL SB media at 1% (v/v) with appropriate antibiotics in 1 L conical flasks. This second seed culture was shaken at 37 °C and 280 rpm for 14–16 h. Cells were then harvested by centrifugation at 9,000×*g* and 20 °C for 10 min and resuspended in 50 mL fresh LB media. The suspended culture was used to inoculate a 1 L stirred tank bioreactor (containing two Rushton radial flow disks as impellers) (CelliGen 115, Eppendorf AG, Hamburg, Germany) at 30 °C and 430 rpm. The semi-defined production medium in the batch bioreactor contained 30 g L^{-1} glycerol, 0.23 g L^{-1} K_2HPO_4, 0.51 g L^{-1} NH_4Cl, 49.8 mg L^{-1} $MgCl_2$, 48.1 mg L^{-1} K_2SO_4, 1.52 mg L^{-1} $FeSO_4$, 0.055 mg L^{-1} $CaCl_2$, 2.93 g L^{-1} NaCl, 0.72 g L^{-1} tricine, 10 g L^{-1} yeast extract, 10 mM $NaHCO_3$, 0.2 μM cyanocobalamin (vitamin B_{12}), and 1000th dilution (i.e., 1 mL l^{-1}) trace elements (2.86 g L^{-1} H_3BO_3, 1.81 g L^{-1} $MnCl_2$• $4H_2O$, 0.222 g L^{-1} $ZnSO_4$• $7H_2O$, 0.39 g L^{-1} Na_2MoO_4• $2H_2O$, 79 μg L^{-1} $CuSO_4$• $5H_2O$, 49.4 μg L^{-1} $Co(NO_3)_2$• $6H_2O$) (Neidhardt et al. 1974), appropriate antibiotics, and supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For fed-batch cultivation, the production strain was first cultivated in batch mode, as described above, followed by three feeding phases, in each of which ~ 15 g L^{-1} glycerol/succinate/fumarate was supplemented for extended cultivation until complete carbon dissimilation. Microaerobic and semiaerobic conditions were maintained by purging air into the headspace and bulk culture, respectively, at either 0.1 or 0.5 vvm, designated as Microaerobic-0.1 (MA-0.1), Semiaerobic-0.1 (SA-0.1), and Semiaerobic-0.5 (SA-0.5). Aerobic conditions were maintained by sparging air into the bulk culture at 1 vvm (AE-1). The pH of the production culture was maintained at 7.0 ± 0.1 with 30% (v/v) NH_4OH and 15% (v/v) H_3PO_4.

Sample processing and analysis

Culture samples were appropriately diluted with 0.15 M saline solution for measuring cell density in OD_{600} using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA). Cell-free medium was prepared by centrifugation of the culture sample at 9000×*g* for 5 min, followed by filter sterilization using a 0.2 μm syringe filter. Extracellular metabolites and glycerol were quantified using high-performance liquid chromatography (HPLC) (LC-10AT, Shimadzu, Kyoto, Japan) with a refractive index detector (RID; RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA). The HPLC column temperature was maintained at 35 °C and the mobile phase was 5 mM H_2SO_4 (pH 2) running at 0.6 mL min^{-1}. The RID signal was acquired and processed by a data processing unit (Clarity Lite, DataApex, Prague, Czech Republic).

Intracellular polymer production was evaluated by gas chromatography (GC) as described by Braunegg et al. (1978). Briefly, culture samples harvested from the bioreactor cultivations were pelleted by centrifugation at 9000×*g* for 10 min, then washed twice with distilled water, and finally dried at 100 °C overnight. The dried cell weight (DCW) was recorded before biopolymer extraction with 1 mL chloroform treatment and filtering of residual biomass. The biopolymer content was then removed from the chloroform solution by evaporation and dried. Subsequently, 2 mL chloroform and 1 mL PHA solution containing 4 g L^{-1} benzoic acid (as an internal standard) and 15% sulfuric acid in methanol was added to dried biopolymer sample and methanolysis was carried out at 100 °C for 3 h. The reaction was then cooled to room temperature, and after the addition of 1 mL distilled water, the mixture was vortexed and allowed to separate into two phases. One microliter of the chloroform phase was injected into Agilent 6890 series GC system (Agilent Technologies, Santa Clara, CA, USA) with a J & W Scientifics DB Wax column (30 m × 0.53 mm, film thickness 1 μM) (Agilent Technologies, Santa Clara, CA, USA). The oven program was set as follows: the initial temperature was set at 80 °C for 5 min, then ramped to 230 °C at 7.5 °C/min, and continued to ramp to 260 °C at a faster rate 10 °C/min followed by maintaining that temperature for the analysis. Pure standards of methyl-3-hydroxybutyrate and methyl-3-hydroxyvalerate (Sigma-Aldrich Co., St Louis, MO, USA) were used to generate calibration curves for the methanolysis assay. The 3-HV fraction was defined as the ratio of 3-HV to 3-HV plus 3-HB in the copolymer, expressed in mole percent.

