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FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Phosphoenolpyruvate:glucose phosphotransferase system modification increases the conversion rate during L-tryptophan production in *Escherichia coli*

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Abstract *Escherichia coli* FB-04(*pta*1), a recombinant L-tryptophan production strain, was constructed in our laboratory. However, the conversion rate (L-tryptophan yield per glucose) of this strain is somewhat low. In this study, additional genes have been deleted in an effort to increase the conversion rate of *E. coli* FB-04(*pta*1). Initially, the *pykF* gene, which encodes pyruvate kinase I (PYKI), was inactivated to increase the accumulation of phosphoenolpyruvate, a key L-tryptophan precursor. The resulting strain, *E. coli* FB-04(*pta*1)*ΔpykF*, showed a slightly higher L-tryptophan yield and a higher conversion rate in fermentation processes. To further improve the conversion rate, the phosphoenolpyruvate:glucose phosphotransferase system (PTS) was disrupted by deleting the *ptsH* gene, which encodes the phosphocarrier protein (HPr). The levels of biomass, L-tryptophan yield, and conversion rate of this strain, *E. coli* FB-04(*pta*1)*ΔpykF/ptsH,* were especially low during fed-batch fermentation process, even though it achieved a significant increase in conversion rate during shake-flask fermentation. To resolve this issue, four HPr mutations (N12S, N12A, S46A, and S46N) were introduced into the genomic background of *E. coli* FB-04(*pta*1)*ΔpykF/ptsH*, respectively. Among them, the strain harboring the N12S mutation (*E. coli* FB-04(*pta*1)*ΔpykF-ptsHN12S*) showed a prominently increased conversion rate of 0.178 g g^{-1} during fed-batch fermentation; an increase of 38.0% compared with parent strain *E. coli* FB-04(*pta*1). Thus, mutation of the genomic of *ptsH* gene provided an alternative method to weaken the PTS and improve the efficiency of carbon source utilization.

Keywords L-Tryptophan · Conversion rate · Pyruvate kinase · HPr · Phosphoenolpyruvate:glucose phosphotransferase system

Introduction

L-Tryptophan, an important amino acid for humans and other animals, is extensively used as a supplement in food and animal feed, as well as for medicinal purposes [12, 15]. Given that the L-tryptophan biosynthetic pathway in microorganisms is fairly long and its regulation is intricate, the underlying regulatory networks have been modified in a variety of ways to improve L-tryptophan yield [1, 17, 28, 34]. L-Tryptophan production through *Escherichia coli* fermentation has been studied extensively, and L-tryptophan production strains with completely defined genetic traits and high yields have been constructed [4, 10, 13, 16]. In recent efforts to reduce productions costs during fermentation, effective utilization of carbon source has attracted considerable attention [6, 18, 30, 31].

Phosphoenolpyruvate, which is a product of glycolysis, is an essential L-tryptophan precursor [18, 33]. In glycolysis, pyruvate kinases (PYKI and PYKII, EC 2.7.1.40, which are encoded by the *pykF* and *pykA* genes, respectively) catalyze the formation of pyruvate from phosphoenolpyruvate [31]. It was well known that knocking out either *pykA* or *pykF* increases the accumulation of phosphoenolpyruvate, which contributes to the production of aromatic amino acids [14]. The phosphoenolpyruvate:glucose

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phosphotransferase system (PTS), which is primarily responsible for the uptake and phosphorylation of sugars, consumes phosphoenolpyruvate. The PTS generally contains two central phosphoryl carriers, EI and HPr, which are encoded by *ptsI* and *ptsH*, respectively [9, 27]. Given that the PTS consumes phosphoenolpyruvate during glucose transfer, the PTS is extensively distributed in eubacteria, forming non-PTS glucose uptake mutants [3, 8]. Moreover, the PTS plays an important role in the regulation of carbon metabolism, which has a close relationship with normal cellular physiology [5, 22, 23, 27]. Remarkably, the effects of genomic mutations (other than deletion) in *ptsI* or *ptsH* on glucose consumption have gone under-reported, even though this strategy may increase the conversion rate without the deleterious effects of PTS disruption.

E. coli FB-04(*pta*1), a recombinant L-tryptophan production strain, was recently constructed and stored in our laboratory [16]. Unfortunately, this strain displays a relatively low conversion rate (L-tryptophan per glucose). Two series of experiments were performed to improve the conversion rate of this strain. In the first, a *pykF* deletion strain (*ΔpykF*), a *pykA* deletion strain (*ΔpykA*), and a *pykF/pykA* double deletion strain (*ΔpykF/pykA*) were constructed, and their ability to produce L-tryptophan was evaluated in the shake-flask fermentations. These results led to a construction of four strains containing the *pykF* deletion, but also harboring a mutation in the *ptsH* gene. The most promising of these strains, *E. coli* FB-04(*pta*1) *ΔpykF-ptsHN12S*, showed a remarkably increased conversion rate.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used or constructed in this study are listed in Table 1. *E. coli* strain JM109, obtained from Novagen (Madison, USA), was used for gene cloning and plasmid construction. Previously, recombinant *E. coli* FB-04(*pta*1) harboring plasmid pSTV-03 was constructed and reserved in our laboratory [16].

The pMD18-T simple vector was obtained from Takara (Dalian, China). Plasmids pKD13, pKD46, and pCP20, which were purchased from the *E. coli* Genetic Stock

