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Construction of Recombinant *Escherichia coli* with a High L-Phenylalanine Production Yield from Glucose

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Abstract: L-phenylalanine is an important aromatic amino acid that is widely used in the area of feed, food additives, and pharmaceuticals. Among the different strategies of L-phenylalanine synthesis, direct microbial fermentation from raw substrates has attracted more and more attention due to its environment friendly process and low-cost raw materials. In this study, a rational designed recombinant *Escherichia coli* was constructed for L-phenylalanine production. Based on wild type *E. coli* MG1655, multilevel engineering strategies were carried out, such as directing more carbon flux into the L-phenylalanine synthetic pathway, increasing intracellular level of precursors, blocking by-product synthesis pathways and facilitating the secretion of L-phenylalanine. During 5 L fed batch fermentation, recombinant *E. coli* MPH-3 could produce 19.24 g/L of L-phenylalanine with a yield of 0.279 g/g glucose. To the best of our knowledge, this is one of the highest yields of L-phenylalanine producing *E. coli* using glucose as the sole carbon source in fed-batch fermentation.

Keywords: *E. coli*; L-phenylalanine; metabolic engineering; glucose utilization; Glf; yield

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1. Introduction

L-phenylalanine is an important aromatic amino acid that is widely used in the area of feed, food additives, pharmaceuticals, or as a precursor for drug synthesis [1]. In particular, L-phenylalanine can act as a precursor for the production of aspartame (L-phenylalanyl-L-aspartyl-methyl ester), a low-calorie sweetener with increasing demand world-wide in soft drinks and confectionary products [2]. Currently, L-phenylalanine is mainly produced through chemical synthesis, enzymatic transformation, or microbial fermentation. Microbial fermentation of L-phenylalanine has attracted more and more attention due to its advantages of an environment friendly process and low-cost raw materials [3]. As *Escherichia coli* possesses advantages of adequate gene information, easily genetic manipulation, and fast growth in several cheap substrates, it is widely employed in the production of valuable chemicals, including L-phenylalanine and other aromatic compounds [4,5].

In *E. coli*, biosynthesis of L-phenylalanine begins with the transformation of two precursors, phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), into 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP). The three aromatic amino acids, L-phenylalanine, L-tryptophan and L-tyrosine, share the same biosynthesis pathway from DAHP to chorismite. As shown in Figure 1, three DAHP synthase isoenzymes encoded by *aroF*, *aroG*, and *aroH* are the first rate-limiting enzymes in the L-phenylalanine biosynthesis pathway [6]. Among them, AroG contributes over 80% of the overall DAHP activity [7]. The overall pathway from PEP and E4P to chorismate is named the common pathway. DAHP then proceeds in turn from DAHP to 3-dehydroquinate, 3-dehydroshikimate, and shikimate, which are catalyzed by *aroB* encoding 3-dehydroquinate synthase, *aroD* encoding 3-dehydroquinate

dehydratase, and *aroE* encoding shikimate dehydrogenase, respectively. Employing an ATP-dependent phosphorylation reaction, shikimate 3-phosphate is formed, which then accepts another PEP to form 5-enolpyruvyl-shikimate 3-phosphate (EPSP). In the last step of the common pathway, chorismite is synthesized by chorismite synthase, which is encoded by *aroC*.

