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Modulation of 3-hydroxyvalerate molar fraction in poly(3-hydroxybutyrate-3-hydroxyvalerate) using *Ralstonia eutropha* transformant co-amplifying *phbC* and NADPH generation-related *zwf* genes

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Abstract

The molar fraction of 3-hydroxyvalerate (3HV) in poly(3-hydroxybutyrate-3-hydroxyvalerate) [P(3HB-3HV)] was modulated through the co-amplification of the *phbC* gene encoding PHB synthase and the *zwf* gene encoding glucose-6-phosphate dehydrogenase generating NADPH from the metabolism of fructose as a cofactor in *Ralstonia eutropha*. The biosynthesis of poly-3-hydroxybutyrate (PHB) and P(3HB-3HV) increased appreciably after the co-amplification of the *phbC* and *zwf* genes due to the accelerated polymerization reaction and enhanced NADPH supplementation. In particular, the 3HV molar fraction in P(3HB-3HV) was effectively modulated. pH-stat fed-batch cultivation of the *R. eutropha* transformant co-amplifying the *phbC* and *zwf* genes was also carried out to increase the biosynthesis of P(3HB-3HV) and modulate the 3HV molar fraction. The content and molar fraction of P(3HB-3HV) achieved up to 60.1% and 71.9 mol%, respectively, through the concurrent feeding of valerate and fructose.

Keywords: *R. eutropha*; Transformation; *phbC* gene; *zwf* gene; NADPH; PHB, P(3HB-3HV); 3-HV molar fraction

1. Introduction

Polyhydroxyalkanoate (PHA) is an energy storage material that accumulates in various microorganisms under abnormal growth conditions such as an excess amount of carbon-source but the limitation of other nutrients. PHB and its copolymers; poly(3-hydroxybutyrate-3-hydroxyvalerate) [P(3HB-3HV)] and poly(3-hydroxybutyrate-4-hydroxybutyrate) [P(3HB-4HB)], have received a lot of attention due to their practical utilization as biodegradable and biocompatible thermoplastics [1,2]. The physical properties of the copolymers are closely connected to the molar fraction of 3-hydroxyvalerate (3HV) in P(3HB-3HV) and 4-hydroxybutyrate (4HB) in P(3HB-4HB). The modulation of the molar fraction has been mainly accomplished by changing the precursor compounds and cultivation conditions, such as, two-stage cultivation [3,4].

The biosynthesis of PHB in *Ralstonia eutropha* is accomplished by three enzymes: β-ketothiolase encoded by *phbA* condensing two acetyl-CoA into acetoacetyl-CoA; NADPH-dependent acetoacetyl-CoA reductase encoded by *phbB* catalyzing the reduction of acetoacetyl-CoA into 3-hydroxybutyryl-CoA using NADPH as the reducing power; and PHB synthase, encoded by *phbC* polymerizing 3-hydroxybutyryl-CoA, 3-hydroxyvaleryl-CoA, and 4-hydroxybutyryl-CoA [1,3,4].

In previous work by the current authors, the overproduction of PHAs was achieved by transforming recombinant plasmids harboring *phbCAB* genes cloned from *R. eutropha* into the parent *R. eutropha* to amplify the enzyme actions related to the PHB biosynthesis pathway [5–8]. The PHA biosynthesis increased significantly after reintroducing the *phbCAB*, *phbAB*, and *phbC* genes, respectively. In addition, the molar fractions of 3HV in P(3HB-3HV) and 4HB in P(3HB-4HB) also substantially increased, especially after amplification of the *phbC* gene [6–8]. The *phbC* gene cloned from *Alcaligenes latus* was also transformed into the parent *A. latus*, and similar results obtained [9]. The biosynthesis rates of PHB and P(3HB-3HV) have been found to be determined by β-ketothiolase and acetoacetyl-CoA reductase [10], plus the current authors identified that the 3HV molar fraction is mainly controlled by PHB synthase under in vivo conditions [7].

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The flux of acetyl-CoA, either towards the TCA cycle for cell growth or the PHB biosynthesis pathway for energy storage, has been controlled by the NADPH/($NADP^+$ + NADPH) ratio in *R. eutropha* [11,12] and recombinant *E. coli* [13]. The effect of NADPH on β-ketothiolase and acetoacetyl-CoA reductase activities has also been investigated after the supplementation of various intermediates of glycolysis and the TCA cycle, including acetyl-CoA, citrate, and NADPH, during cultivation [14–16]. Oxidative pressure also induces the activation of glucose-6-phosphate dehydrogenase (G6PDH), generating NADPH, and the elevated NADPH level significantly enhances PHB accumulation in *R. eutropha* [17].

