

Article

In Silico Design of Engineering Optimization via OptHandle for Effective Synthesis of Adipic Acid Precursor, α -Aminoadipate

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Abstract: The green biosynthesis of adipic acid, an important monomer of nylon 6,6, has become a research hotspot. α -Aminoadipate is a key intermediate in the metabolic pathway that converts L-lysine to produce adipic acid. In addition, metabolic flux analysis has become an important part of metabolic engineering. Many metabolic optimization algorithms have been developed to predict engineering intervention strategies with the aim of improving the production of target chemicals. Here, OptHandle, a new metabolic optimization algorithm, has been developed. And, we use OptHandle to optimize the biosynthesis of α -aminoadipate. Based on the results of OptHandle, an engineered *Escherichia coli* with a 13-fold higher titer was obtained, and 1.10 ± 0.02 g/L of α -aminoadipate was produced. The efficient synthesis of α -aminoadipate lays a foundation for the green production of adipic acid.

Keywords: adipic acid; α -aminoadipate; OptHandle; metabolic optimization algorithm



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1. Introduction

Adipic acid is an important dicarboxylic acid, which is mainly used as a monomer for the production of nylon 6,6, chemical fibers and engineering plastics [1]. The annual output of adipic acid in the world is about 3 million tons, increasing at a compound annual growth rate of 3–5% [2]. At present, the main method to produce adipic acid is nitric acid oxidation using a mixture of cyclohexanol and cyclohexanone (also known as KA oil) as raw material [3,4], which is unrenewable and causes serious environmental pollution.

With an increasing demand for sustainable and environmentally friendly biomanufacturing, the synthesis of chemicals using synthetic biological techniques has attracted more and more attention. The biosynthesis of adipic acid has become the focus of researchers and made some progress in the past years [2,5,6]. However, the synthesis of adipic acid by microbial cell factories has not been industrialized due to low conversion rate and poor economy. Therefore, researchers are trying to develop new and efficient pathway for adipic acid biosynthesis. L-lysine, an essential metabolite, can be produced from glucose using microbial cell factories [7,8] and can be used as a precursor for biosynthesis of many important compounds, such as glutaric acid [9], cadaverine [10,11], 5-aminovaleric acid [12], etc. At present, the biosynthesis pathway of adipic acid with L-lysine as the precursor (Figure 1) has been proposed [13], but the lack of enzymes that catalyze the specific reaction makes it impossible to achieve the synthesis of adipic acid [14]. In the above pathway, α -aminoadipate is an important intermediate (Figure 1). The synthesis of α -aminoadipate by microbial cell factory can lay a foundation for the synthesis of adipic acid with L-lysine as the precursor.

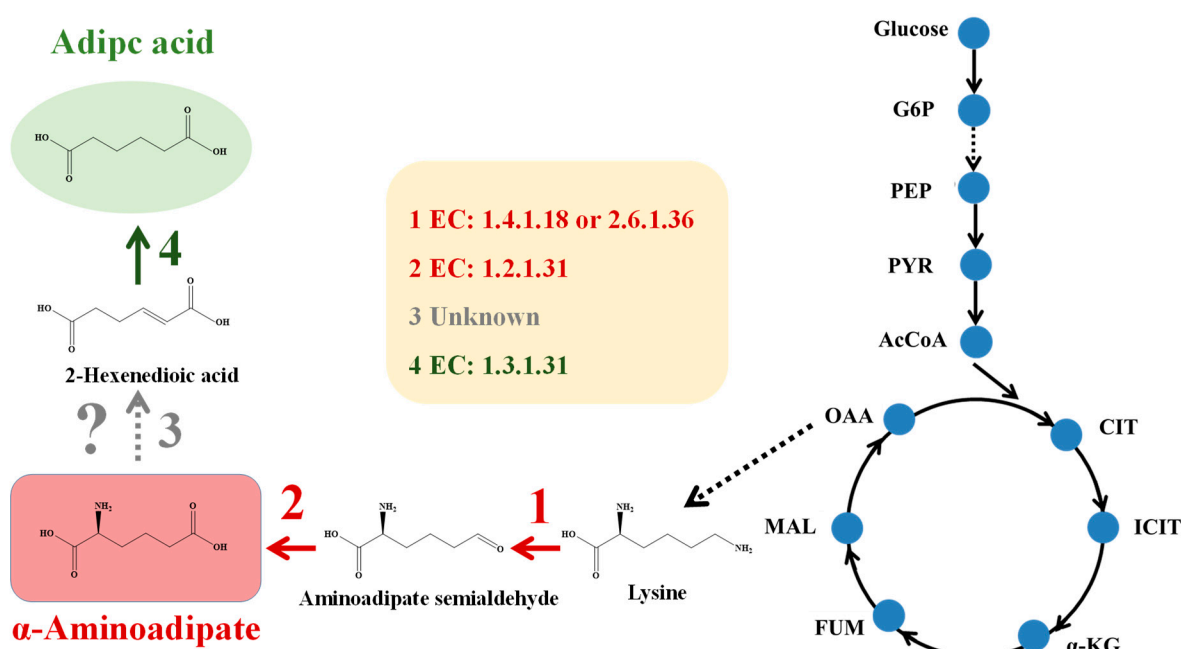


Figure 1. The biosynthesis pathway of adipic acid with *L*-lysine as the precursor. G6P: glucose-6-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl-CoA; CIT: citrate; ICIT: isocitrate; α-KG: α-ketoglutarate; FUM: fumarate; MAL: malate; and OAA: oxaloacetate.

Computational tools provided powerful theoretical guidance for synthetic biology. Research on genome-scale metabolic network is an important part of synthetic biology. Currently, species that have established the genome-scale metabolic network model (GSMM) include *Escherichia coli*, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum*, *Klebsiella pneumoniae*, etc. Metabolic flux analysis algorithms based on the genome-scale metabolic network model are the key to realize flux analysis and prediction. The development of new algorithms is used for more accurate metabolic flux analysis. Flux balance analysis (FBA) is an algorithm widely used to study biochemical reaction networks [15,16]. At present, based on FBA, many optimization algorithms have been developed to predict the regulation strategies of metabolic network, including OptKnock [17], OptStrain [18], OptForce [19] and OptDesign [20]. Currently, the algorithms for strain optimization have been applied to various metabolic engineering modifications for the overproduction of chemicals, such as succinic acid [21], hyaluronic acid [22] and so on. However, some achievements have been made in these algorithms for metabolic engineering, but there are still some problems such as long calculation time and single regulation suggestion. Thus, this paper introduces OptHandle, a new strain design tool. OptHandle combines integer linear programming problem with graph theory, and uses Atom–Atom Mapping (AAM) to reconstruct reaction equations for constructing a regulatory connectivity graph. Then, according to the flux difference between wild-type and overproduction strains, the maximum flow minimum cut theory was used to determine the regulatory suggestions, which can obtain metabolic intervention strategies of up-regulation, down-regulation and knockout.

