# Improvement of Xylose Utilization and L-ornithine Production by Metabolic Engineering of Corynebacterium glutamicum

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**Abstract**

L-ornithine is a basic amino acid, which shows significant value in food and medicine industries. There is a huge space for L-ornithine production with strains available for metabolic engineering, and it is urgent to develop a high-efficiency engineering strain for industrialization. Here, xylose isomerase and xylulose kinase were introduced into Corynebacterium glutamicum S9114 to establish xylose metabolism pathway, and then xylose became a substitute carbon source of glucose. In addition, the optimization and overexpression of phosphoenolpyruvate carboxylase and pentose transporter have been conducted to promote the synthesis of L-ornithine for the first time. Furthermore, though optimizing the concentration ratio of glucose and xylose (7:3), adding biotin and thiamine hydrochloride, we arrived at the highest L-ornithine yield 41.5g/L in shaking flask fermentation so far. Our results demonstrate that the combination of metabolic engineering and the optimization of fermentation process can make great potential for L-ornithine production by lignocellulose hydrolysate.

**Improvement of Xylose Utilization and L-ornithine Production by Metabolic Engineering of *Corynebacterium glutamicum***

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# ABSTRACT

L-ornithine is a basic amino acid, which shows significant value in food and medicine industries. There is a huge space for L-ornithine production with strains available for metabolic engineering, and it is urgent to develop a high-efficiency engineering strain for industrialization. Here, xylose isomerase and xylulose kinase were introduced into *Corynebacterium glutamicum* S9114 to establish xylose metabolism pathway, and then xylose became a substitute carbon source of glucose. In addition, the optimization and overexpression of phosphoenolpyruvate carboxylase and pentose transporter have been conducted to promote the synthesis of L-ornithine for the first time. Furthermore, though optimizing the concentration ratio of glucose and xylose (7:3), adding biotin and thiamine hydrochloride, we arrived at the highest L-ornithine yield 41.5g/L in shaking flask fermentation so far. Our results demonstrate that the combination of metabolic engineering and

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**Keywords:** L-ornithine; *Corynebacterium glutamicum* ; Xylose; Metabolic engineering

# INTRODUCTION

L-ornithine is non-proteinogenic amino acid, which is an important intermediate product of the urea cycle pathway. It is a downstream product of glutamate and a key precursor for the production of L-citrulline, L-proline and L-arginine. L-ornithine has attracted special attention for its biological functions (Sivashanmugam, J, V, & K, 2017), and is widely used in daily health care and disease treatment such as the positive effect on the protection and vitality recovery of human liver and the heart (Butterworth, 2020; Das et al., 2020; Davies et al., 2009), improving visual function (Sakamoto, Mori, Nakahara, Morita, & Ishii, 2015), regulating the secretion of hormones (Matsuo et al., 2015). Moreover, a recent report demonstrates that L-ornithine as raw material has a great prospect for the development of new anticancer drugs (Vargas-Ramírez et al., 2016).

*Corynebacterium glutamicum* is the most important amino acids producing strain in the past 40 years, such as glutamate, L-alanine, L-serine, L-arginine, and L-proline (Gutmann, Hoischen, & Krämer, 1992; Jensen, Eberhardt, & Wendisch, 2015; Jojima, Fujii, Mori, Inui, & Yukawa, 2010; Park et al., 2014; X. Zhang et al., 2018). Although*E. coli* and *Saccharomyces cerevisiae* have ever been engineered to be the producer of L-ornithine (Becker & Wittmann, 2015; Lee & Cho, 2006; Qin et al., 2015), *C. glutamicum,* which produces large amounts of glutamate, the precursor of L-ornithine, still to be the preferred dominant strain (Zhang, Yu, Zhou, & Ye, 2018). The concentration of intracellular glutamate is an important driving force for L-ornithine production.

