

Co-ordinated combination of Embden-Meyerhof-Parnas pathway and pentose phosphate pathway in *Escherichia coli* to promote L-tryptophan production

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Abstract: In this study, phosphoenolpyruvate and erythrose-4-phosphate are efficiently supplied by collaborative design of Embden-Meyerhof-Parnas (EMP) pathway and pentose phosphate (PP) pathway in *Escherichia coli*, thus increasing the L-tryptophan production. Firstly, the effects of disrupting EMP pathway on L-tryptophan production were studied, and the results indicated that the strain with deletion of phosphofructokinase A (i.e., *E. coli* JW-5 Δ pfkA) produced 23.4 ± 2.1 g/L of L-tryptophan production. However, deletion of phosphofructokinase A and glucosephosphate isomerase is not conducive to glucose consumption and cell growth, especially deletion of glucosephosphate isomerase. Next, the carbon flux in PP pathway was enhanced by introduction of the desensitized glucose-6-phosphate dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) and thus increasing the L-tryptophan production (i.e., 26.5 ± 3.2 g/L vs. 21.7 ± 1.3 g/L) without obviously changing the cell growth (i.e., 0.41 h⁻¹ vs. 0.44 h⁻¹) as compared with the original strain JW-5. Finally, the effects of co-modifying EMP pathway and PP pathway on L-tryptophan production were investigated. It was found that the strain with deletion of phosphofructokinase A as well as introduction of the desensitized *zwf* and *gnd* (i.e., *E. coli* JW-5 *zwf*243 *gnd*361 Δ pfkA) produced 31.9 ± 2.7 g/L of L-tryptophan, which was 47.0% higher than that of strain JW-5. In addition, the glucose consumption rate of strain JW-5 *zwf*243 *gnd*361 Δ pfkA was obviously increased despite of the bad cell growth as compared with strain JW-5. The results of this study have important reference value for the following application of metabolic engineering to improve aromatic amino acids producing strains.

Introduction

L-tryptophan, as one of the essential amino acids for human and livestock, plays an important role in various biological activities, which has been widely used as feed additive or nutritional supplement in feed or food industry (Schoppel *et al.*, 2021; Trondle *et al.*, 2020). In addition, it is also widely used as medical intermediate in pharmaceutical industry (Fricke *et al.*, 2017; Trondle *et al.*, 2018). By reason of its commercial importance, more and more researchers are committed to promote the efficient synthesis of L-tryptophan. At present, microbial fermentation using *Escherichia coli* as work-horse is the mainstream method to produce L-tryptophan in industry

because of more environmentally friendly and more economic benefit (Liu *et al.*, 2020). Thus, an L-tryptophan producing strain with excellent fermentability is important to increase the final titer and yield as well as to reduce the production cost. As shown in Fig. 1, the L-tryptophan biosynthetic pathway in *E. coli* can be divided into the following three modules: the central metabolic pathway, the common biosynthetic pathway of aromatic amino acid and the terminal biosynthetic pathway of L-tryptophan. The central metabolic pathway mainly divides into four parts, i.e., Embden-Meyerhof-Parnas (EMP) pathway, tricarboxylic acid (TCA) cycle, pentose phosphate (PP) pathway and glyoxylate cycle. For L-tryptophan biosynthesis, EMP pathway is responsible for providing phosphoenolpyruvate (PEP) while PP pathway provides erythrose-4-phosphate (E4P), phosphoribosyl pyrophosphate (PRPP) and reducing cofactor NADPH. In addition, the other precursor in L-tryptophan biosynthesis, i.e., L-glutamine, is synthesized in the TCA cycle. Many studies have been conducted to increase these above mentioned precursors

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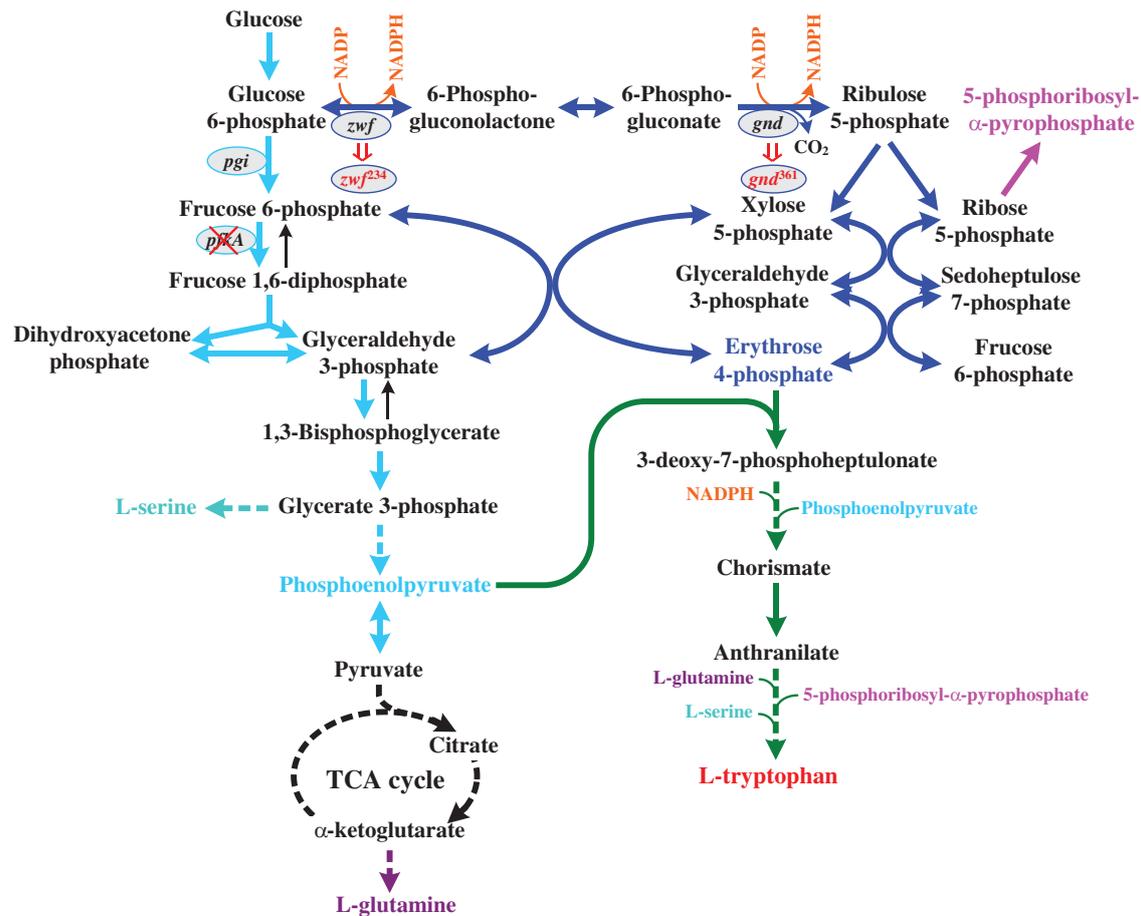


