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Engineering *Corynebacterium glutamicum* for the Efficient Production of 3-Hydroxypropionic Acid from a Mixture of Glucose and Acetate via the Malonyl-CoA Pathway

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Abstract: 3-Hydroxypropionic acid (3-HP) has been recognized as one of the top value-added building block chemicals, due to its numerous potential applications. Over the past decade, biosynthesis of 3-HP via the malonyl-CoA pathway has been increasingly favored because it is balanced in terms of ATP and reducing equivalents, does not require the addition of costly coenzymes, and can utilize renewable lignocellulosic biomass. In this study, gene *mcr* encoding malonyl-CoA reductase from *Chloroflexus aurantiacus* was introduced into *Corynebacterium glutamicum* ATCC13032 to construct the strain Cgz1, which accumulated 0.30 g/L 3-HP. Gene *ldhA* encoding lactate dehydrogenase was subsequently deleted to eliminate lactate accumulation, but this decreased 3-HP production and greatly increased acetate accumulation. Then, different acetate utilization genes were overexpressed to reuse the acetate, and the best candidate Cgz5 expressing endogenous gene *pta* could effectively reduce the acetate accumulation and produced 0.68 g/L 3-HP. To enhance the supply of the precursor acetyl-CoA, acetate was used as an ancillary carbon source to improve the 3-HP production, and 1.33 g/L 3-HP could be produced from a mixture of glucose and acetate, with a 2.06-fold higher yield than from glucose alone. Finally, to inhibit the major 3-HP competing pathway-fatty acid synthesis, 10 μM cerulenin was added and strain Cgz5 produced 3.77 g/L 3-HP from 15.47 g/L glucose and 4.68 g/L acetate with a yield of 187 mg/g substrate in 48 h, which was 12.57-fold higher than that of Cgz1. To our best knowledge, this is the first report on engineering *C. glutamicum* to produce 3-HP via the malonyl-CoA pathway. The results indicate that the innocuous biosafety level I microorganism *C. glutamicum* is a potential industrial 3-HP producer.

Keywords: *Corynebacterium glutamicum*; 3-hydroxypropionic acid; malonyl-CoA pathway; metabolic engineering; acetate

1. Introduction

As an important building block chemical with wide applications in the chemical, food, agricultural and pharmaceutical industries [1–3], 3-hydroxypropionic acid (3-HP) has been designated as one of

the top value-added chemicals by the U.S. Department of Energy (DOE) both in 2004 and 2010 [1,2]. To avoid the serious environmental problems of unsustainable 3-HP synthesis from petrochemicals, increasing attention has been paid to microbial production of 3-HP from renewable feedstocks, such as glycerol, glucose, xylose or even fatty acids [4–6]. The glycerol pathway, malonyl-CoA pathway and β-alanine pathway are the most intensively studied routes for 3-HP biosynthesis [2,3,7]. The highest 3-HP titer was achieved using the glycerol pathway, from a cheap feedstock [8]. However, the dependence of this method on the expensive additive coenzyme B_{12} made it unfeasible for industrial production. Moreover, the imbalance of reducing power would also bring a heavy burden to cellular metabolism [9]. To avoid these shortcomings, the malonyl-CoA pathway and β-alanine pathway were proposed as alternative routes, since both are balanced in terms of ATP and reducing equivalents, independent of costly coenzyme additives, and can utilize renewable lignocellulosic biomass [9–11]. Although both the malonyl-CoA pathway and β-alanine pathway showed great potential for 3-HP production, the β-alanine pathway seems to be relatively complex for engineering, since intermediate metabolites, such as L-glutamate and pyruvate should be timely regenerated. The malonyl-CoA pathway is, therefore, more favored for 3-HP biosynthesis, and has been successfully introduced into *Saccharomyces cerevisiae* [12–14], *Schizosaccharomyces pombe* [15], *Escherichia coli* [5,11,16,17], *Methylobacterium extorquens* [18], *Pyrococcus furiosus* [19,20] and cyanobacteria [21,22].

Corynebacterium glutamicum is one of the most important amino acid industrial producers with the GRAS (generally regarded as safe) status. It has been widely used for the production of various chemicals, fuels and materials over the last decades [23,24], and exhibited excellent performance in the bioconversion of organic acids [25,26]. To evaluate the potential of *C. glutamicum* for 3-HP production, Chen, et al. [27] initially engineered a recombinant *C. glutamicum* strain capable of producing 62.6 g/L 3-HP with a yield of 0.51 g/g glucose in fed-batch fermentation via the glycerol pathway. This was the highest titer and yield of 3-HP from sugars and highlighted *C. glutamicum* as a competitive 3-HP producer. However, the drawbacks of the vitamin B_{12}-dependent glycerol pathway still restricted its industrial application. Therefore, further evaluation of 3-HP production in *C. glutamicum* via the malonyl-CoA pathway was suggested.

