

Article

Biosynthetic Operon Rearrangement for Poly(3-hydroxybutyrate) Production from Wood-Derived Sugars

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Abstract

Poly[(R)-3-hydroxybutyrate] [P(3HB)] is a biological polyester synthesized by microorganisms and can be used as a biodegradable plastic. The higher the molecular weight of P(3HB), the stronger it can be processed into films and fibers. Therefore, high-molecular-weight P(3HB) can be used as high-performance plastics for engineering and specialty applications. The P(3HB) biosynthetic operon consists of three genes, canonically arranged as *phaC-phaA-phaB* (*phaCAB*). This study specifically rearranges the *phaCAB* operon to the *phaBCA* order to investigate P(3HB) production and its molecular weight from glucose and/or xylose, which can be derived from wood hydrolysates, using *Escherichia coli* XL1-Blue as a host. By rearranging the gene order to *phaBCA*, the expression balance of PHA synthase (PhaC, encoded by *phaC*) and 3HB monomer-supplying enzymes (PhaA and PhaB, encoded by *phaA* and *phaB*, respectively) changed, enabling the host to produce P(3HB) with a higher molecular weight than that with the canonical operon. This effect was significant when xylose was used as the carbon source or when a nutritionally rich medium was used. However, the amount of P(3HB) produced by the *phaCAB*-expressing strain was consistently better than that by the *phaBCA*-expressing strain. This study establishes that high-molecular-weight P(3HB) can be produced through an integrated approach of operon engineering and wood-derived sugar bioprocessing, offering a sustainable production route for high-performance biodegradable plastics from non-food biomass.

Keywords: polyhydroxyalkanoate; PHA; poly(3-hydroxybutyrate); molecular weight; *pha* operon; glucose; xylose

1. Introduction

Polyhydroxyalkanoates (PHAs) are microbially synthesized bio-polyesters that have received significant attention as sustainable alternatives to petroleum-based plastics due to their complete biodegradability and derivation from renewable resources [1–11]. Among them, poly[(R)-3-hydroxybutyrate] [P(3HB)] is the most extensively studied homopolymer, naturally accumulated by numerous bacteria as a carbon and energy storage material. P(3HB) possesses desirable properties, such as biocompatibility and thermoplasticity [1–11], yet its industrial deployment remains constrained by high production costs and the relatively low molecular weight of polymers produced in native hosts. Increasing polymer molecular weight is particularly important because high-molecular-weight P(3HB), with a weight-average molecular weight (M_w) of 3×10^6 or more, exhibits markedly improved mechanical strength, enabling its use in performance-driven applications such as specialty films, high-strength fibers, and engineering materials [12–14].



Academic Editor: Igor Polikarpov

Received: 28 May 2026

Revised: 14 July 2026

Accepted: 21 July 2026

Published: 25 July 2026

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In microorganisms, P(3HB) is synthesized through the coordinated action of three enzymes encoded by the *phaC-phaA-phaB* (*phaCAB*) operon: 3-ketothiolase (PhaA, encoded by the *phaA* gene), which condenses two acetyl-CoAs; NADPH-dependent acetoacetyl-CoA reductase (PhaB, encoded by the *phaB* gene), which reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA [(R)-3HB-CoA]; and PHA synthase (PhaC, encoded by the *phaC* gene), which polymerizes (R)-3HB moiety of (R)-3HB-CoA into P(3HB) (Figure 1A) [1]. A previous study showed that the molecular weight and production amount of P(3HB) are strongly influenced by the relative expression levels of these genes [13], which, in turn, depend on their positions within the operon. This is because, for genes in operons, the gene closer to the promoter is more highly expressed [15]. The *phaB-phaC-phaA* (*phaBCA*) operon arrangement substantially increases the molecular weight of P(3HB) but reduces volumetric production compared to the canonical *phaCAB* operon [13]. Rearranging the gene order in the operon can alter the expression balance between PhaC, which catalyzes polymerization, and PhaAB, which supplies (R)-3HB-CoA monomers from acetyl-CoA.