In this study, we firstly evaluated the feasibility and accuracy of OptHandle in metabolic network optimization. Subsequently, the synthesis pathway of α-amino adipate was constructed in *Escherichia coli* (*E. coli*), which is an important precursor for the biosynthesis of adipic acid. Finally, based on OptHandle for the prediction of metabolic network optimization strategies, the titer of α-amino adipate was significantly increased by metabolic engineering modification.

2. Methods and Materials

2.1. The Optimization Procedure of OptHandle

(1) Screening candidate reactions to be regulated by flux variability analysis (FVA)

First, the flux variability analysis (FVA) [23] is used to calculate the flux distribution range of each reaction in the metabolic network model with maximum growth as the target and overproduction as the target (Figure S1). By FVA, we can obtain the reaction sets and regulation range of up-regulation, down-regulation and knockout.

(2) Reconstructing reaction equations by Atom–Atom Mapping (AAM)

The Reaction Decoding Tool (RDT) [24] enables atomic mapping of biochemical reactions. Through RDT, the reaction equation was reconstructed into a new format from a single substrate to a single product using Atom–Atom Mapping (AAM) (Method S1).

(3) Identifying key reactions to be regulated

The reaction equations reconstructed by RDT are used to construct connected graphs according to the reaction sets of up-regulation, down-regulation and knockout. In the connected graphs, the metabolite is used as the node, and the connected edge represents the reaction name. The flux range obtained from FVA is used as the capacity parameter of the connected edge, and maximum flow minimum cut theorem (Method S2) is used to identify optimization strategies and obtain the rate-determining steps (Method S2). Finally, the reaction with the largest proportion of single reaction flux in the sum of multiple reaction fluxes is taken as the reaction to be regulated with the highest priority in the connected graphs. The reaction sets to be regulated are sorted according to the total flux.

2.2. Media, Plasmids, Strains and Culture Conditions

All plasmids and strains used in this study are listed in supporting data (Table S3). Luria–Bertani (LB) medium containing 5 g yeast extract, 5 g NaCl and 10 g tryptone per liter was used to culture *E. coli* Trans 10 and its derivatives. The M9 medium containing 10 g glucose, 5 g yeast extract, 1 g NH₄Cl, 6.78 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl and 2 g MOPS (morpholinepropanesulfonic acid) per liter was used to culture recombinant *E. coli* in a shake flask. Ampicillin, kanamycin, spectinomycin and chloramphenicol were added to the medium when necessary at final concentrations of 100, 50, 50 and 30 µg/mL, respectively. A 1 mM quantity of isopropyl-β-D-thiogalactoside (IPTG) is used to induce the expression of heterologous proteins. L-lysine was added to the medium at a final concentration of 5 g/L for the feeding experiments. And, all primers used for gene cloning are listed in the supporting data (Table S4).

2.3. HPLC Analysis of Metabolites

α-Aminoadipate and L-lysine were analyzed using UltiMate 3000 HPLC equipped with a Kromasil 100-5-C18 column (250 × 4.6 mm) and UV-VIS detector. Methods of sample preparation and detection were obtained by referring to related studies [25]. Glucose and organic acids were analyzed using UltiMate 3000 HPLC equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and RID detector at a column temperature of 40 °C, and the mobile phase used was 5 mM sulfuric acid, and the flow rate was 0.5 mL/min.

3. Results and Discussion

In this section, we benchmark the OptHandle framework by identifying metabolic interventions that lead to the overproduction of succinate and L-threonine using the genome-scale metabolic model of *E. coli*. The prediction results of OptHandle are compared with the results of other algorithms or experimental results to determine its accuracy. Next, we constructed the biosynthetic pathway of α-aminoadipate in *E. coli*, and used OptHandle to predict potential engineering intervention strategies for regulating microbial cell factories and promoting the synthesis of α-aminoadipate.

3.1. Assessment of OptHandle via Two Cases

3.1.1. Case 1: Succinate Overproduction in *E. coli*

Table 1 lists the regulation strategies for succinate overproduction suggested by OptHandle, and Figure 2A shows these design strategies. The results showed that up-regulation of ICL and MALS could directly increase succinate production, and PPC should be up-regulated to indirectly regulate the increase in succinate production. ICL is the initial reaction of glyoxylate cycle, which directly promotes succinate synthesis by strengthening glyoxylate cycle and reducing carbon flux loss. Similarly, k-OptForce [26] was used for computational strain design prediction of succinate overproduction under aerobic condition [27]. ICL, MALS and PPC are also recommended to be up-regulated [27]. In addition, Lin et al. activated glyoxylate pathway by inactivating aceBAK operon repressor (*iclR*) in *E. coli* to promote succinate synthesis [28]. Lin et al. succeeded in increasing succinate production by an up-regulation of PPC, which is essential for increasing the oxalacetate (OAA) pool to enhance the flux of succinate [29]. And Maranas et al. [19] also made use of OptForce to propose the PPC up-regulation for succinate overproduction. The results of OptHandle showed that G6PDH2r can be detected, which can reduce carbon loss by blocking the carbon flux of Pentose Phosphate Pathway (PPP), and introduce more carbon flux into glycolysis and TCA cycle to strengthen succinate synthesis. Shimizu et al. blocked PPP by knocking out *zwf* to increase the flux of TCA cycle [30], and Hager et al. indirectly increased the amount of glucose-6-phosphate (a succinate precursor) by knocking out the *pgl* gene to inhibit the hydrolysis of 6-phosphogluconolactone [31]. In addition, six reactions in Table 1 were suggested to be down-regulated to promote succinate synthesis. The weakening of GLUDy can prevent the formation of by-product of glutamate [19]. GLCPTspp, a reaction in phosphoenolpyruvate-dependent phosphotransferase system (PTS), is recommended to be down-regulated, which converts PEP into pyruvate to achieve glucose uptake and dephosphorylation. Consequently, it improves the availability of PEP, which is an important precursor of succinate synthesis. So PTS is not conducive to succinate production [32]. The down-regulation of AKGDH can not only reduce carbon loss but also increase the flux of glyoxylic acid cycle. OptHandle suggests down-regulation of ATPS4rpp to reduce cleavage of ATP to ADP in order to meet metabolic energy requirements. This prediction has been also suggested by OptDesign [20]. OptHandle also suggests a number of additional modification targets, such as the detection of CYTBO3_4pp and HCO3E, which have not been experimentally implemented for succinate production. Table 1 depicts the regulation strategies for succinate overproduction suggested by OptHandle. Flux ratio = Flux of preferentially regulated reaction/Total flux of reaction sets. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023) and Table S5.

