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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HB-*co*-HHx)) from butyrate using engineered *Ralstonia eutropha*

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Abstract Polyhydroxyalkanoates (PHAs), a promising family of bio-based polymers, are considered to be alternatives to traditional petroleum-based plastics. Copolymers like poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HB-*co*-HHx)) have been shown to exhibit favorable physical and mechanical properties, due to decreased crystallinity resulting from the presence of medium-chain-length 3-hydroxyhexanoate (3HHx) monomers. In this study, we produced P(HB-*co*-HHx) using engineered *Ralstonia eutropha* strains containing deletions of the acetoacetyl-CoA reductase (*phaB*) genes and replacing the native PHA synthase with *phaC2* from *Rhodococcus aetherivorans* I24 and by using butyrate, a short-chain organic acid, as the carbon source. Although the wild-type *R. eutropha* did not produce P(HB-*co*-HHx) when grown on mixed acids or on butyrate as the sole carbon source, we are able to produce polymer containing up to 40 wt% 3HHx monomer with the aforementioned engineered *R. eutropha* strains using various concentrations of just butyrate as the sole carbon source. This is the first report for the production of P(HB-*co*-HHx) copolymer in *R. eutropha* using butyrate.

Keywords Polyhydroxyalkanoate · Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) · Butyrate · Volatile organic acids · *Ralstonia eutropha*

Introduction

The polyhydroxyalkanoate (PHA) family of bio-based, biodegradable polymers is a promising next-generation product that can potentially substitute for petroleum-based plastics and

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is synthesized by many living microorganisms as part of their natural metabolism (Madkour et al. 2013; Steinbuchel and Fuchtenbusch 1998). PHA has many advantages, such as biodegradability and biocompatibility, compared to petroleum-based plastics while exhibiting thermal and mechanical properties similar to existing plastics (Sudesh et al. 2000). As a result, PHA can be used as a raw material to manufacture many different plastic products including biomedical devices, drink bottles, food packaging, etc. (Poirier et al. 1995). Many companies, such as Imperial Chemical Industries, Dupont, ADM, Biomer, DSM, Jiangsu TianAn, Meridian, Metabolix, PHB Industrial, and Tianjin Green Bio-Science, have established pilot or industrial-scale processes to produce PHAs, expecting to achieve global-scale commercialization in the near future (Chen 2009; Verlinden et al. 2007).

There are two major pathways by which organisms typically make specific PHA monomers. The first starts from acetyl-CoA, adding a carbon chain (C_2 or C_3) to the backbone and reducing it, thus resulting in a C_4 (3-hydroxybutyryl-CoA) or a C_5 (3-hydroxyvaleryl-CoA) precursor for polymerization. These are both short-chain-length (*scl*) precursors. PHA produced in this manner is synthesized by the enzymes PhaA or BktB (β-ketothiolases), PhaB (acetoacetyl-CoA reductase), and PhaC (PHA synthase) from various organisms, such as *Vibrio*, *Caulobacter*, *Ralstonia*, and engineered *Escherichia* (Fuchtenbusch et al. 2000; Lee et al. 1994). The second precursor generation pathway starts from fatty acid β-oxidation or fatty acid biosynthesis intermediates and results in PHA precursors, considered medium-chain-length (*mcl*), converted from enoyl-CoA or 3-hydroxyacyl-ACP, respectively. These mcl PHA precursors produced using the second supply pathway are synthesized by PhaJ ((*R*)-specific enoyl coenzyme-A hydratase) or PhaG (3-hydroxyacyl-ACP:CoA transferase), and PhaC (PHA synthase) through fatty acid metabolism in organisms like *Pseudomonas* (Dellomonaco et al. 2011; Fiedler et al. 2002; Lu et al. 2003; Park et al. 2012; Rehm et al. 1998). In most cases, polymers containing C_4–C_5 monomers, such as 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), are made by the first pathway using various sugars, propionate, or valerate as carbon sources. Polymers containing monomers greater than C_6 are usually made by the second pathway (Aldor and Keasling 2003). As a result, production of C_6 units typically requires hexanoate, octanoate, or longer-chain-length fatty acids (Bhubalan et al. 2008; Chen et al. 2001; Doi et al. 1995). When short-chain precursors or substrates such as acetate, propionate, and butyrate were used for PHA production in *Ralstonia eutropha* H16, it resulted in the biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-*co*-HV)), and butyrate (C_4) was not used in *R. eutropha* H16 for the production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HB-*co*-HHx)) (Yang et al. 2010).