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Description/genotype/sequence	Sources
Strains		
FB-04(*pta*1)	*E. coli* W3110 k12, *ΔtrpR*::FRT*ΔtnaA*::FRT*ΔpheA*::FRT*ΔtyrA*::FRT, harboring pSTV-03 plasmid, *pta*1 genomic substitution	[16]
ΔpykA	*ΔpykA*::FRT derived from strain FB-04(*pta*1)	This study
ΔpykF	*ΔpykF*::FRT derived from strain FB-04(*pta*1)	This study
ΔpykF/pykA	*ΔpykF*::FRT and *ΔpykA*::FRT derived from strain FB-04(*pta*1)	This study
ΔpykF/ptsH	*ΔptsH*::FRT derived from strain *ΔpykF*	This study
E. coli JM109	*recA1, thi, endA1, supE44, gyrA96, hsdR17 Δ*(*lac-proAB*)/(*lac-proAB*)/ F′[*traD36,proAB*$^+$, *lacI*^{q}, *lacZΔ*M15]	Takara
ΔpykF-ptsHN12S	*ptsHN12S* genomic substitution in strain*ΔpykF*	This study
ΔpykF-ptsHN12A	*ptsHN12A* genomic substitution in strain*ΔpykF*	This study
ΔpykF-ptsHS46A	*ptsHS46A* genomic substitution in strain*ΔpykF*	This study
ΔpykF-ptsHS46N	*ptsHS46N* genomic substitution in strain*ΔpykF*	This study
Plasmids		
pKD13	Amp and kan markers	[2]
pKD46	Amp markers, helper plasmid	[2]
pCP20	Amp and chl markers, for removal of antibiotic resistance cassette	[2]
pSTV-03	Based on plasmids of pACYC177 and pND707; p15A replicon, kan marker, PR and PL promoters, carrying *aroF*^{fbr} and *trpE*^{fbr}*D*	[31]
pMD-SK	pMD18-T harboring *kan* and *sacB*	[16]
pMD18-T	Cloning vector	Novagen
pMD18-*ptsHN12S*	pMD18-T harboring *ptsHN12S*	This study
pMD18-*ptsHN12A*	pMD18-T harboring *ptsHN12A*	This study
pMD18-*ptsHS46A*	pMD18-T harboring *ptsHS46A*	This study
pMD18-*ptsHS46N*	pMD18-T harboring *ptsHS46N*	This study

Center (Yale University, New Haven, CT, USA), were used for gene knockout procedures. Plasmid pMD-SK, which contains the kanamycin resistance gene (*kan*) and the levansucrase gene (*sacB*), was previously constructed and reserved in our laboratory [16].

Plasmid construction and gene mutation

The sequences of the primers designed for PCR amplification are shown in Table 2. Plasmid and chromosomal DNA were prepared using the Plasmid Mini-Prep Kit and Genomic DNA Isolation Kit, respectively, obtained from BIO Basic Inc. The preparation of competent cells and their transformation with plasmids were carried out using criterion processes [24].

To clone *ptsH*, the *ptsH* gene (GenBank accession number: gi 946886) was first amplified from *E. coli* FB-04(*pta*1) genomic DNA using V-*ptsH* primers (Table 2). The resulting DNA fragment was ligated into the pMD18-T simple vector to form plasmid pMD-*ptsH*. Plasmids pMD-*ptsHN12S*, pMD-*ptsHN12A*, pMD-*ptsHS46A*, and pMD-*ptsHS46N* were constructed using PCR-mediated site-directed mutagenesis, with plasmid pMD-*ptsH* as the template and primers C-*ptsHN12S*, C-*ptsHN12A*, C-*ptsHS46A,* and C-*ptsHS46N*, respectively (Table 2).

Construction of single- or multi-gene knockout mutants was performed using the λ Red recombination method [2]. To delete the *pykF* gene (GenBank accession number gi 946179) or the *pykA gene* (GenBank accession number: gi 946527), a DNA fragment including a *kan* fragment plus homologous sequences was amplified by PCR from plasmid pKD13, using primers P**-***pykF* or P**-***pykA* (Table 2). To delete the *ptsH* gene, a DNA fragment containing a *kan-sacB* fragment plus homologous sequences was amplified from plasmid pMD-SK using P-*ptsH* primers (Table 2). The homologous sequences used for homologous recombination are represented by the underlined letters. The resulting *kan*- or *kan-sacB*-containing fragment was transduced into competent cells harboring plasmid pKD46. The screening of mutant gene was carried out using kanamycin

Table 2 Primers used in this study

Primers		
P-*pykA*	5′-ATGTCCAGAAGGCTTCGCAGAACAAAAATCGTTACCACG TTAGGCCCAGC ATTCCGGGGATCCGTCGACC-3′	This study
	5′-TTACTCTACCGTTAAAATACGCGTGGTATTAGTAGAACCC ACGGTACTCA TGTAGGCTGGAGCTGCTTCG-3	
P**-***pykF*	5′-TAAGACTGTCATGAAAAAGACCAAAATTGTTTGCACCA TCGGACCGAAAAATTCCGGGGATCCGTCGACC-3′	This study
	5′-CAGGACGTGAACAGATGCGGTGTTAGTAGTGCCGCTCG GTACCAGTGCACTGTAGGCTGGAGCTGCTTCG-3	
P-*ptsH*	5′-ATGTTCCAGC AAGAAGTTAC CATTACCGCT CCGGCCGGTC TGCACACCCG ATTCCGGGGATCCGTCGACC-3′	This study
	5′-TTACTCGAGT TCCGCCATCA GTTTAACCAG ATGTTCAACC GCTTTCTGTGCCAATAGGATATCGGCAT -3	
V-*pykA*	5′-CAGAGATAACTTGAAGCGGGTCA -3′	This study
	5′- CCAGCCGTATAAGCGCAAAG -3′	
V-*pykF*	5′-GAGCGAGGCACCACCACTTT-3′	This study
	5′-CCCATCAGGGCGCTTCGATA-3′	
V-*ptsH*	5′-ATGTTCCAGCAAGAAGTTAC -3 -3′	This study
	5′-TTACTCGAGTTCCGCCATCAG -3 -3′	
C-*ptsHN12S*	5′-CATTACCGCTCCGGCCGGTCTGCACACCCGC -3	This study
	5′-GGGTGTGCAGACCGGCCGGAGCGGTAATGGTA -3	
C-*ptsHN12A*	5′-CATTACCGCTCCGAGCGGTCTGCACACCCGC -3	This study
	5′-GCGGGTGTGCAGACCGCTCGGAGCGGTAATG -3	
C-*ptsHS46A*	5′-AGCGCCAGCGCGAAAGCCCTGTTTAAACTG -3	This study
	5′-CAGTTTAAACAGGGCTTTCGCGCTGGCGCT -3	
C-*ptsHS46N*	5′-AGCGCCAGCGCGAAAAACCTGTTTAAACTG -3	This study
	5′-CTGCAGTTTAAACAGGTTTTTCGCGCTGGC -3	