Table 1. The regulation strategies for succinate overproduction suggested by OptHandle. Flux ratio = Flux of preferentially regulated reaction/Total flux of reaction sets. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023) and Table S5.

Serial Number	Reaction Sets of OptHandle	Total Flux of Reaction Sets	Priority Regulated Reaction	Flux Ratio	Regulation Strategy	Validation of Experiments or Other Algorithms
1	ATPS4rpp ^a + ADSS + SADT2 + BPNT + PRATPP	49.32	ATPS4rpp	98.69%	Down-regulation	[20]
2	CYTBO3_4pp	29.05	CYTBO3_4pp	100.00%	Down-regulation	-
3	PPC	8.31	PPC	100.00%	Up-regulation	[19,26,27,29]
4	GLUDy + DDPA	6.50	GLUDy	95.83%	Down-regulation	[19]
5	ACCOAC + AKGDH	5.38	AKGDH	67.29%	Down-regulation	-
6	G6PDH2r	3.51	G6PDH2r	100.00%	Knockout	[30]
7	ICL	2.66	ICL	100.00%	Up-regulation	[26–28]
8	MALS	2.66	MALS	100.00%	Up-regulation	[26,27]
9	CBMKr + HCO3E	2.54	HCO3E	82.03%	Down-regulation	-
10	GLCptspp	2.53	GLCptspp	100.00%	Down-regulation	[32]

^a These similar words represent reaction names, which are defined in the genome-scale metabolic network model from BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023), and their detailed descriptions are shown in Table S5.

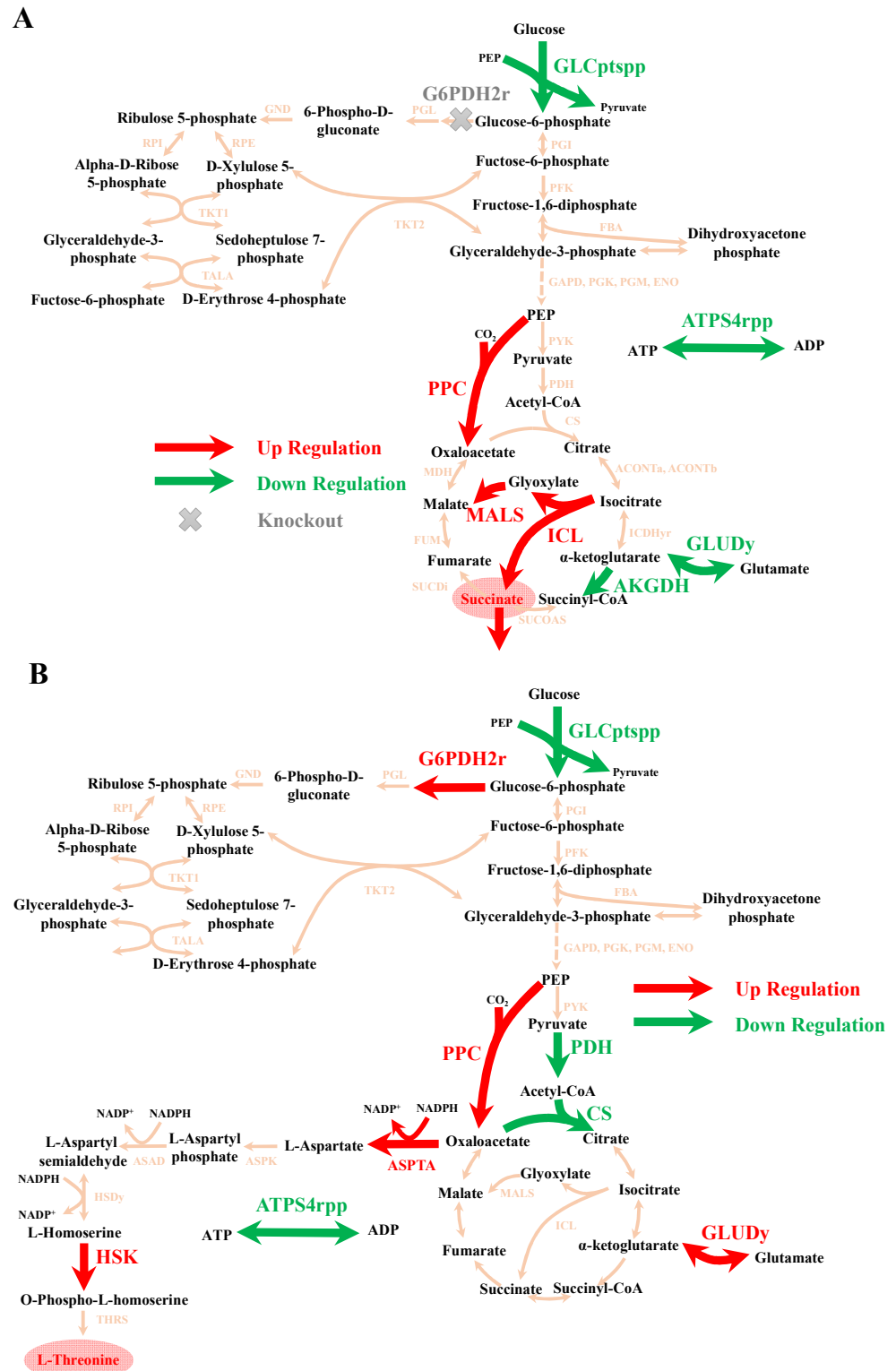


Figure 2. OptHandle's set of reactions for succinate (A) and L -threonine (B) overproduction on a metabolic map of *E. coli*. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023).