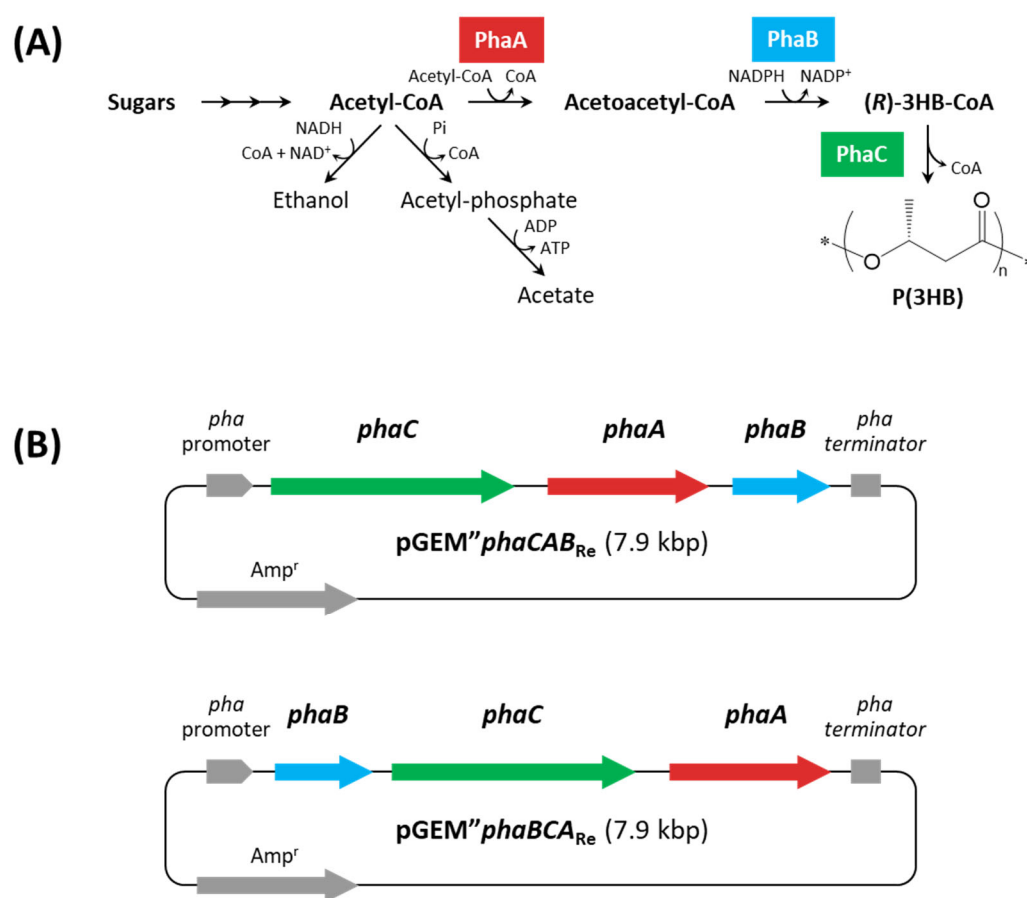


Figure 1. (A) P(3HB) biosynthesis pathway from acetyl-CoA. (B) Plasmid maps of pGEM''*phaCAB_{Re}* (*phaCAB* operon) and pGEM''*phaBCA_{Re}* (*phaBCA* operon). *phaA*: 3-ketothiolase gene (1.2 kb), *phaB*: NADPH-dependent acetoacetyl-CoA reductase gene (0.7 kb), *phaC*: PHA synthase gene (1.8 kb). *Amp^r*: ampicillin resistance gene.

Wood-derived lignocellulosic hydrolysates are non-food biomass and a sustainable carbon source, providing fermentable glucose and xylose in significant proportions. However, most native P(3HB)-producing bacteria exhibit poor xylose utilization [16], thereby limiting their ability to efficiently convert lignocellulosic carbon. Engineered *Escherichia coli* strains, by contrast, are well-suited for mixed-sugar metabolism and thus serve as promising hosts for PHA production from wood-derived hydrolysates [16–21]. Efficient co-utilization of glucose and xylose not only improves carbon conversion efficiency but

also enables the valorization of xylose, a sugar that has traditionally been underutilized in bioplastic fermentation processes [22].

In this study, a recombinant *E. coli* XL1-Blue strain was used to express a rearranged PHA biosynthetic operon (*pha* operon) to synthesize P(3HB) from wood-derived sugars. By employing the *phaBCA* operon, the expression balance between PhaC and PhaAB shifted, enabling the host strain to synthesize P(3HB) with higher molecular weights than with the canonical *phaCAB* operon in media containing glucose and/or xylose. Expression of the *phaBCA* operon reduced overall P(3HB) accumulation compared with the *phaCAB* operon. Crucially, this reduction enhanced monomer availability to sustain chain propagation of P(3HB), thereby enabling the production of high-molecular-weight P(3HB). The increased molecular weight of P(3HB) was notable when xylose was used as the carbon source or a nutritionally rich medium was used. These findings provide important insights for realizing high-molecular-weight P(3HB) production from wood-derived sugars.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids

The PHA synthase gene *phaC*, together with *phaA* and *phaB*, was derived from *Ralstonia eutropha* H16 (ATCC17699). The *pha* operon fragments, including the *phaCAB* and *phaBCA* regions with their native ribosome binding sites from the pGETS109-*pha* series [13], were cloned into the pGEM-T-based vector (Promega, Madison, WI, USA), generating the constructs pGEM''*phaCAB*_{Re} and pGEM''*phaBCA*_{Re}, which were subsequently used to transform *E. coli* XL1-Blue strains (Stratagene, La Jolla, CA, USA) for P(3HB) accumulation.

2.2. Plasmid Construction

The plasmid pGEM''-*phaC*_{Re}*AB* [23] was used as the template for inverse polymerase chain reaction (PCR) [24]. The expected amplicon (vector region) was approximately 4 kb, with an EcoRI and XbaI site introduced to facilitate subsequent ligation of the *phaCAB* and *phaBCA* fragments. Inverse PCR was performed using the primers with the added restriction sites (underlined) as follows: pGEM-EcoRI-Fw-invPCR: 5'-AAAGAATTCCCTGCCGGCCTGGTTCAACCA-3', pGEM-XbaI-Rv-invPCR: 5'-AAATCTAGATTCGAACCGGCTCCGGGCATTG-3'. Then, the insert region *phaBCA* and *phaCAB* fragments, including their native ribosome-binding sites (RBS), were amplified from the pGETS109-*pha* series [13] via polymerase chain reaction (PCR). The primers used for this study incorporate EcoRI and XbaI sites (underlined) and are listed as follows: BCA-XbaI-*phaB* Fw: 5'-AAATCTAGACCAATCAAGGAGTGGACATGACTCAG-3', BCA-EcoRI-*phaA* Rv: 5'-AAAGAATTCTTATTTGCGCTCGACTGCCAGCG-3', CAB-XbaI-*phaC* Fw: 5'-AAATCTAGAAAGAGAGACAATCAAATCATGGCGACC-3', CAB-EcoRI-*phaB* Rv: 5'-AAAGAATTCTCAGCCCATATGCAGGCCG-3'. The amplified *phaBCA* and *phaCAB* fragments were then ligated into the linearized pGEM-T vector using the DNA Ligation Kit (Mighty Mix; Takara Bio, Ohtsu, Japan), yielding the final constructs pGEM''*phaCAB*_{Re} and pGEM''*phaBCA*_{Re}.

The plasmid maps of the two pGEM-T-based expression plasmids containing the *R. eutropha* *pha* operons arranged as *phaCAB* or *phaBCA* are shown in Figure 1B. The *phaCAB* and *phaBCA* fragments, together with their ribosomal binding sites, were PCR-amplified from the previously constructed pGETS109-*pha* series plasmid [13] and cloned into the pGEM-T backbone. Each operon was inserted in the same transcriptional orientation, allowing collinear expression under the control of the *pha*_{Re} promoter. With the promoter and original RBS elements maintained, the primary variable between the two constructs is operon order, enabling a direct assessment of how gene arrangement influences expression levels and polymer production.

2.3. *P(3HB) Biosynthesis in E. coli XL1 Blue*

Recombinant *E. coli* XL1-Blue strains harboring pGEM''*phaCAB*_{Re} (*phaCAB* operon) and pGEM''*phaBCA*_{Re} (*phaBCA* operon) were first cultivated in 2 mL Luria–Bertani (LB) medium (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and 10 g/L NaCl) containing 100 mg/L ampicillin at 30 °C and 160 rpm for 15 h to prepare seed cultures. For P(3HB) production, 100 mL cultures were prepared in either LB or M9Y medium (17.1 g/L Na₂HPO₄·12H₂O, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 2 mL/L of 1 M MgSO₄·7H₂O, 0.1 mL/L of 1 M CaCl₂, and 2.5 g/L Bacto-yeast extract), each in 500 mL shake flasks and supplemented with 100 mg/L ampicillin. For all P(3HB) production experiments, the supplied sugars served as either 20 g/L glucose, 20 g/L xylose, or a mixed substrate containing 10 g/L glucose and 10 g/L xylose. All cultures were incubated at 30 °C and 130 rpm for 72 h, after which cells were harvested by centrifugation and lyophilized for P(3HB) analysis. Culture supernatants collected at harvest were used to quantify residual sugar concentrations and acetate secretion.