Underlined letters represent homologous sequences used for homologous recombination

resistance. PCR using primers V-*pykF*, V-*pykA,* and V-*ptsH* primers was employed to verify the *pykF*, *pykA*, and *ptsH* gene knockout mutations, respectively (Table 2). Finally, the *kan* fragment in the *pykF* or *pykA* deletion strain was eliminated using helper plasmid pCP20. For the scar-less insertion of *ptsH* mutants into the genome, the corresponding *ptsH* mutant fragment, which was amplified from plasmid pMD-*ptsHN12S*, pMD-*ptsHN12A*, pMD-*ptsHS46A*, or pMD-*ptsHS46N*, respectively, using primers V-*ptsH* (Table 2), was transduced into the *ptsH* single-gene knockout mutant harboring plasmid pKD46, and sucrose (15% w/v) was as a selection marker. DNA sequencing was also carried out to verify all genetic mutations.

Media and culture conditions

For shake-flask fermentation, seed cultures of strain FB-04(*pta*1) and its derivatives were grown in Luria–Bertani medium (10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract, and 10.0 g L^{-1} NaCl) supplemented with 30 μg mL^{-1} kanamycin. These cultures were incubated at 37 °C for 10 h in a rotary shaker (200 rpm). Aliquots (200 μL) of these seed cultures were added into flask fermentation medium (100 mL), which contained (per L): 24 g K_2HPO_4, 9.6 g KH_2PO_4, 10 g glucose, 1 g $MgSO_4{\cdot}7H_2O$, 2 g citric acid, 5 g $(NH_4)_2SO_4$, 15 g yeast, and 3 mL of trace element solution [17]. The above-mentioned cultures were incubated in 500-mL flasks supplemented with 30 μg mL^{-1} kanamycin for 48 h at 37 °C with shaking (200 rpm). During the fermentation, 10 g L^{-1} glucose and 20% NH_4OH were added to the medium every 8 h to provide carbon source and maintain the pH (6.5–7.2), respectively.

For fed-batch cultivation in a 3-L fermentor, seed cultures of strain FB-04(*pta*1) and its derivatives were initiated by inoculating LB medium (100 mL supplementing with 30 μg mL^{-1} kanamycin) in a 500-mL shake flask with 200 μL of glycerol stock (kept frozen at −80 °C). The resulting culture was incubated at 37 °C with shaking at 200 rpm for 10 h. These seed cultures (100 mL) were then transferred to a 3-L fermentor (BioFlo 110, New Brunswick Scientific Co., Edison, NJ) charged with 0.9 L of fermentation medium at pH 6.5 containing (per L): 15 g K_2HPO_4, 2 g yeast, 2 g $MgSO_4{\cdot}7H_2O$, 2 g citric acid, 1.6 g $(NH_4)_2SO_4$, 7.5 g glucose, 0.0129 g $CaCl_2$, 0.075 g $FeSO_4{\cdot}7H_2O$ per liter, and 3 mL trace element solution [17], and added with kanamycin at a final concentration of 30 μg mL^{-1}. The 3-L fermentations were performed at 37 °C for about 54 h with the pH at 6.5. The pH and dissolved oxygen (20%) were maintained by automatically adding NH_4OH and adjusting the agitation speed, respectively. Samples were collected every 3–4 h. After the initial glucose was exhausted, additional glucose (800 g L^{-1}) was added to the fermentation medium using an exponential feeding program, which maintained a cell-specific growth (OD_{600}) rate of 0.15 h^{-1}. The term glucose consumption refers to the total amount of glucose consumed by cells per liter, and the ratio of L-tryptophan yield divided by the glucose consumption is termed conversion rate.

Analytical methods

The concentration of cell was determined using the optical density at 600 nm (OD_{600}) after proper dilution. To determine the levels of extracellular metabolites, the fermentation broth was clarified using centrifugation at 14,000×*g* for 10 min, and then reserved at −30 °C until it was analyzed by employing high-performance liquid chromatography (HPLC). The levels of glucose and acetate were measured at 50 °C with an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rid, Hercules, CA). The mobile phase consisted of 50 mM H_2SO_4 and the flow rate was 0.5 mL min^{-1}. The L-tryptophan concentration was determined using a previously described HPLC method [34].

Statistical analyses

All fermentation experiments were repeated at least three times. The fermentation parameters are displayed as the mean ± standard deviation of these replicates. Statistically significant ($P < 0.01$) differences were identified using Student's *t* test.

Results

Inactivation of pyruvate kinase (PYK)

To investigate the effect of pyruvate kinase (PYK) inactivation on L-tryptophan production, the individual genes *pykF* and *pykA,* as well as the combination of *pykF* and *pykA* were deleted from the genome of *E. coli* FB-04(*pta*1) using λ Red recombination. We will refer to the resulting strains as *ΔpykF*, *ΔpykA*, and *ΔpykF/pykA*, respectively. Then, the fermentation performance of these new strains was compared with that of *E. coli* FB-04(*pta*1) in a series of shake-flask cultures. As shown in Fig. 1a, strains *ΔpykF* and *ΔpykA* displayed growth curves similar to that of parent strain FB-04(*pta*1). Among all the strains tested, *ΔpykF* achieved the highest total biomass, while the growth of strain *ΔpykF/pykA* was restricted (Table 4).