3.1.2. Case 2: L -Threonine Overproduction in *E. coli*

Table 2 lists the regulation strategies for L -threonine overproduction suggested by OptHandle, and Figure 2B shows these design strategies. It suggests five reactions for up-regulation, including G6PDH2r, HSK, ASPTA, PPC and GLUDy. A 3 mol quantity of

NADPH is required for the synthesis of 1 mol of L-threonine [33], which involves aspartate transaminase, aspartate semialdehyde dehydrogenase and homoserine dehydrogenase [34]. PPP is the main supply pathway of NADPH. Therefore, OptHandle suggests that G6PDH2r can be up-regulated to increase the flux of PPP, which increases the synthesis of L-threonine by enhancing the supply of NADPH. Xie et al. proved that betaine supplementation up-regulates the expression of *zwf* and increases the NADPH synthesis, which is beneficial for the production of L-threonine. And the detection of *zwf* reduced the supply of NADPH extensively, which led to an inability of recombinant strains to produce L-threonine [33]. L-threonine belongs to the aspartate family of amino acids, so L-aspartate is the key precursor of L-threonine biosynthesis, and aspartate kinase is the key enzyme. The up-regulation of ASPTA may be beneficial for the conversion of L-aspartate to promote L-threonine production. Meanwhile, OptHandle also suggests the overexpression of PPC for increasing the OAA pool, which could not only further disturb the synthesis of L-aspartate, but also fix CO₂ to realize the reuse of CO₂ in cells. Lee et al. enhanced the synthesis of L-aspartate by relieving the feedback inhibition of aspartate kinase, and increased the titer and yield of L-threonine [35]. And Lee et al. proved that enhancement of PPC is necessary for L-threonine production [35]. The availability of co-substrate is an important factor to improve the target product. Aspartate transaminase catalyzes the conversion of OAA to L-aspartate, accompanied by the amino transfer of L-glutamate, which serves as the co-substrate of the reaction. OptHandle suggests the up-regulate of GLUDy, which promotes the synthesis of L-aspartate by strengthening the synthesis of the co-substrate, and the formation of α -ketoglutarate-L-glutamate cycle system is beneficial for the production of L-threonine. Qiao et al. increased L-threonine production by 1.21 times through an overexpression of glutamate dehydrogenase, formate dehydrogenase and pyridine nucleotide transhydrogenase [36]. The up-regulation of HSK shows that overexpression of ATP-dependent homoserine kinase could promote the transformation of L-homoserine to L-threonine. Lee et al. overexpressed a feedback-resistant threonine operon (*thrA*BC*), which showed an increase in L-threonine production [37]. In addition, OptHandle suggests five reactions to be down-regulated, including ATPS4rpp, CYTBO3_4pp, PDH, GLCpt-spp and CS. Among them, the down-regulation of CS can reduce the flux entering the TCA cycle, and the carbon flux is directly introduced into OAA synthesis through the up-regulation of PPC, reducing carbon loss and promoting L-threonine synthesis. The proteomic analysis of L-threonine overproducing *E. coli* showed that the synthesis of citrate synthase was severely inhibited, which inhibited the conversion of OAA to citrate [38]. The accumulation of OAA produced the metabolic flux for the biosynthetic pathway of L-aspartate, the key metabolic precursor of L-threonine. This indirectly indicates that the down-regulation of citrate synthase expression is conducive to promoting L-threonine synthesis [38]. In addition, the down-regulation of PDH can reduce pyruvate consumption, which indicates that an increase in pyruvate pool creates more carbon flux that can be used for L-threonine production [39]. OptHandle also suggests the reduction of GLCpt-spp flux and the down-regulation of PTS system, which may increase the availability of PEP to promote the synthesis of OAA and L-aspartate, thereby increasing the flux of L-threonine [37].

The prediction results of the above two cases, obtained from OptHandle, are highly consistent with relevant experiments or the prediction results of other algorithms. So, OptHandle has a good availability and a high accuracy. More importantly, unlike single strategy prediction algorithms such as Optkonk, OptHandle is a multi-strategy prediction algorithm. Meanwhile, compared with OptForce, it can obtain the more appropriate engineering intervention strategies through priority ranking, and has better operability in experiments. Table 2 shows the regulation strategies for L-threonine overproduction suggested by OptHandle. Flux ratio = Flux of preferentially regulated reaction/Total flux of reaction sets. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023) and Table S5.

Table 2. The regulation strategies for *L*-threonine overproduction suggested by OptHandle. Flux ratio = Flux of preferentially regulated reaction/Total flux of reaction sets. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023) and Table S5.

Serial Number	Reaction Sets of OptHandle	Total Flux of Reaction Sets	Priority Regulated Reaction	Flux Ratio	Regulation Strategy	Validation of Experiments or Other Algorithms
1	ATPS4rpp ^a	42.55	ATPS4rpp	100.00%	Down-regulation	-
2	CYTBO3_4pp	22.86	CYTBO3_4pp	100.00%	Down-regulation	-
3	G6PDH2r	15.22	G6PDH2r	100.00%	Up-regulation	[33]
4	PDH + AKGDH	12.03	PDH	65.46%	Down-regulation	[39]
5	HSK	11.85	HSK	100.00%	Up-regulation	[37]
6	ASPTA	9.76	ASPTA	100.00%	Up-regulation	[35]
7	PPC	9.73	PPC	100.00%	Up-regulation	[35]
8	GLCptspp	8.18	GLCptspp	100.00%	Down-regulation	[37]
9	CS	5.10	CS	100.00%	Down-regulation	[38]
10	GLUDy	4.83	GLUDy	100.00%	Up-regulation	[36]

^a These similar words represent reaction names, which are defined in the genome-scale metabolic network model from BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023), and their detailed descriptions are shown in Table S5.

3.2. Overproduction of α -Aminoadipate via OptHandle in *E. coli*

α -Aminoadipate is a non-protein amino acid, which has potential applications in medicine, chemical industry, feed and other fields. More importantly, α -Aminoadipate is an important intermediate for the synthesis of adipic acid with L -lysine as the precursor (Figure 1). In this study, we aim to construct a biosynthetic pathway of α -aminoadipate in *E. coli* and achieve its efficient synthesis. We use OptHandle to predict the optimization strategies for the overproduction of α -aminoadipate and verify the suggestions provided by OptHandle using experiments.

3.2.1. In Silico Design of an Optimal *E. coli* α -Aminoadipate Producer via OptHandle

The regulation strategies for α -aminoadipate overproduction suggested by OptHandle are listed in Table 3, and Figure 3 shows these optimization strategies. α -Aminoadipate is an L -lysine derivative, which is synthesized with L -lysine as the precursor. The up-regulation of ASPTA and PPC and the down-regulation of PDH and CS enhance the synthesis of L -aspartate, thus introducing more carbon flux into the synthesis of precursor, i.e., L -lysine. The up-regulation of GLUDy provides sufficient co-substrates for aspartate transaminase in the L -lysine synthesis pathway. The down-regulation of PGI or up-regulation of G6PDH2r could introduce carbon flux into PPP, which provides more NADPH for L -lysine synthesis. In the α -aminoadipate synthesis pathway, 2 mol of NAD^+ are required for the synthesis of 1 mol of α -aminoadipate from 1 mol of L -lysine. The up-regulation of THD2pp can not only provide sufficient NADPH for the synthesis of precursor L -lysine, but also provide sufficient NAD^+ for the conversion of L -lysine to α -aminoadipate. Similarly, the knockout of AKGDH could reduce carbon loss and NAD^+ consumption, while increasing the amount of the co-substrate, i.e., α -ketoglutarate. To verify the design strategies for α -aminoadipate overproduction in *E. coli* using OptHandle, some experiments were carried out, and the results are shown below.