In this study, *C. glutamicum* was engineered to produce 3-HP via the malonyl-CoA pathway. Several strategies, including introducing heterogenous malonyl-CoA pathway, blocking by-products synthesis pathway and enhancing acetate reuse pathway were carried out to improve 3-HP production. Strikingly, acetate was found to be a beneficial co-substrate for 3-HP production. The optimal engineered strain Cgz5 (Figure 1) achieved a 3-HP titer of 3.77 g/L with a yield of 187 mg/g substrate in batch fermentation.

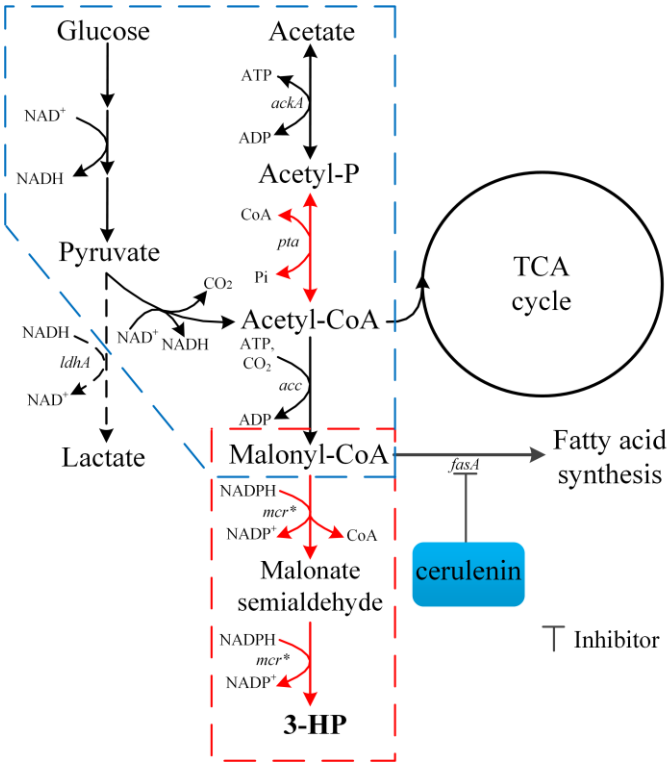

Figure 1. The 3-HP biosynthesis pathway of *C. glutamicum*. Black arrows indicate the native pathways of *C. glutamicum*, black dashed lines indicate deleted pathways, red arrows indicate genes which were overexpressed or introduced, the red dashed box shows the malonyl-CoA reduction module, and the blue dashed box shows the malonyl-CoA synthesis module. Acetyl-P, acetyl phosphate; 3-HP, 3-hydroxypropionic acid; *ldhA*, lactate dehydrogenase A; *ackA*, acetate kinase A; *pta*, acetyl phosphate transferase; *acc*, acetyl-CoA carboxylase; *mcr*, malonyl-CoA reductase; *fasA*, fatty acid synthetase A.

2. Results

2.1. Construction of a Malonyl-CoA Synthesis Pathway and Disruption of Lactate Synthesis

The biosynthesis of 3-HP via the malonyl-CoA pathway can be conceptually separated into two parts, a malonyl-CoA synthesis module and a malonyl-CoA reduction module (Figure 1). *C. glutamicum* has a native metabolic route from glucose to malonyl-CoA [28], but lacks the malonyl-CoA reductase. To produce 3-HP, malonyl-CoA reductase (Mcr) with three known mutations (N940V, K1106W and S1114R) (encoded by gene *mcr**), which could increase the activity of C-terminal of Mcr by 5.54-fold [11], was first introduced into *C. glutamicum*. The *mcr** gene from *C. aurantiacus* was codon-optimized, synthetized and cloned into the pXMJ19 plasmid under the control of the strong IPTG-inducible promoter P_{tac}. The resulting plasmid pX-mcr* was introduced into *C. glutamicum* ATCC 13032 to generate the strain Cgz1 (Table 1).

As shown in Figure 2a, the strain Cgz1 produced 0.28 g/L 3-HP when glucose was exhausted at 24 h, illustrating that the 3-HP synthesis pathway was successfully constructed in *C. glutamicum*. The major by-product lactate reached a high concentration of 9.48 g/L, and was subsequently reused as a secondary carbon source to support cell diauxic-growth. After a further 12 h of cultivation, 3.89 g/L lactate was consumed, but the 3-HP titer was merely increased to 0.30 g/L.

Table 1. Strains and plasmids used in this study.

