Original Research Article

Engineering *Corynebacterium glutamicum* for the efficient production of 3-hydroxypropionic acid from glucose via the β-alanine pathway

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ABSTRACT

3-Hydroxypropionic Acid (3-HP) is recognized as a high value-added chemical with a broad range of applications. Among the various biosynthetic pathways for 3-HP production, the β-alanine pathway is particularly noteworthy due to its capacity to generate 3-HP from glucose at a high theoretical titer. In this study, the β-alanine biosynthesis pathway was introduced and optimized in *Corynebacterium glutamicum*. By strategically regulating the supply of precursors, we successfully engineered a strain capable of efficiently synthesizing 3-HP through the β-alanine pathway, utilizing glucose as the substrate. The engineered strain CgP36 produced 47.54 g/L 3-HP at a yield of 0.295 g/g glucose during the fed-batch fermentation in a 5 L fermenter, thereby attaining the highest 3-HP titer obtained from glucose via the β-alanine pathway.

1. Introduction

3-Hydroxypropionic acid (3-HP) is regarded as a bulk chemical with great potential [1], and a range of chemicals produced from 3-HP are widely used in people's daily life [2]. Based on market forecasts, the market value of 3-HP is projected to surpass $10 billion per year and the size is expected to exceed 3.6 million tons per year [3]. Given its extensive market prospects, various biosynthetic pathways, such as the 1,3-propanediol pathway, β-alanine pathway, malonyl coenzyme A pathway and glycerol oxidation pathway, have been developed for the 3-HP production [2,4].

Recent advances in metabolic engineering have enabled substantive yields of 3-HP using various pathways across different host organisms. Among these, *Halomonas bluephagenesis* utilizing the 1,3-propanediol pathway achieved a high titer of 154 g/L of 3-HP, although the process is costly [5]. A co-culture system involving *Lactobacillus reuteri* and recombinant *Escherichia coli* achieved 125.93 g/L of 3-HP [6]; however, this method suffers from the inevitable byproduct accumulation of 1,3-propanediol. Seo et al. [7] developed a strategy to synthesize 3-HP in *E. coli* via the glycerol oxidation pathway, achieving 3-HP yields of 53.7 g/L. However, this pathway is dependent on vitamin B12, which is

not naturally produced by most microorganisms [8]. Glycerol uptake rates also notably influence 3-HP yields [8]. In *Saccharomyces cerevisiae*, the malonyl-CoA pathway demonstrated a potential, achieving a titer of 71.09 g/L from glucose [9]. Moreover, alternative pathways in *S. cerevisiae* [10] and engineered *E. coli* [11] strains utilizing the β-alanine pathway have demonstrated substantial potential. *S. cerevisiae* engineered to incorporate this pathway produced 25 g/L of 3-HP in fed-batch fermentation [12]. Song et al. [13] reported that an engineered *E. coli* strain producing 3-HP from glucose via heterologously introduced β-alanine/pyruvate transaminase (BAPAT) and overexpressed native hydroxypropionate dehydrogenase (YdfG) achieved a titer of 31.1 g/L. The recent study by Raquel et al. [14] demonstrated that glycerol is a promising substrate for 3-HP production in *E. coli* via the β-alanine pathway, and a titer of 72.2 g/L was achieved in fed-batch fermentation.

While these studies underscore diverse strategies for 3-HP biosynthesis, they also reveal limitations such as costly inputs, substrate utilization and dependence on exogenous additives. In this context, the β-alanine pathway in *C. glutamicum* presents a compelling alternative, especially given its proven capability to produce β-alanine at yields exceeding 160 g/L [15]. Furthermore, β-alanine pathway is capable of

higher theoretical titers compared to other pathways [10]. Despite these advantages, converting β-alanine to 3-HP in *C. glutamicum* has not been studied yet, representing an important gap in the current research landscape. One of the primary challenges to address in leveraging this pathway for 3-HP production is the efficient conversion of β-alanine to 3-HP. This study aims to develop and optimize a genetically engineered *C. glutamicum* strain that efficiently converts glucose to 3-HP via the β-alanine pathway, exploring a more cost-effective, efficient, and scalable method for industrial 3-HP production.

In this study, the β-alanine biosynthetic pathway was introduced into *C. glutamicum*, and further strategies such as optimizing key enzymes expression within the production routes, downregulating the expression of *gltA* and blocking branched-chain metabolism were implemented to increase the yield of 3-HP (Fig. 1). Finally, the metabolically engineered modified strain CgP36 produced 47.54 g/L of 3-HP at a yield of 0.295 g/g after 100 h of fed-batch fermentation, achieving the highest production titer in the current study of 3-HP production via the β-alanine pathway from glucose. This study demonstrated the excellent performance of *C. glutamicum* in the production of 3-HP via the β-alanine pathway, which lays a good foundation for subsequent related researches.