Among various PHAs, (P(HB-*co*-HHx)) is a promising candidate polymer for use in the biomedical field because of its superior biocompatibility, biodegradability, and mechanical properties (Misra et al. 2006; Williams et al. 1999; Wu et al. 2009). As a result, many studies have focused on exploiting microbial fatty acid degradation pathways in order to produce P(HB-*co*-HHx) (Budde et al. 2011a, b; Khanna and Srivastava 2005). Indeed, previous reports have shown P(HB-*co*-HHx), containing greater than 20 % content of 3-hydroxyhexanoate (3HHx) monomer, can be produced using plant oils as the sole carbon source (Budde et al. 2011b; Kahara et al. 2004; Riedel et al. 2012). Although its biosynthetic pathway from butyrate through butyryl-CoA, 3-ketohexanoyl-CoA, and 3-hydroxyhexanoyl CoA to PHA can be predicted in butyrate metabolism (Fig. 1), there is no report on anabolism of 3HHx-CoA and production of P(HB-*co*-HHx) using butyrate in *R. eutropha*.

In this study, we demonstrate that engineered *R. eutropha* can produce P(HB-*co*-HHx) from the volatile organic acid, butyrate, which is prepared as the end product of anaerobic fermentation of organic materials by obligate anaerobic bacteria, such as *Clostridium* and *Fusobacterium* (Lehmann et al. 2012; Potrykus et al. 2007). We had previously demonstrated that *R. eutropha* prefers butyrate to other volatile acids, such as acetate and propionate (Yang et al. 2010). This was made possible by a combination of a marked decrease in the major precursor supply pathway from acetoacetyl-CoA to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB) along with the expression of *phaC2*, a heterologous, broad-specificity PHA synthase gene originating from *Rhodococcus aetherivorans* I24 (Budde et al. 2010).

Materials and methods

Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* H16 (Wilde 1962) and its derivatives were precultured in 3 mL of tryptic soy broth (TSB) supplemented with 10 μg/mL gentamicin for 24 h at 30 °C. The cells were harvested and washed twice with sterilized water and then used to inoculate 5 mL of polyhydroxybutyrate (PHB) minimal media. The PHB minimal medium was prepared as previously described (York et al. 2003). Cell growth was monitored by measuring optical density at 600 nm (OD_{600}), starting from an initial value of 0.05. For cell culture media containing high phosphate buffer (10×), we used 67 mM of Na_2HPO_4, 62.5 mM of NaH_2PO_4, 26 mM of K_2SO_4, 10 mM of NaOH with 0.3 % (*w*/*v*) of organic acids and 0.01 % (*w*/*v*) of NH_4Cl. Cells used for growth experiments were grown initially in 5 mL PHB minimal media in a test tube with different concentrations of butyrate as the main carbon source