Random mutation together with genetic and metabolic engineering are two key methods in the breeding process for L-ornithine production by*C. glutamicum* . However, the random mutation breeding has many disadvantages, including the complex screening process, low success rate, high probability of reverse-mutation. Those made directed genetic metabolic engineering to be the priority option. A series of measures were taken to increase the L-ornithine production by genetic engineering modification (Wu, Guo, Zhang, Jiang, & Ye, 2019). First, focused primarily on the main L-ornithine synthesis pathway (Hao et al., 2016; Jensen et al., 2015; Jiang, Zhang, Li, & Liu, 2013; D. J. Kim, Hwang, Um, & Cho, 2015; Q. Shu et al., 2018; Zhang, Ren, Yu, Zhou, & Ye, 2018; Zhang, Yu, Zhou, Li, & Ye, 2017); second, the transportation of L-ornithine amino acids (B. Zhang, L. Q. Ren, et al., 2018); last, based on glycolysis, acetic acid metabolism, pentose phosphate pathway (S. Y. Kim, Lee, & Lee, 2015; Zhang, Yu, Wei, & Ye, 2018), the tricarboxylic acid cycle and glucose utilization pathway (Ikeda et al., 2011; Lindner et al., 2013; Lindner, Seibold, Henrich, Kramer, & Wendisch, 2011; Xu, Zhang, Liu, & Zhang, 2016; Zhang, Gao, Chu, & Ye, 2019; Zhou, Wang, Xu, Chen, & Cai, 2015). In addition to rational modification, adaptive evolution strategies combined with transcriptional levels analysis provides another strategy to develop a strain with high performance (Jiang, Chen, Zhang, & Liu, 2013). Jensen et al. constructed *C. glutamicum* ORN6 by knocking out *argF* , *argR* and*argG* , attenuating the expression of *pgi* and increasing the copy number of the arginine operon argCJB$^{A49V,\ M54V}$D on the chromosome, the L-ornithine yield was 0.52g/g (Jensen et al., 2015). Hwang et al. knocked out *ncgl2053* , *ncgl0281* and*ncgl2582* that encoding $NADP^+$-dependent oxidoreductase, which resulted in the loss of glucose dehydrogenase activity and the increasing of 6-phosphate gluconate dehydrogenase activity. The production of L-ornithine is 66.3% higher than that of the starting strain (Hwang & Cho, 2014). Shu et al. deleted *proB*and *argF* to block the branch of the L- ornithine synthesis pathway, mutated *ArgB* and expressed heterologous *argA* and*argE* to introduce an artificial linear transacetylation pathway. The production of L-ornithine was 40.4g/L in 5-L bioreactor (Qunfeng Shu et al., 2018). Zhang et al adopted a series of genetic engineering modifications to achieved the maximum L- ornithine yield of 43.6g/L in fed batch fermentation by far (Zhang et al., 2019). Although many strategies have been adapted to increase the production of L-ornithine, how to build a more efficient industrial strain with practical applications is still a long way off.

In order to make good use of the most abundant renewable resources and do not compete with people for food, a large amount of studies have focused on how to construct an efficient microbial cell factory utilizing xylose and glucose as mixed carbon sources in the past fifteen years (Becker, Rohles, & Wittmann, 2018). Thanks to the weak carbon catabolite repression, *C. glutamicum* is regarded as a major industrial force with great potential in recent years (Buschke, Schafer, Becker, & Wittmann, 2013; Wendisch et al., 2016). However, due to*C. glutamicum* lacks of xylose isomerase (XylA), it could not grow on medium containing xylose as the sole carbon source. Buschke et al. and Gopinath et al. used the exogenous xylose isomerase (XylA) and xylulose kinase (XylB) to establish the isomerase pathway in *C. glutamicum* to realize the utilization of xylose (Buschke, Becker, et al., 2013; Gopinath, Meiswinkel, Wendisch, & Nampoothiri, 2011). Five copies of *xylAB* operon from *E. coli* were integrated to*C. glutamicum* R chromosome to generate the strain X5C1, which could consume 40 g/L glucose and 20 g/L xylose in 12h (Sasaki, Jojima, Inui, & Yukawa, 2008). In addition, several other strategies were adopted to improve the xylose utilization, including the introduction of arabinose transporter (Brusseler et al., 2018; H. Kim et al., 2017), overexpressing of TAL/TKT in the pentose phosphate pathway (Jo et al., 2017). Meiswinkel et al. constructed an engineered strain *C. glutamicum* PUT21 by introducing *xylA* from *X. campestris* ,*xylB* from *C. glutamicum* and *argBAD* operon from*E. coli* to produce 2.59 g/L of L-ornithine, and the volumetric ornithine productivity is 43.2 mg/(L[?]h) (Meiswinkel, Gopinath, Lindner, Nampoothiri, & Wendisch, 2013). In addition to xylose isomerase metabolic pathway, Christian et al. introduced the *xylXABCD*operon from *Caulobacter crescentus* into *C. glutamicum*ATCC13032 to establish the Weinberg pathway (Brusseler et al., 2018). Although the utilization of xylose by *C. glutamicum* has already been realized, the utilization rate of xylose is still unsatisfied. More modified strategies are needed to improve the utilization rate of xylose and the production of L-ornithine., which opened the door to the efficient utilization of lignocellulose.