Results

PHBV biosynthesis under different oxygenic conditions

Major metabolic pathways involved in succinate/succinyl-CoA formation in *E. coli*, i.e., reductive TCA branch, oxidative TCA cycle, and glyoxylate shunt, are sensitive to the oxygenic condition and, therefore, can affect PHBV biosynthesis. To investigate the effects of oxygen exposure on PHBV biosynthesis, batch cultivation of the control strain PHBV-C (previously named as PHBV) (Srirangan et al. 2016) in a bioreactor was subject to four levels of aeration (from high to low), i.e., AE-1, SA-0.5, SA-0.1, and MA-0.1 (Fig. 2). While cell growth was favored under higher oxygen exposure, the 3-HV monomeric fraction increased with reduced aeration, i.e., 5.96, 9.32, 15.3, 18.5 mol% under AE-1, SA-0.5, SA-0.1, and MA-0.1, respectively. The results suggest that, with the genetic background of PHBV-C, more dissimilated carbon flux within the TCA cycle was directed into the Sbm pathway via the succinyl-CoA node to form propionyl-CoA and, then, 3-HV-CoA under microaerobic conditions. Note that, among the three metabolic pathways involved in the TCA cycle, only the reductive TCA branch is active under oxygenic limitation (Cheng et al. 2013). On the other hand, the results of superior cell growth and biopolymer synthesis with a lower 3-HV monomeric fraction suggest that a higher intracellular pool of acetyl-CoA existed with a higher oxygen exposure as aerobic conditions can normally favor acetyl-CoA formation via glycolysis and cellular respiration. These results also suggest the feasibility of modulating 3-HV monomeric fraction at the stage of microbial cultivation based on the intracellular distribution of dissimilated carbon flux toward either acetyl-CoA or propionyl-CoA.

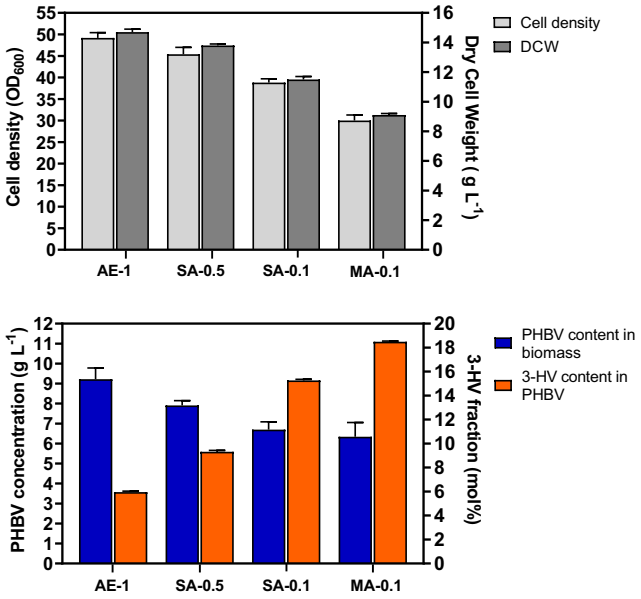

Fig. 2 Effects of varying aeration levels on copolymer characterization in PHBV-C. Results of batch fermentation, presenting cell biomass concentration (OD_{600} and DCW) (top panel) and biopolymer and 3-HV content (represented by mol%) (bottom panel). All values are reported as means ± SD ($n = 3$)