Strains or Plasmids	Relevant Characteristics	Source/Reference
E. coli DH5α	Host for plasmid construction	Invitrogen
ATCC 13032	*C. glutamicum* wild type, biotin auxotrophic	ATCC[a]
Cgz1	ATCC 13032; pX-mcr*	This study
Cgz2	ATCC 13032△*ldhA*	[29]
Cgz3	Cgz2; pX-mcr*	This study
Cgz4	Cgz2; pX-mcr*, pEC-XK99E	This study
Cgz5	Cgz2; pX-mcr*, pEC-pta	This study
Cgz6	Cgz2; pX-mcr*, pEC-pta-ackA	This study
Cgz7	Cgz2; pX-mcr*, pEacsA	This study
Plasmids		
pXMJ19	Cm^R, P_{tac}, *lacIq*, *C. glutamicum*/*E. coli* shuttle vector	[30]
pX-mcr*	pXMJ19 with inserted *mcr** gene	This study
pEC-XK99E	Kan^R; P_{trc}, *lacIq*; *C. glutamicum*/*E. coli* shuttle vector	[31]
pEC-pta	derived from pEC-XK99E, for the overexpression of *pta*	This study
pEC-pta-ackA	derived from pEC-XK99E, for the overexpression of *pta* and *ackA*	This study
pEacsA	derived from pEC-XK99E, for the overexpression of *acsA*	[32]

Cm, chloramphenicol; Kan, kanamycin; [R] resistance; [a] ATCC, American Type Culture Collection.

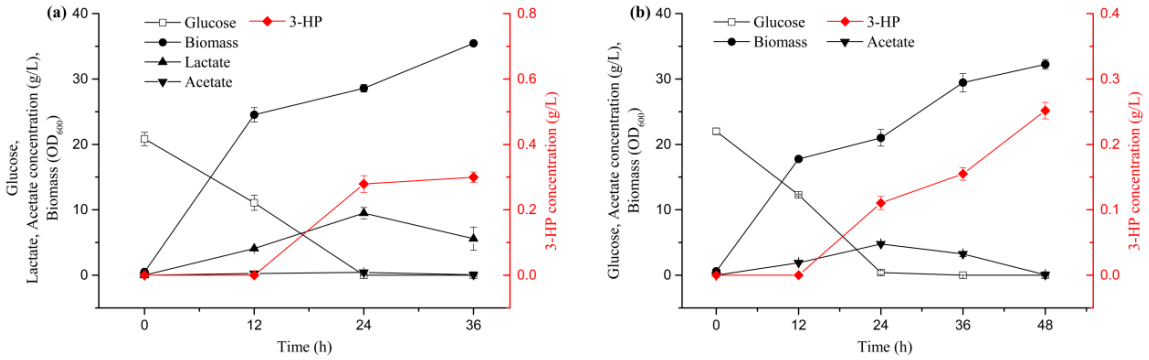

Figure 2. Time profiles of the biomass (filled circles), glucose (open squares), acetate (filled downward triangles), lactate (filled upward triangles) and 3-HP (red filled diamonds) concentrations of strains Cgz1 and Cgz3 grown with 20 g/L glucose. (**a**) Strain Cgz1. (**b**) Strain Cgz3. Error bars indicate the standard deviations from three independent cultures.

To eliminate the accumulation of lactate, the *ldhA* gene encoding the lactate dehydrogenase was deleted to construct the strain Cgz2. The plasmid pX-mcr* was then introduced into Cgz2, resulting in the strain Cgz3. As shown in Figure 2b, strain Cgz3 produced almost no lactate, but the 3-HP titer at 24 h was only 0.11 g/L. In this strain, acetate became the major by-product in place of lactate, with a titer of 4.79 g/L at 24 h, which was 11.19 times higher than that of Cgz1. The accumulated acetate could be reused as a carbon source, and similar diauxic growth was also observed in strain Cgz3 (Figure 2b). The 3-HP titer was increased to 0.15 g/L with the consumption of 1.55 g/L acetate from 24 h to 36 h and the yield of 0.097 g 3-HP/g acetate (0.029 mol-carbon/mol-carbon). While further deletion of major acetate production pathway genes, including *pta-ackA*, *actA* and *pqo*, dramatically lowered acetate accumulation, the 3-HP synthesis remained unacceptably low (data not shown). Notably, the 3-HP production was delayed at the first 12 h in both strain Cgz1 and Cgz3, which might be owing to that the major intracellular carbon fluxes were more easily directed to by-products (lactate or acetate) rather than 3-HP synthesis. While, in the following 12 h, 3-HP started to accumulate and was further produced after glucose was exhausted at 24 h with reuse of lactate or acetate. However, it is noteworthy to mention that 3-HP carbon mole yield from acetate (0.029 mol-C/mol-C, C-moles of increased 3-HP divided by C-moles of consumed acetate from 24 h to 36 h) was drastically higher than from lactate (0.005 mol-C/mol-C, C-moles of increased 3-HP divided by C-moles of consumed lactate from 24 h to 36 h). Consequently, we decided to upregulate the acetate reuse pathway, involving

phosphotransacetylase, acetate kinase and acetyl-CoA synthetase, as an alternative strategy to improve 3-HP production.