Throughout the fermentation process, the L-tryptophan levels of strains *ΔpykF* and *ΔpykA* were higher than that of FB-04(*pta*1), while strain *ΔpykF/pykA* showed a

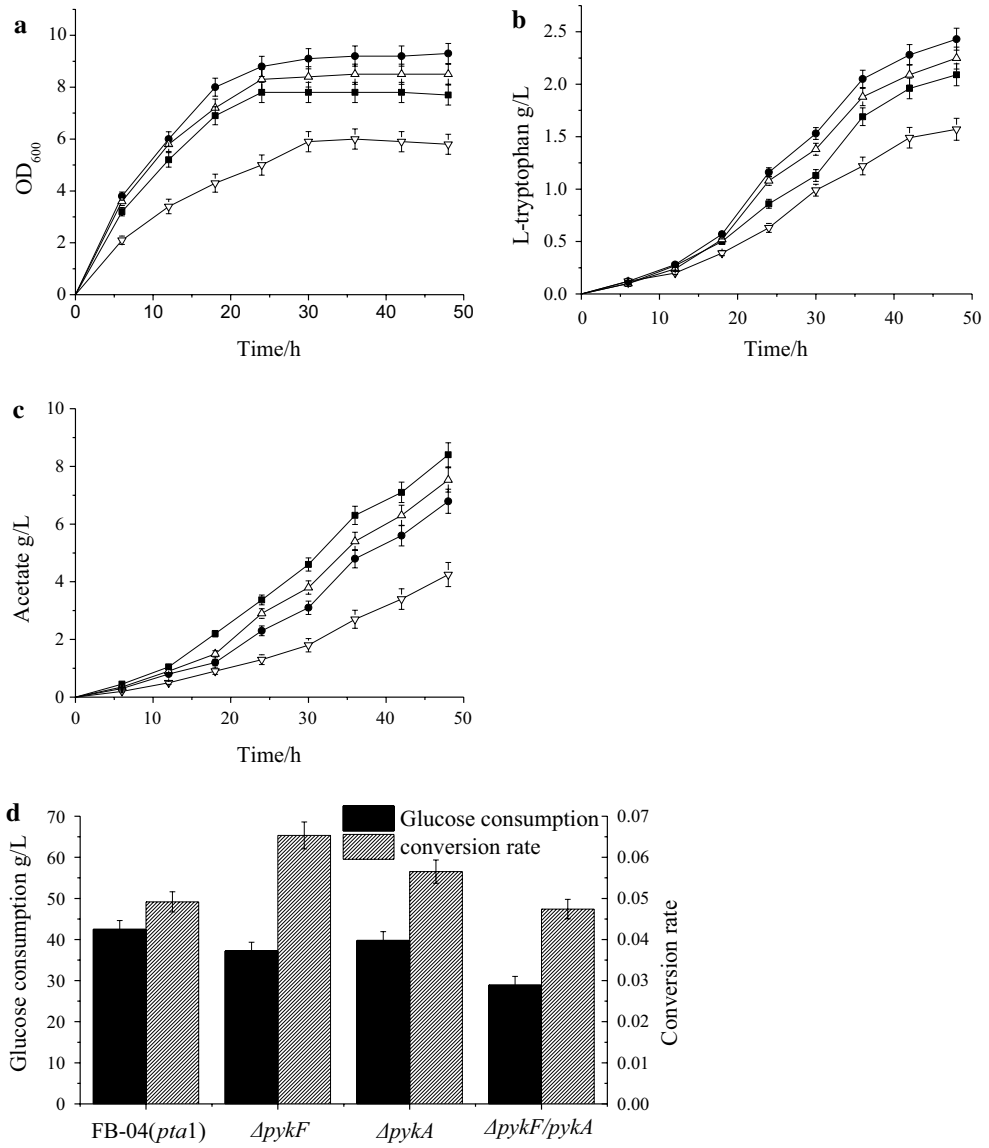

Fig. 1 Shake-flask fed-batch fermentation of strains FB-04(*pta*1), *ΔpykF*, *ΔpykA,* and*ΔpykF/pykA*. **a** Biomass levels (OD_{600}); **b** L-tryptophan levels; **c** acetate levels; **d** glucose consumption levels and conversion rate. FB-04(*pta*1) (*filled square*); *ΔpykF* (*filled circle*); *ΔpykA* (*upright open triangle*); *ΔpykF/pykA* (*inverted open triangle*)

Table 3 Enzyme I kinetic parameters with HPr mutations

HPr variant	K_m (μM)	V_{max} (% wild type)	k_{cat}/K_m (% relative)	Sources
Wild type	6	100	100	[19]
N12S	6	65	65	[20]
N12A	12	50	25	[20]
S46A	15	100	40	[19]
S46N	65	100	9	[19]

significant decrease (Fig. 1b). The final L-tryptophan titers of strains *ΔpykF* and *ΔpykA* were 2.43 and 2.25 g L^{-1}, leading to significant increases of 16 and 8% ($P < 0.01$, Student's *t* test), respectively, compared with FB-04(*pta*1) (Table 3). Acetate levels were also determined (Fig. 1c). Strains *ΔpykF*, *ΔpykA*, and *ΔpykF/pykA* showed 19, 10, and 49% decreases in acetate formation, respectively, compared with that of FB-04(*pta*1) (Table 4). The final glucose consumption of strains *ΔpykF*, *ΔpykA*, and *ΔpykF/pykA*