Modulation of 3-HV monomeric fraction under oxygenic limitation

The 3-HV monomeric fraction in PHBV-C increased with a lower culture aeration, but at the expense of reduced biomass and biopolymer production. To alleviate these culture impacts, we derived single mutants by manipulating two genes involved in the TCA cycle, i.e., PHBVΔ*sdhA* with an inactivated oxidative TCA cycle and PHBVΔ*iclR* with a deregulated glyoxylate shunt (Fig. 3). In addition to more than 50% increase in the final cell density for both PHBVΔ*sdhA* and PHBVΔ*iclR*, the total biopolymer titers were also increased by ~ 56%, compared to PHBV-C under MA-0.1. More importantly, PHBVΔ*sdhA* had a significant increase in the 3-HV monomeric fraction (25.1 vs. 18.5 mol%), whereas a slight reduction was observed for PHBVΔ*iclR* (17.0 vs. 18.5 mol%). The results suggest that the dissimilated carbon flux was directed into the Sbm pathway primarily through the reductive TCA branch under microaerobic conditions. To further verify this metabolic effect, we derived a double mutant of PHBVΔ*iclR*Δ*sdhA* such that the carbon flux channeling could be executed through both the reductive TCA branch and glyoxylate shunt. However, it was observed that the culture performance of PHBVΔ*iclR*Δ*sdhA* was almost identical to that of PHBVΔ*sdhA*, including the 3-HV monomeric fraction (24.6 vs. 25.1 mol%), implying that the above metabolic effect associated with the glyoxylate shunt was minimal under microaerobic conditions.

To further confirm metabolic activity of the reductive TCA branch described above, we conducted fed-batch cultivation of PHBVΔ*sdhA* under MA-0.1 by supplementing the culture with key metabolic intermediates involved in the reductive TCA branch, i.e., succinate and fumarate, and investigated their individual effects on PHBV biosynthesis during the feeding stages (Fig. 4). For comparison purposes, we performed three cycles of pulse-feeding, each one with ~ 15 g L^{-1} glycerol (as a control), succinate or fumarate, after ~ 30 g L^{-1} glycerol was nearly consumed during the initial batch cultivation. Extended cell growth and biopolymer production were observed in the feeding stages, particularly the first two, for all three fed-batch cultures. Importantly, compared to the glycerol fed-batch cultivation, the 3-HV monomeric fractions were significantly higher in the corresponding feeding stages when fumarate or succinate was supplemented, implying that their exogenous supply can enhance carbon flux channeling into the Sbm pathway. Note that the steady increase of succinate titer upon fumarate supplementation further suggests an active

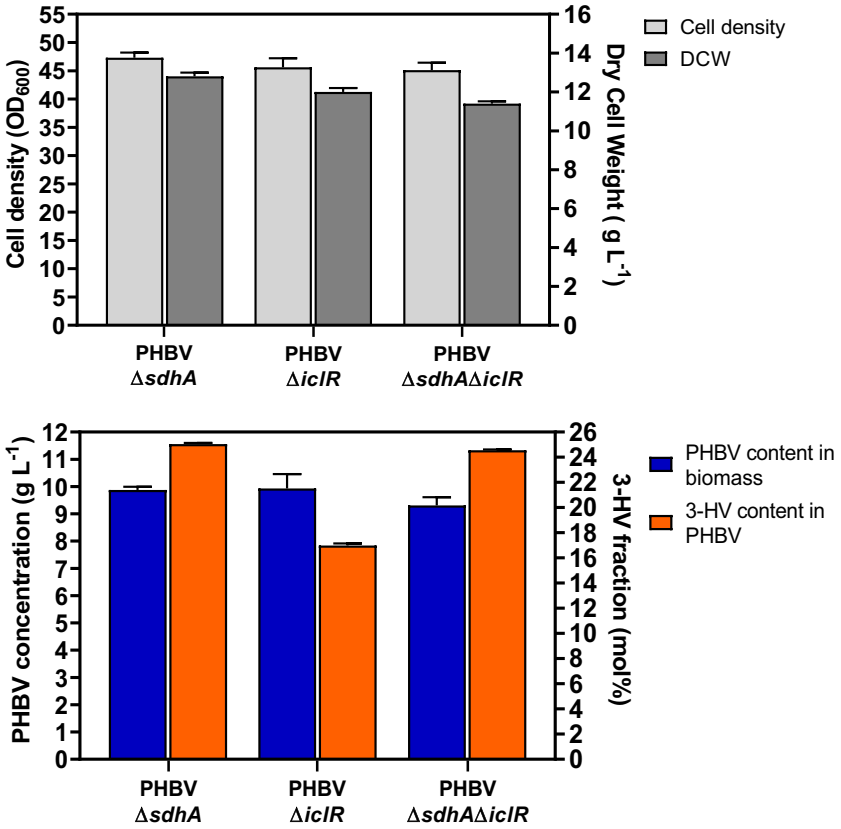

Fig. 3 Engineering of TCA cycle under MA-0.1 conditions. Strains compared include PHBVΔ*sdhA*, PHBVΔ*iclR*, and PHBVΔ*sdhA*Δ*iclR*. Results of batch fermentation, presenting cell biomass concentration (OD_{600} and DCW) (top panel) and biopolymer and 3-HV content (represented by mol%). All values are reported as means ± SD (n = 3)

fumarate-to-succinate conversion in the reductive TCA branch under oxygenic limitation.