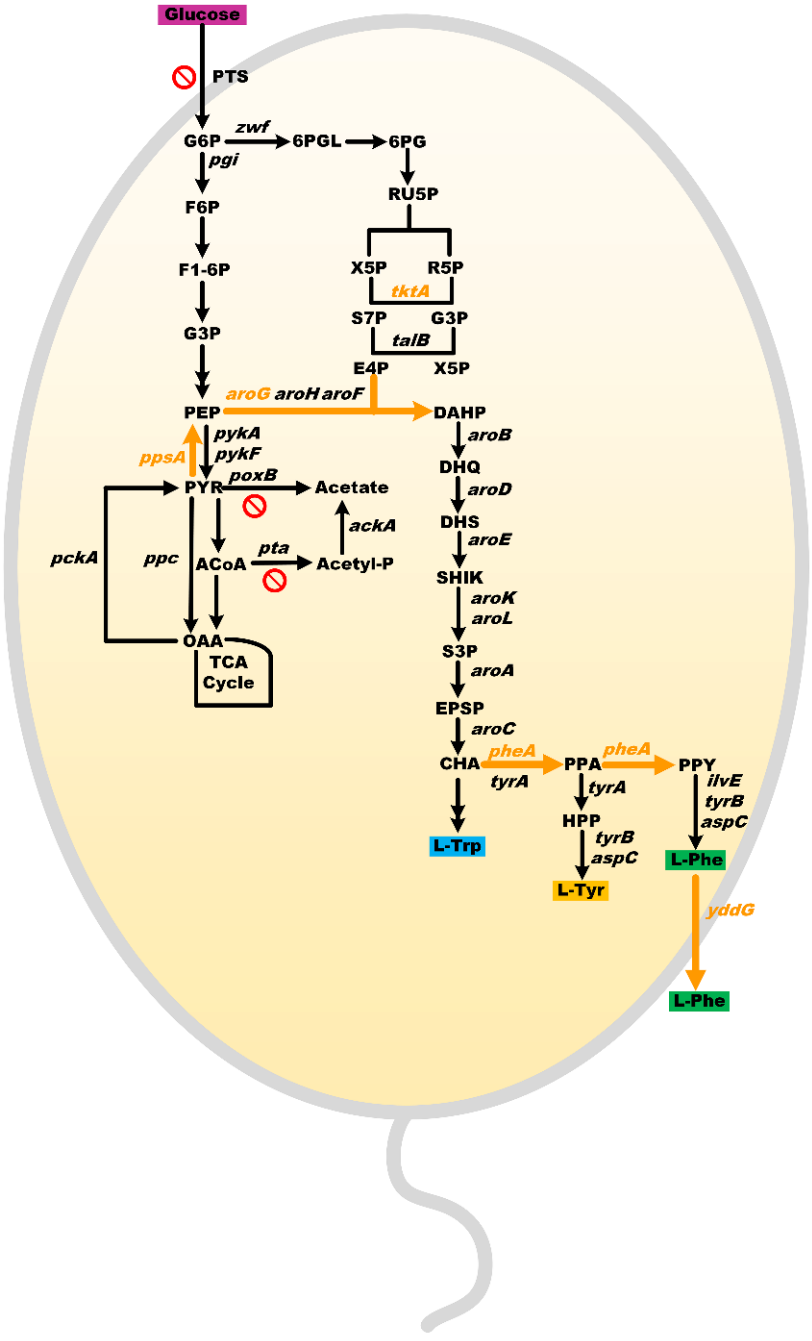

Figure 1. L-phenylalanine biosynthetic pathway in *E. coli*. The red circles indicate the genes that were deleted. The thick yellow arrows and yellow genes indicate the increased flux by directly overexpressing the corresponding genes in the plasmids. Metabolite abbreviations: E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 5-dehydro-quinate; DHS, 5-dehydroshikimate; SHIK, shikimate; S3P, shikimate-5-phosphate; EPSP, 3-enolpyruvyl-shikimate-5-phosphate; CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; HPP, 4-hydroxy-phenylpyruvate; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; L-Phe, L-phenylalanine; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1-6P, fructose-1, 6-diphosphate; G3P, glyceraldehyde-3-phosphate; PYR, pyruvate; OAA,

oxaloacetic acid; 6PGL, 6-phosphoglucono-δ-lactone; 6PG: 6-phosphogluconic acid; Ru5P, ribulose-5-phosphate, X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; R5P ribose-5-phosphate. Genes and enzymes: *aroG*, DAHP synthase feedback inhibited by L-Phe; *aroF*, DAHP synthase feedback inhibited by L-Tyr; *aroH*, DAHP synthase feedback inhibited by L-Trp; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroL* and *aroK*, shikimate kinase; *aroA*, EPSP synthase; *aroC*, chorismite synthase; *pheA*, fused chorismite mutase and prephenate dehydratase; *aspC*, *tyrB* and *ilvE*, aminotransferase; *tyrA*, fused chorismite mutase and prephenate dehydrogenase; PTS, phosphoenolpyruvate-protein phosphotransferase system; *zwf*, glucose-6-phosphate 1-dehydrogenase; *pgi*, glucose-6-phosphate isomerase; *ppsA*, phosphoenolpyruvate synthetase; *tktA*, transketolase; *talB*, transaldolase; *pykA* and *pykF*, pyruvate kinase; *poxB*, pyruvate oxidase; *pta*, phosphate acetyltransferase; *pckA*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase.

As an important intermediate in the synthesis of aromatic amino acids, chorismite then proceeds to branch into pathways of L-phenylalanine, L-tyrosine, and L-tryptophan, respectively. Chorismate mutase-prephenate dehydrogenase (CM-PDT) encoded by *pheA* is another rate-limiting enzyme in the L-phenylalanine pathway that can inhibit feedback by L-phenylalanine. The last step of L-phenylalanine formation is a transamination reaction of phenylpyruvate with glutamate as the amino donor. Several enzymes can accomplish this step, such as tyrosine aminotransferase encoded by *tyrB*, aspartate aminotransferase encoded by *aspC*, and branched-chain-amino-acid aminotransferase encoded by *ilvE*.

In *E. coli*, the biosynthesis of L-phenylalanine is tightly regulated by different regulating factors, which result in a relatively low titer of L-phenylalanine in wild *E. coli*. TyrR and TrpR are main responsible for the transcriptional regulation of L-phenylalanine biosynthesis pathway genes [8]. Among them, TyrR can repress the transcription of *aroF*, *aroL*, and *tyrB* [9,10], while TrpR can repress the transcription of *aroH*, *aroL*, and itself [11]. Accordingly, *tyrR* and *trpR* are often directly inactivated to increase the L-phenylalanine titer. Apart from transcription repression, feedback inhibition is another regulation mechanism for key genes in the L-phenylalanine biosynthetic pathway, such as DAHP synthases encoded by *aroF*, *aroG*, and *aroH* and CM–PDT encoded by *pheA*. The three DAHP synthase isozymes are feedback inhibited by L-phenylalanine, L-tyrosine, and L-tryptophan, respectively, while CM-PDT is only regulated by L-phenylalanine [12]. In addition, shikimate dehydrogenase AroE is feedback inhibited by an intermediate shikimate, which is quite different from most enzymes inhibited by end products [4]. Similar to the tryptophan attenuator, the expression of *pheA* is also regulated by an attenuation mechanism in *E. coli*. It has been reported that 75% of *pheA* transcription was stalled when a rich medium was applied for the cultivation of *E. coli* [13].

The L-phenylalanine titer is also determined by precursor levels, especially the intracellular concentration of PEP and E4P. In *E. coli*, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) represents the main consumption source for PEP when glucose is utilized as carbon source. As one molecule of PEP is needed for transporting and phosphorylating one glucose molecule [14,15], approximately half of the intracellular PEP is consumed by the PTS system. In contrast, less than 2% of PEP is actually employed for aromatic amino acid production, including L-phenylalanine [16,17]. As a result, PTS components, such as *ptsH* encoding phosphocarrier protein HPr, *ptsI* encoding phosphoenolpyruvate-protein phosphotransferase, *ptsG* encoding PTS enzyme IIBC component, and *crr* encoding PTS enzyme EIIA component are often selected as engineering targets for the microbial production of aromatic amino acids. However, PTS-deficient strains often exhibit slow glucose assimilation. To solve this problem, adaptive evolution processes or integration heterogenous glucose transport systems such as Glf glucose-facilitated diffusion protein from *Zymomonas mobilis* are often carried out. Because maintaining a normal glucose utilization rate is as important as improving the intracellular level of PEP, many recently constructed strains for producing aromatic compounds are

based on *E. coli* with wild PTS. For example, Guo et al. engineered a recombinant strain of TRP12 with various modifications to the tryptophan pathway, tryptophan degradation pathway, precursor levels, and tryptophan transport. The final strain of TRP12 could produce 52.1 g/L L-tryptophan with a yield of 0.171 g/g glucose [18]. The engineering of PTS may not be neccessary for further constructing L-phenylalanine producing strains.