2.4. Quantitative Analysis of P(3HB)

PHA accumulation in cells was quantified by gas chromatography using a GC2014 system (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector [25]. Approximately 15 mg of lyophilized cells was subjected to methanolysis by incubation with 2 mL of chloroform and 2 mL of methanol–sulfuric acid (85:15, *v/v*) at 100 °C for 140 min. After cooling to room temperature, 1 mL of distilled water was added, and the biphasic mixture was allowed to stand overnight. The lower chloroform phase was collected, filtered through a 0.45 µm membrane filter, and 0.5 mL of the filtrate was mixed with 0.5 mL of chloroform containing methyl *n*-octanoate (Kanto Chemical Co., Tokyo, Japan) as an internal standard.

2.5. Polymer Extraction

Polymer samples for molecular weight determination by gel permeation chromatography (GPC) were extracted from lyophilized cells [25]. The lyophilized cells were dissolved in chloroform (1 mg PHA:2 mL chloroform) at room temperature for 3 days; the cell residue was then filtered, and the resulting polymer solution was purified by precipitation with cold methanol. The polymers were then collected using filter paper and dried in a fume hood for 2–3 days.

2.6. Gel Permeation Chromatography Analysis

The molecular weight of P(3HB) was determined by gel permeation chromatography (GPC) using a Shimadzu Nexera GPC system equipped with an RI-504 refractive index detector (Shodex, Tokyo, Japan) and two KF-406 LHQ joint columns maintained at 40 °C (Shodex, Tokyo, Japan). Chloroform was used as the mobile phase at a flow rate of 0.3 mL/min. Samples were analyzed at a concentration of 0.1 mg/mL with an injection volume of 50 µL. Polystyrene standards with narrow polydispersity were used to generate a calibration curve.

2.7. High-Performance Liquid Chromatography Analysis

Residual sugars in the hydrolysate samples were quantified using a high-performance liquid chromatograph (HPLC, Nexera XR system, Shimadzu) equipped with a Bio-Rad Aminex HPX 87H Column (300 × 7.8 mm) and a refractive index detector (RID 20A). The column was maintained at 65 °C in a column oven. The mobile phase was 7 mM H₂SO₄, delivered isocratically at a flow rate of 0.6 mL/min. Identification and quantification of glucose, xylose, and acetate were achieved by comparison to external calibration curves prepared from authentic standards under identical HPLC conditions.

2.8. Ethanol Assay

The ethanol concentration in the culture supernatant was enzymatically determined using an ENZYTE Liquid Ethanol (R-Biopharm AG, Darmstadt, Germany), which uses alcohol dehydrogenase to monitor the increase in NADH via absorbance at 340 nm.

3. Results

3.1. P(3HB) Production Using Nutritionally Rich Medium

LB medium, a nutrient-rich medium, was first used by supplementing with glucose and/or xylose for culturing *E. coli* XL1-Blue harboring pGEM''*phaCAB*_{Re} (*CAB* strain) and pGEM''*phaBCA*_{Re} (*BCA* strain). The culture results are shown in Tables 1–3 and Figures 2 and 3.

Table 1. P(3HB) production by *E. coli* XL1-Blue in LB or M9Y medium supplemented with 20 g/L glucose.

Medium	<i>pha</i> Operon	Dry Cell wt. (g/L)	P(3HB) Content (wt%)	P(3HB) (g/L)	Acetate (g/L)	M_w ($\times 10^6$)	PDI
LB	<i>CAB</i>	8.94 $\pm$ 0.07	61 $\pm$ 2	5.44 $\pm$ 0.19	ND	1.61	2.37
	<i>BCA</i>	7.37 $\pm$ 0.06	56 $\pm$ 2	4.09 $\pm$ 0.08	ND	1.83	1.75
M9Y	<i>CAB</i>	4.52 $\pm$ 0.05	67 $\pm$ 3	3.04 $\pm$ 0.15	0.11 $\pm$ 0.01	2.68	1.85
	<i>BCA</i>	1.47 $\pm$ 0.04	14 $\pm$ 1	0.21 $\pm$ 0.01	2.03 $\pm$ 0.03	2.82	1.83

Cells were cultivated in 100 mL culture medium in shake flasks at 30 °C for 72 h. Results represent the mean values of three independent experiments ($n = 3$). ND: not detectable. M_w : weight-average molecular weight, PDI: polydispersity index.

Table 2. P(3HB) production by *E. coli* XL1-Blue in LB or M9Y medium supplemented with 20 g/L xylose.

Medium	<i>pha</i> Operon	Dry Cell wt. (g/L)	P(3HB) Content (wt%)	P(3HB) (g/L)	Acetate (g/L)	M_w ($\times 10^6$)	PDI
LB	<i>CAB</i>	8.66 $\pm$ 0.08	50 $\pm$ 5	3.97 $\pm$ 0.16	Trace	1.49	1.99
	<i>BCA</i>	4.19 $\pm$ 0.13	28 $\pm$ 1	1.16 $\pm$ 0.03	0.95 $\pm$ 0.07	2.24	1.32
M9Y	<i>CAB</i>	3.48 $\pm$ 0.05	66 $\pm$ 3	2.28 $\pm$ 0.07	0.53 $\pm$ 0.02	2.24	1.32
	<i>BCA</i>	1.99 $\pm$ 0.08	34 $\pm$ 2	0.67 $\pm$ 0.06	2.22 $\pm$ 0.02	6.43	1.71

Cells were cultivated in 100 mL culture medium in shake flasks at 30 °C for 72 h. Results represent the mean values of three independent experiments ($n = 3$). Trace: less than 0.01 g/L. M_w : weight-average molecular weight, PDI: polydispersity index.

Table 3. P(3HB) production by *E. coli* XL1-Blue in LB or M9Y medium supplemented with 10 g/L glucose and 10 g/L xylose.