2. Results and discussion

2.1. Introduction of heterologous pathway to produce 3-HP from glucose

The biosynthesis of 3-HP is intricately divided into two main modules: the synthesis of the precursor β-alanine and the synthesis of 3-HP. Despite the inherent metabolic pathway in *C. glutamicum*, which theoretically enables the conversion of glucose into β-alanine, β-alanine was not detected in the culture under standard culture conditions. This suggests the existence of a bottleneck or regulatory mechanism that restricts the accumulation of β-alanine. In addition, aspartate decarboxylase (PanD) - an aminotransferase - catalyzes the conversion of L-aspartate to β-alanine. However, this enzymatic activity is often

reported as being insufficient in prokaryote [16], including *C. glutamicum*, thus representing a significant limitation for β-alanine production in vivo.

In an effort to overcome these limitations, our study has explored the potential of heterologously expressed PanD from two diverse sources: *Bacillus subtilis* [15,17] and *Tribolium castaneum* [10]. Both showed high levels of catalytic activity in β-alanine production studies, providing a novel avenue for improving the catalytic efficiency in β-alanine biosynthesis. Consequently, the introduction of two different sources of PanD into the *ldh* knockout strain CgP1, which was used as a substrate strain, significantly increased the β-alanine production. Notably, the *T. castaneum*-derived PanD exhibited superior efficiency, achieving a β-alanine concentration of 1.26 g/L after 48 h of fermentation, as illustrated in Fig. 2. This observation supports the hypothesis that the introduction of a more efficient aspartate decarboxylase can significantly impact β-alanine biosynthesis.

Additionally, the co-expression strategy involving *C. glutamicum*'s self-derived L-aspartate aminotransferase (AspB) was investigated to further enhance β-alanine production. Contrary to expectations, this approach did not significantly increase β-alanine production. This outcome aligns with previous discoveries by Muhammad et al., which highlighted the already elevated flux of L-aspartate in *C. glutamicum*, suggesting that the bottleneck in β-alanine production may not reside primarily in the availability of L-aspartate [15].

To augment the pathway leading towards 3-HP synthesis, we introduced heterologous genes encoding β-alanine-pyruvate aminotransferase (BAPAT) from *Pseudomonas aeruginosa* and YdfG transaminase from *E. coli* into the bioengineered strains CgP3 and CgP4. This strategic inclusion aimed to leverage β-alanine as a substrate for 3-HP production. Notably, despite overexpressing *aspB* in strain CgP6, engineered to introduce the 3-HP synthesis module, there was no substantial increase in 3-HP production when compared to the strain CgP5. Nevertheless, a slight enhancement in the production rate was evident as detailed in Supplementary Fig. S1. This may be attributed to the robust amino acid production capabilities of *C. glutamicum*, where the supply of the
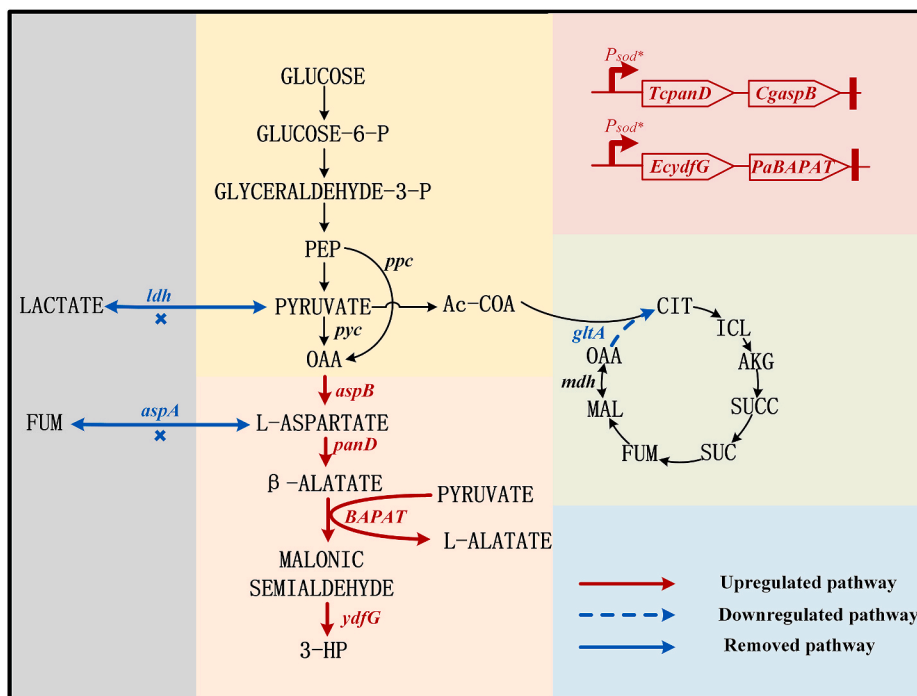

