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Efficient Conversion of Acetate to 3-Hydroxypropionic Acid by Engineered *Escherichia coli*

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Abstract: Acetate, which is an abundant carbon source, is a potential feedstock for microbial processes that produce diverse value-added chemicals. In this study, we produced 3-hydroxypropionic acid (3-HP) from acetate with engineered *Escherichia coli*. For the efficient conversion of acetate to 3-HP, we initially introduced heterologous *mcr* (encoding malonyl-CoA reductase) from *Chloroflexus aurantiacus*. Then, the acetate assimilating pathway and glyoxylate shunt pathway were activated by overexpressing *acs* (encoding acetyl-CoA synthetase) and deleting *iclR* (encoding the glyoxylate shunt pathway repressor). Because a key precursor malonyl-CoA is also consumed for fatty acid synthesis, we decreased carbon flux to fatty acid synthesis by adding cerulenin. Subsequently, we found that inhibiting fatty acid synthesis dramatically improved 3-HP production (3.00 g/L of 3-HP from 8.98 g/L of acetate). The results indicated that acetate can be used as a promising carbon source for microbial processes and that 3-HP can be produced from acetate with a high yield (44.6% of the theoretical maximum yield).

Keywords: metabolic engineering; synthetic biology; 3-hydroxypropionic acid; microbial production; fatty acid synthesis; acetate

1. Introduction

Microbial conversion is a highly promising process for the production of diverse value-added chemicals and as an alternative to petroleum-based processes [1,2]. Specifically, it can utilize a variety of sugars such as glucose, galactose, xylose and glycerol, which are plentiful in nature and readily available as industrial waste, as a feedstock [3–5]. In addition to these sugars, acetate can be used as a carbon source. Acetate is cheap and greatly abundant, as it can be obtained from biomass hydrolysate or from the conversion of various single-carbon gases [6–8]. Therefore, the use of acetate may reduce the cost of feedstock and thereby facilitate the development of more economic processes. In this regard, several recent studies attempted to engineer microorganisms and demonstrated the successful conversion of acetate into value-added chemicals such as itaconic acid, succinic acid and fatty acid [9–11].

3-Hydroxypropionic acid (3-HP) is one of the important platform chemicals that can be produced by microbial fermentation [12,13]. As 3-HP consists of two functional groups (a hydroxyl and carboxylic

group), it can be easily converted to other chemicals (e.g., acrylic acid, acrylamide, and propiolactone) for which there are huge markets [12]. Due to its production from sugars, several metabolic pathways have been suggested to date [12,13]. While the representative route is the coenzyme-B_{12}-dependent dehydration of glycerol [14,15], it is only applicable when the feedstock is glycerol. Alternatively, 3-HP can be produced via the reduction of malonyl-CoA using malonyl-CoA reductase (Figure 1) [16–18]. This pathway is suitable for most carbon sources, including acetate, because malonyl-CoA is a universal intermediate in cells [16,17]. Additionally, this pathway does not require an expensive cofactor, coenzyme B_{12}, which is a potential hurdle for the economic production of 3-HP [18,19].