In microorganisms, the NADPH of a biosynthetic cofactor is mainly generated from the oxidative pentose phosphate pathway by two enzymes: glucose-6-phosphate dehydrogenase encoded by the *zwf* gene and 6-phosphogluconate dehydrogenase encoded by the *gnd* gene [18,19]. The acetoacetyl-CoA reductase in *R. eutropha* is a NADPH-dependent type, requiring NADPH as the reducing power [20]. As such, PHB biosynthesis in *R. eutropha* could possibly be enhanced through the amplification of the *phbC* gene or NADPH generation-related *zwf* gene encoding G6PDH, either separately or concurrently.

Accordingly, in the current work, the *phbC* gene cloned from *R. eutropha* and *zwf* gene cloned from *E. coli* were introduced into the parent *R. eutropha* to enhance the PHB synthase and G6PDH activities. The characteristics of the PHB and P(3HB-3HV) biosynthesis by the transformants amplifying the above two genes, either separately or concurrently, were then analyzed. The molar fraction of 3HV in P(3HB-3HV) was also modulated based on controlling the cultivation conditions, including the mixing ratio of the substrate and precursor compound. One of the *R. eutropha* transformants was also cultivated using a pH-stat fed-batch strategy in an attempt to overproduce P(3HB-3HV) and modulate its 3-HV molar fraction. Consequently, this work will facilitate new strain and process developments for the industrial production of the commercially valuable biopolymer PHA.

2. Materials and methods

2.1. Strains, plasmids, and gene sources

Ralstonia eutropha H16 (ATCC 17699) was used as the parent strain and gene source for the *phbCAB* operon, while *E. coli* DH5α was used as the source for the *zwf* gene. The plasmid pKT230 was used as the *E. coli–R. eutropha* shuttle vector for the transformation of the *phbC* and *zwf* genes into *R. eutropha*. The *zwf* gene was isolated from the plasmid pZWF carrying the *zwf* gene [21], while the *phbC* gene was isolated from the plasmid pHB523 carrying the *phbCAB* operon [6]. The plasmid pHB525 harboring *phbC* was constructed from the plasmid pHB523 after discarding the *phbAB* gene. The plasmid pKTZ harboring the *zwf* gene was constructed by inserting the *zwf* gene into the plasmid pKT230. The plasmid pHBCZ co-harboring the *phbC* and *zwf* genes was obtained by inserting the *zwf* gene into the plasmid pHB523 after removing the *phbAB* gene. The constructed plasmids, pHB525, pKTZ and pHBCZ, were transformed into *R. eutropha* H16 by electroporation [5] to obtain the transformants *R. eutropha* AER5, REZ, and RCZ, respectively.

2.2. Medium and cultivation

The one-stage cultivation for cell growth and PHB biosynthesis was carried out in a minimal medium containing 3.32 g l^{-1} of Na_2HPO_4, 0.83 g l^{-1} of KH_2PO_4, 3.0 g l^{-1} of $(NH_4)_2SO_4$, 0.2 g l^{-1} of $MgSO_4$, 20.0 g l^{-1} of fructose, and 1.0 ml l^{-1} of trace minerals in a 2.5 l fermentor with an inoculum size of 5.0% (v/v), aeration rate of 1.0 VVM, and impeller speed of 300 rpm.

The biosynthesis of P(3HB-3HV) was carried out using two-stage cultivation. First, the cell growth was conducted using a nutrient-rich medium containing 10.0 g l^{-1} of polypeptone, 10.0 g l^{-1} of yeast extract, 5.0 g l^{-1} of meat extract, and 5.0 g l^{-1} $(NH_4)_2SO_4$ for 36 h. Next, the cultivation of the harvested cells was conducted using a nitrogen-free minimal medium, supplemented with 10.0 g l^{-1} of valerate and fructose as the precursors to P(3HB-3HV) biosynthesis. The pH-stat fed-batch cultivation was carried out under similar conditions as the one-stage cultivation, except for the feeding of a medium composed of 10 g l^{-1} of valerate and fructose to maintain a constant pH of 7.0. A kanamycin concentration of 200 mg ml^{-1} was used as the selective pressure for the cultivation of the transformants.