Modulation of 3-HV monomeric fraction under high oxygen exposure

The current observation of minimal metabolic effects of Δ*iclR* on the 3-HV monomeric fraction was likely associated with a low oxygen exposure since the glyoxylate shunt is typically operational under aerobic conditions in *E. coli* (Cheng et al. 2013). Hence, we re-evaluated these effects by conducting three cultivations of PHBVΔ*sdhA*, PHBVΔ*iclR*, and PHBVΔ*sdhA*Δ*iclR* under aerobic conditions (Fig. 5). Compared to PHBV-C, mutating *sdhA* in PHBVΔ*sdhA* led to defective cell growth (32.1 vs. 49.2 OD_{600}) with a significant reduction in total biopolymer titer (6.23 vs. 9.22 g L^{-1}) under AE-1. On the other hand, inactivation of *iclR* in PHBVΔ*iclR* only led to slightly reduced cell growth (46.1 vs. 49.2 OD_{600}), but with a significant increase in total biopolymer titer (12.4 vs. 9.22 g L^{-1}) under AE-1. Most importantly, the 3-HV monomeric fraction was highly increased in PHBVΔ*iclR* under AE-1 (28.0 vs. 5.96 mol%) and this fraction level was comparable to those obtained (e.g., in PHBVΔ*sdhA*) under MA-0.1. These results indicate efficient redirection of dissimilated carbon flux into the Sbm pathway for propionyl-CoA/3-HV-CoA biosyntheses via the glyoxylate shunt under aerobic conditions.

To further enhance the carbon flux redirection under anaerobic conditions, we conducted cultivation of the double mutant PHBVΔ*sdhA*Δ*iclR* under AE-1. While both cell growth and total biopolymer titer were slightly impacted compared to either PHBV-C or PHBVΔ*iclR*, the 3-HV monomeric fraction reached to a high level at 40.9 mol%. The results also suggest the Δ*sdhA* mutation could prevent the carbon escape via the oxidative TCA cycle at the succinate node when glyoxylate shunt was active under aerobic conditions. This observation was further evidenced by much higher propionate production (up to 3.57 g L^{-1}) with no acetogenesis in PHBVΔ*sdhA*Δ*iclR*, signifying an increased carbon flux redirected into the Sbm pathway.

To explore the full capacity of PHBVΔ*sdhA*Δ*iclR* for PHBV biosynthesis, we further conducted fed-batch cultivation by extending the existing batch with three pulse feedings, each at 15 g L^{-1} glycerol, under AE-1 (Fig. 6). Basically, cells remained viable and metabolically active for biopolymer production in all three feeding stages, reflected by steady increases in biomass concentration and total biopolymer titer, achieving high levels of 97.6 OD_{600} and 22.5 g L^{-1}, respectively, at the end of the fed-batch cultivation. Note that, in the feeding 1 phase, carbon flux redirection into the Sbm pathway was even more effective than that in the initial batch phase, achieving a record high level of the 3-HV monomeric fraction at 48.8 mol%. However, in subsequent feeding 2 and feeding

3 phases, carbon flux redirection was less effective as the 3-HV monomeric fraction steadily decreased to 43.3 and 39.4 mol%, respectively, even though the supplemented glycerol was effectively used for cell growth and biopolymer production. Also, note that such reduced 3-HV monomeric fraction occurred simultaneously with both retarded propionate production and increased carbon spill in terms of acetate secretion, further confirming the less effective carbon flux redirection.