To alleviate these limiting factors, here, several different engineering strategies were performed for *E. coli* strains, and various L-phenylalanine producing strains with high titers and yields were constructed. After replacing the phosphotransferase system (PTS) with combinatorial expressed GalP and Glk, engineering the HTH domain of TyrR, and optimizing the *aroD* promoter strength, *E. coli* strain Xllp21 could produce 72.9 g/L of L-phenylalanine in a 5 L fermenter [19]. By overexpression of $pheA^{fbr}$ and wild-type $aroF^{wt}$ in an *E. coli* K12 mutant, the L-phenylalanine titer of the final *E. coli* strain WSH-Z06 (pAP-B03) could reach 35.38 g/L in 3 L fed-batch fermentation [1]. Encouraged by these successful examples, multilevel engineering strategies were combined in wild *E. coli* MG1655 in this study. The *poxB* encoding pyruvate dehydrogenase and *pta* encoding phosphate acetyltransferase were first deleted to decrease the accumulation of acetate. Then, the *ptsI* encoding phosphoenolpyruvate phosphotransferase enzyme I was deleted to save more PEP for L-phenylalanine production. Afterwards, five key genes for L-phenylalanine biosynthesis, including $pheA^{fbr}$ encoding feedback inhibition resistant chorismate mutase/prephenate dehydratase, *tktA* encoding transketolase, $aroG^{fbr}$ encoding feedback inhibition resistant 3-deoxy-7-phosphoheptulonate synthase, *ppsA* encoding phosphoenolpyruvate synthetase, and *yddG* encoding aromatic amino acid exporter, were overexpressed in the plasmid pCL1920. Finally, fed-batch fermentation was performed to explore the production potential of the constructed *E. coli*. The final strain MPH-3 could produce 19.24 g/L of L-phenylalanine with a yield of 0.279 g/g glucose, representing one of the highest yields of L-phenylalanine producing *E. coli* using glucose as the sole carbon source.

2. Materials and Methods

2.1. Bacterial Strains

All of the strains, plasmids, and oligonucleotides used in this study are listed in Table 1, Table 2, and Table S1, respectively. *E. coli* MG1655 was employed for constructing the L-phenylalanine producing strain. *E. coli* DH5α was selected as a host strain for constructing different plasmids.

Table 1. *E. coli* strains used in this study.

Name	Relevant Genotype	Reference
DH5α	*F−, endA1, hsdR17* (*rK−, mK+*), *supE44, thi-l, λ−, recA1, gyrA96,* Δ*lacU169* (*Φ80lacZ* Δ*M15*)	Lab stock
MG1655	*F−, λ−, rph-1*	Lab stock
MG-1	MG1655 (Δ*poxB*)	This study
MG-2	MG1655 (Δ*poxB*Δ*pta*)	This study
MG-3	MG1655/pT1	
MG-4	MG-1/pT1	This study
MG-5	MG-2/pT1	This study
MPH-1	MG1655 (Δ*poxB*Δ*pta*Δ*ptsI*)	This study
MPH-2	MPH-1/pT1	This study
MPH-3	MPH-1/pT1/pT2	This study
MPH-4	MPH-1 with *tyrA* replacement by *glf* containing plasmid pT1	This study

Table 2. Plasmids used in this study.

Name	Relevant Genotype	Reference
pKD3	*bla*, FRT-*cat*-FRT	[20]
pCP20	*bla* and *cat*, helper plasmid	[21]
pTKRed	SpcR, IPTG induced λRed enzymes	[22]
pCL1920	SpcR	[23]
pTrc99a	*bla*	Lab stock
pT1	pCL1920-*pheA*^{fbr}-*tktA*-*aroG*^{fbr}-*ppsA*-*yddG*	Synthesized by TSINGKE Biological Technology
pT2	pTrc99a-*glf*	Synthesized by TSINGKE Biological Technology

2.2. Gene Deletion

Three genes, *poxB* encoding pyruvate dehydrogenase, *pta* encoding phosphate acetyltransferase, and *ptsI* encoding phosphoenolpyruvate phosphotransferase enzyme I, were deleted in turn using the one-step inactivation method [19]. Primers poxB-QF/poxB-QR, pta-QF/pta-QR, and ptsI-QF/ptsI-QR and template plasmids pKD3 were employed to obtain the DNA fragment flanked by homologous arms for *poxB*, *pta*, and *ptsI* deletion, respectively. The PCR product was purified and electroporated into electrocompetent *E. coli* strains with plasmid pTKRed containing isopropyl β-D-1-thiogalactopyranoside (IPTG) induced Red recombinase. Electroporation was carried out according to the manufacturer's instructions using 25 mL of cells and 50–100 ng of PCR product. Shocked cells were added to 1 mL of SOC culture medium (2% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, 0.05% (*w*/*v*) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose), incubated for 1–2 h at 37 °C, and spread onto agar to select the chloramphenicol or kanamycin resistant transformants. Positive clones were verified by PCR employing poxB-JF/poxB-JR, pta-JF/pta-JR, and ptsI-JF/ptsI-JR separately. The chloramphenicol-resistant marker was removed with the helper plasmid pCP20. The resulting recombinant *E. coli* with inactivated *poxB*, *pta*, and *ptsI* was named MG-1, MG-2, and MPH-1, respectively. To replace *tyrA* with *glf* in MPH-1, a replacement fragment containing homologous arms of *tyrA*, *glf* gene, and chloramphenicol resistant gene *cmr* was obtained through overlapped PCR. Firstly, DNA fragment 1 containing an upstream homologous arm of *tyrA* and *cmr* was obtained using pKD3 as a template and TyrA-cmr-NF/cmr-R as the primers. Similarly, DNA fragment 2 containing a downstream homologous arm of *tyrA* and *glf* was obtained using pT2 as a template and cmr-glf-F and glf-tyrA-R as the primers. Next, DNA fragment 1 and 2 were assembled by overlapped PCR. To increase Red recombination efficiency, the homologous arms of *tyrA* were further extended by the primers TyrA-cmr-NF/glf-tyrA-NR. Then, the replacement of *tyrA* was carried out through the one-step inactivation method [20]. Positive clones were verified by PCR using the primers tyrA-JF/tyrA-JR. The chloramphenicol cassette was removed with helper plasmid pCP20, and the resulting strain was named MPH-4.