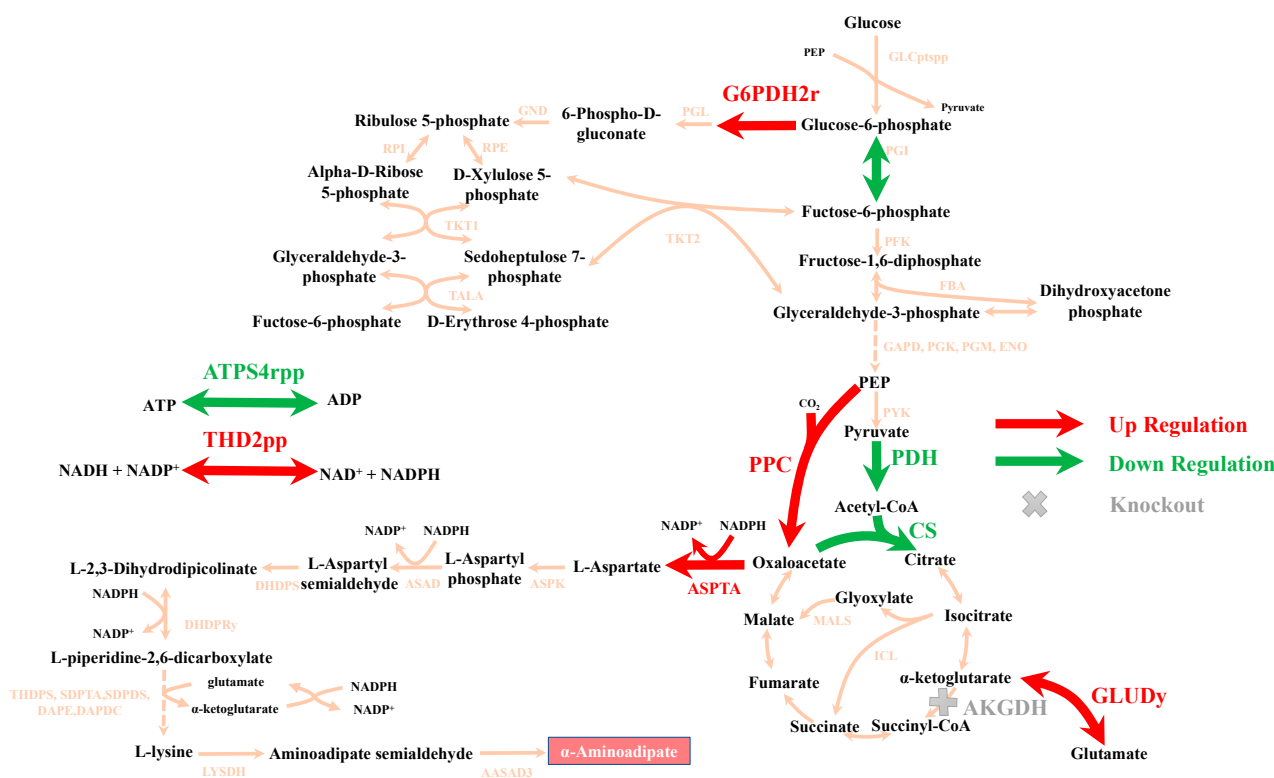


Figure 3. OptHandle's set of reactions for α -aminoadipate overproduction on a metabolic map of *E. coli*. LYSDH: $h2o_c + lys_L_c + nad_c \rightarrow L2adp6sa_c + h_c + nadh_c + nh4_c$. Details of relevant metabolites and reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023).

Table 3. The regulation strategies for α -aminoadipate overproduction suggested by OptHandle. Flux ratio = Flux of preferentially regulated reaction/Total flux of reaction sets. Details of relevant reactions in the table are shown in BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023) and Table S5.

Serial Number	Reaction Sets of OptHandle	Total Flux of Reaction Sets	Priority Regulated Reaction	Flux Ratio	Regulation Strategy
1	ATPS4rpp ^a + ADSS + SADT2	52.34	ATPS4rpp	98.58%	Down-regulation
2	+ BPNT + PRATPP				
3	CYTBO3_4pp	26.40	CYTBO3_4pp	100.00%	Down-regulation
4	THD2pp	15.64	THD2pp	100.00%	Up-regulation
5	PTAr + PDH	9.99	PDH	95.15%	Down-regulation
6	GLUDy	9.27	GLUDy	100.00%	Up-regulation
7	DDPA + CS	7.18	CS	95.58%	Down-regulation
8	PGI	6.49	PGI	100.00%	Down-regulation
9	G6PDH2r	6.49	G6PDH2r	100.00%	Up-regulation
10	AKGDH	5.97	AKGDH	100.00%	Knockout
11	ASPTA	5.76	ASPTA	100.00%	Up-regulation
	PPC	5.73	PPC	100.00%	Down-regulation

^a These similar words represent reaction names, which are defined in the genome-scale metabolic network model from BiGG Models (<http://bigg.ucsd.edu/>, accessed on 1 July 2023), and their detailed descriptions are shown in Table S5.

3.2.2. Tuning the Architecture of the Biosynthetic Pathway and Selecting Gene Orthologues to Increase α -Aminoacidipate Production

The biosynthesis of α -aminoadipate can be carried out through the saccharopine pathway and the direct transamination pathway [40,41]. We first compared the two pathways (Figure 4A) and screened the appropriate enzymes that catalyze the conversion of L-lysine to α -aminoadipate (Table S1). First, aminoacidipate semialdehyde dehydrogenase from *Pseudomonas fulva* 12-X (encoded by the *Psefu_1272* gene) and *R. erythropolis* (encoded by the *reAASADH* gene) were compared. The results showed that strain BWpETN_re carrying *reAASADH* gene converted L-lysine to α -aminoadipate with a titer of 935 mg/L in feeding experiments, which is 1.97 times higher than that of the strain BWpETN (Figure 4B). Subsequently, the saccharopine pathway from yeast and the direct transamination pathway from *Bacillus thermoamylovorans* 1A1 were compared, and the titer of α -aminoadipate produced by the strain BWpETN_19re with the saccharopine pathway is low. Thus, the aminoacidipate semialdehyde dehydrogenase from *R. erythropolis* and the direct transamination pathway are more suitable for α -aminoadipate production. To achieve efficient catalysis of the above enzymes, their expression was modulated stepwise using plasmids with different copy numbers. The results of feeding experiments showed that the low-copy plasmid (pACYC-trc-*lysDH-reAASADH*) is more suitable for α -aminoadipate synthesis. Strains BWpACYCN_re effectively converted L-lysine to α -aminoadipate with a titer of 1.8 g/L (Figure 4C). Different RBS and N-terminal fusion tags were also used to regulate gene expression, but the results were inferior to that of strain BWpACYCN_re (Figure S2). To explore de novo biosynthesis of α -aminoadipate, strain BWpACYCN_re was cultivated in M9 medium with 10 g/L of glucose, which produced 77 mg/L of α -aminoadipate at 72 h (Figure 4D), and acetic acid was the main by-product (Figure 4E).