FIGURE 1. The biosynthetic pathway of L-tryptophan in *E. coli*. Different color lines represent the different pathway. The symbols “x” and “=>” represent the deletion and replacement, respectively.

supply by modification of EMP pathway or PP pathway. For example, Liu *et al.* (2017) pointed out that modification of phosphoenolpyruvate:glucose phosphotransferase system (PTS) increases the supply of PEP and thus to increase the L-tryptophan production in *E. coli*. To increase the E4P supply, moreover, the gene *zwf1* (encoding glucose 6-phosphate dehydrogenase) was deleted and the gene *tk1* (encoding transketolase) was overexpressed at the same time to enhance the carbon flux in non-oxidative PP pathway (Trondle *et al.*, 2020). However, the initial step for L-tryptophan biosynthesis is a stereo-specific condensation of E4P and PEP to generate 3-deoxy-d-arobino-heptulosonate 7-phosphate (DAHP) (Fig. 1). Given that the low concentration of intracellular PEP would result in declining the carbon flux in the TCA cycle, the final titer would be decreased because of the poor cell growth (Li *et al.*, 2020). By contrast, the weak PP pathway results in the shortage of E4P (Ikeda *et al.*, 1999; Koendjibiharie *et al.*, 2020). To construct an L-tryptophan high-yielding strain, therefore, it not only needs to increase the carbon flux in EMP pathway, but also needs to enhance the PP pathway. However, how to balance the carbon flux in EMP pathway and PP pathway is important for constructing L-tryptophan producing strains with excellent fermentability.

In order to address the above mentioned problem, the objective of this study was to try to construct an L-tryptophan high-producing strain based on coordinated

combination of EMP pathway and PP pathway in an L-tryptophan producing strain *E. coli* JW-5. The main methods of this study involved in three aspects: (1) Individually reconstructing the EMP pathway or PP pathway in strain *E. coli* JW-5 via deletion of the key enzymes in EMP pathway or introduction of the desensitized *zwf* (i.e., *zwf^{A243T}*, encoded by gene *zwf243*) and *gnd* (i.e., *gnd^{S361F}*, encoded by gene *gnd361*) from *C. glutamicum*. These results indicated that disruption of EMP pathway or enhancement of PP pathway plays a positive effect on increasing L-tryptophan production but individual disruption of EMP pathway limits the glucose consumption rate and cell growth; (2) Collaboratively modifying the EMP pathway and PP pathway via introduction of the *zwf^{A243T}* and *gnd^{S361F}* in the recombinant strains with the disturbed EMP pathway. Compared with the strain with the only modification of EMP pathway or PP pathway, the strain with the co-modification of EMP pathway and PP pathway showed the excellent fermentability, i.e., the high L-tryptophan yield and cell growth as well as the quick glucose consumption rate. As a result, the resultant high-yielding strain *E. coli* JW-5 *zwf243 gnd361 ΔpfkA* produced 31.9 ± 2.7 g/L of L-tryptophan in 40-h fed-batch fermentation. Here, we report an effective strategy to construct an L-tryptophan high-producing strain by collaboratively modifying EMP pathway and PP pathway. The design-based strategy for constructing L-tryptophan high-yielding strain reported here could serve as a general

concept for constructing the other fine chemicals high-yielding strain, in which the biosynthesis of this fine chemical needs no fewer than two precursors.

Materials and Methods

Strains, growth medium and culture conditions

Strains used in this study are listed in [Table 1](#). The parental strain *E. coli* JW-5 (i.e., *E. coli* PFPr 5-FTr AzaSerr SGr Tyr⁻) was an L-tryptophan producing strain derived from the wild-type strain *E. coli* MG1655, which was mutagenized by Atmospheric and Room Temperature Plasma (ARTP) mutagenesis breeding system (Wuxi Institute of Applied Technology, Tsinghua University, Wuxi, China) and screened from p-fluorophenylalanine (PFP)-, 5-fluorotryptophan (5-FT)-, azaserine (AzaSer) and sulfanidine (SG)-resistance M9 minimal agar medium without L-tyrosine (Tyr). *E. coli* was cultivated in Luria-Bertani (LB) medium at 37°C ([Xu et al., 2016](#)). M9 minimal medium was prepared according to the previous report ([Siedler et al., 2012](#)). The SOC medium was used for cell recovery after electro-transformation, which was prepared according to the description of [Chung et al. \(2017\)](#). Appropriately, 50 µg/mL kanamycin (Kan) and 50 µg/mL streptomycin (Sm) solution was used to screen the target plasmids and strains.

Fed-batch fermentation was carried out in 5-L fermenter with 2.5 L of fermentation medium, and was performed according to the methods described by [Gu et al. \(2013\)](#). The seed medium was prepared according to the following formula (per liter): 15 g glucose, 2.5 g (NH₄)₂HPO₄, 5 g yeast extract powder, 1.5 g KCl, 1.5 g MgSO₄·7H₂O, 30 mg FeSO₄, 2 mg

thiamine-HCl. Inoculum was from a seed culture with ΔOD₅₆₂ = 0.45–0.50 (at a dilution of 25-fold), and the inoculation amount was 10%. The fermentation medium was prepared according to the following formula (per liter): 30 g glucose, 5 g corn steep powder, 5 g (NH₄)₂SO₄, 2 g yeast extract powder, 2 g citric acid, 1 g KH₂PO₄, 2 g MgSO₄·7H₂O, 0.01 g MnSO₄·H₂O, 50 mg FeSO₄, 160 µg thiamine-HCl, 50 µg biotin and 5 g CaCO₃.

Gene deletion

Two genes (i.e., *pgi* and *pfkA*) were individually deleted via CRISPR-Cas9-assisted genome-editing technique ([Marco et al., 2022](#)). The oligonucleotide primers used in this study are listed in [Table 2](#). The two-plasmid system including pCas and pTarget was used for genome editing. pCas was initially introduced into the L-tryptophan producers. 20 mmol/L of L-arabinose was supplemented in twice when competent cell was prepared to balance cell growth and RED proteins. pTarget-Δ*pgi* and pTarget-Δ*pfkA* were separately constructed by whole plasmid PCR using the original pTargetF as template, just adjusting N20 sequence embedded into primers at 5' end. Homologous arms including the upstream and downstream regions of the targeted locus were fused by overlap extension PCR. The detail progress of gene deletion was referred to the previous methods ([Jiang et al., 2015](#)).