2.3. Determination of PHB and P(3HB-3HV) concentrations

The concentrations of PHB and P(3HB-3HV) were measured following the method of Braunegg et al. [22]. The harvested cells were treated with acidified methanol to obtain methylated monomers. Next, the methylated monomer composition was determined by gas chromatography using an FID detector (PerkinElmer Instruments, Norwork, USA) and Carbowax 20 M column (Hewlett-Packard Co., Palo Alto, USA). The detection conditions were an initial temperature of 100 °C, final temperature of 150 °C, and temperature increment rate of 5 °C min^{-1}. The standards were the PHB and P(3HB-3HV) powders of *R. eutropha* (Sigma Chemical, St. Louis, USA).

2.4. Determination of NADPH concentration

The NADPH concentration was determined by measuring the absorbance of oxidized and reduced forms of nicotinamide nucleotides at $A_{570\,nm}$ according to the method of Zerez et al. [23]. The sonificated cell extract

was mixed with a coloring reagent containing 5.0 mmol Na_4EDTA, 2.0 mmol phenazine-ethosulfate, 0.5 mmol thiazolyl blue, 1.3 U of glucose-6-phosphate dehydrogenase, and 1.0 mmol of glucose-6-phosphate dissolved in a 100 mM Tris–HCl buffer (pH 8.0). Then, the slope of the absorbance was followed for 5 min to determine the NADPH concentration.

2.5. Measurement of glucose-6-phosphate dehydrogenase activity

The glucose-6-phosphate dehydrogenase activity was determined using the method of Deutsch [24]. The sonificated cell extract was mixed with a reaction solution containing 1 mM glucose-6-phosphate, 1 mM $NADP^+$, and 10 mM $MgCl_2$ dissolved in a 50 mM Tris–HCl buffer (pH 7.5). Then, the NADPH produced in 5 min was measured at an absorbance of 340 nm. The intrinsic activity of G6PDH was expressed as the mmol of NADPH produced per min per mg of cellular soluble protein.

2.6. Measurement of enzyme activities related to PHB biosynthesis

The β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase activities were analyzed according to the methods of Haywood et al. [20,25], where the NADPH generated was measured in a sonificated cell extract at $A_{340\,nm}$, and the PHB synthase activity was determined by measuring the change in absorbance caused by the release of CoA at $A_{412\,nm}$ [26]. The protein concentration was measured using the Bradford method [27]. The intrinsic activity was expressed as the mmol of product per min per mg of cellular soluble protein.

3. Results and discussion

3.1. Construction and transformation of plasmids expressing phbC or zwf gene, and co-expressing phbC and zwf genes

Fig. 1 illustrates the construction of the plasmids expressing the *phbC* or *zwf* gene, and co-expressing the *phbC* and *zwf* genes. The plasmid pZWF [21], carrying the *zwf* gene encoding G6PDH cloned from *E. coli* DH5α, was digested with *Eco*RI and a *zwf* gene fragment inserted into *Eco*RI-digested pKT230 to construct the plasmid pKTZ. The plasmid pHB523 [6,7], carrying the *phbCAB* operon of *R. eutropha* H16, was digested with *Eco*RI and *Stu*I to discard the *phbAB* genes. The residual plasmid harboring only the *phbC* gene was self-ligated to obtain the plasmid pHB525. To construct the plasmid pHBCZ co-integrating the *phbC* and *zwf* genes, a *zwf* gene fragment obtained from the plasmid pZWF [21] by digestion with *Eco*RI and *Pvu*II was inserted into the *Eco*RI and *Stu*I site of pHB523 [6,7] after discarding the *phbAB* gene.

The *phbC* gene operates according to its own σ^{70}-like promoter sequence located about 300 bp upstream of the structure gene [28], while the *zwf* gene operates based on its