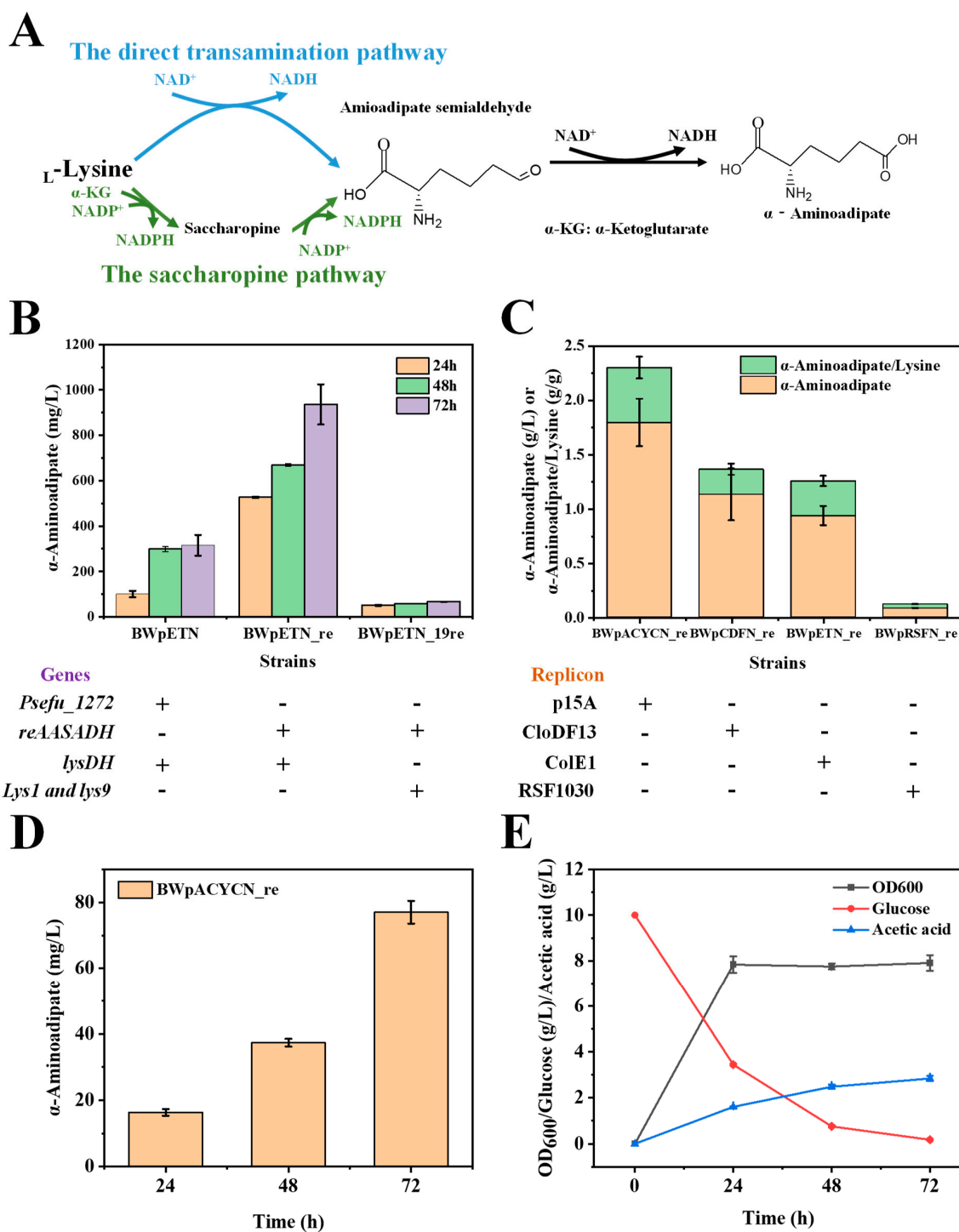


Figure 4. α -Aminoadipate production in *E. coli*. (A) The biosynthesis of α -aminoadipate from L-lysine. (B) Gene screen for α -aminoadipate production. (C) Plasmids with different copy numbers for α -aminoadipate production. (D) The de novo biosynthesis of α -aminoadipate from glucose by the strain BWpACYC_re. (E) The biomass and the concentrations of glucose and acetic acid.

3.2.3. Strengthening Synthesis of Precursors to Increase α -Aminoadipate Production

The result of OptHandle suggested the up-regulation of ASPTA to increase α -aminoadipate production. However, an overexpression of *aspC* (encoding aspartate transaminase) cannot increase the titer of α -aminoadipate, and the data were not shown. Nevertheless, the results of OptHandle implied that the intensification of L-lysine synthesis is conducive for promoting the synthesis of α -aminoadipate. The feeding experiment

of L-lysine with different concentrations proved the above viewpoint (Figure S3). A recombinant strain $\Delta A\Delta C$ -dapA for L-lysine overproduction was obtained, and the results showed that the knockout of L-lysine degradation pathway (the deletion of *cadA* and *ldcC* genes) and overexpression of 4-hydroxy-tetrahydropoligine synthesis (encoded by *dapA*) can lead to the effective synthesis of L-lysine (Figure S4). Thus, strain $\Delta A\Delta C$ -dapA was used for the production of α -aminoadipate, and the plasmid pACYC-trc-*lysDH*-*reAASADH* was electrotransformed into the strain $\Delta A\Delta C$ -dapA to obtain the strain pA-pA. Meanwhile, we investigated the effect of different expression levels of *dapA* on the synthesis of α -aminoadipate. The results of fermentation in the shake flasks showed that the titer of α -aminoadipate produced by the strain pA-pET was higher than that produced by the strain BWpACYCN_re, an increase of 3.9-fold, reaching a concentration of 377.6 mg/L (Figure 5B). These results demonstrated that a high level of expression of *dapA* and a knockout of L-lysine degradation pathway promoted the synthesis of α -aminoadipate.