In our previous studies, we have successfully constructed the *C. glutamicum* SO26 with high L-ornithine yield (Zhang et al., 2019; B. Zhang, L. Q. Ren, et al., 2018). In this study, we attempted to utilize the most abundant carbon source in lignocellulose hydrolysate - glucose and xylose. We adopted the approaches of metabolic engineering and fermentation process control to accelerate the xylose consumption rate and the yield of L-ornithine. First, a more efficient *xylAB*operon was screened out from different strains, and the arabinose transporter araE from *Bacillus subtilis* was knocked into the*iolR* locus under the promoter $P_{eftu}$. Second, the acetylation of phosphoenolpyruvate carboxylase (PEPC) was reduced to release the feedback inhibition of aspartic acid, and a strong constitutive promoter $P_{H36}$ was introduced in the upstream of *pepc* . The strain after a series of modulations is named *C. glutamicum* XAB03. Finally, though the optimization of fermentation process, we found the concentration ratio of glucose and xylose (7:3) and the coenzyme addition (biotin 0.9 $\mu$M and thiamine-HCl 15 $\mu$M respectively) can realize the highest L-ornithine yield 41.5g/L in shaking flask fermentation up to date. All the metabolic engineering process are shown in Fig.1. Schematic diagram.

# MATERIALS AND METHODS

## Bacterial strains, plasmids and primers

The strain SO26 originated from *C. glutamicum* S9114 underwent a series of modifications (deletion of *argF* , *ncgl1221* ,*argR* , *putP* , *iolR* , and *mscCG2* ; attenuation of*odhA* , *proB* , *pta* , *cat* , and *ncgl2228* ; and overexpression of *lysE* , *gdh* , *gdh2* ,*cg3035* , *pfkA* , *pyk* , *glt* , *tkt* ,*argCJBD* , and *iolT1* ) was used as a starting strain for further metabolic engineering in this study. *E. coli* DH5α was used as host for rapid replication of recombinant plasmids. Xylose isomerase (*xylA* ) and xylulose kinase (*xylB* ) were amplified from *E. coli* K-12 MG1655 and *X. campestris* . The arabinose transporter (araE) were derived from *Bacillus subtilis* . All the strains and plasmids used in this study are presented in Table 1.

## Construction of plasmids and strains

The basic DNA manipulation and strain construction was operated according to the standard molecular cloning manual.*E. coli* DH5α was used as the host of gene cloning. All the primers used in this study are presented in Table S1 (Supplementary Information). The suicide vector pK18*mobsacB* containing the sucrose lethal gene *sacB* was used to delete or integrate gene on chromosome.

The *xylAB* gene clusters from *E. coli* and *X. campestris* were amplified by primers EAB-F/EAB-R and XAB-F/XAB-R respectively, the inserted restriction sites are*Hind* III/*EcoR* I and *Hind* III/*Sac* I of pXMJ19. The plasmids pXMJ19, p19$P_{lac}$-EcoAB, and p19$P_{lac}$-XcaAB were transformed into *C. glutamicum*SO26 to produce strains *C. glutamicum* pX、EAB、XAB. *AraE*from *B. subtilis* with a constitutive $P_{eftu}$promoter were amplified by araE-F/araE-R and up-eftu-F/araE-eftu-R respectively. The product of fusion PCR was inserted into the*EcoR* V site of plasmid pk18-$^{iolR}$*byGibsonassembly*, *andthengeneratedtherecombinantplasmidpk*18 − $P_{\mathrm{eftu}}$ $-^{i}$ *olR* :: *araEandcorrespondingstrainXAB*01.