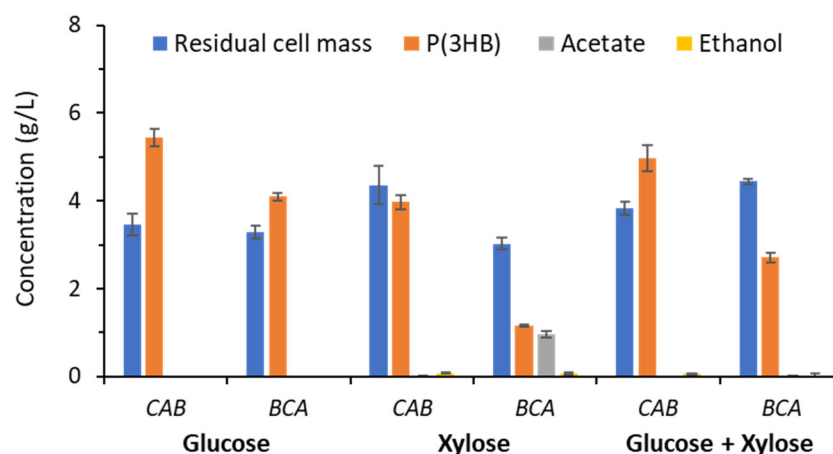
Medium	<i>pha</i> Operon	Dry Cell wt. (g/L)	P(3HB) Content (wt%)	P(3HB) (g/L)	Acetate (g/L)	M_w ($\times 10^6$)	PDI
LB	<i>CAB</i>	8.79 $\pm$ 0.30	56 $\pm$ 2	4.96 $\pm$ 0.30	ND	1.68	2.02
	<i>BCA</i>	7.14 $\pm$ 0.07	38 $\pm$ 1	2.71 $\pm$ 0.11	Trace	2.88	1.58
M9Y	<i>CAB</i>	3.76 $\pm$ 0.05	70 $\pm$ 4	2.63 $\pm$ 0.13	0.11 $\pm$ 0.01	2.97	1.96
	<i>BCA</i>	1.29 $\pm$ 0.02	11 $\pm$ 1	0.14 $\pm$ 0.01	2.93 $\pm$ 0.01	3.14	1.90

Cells were cultivated in 100 mL culture medium in shake flasks at 30 °C for 72 h. Results represent the mean values of three independent experiments ($n = 3$). Trace: less than 0.01 g/L. M_w : weight-average molecular weight, PDI: polydispersity index.

For glucose as a carbon source, the *CAB* strain produced 5.44 g/L P(3HB) with no detectable acetate and ethanol, whereas the *BCA* strain produced 4.09 g/L P(3HB). The residual cell mass (dry cell weight minus polymer weight) was similar for the *CAB* and *BCA* strains, at 3.46 g/L and 3.28 g/L, respectively. The P(3HB) content of the *CAB* strain

(61 wt%) was slightly higher than that of the *BCA* strain (56 wt%). In LB medium, all the glucose was consumed during the cultivation. Consistent with the previous study [13], the *BCA* strain produced P(3HB) from glucose, but the amount was slightly lower than that produced by the *CAB* strain.

(A) LB medium



(B) M9Y medium

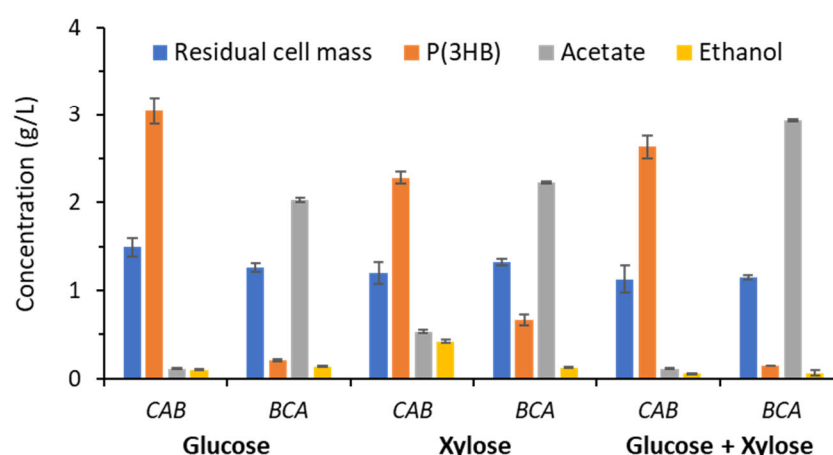


Figure 2. P(3HB) production from glucose and/or xylose by *E. coli* XL1-Blue harboring pGEM''*phaCAB*_{Re} (*CAB* strain) and pGEM''*phaBCA*_{Re} (*BCA* strain). Cells were cultured in (A) LB medium and (B) M9Y medium at 30 °C for 72 h. Residual cell mass: dry cell weight minus polymer weight. The culture supernatant was used to measure the concentrations of acetate and ethanol produced by *E. coli* XL1-Blue. Results are shown as mean $\pm$ SD ($n = 3$).

For xylose, the *CAB* strain again produced a higher amount of P(3HB) (3.97 g/L, 50 wt% polymer content) than the *BCA* strain (1.16 g/L, 28 wt%). The residual cell mass for the *CAB* strain was as high as 4.36 g/L, whereas that of the *BCA* strain was 3.02 g/L. The *CAB* strain produced trace amounts of acetate, whereas the *BCA* strain produced 0.95 g/L acetate. Both strains produced ethanol at low levels (0.06–0.08 g/L). The xylose consumption by *CAB*- and *BCA*-strains was 100% and 71%, respectively. Unexpectedly, the *BCA* strain produced acetate from xylose, in addition to P(3HB).

For the glucose–xylose mixture, the *CAB* strain produced 4.96 g/L P(3HB) and 3.83 g/L residual cell mass, with no detectable acetate. On the other hand, the *BCA* strain produced 2.71 g/L P(3HB) and 4.44 g/L residual cell mass, with a trace amount of acetate. Both strains produced very small amounts of ethanol (0.02–0.07 g/L).

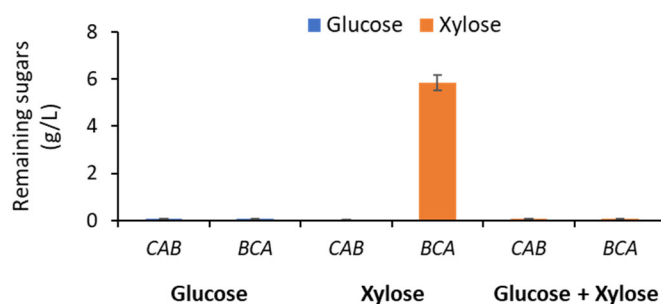
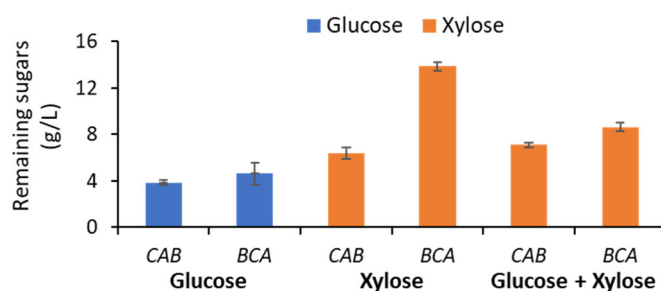
(A) LB medium**(B) M9Y medium**

Figure 3. Remaining sugar concentration in the culture supernatant after 72 h of culture. **(A)** LB medium, **(B)** M9Y medium. Results are shown as mean $\pm$ SD ($n = 3$). The product concentrations are shown in Figure 2.