2.2. Enhancement of Acetate Reuse for 3-HP Production

Phosphotransacetylase and acetate kinase, encoded by the endogenous *pta-ackA* operon, are considered to constitute the main acetate utilization pathway in *C. glutamicum* [33]. Moreover, our previous work showed that the introduction of *acsA* from *B. subtilis*, encoding acetyl-CoA synthetase, can effectively improve acetate recycling in *C. glutamicum* [32]. Therefore, *pta*, *pta-ackA* and *acsA* were respectively overexpressed in the strain Cgz3 using the plasmid pEC-XK99E with the strong inducible promoter P_{trc}. The resulting strains Cgz5, Cgz6, and Cgz7, as well as the control strain Cgz4 harboring pEC-XK99E, were evaluated for 3-HP production.

As shown in Figure 3a, the strains Cgz5 and Cgz6 showed reduced acetate accumulation at both 24 h and 36 h, which indicated that acetate reuse might be successfully enhanced. However, when using acetate alone as substrate, no obvious difference was observed in strains Cgz4, Cgz5 and Cgz6 (Figure S1). Compared with simultaneously overexpressing the *pta* and *ackA* genes, simply overexpressing *pta* led to a 10% higher 3-HP titer (0.66 versus 0.60 g/L) at 48 h. Unexpectedly, the acetate reuse of strain Cgz7 was not improved, and its acetate accumulation at 36 h was even 14% higher than that of strain Cgz4. Moreover, the cell growth of strain Cgz7 was obviously inhibited, and the OD_{600} at 48 h was 23.43, which was 30.1% lower than that of Cgz4 (Figure 3b). This inhibitory effect on cell growth was consistent with our previous result in a succinate-producing strain [32], indicating that overexpression of *acsA* would bring a heavy metabolic burden on cell metabolism. However, all the strains achieved an increased 3-HP titer compared with the control strain Cgz4, and strain Cgz5 with the highest 3-HP titer was chosen for further investigation.

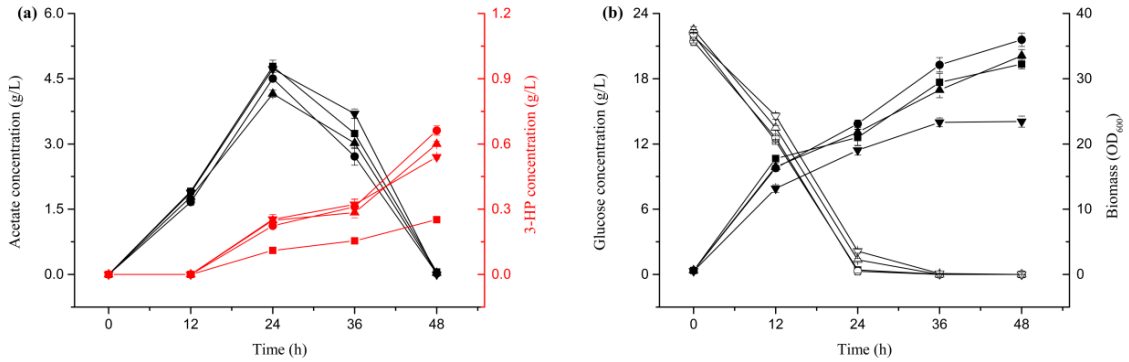

Figure 3. Time profiles of batch fermentations of strains Cgz4 (squares), Cgz5 (circles), Cgz6 (upward triangles) and Cgz7 (downward triangles) with 20 g/L glucose. (**a**) Time profiles of acetate accumulation (black filled symbols) and 3-HP production (red filled symbols). (**b**) Time profiles of glucose consumption (open symbols) and cell growth (filled symbols). Error bars indicate the standard deviations from three independent cultures.

2.3. 3-HP Production from a Mixture of Glucose and Acetate

It has been reported that the intracellular concentration of acetyl-CoA, the key precursor of malonyl-CoA, increased 6-fold when using acetate instead of glucose as the sole carbon source [34]. To evaluate the effect of acetate on 3-HP production, strain Cgz5 was cultured in a series of media containing 20 g/L total carbon sources with different ratios of glucose to acetate.

As shown in Table 2, the 3-HP titers and yields at 48 h were increased to some degree with the addition of acetate at all tested concentrations. The 3-HP titer and yield were significantly increased to 1.33 g/L (1.96-fold) and 66 mg/g substrate (2.06-fold) when the initial percentage of acetate in total carbon sources was increased from 0 to 25%. At higher acetate ratios, a significant inhibitory effect on cell growth was observed, and the substrate utilization was impeded at the same time. The 3-HP

yield at 48 h was improved from 66 to 73 mg/g substrate when the initial acetate ratio was increased from 25 to 50%, but the corresponding titer was deceased to 1.21 g/L and was not further increased when all the carbon sources were consumed at 60 h (data not shown). The cell growth and substrate consumption rate decreased concomitantly with the increasing ratio of acetate, which was consistent with a previous report [34]. Considering substrate utilization and 3-HP production, an initial acetate ratio of 25% was chosen for further investigation.