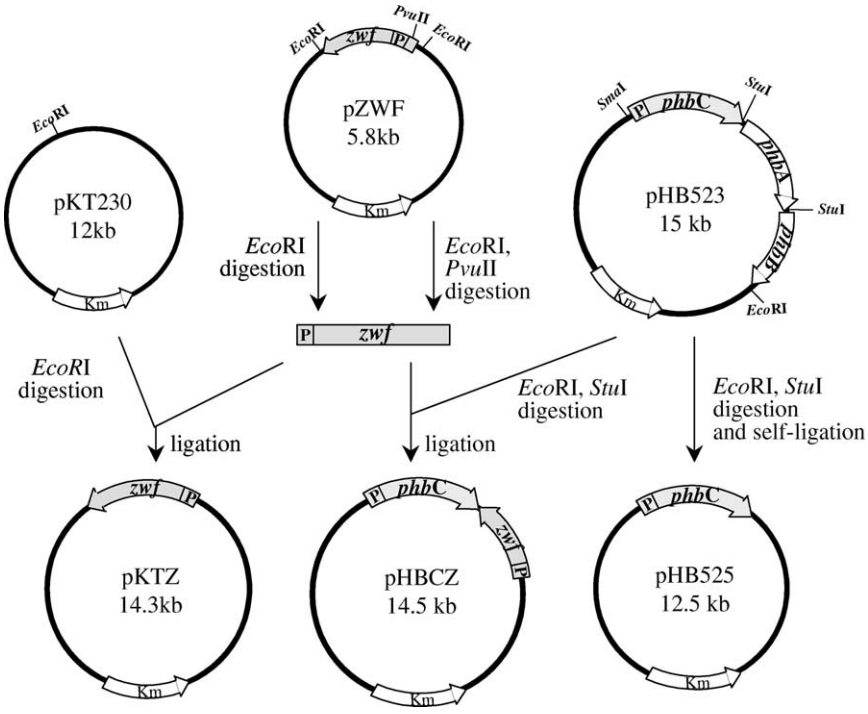

Fig. 1. Construction of the recombinant plasmids pHB525 containing the *phbC* gene, pKTZ containing the *zwf* gene, and pHBCZ co-integrating *phbC* and *zwf* genes, respectively.

Table 1
Comparison of the intrinsic activities of PHB synthase and glucose-6-phosphate dehydrogenase

Strains	Intrinsic activity[a]	
	PHBs[b]	G6PDH[c]
Parent *R. eutropha* H16	1.70 ± 0.13	1.22 ± 0.21
Transformant *R. eutropha* AER5 amplifying *phbC* gene	2.97 ± 0.17	1.67 ± 0.19
Transformant *R. eutropha* REZ amplifying *zwf* gene	1.95 ± 0.12	3.85 ± 0.23
Transformant *R. eutropha* RCZ co-amplifying *phbC* and *zwf* gene	3.22 ± 0.16	4.10 ± 0.21

Cells are cultivated in the minimal medium, 20 g l^{-1} of fructose, pH 7.0, 30 °C, for 36 h.

[a] Intrinsic activities are expressed as μmol of product generated per min per mg of cellular soluble protein and each value is an average (±standard deviation) of three cultures.

[b] PHBs; PHB synthase.

[c] G6PDH; glucose-6-phosphate dehydrogenase.

own σ^{70} promoter sequence located about 100 bp upstream [18]. The constructed plasmids pHB525, pKTZ, and pHBCZ were confirmed by Southern hybridization (data not shown) and then transformed into *R. eutropha* by electroporation to obtain the transformants *R. eutropha* AER5 amplified by the *phbC* gene, REZ amplified by the *zwf* gene, and RCZ co-amplified by the *phbC* and *zwf* genes, respectively.

The expression of the amplified *phbC*, *zwf*, and *phbC* and *zwf* genes was confirmed by measuring the intrinsic activities of PHB synthase and G6PDH in the transformants after cultivation in a minimal medium containing fructose as the carbon source for 36 h. As shown in Table 1, the PHB synthase activity increased 1.75 times after enforcing the *phbC* gene and 1.89 times after enforcing the *phbC* and *zwf* genes concurrently. The G6PDH activity was also elevated 2.15 times by the integrated *zwf* gene and 2.31 times by the co-integrated *phbC* and *zwf* genes. This indicates that the transformed *phbC* and *zwf* genes were successfully over-expressed both separately and concurrently by their own strong σ^{70} promoters, located upstream of the *phbC* and *zwf* genes.

3.2. PHB biosynthesis characteristics of *R. eutropha* transformants amplifying *phbC* or *zwf*, and co-amplifying *phbC* and *zwf* genes

The PHB biosynthesis characteristics of the transformants *R. eutropha* AER5, REZ, and RCZ amplifying *phbC*, *zwf*, and both *phbC* and *zwf* genes, respectively, can be seen in Fig. 2, where the variation in the rest-biomass, concentration of PHB accumulated, and PHB content are all compared with those of the parent strain.

The total cell mass, including the rest-biomass and accumulated PHB, increased appreciably after transformation of the cloned gene mainly due to the accumulation of PHB (data not shown). However, the rest-biomass, based on subtracting the PHB content from the total cell mass, decreased significantly after the over-expression of the cloned genes in the order of the co-integrated *phbC* and *zwf* genes, *phbC*, and *zwf* genes, respectively. This may have been due to two reasons: (1) the shifting of the carbon flux to PHB biosynthesis rather than the TCA cycle resulting from enforced PHB synthase and G6PDH activities; and (2) the inhibitory effect on the TCA cycle due to over-generated NADPH caused by amplified G6PDH, as reported in previous studies, including cultivation under oxidative pressure using methyl viologen [17], the cultivation of an isocitrate dehydrogenase leaky mutant [29], and cultivation of the strain knock-out *pgi* gene located next to the *zwf* gene, thereby indirectly activating G6PDH [30].