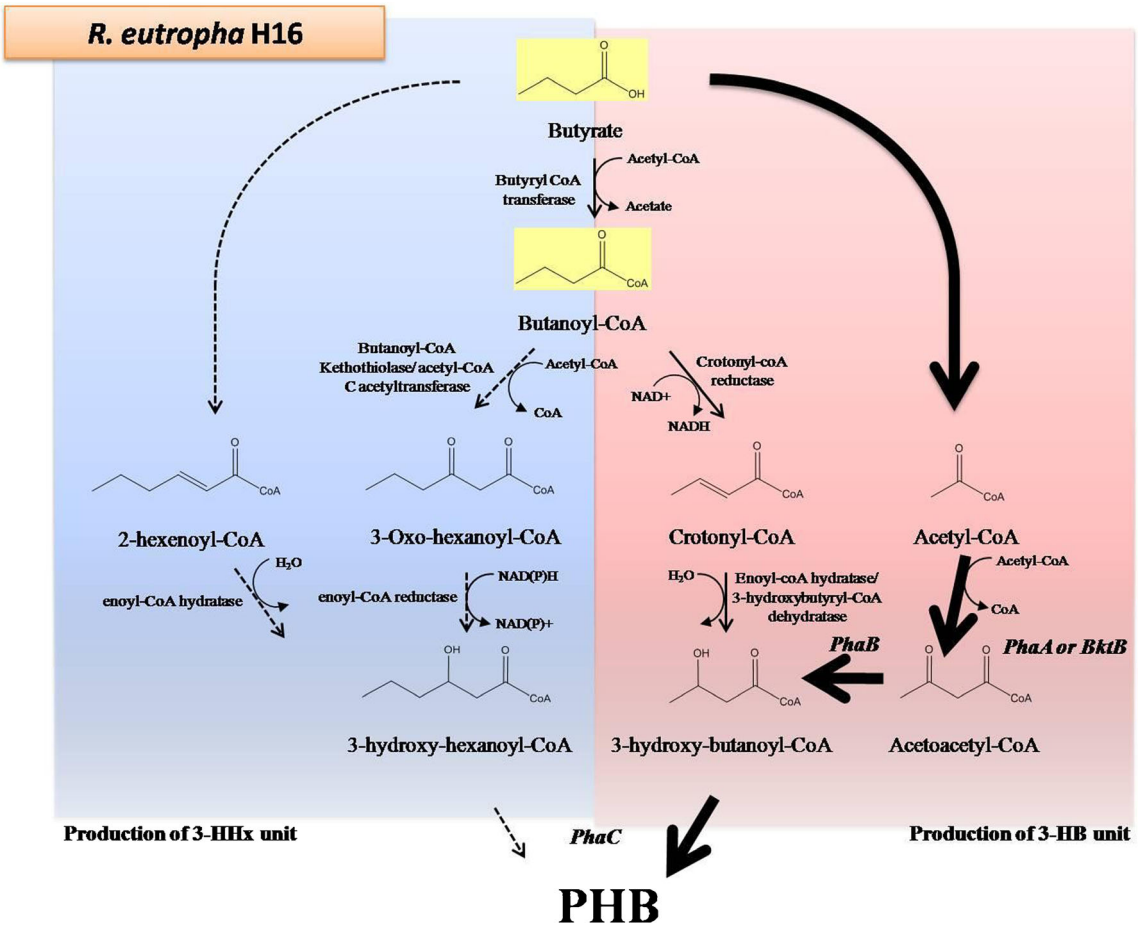

Fig. 1 Putative scheme of butyrate utilization for PHB production in *R. eutropha* H16. The pathway involving PhaA (or BktB) and PhaB is regarded as the main pathway for the biosynthesis of PHB homopolymer (*bold arrows*) (Dellomonaco et al. 2011)

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
R. eutropha strains		
H16	Wild-type strain, Gm resistant	ATCC17699
Re2115	H16 Δ*phaB1* Δ*phaB2* Δ*phaB3*	Budde et al. (2010)
Re1031	Re1034 expressing *Allochromatium vinosum phaEC*	York et al. (2001)
Re2000	Re1034::$phaC1_{Ra}$, made with pINY3	Budde et al. (2011b)
Re2001	Re1034::$phaC2_{Ra}$, made with pINY4	Budde et al. (2011b)
Re2133	Re2115 Δ*phaC1*, made with pGY46	Budde et al. (2011b)
Re2135	Re2133::$phaC2_{Ra}$, made with pINY4	Budde et al. (2011b)
E. coli strains		
DH5α	General cloning strain	Invitrogen
Plasmids		
pBBR1MCS-2	Vector for plasmid-based gene expression in *R. eutropha*, confers Km resistance	Kovach et al. (1995)
pCB81	pBBR1MCS-2 with the PHA operon from Re2152 cloned between *Kpn*I and *Hin*dIII sites	Budde et al. (2011b)
pJV7	pGY46 with *Swa*I site between regions of DNA upstream and downstream of *phaC1*, used to insert new genes at the *phaC1* locus	Budde et al. (2011b)
pINY4	pJV7 with $phaC2_{Ra}$ cloned into *Swa*I site	Budde et al. (2011b)

for 48 h by a roller drum or shaker. For 50-mL volume experiments with high phosphate media containing 0.1–0.5 % butyrate (Yang et al. 2010), 1 mL of cells from an overnight culture grown in TSB medium, supplemented with 10 μg/mL gentamicin, was used to inoculate 50 mL of high-phosphate minimal media containing 0.1–0.5 % butyrate in 250-mL flasks and then grown for 48 h at 30 °C. The cells were harvested, washed twice with cold water, and lyophilized with a FreeZone 4.5 (Labconco Corp., MO, USA) for 48 h.

Analytical methods

PHA quantity and composition were determined by gas chromatography using a slight modification of a method described previously (Braunegg et al. 1978). Approximately 10 mg of freeze-dried cells from each experiment was weighed and placed in Teflon-stoppered glass vials, and methanolysis of PHA samples was performed as described previously (Yang et al. 2010). These samples were then injected into a gas chromatograph (Young-lin, Seoul, South Korea) equipped with a fused silica capillary column (Agilent HP-FFAP, 30 m×0.32 mm, i.d. 0.25 μm film) with hydrogen as the carrier gas. A 1-μL portion of the organic phase was injected using an autosampler. The inlet was maintained at 250 °C. The oven was held at 80 °C for 5 min, heated to 220 °C at 20 °C/min, and then held at 220 °C for 5 min. Peak detection was performed by a flame ionization detector, which was maintained at 300 °C. Butyrate and other organic compounds present in the growth media were monitored by high-performance liquid chromatography (HPLC, Young-lin, Seoul, South Korea) isocratically using an Aminex HPX-87H column (Bio-Rad, CA, USA) at 60 °C with a diode array detector at a wavelength of 210 nm using 0.008 N sulfuric acid as the mobile phase with a flow rate of 0.6 mL/min, as described (York et al. 2003).