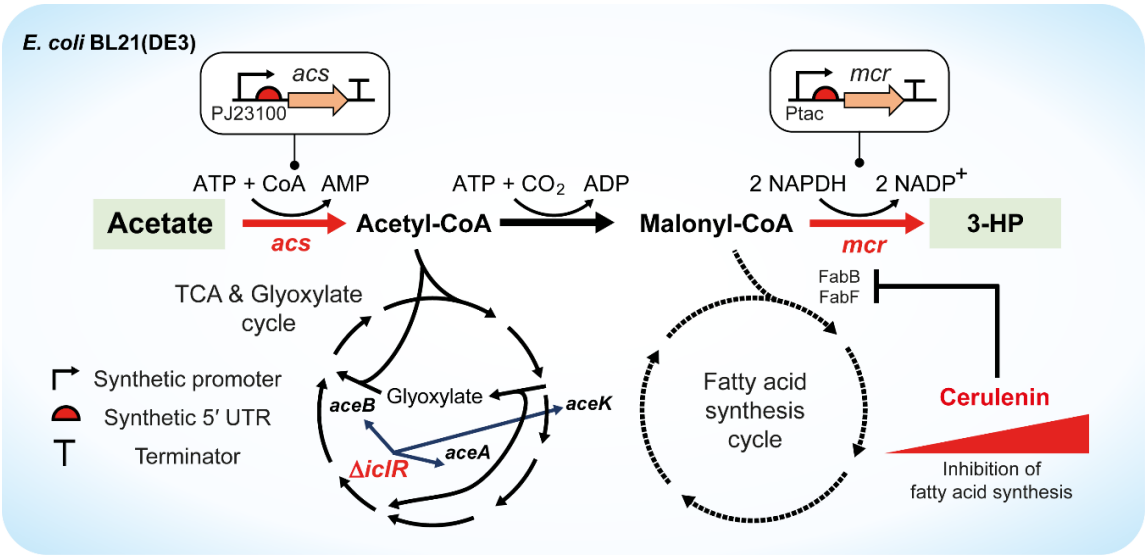

Figure 1. Schematic diagram of a metabolic pathway for the 3-HP production from acetate. 3-HP was synthesized by heterologous overexpression of *mcr* (encoding malonyl-CoA reductase from *C. aurantiacus*). For accelerated acetate assimilation, *acs* (encoding acetyl-CoA synthetase) was overexpressed and *iclR* (encoding glyoxylate shunt pathway repressor) was deleted. Deletion of *iclR* upregulates the expression of *aceA* (encoding isocitrate lyase), *aceB* (encoding malate synthase), and *aceK* (encoding isocitrate dehydrogenase kinase/phosphatase). Phosphorylation of isocitrate dehydrogenase (encoded by *icd*) results in its reduced activity. Different amounts of cerulenin were added to inhibit fatty acids biosynthesis.

To achieve the efficient conversion of acetate to 3-HP, acetate should be rapidly utilized. However, microorganisms slowly utilize acetate as a carbon source and exhibit reduced cell growth [20,21]. Therefore, acetate assimilation and biomass formation should be accelerated via genetic engineering [11,22–24]. Furthermore, once acetate is assimilated, malonyl-CoA has to be sufficiently converted to 3-HP. However, the primary use of malonyl-CoA in microorganisms is to synthesize fatty acids, which significantly reduces 3-HP production [25–27]. Thus, acetate consumption for fatty acid synthesis should be reduced to improve 3-HP production.

In this study, we demonstrated the efficient conversion of acetate to 3-HP by engineering a representative microorganism, *Escherichia coli*. Initially, we constructed a synthetic 3-HP production pathway with maximal expression of heterologous *mcr* (encoding malonyl-CoA reductase) from *Chloroflexus aurantiacus*. To accelerate acetate consumption, we activated both the acetate assimilating pathway and glyoxylate shunt pathway by amplifying *acs*, which encodes acetyl-CoA synthetase, and deleting *iclR*, which encodes the transcriptional repressor of the glyoxylate shunt pathway operon. Additionally, to enhance the conversion of malonyl-CoA to 3-HP, carbon flux into a competing pathway (i.e., fatty acid biosynthesis) was inhibited by adding cerulenin at different concentrations. Consequently, we demonstrated that 3-HP could be efficiently produced from acetate using the engineered microbial process.

2. Results

2.1. Heterologous Expression of mcr for 3-HP Production from Acetate

For 3-HP production, we introduced *mcr* from *C. aurantiacus* into the *E. coli* strain BL21(DE3) (Figure 1). To increase carbon flux toward 3-HP biosynthesis, we expressed *mcr* at a maximum level. Specifically, we developed a synthetic cassette with a strong inducible promoter (P_{tac}) and a synthetic 5′ UTR (Table 1) designed using UTR Designer [28] to ensure high transcription and translation levels. Furthermore, we used the pETDuet plasmid, which has a high copy number (~40 copies per cell), to ensure its overexpression. Additionally, we introduced 3 point mutations (N940V, K1106W, S1114R) known to enhance the activity of malonyl-CoA reductase [16].