Fig. 1. Strategies for metabolic engineering of *Corynebacterium glutamicum* to produce 3-HP. *aspB*, encoding L-aspartate aminotransferase; BAPAT, encoding β-alanine pyruvate transaminase; *aspA*, encoding aspartate ammonia-lyase; *gltA*, Citrate synthase; *ldh*, D-lactate dehydrogenase; *mdh*, malic dehydrogenase; *ppc*, encoding phosphoenolpyruvate carboxylase; *panD*, encoding aspartate-α-decarboxylase; *pyc*, encoding pyruvate carboxylase; *ydfG*, encoding 3-hydroxypropanoic acid dehydrogenase.
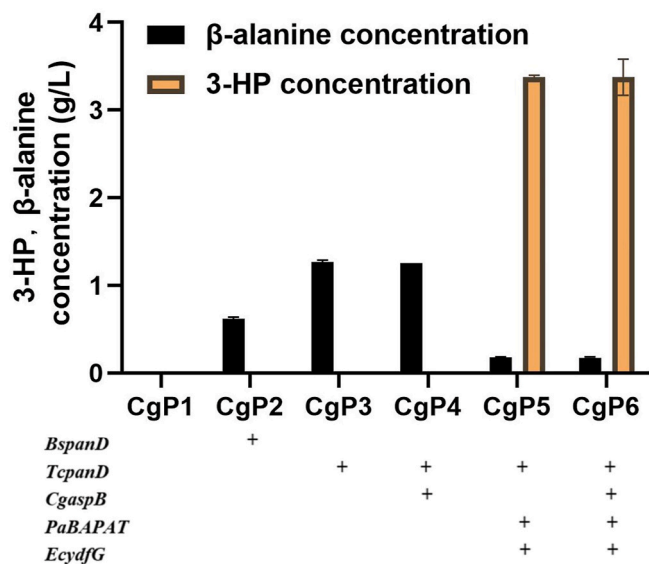
Fig. 2. Production of β-alanine and 3-HP by overexpression of genes related to the β-alanine production pathway.

precursor aspartic acid is not a major limiting factor for 3-HP synthesis. It is speculated that an imbalance between the 3-HP synthesis module and the β-alanine synthesis module or the supply of other key precursors may have limited the further increase in 3-HP production. The findings highlight the complex interplay between β-alanine synthesis and its conversion to 3-HP.

Finally, the bioengineered strain CgP6, specifically developed to optimize the 3-HP biosynthetic pathway, generated 3.37 g/L of 3-HP after 48 h of fermentation. It is noteworthy that during this process, only a minor accumulation of β-alanine (0.17 g/L) was detected, illustrating the pathway's efficiency in rapidly converting β-alanine to the targeted end product, 3-HP. This finding highlights the engineered strain's capability to effectively channel β-alanine into the 3-HP biosynthetic route, thereby minimizing the intermediary accumulation of β-alanine and emphasizing the potential of metabolic engineering in redirecting central metabolites towards desired bioproducts.

2.2. Optimizing the expression of key enzymes improves 3-HP production

In this subsection, we employed promoter engineering approaches to fine-tune the expression levels of genes involved in the β-alanine synthesis module and the 3-HP synthesis module, thereby enhancing the efficiency of the metabolic pathway (Fig. 3a). We selected four constitutive promoters with varying strengths, including P_{sod*}, P_{tuf}, P_{H36} and P_{H30}, which were utilized to replace the original P_{tac} and P_{trc} promoters. The P_{sod} and P_{tuf} promoters are well-established, robust constitutive promoters frequently employed in *C. glutamicum* studies. In a prior investigation, we obtained a mutated, highly active constitutive promoter, P_{sod*}, which demonstrated a 5-fold increase in gene expression levels when compared to its unmutated counterpart, P_{sod} [18]. The P_{H36} and P_{H30} promoters were initially isolated from a 70 bp random sequence promoter library in *C. glutamicum* by employing fluorescence-activated cell sorting (FACS) techniques, with the green fluorescent protein (GFP) serving as a reporter gene in the study conducted by Yim et al. [19] According to the literature, the transcriptional strengths of the aforementioned promoters can be ranked as follows: $P_{sod*}>P_{tac}>P_{tuf}$, $P_{H36}>P_{H30}>P_{trc}$.

The results of the fermentation tests conducted on the engineered strains formed through promoter engineering strategies are presented in Fig. 3b. Remarkable 3-HP production titers were observed in the strains CgP11-CgP15, all of which employed an upregulated β-alanine synthesis module under the control of the P_{sod*} promoter. Thus, when producing 3-HP via the β-alanine pathway, increasing the supply of β-alanine serves as a crucial factor in enhancing 3-HP yields, thereby facilitating a more efficient carbon flux towards 3-HP production.