Mechanical properties

To obtain P(HB-*co*-HHx) film, Re2133/pCB81 cells grown in a 3-L volume of high-phosphate minimal media liquid cultures were collected by centrifugation (5,000×*g* for 6 min) and washed twice with 100 % ethanol and distilled water, respectively. After lyophilization overnight, intracellular PHA polymers were extracted from dried cells using methyl isobutyl ketone (MIBK), at a cell dry weight (CDW)/MIBK ratio of 1 g/66 mL, at 60 °C for 4 h. After cooling to room temperature, the PHA was filtered through a Whatman no. 1 paper filter to remove any residual cell debris; then, the polymer dissolved in MIBK was precipitated with 10-fold volume of ice-cold ethanol. The polymer solution was then spread on the glass as a thin film, and the solvent was evaporated at room temperature for 3 h. Prepared samples were cut into dumbbell shapes with a width of 7 mm and a thickness of approximately 158 μm. Prior to mechanical property studies, the samples were maintained at the room temperature for 1 week to allow stable crystallization. Tensile mechanical properties of the polymer were studied using ASTM D638 at room temperature at a speed of 10 mm/min.

Results

Examination of *R. eutropha* strains producing 3HV and 3HHx from volatile organic acids

Initially, we examined growth and PHA production of different engineered *R. eutropha* strains using an organic acid mixture, similar to that obtained from treated palm oil mill effluent (POME), containing a ratio of acetate, propionate, and butyrate (Yang et al. 2010). In most cases, P(HB-*co*-HV) was produced, because propionate is a precursor of the 3HV monomer in P(HB-*co*-HV), although the percentage of 3HV in the resulting

Table 2 PHA production by different *R. eutropha* strains

Strain	CDW (g/L)	% PHAs in the cell (% of CDW)	% 3HB (wt%)	% 3HV (wt%)	% 3HHx (wt%)
H16	1.05±0.04	64.0±0.8	89.7±0.2	10.3±± 0.2	N.D.
Re1031	0.76±0.01	66.4±0.3	92.7±0.3	7.3±0.3	N.D.
Re2000	0.79±0.04	65.9±6.4	87.4±0.4	12.6±0.4	N.D.
Re2001	0.17±0.01	17.7±0.7	100.0±2.4	0.0±2.4	N.D.
Re2115	0.53±0.00	10.2±0.0	73.7±0.0	26.3±0.0	N.D.
Re2135	0.10±0.01	4±0.1	71±0.4	16.5±0.4	12.5±0.4
Re2133/pCB81	0.17±0.01	12.5±0.2	82.5±0.3	11±0.2	6.5±0.1

All strains were cultured in minimal media containing 0.3 % mixed acids composed of acetate, propionate, and butyrate (3:1:1) for 48 h at 30 °C (Yang et al. 2010)

CDW cell dry weight, *N.D.* not detected

copolymers was different, depending on the strains used (Table 2). The 3HHx monomer was not detected in PHA produced from most strains grown with mixed organic acids as the main carbon source. Although butyrate has been regarded as a potential precursor of 3HHx monomer (Park et al. 2012), no production of P(HB-*co*-HHx) from butyrate was observed. This is an interesting observation, especially considering that Re2001, containing the PHA synthase gene from *R. aetherivorans* strain I24 ($phaC2_{Re}$) (Budde et al. 2010), demonstrated the ability to polymerize 3-hydroxyhexanoyl-CoA precursors, resulting in the biosynthesis of P(HB-*co*-HHx) when cells were grown on palm oil as the sole carbon source (Budde et al. 2011b). *R. eutropha* strain Re2001 was expected to produce P(HB-*co*-HHx) or poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) terpolymer (P(HB-*co*-HV-*co*-HHx)) when grown in the presence of butyrate. However, no production of P(HB-*co*-HHx) or P(HB-*co*-HV-*co*-HHx) was detected using Re2001 cells when grown on mixed acids (Table 2).

We discovered that strains with deletions of the acetoacetyl-CoA reductase genes *phaB1*, *phaB2*, and *phaB3*, and also containing the $phaC2_{Re}$ gene, did produce P(HB-*co*-HV-*co*-HHx) terpolymer. As shown in Table 2, both strain Re2135, containing $phaC2_{Re}$ but with gene deletions in *phaB1*, *phaB2*, and *phaB3*, as well as strain Re2133/pCB81, containing *phaJ* from *Pseudomonas* and $phaC2_{Re}$ but also containing deletions of *phaB1*, *phaB2*, and *phaB3* genes, produced P(HB-*co*-HV-*co*-HHx). Strain Re2115, without *phaB1*, *phaB2*, and *phaB3*, could produce P(HB-*co*-HV), but notably not P(HB-*co*-HV-*co*-HHx), suggesting that $phaC2_{Re}$ is important for incorporation of 3HHx monomer. These data suggest that the deletion of acetoacetyl-CoA reductase genes is important for de novo biosynthesis of 3HHx monomer from butyrate for incorporation into PHA by broad-substrate specificity PHA synthases like $PhaC2_{Re}$.