Table 2. Production of 3-HP by strain Cgz5 in media with different initial acetate ratios.

Initial Actate Ratio (%)	Consumption of Substrates (g/L)		OD_{600}	3-HP Titer (g/L)	3-HP Yield (mg/g Substrate)
	Glucose	Acetate			
0	21.56 ± 0.16	0	34.52 ± 0.39	0.68 ± 0.01	32 ± 0.46
25	15.10 ± 0.31	5.02 ± 0.12	33.56 ± 0.80	1.33 ± 0.04	66 ± 3.60
50	10.04 ± 0.47	6.56 ± 0.35	27.14 ± 0.47	1.21 ± 0.05	73 ± 2.10
75	5.67 ± 0.20	10.11 ± 0.12	21.38 ± 0.12	0.89 ± 0.05	56 ± 3.40
100	0	13.70 ± 0.22	16.45 ± 0.11	0.63 ± 0.01	46 ± 3.20

Error bars indicate the standard deviations from three independent cultures.

2.4. Enhancement of 3-HP Production by Inhibiting the Fatty Acid Synthesis Pathway

Despite the fact that 3-HP production was improved by co-feeding glucose and acetate, its titer was still low. Fatty acid synthesis, which is essential for cell growth, competes for intracellular malonyl-CoA during microbial 3-HP production via the malonyl-CoA pathway [9]. The addition of cerulenin, which can selectively inhibit fatty acid synthesis, was reported to be an effective method to balance the synthesis of fatty acids and target products derived from malonyl-CoA in *C. glutamicum* [35,36]. To evaluate whether fatty acid synthesis is also a competing pathway for 3-HP production in *C. glutamicum*, different concentrations of cerulenin (10, 30, 60 and 100 μM) were added during the fermentation of strain Cgz5 at 6 h.

As expected, with the increase of cerulenin concentration, cell growth was gradually inhibited (Figure 4). However, the 3-HP concentration was drastically increased to 3.77 g/L when 10 μM cerulenin was added, representing a 2.83-fold increase over the result obtained without the inhibitor. While a further increase of cerulenin concentration no longer improved the 3-HP titer, 3-HP production was enhanced by the addition of cerulenin at all concentrations compared to the condition without cerulenin addition, which indicated that inhibition of fatty acid synthesis indeed increased 3-HP production significantly.

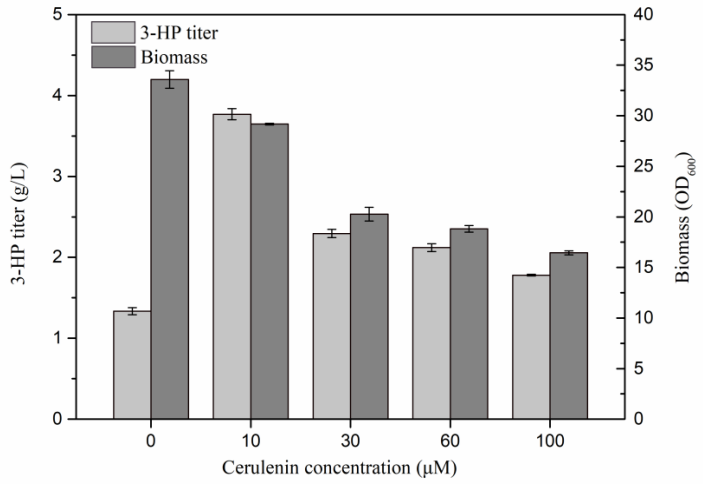

Figure 4. 3-HP titers and biomass (OD_{600}) of strain Cgz5 grown in a medium comprising 15 g/L glucose and 5 g/L acetate with different cerulenin concentrations at 48 h. Error bars indicate the standard deviations from three independent cultures.

As shown in Figure 5a, without cerulenin addition, strain Cgz5 preferentially consumed glucose, however, with the addition of 10 μM cerulenin, strain Cgz5 effectively co-utilized glucose and acetate (Figure 5b), and produced 3.77 g/L 3-HP with a yield of 187 mg/g substrate from a mixture of 15.47 g/L glucose and 4.68 g/L acetate.