Discussion

In this study, we explored metabolic redirection of dissimilated carbon flux with adjusted culture aeration to modulate 3-HV monomeric fraction, which correlates copolymer properties, while enhancing cell growth and overall biopolymer production during microbial cultivation for PHBV biosynthesis. Theoretically, the 3-HV monomer fraction of PHBV synthesized in *E. coli* is determined by the relative intracellular abundance of two monomers, i.e., 3-HB-CoA and 3-HV-CoA, and, therefore, two key precursors, i.e., acetyl-CoA and propionyl-CoA. In the control strain PHBV-C, while cell growth and biopolymer production were favored by high oxygen exposure, the 3-HV monomeric fraction was increased with oxygenic limitation, an outcome also described previously in *C. necator* (Lefebvre et al. 1997). This finding suggests that dissimilated carbon flux within the TCA cycle was directed into the Sbm pathway primarily through the reductive TCA branch. In *E. coli*, succinate/succinyl-CoA, the central metabolites predating the Sbm pathway, can be derived via three oxygen-dependent pathways in the TCA cycle, i.e., (i) reductive TCA branch, (ii) oxidative TCA cycle, and (iii) glyoxylate shunt (Fig. 1) (Cheng et al. 2013). Under low oxygenic conditions, succinate is synthesized as an end-product of mixed acid fermentation via the reductive TCA branch (Thakker et al. 2012), which is often metabolically unfavorable due to the restricted availability of reducing equivalents (Skorokhodova et al. 2015). Under high oxygenic conditions, succinate does not normally accumulate as it is readily consumed in the active oxidative TCA cycle, except for the conditions of oxidative stress and/or acetate/fatty-acid consumption under which succinate can be aerobically derived via the operational glyoxylate shunt (Thakker et al. 2012). Hence, selected genes involved in the TCA cycle were manipulated for regulation of carbon flux channeling into the Sbm

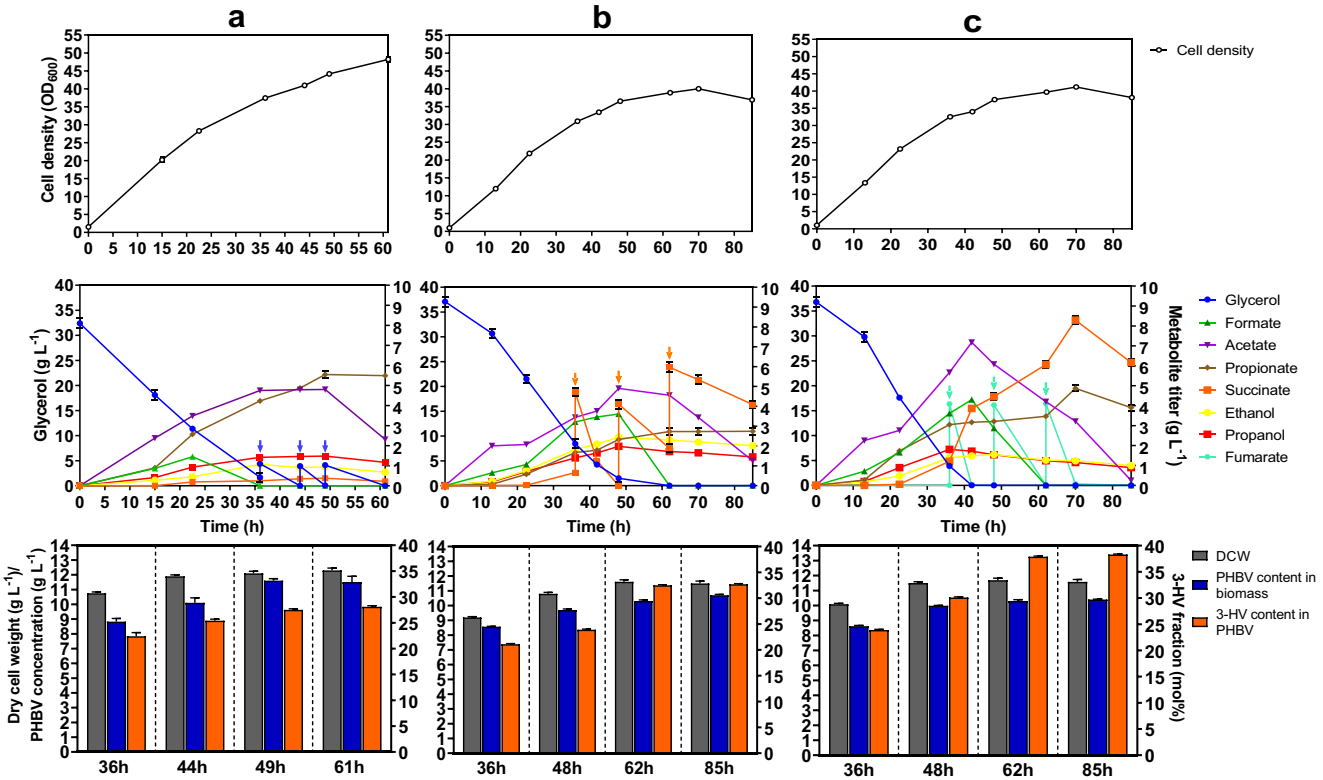

Fig. 4 Physiological effects of succinate and fumarate supplementations on biosynthesis of copolymer in PHBVΔ*sdhA* under MA-0.1 conditions. Time profiles of **a** glycerol, **b** succinate, and **c** fumarate supplementations, in which each addition is represented by a blue-, orange-, and aqua-colored arrow, respectively. Results of fed-batch fermentation, presenting cell density (OD_{600}) (top panel), glycerol consumption, and metabolite production (middle panel), and dry cell weight, biopolymer, and 3-HV content (represented by mol%) (bottom panel). Dotted vertical lines in the bottom panel separate batch, feeding 1, feeding 2, and feeding 3 stages of fermentation. All values are reported as means ± SD (n = 3)