Table 1. Synthetic 5′ UTR for gene expression.

Gene	5′ UTR sequence (5′—3′) [a]	Predicted Expression Level (a.u.)
mcr	AACAATTACTAGTAAGGAGAGGAGT	3,110,669.92
acs	AAAATCAGCGCCCAAGGAGTCACCG [b]	1,074,836.02

[a] 5′ UTR sequences were designed using UTR Designer [26]. [b] This sequence was originally designed in a previous study [9].

Following this, we cultivated the HJ1 strain (Table 2), which is BL21(DE3) with the constructed plasmid (pET-*mcr**), in modified minimal medium. After 48 h of fermentation, the HJ1 strain consumed 8.55 g/L of acetate and produced 95.7 mg/L of 3-HP (Figure 2). Although we successfully produced 3-HP from acetate with this engineered *E. coli* strain, the achieved titer was too low (1.49% of the theoretical maximum yield). Thus, the strain required further engineering to improve 3-HP production with efficient acetate utilization.

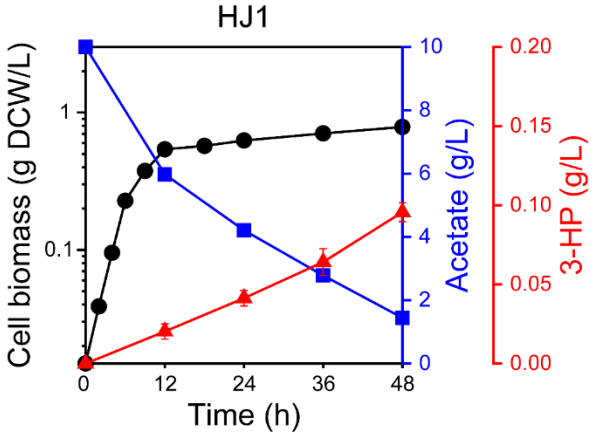

Figure 2. Time-course fermentation profile of the HJ1 strain. The left y-axis, right y-axis and right y-offset represent the cell biomass (g DCW/L), acetate (g/L) and 3-HP (g/L), respectively. The x-axis denotes time (h). Symbols: circles, cell biomass; squares, acetate; triangles, 3-HP. The error bars indicate standard deviations for measurements from three independent cultures. One OD_{600} unit corresponds to 0.31 g dry cell weight (g DCW/L).

2.2. Engineering the Acetate Assimilation and Glyoxylate Shunt Pathways

Compared to that of other sugars, the assimilation rate of acetate in microorganisms is relatively low [8]. Therefore, we investigated the effect of activating the acetate assimilation pathway on 3-HP production. To improve acetate uptake during the entire fermentation period, we expressed *acs* (encoding acetyl-CoA synthetase) with a synthetic expression cassette consisting of the strong constitutive promoter (P_{J23100}) and a synthetic 5′ UTR (Table 1). The synthetic expression cassette was inserted into the pACYCDuet plasmid, which has a moderate copy number (10–12 copies/cell), and

the pACYC-Acs plasmid was introduced into the HJ1 strain, resulting in the HJ2 strain. Compared to the HJ1 strain, the HJ2 strain showed increased acetate consumption (a 1.11-fold increase) and cell biomass (a 1.90-fold increase), indicating that acetate assimilation was successfully expedited by *acs* overexpression (Figure 3A,B). Furthermore, the improved acetate assimilation in the HJ2 strain led to a 1.75-fold increase in 3-HP production (0.17 g/L, Figure 3C).