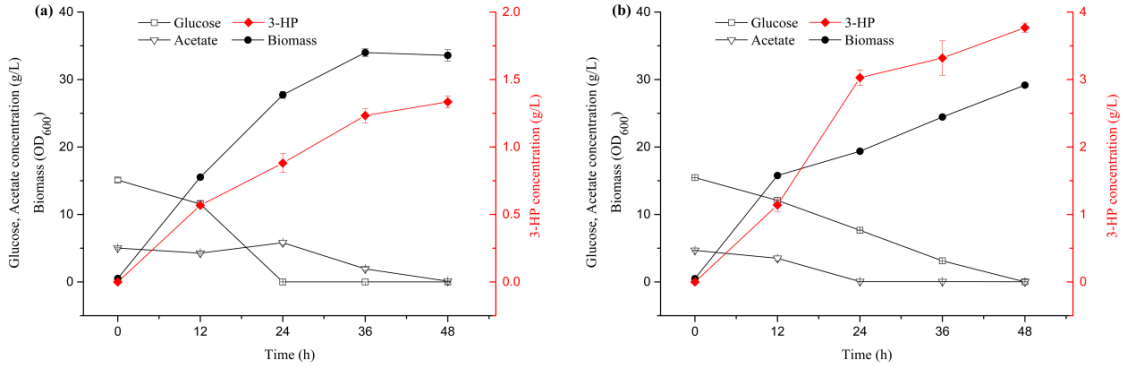

Figure 5. Time profiles of the biomass (filled circles), glucose (open squares), acetate (open downward triangles) and 3-HP (red filled diamonds) concentrations of strain Cgz5 grown in medium with an initial 15 g/L glucose and 5 g/L acetate (**a**) without cerulenin, and (**b**) with 10 μM cerulenin being added at 6 h. Error bars indicate the standard deviations from three independent cultures.

3. Discussion

To construct the malonyl-CoA pathway in *C. glutamicum*, a codon-optimized *mcr* gene with three reported mutations [11] was introduced to enable 3-HP biosynthesis from glucose. Subsequently, the major by-product lactate was eliminated by deleting the *ldhA* gene, but acetate rather than 3-HP increased dramatically. Deletion of acetate synthesis genes was initially tested in an attempt to redirect the carbon flux toward 3-HP, but the 3-HP titer of the corresponding knockout strains was even lower (data not shown). As shown in Figure 2, *C. glutamicum* was able to reuse both the accumulated lactate and acetate to support cell growth and 3-HP production after glucose was exhausted. It is worth mentioning that acetate was significantly more conducive to 3-HP production than lactate (0.029 versus 0.005 mol-C/mol C). The reason might be that lactate was redirected to pyruvate, while acetate was converted to acetyl-CoA [33,37], which is a direct precursor of malonyl-CoA, and therefore, more favorable for 3-HP production.

To accelerate acetate reuse, the acetate kinase-phosphotransacetylase route and acetyl-CoA synthetase route were individually overexpressed. Overexpression of *pta* indeed accelerated acetate reuse and improved 3-HP production. However, further overexpression of *ackA* did not result in a better performance, indicating that acetate kinase might not be a limiting step. The strain overexpressing *acsA* did not show any improvement of acetate reuse, which was different from our previous result in the succinate producer ZX1 [32]. The major acetate metabolism genes (*pta-ackA*, *catA* and *pqo*) were deleted in strain ZX1, leading to decreased acetate accumulation, as well as the ability to reuse acetate. By contrast, strain Cgz5 still harbors the complete set of acetate utilization genes. Therefore, the expression of a heterologous acetyl-CoA synthetase would play a more important role in the acetate utilization pathway of strain ZX1 than that of strain Cgz5.

Acetate, once a nuisance during microbial fermentation from sugars, is increasingly favored as an alternative carbon source for bioconversion process, since it is cheap, and could be obtained from cheap or freely available resources, such as methane or CO_2 [38,39]. Recently, Lee, et al. [40] constructed a recombinant *E. coli* strain capable of producing 0.25 g/L 3-HP from acetate with a yield of 25 mg/g acetate via the malonyl-CoA pathway. Notably, the recombinant *C. glutamicum* strain Cgz5 developed in this study was a better 3-HP producer from acetate, with a titer of 0.63 g/L and a yield of 46 mg/g acetate. Strikingly, mixtures of glucose and acetate were better for 3-HP production than acetate alone (Table 2). Different from the result that almost no 3-HP was detected at 12 h in Cgz5 on glucose alone

(Figure 3a), the delay in 3-HP production in Cgz5 was eliminated, and 0.57 g/L 3-HP was accumulated at 12 h when using glucose and acetate as co-substrate (Figure 5a). This might be owing to that the initial carbon fluxes were more easily directed to acetate synthesis in Cgz5, while, when using glucose and acetate as co-substrate, the acetate synthesis was inhibited, and intracellular acetyl-CoA was, therefore, saved for 3-HP production.