Meanwhile, the biosynthesis of PHB enhanced appreciably after transformation of the *phbC* and *zwf* genes either separately or concurrently. The PHB content in the cells also increased proportionally in the order of the co-integrated *phbC* and *zwf* genes, *phbC*, and *zwf* genes, respectively. This was due to the accelerated polymerization reaction of 3HB by the over-expressed PHB synthase, along with the sufficient supplementation of NADPH from the metabolism of fructose by the enhanced G6PDH activity. The PHB synthase encoded by the *phbC* gene had a more critical influence on the PHB biosynthesis compared to the enhanced G6PDH encoded by the *zwf* gene generating NADPH as a cofactor.

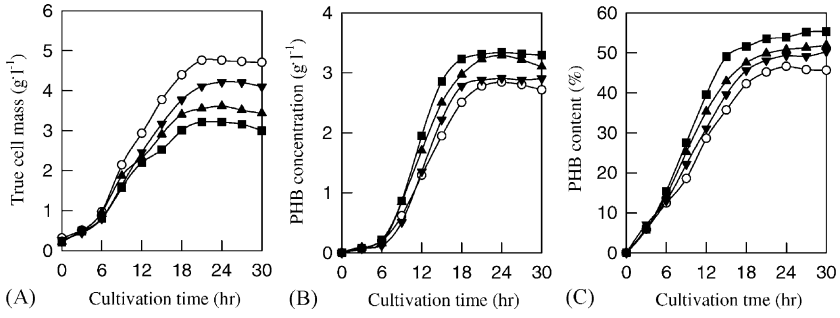

Fig. 2. Comparison of gene amplification in transformants *R. eutropha* for biosynthesis of PHB; the true cell growth (A), concentration of PHB (B), and content of PHB (C). Cells were cultivated in minimal medium, 20 g l^{-1} of fructose, pH 7.0, 30 °C for 30 h in a 2.5 l fermentor. (○) *R. eutropha* H16, (▲) *R. eutropha* AER5 enforcing the *phbC* gene, (▼) *R. eutropha* REZ enforcing the *zwf* gene, (■) *R. eutropha* RCZ co-enforcing the *phbC* and *zwf* genes.

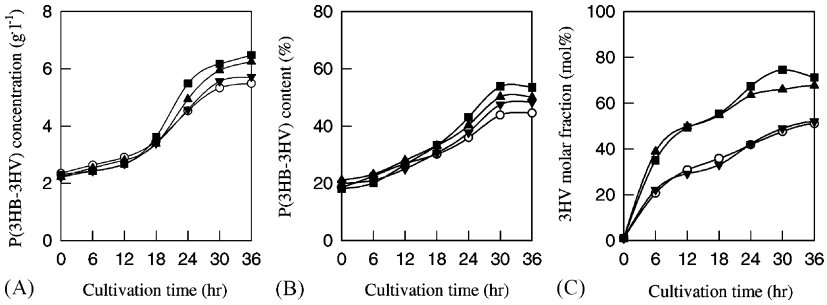

Fig. 3. Comparison of amplified genes in transformants for biosynthesis of P(3HB-3HV); concentration of P(3HB-3HV) (A), content of P(3HB-3HV) (B), and 3HV molar fraction (C). Cells were cultivated in nutrient-rich medium at the first stage, and then in nitrogen-free minimal medium containing 10 g l^{-1} of valerate as a precursor at the second stage, pH 7.0, 30 °C for 36 h. (○) *R. eutropha* H16, (▲) *R. eutropha* AER5 enforcing the *phbC* gene, (▼) *R. eutropha* REZ enforcing the *zwf* gene, (■) *R. eutropha* RCZ co-enforcing the *phbC* and *zwf* genes.