2.3. Plasmids Construction

Five genes, *pheA*^{fbr} encoding feedback inhibition resistant chorismate mutase/prephenate dehydratase, *tktA* encoding transketolase, *aroG*^{fbr} encoding feedback inhibition resistant 3-deoxy-7-phosphoheptulonate synthase, *ppsA* encoding phosphoenolpyruvate synthetase, and *yddG* encoding aromatic amino acid exporter were overexpressed in plasmid pCL1920. The resulting recombinant plasmid was named pT1. In addition, the *glf* encoding glucose-facilitated diffusion protein from *Z. mobilis* was overexpressed in plasmid pTrc99a and the recombinant plasmid pT2 was obtained. To save on construction time, plasmids pT1 and pT2 were directly synthesized using TSINGKE Biological Technology. Finally, plasmid pT1 was transformed into MG1655, MG-1, MG-2, and MPH-1 to obtain MG-3, MG-4, MG-5, and MPH-2, respectively. Plasmids pT1 and pT2 were co-transformed into MPH-1 to obtain MPH-3.

2.4. Growth Conditions

Strains for cloning and inoculums were grown in Luria−Bertani media (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37 °C for 8–12 h supplemented with appropriate antibiotics (ampicillin (100 mg/L), spectinomycin (50 mg/L), and chloramphenicol (17 mg/L)) when necessary. The batch fermentation and fed-batch fermentation were carried out according to a previous report, with slight modifications [19]. For shake flask fermentation, a single colony was inoculated into 5 mL of LB medium and cultured at 37 °C for 12–16 h. Then, the strains were inoculated into 50 mL of seed culture medium (8 g/L yeast extract, 14 g/L $(NH_4)_2SO_4$, 2 g/L sodium citrate, 4 g/L KH_2PO_4, 20 g/L glucose, 8 mg/L $FeSO_4$, 40 mg/L thiamine, and 2 g/L $MgSO_4$) with an inoculation ratio of 1%. After strain incubation at 37 °C and 220 rpm for 12 h, the seed culture was transferred into the fermentation medium (20 g/L glucose, 10 g/L $(NH_4)_2SO_4$, 5 g/L KH_2PO_4, 5 g/L $MgSO_4$, 4 g/L yeast extract, 0.015 g/L $FeSO_4$, 0.015 g/L $MnSO_4$, and 10 μg/L biotin) at a ratio of 2–3%. Antibiotics were added into the culture medium at a suitable concentration. Three parallel experiments were performed for each strain and the error bars represent standard deviations from three replicate fermentations. For 5 L fed-batch fermentation, a single colony was inoculated into 5 mL of LB medium and cultured at 37 °C for 12–16 h. Then, *E. coli* strains were cultivated in the seed culture medium for 8–12 h at 37 °C and then inoculated into a 5 L fermenter containing 3 L seed culture medium with an inoculation ratio of 5%. When the glucose concentration in the medium was below 5 g/L, feeding solution containing 500 g/L glucose was added into the medium. The culture temperature was 37 °C, and the pH was controlled at 7.0 with an ammonia solution containing 25–28% NH_3. The dissolved oxygen (DO) was maintained at 30–40% saturation by manipulating the agitation speed (400–700 rpm) and aeration rate (2.0–5.0 vvm). The samples were collected every 6 h to determine the cell density (OD_{600}), residual glucose, and L-phenylalanine production. IPTG was added at a final concentration of 0.2 mM. Three parallel experiments were performed for each strain. The production titer (g/L) was exhibited as the average value of three replicate fermentations and the error bars represent standard deviations from three replicate experiments. The yield (g/g) was calculated using the maximum L-phenylalanine titer divided by the total consumed glucose until the time point of reaching the maximum L-phenylalanine titer.

2.5. Analytical Methods

Cell growth was monitored by OD_{600} using a UV5100H spectrophotometer (METASH, Shanghai, China). Glucose was analyzed by an SBA-40E biosensor (Biology Institute of Shandong Academy of Sciences, Jinan, China). L-phenylalanine production was determined by high-performance liquid chromatography (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a column of Eclipse Plus C18 (250 mm × 4.6 mm, Agilent, Palo Alto, CA, USA). The mobile phase was comprised of 0.05% (*v*/*v*) trifluoroacetic acid methanol solution (A) and 0.05% (*v*/*v*) trifluoroacetic acid water solution (B) with a gradient elution as follows: 0–20 min, 10–100% A; 20–23 min, 100% A; and, finally, 23–25 min, 10% A. The column was maintained at 30 °C and a UV detector was employed at 210 nm with an injection volume of 10 μL. The acetate was quantitatively analyzed by HPLC equipped with an HPX-87H column (300 × 7.8 mm; 9 μm; Bio-Rad, Hercules, CA, USA) maintained at 50 °C and a refractive index detector. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

3. Results and Discussion

3.1. Engineering a Base E. coli Strain for L-Phenylalanine Production

In a previous study, we found acetate was a main byproduct when *E. coli* was employed for aromatic amino acid production [24]. Accordingly, *poxB* encoding pyruvate dehydrogenase and *pta* encoding phosphate acetyltransferase were first deleted in wild *E. coli* MG1655 to block the main acetate secretion pathway and direct more carbon source into biomass accumulation and L-phenylalanine production (Figures S1 and S2). In addition, the carbon flux distribution analysis indicated that the phosphoenolpyruvate:carbohydrate phospho-

transferase system (PTS) is the largest consumer of PEP in *E. coli* [17]. Accordingly, *ptsI* encoding phosphoenolpyruvate phosphotransferase enzyme I was deleted to save more PEP for L-phenylalanine production (Figure S3). As a result, recombinant *E. coli* MPH-1 was obtained.