It was reported that wild-type *C. glutamicum* could simultaneously utilize 6.3 g/L glucose and 7.2 g/L acetate [34]. However, as shown in Figure 5a, an obvious carbon catabolite repression (CCR) effect was observed when strain Cgz5 was grown on 15.10 g/L glucose and 5.02 g/L acetate. Acetate was initially consumed as a co-substate with glucose during the first 12 h, but its concentration was increased by 36.0% when glucose was exhausted in the following 12 h (Figure 5a). Hence, it appeared that the high initial glucose concentration might have caused an acetate overflow metabolism. To confirm whether the ratio of glucose to acetate or the concentration of glucose should be responsible for this, batch fermentation of strain Cgz5 without cerulenin addition from 5 g/L glucose and 5 g/L acetate or 10 g/L glucose and 10 g/L acetate was carried out. As shown in Figure S2a, the two substrates could be simultaneously utilized with low glucose and acetate concentrations. However, when feeding 10 g/L glucose and 10 g/L acetate, the sequential usage of glucose and acetate was clearly observed. Therefore, it seemed like that the concentrations of glucose and acetate played an important role in simultaneous co-utilization of glucose and acetate in *C. glutamicum*. However, the problems of acetate re-accumulation (Figure 5a), as well as the inhibitory effects of high acetate concentrations (Table 2), could likely be solved by maintaining a low total substrate concentration using continuous fermentation control.

The inhibitor to fatty acid synthesis cerulenin was used to improve 3-HP production in strain Cgz5. Unexpectedly, the addition of 10 μM cerulenin eliminated the effect of CCR in strain Cgz5 (Figure 5b), so that glucose and acetate could be simultaneously utilized. Acetate was exhausted before glucose, and was not further re-accumulated during the following metabolism of residual glucose. Similar results were also observed at other cerulenin concentrations (Figure S3–S5), but the underlying mechanism remains unknown. Although the 3-HP titer was dramatically increased by adding 10 μM cerulenin (Figure 4), it may be possible to further increase the 3-HP titer by optimizing the amount and timing of cerulenin addition. However, cerulenin is too expensive for industrial application, and future studies should concentrate on weakening fatty acid synthesis through genetic engineering.

Until now, such efforts had been paid to malonyl-CoA pathway on 3-HP production. As reviewed by Liu et al. [9], early researches on malonyl-CoA-mediated 3-HP production using model microorganisms, such as *E. coli* and *S. cerevisiae*, could only obtain strains whose titers were below 1 g/L and productivities were below 10 mg/L/h. Besides proof of concept work on milligram per liter level of 3-HP titer using methanol or CO_2 [18,21,22], new host, such as *S. pombe* with good 3-HP tolerance, was recently engineered to produce 3-HP from glucose and acetate via malonyl-CoA pathway [15]. The optimal strain SPHP334 could accumulate 3.5 g/L 3-HP at 102 h in a flask culture, while its yield was below 23 mg/g substrate from initial 150 g/L glucose and 1.2 g/L acetate. Therefore, the final strain Cgz5 in this study demonstrated its potential in 3-HP production via malonyl-CoA pathway with a titer of 1.33 g/L, a yield of 66 mg/(g mixture of glucose and acetate) and productivity of 28 mg/L/h.

4. Materials and Methods

4.1. Reagents

Primers were synthesized by GENEWIZ (Suzhou, China). Plasmids were extracted using the Axyprep^{TM} Plasmid Miniprep Kit (Axygen, New York, NY, USA). DNA was purified using the SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China). The 3-HP standard (30% in water) was purchased from Tokyo Chemical Industry (Tokyo, Japan). BHI Broth was purchased from Hopebio (Qingdao, China). Yeast extract and tryptone were purchased from OXOID (Hants, UK).

MOPS (3-morpholinopropanesulfonic acid) was purchased from Solomen (Tianjin, China). Other reagents were purchased from Sangon Biotech (Shanghai, China).

4.2. Construction of Strains and Plasmids

The original strain was *C. glutamicum* ATCC 13032. All engineered strains used in this study are listed in Table 1. All primers are listed in Table 3. *E. coli* DH5α was used as the host for plasmid construction, and was cultivated in lysogeny broth (LB) medium.

Table 3. Primers used in this study.

Name	Sequence (5′-3′)
pX-mcr-F	TTCGGTCTAGAAAAGGAGGACAACCATGTCCGGCACTGGCCGTTTAGCTG
pX-mcr-R	TCCAAGAGCTCTTACACGGTGATAGCACGACCAC
pEC-pta-F	ATTCGGAGCTCAAAGGAGGACAACCATGTCTGACACACCGACCTCAGCTCT
pEC-pta-R	TTCAAGGTACCTTAGCTGCGTCCTCCTGCCTGAATTGCTG
pEC-ackA-F	TCAGGGGATCCAAAGGAGGACAACCATGGCATTGGCACTTGTTTTGAA
pEC-ackA-R	AATCGTCTAGACTAAGCGAACTTCACCGCGTACCTAGC

The restriction sites are underlined.