To increase PEPC expression by lowing the acylation of PEPC, a synthesized strong promoter H36 was inserted in the upstream of*pepc* gene (Yim, An, Kang, Lee, & Jeong, 2013), and then the AAG bases from 1957 bp to 1959 bp were mutated to CGC by overlap PCR, the primer up-HA-F1 / up-HA-H36-R2 amplified *pepc* upstream homology arm sequence, primer up-HA-H36-F3 / pepc-H36-R4 amplified H36 promoter, primer mutant-(KR)-F/down-HA-pepc-R8 realizes the base mutation of K653R, pepc-H36-F5/mutant-(KR)-R amplifies the *pepc* upstream homology arm sequence, primer down-HA-pepc-F9/down-HA-R10 amplified the homology arm sequence downstream of *pepc* . Finally, the entire fragment was combined by Gibson assembly under the primer up-HA-F1/down-HA-R10, resulted the recombinant plasmid pk18-$P_{H36}$-*pepc* T1. Furthermore, to reduce the feedback inhibition of aspartic acid on PEPC, the same operation has been done at 895-897 bp AAC to replace GAT, amplification using pepc-H36-F5/D299N-R6 and D299N-F7/down-HA-pepc-R8 with pk18-$P_{H36}$-*pepc* T1 as a template can realize base mutation of D299N, forming the pk18-$P_{H36}$ -*pepc* T2, resulted in the strain*C. glutamicum* XAB03. Primers pXMJ19-F/pXMJ19-R and M13 fwd/M13 rev are verification primers for pXMJ19 and pK18*mobsacB* , respectively.

All the recombinant plasmids were constructed in *E. coli* DH5α and transformed into *C. glutamicum* by electroporation. The mutant strains were screened through two rounds of homologous recombination, and further confirmed by colony PCR and sequencing.

## Cultivation medium and conditions

Luria–Bertani (LB) medium containing NaCl 10 g/L, tryptone 10 g/L and yeast extract 5 g/L were used for cultivation of *C. glutamicum*and *E. coli* . Antibiotic was added to the medium for mutants screening when needed: 50 $\mu$g/mL kanamycin or 30 $\mu$g/mL chloramphenicol for *E. coli* , 10 $\mu$g/mL kanamycin or 15 $\mu$g/mL chloramphenicol for*C. glutamicum* .

For the shaking flask fermentation experiment, the correct monoclonal strains were activated twice on the LB medium for 36 h, and then the appropriate amount of bacterial seed was inoculated into a 100 mL flask containing 10 mL of seed solution. The seed medium contains glucose 30 g/L, corn steep liquor 10 g/L, yeast extract 10 g/L, $(NH_4)_2SO_4$ 15 g/L, $MgSO_4$ 2.5 g/L, $KH_2PO_4$ 1 g/L, $K_2HPO_4$ 0.5 g/L, $Na_2HPO_4{\cdot}12H_2O$ 1.26 g/L and $CaCO_3$ 10 g/L, the pH was adjusted to 6.7 (Supplementary Information: Figure S1). The monoclonal strains were incubated at 30°C, 220 rpm for 11 h. Take an appropriate amount of seed solution and inoculate it in a 250 mL Erlenmeyer flask containing 20 mL of fermentation medium so that the $OD_{600}$ of the initial fermentation solution is 1 at 600 nm. The fermentation medium contains xylose 100 g/L or glucose 70 g/L and xylose 30 g/L, yeast extract 6 g/L, $(NH_4)_2SO_4$ 50 g/L, $MgSO_4$ 2.5 g/L, $KH_2PO_4$ 1 g/L, $K_2HPO_4$ 0.5 g/L, $Na_2HPO_4{\cdot}12H_2O$ 1.26 g/L, $MnSO_4{\cdot}H_2O$ 0.02 g/L, $FeSO_4{\cdot}7H_2O$ 0.02 g/L, biotin 0.9 $\mu$M,

thiamine-HCl 15 $\mu$M and $CaCO_3$ 10 g/L, while the pH was adjusted to 6.7. The strains in fermentation medium were incubated at 31.5°C, 250 rpm for 72 h.