Site-directed mutagenesis

Site-directed mutagenesis of *zwf* and *gnd* gene was performed using a commercial Site-Directed Mutagenesis Kit (Mut Express[®] MultiS Fast Mutagenesis Kit V2, Vazyme, Nanjing, China). Base replacement was introduced into oligonucleotide primers. The specific steps of the method are referred to the

TABLE 1

The strains and plasmids used in this study

Strains and plasmids	Genotype description	Source
<i>E. coli</i> strains		
JW-5	An L-tryptophan producing strain, derived from <i>E. coli</i> MG1655	Our Lab
JW-5 Δ <i>pgi</i>	Strain JW-5 with deletion of <i>pgi</i>	This study
JW-5 Δ <i>pfkA</i>	Strain JW-5 with deletion of <i>pfkA</i>	This study
JW-5 <i>zwf</i> 243	Strain JW-5 with mutated <i>zwf</i> 243	This study
JW-5 <i>gnd</i> 361	Strain JW-5 with mutated <i>gnd</i> 361	This study
JW-5 <i>zwf</i> 243 <i>gnd</i> 361	Strain JW-5 with mutated <i>zwf</i> 243 and <i>gnd</i> 361	This study
JW-5 <i>zwf</i> 243 <i>gnd</i> 361 Δ <i>pgi</i>	Strain JW-5 with mutated <i>zwf</i> 243, <i>gnd</i> 361 and deletion of <i>pgi</i>	This study
JW-5 <i>zwf</i> 243 <i>gnd</i> 361 Δ <i>pfkA</i>	Strain JW-5 with mutated <i>zwf</i> 243, <i>gnd</i> 361 and deletion of <i>pfkA</i>	This study
Plasmids		
pCas	Contains the Cas9 gene with constitutive promoter and λRed recombination genes controllable by arabinose promoter	Jiang et al., 2015
pTargetF	Transcribes sgRNA under the control of an constitutive j23119 promoter	Jiang et al., 2015
pTarget-Δ <i>pgi</i>	Transcribes the sgRNA targeting <i>pgi</i>	This study
pTarget-Δ <i>pfkA</i>	Transcribes the sgRNA targeting <i>pfkA</i>	This study
pTarget- <i>zwf</i>	transcribes the sgRNA targeting <i>zwf</i>	This study
pTarget- <i>gnd</i>	transcribes the sgRNA targeting <i>gnd</i>	This study

TABLE 2

Primers sequences used in this study

Primers	Sequences ^a
ΔpfkA-1	ctccaacaccgatgacagatc
ΔpfkA-2	ttttccgaatcaccggaagtcttctgacat
ΔpfkA-3	agaagactccgggtatttcgaaaaaggcagattctc
ΔpfkA-4	caccggtttaacgatcgttcc
N20-pfkA-F	tggagactgacgttggtggttttagagctagaaatagcaag
N20-pfkA-R	cagccaacgtcagatctccaactagtattatactaggactgag
Δpgi-1	cactctggcggctcagtag
Δpgi-2	cggcaccaccagcacttccgcatgtg
Δpgi-3	gaagtgtggtggtgccgttaaagtattcg
Δpgi-4	cgagtatacaactaaagcatcgc
N20-pgi-F	tgctggcgtgattggcatcgttttagagctagaaatagcaag
N20-pgi-R	gatgccaatcagcggcgaactagtattatactaggactgag
gnddown-R	cggcagacaataaccacat
EcoRI-gnddown-F	tttGAATTCaaccaacaataaaattgaggcc
gndS361F-F	tggcTTCgacgagaacaactgggacgttgacc
gndS361F-R	tgttctccgtcGAAgccagcctgatctcgtc
N20-gnd-F	aactcagcctgtgcctcgttttagagctagaaatagcaag
N20-gnd-R	gcaggcgacaaggctgattactagtattatactaggactgag
EcoRI-Cgnd-R	tttGAATTCtaagcttcaacctcggagcggtc
XbaI-Cgnd-F	tttTCTAGAatgccgtcaagtagcatcaataacatgacta
gnd361-F	atgccgtcaagtagcatcaataacatgac
gnd361-R	ttaagctcaacctcggagcggtc
gndup-F	cctcaatcagctgggtaactc
XbaI-gndup-R	tttTCTAGAtacatactctgtcaggtgtg
zwfA243T-F	gatccatgACAGaagatattggcttgggtggac
zwfA243T-R	cttcTGTcatggtgatctggacgtggtcaacg
HindIII-Czwf-F	tttAAGCTTgtgagcacaacacgacccc
xbaI-Czwf-R	tttTCTAGAttatggcctgcgccagg
N20-zwf-F	gatcatgtcgcgatctgacgttttagagctagaaatagcaag
N20-zwf-R	gtcagatgcccgcgatgatcactagtattatactaggactgag
zwfdown-R	gagaatccggatgaacaaaagaagagc
XbaI-zwfdown-F	tttTCTAGAtatctcgccttatcctttatggtattttaccg
zwf243-F	gtgagcacaacacgaccc
zwf243-R	ttatggcctgcgccagtg
HindIII-zwfup-R	tttAAGCTTgtcattctccttaagtaactaaccgg
zwfup-F	taattgccagcgttctccc

Note: ^a The capital letters represent the mutated bases, and the capital letters with italic represent the restriction enzyme sites.

manufacturer's instructions. The oligonucleotide primers used in this study are listed in Table 2. The base replacements in genes *zwf* and *gnd* were confirmed via sequencing by GENEWIZ (Suzhou), Inc. (Suzhou, China).

Construction of *E. coli* recombinant strains

Plasmids used in this study are listed in Table 1. Plasmid pTarget-Δ*pgi*, pTarget-Δ*pfkA* were used for gene *pgi* and *pfkA* deletion, respectively. In addition, plasmids

pTarget-*zwf* and pTarget-*gnd* were used for replacing the native genes *zwf* and *gnd* in *E. coli*. The detail methods used for gene deletion and gene replacement were referred to the published method (Jiang *et al.*, 2015). The resultant plasmids were transferred into *E. coli* by electroporation, and the target recombinants were selected referring to the published method (Fang *et al.*, 2020). In order to confirm the gene replacements in *E. coli* genome, the genes *zwf* and *gnd* were cloned by PCR using the target *E. coli* recombinant genome as template and using *zwf*234-F/*zwf*234-R and *gnd*361-F/*gnd*361-R as primer pairs, respectively.

Preparation of crude enzyme and enzyme activity assays

The cells in the logarithmic post-growth were collected by centrifuge for 10 min at 6000 r/min, and then disrupted using sonication. Following, fragmented liquid was separated by centrifuge at 10000 r/min and 4°C for 30 min. Thus, the cell-free supernatants were used as crude enzyme to determine the enzyme activities. The enzyme activity assays were done in triplicate, and the analytical methods of *zwf* and *gnd* were based on the protocol of Wang *et al.* (2011).