The *mcr* gene (NCBI-protein ID: AAS20429.1) was synthesized by GENEWIZ (Suzhou, China), and three reported mutations (N940V, K1106W, S1114R) [11] were introduced to improve enzyme activity. To construct the pX-mcr* plasmid, the primer pair pX-mcr-F/R was used to amplify the mutant *mcr* gene. The resulting fragment was digested with *Xba*I and *Sac*I, and ligated into the pXMJ19 plasmid cut with the same endonucleases. The pEacsA plasmid was preserved in our lab [32]. The *pta* and *ackA* genes were amplified from the *C. glutamicum* genome. The *pta* coding sequence was cloned between the *Sac*I/*Bam*HI restriction sites of pEC-XK99E to obtain pEC-pta; that of *ackA* was cloned between the *Bam*HI/*Xba*I restriction sites of pEC-pta to obtain pEC-pta-ackA. All constructs used the ribosome binding site AAAGGAGGACAACC.

4.3. Culture Conditions

E. coli DH5α was incubated at 37 °C and 220 rpm in test-tubes containing 5 mL of LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl). For strains containing plasmids, 40 μg/mL kanamycin and/or 10 μg/mL chloramphenicol was added to the medium.

For the pre-cultivation of *C. glutamicum*, a single colony was used to inoculate 5 mL of BHI broth (74 g/L). After overnight cultivation, 1 mL of the resulting seed culture was transferred into a 500 mL flask containing 50 mL CGIII medium (10 g/L yeast extract, 10 g/L tryptone, 21 g/L MOPS, 2.5 g/L NaCl, pH 7.0) supplemented with 20 g/L glucose. When the OD_{600} reached 15–20, this culture was used to inoculate a 500 mL flask containing 50 mL of modified CGXII medium (20 g/L $(NH_4)_2SO_4$, 5 g/L urea, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 21 g/L MOPS, 10 mg/L $CaCl_2$, 10 g/L yeast extract, 10 mg/L $FeSO_4{\cdot}7H_2O$, 0.1 mg/L $ZnSO_4{\cdot}7H_2O$, 0.2 mg/L $CuSO_4{\cdot}5H_2O$, 20 μg/L $NiCl_2{\cdot}H_2O$, 0.2 mg/L biotin, pH 7.0) supplemented with 20 g/L glucose or mixtures of glucose and acetate to an initial OD_{600} of 0.5. When required, 25 μg/mL kanamycin and/or 5 μg/mL chloramphenicol was added to the medium. Expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0 h. All fermentations were performed at 30 °C and 220 rpm.

4.4. Analytical Techniques

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). Glucose was measured using an SBA sensor machine (Shandong, China). Organic acids were quantified using a high-performance liquid chromatography (HPLC) system (Agilent Technologies, USA) equipped with a cation-exchange column (HPX-87H; BioRad, USA). Organic acids were quantified, as described previously [24,26]. The temperature of the cation-exchange column was set at 65 °C, and 5 mM H_2SO_4

was used as the mobile phase at a flow rate of 0.4 mL/min. All data represent the average values and standard deviations from three independent replicates.

5. Conclusions

To our best knowledge, this is the first report on the engineering of *C. glutamicum* for 3-HP production via the malonyl-CoA pathway. The engineered *C. glutamicum* Cgz5 was able to utilize glucose and acetate simultaneously, and produced 3.77 g/L 3-HP with a yield of 187 mg/g substrate, which indicates that the GRAS organism *C. glutamicum* is a promising 3-HP producer.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/2/203/s1, Figure S1: Time profiles of acetate consumption (open symbols) and biomass (red filled symbols) of strains Cgz4 (squares), Cgz5 (circles), Cgz6 (upward triangles) and Cgz7 (downward triangles) with 10 g/L acetate, Figure S2: Time profiles of the biomass (OD_{600}), glucose, acetate, and 3-HP concentrations of strain Cgz5 grown (a) with 5 g/L glucose and 5 g/L acetate, (b) with 10 g/L glucose and 10 g/L acetate, Figure S3: Time profiles for biomass (OD_{600}), glucose, and organic acids in the fermentation broth of strain Cgz5 grown in medium with an initial 15 g/L glucose and 5 g/L acetate with 30 μM cerulenin being added at 6 h, Figure S4: Time profiles for biomass (OD_{600}), glucose, and organic acids in the fermentation broth of strain Cgz5 grown in medium with an initial 15 g/L glucose and 5 g/L acetate with 60 μM cerulenin being added at 6 h, Figure S5: Time profiles for biomass (OD_{600}), glucose, and organic acids in the fermentation broth of strain Cgz5 grown in medium with an initial 15 g/L glucose and 5 g/L acetate with 100 μM cerulenin being added at 6 h.

Author Contributions: Z.C. (Zhishuai Chang), Y.M., and T.C. conceived and designed the experiments; Z.C. (Zhishuai Chang), W.D. and Z.C. (Zhenzhen Cui) performed the experiments; Y.M. and Z.C. (Zhishuai Chang) analyzed the data; Y.M., Z.C. (Zhishuai Chang), and T.C. wrote this manuscript; T.C., Z.W. and Y.M. supervised the work. All authors have read and agreed to the published version of the manuscript.

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