## Analytical methods of cell growth and fermentation products

Every 12 hours, three parallel samples (100 $\mu$L) were taken to monitor the bacteria density, consumption of glucose and xylose, and L-ornithine production. The $OD_{600}$ value was detected to assess the cell growth by a microplate reader (BioTek Instruments, Winooski, VT, USA) after adding 0.125 mol/L HCl to dissolve $CaCO_3$ (Zhang et al., 2019). The samples were centrifuged to obtain fermentation supernatant. The glucose concentration was analyzed by SBA-40E biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, Jinan, Shandong, China). The level of xylose consumption was determined by high-performance liquid chromatography (HPLC) (Chen, Zhu, & Xia, 2014; Yim et al., 2013). The content of L-ornithine was determined by ninhydrin colorimetry (B. Zhang, M. Yu, Y. Zhou, et al., 2018). All experiments had triple parallels, and data are repressed as mean and standard deviation (SD).

# RESULTS AND DISCUSSION

Comparison with the xylose utilization capacity of xylose isomerases and xylulose kinases from different sources

Xylose isomerase (*xylA* ) and xylulose kinase (*xylB* ) exist in the form of gene clusters. The xylose isomerase metabolic pathway from different sources established in *C. glutamicum* have different xylose utilization capacities, this might be the result of the genetic codon preference between the *xylAB* source strain and*C. glutamicum* (Meiswinkel et al., 2013). The *xylAB* genes derived from *E. coli* MG1655 and *X. campestris* were expressed under IPTG induced vector pXMJ19, in which the $P_{tac}$ is the promoter for gene expression. The resulting expression plasmids were labeled p19$P_{lac}$-EcoAB and p19$P_{tac}$-XcaAB. The two plasmids were transformed into *C. glutamicum* SO26, resulted the *C. glutamicum* EAB and XAB. Fig.2a showed the growth curve and xylose consumption of strain EAB and XAB during 72h fermentation. The results indicated that *xylAB* from *X. campestris* achieved higher xylose consumption (45.1 g/L) compared with *xylAB* from*E. coli* MG1655 (25.0 g/L). The growth $OD_{600}$increased from 12.05 (EAB) to 12.95 (XAB). The L-ornithine concentration in the supernatant of fermentation was determined (Fig.2b). The L-ornithine production titer of the strain XAB (21.6 $\pm$ 0.19 g/L) is 18.7% higher than strain EAB (18.2 $\pm$ 0.35 g/L), and the corresponding xylose yield of XAB is 0.48 g/g. The *C. glutamicum* XAB demonstrated the better capacity of xylose utilization and L-ornithine synthesis than strain EAB.

Knocking-in the pentose transporter demonstrates the promotion of xylose utilization and L-ornithine production

In order to increase the rate of xylose consumption, the pentose transporter gene ( *araE* ) from *Bacillus subtilis* was integrated into the genome locus of*iolR* with the strong $P_{eftu}$ promoter, to generate the strain termed as XAB01. The pentose transporter AraE is not only extremely significant for the arabinose transportation, but also promotes the transportation of xylose to cells (Mao et al., 2018). As expected, the results showed that strain XAB01 demonstrated superior xylose consumption and L-ornithine synthesis than strain XAB (Fig.3a and 3b). strain XAB01 consumed 47.9 g/L xylose after 72h fermentation, with an average consumption rate of 0.665 g/(L·h). Through the rapid utilization of xylose, the growth and L-ornithine yield of strain were also improved. The output of L-ornithine increased by 12.5% (24.3 $\pm$ 0.23g/L) compared with strain XAB, and the xylose yield is 0.51 g/g.