3HHx production is dependent on butyrate concentration

To confirm the production of P(HB-*co*-HHx) from butyrate and to compare the effect on P(HB-*co*-HHx) production using different amounts of butyrate in culture media, measurements of growth, PHA content, and 3HHx content of PHA in strains H16 and Re2133/pCB81 were taken in cultures with different concentrations of butyrate as the sole carbon source. Compared with wild-type H16, the strain Re2133/pCB81 showed less robust growth (Fig. 2a) and produced a lower amount of PHA (69.3 wt%, Fig. 2b). However, Re2133/pCB81 produced P(HB-*co*-HHx) copolymer containing a surprising 40 wt% of 3HHx, whereas H16 produced only PHB homopolymer, containing no 3HHx monomer (Fig. 2c). Use of butyrate in the PHA production media, in the absence of other volatile acids like acetate and propionate, resulted in the biosynthesis of P(HB-*co*-HHx) by Re2133/pCB81 and

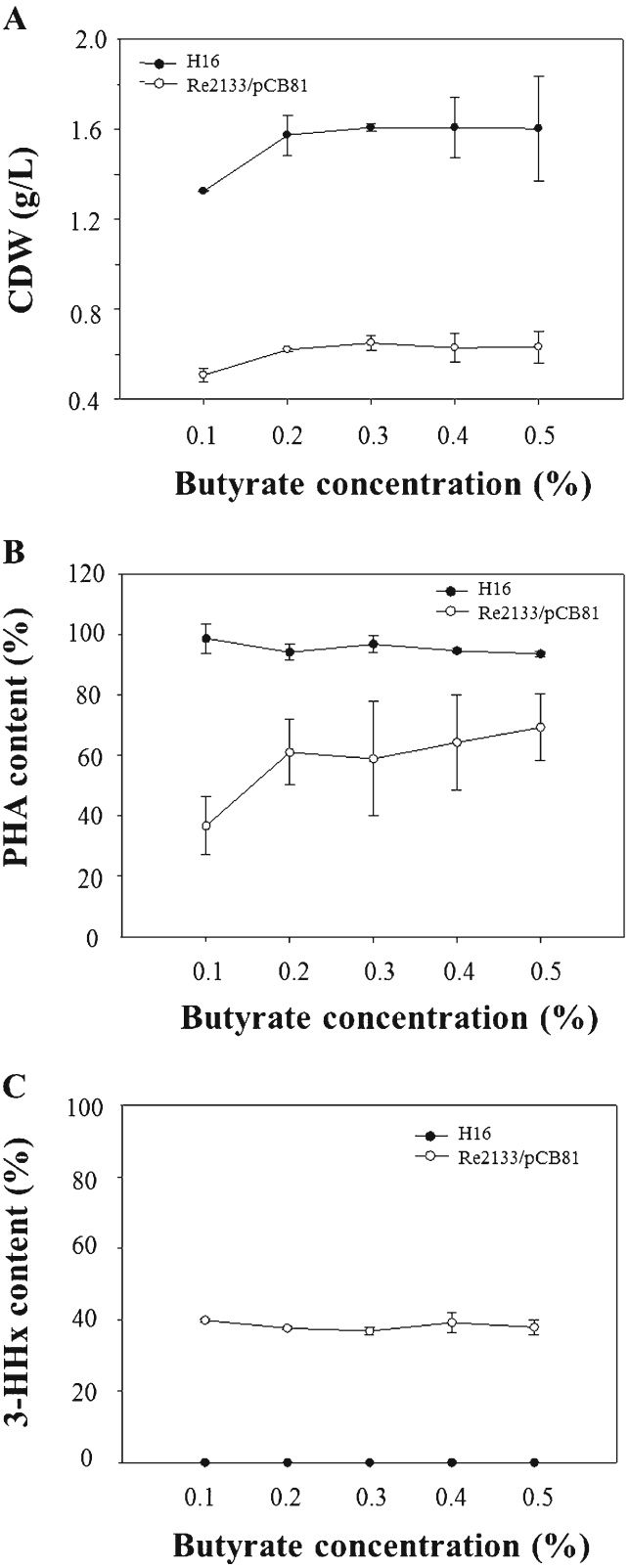

Fig. 2 Cell growth (**a**), PHA content (**b**), and 3HHx composition of PHA (**c**) of *R. eutropha* strain H16 and Re2133/pCB81 in minimal medium cultures containing varying concentrations of butyrate. Cultures were grown for 72 h at 30 °C

exhibited a higher 3HHx content, between 20 and 40 wt%, depending on culture time and volume. Deletion of acetoacetyl-CoA reductases and replacement of the native PHA synthase with a broad-specificity synthase resulted in lower amounts of final biomass and PHA content. However, these genetic modifications clearly established the incorporation of 3HHx, resulting in P(HB-*co*-HHx) copolymer production when butyrate was the sole carbon source present in the media.