Table 4 Fermentation parameters of different strains

Strains	Glucose consumption (g/L)	Maximum biomass (OD_{600})	Maximum L-tryptophan (g L^{-1})	L-Tryptophan productivity (g L^{-1} h^{-1})	L-Tryptophan yield per glucose (g g^{-1})	Maximum acetate (g L^{-1})
Flask fermentation						
FB-04(*pta*1)	42.5 ± 4.3	7.5 ± 0.6	2.09 ± 0.2	0.044 ± 0.005	0.049 ± 0.003	8.40 ± 0.5
ΔpykF	37.2 ± 3.0	9.0 ± 0.9	2.43 ± 0.2	0.051 ± 0.004	0.065 ± 0.004	6.79 ± 0.6
ΔpykA	39.8 ± 3.7	8.6 ± 0.6	2.25 ± 0.1	0.047 ± 0.003	0.056 ± 0.003	7.53 ± 0.3
ΔpykF/pykA	26.9 ± 2.4	5.8 ± 0.4	1.37 ± 0.1	0.029 ± 0.001	0.051 ± 0.001	4.25 ± 0.2
ΔpykF/ptsH	6.1 ± 1.0	4.4 ± 0.3	0.78 ± 0.1	0.016 ± 0.001	0.128 ± 0.004	0.97 ± 0.1
ΔpykF-ptsHN12S	34.4 ± 2.7	9.2 ± 0.7	2.65 ± 0.2	0.055 ± 0.003	0.077 ± 0.002	5.87 ± 0.2
ΔpykF-ptsHN12A	34.8 ± 2.2	9.8 ± 0.8	3.28 ± 0.3	0.067 ± 0.002	0.094 ± 0.001	3.19 ± 0.2
ΔpykF-ptsHS46A	35.0 ± 2.9	9.5 ± 0.6	2.84 ± 0.4	0.059 ± 0.003	0.081 ± 0.001	5.01 ± 0.4
ΔpykF-ptsHS46N	8.3 ± 1.2	5.2 ± 0.4	0.99 ± 0.1	0.021 ± 0.001	0.119 ± 0.003	1.23 ± 0.1
Fed-batch fermentation						
FB-04(*pta*1)	341 ± 27	82.8 ± 6.5	44.0 ± 2.8	0.820 ± 0.06	0.129 ± 0.01	2.1 ± 0.3
ΔpykF	327 ± 21	72.2 ± 4.1	45.5 ± 3.1	0.834 ± 0.06	0.140 ± 0.01	1.9 ± 0.3
ΔpykF/ptsH	52 ± 4	21.6 ± 1.7	3.8 ± 0.2	0.070 ± 0.01	0.073 ± 0.01	0.2 ± 0.1
ΔpykF-ptsHN12S	146 ± 12	48.6 ± 2.2	26.0 ± 1.5	0.477 ± 0.03	0.178 ± 0.02	0.6 ± 0.1
ΔpykF-ptsHN12A	117 ± 11	39.5 ± 2.0	9.6 ± 0.8	0.176 ± 0.01	0.082 ± 0.01	0.3 ± 0.1
ΔpykF-ptsHS46A	164 ± 13	66.4 ± 3.8	14.1 ± 1.1	0.259 ± 0.02	0.086 ± 0.01	0.9 ± 0.3

was lower than that of parent strain FB-04(*pta*1), resulting in decreases of 13, 11, and 37%, respectively (Fig. 1d). In addition, the conversion rate (L-tryptophan yield per glucose) of strain *ΔpykF* was higher than that of strains FB-04(*pta*1), *ΔpykA*, and *ΔpykF/pykA* (Fig. 1d), leading to significant increase of 33, 16, and 27% ($P < 0.01$, Student's *t* test), respectively.

Deletion of gene *ptsH*

To abolish the consumption of phosphoenolpyruvate in the glucose uptake process, the phosphoenolpyruvate:glucose phosphotransferase system (PTS) of strain *ΔpykF* was disrupted by genomic deletion of the *ptsH* gene, forming strain FB-04(*pta*1)*ΔpykF/ptsH*, which we will refer to as *ΔpykF/ptsH*. During shake-flask fermentation, the biomass level, L-tryptophan production, and glucose consumption of strain *ΔpykF/ptsH* were significantly lower than those of *ΔpykF* (Fig. 2a–c). The final glucose consumption of strain *ΔpykF/ptsH* was significantly lower than that of strain *ΔpykF* (Fig. 2d). However, the conversion rate of strain *ΔpykF/ptsH* was about twice that of the strain *ΔpykF* (Table 4).

Genomic substitution of *ptsH* mutants

The phosphocarrier protein (HPr) of the PTS has been thoroughly studied, and a series of HPr mutants which led to different kinetic properties of enzyme I (EI) were found [19, 20]. In this study, HPr mutants N12A, N12S, S46A, and S46N were introduced into the genomic background of *ΔpykF/ptsH* to restore the growth and glucose uptake restrictions of *ΔpykF/ptsH* caused by its *ptsH* deletion. This process produced strains FB-04(*pta*1)*ΔpykF-ptsHN12A*, FB-04(*pta*1)*ΔpykF-ptsHN12S*, FB-04(*pta*1)*ΔpykF-ptsHN46A*, and FB-04(*pta*1)*ΔpykF-ptsHN46S*, which we will refer to as *ΔpykF-ptsHN12A*, *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN46A*, and *ΔpykF-ptsHN46S*, respectively. The different kinetic properties of EI caused by HPr mutants N12A, N12S, S46A, and S46N are displayed in Table 3. To determine the performance of the mutant strains, they were cultivated in shake flasks. *ΔpykF-ptsHN12S* grew slightly faster than *ΔpykF* during the entire fermentation process (Fig. 3a). The final biomass of strain *ΔpykF-ptsHN12A* was the highest among all the strains. Strain *ΔpykF-ptsHS46A* achieved the second-highest biomass, even though the growth of *ΔpykF-ptsHN12A* and *ΔpykF-ptsHS46A* was lower than that of *ΔpykF* at the first 24 h. The growth of *ΔpykF-ptsHS46N* was restricted, although it was slightly higher than that of *ΔpykF/ptsH* during the entire fermentation process.

The L-tryptophan titers of all strains increased with time during the entire shake-flask fermentation process (Fig. 2b). Compared with strain *ΔpykF*, the final L-tryptophan titers of *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*,