3.2. P(3HB) Production Using Mineral-Based Medium

M9Y medium, which is an M9 mineral medium supplemented with 2.5 g/L yeast extract to promote bacterial cell growth, was then used for P(3HB) production from glucose and/or xylose. The culture results are shown in Tables 1–3 and Figures 2 and 3.

For glucose as a carbon source, the CAB strain achieved a far higher P(3HB) titer (3.04 g/L) than the BCA strain (0.20 g/L). However, the residual cell mass was similar for the CAB and BCA strains, at 1.48 g/L and 1.26 g/L, respectively. Thus, the P(3HB) contents of the CAB and BCA strains resulted in 67 wt% and 14 wt%, respectively. The CAB strain consumed 81% of the glucose, whereas the BCA strain consumed 77% of the glucose. Acetate production was 0.11 g/L and 2.03 g/L for CAB and BCA strains, respectively. Ethanol production by the CAB and BCA strains was at low levels of 0.10–0.14 g/L. In M9Y medium, the BCA strain produced acetate from glucose rather than P(3HB).

For xylose, the CAB strain again produced a higher amount of P(3HB) (2.28 g/L, 66 wt% of polymer content) than the BCA strain (0.67 g/L, 34 wt%). The residual cell mass of the BCA strain was 1.32 g/L, being similar to that of the CAB strain (1.20 g/L). The CAB strain consumed 68% of the xylose, whereas the BCA strain consumed only 31%. Acetate production by the CAB and BCA strains was 0.53 and 2.22 g/L, respectively. Ethanol production by the CAB strain was relatively higher (0.42 g/L) than that by the BCA strain (0.13 g/L). The BCA strain also produced a large amount of acetate from xylose in M9Y medium.

For the glucose–xylose mixture, the CAB strain produced more P(3HB) (2.63 g/L, 70 wt%) than the BCA strain (0.14 g/L, 11 wt%). The residual cell mass of the CAB and BCA strains was similar, at 1.13 g/L and 1.15 g/L, respectively. Both strains completely consumed glucose, but xylose consumption was limited (14–30%). The CAB strain produced 0.11 g/L acetate, whereas the BCA strain produced an excessive 2.93 g/L of acetate. Both strains produced a small amount of ethanol (0.06 g/L) from the glucose–xylose mixture.

3.3. Molecular Weight of P(3HB)

The molecular weights of P(3HB) were determined by GPC, and their molecular weights and distributions are shown in Tables 1–3 and Figure 4.

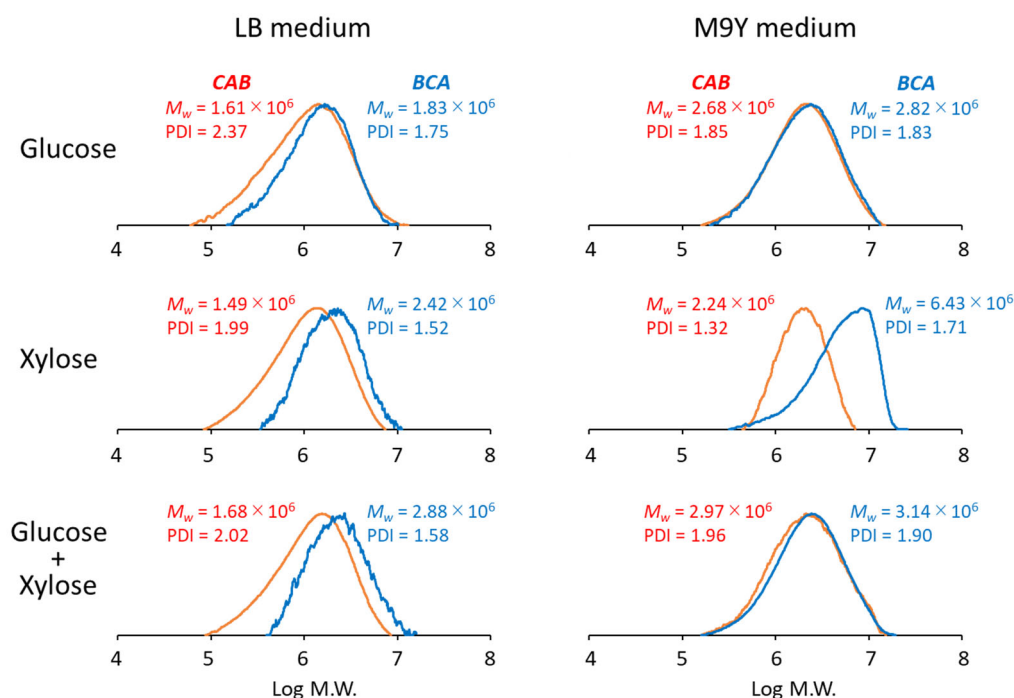


Figure 4. Molecular weight distribution of P(3HB) produced from glucose and/or xylose. The strains are *E. coli* XL1-Blue harboring pGEM''*phaCAB*_{Re} (CAB strain, orange line) and pGEM''*phaBCA*_{Re} (BCA strain, blue line).

For LB medium, the M_w s of P(3HB) produced by the CAB strain were 1.49 – 1.68×10^6 , whereas those produced by the BCA strain were 1.83 – 2.88×10^6 . The BCA strains tend to produce P(3HB) with higher molecular weights than the CAB strains. The polydispersity index of P(3HB) was narrower in the BCA strain than in the CAB strain.

For M9Y medium, M_w s of P(3HB) produced by the CAB strain were 2.24 – 2.97×10^6 whereas those by the BCA strain were 2.82 – 6.43×10^6 . The BCA strain tended to produce P(3HB) with a slightly higher molecular weight than the CAB strain when glucose and mixed sugars were used. Moreover, the BCA strain synthesized P(3HB) with a very high molecular weight when xylose was used as the carbon source. There was no difference in the polydispersity index of P(3HB) between the two strains except when xylose was used as the carbon source.