## Effect of modification of phosphoenolpyruvate carboxylase on the production of L-ornithine

Phosphoenolpyruvate carboxylase (PEPC) is an enzyme in the glycolysis pathway of *C. glutamicum* and plays an important role in the regulation of the TCA cycle (Fig.1). Phosphoenolpyruvate and carbon dioxide synthesize oxaloacetic acid under the catalysis of PEPC, and then oxaloacetic acid enters the TCA cycle for further metabolism. The expression and activity of PEPC affect the synthesis of glutamic acid in *C. glutamicum* (Nagano-Shoji et al., 2017; Wada et al., 2015). Lysine at position 653 (K653) is essential for the regulation of PEPC acetylation. Megumi ed al. found that acetylation of PEPC at K653 decreased enzymatic activity and reduced glutamate production, this means K653-acetylation regulates PEPC activity negatively. Mutated K653 into arginine can lower the level of acetylation on PEPC, which correspondingly improve its activity (Nagano-Shoji et al., 2017). In addition, PEPC will be feedback suppressed by aspartic acid when it is overexpressed in *C. glutamicum* , mutated aspartic acid at position 299 of PEPC to asparagine with a similar structure can effectively reduce the inhibitory effect (Wada et al., 2015). In our study, we attempt to combine the weakening acetylation by K653R and the attenuating feedback inhibition by D299N of PEPC to enhance the activity of this enzyme, leading to promote the glutamate synthesis and the L-ornithine yield. We selected the more preferred CGC and AAC codons in *C. glutamicum* S9114 to mutate K653 and D299. At the same time, the *pepc* gene was overexpressed by adding a strong constitutive promoter H36 (Yim et al., 2013). Multiple gene fragments are fused into a complete fragment by PCR overlapping amplification technology. After two point mutations in *pepc* , strain XAB03 was constructed. The shaking flask fermentation of strain XAB03 showed that the production of L-ornithine was 27.1 ± 0.32 g/L, which was 11.5% higher than that of strain XAB01 (Fig.4b). Moreover, it has a positive effect on the growth of *C. glutamicum* (Fig.4a). These results indicate the importance of PEPC in L-ornithine synthesis, and this modification strategy shows value for the synthesis of L-glutamate, L-citrulline and L-arginine.

## Addition of biotin and thiamine hydrochloride accelerates the synthesis of L-ornithine

In addition to the modification of key enzymes in metabolic pathways, the addition of key coenzymes is also an important method to promote the synthesis of products (Y. Cao, Duan, & Shi, 2014). Biotin and thiamine hydrochloride are the coenzymes of carboxylase in the metabolic process. Biotin plays an important role in the metabolism of bacterial proteins, which could change the content of cell membrane components and permeability. Different concentrations of biotin affect the transcription levels of enzymes and promote the synthesis of glutamate (Y. Cao et al., 2014; Yan Cao, Mpofu, Jian, & Zuoying, 2012). In order to further optimize the fermentation process, we attempted to adding the coenzyme during the L-ornithine production. The concentration of biotin and thiamine hydrochloride in the fermentation medium was 0.9 $\mu$M and 15 $\mu$M (Hyun Jin, Myung Cho, & Hoon Park, 2007), and the L-ornithine production increased to 33.4 g/L compared with no adding coenzyme fermentation (27.1 g/L) (Fig.5). During the 0-48 h fermentation period in shake flasks, the strain XAB03 grows with abundant nutrients, the energy distribution mainly focuses on the growth of the bacteria while the production of L-ornithine is weak. From 48-72 h, the growth tends to be stable and the L-ornithine is synthesized rapidly. The results provide a research direction for the promotion of certain target products through the addition of coenzymes and some small molecules.

## Effects of a combination of glucose and xylose on L-ornithine

Metabolism of different sugars is an important basement for the lignocellulose utilization. Glucose and xylose are the most important six- and five-carbon sugars in the hydrolysis of lignocellulose. We compared L-ornithine yield in different ratios of glucose and xylose in the case of the total sugar concentration was

constant. On the premise that the total sugar concentration is 100 g/L, seven groups of different glucose concentration gradients were chosen (Fig.5). The mixed carbon sources showed the advantage of L-ornithine yield compared with using glucose or xylose as the sole carbon source. The results showed that the maximum L-ornithine production could be obtained (41.5 ± 0.02 g/L) when glucose is 70 g/L and xylose is 30 g/L (Fig.5 b, c).