When compared to engineered strain CgP6, the 3-HP production in strains CgP12 and CgP13 increased by 31.7 % and 25.8 %, reaching concentrations of 4.44 and 4.24 g/L, respectively. In strain CgP12, both the β-alanine synthesis module and the 3-HP synthesis module were overexpressed using the P_{sod*} promoter; whereas, in strain CgP13, the β-alanine synthesis module was upregulated by the P_{sod*} promoter, and the 3-HP synthesis module was regulated by the P_{tuf} promoter. To explore the underlying causes for the enhanced production observed in strains CgP12 and CgP13, relative transcript levels of the β-alanine
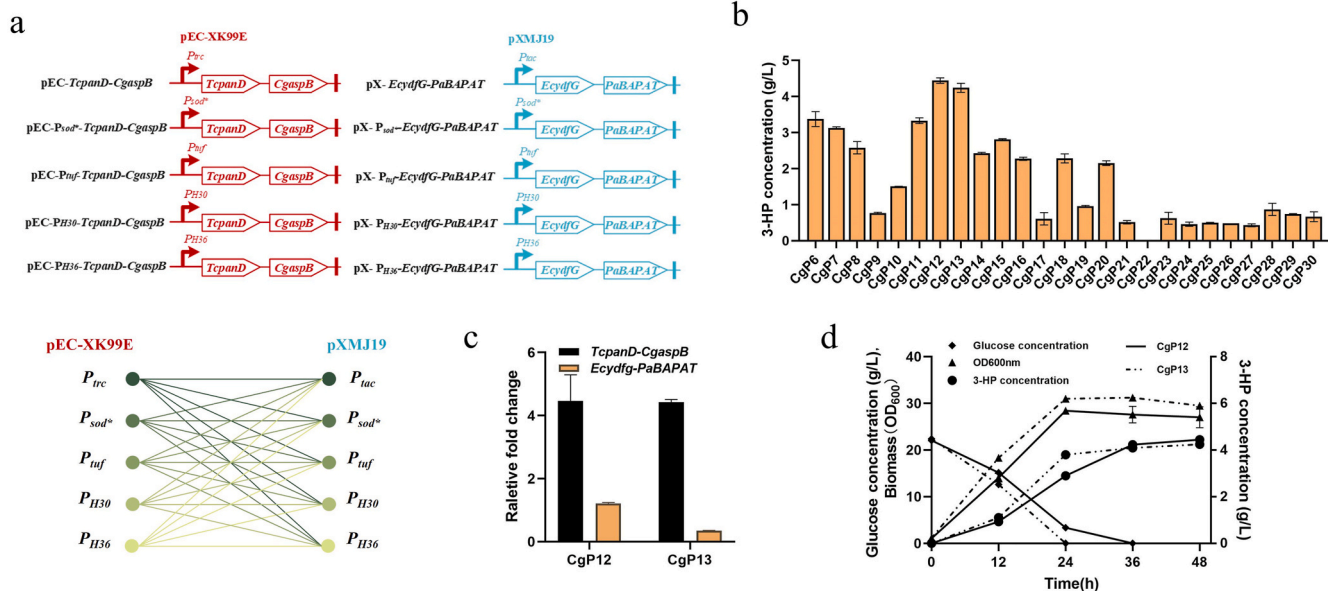

Fig. 3. Effects of optimization of Key Enzymes expression level. (a) Structures of different expression plasmids and (b) their effects on 3-HP production. (c) Relative mRNA levels of gene *TcpanD-CgaspB* and *EcydfG-PaBAPAT*. Transcription level of genes in CgP6 was considered to be 1.0. (d) Shake flask fermentation of strains CgP12 and CgP13.

synthesis module and the 3-HP synthesis module were measured via RT-qPCR. The results are depicted in Fig. 3c. There was a 3.46- and 3.42-fold increase in transcript levels of the genes correlated with the β-alanine synthesis module in strains CgP12 and CgP13, respectively, when compared to strain CgP6. Moreover, the transcript levels of the genes linked to the 3-HP synthesis module were elevated by 21 % in strain CgP12; conversely, strain CgP13 exhibited a reduction to 38 % of that observed in strain CgP6. In Fig. 3d, this comparison between CgP12 and CgP13 indicates that CgP13 exhibited better growth metrics and a faster rate of 3-HP production. This suggests that moderately reducing the expression of the *EcydfG-PaBAPAT* operon helps lighten the metabolic burden on cells, thereby boosting production rates. Therefore, while there is merit in increasing the supply of β-alanine precursors, it is crucial to strategically balance the synthesis of precursors with the downstream production of the 3-HP. The objective should be to refine the interaction between these two modules to optimize overall pathway efficiency and maximize flux, ensuring that the host cells are not subjected to undue metabolic stress.

To alleviate the metabolic burden of the strain due to the dual plasmid expression system, we attempted to express both P_{sod*}-*TcpanD-CgaspB* and P_{tuf}-*EcydfG-PaBAPAT* manipulators on the pEC-XK99E plasmid. However, the strain CgP31 using a single plasmid produced only 1.55 g/L of 3-HP, which is less than half the yield of strain CgP6 using the dual plasmid expression system (Supplementary Fig. S2a). As shown in Figs. S2a and S2b, the growth status of strain CgP31 and the transcript levels of the two modular of the β-alanine pathway were examined, and it was found that the decrease production of strain CgP31 appeared to be due to a greater flow of carbon fluxes to biomass synthesis. Therefore, reducing TCA cyclic flux to limit biomass synthesis could be considered in subsequent studies, which might be an effective strategy to increase 3-HP production.