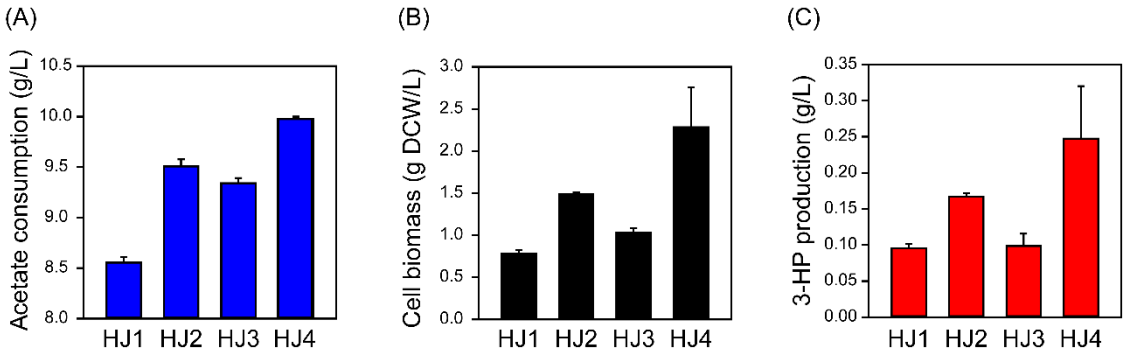

Figure 3. Genetic engineering to improve 3-HP production from acetate. Acetate consumption (g/L), cell biomass (g DCW/L) and 3-HP (g/L) production of the HJ1—4 strains after 48 h of fermentation. (**A**) Acetate consumption (g/L), (**B**) cell biomass (g DCW/L), and (**C**) 3-HP production (g/L) of engineered strains were compared. The error bars indicate standard deviations for measurements from three independent cultures.

We then evaluated the effect of activating the glyoxylate shunt pathway. Because the glyoxylate shunt pathway is responsible for a key anaplerotic reaction during acetate utilization, we expected that its activation would facilitate biomass formation and improve acetate consumption. To investigate this effect, we deleted chromosomal *iclR*, which is known to repress the expression of *aceBAK* in the glyoxylate shunt pathway [11,29,30], in the HJ1 strain. Similar to the results for the overexpression of *acs*, the acetate consumption and cell biomass of the resultant HJ3 strain were enhanced 1.09-fold and 1.31-fold, respectively (Figure 3A,B). These results indicated that *iclR* deletion successfully enhanced cell biomass synthesis from acetate, which resulted in increased overall acetate consumption.

When the overexpression of *acs* and deletion of *iclR* were combined, the acetate uptake rate (1.16-fold increase) and cell biomass (2.61-fold increase) were further enhanced (HJ4 strain, Figure 3A,B). Moreover, its 3-HP production was synergistically improved 2.54-fold (0.25 g/L) compared to that of the HJ1 strain (Figure 3C). Consequently, the combination of *acs* overexpression and *iclR* deletion resulted in the most significant improvement in acetate assimilation and 3-HP production. However, despite the improvement, only a small amount of acetate was converted to 3-HP (3.35% of the theoretical maximum yield), indicating that further flux control is required for the efficient conversion of acetate to 3-HP.

2.3. Improved 3-HP Production from Acetate by Inhibiting Fatty Acid Synthesis

Despite the elevated acetate consumption level, the 3-HP titer was still low, indicating the inefficient conversion of malonyl-CoA due to leakage toward fatty acid synthesis. Therefore, we decided to inhibit fatty acid synthesis to increase the intracellular malonyl-CoA pool for 3-HP production (Figure 1). Bacterial fatty acid synthesis can be inhibited by the addition of cerulenin, which binds to FabB and FabF and irreversibly inactivates them [31–33]. Thus, we cultivated the HJ4 strain with the addition of different levels of cerulenin (10, 25, 50 and 100 μM) to gradually reduce carbon flux from malonyl-CoA to fatty acids. As expected, the addition of cerulenin was unfavorable for cell growth (Figure 4A–C). On the other hand, notably, it was highly beneficial for 3-HP production, as the titers and yields were dramatically increased. Specifically, when 50 μM of cerulenin was added, the HJ4 strain produced 3.00 g/L of 3-HP while consuming 8.98 g/L of acetate (0.30 g/g, 44.6% of the maximum theoretical yield, Figure 4D). This titer was 12.0-fold higher compared to the 3-HP titer produced by the same