Analytical methods

A sample was taken from the fermenter at the right time. These samples were used to analyze cell growth, glucose concentration and L-tryptophan concentration. For analyzing cell growth, optical density at 562 nm (i.e., OD₅₆₂) was detected using a spectrophotometer after an appropriate dilution. In addition, glucose concentration was detected using an SBA-40E immobilized enzyme biosensor (Shandong, China). The concentration of L-tryptophan was monitored by high performance liquid chromatography (HPLC) using Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) according to the procedure described by Trondle *et al.* (2020)

Results

Disturbance of EMP pathway to increase L-tryptophan production but to limit glucose consumption and cell growth

To disturb the EMP pathway, glucosephosphate isomerase (encoded by gene *pgi*) or phosphofructokinase A (encoded by *pfkA*) were deleted. The resultant strains *E. coli* JW-5 Δ*pgi* and *E. coli* JW-5 Δ*pfkA* showed the bad glucose consumption and cell growth as compared with the original strain *E. coli* JW-5 in M9 minimal medium with 0.1 g/L L-tyrosine, especially for strain JW-5 Δ*pgi* (Fig. 2). The similar results were also found in the next study, in which strains JW-5 Δ*pgi* and JW-5 Δ*pfkA* were incubated in the fermentation medium by fed-batch culture. As can be seen from Figs. 3a and 3b and Table 3, the glucose consumption and cell growth of strains JW-5 Δ*pgi* and JW-5 Δ*pfkA* were obviously decreased as compared with the strain JW-5. It is worth noting that deletion of phosphofructokinase A has lower effect on glucose consumption and cell growth than deletion of glucosephosphate isomerase, especially cultivated in M9 minimal medium (Fig. 2, Figs. 3a and 3b). In fed-batch fermentation, the highest cell growth rate (μ) was found in original strain JW-5 (i.e., 0.44 h⁻¹), and the next

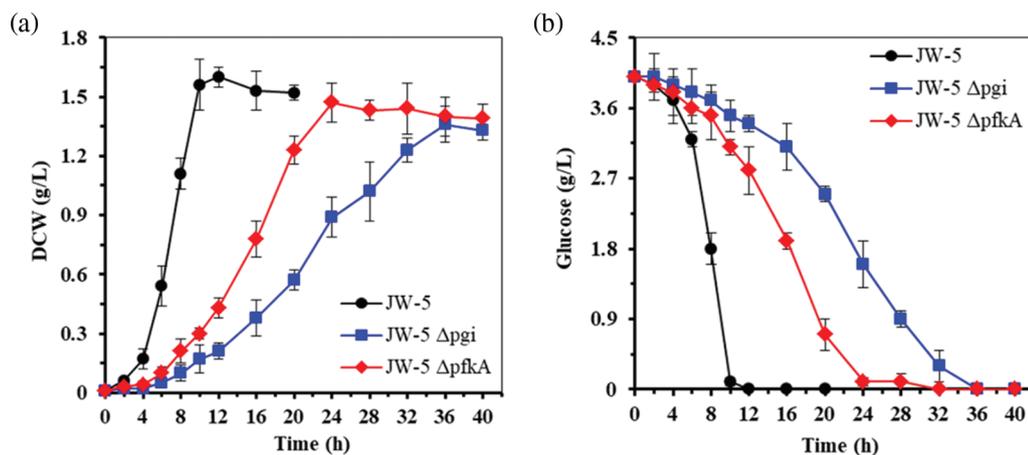


FIGURE 2. Growth situation of recombinant and original strains in M9 medium. (a) Dry cell weight (DCW) of the test strains. (b) The glucose consumption of the test strains. The data represent mean values and standard deviations obtained from three independent cultivations.

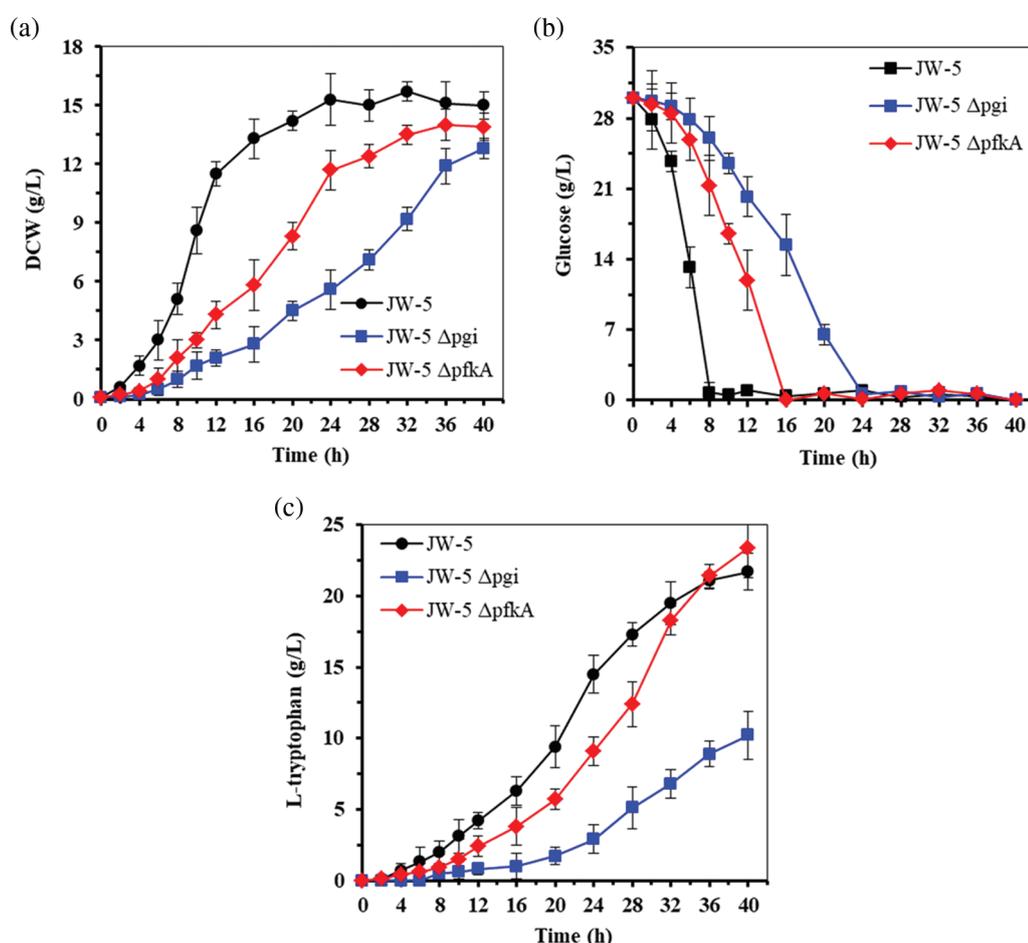


FIGURE 3. Fermentation performance of original strain and recombinant strains in fermentation medium. (a) DCW of the test strains. (b) Glucose consumption of the test strains. (c) L-tryptophan production of the test strains. The data represent mean values and standard deviations obtained from three independent cultivations.

was strain JW-5 Δ pfkA (i.e., 0.29 h^{-1}) and strain JW-5 Δ pgi (i.e., 0.12 h^{-1}) (Table 3). In addition, the original strain JW-5 showed the highest glucose consumption rate (i.e., $0.75 \pm 0.05 \text{ g}/(\text{h-g DCW})$) followed by strain JW-5 Δ pfkA (i.e., $0.63 \pm 0.04 \text{ g}/(\text{h-g DCW})$) and strain JW-5 Δ pgi (i.e., $0.32 \pm 0.07 \text{ g}/(\text{h-g DCW})$) (Table 3). Although strain JW-5 Δ pfkA showed the worse glucose consumption and cell growth than that of strain JW-5, it accumulated the highest L-tryptophan titer (i.e., $23.4 \pm 2.1 \text{ g/L}$) (Fig. 3c and Table 3). However, the strain JW-5 Δ pgi showed the lowest L-tryptophan titer (i.e., $10.2 \pm 1.6 \text{ g/L}$) because of the worst glucose consumption and cell growth (Fig. 3c and Table 3). Thus, deletion of

phosphofructokinase A is the optional strategy to disturb the EMP pathway for constructing L-tryptophan high-producing strain.