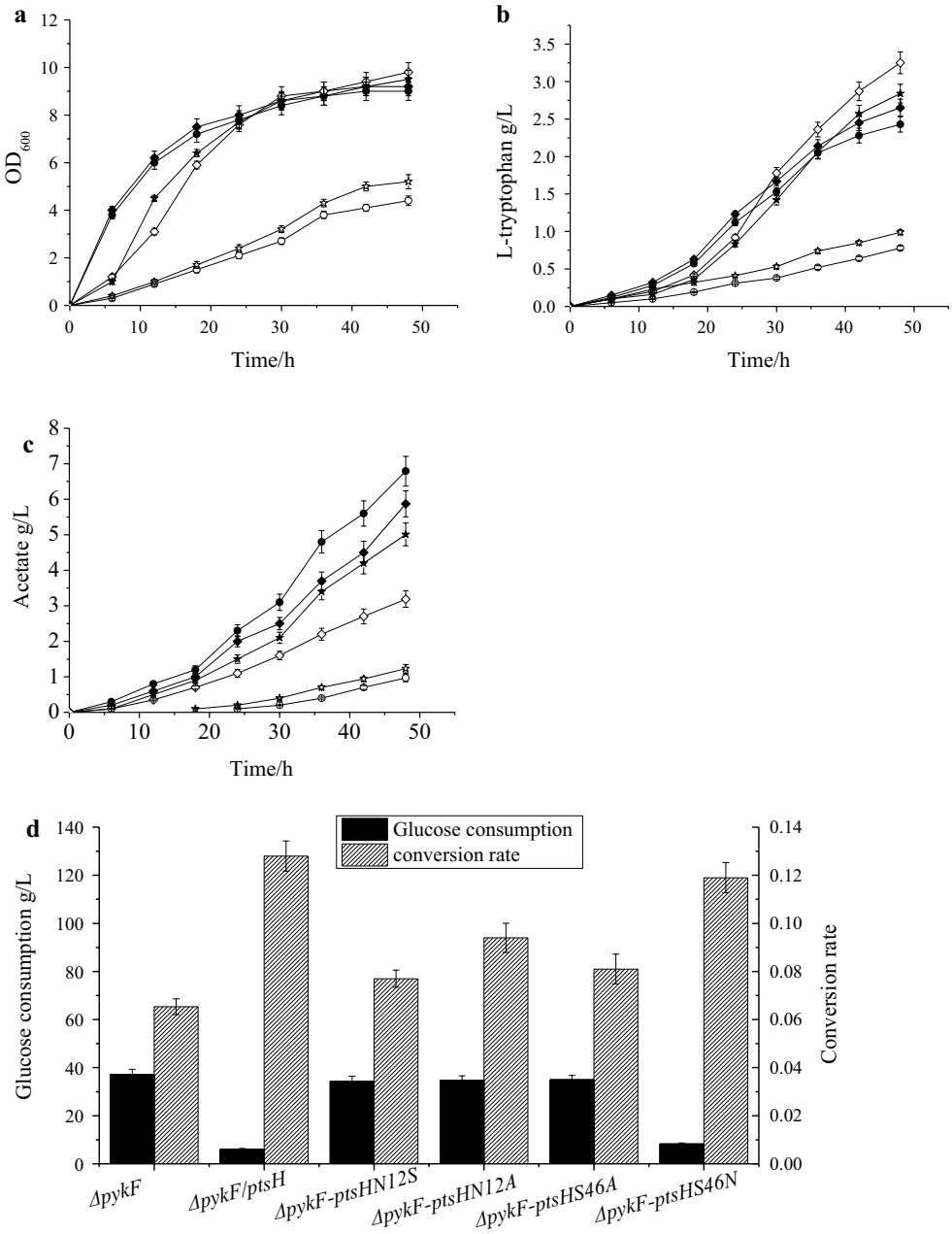

Fig. 2 Effect of *ptsH* modification on L-tryptophan production. **a** Biomass levels (OD_{600}); **b** L-tryptophan levels; **c** acetate levels; **d** glucose consumption levels and conversion rate. *ΔpykF* (*filled circle*); *ΔpykF/ptsH* (*open circle*); *ΔpykF-ptsHN12S* (*filled rhombus*); *ΔpykF-ptsHN12A* (*open rhombus*); *ΔpykF-ptsHS46A* (*filled pentagon*); *ΔpykF-ptsHS46N* (*open pentagon*)

and *ΔpykF-ptsHS46A* were 2.65, 3.25, and 2.84 g L^{-1}, corresponding to significant increases of 9, 34, and 17% ($P < 0.01$, Student's *t* test), respectively, compared with *ΔpykF*. The final L-tryptophan titer of *ΔpykF-ptsHS46N* was extremely low and did not represent a significant improvement over *ΔpykF/ptsH* (Table 4). The final acetate levels of *ΔpykF-ptsHN12S*, *ΔpykF-ptsHS46A*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46N* were successively lower than that of *ΔpykF* (Table 4). The glucose consumption of strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* was lower than that of *ΔpykF* (Fig. 2d), while the conversion rates of *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* increased by 18, 45, and 25% ($P < 0.01$, Student's *t* test), respectively (Table 4). However, the glucose consumption and conversion rate of *ΔpykF-ptsHS46N* were extremely low and did not represent a significant improvement over *ΔpykF/ptsH* (Table 4).