Production of P(HB-*co*-HHx) over time from butyrate as the main carbon source

Production of P(HB-*co*-HHx) was monitored at different time points using 0.3 % (*w*/*v*) butyrate as the sole carbon source in 250-mL flasks. When 0.01 % (*w*/*v*) of NH_4Cl was used, PHA production was detected at 24 h, with *R. eutropha* H16 accumulating up to 80 wt% PHA and Re2133/pCB81 accumulating 20 wt% PHA (Fig. 3a, b). When the cells were grown for 48 h, PHA content reached up to 90 % with H16 and 60 % with Re2133/pCB81. Strain H16 produced only PHB, whereas Re2133/pCB81 synthesized P(HB-*co*-HHx) containing about 20 wt% of 3HHx (Fig. 3c). The pattern of butyrate consumption showed slight differences between the two strains, although total butyrate consumption was observed to be similar (Fig. 3d).

H16 and Re2133/pCB81 both produced fumarate and an unidentified organic acid (retention time is 25 min) during cultivation, but only Re2133/pCB81 produced glycoxylic acid and acetate by-products, as monitored by HPLC (Fig. S1). When PHA production on fructose was compared with that on butyrate, Re2133/pCB81 could only produce PHB with fructose as the main carbon source (data not shown). This suggests that fatty acid metabolism is not involved in facilitating PHA production using fructose as the main carbon source, since P(HB-*co*-HHx) is not synthesized. Also, butyrate appears to be used as a direct precursor to produce P(HB-*co*-HHx) through an as-yet unidentified ketothiolase, an acetyl-CoA C acetyltransferase enzyme, or a reductase, as well as the heterologously expressed PHA synthase.

Mechanical properties of P(HB-*co*-HHx) produced from butyrate

When purified P(HB-*co*-HHx) polymer, containing 42 wt% 3HHx (see Fig. 2), was examined for its mechanical properties, the Young's modulus, tensile strength, and elongation to