4. Discussion

Generally, there is a trade-off between molecular weight and yield of P(3HB) produced by bacteria [13,26–29]. Therefore, producing large amounts of high-molecular-weight P(3HB) is challenging. One solution to this task may be the P(3HB) production via rearrangements of the *pha* operon. The molecular weight and production amount of P(3HB) are influenced by the ratio of PhaC, which catalyzes polyester polymerization, to PhaAB, which supplies (R)-3HB-CoA monomer. Therefore, differences in the expression levels of these proteins could lead to variations in P(3HB) production. For genes in operons, it is also known that the gene closer to the promoter is more highly expressed [15]. Many studies have employed the native *pha* operon (gene order: *phaC-phaA-phaB*) from *R. eutropha* to study P(3HB) production in *E. coli* [13,16,23,25,30–33]. Our previous study showed that rearranging the *phaCAB* operon to *phaBCA* enabled relatively efficient biosynthesis of high-

molecular-weight P(3HB) in recombinant *E. coli* DH5 α using LB medium supplemented with glucose [13]. However, it has not been investigated whether the increase in molecular weight generalizes across different culture media or carbon sources. Furthermore, the plasmid used in the previous study was not optimized for *E. coli* to produce P(3HB); therefore, the maximum P(3HB) content was relatively low, at 57 wt% [13].

In this study, we investigated the effects of the *pha* operon structure on P(3HB) production from glucose and/or xylose, as well as on medium composition. Based on previous studies [23,31], we here used *E. coli* XL1-Blue as the host and a pGEM-T-based plasmid to construct a stable P(3HB) production system. As a result, the *phaCAB* operon consistently outperformed the *phaBCA* operon in P(3HB) titer. However, the *phaBCA* operon tends to produce P(3HB) with a higher molecular weight than the *phaCAB* operon. Xylose, in combination with glucose, can be converted to P(3HB) using the nutrient-rich LB medium, even by the *BCA* strain. However, in the mineral-based M9Y medium, the *BCA* strain produced acetate from xylose, instead of P(3HB).

The pronounced performance gap in P(3HB) production between the *phaCAB* and *phaBCA* operons can be attributed to differences in the balance of enzyme expression and metabolic flux toward P(3HB) biosynthesis. The *phaCAB* operon ensures that acetyl-CoA condensation by PhaA and the generation of (*R*)-3HB-CoA by PhaB occur upstream of polymerization by PhaC, thereby maximizing P(3HB) biosynthesis. P(3HB) synthesis from sugars is critically dependent on the availability of acetyl-CoA and reducing equivalents [13]. Acetate overflow is a common phenomenon in aerobic *E. coli* cultures under high glycolytic flux, diverting acetyl-CoA away from P(3HB) synthesis [34–36]. It has been demonstrated that the expression level of PhaC from the *phaBCA* operon is lower than that of *phaCAB* [13]. Lower PhaC expression reduces the number of active polymerization sites in the cell, thereby decreasing competition for available monomer and enabling the synthesis of higher-molecular-weight P(3HB). Meanwhile, decreased PhaC expression led to fewer polymerizable 3HB monomers, thereby allowing excess acetyl-CoA to be metabolized as acetate.

Xylose has been shown to serve as an efficient substrate for P(3HB) synthesis, supplying both acetyl-CoA and the necessary reducing equivalents, and enabling substantial P(3HB) accumulation in engineered microbial systems [37–39]. Mixed-sugar fermentations by *E. coli* with glucose and xylose often experience carbon catabolite repression, leading to preferential glucose consumption, delayed xylose uptake, and impaired redox balance. In this study, residual xylose in the culture broth was also observed under some culture conditions (Figure 3). Co-utilization of sugars often reduces P(3HB) production compared with single-sugar fermentations [39]. However, in nutrient-rich LB medium, sufficient availability of nitrogen source, cofactors, and precursors alleviates metabolic constraints, suppresses acetate overflow, and enables higher P(3HB) titers in this study. Activation of acetate assimilation and TCA cycle pathways has been shown to improve carbon utilization efficiency and product yields [34]. This study's observations also indicate that the *CAB* strain produced more P(3HB) and less acetate from a glucose–xylose mixture than from xylose alone.

Endogenous ethanol is known to induce a chain transfer (CT) reaction during PhaC-catalyzed polymerization, acting as a CT agent [40–47]. When the CT reaction occurs during P(3HB) polymerization, low-molecular-weight P(3HB) is formed. The highest ethanol production, at 0.42 g/L, was observed for the *CAB* strain cultured in M9Y medium supplemented with xylose. The P(3HB) produced under these conditions had M_w of 2.24×10^6 , which was not particularly low, contrary to our expectations. This may be because the production phases of P(3HB) and ethanol differ, and ethanol may not have been present during P(3HB) polymerization.

5. Conclusions

This study demonstrated that operon architecture and culture medium selection are critical determinants of P(3HB) yield and molecular weight in recombinant *E. coli*. By rearranging the canonical *phaCAB* to the *phaBCA* configuration, the intracellular balance of P(3HB) biosynthetic enzymes was altered, enhancing polymer chain elongation. In particular, the *BCA* strain effectively used glucose and xylose in LB medium, converting both sugars into high-molecular-weight P(3HB), highlighting its potential for sustainable bioplastic production from wood-derived lignocellulosic hydrolysates. Collectively, this work establishes a strategic framework that integrates operon engineering and wood-derived sugar bioprocessing, offering a sustainable production route for high-performance biodegradable plastics from non-food biomass.