pathway, and thereby, relative abundance of acetyl-CoA and propionyl-CoA, and finally, 3-HV monomeric fraction.

The contrasting oxygenic conditions associated with culture performance (in terms of cell growth and overall PHBV production) and 3-HV monomeric fraction in PHBV-C could be partially resolved by blocking the oxidative TCA cycle in PHBVΔ*sdhA*. While still channeling dissimilated carbon flux into the Sbm pathway via the reductive TCA branch to achieve a much higher 3-HV monomeric fraction than PHBV-C under MA-0.1 (25.1 vs. 18.5 mol%), the arrested cell growth and biopolymer production observed in PHBV-C were lifted in this mutant. These results also suggest that the contribution from the oxidative TCA cycle for carbon flux channeling into the Sbm pathway was minimal, a claim that is further corroborated by an observed increase in the 3-HV monomeric fraction upon exogenous supplementation of succinate and fumarate in PHBVΔ*sdhA* under MA-0.1. The supplemented succinate and fumarate appeared to be readily converted to succinyl-CoA via the active reductive TCA branch, resulting in an increased carbon flux into the Sbm pathway for biosynthesis of propionyl-CoA and 3-HV-CoA. While mutating *iclR* to deregulate glyoxylate shunt in PHBVΔ*iclR* also lifted the arrested cell growth and biopolymer production observed in PHBV-C under MA-0.1, the 3-HV monomeric fraction remained relatively unchanged (17.0 vs. 18.5 mol%), suggesting that the glyoxylate shunt minimally contributed carbon flux channeling into the Sbm pathway under low oxygen exposure. These observations are further corroborated by the similar culture performance (in terms of cell growth and overall biopolymer production) and 3-HV monomeric fraction between the two cultures of PHBVΔ*sdhA*Δ*iclR* and PHBVΔ*sdhA* under MA-0.1.

Given that the glyoxylate shunt is normally functional under aerobic conditions, we also explored this metabolic route for carbon flux redirection from the TCA cycle into the Sbm pathway to further increase propionyl-CoA pool and, then, 3-HV monomeric fraction. With the deregulated glyoxylate shunt in PHBVΔ*iclR* under AE-1, we obtained a relatively high 3-HV monomeric fraction at 28.0 mol% with active cell growth and biopolymer production, suggesting successful carbon flux redirection. To enhance such carbon flux redirection, we further inactivated the oxidative TCA cycle to eliminate carbon flux split at the succinate node (to either the oxidative or reductive TCA direction) such that all the carbon flux was redirected from succinate to succinyl-CoA and, then, into the Sbm pathway, achieving a high 3-HV monomeric fraction at 40.9 mol% in PHBVΔ*sdhA*Δ*iclR* under AE-1 even though cell growth and biopolymer production were somewhat arrested.