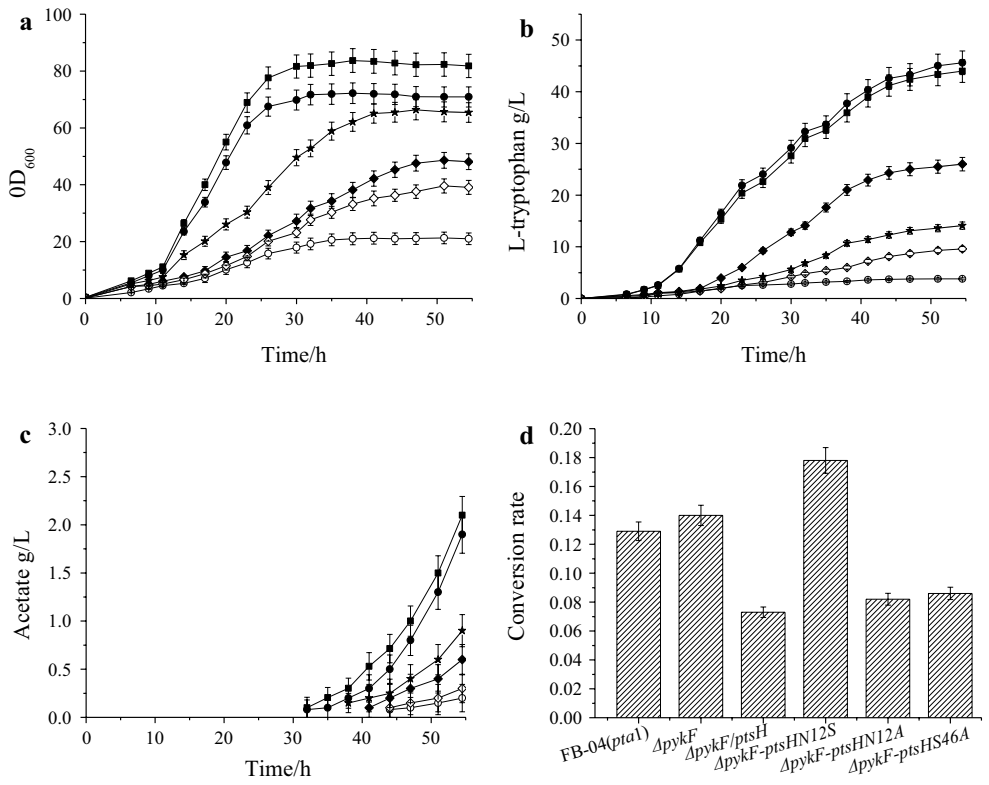

Fig. 3 3-L fed-batch fermentation. **a** Biomass levels (OD_{600}); **b** L-tryptophan levels; **c** acetate levels; **d** conversion rate. FB-04(*pta*1) (*filled square*); *ΔpykF* (*filled circle*); *ΔpykF/ptsH* (*open circle*); *ΔpykF-ptsHN12S* (*filled rhombus*); *ΔpykF-ptsHN12A* (*open rhombus*); *ΔpykF-ptsHS46A* (*filled pentagon*)

3-L fed-batch fermentation

To further investigate L-tryptophan production by the strains containing *ptsH* mutations, *ΔpykF*, *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were cultivated in separate 3-L fermentations, and the results were compared with those of the parent FB-04(*pta*1) and *ΔpykF/ptsH* (Fig. 3). The growth curves of FB-04(*pta*1) and *ΔpykF* were similar, while the growth curves of *ΔpykF/ptsH*, *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were similar (Fig. 3a). Among all strains, *ΔpykF* achieved the highest total biomass, while the growth of *ΔpykF/ptsH* was extremely impaired.

L-Tryptophan production levels were determined (Fig. 3b). L-Tryptophan production by FB-04(*pta*1), *ΔpykF*, *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* increased with time, while the L-tryptophan yield of *ΔpykF/ptsH* increased continuously over the first 24 h of fermentation, then remained almost unchanged. Throughout the entire fermentation process, the L-tryptophan yield of *ΔpykF* was slightly higher than that of the parent strain FB-04(*pta*1), while the L-tryptophan yields of *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were lower than that of the parent strain, and the L-tryptophan yield of *ΔpykF/ptsH* was the lowest. The final L-tryptophan titers of *ΔpykF*, *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were 45.5, 26.0, 9.6, and 14.1 g L^{-1}, which do not represent a significant improvement over that of FB-04(*pta*1) (44.0 g L^{-1}) (Table 4). Even so, the conversion rates of *ΔpykF* and *ΔpykF-ptsHN12S* were significantly higher than that of FB-04(*pta*1) (Fig. 3c), leading to increases of 8.5 and 38.0% ($P < 0.01$, Student's t test), respectively. The conversion rates of *ΔpykF-ptsHN12A*, *ΔpykF-ptsHS46A*, and *ΔpykF/ptsH* were lower (Table 4). Moreover, compared with parent strain FB-04(*pta*1), the final acetate levels of all of the mutants were diminished (Table 4).

Discussion

The L-tryptophan biosynthetic pathway and its complicated regulation mechanism have been studied for a long time, and various L-tryptophan-producing strains with defined

genetic modifications have been constructed [4, 10, 12, 13, 28, 29, 34]. In a previous effort to produce a useful L-tryptophan production strain, feedback-resistant variants of the enzymes of the L-tryptophan biosynthetic pathway were overexpressed in the L-tryptophan production strain *E. coli* FB-04(*pta*1), which harbors plasmid pSTV-03 [16]. However, this strain has a lowered conversion rate (L-tryptophan yield per glucose), which diminishes its utility. Increasing the supply of the crucial L-tryptophan precursor phosphoenolpyruvate and erythrose-4-phosphate is an additional strategy commonly used to boost L-tryptophan production [18].

To overcome possible limitations in the supply of phosphoenolpyruvate, a *pykF* deletion strain (*ΔpykF*), a *pykA* deletion strain (*ΔpykA*), and a *pykF/pykA* double deletion strain (*ΔpykF/pykA*) were constructed, and their ability to produce L-tryptophan was evaluated in the shake-flask fermentations. The results showed that strain *ΔpykF* achieved a higher L-tryptophan yield and conversion rate than that of strains FB-04(*pta*1), *ΔpykA*, and *ΔpykF/pykA* (Table 3). Of the two PYK isoenzymes, PYKI, which is encoded by *pykF* gene, plays the major role in catalyzing the conversion of phosphoenolpyruvate to pyruvate [31]. In one *pykF* knockout mutant, the metabolic flux through glycolysis was reduced by 45% and the flux through the pentose phosphate pathway was increased by 45%, compared with a wild-type parent [25]. Moreover, the inactivation of PYK can decrease the metabolic flow through the TCA cycle and diminish the formation of acetate, a by-product which could inhibit the growth of cells [11, 26, 35]. The results obtained with strain *ΔpykF* in this study are consistent with these previous reports. Double deletion of *pykF* and *pykA* may have a significant impact on the normal cellular physiology; thus, the L-tryptophan yield and biomass production of strain *ΔpykF/pykA* were lower than that of its parent.