Author Contributions: Conceptualization, R.M.S. and T.T.; methodology, R.M.S., Z.Q., Y.M. and T.T.; software, R.M.S. and T.T.; validation, R.M.S., Z.Q., Y.M. and T.T.; formal analysis, R.M.S., Z.Q. and Y.M.; investigation, R.M.S., Z.Q. and Y.M.; resources, T.T.; data curation, R.M.S., Z.Q. and Y.M.; writing—original draft preparation, R.M.S.; writing—review and editing, T.T.; visualization, R.M.S., Y.M. and T.T.; supervision, T.T.; project administration, T.T.; funding acquisition, T.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors also thank the Biomaterials Analysis Division of the Institute of Science Tokyo for the DNA sequencing analysis.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Sudesh, K.; Abe, H.; Doi, Y. Synthesis, structure, and properties of polyhydroxyalkanoates: Biological polyesters. *Prog. Polym. Sci.* **2000**, *25*, 1503–1555. [\[CrossRef\]](#)
2. Meereboer, K.W.; Misra, M.; Mohanty, A.K. Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites. *Green Chem.* **2020**, *22*, 5519–5558. [\[CrossRef\]](#)
3. Zytner, P.; Kumar, D.; Elsayed, A.; Mohanty, A.; Ramarao, B.V.; Misra, M. A review on polyhydroxyalkanoate (PHA) production through the use of lignocellulosic biomass. *RSC Sustain.* **2023**, *1*, 2120–2134. [\[CrossRef\]](#)
4. Getino, L.; Martín, J.L.; Chamizo Ampudia, A. A review of polyhydroxyalkanoates: Characterization, production, and application from waste. *Microorganisms* **2024**, *12*, 2028. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Yao, J.; Xiao, X.Y.; Wang, M.; Zhang, Q.; Chen, Y.; Gou, M.; Xia, Z.Y.; Tang, Y.Q. A review of low-cost production of polyhydroxyalkanoates: Strategies, challenges, and perspectives. *Bioresour. Technol.* **2025**, *433*, 132745. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Paduvari, R.; Somashekara, D.M. Advancements in genetic engineering for enhanced polyhydroxyalkanoates (PHA) production: A comprehensive review of metabolic pathway manipulation and gene deletion strategies. *Bioengineered* **2025**, *16*, 2458363. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Muigano, M.N.; Mauti, G.O.; Anami, S.E.; Onguso, J.M. Advances and challenges in polyhydroxyalkanoates (PHA) production using *Halomonas* species: A review. *Int. J. Biol. Macromol.* **2025**, *309*, 142850. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Jia, L.; Zhang, M.; Kumar, D.; Zhao, J. A review of polyhydroxybutyrate biosynthesis by different microorganisms. *ChemBioChem* **2025**, *26*, e202500562. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Subramanian, B.; Basak, S.; Thirumurugan, R.; Saleena, L.M. Metabolic flux analysis and metabolic engineering for polyhydroxybutyrate (PHB) production. *Polym. Bull.* **2024**, *81*, 10589–10608. [\[CrossRef\]](#)
10. Guimarães, T.C.; Araújo, E.S.; Hernández-Macedo, M.L.; López, J.A. Polyhydroxyalkanoates: Biosynthesis from alternative carbon sources and analytic methods: A short review. *J. Polym. Environ.* **2022**, *30*, 2669–2684. [\[CrossRef\]](#)
11. Hwang, D.K.; Park, J.; Oh, D.X.; Jeon, H.; Koo, J.M. Assessing the role of compostable plastics in circular economy transition. *ChemSusChem* **2026**, *19*, e202501938. [\[PubMed\]](#)
12. Iwata, T. Strong fibers and films of microbial polyesters. *Macromol. Biosci.* **2005**, *5*, 689–701. [\[CrossRef\]](#) [\[PubMed\]](#)

13. Hiroe, A.; Tsuge, K.; Nomura, C.T.; Itaya, M.; Tsuge, T. Rearrangement of gene order in the *phaCAB* operon leads to effective production of ultrahigh molecular weight poly[(R)-3-hydroxybutyrate] in genetically engineered *Escherichia coli*. *Appl. Environ. Microbiol.* **2012**, *78*, 3177–3184. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Kabe, T.; Sugiura, T.; Hikima, T.; Takata, M.; Iwata, T. Effect of molecular weight on physical and crystallization properties of UHMW poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyhexanoate]. *J. Fiber Sci. Technol.* **2018**, *74*, 30–39. [\[CrossRef\]](#)
15. Lim, H.N.; Lee, Y.; Hussein, R. Fundamental relationship between operon organization and gene expression. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 10626–10631. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Lee, S.Y. Poly(3-hydroxybutyrate) production from xylose by recombinant *Escherichia coli*. *Bioprocess Eng.* **1998**, *18*, 397–399. [\[CrossRef\]](#)
17. Hori, C.; Yamazaki, T.; Ribordy, G.; Takisawa, K.; Matsumoto, K.I.; Ooi, T.; Taguchi, S. High-cell density culture of poly(lactate-co-3-hydroxybutyrate)-producing *Escherichia coli* by using glucose/xylose-switching fed-batch jar fermentation. *J. Biosci. Bioeng.* **2019**, *127*, 721–725. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Lopes, M.S.G.; Rocha, R.C.S.; Zannotto, S.P.; Gomez, J.G.C.; Silva, L.F.D. Screening of bacteria to produce polyhydroxyalkanoates from xylose. *World J. Microbiol. Biotechnol.* **2009**, *25*, 1751–1756. [\[CrossRef\]](#)
19. Huo, G.; Zhu, Y.; Liu, Q.; Tao, R.; Diao, N.; Wang, Z.; Chen, T. Metabolic engineering of an *Escherichia coli ndh* knockout strain for PHB production from mixed glucose–xylose feedstock. *J. Chem. Technol. Biotechnol.* **2017**, *92*, 2739–2745. [\[CrossRef\]](#)
20. Silva, L.F.; Taciro, M.K.; Michelin Ramos, M.E.; Carter, J.M.; Pradella, J.G.C.; Gomez, J.G.C. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 245–254. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Mizuno, S.; Enda, Y.; Saika, A.; Hiroe, A.; Tsuge, T. Biosynthesis of polyhydroxyalkanoates containing 2-hydroxy-4-methylvalerate and 2-hydroxy-3-phenylpropionate units from a related or unrelated carbon source. *J. Biosci. Bioeng.* **2018**, *125*, 295–300. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Gao, J.; Yu, W.; Li, Y.; Jin, M.; Yao, L.; Zhou, Y.J. Engineering co-utilization of glucose and xylose for chemical overproduction from lignocellulose. *Nat. Chem. Biol.* **2023**, *19*, 1524–1531. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Agus, J.; Kahar, P.; Abe, H.; Doi, Y.; Tsuge, T. Molecular weight characterization of poly[(R)-3-hydroxybutyrate] synthesized by genetically engineered strains of *Escherichia coli*. *Polym. Degrad. Stab.* **2006**, *91*, 1138–1146. [\[CrossRef\]](#)
24. Ochman, H.; Gerber, A.S.; Hartl, D.L. Genetic applications of an inverse polymerase chain reaction. *Genetics* **1988**, *120*, 621–623. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Sivashankari, R.M.; Miyahara, Y.; Tsuge, T. Poly(3-hydroxybutyrate) biosynthesis from [U-¹³C₆] D-glucose by *Ralstonia eutropha* NCIMB 11599 and recombinant *Escherichia coli*. *Microbiol. Res.* **2023**, *14*, 1894–1906. [\[CrossRef\]](#)
26. Sim, S.J.; Snell, K.D.; Hogan, S.A.; Stubbe, J.; Rha, C.; Sinskey, A.J. PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate in vivo. *Nat. Biotechnol.* **1997**, *15*, 63–67. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Sim, S.J.; Snell, K.D.; Kim, B.W.; Rha, K.C.; Sinskey, A.J. Increased poly-β-hydroxybutyrate (PHB) chain length by the modulation of PHA synthase activity in recombinant *Escherichia coli*. *Biotechnol. Lett.* **2001**, *23*, 2057–2061. [\[CrossRef\]](#)
28. Shi, F.; Ashby, R.; Gross, R.A. Use of poly(ethylene glycol)s to regulate poly(3-hydroxybutyrate) molecular weight during *Alcaligenes eutrophus* cultivations. *Macromolecules* **1996**, *29*, 7753–7758. [\[CrossRef\]](#)
29. Tsuge, T. Fundamental factors determining the molecular weight of polyhydroxyalkanoate during biosynthesis. *Polym. J.* **2016**, *48*, 1051–1057. [\[CrossRef\]](#)
30. Choi, J.; Lee, S.Y. High level production of supra molecular weight poly(3-hydroxybutyrate) by metabolically engineered *Escherichia coli*. *Biotechnol. Bioprocess Eng.* **2004**, *9*, 196–200. [\[CrossRef\]](#)
31. Kahar, P.; Agus, J.; Kikkawa, Y.; Taguchi, K.; Doi, Y.; Tsuge, T. Effective production and kinetic characterization of ultra-high-molecular-weight poly[(R)-3-hydroxybutyrate] in recombinant *Escherichia coli*. *Polym. Degrad. Stab.* **2004**, *87*, 161–169.
32. Kusaka, S.; Abe, H.; Lee, Y.; Doi, Y. Molecular mass of poly[(R)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 140–143. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Kusaka, S.; Iwata, T.; Doi, Y. Properties and biodegradability of ultra-high-molecular-weight poly[(R)-3-hydroxybutyrate] produced by a recombinant *Escherichia coli*. *Int. J. Biol. Macromol.* **1999**, *25*, 87–99. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Millard, P.; Enjalbert, B.; Uttenweiler-Joseph, S.; Portais, J.C.; Létisse, F. Control and regulation of acetate overflow in *Escherichia coli*. *eLife* **2021**, *10*, e63661. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Valgepea, K.; Adamberg, K.; Nahku, R.; Lahtvee, P.J.; Arike, L.; Vilu, R. Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by repression of acetate assimilation under certain carbon source conditions. *BMC Syst. Biol.* **2010**, *4*, 166. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Sandström, A.G.; Muñoz de las Heras, A.; Portugal-Nunes, D.; Gorwa-Grauslund, M.F. Engineering of *Saccharomyces cerevisiae* for the production of poly-3-D-hydroxybutyrate from xylose. *AMB Express* **2015**, *5*, 63. [\[CrossRef\]](#)