2.3. Optimization of the intracellular supply of oxaloacetate

The tricarboxylic acid (TCA) cycle plays a pivotal role in cellular metabolism by serving as a source of both energy and biosynthetic precursors. However, the TCA cycle competes with 3-HP synthesis for the key precursor oxaloacetate, and decreasing the flux of the TCA cycle is critical for increasing the titer of oxaloacetate-derived compounds. It is understood that while the TCA cycle is essential for providing the metabolites necessary for cell growth and amino acid synthesis, excessive down-regulation of this cycle could detrimentally impact the cell's metabolic balance [20]. Thus, fine-tuning the carbon flux entering the TCA cycle emerges as a promising approach to optimize the synthesis of desired metabolites such as 3-HP.

Citrate synthase (CS), encoded by the gene *gltA*, is acknowledged as the rate-limiting enzyme of the TCA cycle, facilitating the conversion of oxaloacetate and acetyl-CoA to citrate [21]. Prior studies by Xu et al. [20] have demonstrated that diminishing CS activity can indeed favor the accumulation of oxaloacetate-derived compounds by conserving oxaloacetate for pathways other than the TCA cycle. Thereby, our research has aimed to decrease the flux through the TCA cycle by specifically down-regulating *gltA* expression, therefore allocating a greater share of oxaloacetate for 3-HP production.

In previous work, Chang et al. [22] successfully generated a collection of engineered strains exhibiting varying citrate synthase (CS) enzyme activities. This change was achieved by strategically replacing the native *gltA* promoter with three weak promoters, P1, P5 and P7 (1 %, 6 %, and 13 % of the strength of P_{trc}, respectively). These promoters were selected from the *C. glutamicum* promoter library constructed in previous work [23]. The promoter sequences are detailed in Supplementary Table 1. Further modifications included altering the start codon of *gltA* to TTG in conjunction with the weakest promoter P1, aiming to systematically reduce CS enzyme activity. The series of strains were named Cgz2, Cgz3, Cgz4, and Cgz5, and their CS enzyme activities were 8.8 %, 12.3 %, 15.8 %, and 3.62 % of strain Cgz1, respectively [22]. The

engineered strains, harboring the 3-HP production pathway and ordered by decreasing CS activity, exhibited a correlation between reduced CS activity and increased 3-HP production, as evidenced in fermentation outcomes presented in Fig. 4a. Among the strains, CgP35, which had the lowest CS enzyme activity, displayed the highest 3-HP production titer of 5.94 g/L and a yield of 0.273 g/g. This result was 33 % higher compared to that of CgP12, a strain unmodified in terms of CS activity. Further analysis, as illustrated in Fig. 4b, revealed that the reduction in CS enzyme activity had a negative impact on the growth rate and maximum biomass achieved by the engineered strains. Specifically, the maximal and 12-h incubation optical density (OD_{600}) values for strain CgP12 were 29 and 15, respectively. In contrast, the corresponding OD_{600} values for the engineered CgP35 strain were observed to be remarkably lower, at 23 and 7, respectively.

Furthermore, enhancing the intracellular availability of oxaloacetate via the overexpression of key metabolic enzymes like phosphoenolpyruvate carboxylase (PPC) or pyruvate carboxylase (PYC) has been explored as a strategy to boost the production of oxaloacetate-derived compound [10,11,20]. In our investigations, we used the P_{tuf} promoter to overexpress various forms of the *pyc* and *ppc* genes from *C. glutamicum* and *E. coli*, along with their feedback inhibition-resistant mutant. A series of plasmids overexpressing *pyc* and *ppc* were constructed to enhance the biosynthetic pathways. Despite these efforts, the overexpression of *pyc* and *ppc* in engineered *C. glutamicum* strains did not yield a positive impact on 3-HP production. A range of engineered strains overexpressing *pyc* and *ppc* showed a trend towards increased maximum cell OD_{600} and reduced growth rates (Supplementary Fig. S3), the exact mechanism by which overexpression of these enzymes affects cell growth and 3-HP synthesis remains to be elucidated. This observation highlights the complexity of metabolic interactions and the need for a nuanced understanding of metabolic engineering strategies aimed at optimizing the production of value-added chemicals such as 3-HP.

2.4. Increase of 3-HP production flow by blocking of the branch pathway

The ineffective oxaloacetate-aspartate-fumarate cycle could result in a metabolic bottleneck in the production of L-aspartic acid and its derivatives, as shown by Piao et al. [24] In the previous section, we found that the main competing pathway for oxaloacetate was into the TCA cycle for cell metabolism. Malate dehydrogenase, encoded by *mdh*, catalyzes the reversible conversion of oxaloacetate to malate. This reaction, while integral to cellular metabolism, can inadvertently lead to the diversion of oxaloacetate away from desired biosynthetic pathways, effectively reducing the efficiency of cofactor utilization and thereby impacting reactions dependent on these cofactors.