As precursor levels are pivotal for L-phenylalanine production, *ppsA* encoding phosphoenolpyruvate synthetase was overexpressed to direct more pyruvate into PEP. To decrease the possible metabolic burden generated by the plasmid, pCL1920 with only 5 copies per cell was applied to overexpress key genes. In addition, *tktA* encoding a transketolase in the pentose phosphate pathway was also overexpressed in *E. coli* to supply more E4P for L-phenylalanine production. Next, two key genes in the L-phenylalanine synthetic pathway, $aroG^{fbr}$ encoding feedback inhibited resistant 3-deoxy-7-phosphoheptulonate synthase [25] and $pheA^{fbr}$ [1] encoding feedback inhibited resistant chorismate mutase/prephenate dehydratase, were overexpressed by pCL1920. Otherwise, the inner membrane protein YddG of *E. coli* was also overexpressed. It was reported YddG overexpression could increase the production titer of L-phenylalanine, L-tyrosine or L-tryptophan in *E. coli*. On the other hand, the inactivation of *yddG* decreased the aromatic amino acid accumulation by these strains [26]. Accordingly, improving the intracellular expression level of *yddG* would be advantages for decreasing the intracellular L-phenylalanine titer and alleviating the feedback inhibition of L-phenylalanine for key enzymes in the L-phenylalanine biosynthetic pathway. The resulting plasmid pT1 (Figure S4) containing $aroG^{fbr}$, *tktA*, $pheA^{fbr}$, *ppsA*, and *yddG* was transformed into MPH-1 to generate MPH-2, and batch fermentation was performed for this strain.

As shown in Figure 2, MPH-2 exhibited slow growth before 36 h. Then, MPH-2 entered into a relative rapid growth with a maximum OD_{600} of 10.38 at 54 h. In addition, 20 g/L glucose was used up after 60 h for MPH-2. In comparison, the wild *E. coli* MG1655 strain could completely consume 20 g/L before 24 h [27]. In *E. coli*, PTS^{Glu}, PTS^{Man}, MglABC, and GalP are mainly responsible for glucose assimilation. All of the PTS systems share EI and HPr transporter proteins, which are encoded by *ptsI* and *ptsH*, respectively [28]. As a result, the *E. coli* strain with *ptsI* deletion must employ a non-PTS transport system, which could result in a relatively slow glucose consumption rate. In addition, the EI transporter protein also plays a crucial role in physiological signaling [29]. Accordingly, MPH-2 exhibited a slow glucose consumption rate, which may mainly have resulted from the inactivation of *ptsI*. Nevertheless, MPH-2 produced 1.46 g/L L-phenylalanine in batch fermentation, indicating it was a suitable base strain for L-phenylalanine production. However, during the first 24 h, L-phenylalanine synthesis was extremely weak. To further increase the L-phenylalanine titer, additional strain engineering was needed, especially for improving the glucose assimilation rate.

In addition, plasmid pT-1 was also introduced into wild MG1655, MG-1, and MG-2 to obtain recombinant *E. coli* MG-3, MG-4m and MG-5, respectively. As shown in Table S2, the deletion of *poxB* and *pta* brought about no obvious interference in strain growth, while *ptsI* inactivation led the maximum OD_{600} to decrease to 10.38 ± 0.40. The acetate accumulation decreased from 4.64 ± 0.13 g/L to 3.13 ± 0.23 g/L with the deletion of *poxB* in MG-4. Further inactivation of *pta* in MG-5 could achieve only 1.02 ± 0.16 g/L acetate secretion. These results indicate that the inactivation of *pta* was more efficient than *poxB* at decreasing acetate generation, which was consistent with a previous report [30]. MPH-2 with deletion of *ptsI* in MG-5 exhibited least acetate generation among these strains. The growth interfere and less acetate secretion may both result from slow glucose assimilation rate. MG-3 and MG-4 exhibited similar L-phenylalanine production, while deletion of *pta* in MG-5 could slightly increase the L-phenylalanine titer to 0.77 ± 0.05 g/L. We thought that decreasing the acetate secretion may save more carbon source into the L-phenylalanine biosynthetic pathway. Compared with MG-3, MG-4, and MG-5, MPH-2 showed a greatly increased L-phenylalanine titer, demonstrating positive effect of *ptsI* deletion for L-phenylalanine production in *E. coli*.

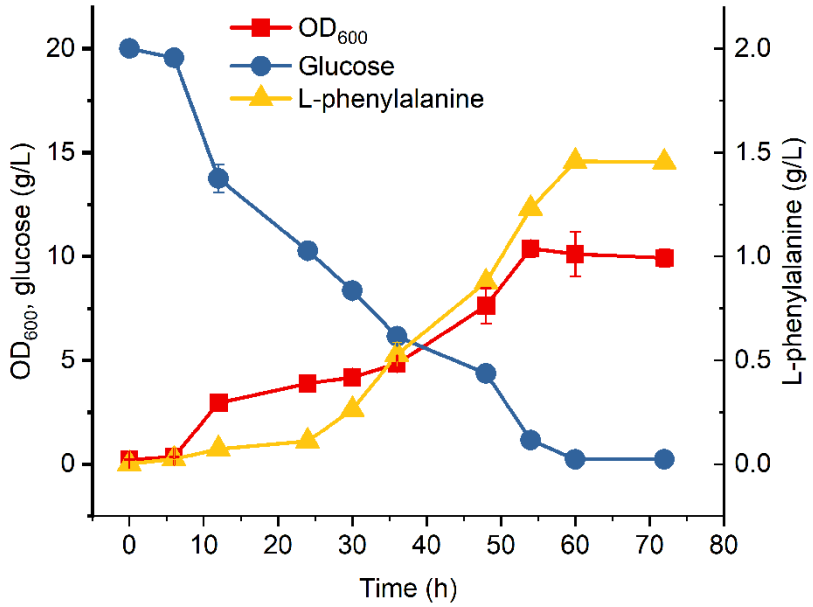

Figure 2. Batch fermentation of MPH-2. The error bars represent standard deviations from three replicate fermentations.