Given that the PTS consumes phosphoenolpyruvate for glucose uptake and the constitutively expressed galactose transporter also enables glucose transport [29], the PTS encoded within the genome of *ΔpykF* was inactivated by constructing the strain *ΔpykF/ptsH*. The conversion rate of *ΔpykF/ptsH* was remarkably higher than that of strain *ΔpykF* in shake-flask fermentation (Table 3), but the deletion of *ptsH* caused significant decreases in biomass, glucose consumption, and L-tryptophan production (Table 3). It has been reported that, in addition to its role in the transport of sugars, the PTS also plays an important role in the regulation of carbon metabolism [5, 22, 23, 27]. Thus, disruption of the PTS may have a detrimental effect on normal cellular physiology. To increase the L-tryptophan conversion rate without having this detrimental effect on cellular physiology, the PTS activity should, therefore, be weakened, not completely disrupted. When this study was initiated, it was known that HPr mutation could impair the phosphohydrolysis activity of EI and decrease PTS activity, and a series of mutants that altered the kinetic properties of EI had been reported [19, 20]. In this study, HPr mutants N12S, N12A, S46A, and S46N were introduced into the strain *ΔpykF/ptsH* to weaken, but not disrupt, PTS activity. L-Tryptophan production by the resulting strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, *ΔpykF-ptsHS46A*, and *ΔpykF-ptsHS46N* was first investigated in shake-flask fermentations. The results showed that the L-tryptophan yields and conversion rates of strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were higher than that of *ΔpykF* (Table 4). The levels of acetate produced by strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* were significantly lower than that produced by *ΔpykF*, which is consistent with the higher biomass they achieved (Fig. 2a). In shake-flask fermentations, the biomass, acetate, L-tryptophan production, and conversion rate of strain *ΔpykF-ptsHS46N* were not significantly different than that of strain *ΔpykF/ptsH* (Table 4). However, since the HPr mutants N12S, N12A, S46A, and S46N lead to different EI kinetic properties, these mutations may affect the fermentation performance of strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, *ΔpykF-ptsHS46A*, and *ΔpykF-ptsHS46N* differently. The weakened PTS could lower the glucose transport rate, lowering the acetate formation rate. In shake-flask cultivation, the dissolved oxygen level was lower, which favors the formation of acetate, a by-product that hampers cell growth and L-tryptophan production. Thus, the significantly decreased acetate levels, compared with the parent stain, of PTS-modified strains in shake-flask cultivation contributed to cell growth and L-tryptophan production.

It was worth noting that the fermentation performances of strains *ΔpykF-ptsHN12S*, *ΔpykF-ptsHN12A*, and *ΔpykF-ptsHS46A* in fed-batch fermentation were different from the fermentation performances observed for these strains in shake flasks (Fig. 3). In fed-batch cultivation, the dissolved oxygen levels of all strains were maintained at 20%. Under these conditions, the acetate levels of all strains were low enough throughout fed-batch fermentation process (Fig. 3c) that there was no effect on cell growth or L-tryptophan production. The conversion rate of *ΔpykF-ptsHN12S*, which reached 0.178, was 27.1% greater than that of *ΔpykF*, even though its L-tryptophan yield was lower (Table 4). The L-tryptophan yields and conversion rates of strains *ΔpykF-ptsHN12A* and *ΔpykF-ptsHS46A* were significantly lower (Table 4). HPr mutations N12A, S46A, and S46N increase K_m of EI levels that are 2, 2.5, and 11 times that of the wild type, respectively (Table 3). Given that the phosphoenolpyruvate content of these cells is relatively low, the altered EI K_ms may have remarkable

effects on normal physiological functions. This is consistent with fermentation performances of strains *ΔpykF-ptsHN12A*, *ΔpykF-ptsHS46A*, and *ΔpykF-ptsHS46N* observed in this study (Fig. 3). Moreover, mutant N12S had no effect on K_m of EI, and only decreased V_{max} of EI to 65% that of the wild type (Table 3). The altered EI V_{max} may influence the consumption of glucose and the growth rate, which is consistent with the observed fermentation performance of *ΔpykF-ptsHN12S* (Fig. 3).

Measuring the conversion rate is a standard way to evaluate the efficiency industrial fermentation processes. Increasing the conversion rate reduces production costs and improves the efficiency of carbon source utilization. Various strategies have been used to increase the carbon flux through the aromatic pathway in efforts to improve the production and conversion rate of aromatic amino acids. PEP synthase and transketolase were overexpressed to enhance the availability of the precursor substrates PEP and E4P, which improved aromatic amino acid production and the conversion rate [18, 21]. Carbon storage regulator (Csr) was overexpressed to increase PEP levels and channel carbon flux into the aromatic pathway [32]. In addition, fructose repressor (FruR) has been inactivated to enhance the carbon flux through glycolysis and the pentose phosphate pathway, improving the conversion rate by 52.3% [17]. However, inactivating FruR also led to increased levels of acetate [17]. In this study, introducing mutations into the *ptsH* gene provided a novel way to increase the conversion rate as well as decreasing acetate formation by *E. coli* FB-04(*pta*1) during the L-tryptophan fermentation process, even though the L-tryptophan yield of the final strain, *ΔpykF-ptsHN12S*, decreased. One potential method to increase the L-tryptophan yield and conversion rate at the same time would be to introduce mutations into the *ptsH* gene using error-prone PCR and screening the resulting strains for superior L-tryptophan fermentative capability. Alternatively, given that both erythrose-4-phosphate, another crucial L-tryptophan precursor [18, 32–34], and NADPH, a substrate in the common aromatic pathway [7, 16], come from the pentose phosphate pathway, L-tryptophan production, and the conversion rate should be enhanced by increasing the metabolic flow through the pentose phosphate pathway using genetic engineering methods. In addition, considering that the glucose feeding rate and fermentation temperature can be used as optimization factors [6, 33], a series of optimization strategies based on these two factors should be undertaken to improve L-tryptophan production. Finally, it was well known that glycerol was not transported by PTS; thus, glycerol could be used as a supplementary carbon source during the fermentation process to try to increase L-tryptophan production.

Conclusions

In efforts to increase the conversion rate of L-tryptophan production by *E. coli* FB-04(*pta*1), the *pykF* gene encoding pyruvate kinase I (PYKI) was deleted. The resulting strain (*ΔpykF*) showed a slightly higher L-tryptophan yield and conversion rate in fermentation processes. To further improve the conversion rate, the PTS was disrupted by deleting the *ptsH* gene, which encodes phosphocarrier protein HPr. The resulting strain *(ΔpykF/ptsH*) achieved a significant increase in conversion rate during shake-flask fermentations. However, the biomass level, L-tryptophan yield, and conversion rate of *ΔpykF/ptsH* were especially low during the fed-batch fermentation process. Finally, HPr mutant N12S was introduced in the genomic background of *ΔpykF/ptsH*. The resulting strain (*ΔpykF-ptsHN12S*), which showed prominent fermentation performance, displayed a conversion rate 38.0% higher than that of the parent strain *E. coli* FB-04(*pta*1). The strategies employed in this study improved the efficiency of carbon source utilization and are of great value for industrial L-tryptophan production.

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