In addition, L-aspartate aminotransferase (AspA) naturally occurs in *C. glutamicum* and facilitates the reversible conversion between L-aspartate and fumarate, an intermediate in the TCA cycle. In strains engineered to have reduced citrate synthase (CS) activity, there is a tendency for AspA to favor the conversion of L-aspartate to fumarate over its reverse reaction. This preference potentially detracts from the metabolic flux towards the β-alanine synthesis pathway, highlighting a critical area for metabolic engineering interventions. In an effort to address these challenges, we engineered strains with targeted knockouts of *aspA* and *mdh*, aiming to optimize the OAF cycle and, by extension, enhance 3-HP production.

As depicted in Fig. 5, a comparative analysis was conducted on the engineered strains CgP36, CgP37, and CgP38, which disrupted the suboptimal oxaloacetate-aspartate-fumarate cycle. Among these strains, CgP36, which features a knockout of *aspA* alone, achieved the most notable increase in 3-HP production, registering a titer of 6.20 g/L and a yield of 0.292 g/g, alongside an accumulation of 0.63 g/L of β-alanine. Interestingly, the strain with both *aspA* and *mdh* knockouts (CgP38) also showed enhanced 3-HP production metrics relative to the control (CgP35), albeit with a slightly lesser titer of 6.13 g/L and a yield of 0.290 g/g. This indicates a nuanced interplay between these metabolic
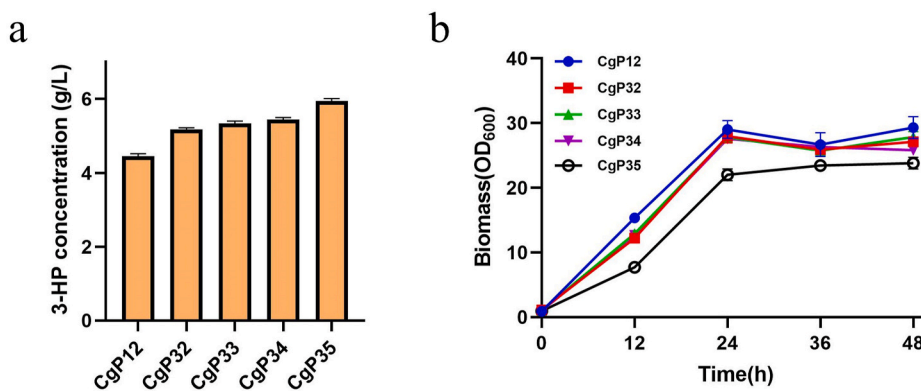
Fig. 4. The effect of weakened CS enzyme activity on 3-HP concentration (a) and growth (b).
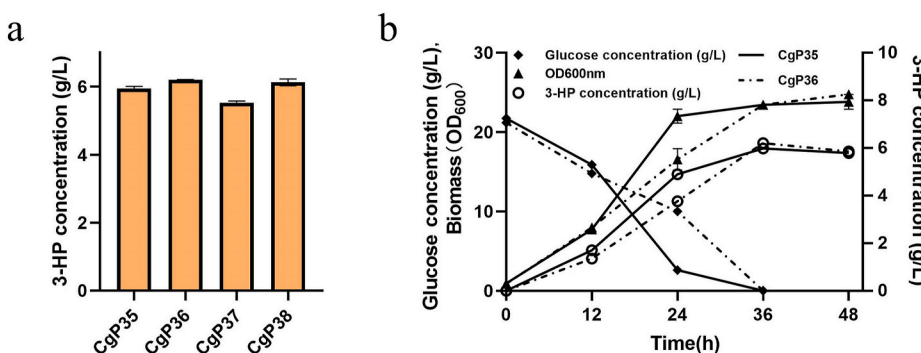

Fig. 5. (a) The effect of knockdown of the oxaloacetate-aspartate-fumarate cycle on 3-HP production. (b) The effect of knockdown of *aspA* on cell growth.

modifications and 3-HP production. Conversely, the strain with a sole knockout of *mdh* (CgP37) did not display a beneficial effect on 3-HP yields, underscoring the more critical role of AspA in this context.

In the genetically engineered strain CgP36, the production of 3-HP was increased due to the knockout of *aspA*. However, the sugar consumption rate, growth rate and 3-HP production rate were all diminished. This observation lends support to our hypothesis that, in engineered strains possessing a weakened TCA cycle, AspA demonstrates a stronger propensity to catalyze the conversion of L-aspartic acid into fumaric acid, a TCA cycle intermediate metabolite, rather than promoting the reverse reaction. Consequently, the downregulation of *aspA* would curtail the levels of TCA cycle intermediates, thereby further impeding the TCA flux and resulting in the deceleration of the engineered strains' growth. Such findings illuminate the intricate balance between metabolic pathway optimization and the broader impacts on cellular metabolism.