3.2. Introduction of Glf to Improve Glucose Consumption of MPH-2

To accelerate glucose assimilation, *glf* encoding a glucose-facilitated diffusion protein from *Z. mobilis* was introduced into MPH-2. To facilitate the construction process, plasmid pTrc99a compatible with pCL1920 was utilized to overexpress *glf* (Figure S4). Consistent with our expectations, MPH-3 with overexpressed *glf* exhibited a faster glucose utilization rate than MPH-2. The residual glucose was below 4 g/L at 30 h and 20 g/L of glucose was nearly used up after 48 h of batch fermentation (Figure 3). This positive function of *glf* on glucose utilization was also verified by other reports [31,32]. In addition, the maximum OD_{600} of MPH-3 was 28.8% higher than MPH-2, suggesting that the expression of *glf* did not bring an additional metabolic burden for MPH-3. Based on the faster glucose consumption and better growth than MPH-2, MPH-3 could produce 1.76 g/L L-phenylalanine, which was 20.5% higher than MPH-2. Interestingly, we noticed strain growth and L-phenylalanine production were still increased after 54 h, while glucose was nearly entirely consumed. To investigate the possible reasons for this phenomenon, acetate secretion of MPH-3 was explored. As shown in Figure S6, the acetate accumulation was increased before 48 h. Afterwards, it began to sharply decrease when the glucose concentration was below 0.1 g/L. Acetate might have become a supplementary carbon source for MPH-3, which supported strain growth and L-phenylalanine production in a short time. This phenomenon was also observed by other groups [33,34].

Next, the effect of directly integrating *glf* into the chromosome of MPH-2 was investigated. In *E. coli*, the synthesis of L-phenylalanine and L-tyrosine share the same intermediate prephenate. Accordingly, blocking the L-tyrosine synthetic pathway may redirect more prephenate into the L-phenylalanine pathway and improve the L-phenylalanine production titer. To achieve these two goals in one step, *tyrA* encoding chorismate mutase/prephenate dehydrogenase of MPH-2 was replaced by *glf* and *E. coli* MPH-4 was thus obtained (Figure S5). In batch fermentation, MPH-4 exhibited a worse growth compared with MPH-2 and MPH-3, indicated by the maximum OD_{600} of 5.66 (Figure 4). In addition, the glucose utilization was also poor for MPH-4. After 72 h of batch cultivation, 8.52 g/L glucose still remained, meaning 42.6% of the initial glucose was still not consumed. The L-phenylalanine production of MPH-4 was 0.57 g/L, which was only 39% and 32.3% of MPH-2 and MPH-3, respectively. In another report, Tyagi et al. also constructed a series of recombinant *E. coli* strains with *tyrA* deletion. They also observed a reduced growth and L-phenylalanine production when *tyrA* was deleted in *E. coli* [35]. Interestingly, they

found that the simultaneous inactivation of *pykF* and *tyrA* could significantly increase the L-phenylalanine titer by six-fold compared with the wild type [35]. This phenomenon suggested multiple modifications and carefully engineering is required to ensure a higher L-phenylalanine titer in *E. coli*. In addition, for an exogenous supply of tyrosine, optimizing the supplement concentration of yeast extract may be another effective strategy for further increasing the L-phenylalanine production of MPH-4.

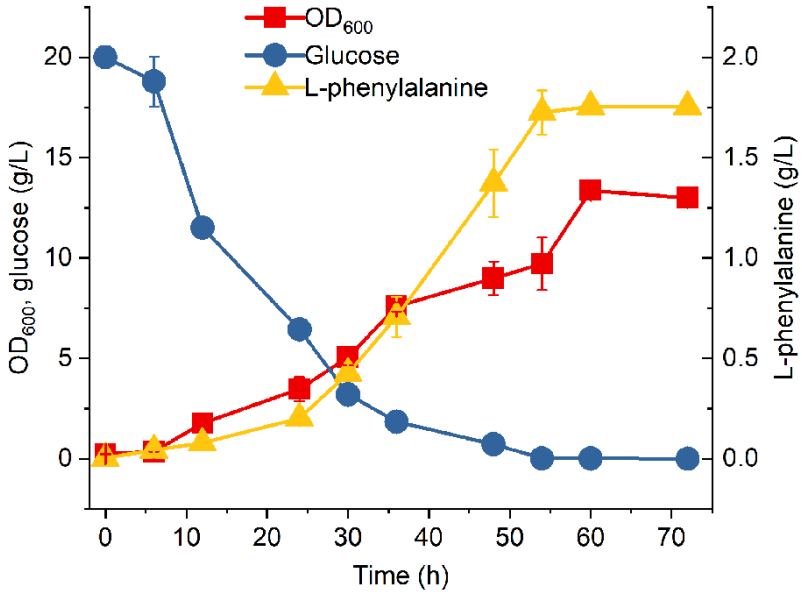

Figure 3. Batch fermentation of MPH-3. The error bars represent standard deviations from three replicate fermentations.

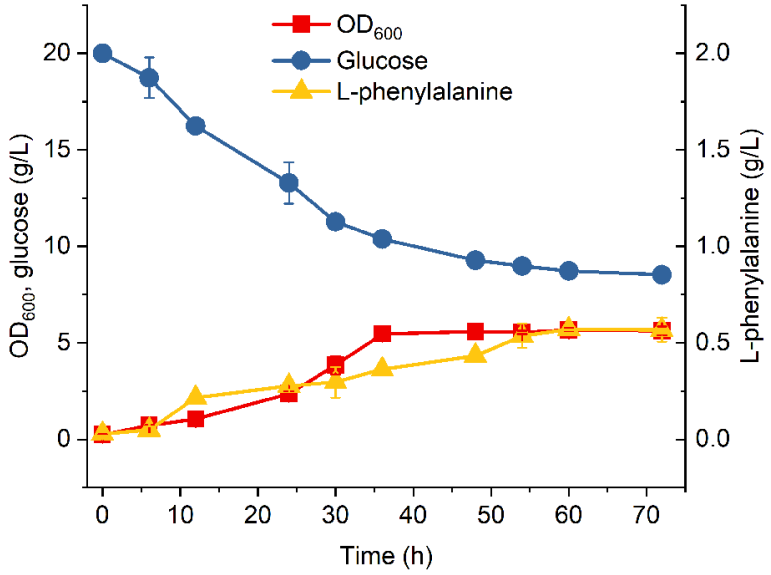

Figure 4. Batch fermentation of MPH-4. The error bars represent standard deviations from three replicate fermentations.