strain without cerulenin addition. These results indicated that 3-HP production from acetate was successfully improved by inhibiting fatty acid biosynthesis and that flux control around malonyl-CoA was critical for the efficient conversion of acetate to 3-HP.

Table 2. Bacterial strains and plasmids used in this study.

Name	Description	Source
Strains		
Mach1-T1^{R}	*E. coli* F^{-} φ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R($r_K{}^{-}m_K{}^{+}$) Δ*rec*A1398 *end*A1 *ton*A	Invitrogen
BL21(DE3)	*E. coli* F^{-} *omp*T *gal dcm lon hsd*SB (rB^{-} mB^{-}) λ(DE3)	Invitrogen
HJ1	BL21(DE3)/pET-*mcr**	This study
HJ2	BL21(DE3)/pET-*mcr**/pACYC-*acs*	This study
HJ3	BL21(DE3) Δ*iclR*/pET-*mcr**	This study
HJ4	BL21(DE3) Δ*iclR*/pET-*mcr**/pACYC-*acs*	This study
Plasmids		
PLB0110	Source of *mcr*	[15]
pETDuet	Expression vector, ColE1 ori, Amp^{R}	Novagen
pACYCDuet	Expression vector, p15A ori, Cm^{R}	Novagen
pKD46	Red recombinase expression vector, Amp^{R}	[32]
pFRT72$_{variant}$	Source of mutant FRT-kan^{R}-FRT	[33]
pCP20	FLP expression vector, Amp^{R}, Cm^{R}	[32]
pET-*mcr**	pETDuet/P_{tac}-SynUTR$_{mcr}$-*mcr* $^{N940V, K1106W, S1114R}$	This study
pACYC-*acs*	pACYCDuet/P_{BBa_J23100}-SynUTR$_{acs}$-*acs*	This study