Enhancement of PP pathway to increase the L-tryptophan production rather than to obviously change glucose consumption and cell growth

To enhance the carbon flux in PP pathway, the desensitized *zwf* (i.e., *zwf*^{A243T}) and/or *gnd* (i.e., *gnd*^{S361F}) from *C. glutamicum* replaced the native *zwf* and *gnd* in *E. coli* JW-5. The results of enzymes analysis confirmed that *zwf*^{A243T}-coding gene *zwf243* and/or *gnd*^{S361F}-coding gene

TABLE 3

Cell growth, glucose consumption and L-tryptophan production of the original strain *E. coli* JW-5 and the other modified strains used in this study in fermentation medium

<i>E. coli</i> strains	Growth rate μ (h^{-1})	Glucose consumption rate ($\text{g}/(\text{h}\cdot\text{g DCW})$)	L-tryptophan titer (g/L)	L-tryptophan yield YX/S (g/g)
JW-5	0.44	0.75 ± 0.05	21.7 ± 1.3	0.146
JW-5 Δpgi	0.12	0.32 ± 0.07	10.2 ± 1.6	0.155
JW-5 $\Delta pfkA$	0.29	0.63 ± 0.04	23.4 ± 2.1	0.198
JW-5 <i>zwf</i> 243	0.43	0.75 ± 0.10	24.8 ± 1.8	0.183
JW-5 <i>gnd</i> 361	0.44	0.74 ± 0.06	23.3 ± 2.5	0.172
JW-5 <i>zwf</i> 243 <i>gnd</i> 361	0.41	0.76 ± 0.08	26.5 ± 3.2	0.190
JW-5 <i>zwf</i> 243 <i>gnd</i> 361 $\Delta pfkA$	0.32	0.83 ± 0.04	31.9 ± 2.7	0.213

Note: The data was calculated based on at least three independent experiments (\pm SD).

*gnd*³⁶¹ were successfully introduced into *E. coli* JW-5 chromosome (Table 4). Michaelis-Menten affinity constants of *zwf* for glucose-6-phosphate (G6P) and NADP ($K_m(\text{zwf})_{\text{G6P}}$ and $K_m(\text{zwf})_{\text{NADP}}$) of strains JW-5 *zwf*243 and JW-5 *zwf*243 *gnd*361 were all significantly lower than that of strain JW-5 (Table 4). Similar results were also found on Michaelis-Menten affinity constants of *gnd* for 6-phosphogluconate (6GP) and NADP ($K_m(\text{gnd})_{\text{6GP}}$ and $K_m(\text{gnd})_{\text{NADP}}$) of strains JW-5 *gnd*361 and JW-5 *zwf*243 *gnd*361 (Table 4). Although the activity of *zwf*^{A243T} and *gnd*^{S361F} slight lower than that of the native *zwf* and *gnd* in strain JW-5, they showed the lower sensitivity against inhibition by ATP, NADPH and fructose-1, 6-diphosphate (FBP) than that of the native *zwf* and *gnd*, respectively (Fig. 4).

The cell growth of the original and modified strains was monitored in fermentation medium and M9 minimal medium with 0.1 g/L L-tyrosine, and there is not a sharp distinction between the original strain and the modified strains, whether in fermentation medium or in M9 medium (Figs. 5a and 5b). The original strain JW-5 and strain JW-5 *gnd*361 had the highest μ (i.e., 0.44 h^{-1}) and was followed by the strain JW-5 *zwf*243 (i.e., 0.43 h^{-1}) and the strain JW-5 *zwf*243 *gnd*361 (i.e., 0.41 h^{-1}) in fermentation medium (Table 3). In addition, the modified strains also showed the similar glucose consumption to the original strain in M9 medium (Fig. 5c and Table 3). The modified strain JW-5 *zwf*243 *gnd*361 had the highest glucose consumption rate

(i.e., $0.76 \pm 0.08 \text{ g}/(\text{h}\cdot\text{g DCW})$), which was increased no more than 1% than that of the original strain JW-5 (i.e., $0.75 \pm 0.05 \text{ g}/(\text{h}\cdot\text{g DCW})$) in fermentation medium (Table 3). However, the L-tryptophan production was significantly increased with introduction of the desensitized *zwf*^{A243T} and/or *gnd*^{S361F} in strain JW-5 in fermentation medium (Fig. 5d). L-tryptophan production of strain JW-5 *zwf*243 *gnd*361 increased by 22.1% over the strain JW-5 (i.e., $26.5 \pm 3.2 \text{ g/L}$ vs. $21.7 \pm 1.3 \text{ g/L}$), while that of strain JW-5 *zwf*243 (i.e., $24.8 \pm 1.8 \text{ g/L}$) and strain JW-5 *gnd*361 (i.e., $23.3 \pm 2.5 \text{ g/L}$) increased by 14.3% and 7.4%, respectively (Table 3). As can be seen from Table 3, the strain JW-5 *zwf*243 *gnd*361 showed the highest L-tryptophan yield (i.e., 0.190 g/g) among the above mentioned strains including the strains with disruption of EMP pathway and the other strains with enhancement of PP pathway.

Collaboratively modification of the EMP pathway and PP pathway to balance the cell growth and erythrose-4-phosphate supply

As the mention above (Figs. 3 and 5 and Table 3), disruption of EMP pathway (e.g., strain JW-5 $\Delta pfkA$) limited the cell growth and glucose consumption, whereas enhancement of PP pathway (e.g., strain JW-5 *zwf*243 *gnd*361) did not obviously change the cell growth and glucose consumption. It is worth noting that disruption of EMP pathway (i.e., deletion of *pfkA*) and enhancement of PP pathway (i.e., introduction of the desensitized *zwf*234 and *gnd*361)

TABLE 4

Kinetic characterization of *zwf* and *gnd* in *E. coli* strains

<i>E. coli</i> strains	$K_m(\text{zwf})_{\text{G6P}}$ ($\mu\text{mol/L}$)	$K_m(\text{zwf})_{\text{NADP}}$ ($\mu\text{mol/L}$)	$K_m(\text{gnd})_{\text{G6P}}$ ($\mu\text{mol/L}$)	$K_m(\text{gnd})_{\text{NADP}}$ ($\mu\text{mol/L}$)
JW-5	372.6 ± 16.5	87.1 ± 5.8	436.5 ± 37.2	98.4 ± 12.7
JW-5 <i>zwf</i> 243	248.9 ± 14.3	52.4 ± 6.3	ND a	ND a
JW-5 <i>gnd</i> 361	ND a	ND a	325.2 ± 32.4	64.2 ± 4.8
JW-5 <i>zwf</i> 243 <i>gnd</i> 361	250.2 ± 17.2	49.3 ± 2.6	313.6 ± 26.9	69.5 ± 8.3
JW-5 <i>zwf</i> 243 <i>gnd</i> 361 $\Delta pfkA$	258.7 ± 15.6	50.7 ± 3.9	326.3 ± 40.5	68.7 ± 5.4

Note: a Not detected. The data was calculated based on at least three independent experiment.