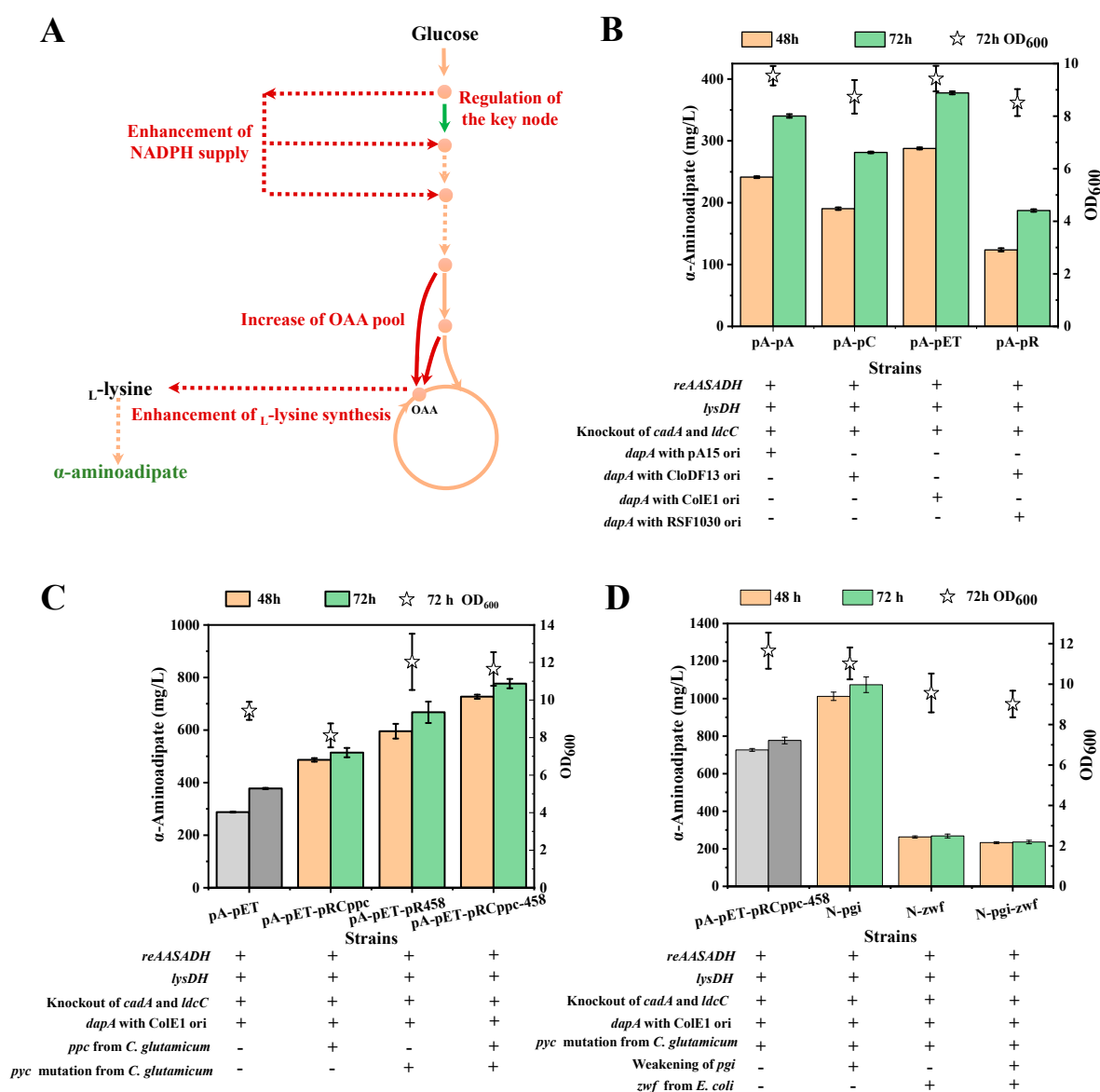


Figure 5. The intensification of precursor synthesis and regulation of the key node for α -aminoadipate production. (A) Scheme of the α -aminoadipate biosynthesis suggested by Ophandle. (B) The enhancement of L-lysine synthesis for α -aminoadipate production. (C) The enhancement of OAA synthesis for α -aminoadipate production. (D) The regulation of the key node for α -aminoadipate production.

Next, the overexpression of PPC was facilitated to increase the OAA pool based on OptHandle, and another heterologous OAA supplementation pathway was compared, which is the conversion of pyruvate to OAA catalyzed by pyruvate carboxylase from *Corynebacterium glutamicum* [42]. The results showed that the up-regulation of PPC (strain pA-pET-pRCppc) increased the titer of α -aminoadipate to 514.1 mg/L, which is 36.2% higher than that of the strain pA-PET. In addition, the introduction of heterogeneous OAA supplementation pathway further improved α -aminoadipate production, and strain pA-pET-pRCppc-458 had the highest α -aminoadipate titer of 776.84 mg/L, which is 1.06 times higher than the strain pA-pET and 9.1 times higher than the parent strain BWpACYCN_re (Figure 5C). The above results on precursor synthesis enhancement showed that the prediction results of OptHandle were effective for the synthesis of α -aminoadipate.

3.2.4. Regulating the Flux of the Key Node to Increase α -Aminoadipate Production

Maintaining the coupling balance of molecules and energy in cells is the key to achieve efficient synthesis of target chemicals [42]. The biosynthetic pathway of α -aminoadipate involves many cofactor-dependent reactions, especially NADPH-dependent reactions. NADPH plays an important role in the synthesis of L-lysine and its derivatives. Most NADPH can be obtained from PPP and used in the synthesis of related chemicals. Glucose-6-phosphate is the key node of glycolysis and PPP. So, PGI and G6PDH2r are the two key reactions. OptHandle suggests the down-regulation of PGI or up-regulation of G6PDH2r to strengthen the supply of NADPH. We compared three regulation modes of the key node to evaluate the influence of different NADPH levels on the synthesis of α -aminoadipate. The up-regulation of G6PDH2r (overexpression of *zwf* gene) caused a serious decrease in the titer of α -aminoadipate (the strains N-*zwf* and N-*pgi-zwf*, Figure 5D). This may be due to an excessive intracellular level of NADPH caused by high level of expression of glucose 6-phosphate dehydrogenase, which leads to serious damage of the redox balance in a cell, and thus it has an adverse effect on the synthesis of α -aminoadipate (Table S2). The result of weakening of *pgi* gene showed that inhibition of 6-phosphate glucose isomerase activity is more conducive to the synthesis of α -aminoadipate. Strain N-*pgi* increased α -aminoadipate titer to 1.1 g/L, which is 12.95 times higher than the parent strain BWpACYCN_re and 38% higher than strain pA-pET-pRCppc-458 (Figure 5D). The above results show that the intensification of lysine synthesis, the increase in OAA pool and the enhancement of NADPH supply promote the utilization of glucose and reduce the synthesis of by-product, i.e., acetic acid, promoting the carbon flux into the synthesis of α -aminoadipate (Figure S5).