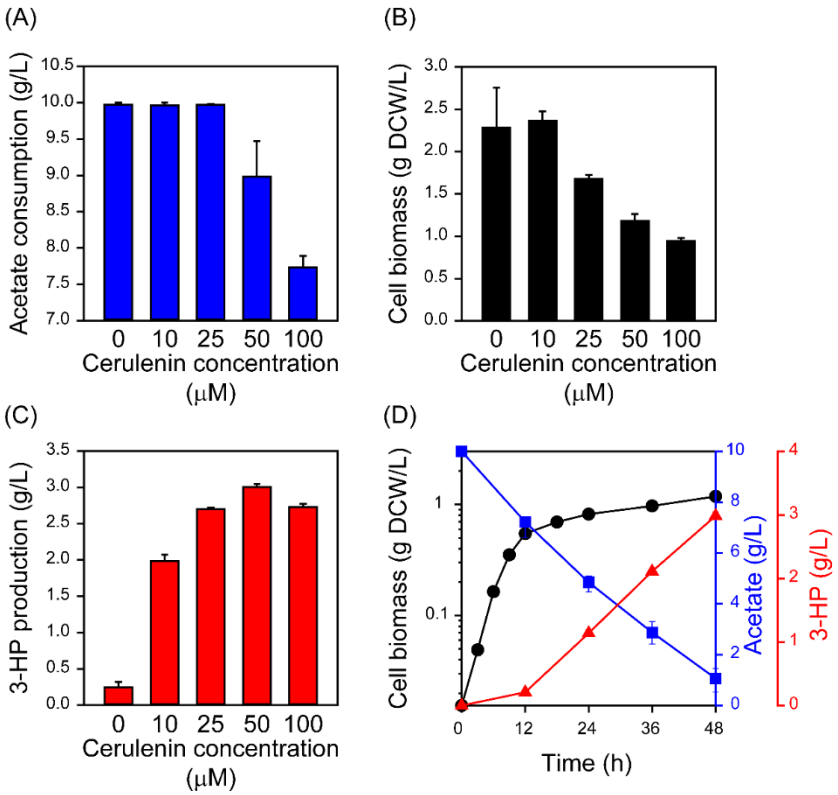

Figure 4. Improved 3-HP production with the addition of cerulenin. (**A**) Acetate consumption (g/L), (**B**) cell biomass (g DCW/L) and (**C**) 3-HP (g/L) production of the HJ4 strain with different cerulenin concentrations after 48 h fermentation. (**D**) Time-course fermentation profile (48 h) of the HJ4 strain with 50 μM of cerulenin in a modified acetate minimal medium. The left y-axis, right y-axis and right y-offset represent the cell biomass (g DCW/L), acetate (g/L) and 3-HP (g/L), respectively. The x-axis denotes time (h). Symbols: circles, cell biomass; squares, acetate; triangles, 3-HP. The error bars indicate standard deviations for measurements from three independent cultures.

3. Discussion

In this study, we developed a microbial process for the conversion of acetate to 3-HP. Initially, we introduced *mcr* at a maximum expression level to enable 3-HP production by an *E. coli* strain. In addition, acetate consumption was expedited by engineering the acetate assimilating pathway and glyoxylate shunt pathway. Furthermore, we increased carbon flux to the 3-HP production pathway by inhibiting fatty acid synthesis with the addition of cerulenin. These efforts allowed us to efficiently convert 8.98 g/L of acetate to 3.00 g/L of 3-HP. This is the first report on producing 3-HP from acetate, and the achieved yield (0.30 g/g, 44.6% of the maximum theoretical yield) was superior to the previously reported value (0.19 g/g) with glucose utilization [16].

To further improve the 3-HP production in *E. coli*, it could be more engineered. For example, the activity of heterologous malonyl-CoA reductase from *C. aurantiacus* could be improved by codon optimization of the *mcr* coding sequence. A number of previous studies have shown that codon optimization can elevate the activities of heterologous enzymes [34–36]. Thus, codon optimization of *mcr* would further enhance 3-HP production by expediting the conversion of malonyl-CoA to 3-HP. Furthermore, because production of 3-HP from acetate requires energy and reducing cofactor, their stable supplementation should be achieved by balancing between the TCA cycle and 3-HP synthesis. Therefore, the fine-tuning of flux toward the TCA cycle should be critical for 3-HP production. Moreover, based on the results obtained in this study, reduction of the flux to fatty acid synthesis may enhance the production of diverse malonyl-CoA-derived biochemicals in *E. coli*.

4. Materials and Methods

4.1. Reagents

Oligonucleotides, which are listed in Table 1, were synthesized by Cosmogenetech (Seoul, Korea). Plasmid DNA and genomic DNA were purified using Expin^{TM} Plasmid SV and Expin^{TM} Cell SV kits (GeneAll Biotechnology, Seoul, Korea). PCR products were purified using Expin^{TM} Gel SV kits (GeneAll). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Cerulenin was purchased from Cayman Chemical (Ann Arbor, MI, USA). 3-HP was obtained from Tokyo Chemical Industry (Tokyo, Japan). Other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Plasmid Cloning and Bacterial Strain Construction