2.5. Fed-batch fermentation in a 5 L bioreactor

To further evaluate the 3-hydroxypropionic acid production capabilities of the engineered microbial strain CgP36, an extensive cultivation experiment was conducted in a 5-litre bioreactor containing semi-synthetic CGXIIM medium supplemented with 3 g/L citric acid and 10 g/L yeast extract to promote optimal growth conditions. The resulting fermentation data are systematically presented in Fig. 6.

The recombinant strain CgP36 successfully produced 47.54 g/l of 3-HP during a fermentation cycle lasting 100 h. This yield corresponds to a conversion yield of 0.295 g/g glucose and achieved a volumetric productivity of 0.475 g/l/h, highlighting the efficiency of this engineered strain in the biosynthesis of 3-HP. Notably, at the end of the fermentation phase, CgP36 produced β-alanine at a concentration of 1.70 g/l, suggesting a targeted shift of its metabolic flux towards the target compound, 3-HP. Moreover, the analysis revealed the negligible
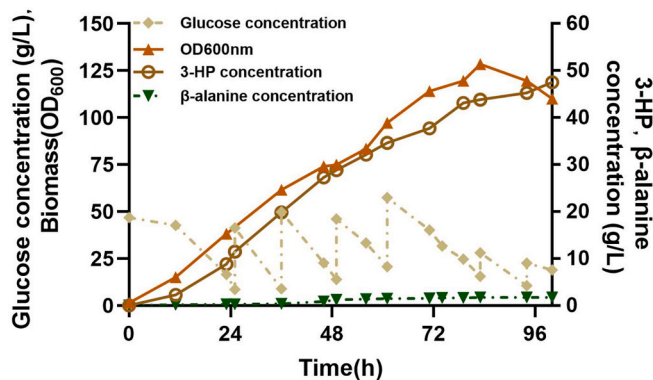

Fig. 6. Time profiles of cell growth (OD$_{600}$), glucose, β-alanine and 3-HP concentrations during the fed-batch culture of recombinant strain CgP36 in a 5-L bioreactor.

presence of secondary by-products such as lactic and acetic acids, which are often observed in microbial fermentations. These findings suggest that the metabolically engineered strain CgP36 is capable of efficiently producing 3-HP through the β-alanine-dependent biosynthetic route.

3. Conclusions

In the present investigation, we focused on the de novo biosynthesis of 3-HP in *C. glutamicum* by engineering the β-alanine pathway. While the achieved production titers of 3-HP were encouraging, this highlighted several points for further enhancement. Firstly, the efficiency of the enzymes involved in converting β-alanine to 3-HP remains suboptimal. Future efforts should focus on modifying these enzymes to improve their catalytic actions. Additionally, optimizing the balance

between precursor synthesis and product generation is crucial for maximizing the overall production efficiency. Secondly, the biosynthesis of 3-HP via the β-alanine pathway entails the consumption of NADPH, a critical cofactor whose availability limits production rates. By redirecting carbon flux to the pentose phosphate pathway (PPP) [25] or over-expression of the pyridine nucleotide transhydrogenase gene (*pntAB*) [26] have been shown to improve NADPH supply. Moreover, as 3-HP is an organic acid, the discovery of an effective export mechanism could further increase its tolerance by the host strain. Currently, the *yohJK* manipulator-encoded transport proteins have been found to be used for 3-HP export in *E. coli* [27], however the mechanism of 3-HP transport in *C. glutamicum* remains unclear.

In this study, the titer of 3-HP was elevated by 31.7 % using the strongest promoter, P_{sod*}, to overexpress genes related to the production pathway. Down-regulation of *gltA* expression to appropriately attenuate TCA cyclic flux and knocking out the branch metabolic pathways to prevent metabolic overflow were both effective strategies to elevate 3-HP production of the via β-alanine pathway, with a 39.6 % increase in this study. Remarkably, our engineered strain, designated as CgP36, notably generated 47.54 g/L of 3-HP, with an associated yield of 0.295 g/g. To the best of our knowledge, these results represent the most substantial 3-HP yield attained from glucose via the β-alanine biosynthetic pathway to date.

4. Materials and methods

4.1. Construction of plasmids and mutant strains of C. glutamicum

All plasmids and strains used in this study are listed in Supplement Table 2. Plasmid construction was carried out using *E. coli* DH5α. The gene encoding aspartate beta-decarboxylase (*aspB*) was amplified directly from the genome of *C. glutamicum*, while the *ydfg* gene was sourced from *E. coli*. To construct the complete β-alanine pathway, *T. castaneum*-derived PanD, *P. aeruginosa*-derived BAPAT (codon-optimized) were synthesized at Azenta (Suzhou, China). Gene editing methods and plasmid expression in *C. glutamicum* consistent with previously published studies [22].