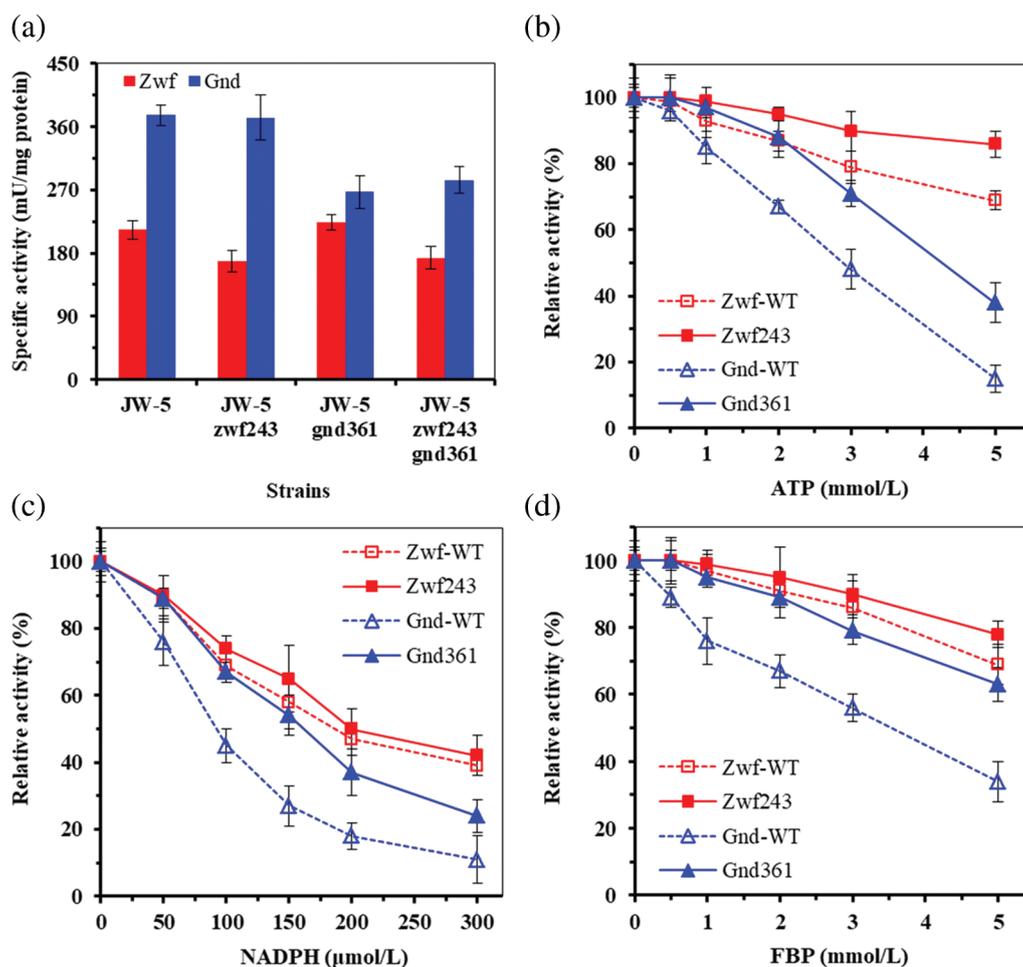


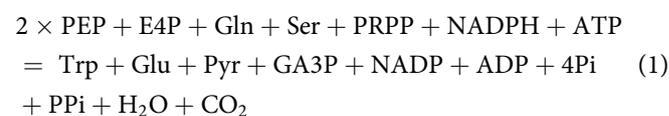
FIGURE 4. The enzymatic properties of the wild-type and mutated *zwf* and *gnd*. (a) The specific activity of *zwf* and *gnd* in different strains. (b) Relative activity (%) of the wild-type and the mutated variant by addition of ATP as inhibitor. (c) Relative activity (%) of the wild-type and the mutated variant by addition of NADPH as inhibitor. (d) Relative activity (%) of the wild-type and the mutated variant by addition of FBP as inhibitor. The data represent mean values and standard deviations obtained from three independent cultivations.

benefits to increase L-tryptophan production because of increasing the carbon flux in PP pathway and thus increasing of the E4P supply. These results implied that the E4P supply is an important limited factor for increasing L-tryptophan production in strain JW-5. As the other precursor for L-tryptophan biosynthesis, PEP is synthesized at the end of the EMP pathway (Fig. 1). In theory, increasing the carbon flux in PP pathway did not change the concentration of PEP (Patnaik and Liao, 1994). Thus, in order to efficiently supply E4P for L-tryptophan production, EMP pathway and PP pathway were co-modified to increase E4P supply rather than to significantly limit the cell growth. In this study, the phosphofructokinase A-coding gene *pfkA* in strain JW-5 *zwf243 gnd361* was deleted to disturb the EMP pathway. And the resultant strain JW-5 *zwf243 gnd361 ΔpfkA* showed the decreased cell growth rate as compared with the strain JW-5 *zwf243 gnd361* in fermentation medium (i.e., 0.32 h^{-1} vs. 0.41 h^{-1}) (Table 3). However, the final dry cell weight (DCW) of strain JW-5 *zwf243 gnd361 ΔpfkA* (i.e., $14.2 \pm 1.3 \text{ g/L}$) was similar with the strain JW-5 *zwf243 gnd361* (i.e., $13.9 \pm 0.8 \text{ g/L}$) (Fig. 6a). In addition, the strain JW-5 *zwf243 gnd361 ΔpfkA* had the highest glucose consumption rate (i.e., $0.83 \pm 0.04 \text{ g/(h·g DCW)}$) followed by the strain JW-5 *zwf243 gnd361* and the strain JW-5 with $0.76 \pm 0.08 \text{ g/(h·g DCW)}$ and 0.75 ± 0.05

g/(h·g DCW) , respectively (Table 3 and Fig. 6b). As can be seen from Fig. 6c and Table 3, the strain JW-5 *zwf243 gnd361 ΔpfkA* produced $31.9 \pm 2.7 \text{ g/L}$ of L-tryptophan, which was 20.4% and 47.0% higher than that of strain JW-5 *zwf243 gnd361* and strain JW-5, respectively.

Discussion

To develop an L-tryptophan high-producing strain, increasing the accessible amounts of precursors has been considered as an indispensable strategy. According to Eq. (1) reported by Bai *et al.* (2019), there are five precursors (i.e., PEP, E4P, Gln, Ser and PRPP) and two co-factors (i.e., NADPH and ATP) involved in L-tryptophan biosynthesis. Normally, increasing the supply of PEP or E4P was deemed as a preferential option for enhancing



production of DAHP, which was the key intermediate for L-tryptophan biosynthesis (Xiong *et al.*, 2021). Two categories of metabolic engineering strategies are used to increase PEP supply in developing L-tryptophan high-producing strain, i.