All bacterial strains and plasmids used in this study are summarized in Table 2. Synthetic 5′ untranslated regions (5′ UTRs) were generated by UTR Designer [28] and are listed in Table 3. *E. coli* Mach1-T1^{R} (Invitrogen, Carlsbad, CA, USA) was used as a cloning host. To construct the pET-*mcr** plasmid, *mcr* was initially amplified from the PLB0110 plasmid [17] using the O-mcr-F1, O-mcr-F2, and O-mcr-B primers to attach a strong inducible promoter (P_{tac}) and a synthetic 5′ UTR. Then, the amplified fragment was digested with *BamH*I and *Xho*I endonucleases and inserted into the pETDuet plasmid. It should be noted that the coding sequence of *mcr* was modified from its original sequence via conventional site-directed mutagenesis using the O-N940V-F and O-N940V-B, O-K1106W-F and O-K1106W-B, and O-S1114R-F and O-S1114R-B primer pairs for the introduction of N940V(AAT to GTG), K1106W (AAG to TGG), and S1114R (AGT to CGT) mutations, respectively [16].

To construct the pACYC-*acs* plasmid, *acs* was amplified from the genomic DNA of *E. coli* BL21(DE3) and attached to the BBa_J23100 promoter obtained from the Registry of Standard Biological Parts (http://parts.igem.org) and a synthetic 5′ UTR using the O-Acs-F and O-Acs-B primers. The amplified fragment was digested using *EcoRI* and *SacI* restriction enzymes and introduced into the pACYCDuet plasmid.

Genome manipulation was conducted using the Lambda-Red recombination method with the pKD46 plasmid [37]. Chromosomal *iclR* was deleted by inserting a FRT-kan^{R}-FRT fragment that was

amplified using the R-iclR-F and R-iclR-B primer pair. The integrated kanamycin resistance gene was removed by expression of the flippase from the pCP20 plasmid.

Table 3. Oligonucleotides used in this study.

Name	Sequence (5′−3′)
O-mcr-F1	GGAATTGTGAGCGGATAACAATTACTAGTAAGGAGAGGAGT ATGAGCGGAACAGGACGACT
O-mcr-F2	GGATCCTTGACAATTAATCATCGGCTCGTATAATGTGTG GAATTGTGAGCGGATAACAATT
O-mcr-B	CTCGAGTGCGAAAAAACCCCGCCGAAGCGGGG TTTTTTGCGGCATGCTTACACGGTAATCGCCCGT
O-N940V-F	TATTACCTTGCCGACCGCAATGTCAGTGGTGAGACATTCC
O-N940V-B	GCGGTCGGCAAGGTAATAG
O-K1106W-F	ATTTCCGGGTAGCGCGCAAGATTGCCCTGAGTGATGGTG
O-K1106W-B	GCGCGCTACCCGGAAATG
O-S1114R-F	TGAGTGATGGTGCCAGTCTCGCGCTGGTCACTC
O-S1114R-B	AGACTGGCACCATCACTCAGGGC
O-acs-F	GAATTCTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC AAAATCAGCGCCCAAGGAGTCACCGATGAGCCAAATTCACAAACACA
O-acs-B	GAGCTCAAAAAAAACCCCGCCCTGTCAGGGGCGGGG TTTTTTTTTTTACGATGGCATCGCGATAG
R-iclR-F	TGCCACTCAGGTATGATGGGCAGAATATTGCC TCTGCCCGCCAGAAAAAGGCATGACCGGCGCGATGC
R-iclR-B	TAACAATAAAAATGAAAATGATTTCCACGAT ACAGAAAAAGGAGACTGTCGCTCAGCGGATCTCATGCGC
C-iclR-F	CAACATTAACTCATCGGATCAG
C-iclR-B	TCTATTGCCACTCAGGTATGATGGGC