Based on all the above conditions, the $OD_{600}$ and L-ornithine production of strain XAB03 could reach to 16.8 ± 0.19 and 41.5 g/L (Fig.6c) respectively after shake flask fermentation for 72h, which is the highest titer so far to the best of our knowledge (Table 2), 7.8% higher than Zhang et al. had reported, and increased by 43.4% compared with the sole xylose carbon source (Zhang et al., 2019). The 2:1 ratio is close to the ratio of glucose to xylose after lignocellulose hydrolysis. This result lays a foundation for the feasibility and superiority of L-ornithine synthesis from lignocellulose hydrolysate.

# CONCLUSION

In this study, *xylAB* operon was introduced into *C. glutamicum* SO26 to achieve the consumption of xylose for the production of L-ornithine. And then, we verified the AraE, the reduction in the degree of acetylation and the release of feedback inhibition of aspartic acid of PEPC, the addition of biotin and thiamine hydrochloride, the resulting strain *C. glutamicum* XAB03 has reached 41.5 g/L shaker flask output from glucose and xylose. This work also shows the possibility of making full use of lignocellulose for the synthesis of L-ornithine and lays the foundation for the further realization of industrialized strain production.

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## GRAPHICAL ABSTRACT

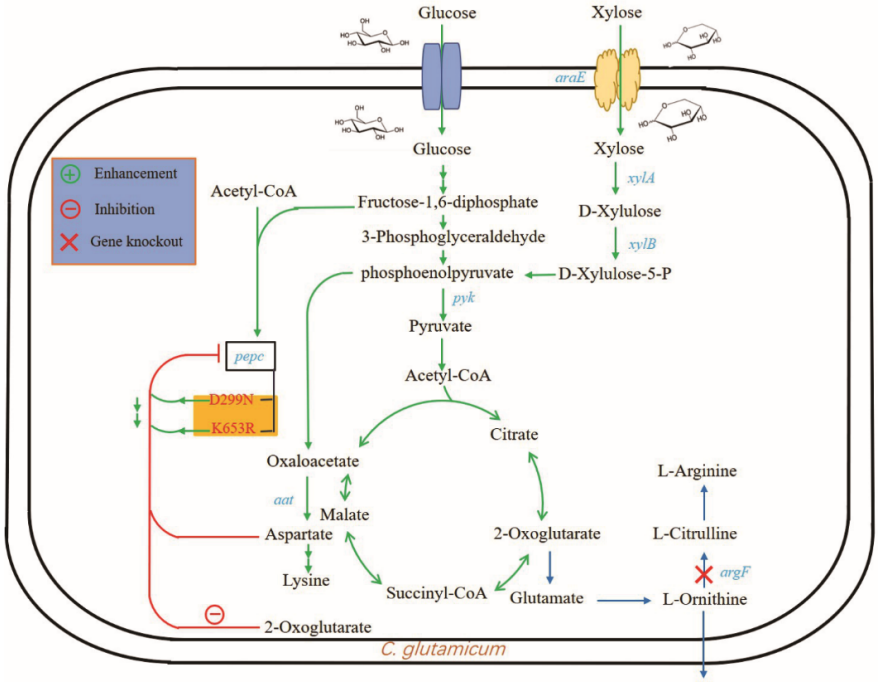


Fig.1. Schematic diagram of the metabolic engineering process of*C. glutamicum* .

*pepc* encodes phosphoenolpyruvate carboxylase; *pyk* encodes pyruvate kinase; *xylA* encodes xylose isomerase; *xylB*encodes xylulose kinase; *aat* encodes aspartate aminotransferase;*araE* encodes pentose transporter; *argF* encodes N-ornithine carbamoyl transferase.