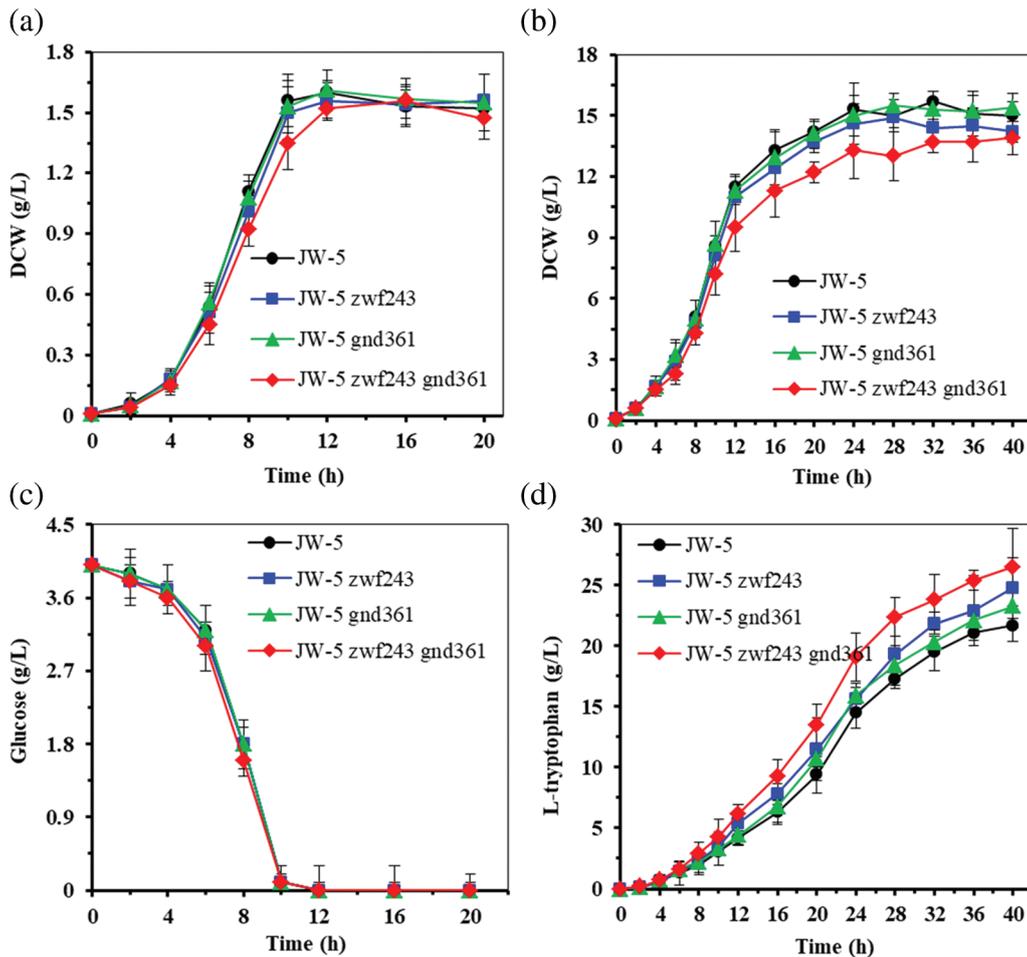


FIGURE 5. Fermentation performance of original strain and recombinant strains in M9 medium and fermentation medium. (a) DCW of the test strains in M9 medium. (b) DCW of the test strains in fermentation medium. (c) The glucose consumption of the test strains in M9 medium. (d) The L-tryptophan production of the test strains in fermentation medium. The data represent mean values and standard deviations obtained from three independent cultivations.

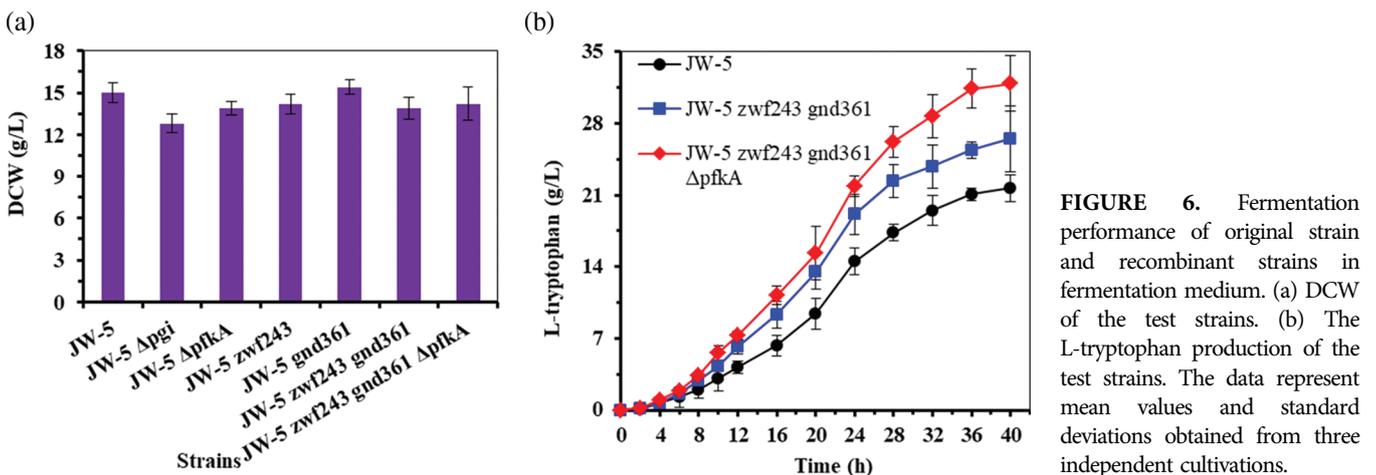


FIGURE 6. Fermentation performance of original strain and recombinant strains in fermentation medium. (a) DCW of the test strains. (b) The L-tryptophan production of the test strains. The data represent mean values and standard deviations obtained from three independent cultivations.

e., reasonable control the local PEP-Pyruvate metabolic note (e.g., overexpression of *ppsA* and deletion of *pykAF* or *ppc*) and reasonable control the PTS (e.g., replacement of PTS transport system by PTS-independent transport system) (Chen et al., 2018; Liu et al., 2017; Ruan et al., 2020; Xiong et al., 2021). In order to increase E4P supply, inactivation or suppression of glucosephosphate isomerase and/or

overexpression of glucose-6-phosphate dehydrogenase and phosphoketolase have been reported (Li et al., 2021; Na et al., 2013; Xiong et al., 2021). However, few researches focused on maintaining the supply of PEP and E4P by collaboratively modifying EMP pathway and PP pathway in *Escherichia coli* to promote L-tryptophan production as far as we know. In this study, we collaboratively modified EMP