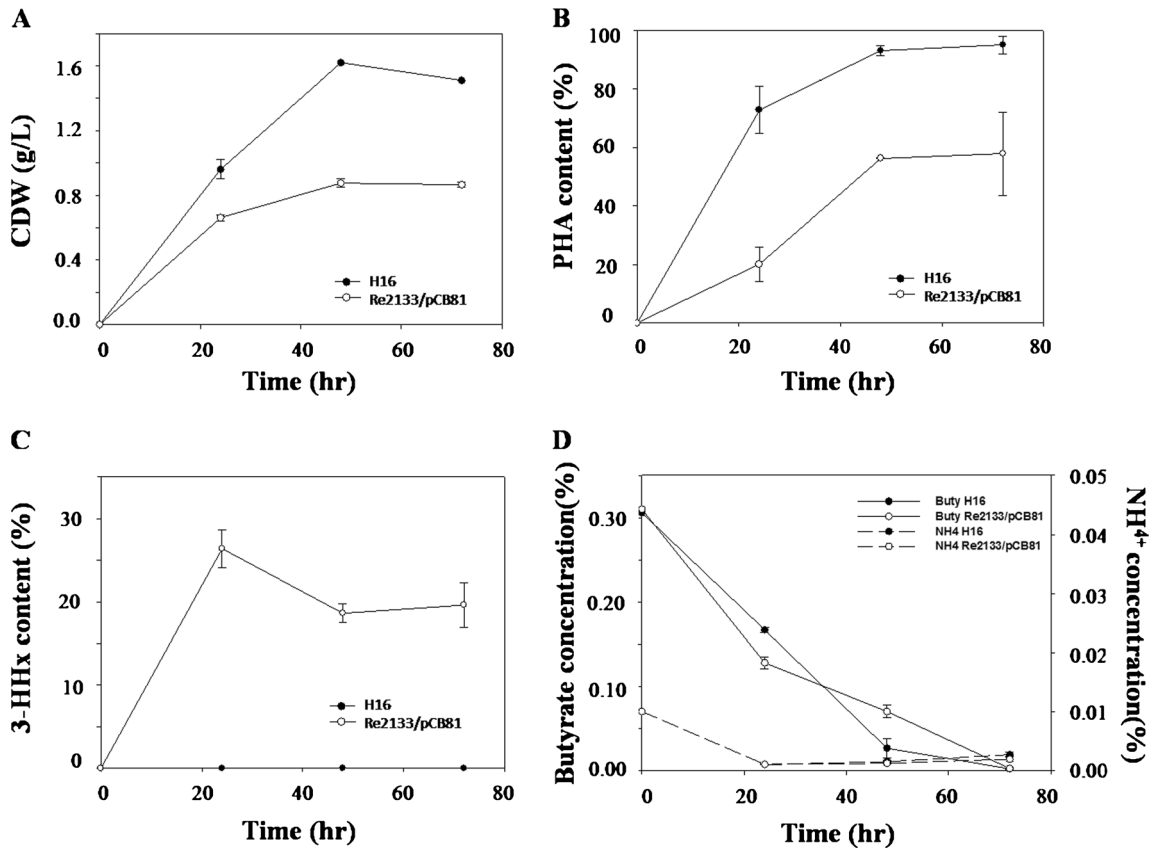

Fig. 3 Growth (**a**), PHA content (**b**), 3HHx composition of PHA (**c**), and butyrate and NH_4^+ concentrations in the culture media (**d**) of *R. eutropha* strains H16 and Re2133/pCB81 at different time points. Cultures were grown for 72 h at 30 °C

break were measured to be 0.2 GPa, 72 MPa, and 690 %, respectively (Fig. S2). Considering that the Young's modulus, tensile strength, and elongation to break low-density polyethylene are 0.2 GPa, 10 MPa, and 620 %, respectively, the mechanical properties of the P(HB-*co*-HHx) obtained here show similar results to polyethylene, except for higher tensile strength, as an elastomer (Brigham et al. 2011; Doi et al. 1995).

Discussion

P(HB-*co*-HHx) has superior physical and mechanical properties, such as lower melting point, enhanced flexibility, and better impact strength than PHB homopolymer, due to the presence of the longer-chain-length 3HHx monomer fraction (Feng et al. 2002; Qiu et al. 2005). P(HB-*co*-HHx) is mainly produced from related carbon sources such as fatty acids or plant oils in wild-type *Aeromonas*, *Pseudomonas*, as well as engineered *Ralstonia*, *Escherichia*, and others (Chen et al. 2001; Doi et al. 1995; Lee et al. 2000; Park et al. 2001; Qiu et al. 2005). However, long-chain fatty acids or plant oils, when used as carbon sources during fermentation, can cause some undesirable problems, such as severe foaming in the fermentation process and challenge in downstream processing of the removal of residual fatty acids (Qiu et al. 2005; Riedel et al. 2013). In addition, plant oils are more expensive in comparison to waste organic acids and are often considered controversial due to the conflicting production of biochemicals versus food, similar to the issue of corn being used for biofuel versus food (Flammini 2008). Therefore, using unrelated carbon sources such as glucose, fructose, and gluconate for P(HB-*co*-HHx) synthesis will be helpful to overcome those problems (Agnew et al. 2012; Wang et al. 2012). However, to achieve them, many pathways in wild-type strains must be modified to synthesize P(HB-*co*-HHx) from unrelated carbon sources and the resulting 3HHx incorporation is very low (Fukui et al. 2002; Qiu et al. 2005). Interestingly, although butyrate would be the most straightforward carbon substrate for P(HB-*co*-HHx) production based on the reaction of PHA biosynthetic enzymes such as PhaA and PhaB (Fig. 1), there is no prior report of the production of P(HB-*co*-HHx) from butyrate in *R. eutropha*. In neither