4.3. Cultivation Methods

Cells were cultivated in a modified minimal medium consisting of 100 mM phosphate buffer (pH 7.0), 1.0 g/L NaCl, 1.0 g/L NH_4Cl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 1.0 g/L yeast extract. Pre-neutralized 10 g/L acetate with NaOH was used as a carbon source. To initiate a culture, a single colony was inoculated into 3 mL of the medium in a test-tube. After an overnight incubation, the turbid culture was refreshed by re-inoculating into fresh medium. When the OD_{600} reached ~1.0, the refreshed seed was transferred to a 25 mL medium in a 300 mL flask until an OD_{600} of 0.05 was obtained. Genes under the P_{tac} promoter were expressed by the addition of 0.1 mM IPTG when the OD_{600} reached 1. At this time, different amounts of cerulenin were also added to inhibit fatty acid synthesis. The pH was adjusted by adding 5 M of HCl with a 12 h interval during 48 h of culture. The plasmids were maintained by adding 50 μg/mL ampicillin, 34 μg/mL chloramphenicol, and 50 μg/mL streptomycin. All cell cultures were conducted with three biological replicates.

4.4. Analytical Methods

To monitor the cell growth, OD_{600} was measured using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). Acetate and 3-HP were quantified using an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Sunnyvale, CA, USA). To separate the metabolites, Aminex HPX-87H (Bio-Rad Laboratories, Richmond, CA, USA) was used with 5 mM H_2SO_4 solution as a mobile phase (0.6 mL/min). The temperature of the column oven was set to 14 °C. Refractive index signals were measured using a Shodex RI-101 detector (Shodex, Klokkerfaldet, Denmark).

4.5. Calculation of the Theoretical Maximum Yield

Initially, 1 mol acetate could be converted into 1 mol acetyl-CoA with the generation of 1 mol AMP from 1 mol ATP via the Acs pathway. The generation of 1 mol AMP was equivalent to the consumption of 2 mol ATP (Equation (1)). Next, 1 mol malonyl-CoA was produced from 1 mol acetyl-CoA with the consumption of an additional 1 mol ATP. Finally, 1 mol malonyl-CoA was converted into 1 mol 3-HP with the consumption of 2 mol NADPH (Equation (3)). Therefore, 3 mol ATP and 2 mol NADPH were required to produce 3-HP from acetate (Equation (4)). They could be obtained from the TCA cycle with the oxidation of 1 mol acetate (1 mol NADH is equivalent to 2.5 mol ATP; 1 mol FADH is equivalent to 1.5 mol ATP; 1 mol GTP is equivalent to 1 mol ATP; 1 mol NADH is equivalent to 1 mol NADPH) (Equations (1), (5) and (6)). Consequently, the production of 1 mol 3-HP required 2 mol acetate (Equation (7), 50% mol/mol and 0.75 g/g).

$$\text{Acetate} + 2\ \text{ATP} + \text{CoA} \rightarrow \text{Acetyl-CoA} + 2\ \text{ADP} + 2\ \text{P}_\text{i} \quad (1)$$

$$\text{Acetyl-CoA} + \text{ATP} + \text{CO}_2 \rightarrow \text{Malonyl-CoA} + \text{ADP} + \text{P}_\text{i} \quad (2)$$

$$\text{Malonyl-CoA} + 2\ \text{NADPH} \rightarrow \text{3-HP} + 2\ \text{NADP}^+ + \text{CoA} \quad (3)$$

$$\text{Acetate} + \text{CO}_2 + 3\ \text{ATP} + 2\ \text{NADH} \rightarrow \text{3-HP} + 3\ \text{ADP} + 3\ \text{P}_\text{i} + 2\ \text{NAD}^+ \quad (4)$$

$$\text{Acetyl-CoA} + 3\ \text{NAD}^+ + \text{FAD}^+ + \text{GDP} + \text{P}_\text{i} \rightarrow 2\ \text{CO}_2 + 3\ \text{NADH} + \text{FADH} + \text{GTP} + \text{CoA} \quad (5)$$

$$\text{Acetate} + 2\ \text{NADP}^+ \rightarrow 2\ \text{CO}_2 + 2\ \text{NADPH} + 3\ \text{ATP} \quad (6)$$

$$2\ \text{Acetate} \rightarrow \text{3-HP} + \text{CO}_2 \quad (7)$$

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

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