4. Conclusions

In summary, adipic acid is an important monomer of nylon 6, 6. At present, the biosynthesis of adipic acid has not been industrialized due to a low conversion rate and a poor economy. Adipic acid synthesis with L-lysine as a precursor is a potential synthesis route, and α -aminoadipate is a key intermediate in this route (Figure 1). In this study, we successfully constructed the biosynthetic pathway of α -aminoadipate, and the titer of α -aminoadipate was significantly increased based on OptHandle, a novel metabolic optimization algorithm. Firstly, we evaluated the feasibility and accuracy of OptHandle in the prediction of engineering intervention strategies through two cases (succinate overproduction and L-threonine overproduction in *E. coli*). The results show that OptHandle is an excellent optimization algorithm. Subsequently, we used OptHandle to predict the metabolic optimization strategies for the biosynthesis of α -aminoadipate in *E. coli*. According to the prediction results, we successfully achieved a significant increase in the titer of α -aminoadipate by strengthening the precursor synthesis and regulating the flux of the key metabolic node. The titer of α -aminoadipate was increased from 77 mg/L to 1.1 g/L, which is an increase of 12.95-fold (Figure 6). This lays a foundation for the application of OptHandle and the synthesis of adipic acid with L-lysine as the precursor.

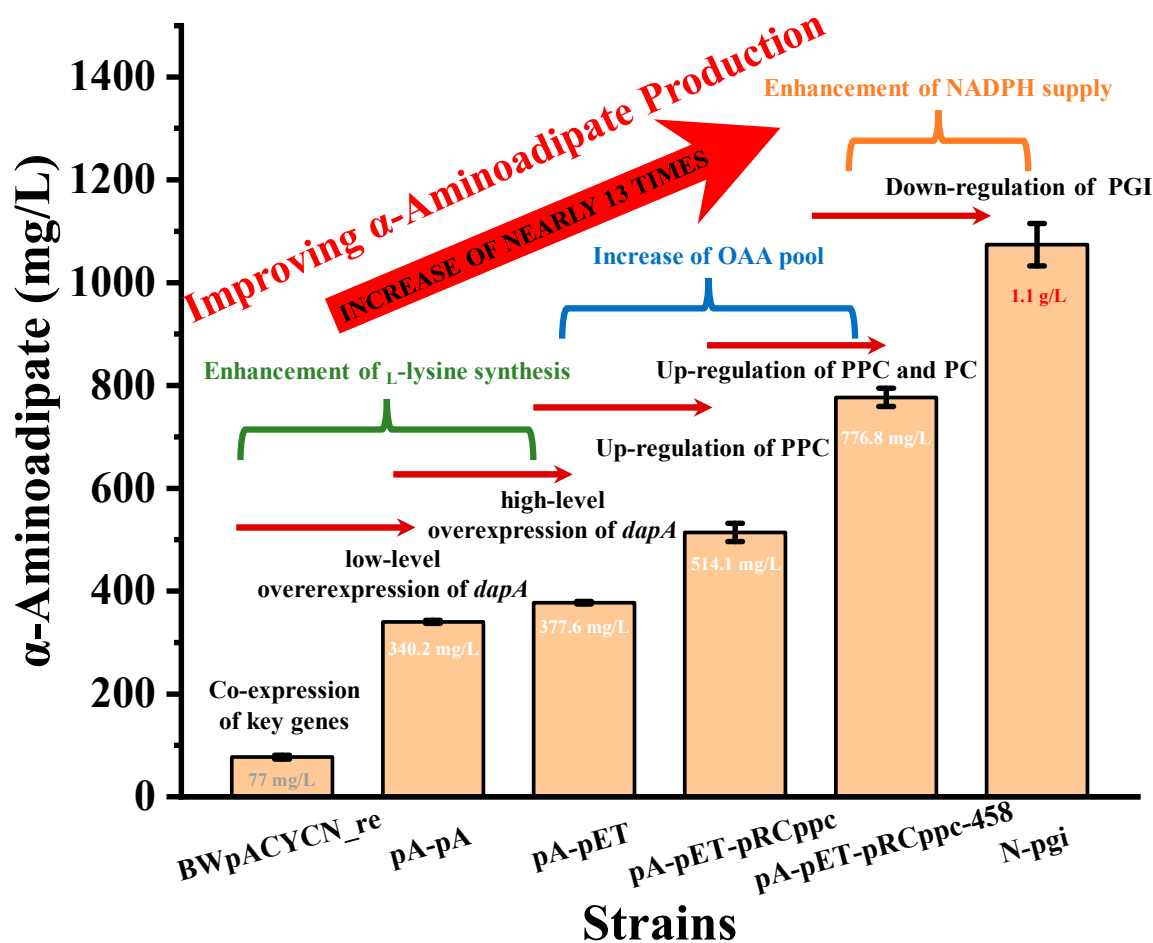


Figure 6. Summary of α -amino adipate production via OptHandle.

At present, the tool does not introduce more precise constraints such as enzyme kinetics constraints and thermodynamic constraints, which could lead to a wide range of prediction results or some omissions. In the future, we will further optimize the algorithm. In addition, we used only a few engineering intervention strategies suggested by OptHandle to optimize the synthesis of α -amino adipate. The application of other strategies suggested by OptHandle, such as the down-regulation of PDH and CS, the up-regulation of THD2pp and GLUDy, etc., may further promote the synthesis of α -amino adipate, which will be investigated in subsequent studies. Furthermore, we can identify and modify the unknown enzymes in the adipic acid synthesis pathway using L-lysine as a precursor by the means of big data screening and computer-aided design, so as to realize the green and efficient synthesis of adipic acid.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation9090859/s1>, Figure S1: The Optimization Procedure of OptHandle; Figure S2: Different RBS and N-terminal fusion tag for α -amino adipate production; Figure S3: The feeding experiment of L-lysine with different concentrations for α -amino adipate production; Figure S4: L-lysine overproduction; Figure S5: Concentrations of glucose and acetic acid at different strains; Table S1: The candidate enzymes that catalyze the conversion of L-lysine to α -amino adipate.; Table S2: Intracellular ratio of NADPH to NADP+ of the recombinant strains; Table S3: Strains and plasmids used in this study; Table S4: List of primers used in this study; Table S5: List of abbreviations used in this study. Method S1: Reconstructing reaction equations by Atom-Atom Mapping (AAM); Figure MS1: The atom matching map of DHDPs. Method S2: The solution of maximum flow and minimum cut. Figure MS2: The solution of maximum flow and minimum cut.

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