3.3. P(3HB-3HV) biosynthesis characteristics of R. eutropha transformants amplifying phbC or zwf, and co-amplifying phbC and zwf genes

The P(3HB-3HV) biosynthesis characteristics were investigated after the two-stage cultivation, where cell growth in a nutrient-rich medium was followed by the induction of P(3HB-3HV) biosynthesis in a nitrogen-free minimal medium using valerate as the precursor compound. The rest-biomass decreased slightly during the second stage of cultivation, yet the biosynthesis of P(3HB-3HV) increased appreciably, in the order of the co-amplified *phbC* and *zwf*, amplified *phbC*, and amplified *zwf* genes, as shown in Fig. 3. In particular, the 3HV molar fraction of P(3HB-3HV) increased significantly up to a maximum value of 62.2 mol% after enforcing *phbC* and up to 74.5 mol% after the co-expression of the *phbC* and *zwf* genes. The amplified *zwf* gene alone did not seriously affect the 3HV molar fraction, as observed in a previous study where the G6PDH in *R. eutropha* was activated under oxidative pressure using methyl viologen [17].

The amplified *phbC* gene exhibited a more noticeable influence on the 3HV molar fraction compared to the *zwf* gene amplification, indicating a stronger direct effect from the PHB synthase compared to the effect of the β-ketothiolase and acetoacetyl-CoA reductase indirectly activated by the amplified *zwf* gene. The PHB synthase located in the membrane of the PHA granule would also seem to play a more critical role compared to the other two enzymes, β-ketothiolase and acetoacetyl-CoA reductase, located in the cytoplasm [1].

3.4. Effect of amplified phbC and zwf, and co-amplified phbC and zwf genes on intrinsic activities of enzymes related to P(3HB-3HV) biosynthesis and intrinsic NADPH level

The effect of the amplified *phbC* and *zwf* and co-amplified *phbC* and *zwf* genes on the PHB and P(3HB-3HV) biosynthesis was elucidated by measuring the intrinsic activities of β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, PHB synthase, and G6PDH after 30 h when the most active P(3HB-3HV) accumulation occurred. The NADPH level was also indirectly measured by the NADPH/(NADPH + $NADP^+$) ratio. The amplified *phbC* gene only influenced the intrinsic PHB synthase activity, which was 2.3 times higher compared to the parent strain, as shown in Table 2. The amplified *zwf* gene had a direct influence on the G6PDH activity, which exhibited a significant 3.3-fold increase, while indirectly influencing the β-ketothiolase and

Table 2
Intrinsic activities of enzyme related to P(3HB-3HV) biosynthesis, G6PDH, and the level of NADPH[a]

Strains	β-KT[b]	AACR[c]	PHBs[d]	G6PDH[e]	NADPH[f]
Parent *R. eutropha* H16	0.24 ± 0.03	0.28 ± 0.01	1.29 ± 0.14	0.88 ± 0.22	1.05 ± 0.11
Transformant *R. eutropha* AER5 amplifying *phbC* gene	0.25 ± 0.02	0.30 ± 0.01	2.88 ± 0.16	0.81 ± 0.19	0.97 ± 0.19
Transformant *R. eutropha* REZ amplifying *zwf* gene	0.31 ± 0.03	0.36 ± 0.01	1.25 ± 0.12	2.63 ± 0.25	1.61 ± 0.22
Transformant *R. eutropha* RCZ co-amplifying *phbC* and *zwf* gene	0.33 ± 0.03	0.40 ± 0.02	3.05 ± 0.18	2.50 ± 0.23	1.59 ± 0.20

Cells are cultivated in the nutrient-rich medium at the first stage, and then further cultivated in the nitrogen-free minimal medium containing valerate at pH 7.0, 30 °C for 30 h.

[a] Intrinsic activities are expressed as μmol of product generated per min per mg of cellular soluble protein and each value is average (±standard deviation) of three cultures.

[b] β-KT; intrinsic activity of β-ketothiolase.

[c] AACR; intrinsic activity of acetoacetyl-CoA reductase.

[d] PHBs; intrinsic activity of PHB synthase.

[e] G6PDH; intrinsic activity of glucose 6-phosphate dehydrogenase.

[f] NADPH; NADPH/(NADPH + $NADP^+$) ratio.

acetoacetyl-CoA reductase activities, which were 1.3 and 1.4 times higher, respectively. The enforcement of the *zwf* gene also made the NADPH level around 1.6 times higher during the fructose metabolism in the transformants *R. eutropha* REZ and RCZ.

The activation of the NADPH-dependent acetoacetyl-CoA reductase requiring NADPH as a cofactor seemed to be induced by the elevated NADPH level inside the *R. eutropha* transformants. The elevated NADPH level also indirectly activated the β-ketothiolase, inducing an acetyl-CoA flux toward the PHA biosynthetic pathway, which has a reversible relationship with acetoacetyl-CoA reductase.