pathway and PP pathway to construct an L-tryptophan high-producing strain, and the resultant strain *E. coli* JW-5 *zwf243 gnd361 ΔpfkA* produced 31.9 ± 2.7 g/L of L-tryptophan with a yield of 0.213 g/g in 40-h fed-batch fermentation. The results of this study have important reference value for constructing the other fine chemicals high-yielding strain, in which the biosynthesis of this fine chemical needs no fewer than two precursors Glucosephosphate isomerase (encoded by *pgi*) and phosphofructokinase A (encoded by *pfkA*) are key enzymes in EMP pathway (Fig. 1). Deletion of *pgi* or *pfkA* strongly decreased the cell growth and glucose consumption rate (Fig. 2). The similar results were also found in previous results (Li *et al.*, 2021; Siedler *et al.*, 2012), and this is because the high glucose-6-phosphate pool will destabilize the *ptsG* mRNA in *pgi*-deleted strain (Morita *et al.*, 2003). In addition, deletion of *pgi* or *pfkA* reduces PEP concentration and thus limits the PTS (Roehl and Vinopal, 1976). As compared with the strain JW-5 Δ *pgi*, the strain JW-5 Δ *pfkA* showed the high cell growth, glucose consumption rate and L-tryptophan production in fermentation medium (Fig. 3). According to the flux analysis reported by Siedler *et al.* (2012), the *pfkA*-deleted strain possessed the higher carbon flux in EMP pathway and PP pathway than that in *pgi*-deleted strain. Therefore, this may be the reason why the strain JW-5 Δ *pfkA* shows the better fermentability than strain JW-5 Δ *pgi*. *zwf* (encoded by *zwf*) and *gnd* (encoded by *gnd*) are the limit enzymes in PP pathway, which involve in the NADPH regeneration (Chen *et al.*, 2019). However, the activity of *zwf* and *gnd* was inhibited by NADPH, ATP and fructose-1,6-bisphosphate (Becker *et al.*, 2007; Ohnishi *et al.*, 2005). In addition, the activity of *gnd* was additionally inhibited by glyceraldehyde-3-phosphate, E4P and ribulose 5-phosphate (Wang *et al.*, 2011). Becker *et al.* (2007) pointed out that replacement of L-alanine residue by L-threonine residue at 243 site (i.e., A243T) relieves the inhibition by ATP, PEP and fructose-1,6-bisphosphate as well as increases the affinity of *zwf* towards NADP⁺. In addition, introduction of point mutation (i.e., S361F) in *gnd* resulted in reducing the sensitivity against inhibition by fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate, PRPP, ATP, and NADPH (Ohnishi *et al.*, 2005). Based on these findings, the native *zwf* and *gnd* were replaced by the mutated *zwf* (i.e., *zwf*^{A234T}) and *gnd* (i.e., *gnd*^{S361F}) from *C. glutamicum* in *E. coli* JW-5, and the resultant strains showed the significantly lower Km for substrate than that of the originals strain JW-5 (Table 4). The similar results were also found in previous results, in which the *zwf*^{A234T} and *gnd*^{S361F} were introduced into *Bacillus subtilis* (Wang *et al.*, 2011). Our results (Table 3 and Fig. 5) and previous results (Meng *et al.*, 2016; Wang *et al.*, 2011) confirmed that the introduction of *zwf*^{A234T} and *gnd*^{S361F} did not obviously change glucose consumption rate and cell growth rate but significant increase the production of the target products. These results indicated that increase the carbon flux in PP pathway benefits to increase L-tryptophan production and the E4P supply is an important limited factor for increasing L-tryptophan production in strain JW-5. In addition, Xiong *et al.* (2021) found that heterologous expression of phosphoketolase from *Bifidobacterium adolescentis* to strengthen E4P formation benefits to increase L-tryptophan

production, and these results confirmed E4P supply is an important limited factor for L-tryptophan biosynthesis. In order to enhancement the PP pathway, EMP pathway and PP pathway were co-modified to increase E4P supply. As can be seen from Fig. 6c and Table 3, the resultant strain JW-5 *zwf243 gnd361 ΔpfkA* produced 31.9 ± 2.7 g/L of L-tryptophan, which was 20.4% and 47.0% higher than that of strain JW-5 *zwf243 gnd361* and strain JW-5, respectively. Although the strain JW-5 *zwf243 gnd361 ΔpfkA* showed the decreased cell growth rate as compared with the strain JW-5 *zwf243 gnd361* in fermentation medium (Table 3), its final DCW was similar with the strain JW-5 *zwf243 gnd361* (Fig. 6a). This is probably due to the low PEP supply for cell growth. Li *et al.* (2021) indicated that the mutated *E. coli* strains show the different growth situation during suppressing of *pfkA* with different repression range using CRISPRi. It should be noted that the strain JW-5 *zwf243 gnd361 ΔpfkA* had the highest glucose consumption rate (i.e., 0.83 ± 0.04 g/(h-g DCW)) among these tested strains (Table 3). In addition, the cell growth rate and DCW of strain JW-5 *zwf243 gnd361 ΔpfkA* were also higher than that of strain JW-5 Δ *pfkA* in fermentation medium (Fig. 3a, Fig. 6a and Table 3). Morita *et al.* (2003) pointed out that the high glucose-6-phosphate pool will destabilize the *ptsG* mRNA in *pgi*-deleted strain and thus limit the PTS. However, the strain JW-5 *zwf243 gnd361 ΔpfkA* with the desensitized *zwf*^{A234T} and *gnd*^{S361F} ensure that glucose-6-phosphate could effectively entry in PP pathway. As compared with original strain JW-5, the strain JW-5 *zwf243 gnd361 ΔpfkA* with co-modification of EMP pathway and PP pathway showed the high L-tryptophan titer (i.e., 31.9 ± 2.7 g/L vs. 21.7 ± 1.3 g/L), the high L-tryptophan yield (i.e., 0.213 g/g vs. 0.146 g/g) and the high glucose consumption rate (i.e., 0.83 ± 0.04 g/(h-g DCW) vs. 0.75 ± 0.05 g/(h-g DCW)) despite of the bad cell growth rate (i.e., 0.32 h⁻¹ vs. 0.44 h⁻¹). These are because enhancement of PP pathway results in the increase of intermediate metabolites (e.g., NADPH, PRPP and E4P) supply and thus to increase the production of L-tryptophan and the other products, for example, xylitol (Yuan *et al.*, 2021).

Conclusions

In conclusion, an L-tryptophan high-yielding strain *E. coli* JW-5 *zwf243 gnd361 ΔpfkA* was constructed by collaboratively modifying EMP pathway and PP pathway for the first time, which produced 31.9 ± 2.7 g/L of L-tryptophan with a yield of 0.213 g/g in 40-h fed-batch fermentation. Compared with the strain with the only modification of EMP pathway or PP pathway, the strain *E. coli* JW-5 *zwf243 gnd361 ΔpfkA* showed the excellent fermentability, i.e., the high L-tryptophan titer and yield as well as the quick glucose consumption rate.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: Shuai Liu, Jian-Zhong Xu, Zhi-Ming Rao, Wei-Guo Zhang; data collection: Shuai Liu, Ting-Shan Liu; analysis and interpretation

of results: Shuai Liu; Jian-Zhong Xu; draft manuscript preparation: Jian-Zhong Xu, Shuai Liu. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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