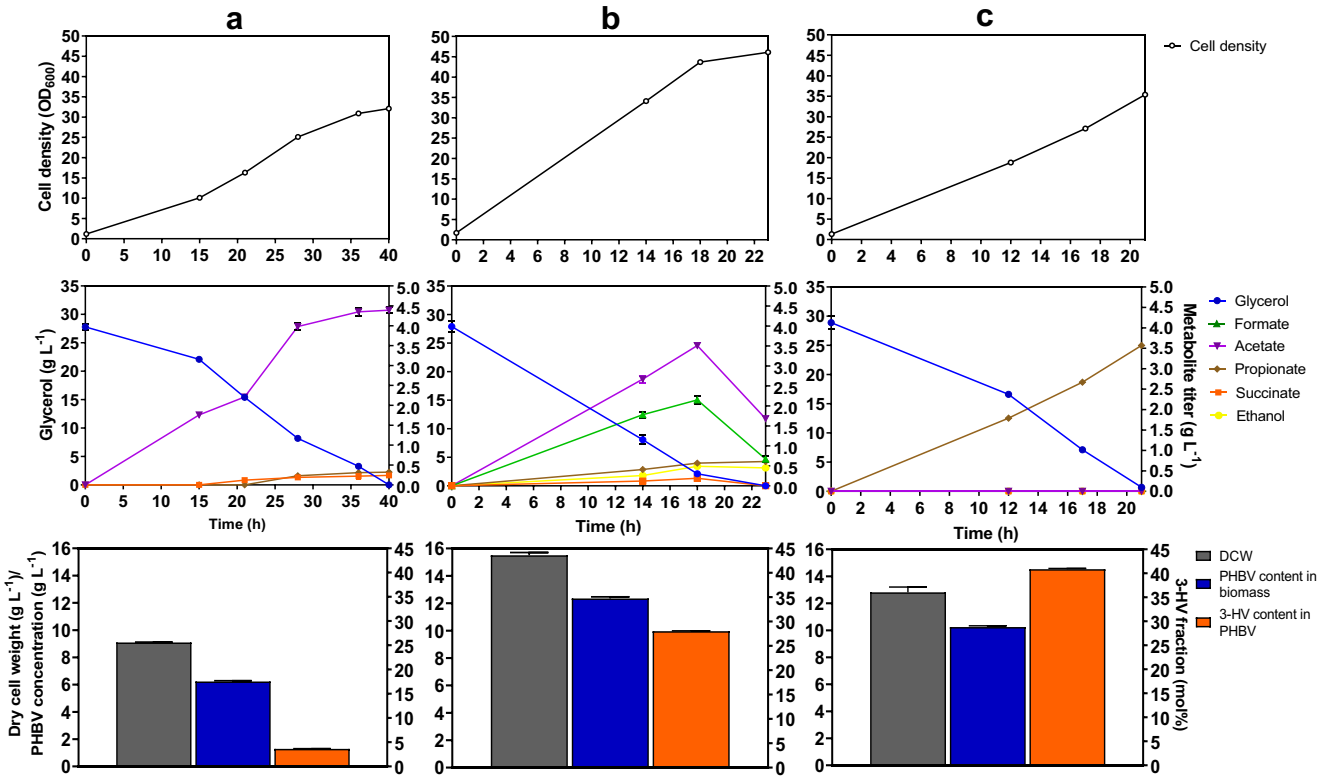

Fig. 5 Comparing the effects of redirecting carbon flux in TCA cycle for copolymer characterization under AE-1 conditions. Strains compared include **a** PHBVΔ*sdhA*, **b** PHBVΔ*iclR*, and **c** PHBVΔ*sdhA*Δ*iclR*. Results of batch fermentation, presenting cell density (OD_{600}) (top panel), glycerol consumption, and metabolite production (middle panel), and dry cell weight, biopolymer, and 3-HV content (represented as mol%) (bottom panel). All values are reported as means ± SD ($n = 3$)

The physiological impacts of inactivating the oxidative TCA cycle could be clearly observed in PHBVΔ*sdhA* with significantly retarded cell growth and biopolymer production and a low 3-HV monomer fraction under AE-1. These results suggest critical metabolic roles of the deregulated glyoxylate shunt for carbon flux redirection into the Sbm pathway, similar to our recent reports for the production of several metabolites derived from propionyl-CoA (Miscevic et al. 2019, 2020a, b). Finally, note that there was hardly any secretion of major metabolites, particularly acetate, other than propionate in PHBVΔ*sdhA*Δ*iclR* under AE-1, suggesting rather limited formation of acetyl-CoA compared to propionyl-CoA under such culture conditions. It was reported that increasing culture aerobicity can potentially limit acetate secretion in *E. coli* via recycling of accumulated acetyl-CoA through enhanced glyoxylate shunt, which involves acetyl-CoA as a co-substrate and is typically active under aerobic conditions (Ahn et al. 2016; Renilla et al. 2012).

Using the double mutant PHBVΔ*sdhA*Δ*iclR* for fed-batch cultivation, we further demonstrated its capacity for PHBV biosynthesis with high-level 3-HV-CoA incorporation under aerobic conditions. In the feeding 1 stage, cell growth and biopolymer production were continually effective with minimal carbon spill, achieving a record high 3-HV monomeric fraction at 48.8 mol%, which is among the highest levels reported so far for microbial production of PHBV from unrelated carbons (Moorkoth and Nampoothiri 2016; Urtuvia et al. 2020; Wang et al. 2013). However, the 3-HV monomeric fraction slightly decreased in the feeding 2 and 3 phases with an onset of acetate secretion being observed toward the late feeding 1 phase. This observed carbon spill in terms of acetogenesis was likely associated with the oxygenic limitation in such dense and viscous culture. While 3-HV-CoA incorporation into PHBV was somewhat limited in the feeding 2 and 3 stages, cells continued to grow with high-level biopolymer production, achieving a final cell density at 97.6 OD_{600}