The target fragment was amplified using Phanta Max high-fidelity DNA polymerase followed by ligation of the multiple fragments using a Seamless Cloning Kit (ABclonal Technology), and the ligation products were transformed into *E. coli* DH5α cells. As previously described, the recombinant vectors were transformed into *C. glutamicum*.

4.2. Media and culture conditions

For plasmid host *E. coli* DH5α: colonies were inoculated in test tubes containing 5 mL of LB medium at 37 °C and 220 rpm, with 40 μg/mL kanamycin or 10 μg/mL chloramphenicol added as needed.

For shake flask cultures of *C. glutamicum*: 1) single colonies were inoculated into test tubes containing 5 ml of BHI medium for overnight incubation; 2) 1 mL of seed cultures were transferred into 250 mL shake flasks containing 50 mL of CGIII medium (Media formulations are detailed in previous studies [22]) and incubated for 12 h; 3) seed inoculations into 250 mL of CGXIIM medium containing 25 mL of CGXIIM medium (3 g/L yeast extract, 20 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.25 g/L $MgSO_4–7H_2O$, 10 mg/L $FeSO_4–7H_2O$, 0.1 mg/L $ZnSO_4–7H_2O$, 0.2 mg/L $CuSO_4–5H_2O$, 20 μg/L $NiCl_2–H_2O$, 5 g/L urea, 10 mg/L $CaCl_2$, 21 g/L MOPS, 0.2 mg/L biotin, pH 7.0) to achieve an initial OD_{600} of 1. All of the above were incubated at 220 rpm at 30 °C and supplemented with 1 mM isopropyl-β-D-1-thiogalactoside glycan (IPTG), 25 μg/mL kanamycin and/or 10 μg/mL chloramphenicol, as required.

For fermenter culture of strain: 1) The seed culture for fed-batch cultivation was prepared by the same method. 2) 200 mL of seed solution cultured in CGIII medium was transferred to a 5 L bioreactor (Baoxing Bio, Shanghai, China) containing 1.7 L CGXIIM2 medium (10 g/L yeast extract, 3 g/L citric acid, 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 $MgSO_4–7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4–7H_2O$, 0.1 mg/L $ZnSO_4–7H_2O$, 0.2 mg/L $CuSO_4–5H_2O$, 20 μg/L $NiCl_2–H_2O$, 0.2 mg/L biotin, pH 7.0). Add the appropriate antibiotic and 50 g/L glucose. The temperature was maintained at 30 °C and the air flow rate was set at 1 vvm. The initial stirring speed was 300 rpm and was automatically adjusted to keep the dissolved oxygen above 30 % saturation. Feeding with 750 g/L glucose and 10 g/L yeast extract was performed manually when the glucose concentration in the fermentation broth fell below 10 g/L, and was restored to 50 g/L.

4.3. Analytical methods

A conventional spectrophotometer (Puxi, Beijing) was used to monitor growth of engineered *C. glutamicum* by measuring the optical density at 600 nm (OD_{600}).

For analysis of β-alanine, 100 μL of fermentation supernatant was mixed with freshly prepared derivatization reagent (100 μL of 15 mg/mL 2,4-dinitrobiphenyl (DNFB) dissolved in acetonitrile and 100 μL of 0.5 M $NaHCO_3$ solution with pH 9.0), incubated for 1 h at 60 °C. Then 700 μL of 0.05 M phosphate buffer was added, centrifuged at 12000 r/min for 30 min, and the supernatant was taken for detection. The supernatant was filtered through a 0.22 μm membrane and immediately analyzed on Dionex UltiMate 3000 HPLC using a Diamonsil C18 column (4.6 × 250 mm, 5 μm). The column temperature was set at 30 °C, the detection wavelength was set at 360 nm, the constant flow rate was set at 1 mL/min, the mobile phase was 0.02 M NaAc-HAc buffer or acetonitrile, and the conditions throughout the 55-min sequence were as follows: organic solvent (v/v) 5%–40 % for 30 min, 40%–70 % for 5 min, 70 % organic solvent for 10 min, and 70 %–5 % organic solvent for 5 min each followed by 5 % organic solvent for 5 min. The concentrations of glucose and organic acids in the fermentation broth were determined by high performance liquid chromatography (HPLC), and the details of the determination were referred to the previous description [22].

4.4. Real-time quantitative PCR

RNA extraction, cDNA synthesis and qRT-PCR procedures followed previous descriptions [22]. The internal reference was the transcript level of 16S ribosomal RNA of *C. glutamicum*. Three measurements were performed for the target gene of each strain.

Data availability statement

All data generated or analyzed during this study are included in this research article.

CRediT authorship contribution statement

Xiaodi Wang: Writing – original draft. **Junyuan Hou:** Writing – original draft. **Jieyao Cui:** Writing – original draft. **Zhiwen Wang:** Writing – review & editing, All authors have read and agreed to the published version of the manuscript. **Tao Chen:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.06.003.

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