3.3. Fed-Batch Fermentation of MPH-3

To explore the L-phenylalanine production potential of MPH-3, 5 L fed-batch fermentation was performed for this strain. Compared with batch fermentation, no obvious lag phase was exhibited for MPH-3, and the maximum OD_{600} could reach 26.88 (Figure 5). In addition, the L-phenylalanine production titer of MPH-3 could achieve 19.24 g/L with a

yield of 0.279 g/g glucose. In 2004, through two protein-evolutionaries of PheA, two mutant genes, *pheAev1* and *pheAev2*, were obtained. *E. coli* strain PB12 with overexpressed *tktA*, $aroG^{fbr}$, and $pheA^{ev2}$ could produce 0.33 g L-phenylalanine per gram of glucose, representing 60% of the maximum theoretical yield (0.55 g/g) [36]. However, for investigation of the resting cell experiments, direct fermentation was not employed. Compared with several previous constructed L-phenylalanine producing *E. coli* strains (Table 3), the L-phenylalanine titer of MPH-3 was less prominent. However, to the best of our knowledge, the yield of MPH-3 for glucose reached one of the highest levels in fed-batch fermentation compared with other L-phenylalanine producing *E. coli* strains. As MPH-3 exhibited a high yield of L-phenylalanine, while the production titer was not satisfactory, increasing the supplemented glucose concentration may be a possible effective strategy for further improving the L-phenylalanine titer.

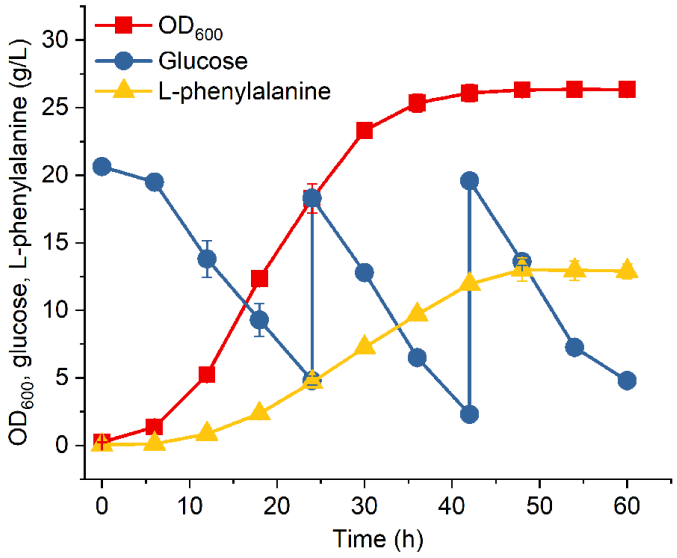

Figure 5. Fed-batch fermentation of MPH-3. The error bars represent standard deviations from three measurements.

Table 3. Summary of L-phenylalanine production from glucose in recombinant *E. coli* with relative high titer and yield.

E. coli Strains	Genes Overexpressed in Plasmids	Promoters and Replicon in Plasmids	Host Engineering	Culture Methods	L-Phenylalanine Production		References
					Titer (g/L)	Yields (g/g)	
Xllp21	*pheA* (Thr326Pro), *aroF*, *galP*, *glk*, and *aroD*	pBR322 replicon and BBa_J23106 promoter	W3110 mutant with L-tyrosine auxotrophic (Δ*ptsH* and *tyrR* (T495I))	5 L fed-batch fermentation	72.9	0.26	[19]
PAPV	*aroF*, $pheA^{fbr}$, and *vgb* from *Vitreoscilla*	$P_LP_R$promoters and replicon was not indicated	Derived from *Escherichia coli* K-12, Hfr (PO1), λ⁻, el4-, *tyrA4*, *relA1*, *spoT1*, *thiE1*	3 L fed-batch fermentation	44.21	0.071	[37]
W14 (pR15BABKG)	*aroG15*, $pheA^{fbr}$, *aroK*, *ydiB*, *yddG*, and *tyrB*	$\lambda cI^{ts}857$ replicon and P_LP_R promoters	W3110 mutant with L-tyrosine auxotrophic (Δ*crr*)	15 L fed-batch fermentation	47	0.252	[38]
W3110 (pNpheABK15)	$pheA^{fbr}$ and *aroG15*	pBR322 replicon and P_{N25} promoter	Wild W3110	15 L fed-batch fermentation	23.8	0.154	[39]
W3110 (pQPTABG8/15)	AroG (A202T and M147I)	pBR322 replicon and P_{tac}promoter	Wild W3110	3 L fed-batch fermentation	26.78	0.231	[40]
WSH-Z06 (pAP-B03)	$pheA^{fbr}$ and $aroF^{wt}$	p15A replicon and P_LP_R promoters	W3110 mutant with L-tyrosine auxotrophic	3 L fed-batch fermentation	35.38	0.238	[1]
MPH-3	$pheA^{fbr}$, *tktA*, $aroG^{fbr}$, *ppsA*, and *yddG*	pBR322 replicon with P_{trc} promoter and pSC101 replicon with P_{lac} promoter	MG1655 (Δ*poxB*Δ*pta*Δ*ptsI*)	5 L fed-batch fermentation	19.24	0.279	This study

Similar to other aromatic amino acids, we believed the L-phenylalanine production titer of MPH-3 could be further increased by careful optimization of the fermentation conditions, including media components, cultivation temperature and pH, initial glucose concentration, and feed strategy [41,42]. The dissolved oxygen (DO) manipulation in fed-batch fermentation is considered to be important for bacterial biomass accumulation and L-phenylalanine production. The co-expression of a hemoglobin gene from *Vitreoscilla* with *aroF* and $pheA^{FBR}$ in *E. coli* CICC10245 could result in 21.9% more biomass and 16.6% more L-phenylalanine production, while only approximately 5% more glucose was consumed [37]. In addition, carefully on-line glucose control could also be employed for improving L-phenylalanine titer and avoiding acetate by-product formation [43].