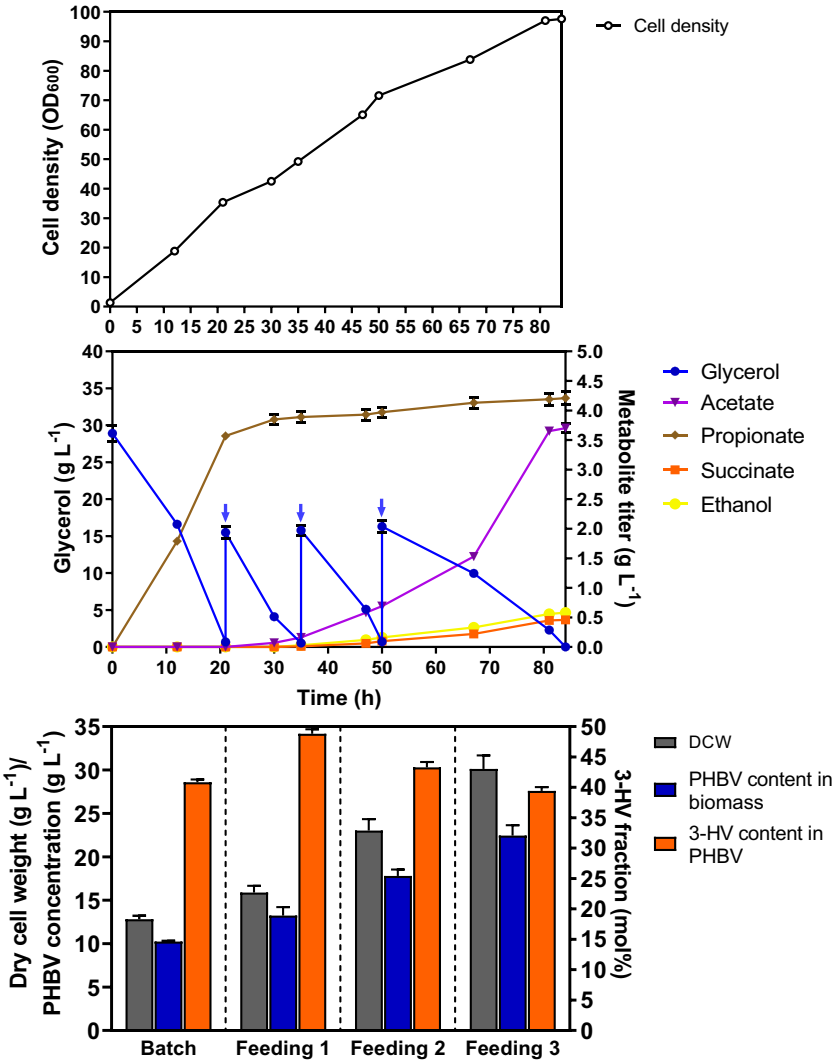

Fig. 6 Fed-batch cultivation of PHBVΔ*sdhA*Δ*iclR* under extended AE-1 conditions. Results of fed-batch fermentation, presenting cell density (OD_{600}) (top panel), glycerol consumption, and metabolite production (middle panel), and dry cell weight, biopolymer, and 3-HV content (represented as mol%) (bottom panel). Blue arrows indicate points of glycerol supplementation. Dotted vertical lines in the bottom panel separate batch, feeding 1, feeding 2, and feeding 3 stages of fermentation. All values are reported as means ± SD ($n = 3$)

with a total biopolymer titer at 22.5 g L^{-1} and a relatively high 3-HV monomeric fraction at 39.4 mol% toward the end of the fed-batch cultivation. Given the effective carbon flux redirection demonstrated herein, we envision that similar metabolic engineering and bioprocessing strategies can be applied for bio-based production of chemicals derived from succinyl-CoA.

Authors' contributions D.M. conceived the study, formulated research plan, coordinated research team, carried out experiments, performed result interpretation and data analysis, and drafted the manuscript. J.M., B.M., and K.S. helped to carry out experiments and data analysis. M.M.Y., D.A., and C.P.C. conceived, planned, supervised, and managed the study, as well as helped to draft the manuscript. All authors read and approved the final manuscript.

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Data availability All experimental data in this study will be made available upon reasonable request from readers.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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