The acetoacetyl-CoA reductase activated through the amplification of the *zwf* gene facilitated the supply of both monomers, i.e. 3HB from acetyl-CoA and 3HV from valerate. Meanwhile, the PHB synthase over-expressed by the *phbC* gene accelerated the polymerization reaction of 3HV with the 3HB monomer based on its broader monomer specificities, as observed in previous studies by the current authors [7,8]. It also indirectly facilitated the versatile conversion of the precursor compound valerate to 3HV, which remained at a low concentration because of its accelerated consumption. Finally, the co-enforcement of the *phbC* and *zwf* genes significantly influenced not only the enzymes related to P(3HB-3HV) biosynthesis, but also the NADPH level, thereby enhancing the biosynthesis of P(3HB-3HV).

3.5. Modulation of molar fraction of P(3HB-3HV) in R. eutropha transformants through control of fructose and valerate mixing ratios

Normally, valerate is supplemented as a precursor during the second stage of cultivation in the biosynthesis of P(3HB-3HV) as a source of the 3-HV monomer, while only a small amount of fructose is applied for the purpose of cell maintenance [8,29]. However, in the current work, a relatively large amount of fructose was supplied in the second stage to generate additional NADPH in the transformants REZ and RCZ, in which the pentose phosphate pathway had been activated based on the *zwf* gene dosage, generated from the metabolism of fructose.

Table 3 shows the effects of the valerate and fructose mixing ratios. The concentration and content of P(3HB-3HV) tended to increase when the fructose supplementation was increased, although the 3-HV molar fraction decreased. The amplified *zwf* gene was also less influential on both the P(3HB-3HV) content and the 3HV molar fraction, when compared to the *phbC* gene enforcement or *phbC* and *zwf* genes co-enforcement.

The transformant *R. eutropha* AER5 amplifying the *phbC* gene accumulated P(3HB-3HV) with a 3HV molar fraction of higher than 43.3 mol%, even with extremely high fructose supplementation corresponding to fructose and valerate mixing ratios of 15 and 5 g l^{-1}. Furthermore, a noticeably higher 3HV molar fraction of up to around 66.9 mol%, which was unobtainable from the parent strain *R. eutropha* H16, was achieved by enforcing the *phbC* gene with valerate as the only feed. Moreover, the 3HV molar fraction increased as high as 73.4 mol% in the transformant *R. eutropha* REZ co-expressing the *phbC* and *zwf* genes. Accordingly, a high molar fraction was achieved even when a considerable amount of fructose was supplied in the second stage of cultivation.

The over-supplemented fructose in the second stage can also be utilized for both maintaining the cells through the TCA cycle and the biosynthesis of 3HB through the

Table 3
Effect of mixing ratios of fructose and valerate on total cell mass, concentration and content of P(3HB-3HV), and the 3HV molar fraction

Fructose (g l^{-1})	Valerate (g l^{-1})	Strains	Total cell mass (g l^{-1})	P(3HB-HV) (g l^{-1})	P(3HB-HV) (%)	3HV fraction (mol%)
15.0	5.0	Parent *R. eutropha* H16	8.83	5.20	59.0	20.1
		R. eutropha with *phbC* gene	9.11	5.56	61.0	43.4
		R. eutropha with *zwf* gene	8.78	5.30	60.4	22.3
		R. eutropha with *phbC* and *zwf* gene	8.70	5.55	63.8	58.5
10.0	10.0	Parent *R. eutropha* H16	8.56	4.73	55.3	31.5
		R. eutropha with *phbC* gene	8.89	4.99	56.1	55.1
		R. eutropha with *zwf* gene	8.50	4.75	55.9	31.2
		R. eutropha with *phbC* and *zwf* gene	8.47	4.91	58.0	66.8
5.0	15.0	Parent *R. eutropha* H16	8.46	3.88	45.9	41.9
		R. eutropha with *phbC* gene	8.61	4.07	47.3	62.2
		R. eutropha with *zwf* gene	8.39	3.90	46.5	42.3
		R. eutropha with *phbC* and *zwf* gene	8.33	4.03	48.4	70.8
0.0	20.0	Parent *R. eutropha* H16	7.50	2.86	38.1	44.1
		R. eutropha with *phbC* gene	7.59	3.10	40.9	66.9
		R. eutropha with *zwf* gene	7.44	2.99	40.2	46.5
		R. eutropha with *phbC* and *zwf* gene	7.41	3.05	41.2	73.4

Cells were cultivated in the nutrient-rich medium at the first stage, and then further cultivated in the nitrogen-free minimal medium containing different mixing ratios of feeding solution at the second stage, pH 7.0, 30 °C for 30 h.