37. de las Heras, A.M.; Portugal Nunes, D.J.; Rizza, N.; Sandström, A.G.; Gorwa-Grauslund, M.F. Anaerobic poly-3-D-hydroxybutyrate production from xylose in recombinant *Saccharomyces cerevisiae* using a NADH-dependent acetoacetyl-CoA reductase. *Microb. Cell Fact.* **2016**, *15*, 117. [[CrossRef](#)]
38. Guamán, L.P.; Barba-Ostria, C.; Zhang, F.; Oliveira-Filho, E.R.; Gomez, J.G.C.; Silva, L.F. Engineering xylose metabolism for production of polyhydroxybutyrate in the non-model bacterium *Burkholderia sacchari*. *Microb. Cell Fact.* **2018**, *17*, 131. [[CrossRef](#)]
39. Nichols, N.; Dien, B.; Bothast, R. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 120–125. [[CrossRef](#)] [[PubMed](#)]
40. Hiroe, A.; Hyakutake, M.; Thomson, N.M.; Sivaniah, E.; Tsuge, T. Endogenous ethanol affects biopolyester molecular weight in recombinant *Escherichia coli*. *ACS Chem. Biol.* **2013**, *8*, 2568–2576. [[CrossRef](#)] [[PubMed](#)]
41. Doi, Y.; Kawaguchi, Y.; Koyama, N.; Nakamura, S.; Hiramitsu, M.; Yoshida, Y.; Kimura, H. Synthesis and degradation of polyhydroxyalkanoates in *Alcaligenes eutrophus*. *FEMS Microbiol. Rev.* **1992**, *9*, 103–108. [[CrossRef](#)]
42. Kawaguchi, Y.; Doi, Y. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. *Macromolecules* **1992**, *25*, 2324–2329. [[CrossRef](#)]
43. Koizumi, F.; Abe, H.; Doi, Y. Molecular weight of poly(3-hydroxybutyrate) during biological polymerization in *Alcaligenes eutrophus*. *J. Macromol. Sci. A* **1995**, *32*, 759–774. [[CrossRef](#)]
44. Madden, L.A.; Anderson, A.J.; Shah, D.T.; Asrar, J. Chain termination in polyhydroxyalkanoate synthesis: Involvement of exogenous hydroxy-compounds as chain transfer agents. *Int. J. Biol. Macromol.* **1999**, *25*, 43–53. [[CrossRef](#)] [[PubMed](#)]
45. Lawrence, A.G.; Choi, J.; Rha, C.; Stubbe, J.; Sinskey, A.J. In vitro analysis of the chain termination reaction in the synthesis of poly-(R)- β -hydroxybutyrate by the class III synthase from *Allochromatium vinosum*. *Biomacromolecules* **2005**, *6*, 2113–2119. [[CrossRef](#)] [[PubMed](#)]
46. Ashby, R.D.; Solaiman, D.K.; Strahan, G.D.; Levine, A.C.; Nomura, C.T. Methanol-induced chain termination in poly(3-hydroxybutyrate) biopolymers: Molecular weight control. *Int. J. Biol. Macromol.* **2015**, *74*, 195–201. [[CrossRef](#)] [[PubMed](#)]
47. Ashby, R.D.; Shi, F.; Gross, R.A. Use of poly(ethylene glycol) to control the end group structure and molecular weight of poly(3-hydroxybutyrate) formed by *Alcaligenes latus* DSM 1122. *Tetrahedron* **1997**, *53*, 15209–15223. [[CrossRef](#)]

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