Apart from fermentation optimization, another possible strategy for further improving the L-phenylalanine titer was exploring novel and unreported engineering targets. For example, flux variability analysis could be used for analyzing intracellular flux distribution during L-phenylalanine production [44]. According to the results, the inactivation of malic enzyme was advantageous for L-phenylalanine production. In 2023, Sun et al. performed transcriptomics and metabolomics analysis for a L-phenylalanine producing strain [45]. They found that precursor supply was not a major limiting factor in this strain, while the rational distribution of metabolic fluxes was achieved by redistributing the carbon flux. On other hand, more and more tools can be used for constructing large diversified mutant libraries of L-phenylalanine producers, such as *mtr* promoter-based biosensor combined with FACS high-throughput screening [46]. The best mutant strain exhibited 4.3-fold higher L-phenylalanine levels compared with the wild-type strain. This suggests two overall strategies can be performed to obtain L-phenylalanine producing *E. coli* with relatively high titers and yields: rational engineering strategies focusing on traditional targets or novel targets explored using flux or omics analysis or random mutation and high-throughput screening. These two strategies can also be combined; for example, whole genome sequencing of the mutant strain could provide various possible novel targets for rational engineering.

In addition, global regulators may represent another potential engineering target for constructing a further increase in the L-phenylalanine titer. Yakandawala et al. indicated that the manipulation of *csrB* and/or *csrA* combined with *tktA* could achieve more ideal results in the L-phenylalanine titer than traditional strategies targeting the glycolytic or aromatic pathway enzymes [47]. Similarly, *fis* encoding a nucleoid-associated protein was found to be abundant during the exponential phase and become sharply reduced in the stationary phase. Tyagi et al. increased the expression of Fis in recombinant strain WF123456. Consistent with expectations, they found the L-phenylalanine titer was increased 1.2-fold compared with the control strain [35]. Apart from *csrA* and *fis*, Ojima et al. found that a stress-responsive gene, *yggG*, also regulated acetate secretion in *E. coli*, which could direct more carbon sources into the L-phenylalanine biosynthetic pathway and increase the L-phenylalanine titer [48]. Afterwards, they integrated wild *yggG* into an L-phenylalanine base strain, AJ12741, with feedback resistant AroG. In the batch fermentation, the recombinant strain could produce 6.4 g/L L phenylalanine, which was 73% higher than the original strain. These successful examples indicate that engineering of global regulators that are not directly related to aromatic amino acid biosynthetic pathways may exhibit a positive effect for L-phenylalanine production in *E. coli*. To facilitate the potential application of industrial L-phenylalanine production, the development of effective extraction strategies is also important. In situ removal of isobutanol has been demonstrated as an effective strategy to alleviate the toxicity of isobutanol for *E. coli*, and it allowed 50 g/L of isobutanol to be produced by *E. coli* [49]. This example could provide a reference for L-phenylalanine production.

4. Conclusions

In this study, a rational designed recombinant *Escherichia coli* was constructed for L-phenylalanine production and multilevel engineering strategies were carried out, such

as directing more carbon flux into the L-phenylalanine synthetic pathway, increasing the intracellular level of precursors, blocking the by-product synthesis pathway and facilitating the secretion of L-phenylalanine. During 5 L fed batch fermentation, recombinant *E. coli* MPH-3 could produce 19.24 g/L of L-phenylalanine with a 0.279 g/g yield of glucose. This work could offer some references for L-phenylalanine production in *E. coli* with glucose as the sole carbon source. The L-phenylalanine production titer of MPH-3 can be further increased by careful optimization of the fermentation conditions.

Although many L-phenylalanine producers have been developed by different research groups, the highest yield (0.33 g/g glucose by resting cell experiments [36] and 0.279 g/g glucose (this study) and productivity (2.76 g/L/h) [45] still exhibited a distance from the ideal level. As a result, further efforts should be carried out to facilitate large-scale industrial production and widescale application. For example, several genetic engineering strategies have been performed on the most recent L-phenylalanine producing strains, including gene inactivation, overexpression, or dynamic regulation. While these genetic modifications bring about a high production titer and yield, they often affect the normal growth of L-phenylalanine producing strains. It was found that wild-type *aroF* could achieve even higher final L-phenylalanine titers (34 g/L) than the $aroF^{FBR}$-containing strain (28 g/L) due to the higher activity of wild-type AroF [43], which suggests genetic engineering did not always exhibit a positive effect for L-phenylalanine production. Accordingly, obtaining L-phenylalanine producing strains with high titers and yields by minimal genetic modification may be an alternative direction. In addition, expanding the substrate utilization range would be beneficial for further decreasing the substrate cost for L-phenylalanine production, especially industrial and agricultural waste, such as acetate, waste protein, or lignocellulose.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14030079/s1, Figure S1: Agarose gel electrophoresis for *poxB* deletion in *E. coli*; Figure S2: Agarose gel electrophoresis for *pta* deletion in *E. coli*; Figure S3: Agarose gel electrophoresis for *ptsI* deletion in *E. coli*; Figure S4: The organization of plasmids pT1 and pT2; Figure S5: Agarose gel electrophoresis for replacing *tyrA* with *glf* in *E. coli*; Figure S6: Acetate accumulation of MPH-3 in batch fermentation. Table S1: Primers used in this study; Table S2: Batch-fermentation parameters for constructed *E. coli*.

Author Contributions: P.G. conceived the study, participated in its design, and drafted the manuscript; P.G. and S.Z. constructed recombinant plasmids and strains; C.L., S.J., H.Z. and Q.L. performed batch and fed-batch fermentation. All authors have read and agreed to the published version of the manuscript.

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