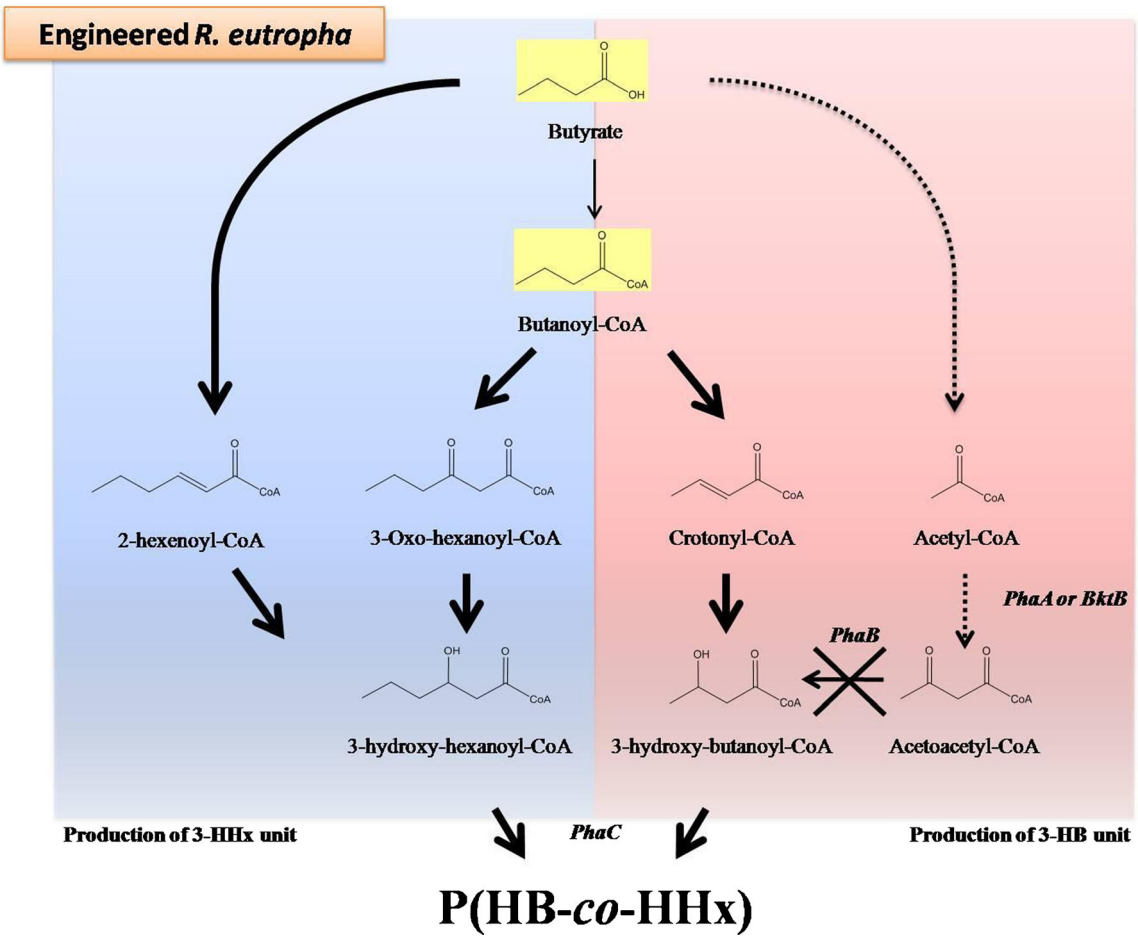

Fig. 4 Potential scheme of butyrate utilization for P(HB-*co*-HHx) production in engineered *R. eutropha* strains, such as Re2135 and Re2133/pCB81. The production of 3-hydroxyhexanoyl-CoA is regarded as a major pathway, along with the production of 3-hydroxybutyryl-CoA, without functional PhaB enzymes, in order to make P(HB-*co*-HHx) copolymer (*bold arrows*)

R. eutropha H16 nor Re2001 containing $phaC2_{Re}$ could P(HB-*co*-HHx) be produced (Fig. 1). However, when butyrate is fed to cultures of strains Re2135 and Re2133/pCB81, neither of which contains functional *phaB1*, *phaB2*, nor *phaB3* genes, P(HB-*co*-HHx) copolymer was produced, likely as a result of a decrease in 3HB monomer production (Fig. 4). Considering butyrate is easily prepared as the end product of anaerobic fermentation of organic materials by obligate anaerobic bacteria such as *Clostridium* and *Fusobacterium* (Lehmann et al. 2012; Potrykus et al. 2007) and that *R. eutropha* also prefers butyrate to other volatile acids such as acetate and propionate (Yang et al. 2010), P(HB-*co*-HHx) production from butyrate alone could potentially be more straightforward and cost-effective than the use of oils or refined sugars.

Although the exact identity of the gene encoding the enzyme, the 3-hydroxybutyryl-CoA dehydrogenase in *R. eutropha* (Dekishima et al. 2011), that utilizes 2-hexenoyl-CoA or 3-ketohexanoyl-CoA as a substrate to make 3-hydroxyhexanoyl-CoA in the absence of PhaB1, PhaB2, and PhaB3 has not yet been revealed, we have discovered that engineered *R. eutropha* strains, such as Re2135 and Re2133/pCB81 (Table 1), could produce P(HB-*co*-HHx) from butyrate. Further, we have shown that the resulting 42 wt% of the HHx monomer in the biopolymer produced on this sole carbon source confers highly favorable mechanical properties to the resulting PHA. This copolymer was not only possible by simply substituting the native PHA synthase from *R. eutropha* for $phaC2_{Re}$ but also by deleting the reductase genes *phaB1*, *phaB2*, and *phaB3*. Our results suggest that copolymer biosynthesis was possible due to a marked decrease in the major precursor supply pathway from acetoacetyl-CoA to 3-hydroxybutyl-CoA by acetoacetyl-CoA reductase (PhaB). To our knowledge, this is the first report for the production of P(HB-*co*-HHx) using butyrate in *R. eutropha*.

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