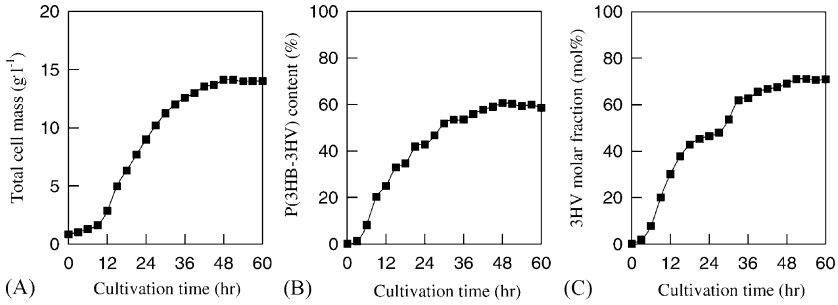

Fig. 4. Postulated mechanism for the modulation of the 3HV molar fraction in P(3HB-3HV) in the transformant *R. eutropha* co-enforcing the *phbC* and *zwf* genes.

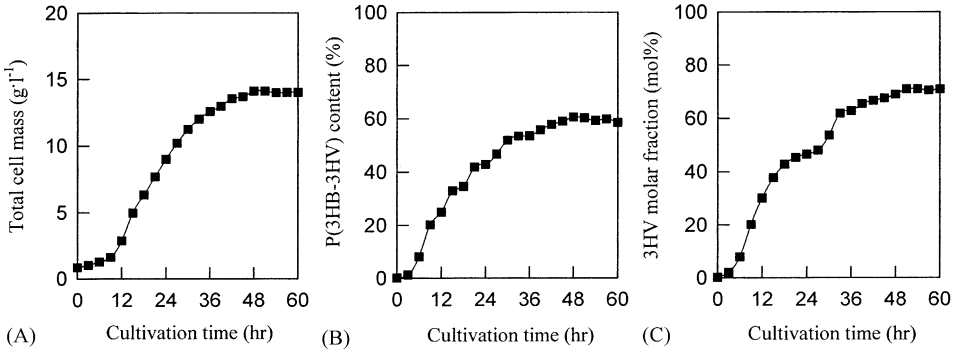

Fig. 5. pH-stat fed-batch cultivation of transformant *R. eutropha* co-enforcing *phbC* and *zwf* genes; total cell growth (A), content of P(3HB-3HV) (B), and 3HV molar fraction (C). Cells were cultivated in minimal medium, 30 °C, for 60 h in a 2.5 l fermentor, and feeding solution, composed of 10 g l^{-1} of valerate and fructose, was injected to maintain the culture broth at pH 7.0 constantly.

glycolysis pathway. The over-generated NADPH from the pentose phosphate pathway during the utilization of fructose by the amplified G6PDH seemed to activate the acetoacetyl-CoA reductase, thereby leading to the reduction of valeryl-CoA into the 3HV monomer, a higher generation of 3HV, and ultimately an increased biosynthesis of P(3HB-3HV). Consequently, this can play a significant role in reducing the production costs of P(3HB-3HV) based on diminishing the supplementation of the costly precursor compound valerate.

3.6. pH-stat fed-batch cultivation of transformant R. eutropha co-expressing phbC and zwf genes

To achieve the overproduction of P(3HB-3HV) with a high 3HV molar fraction, the transformant *R. eutropha* REZ co-expressing the *phbC* and *zwf* genes was cultivated using pH-stat fed-batch strategy. As such, the transformant was cultivated in a minimal medium, while feeding a solution composed of 10 g l^{-1} valerate and fructose to maintain a constant pH level of 7.0. As seen in Figs. 4 and 5, the total cell mass, P(3HB-3HV) content, and 3HV molar fraction in the P(3HB-3HV) after 60 h of pH-stat fed-batch cultivation increased up to maximum values of 14.0 g l^{-1}, 60.1%, and 71.9 mol%, respectively.

Previous efforts to achieve concomitant increments in the biosynthesis of P(3HB-3HV) and the modulation of its 3HV molar fraction were unsuccessful when only feeding the precursor valerate to the parent strain *R. eutropha* H16, whose genetic sources had not been manipulated [31,32]. However, these constraints of the parent strain were overcome when using the transformant *R. eutropha* RCZ, co-amplified polymerization, and NADPH over-generation reactions, which facilitated the simultaneous feeding of fructose and valerate. Optimized culture conditions for pH-stat fed batch cultivation still need to be determined for practical application of the transformant co-enforcing the *phbC* and *zwf* genes in the commercial production of the biodegradable copolymer P(3HB-3HV).

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