Table 1. Strains and plasmids

| Strains/plasmids | Relevant characteristics |
|---|---|
| **Strains** | |
| *E. coli* DH5a | Clone host strain |
| *E. coli* K-12 MG1655 | The source of Eco-*xylAB* genes |
| *X. campestris* | The source of Xca-*xylAB* genes |
| *C. glutamicum* SO26 | Deletion of *argF*, *ncgl1221*, *argR*, *putP*, *iolR*, and *mscCG2*; attenuation of *odhA*, *proB*, *pta*, *cat*, ar |
| *Bacillus subtilis* | The source of pentose transporter *araE* gene |
| *C. glutamicum* pX | *C. glutamicum* SO26 with pXMJ19 |
| *C. glutamicum* EAB | *C. glutamicum* SO26 with p19$P_{lac}$-EcoAB |
| *C. glutamicum* XAB | *C. glutamicum* SO26 with p19$P_{lac}$-XcaAB |

| Strains/plasmids | Relevant characteristics |
|---|---|
| *C. glutamicum* XAB01 | XAB with the knocking of *araE* gene into the locus of *iolR* |
| *C. glutamicum* XAB02 | XAB01with $P_{H36}$ promoter in the upstream of *pepc* gene and 1957-1959 bp CGC replaces AAG in |
| *C. glutamicum* XAB03 | XAB02 with 895-897 bp AAC replaces GAT in *pepc* gene |
| **Plasmids** | |
| pK18mobsacB | The suicide vector containing the *B. subtilis sacB* gene; $kan^R$, allows for selection of double crosso |
| pXMJ19 | High copy expression vector,$Cm^R$,LacIq promoter,tac promoter |
| p19$P_{lac}$-EcoAB | A derivative of pXMJ19, harboringthe *xylAB* operon from *E. coli* MG1655 |
| p19$P_{lac}$-XcaAB | A derivative of pXMJ19, harboringthe *xylAB* operon from *X. campestris* |
| pk18-$P_{eftu}$-$^{i}olR :: araE$ | Using pK18*mobsacB* and *araE* to connect through the upper and lower homology arms of $^{iolR}$, *an* |
| pk18-$P_{H36}$-pepcTI | A originated from pK18*mobsacB*, add $P_{H36}$ promoter in the upstream of pepc gene and 1957-1959 |
| pk18-$P_{H36}$-pepcT2 | On the basis of pk18-$P_{H36}$-pepcTI, harboring 895-897 bp AAC replaces GAT in *pepc* gene |

Table 2. Synthesis of L-ornithine by *C. glutamicum*

| Strains | L-ornithine concentration ($g{\cdot}L^{-1}$)/yield | Cultivation | Sugar source | Modulations | References |
|---|---|---|---|---|---|
| *C. glutamicum* SJC8514 | 12.48/ND | Shake flask; batch | Glucose | Overexpression of *ncgl0452* and *argCJBD*mut | (D. J. Kim et al., 2015) |
| *C. glutamicum* SJC8039 | 14/ND | Shake flask; batch | Glucose | Deletion of *argF*, *argR*, and *proB*; Blocking gluconate biosynthesis | (Hwang & Cho, 2014) |
| *C. glutamicum* YW06 | 51.5/0.240 | Bioreactor; fed-batch | Glucose | Deletion of *argF*, *argR* and *proB*; Reinforcement of the PPP pathway flux; The use of a feedback-resistant enzyme | (S. Y. Kim et al., 2015) |
| *C. glutamicum* ORN1 (pVWEx1-*araBAD*) | 25.8/0.78 | Shake flask | Glucose, arabinose | Deletion of *argF*, *argR*; in-frame deletion of *argF* and *argR*, auxotrophic for l-arginine | (Schneider, Niermann, & Wendisch, 2011) |

| Strains | L-ornithine concentration ($g \cdot L^{-1}$)/yield | Cultivation | Sugar source | Modulations | References |
|---|---|---|---|---|---|
| *C. glutamicum* [?]APE6937R42 | 24.1/0.298 | Bioreactor; batch | Glucose | Deletion of *argF*, *argR*, and *proB*; Adaptive evolution in presence of L-ornithine | (Jiang, Chen, et al., 2013) |
| *C. glutamicum* [?]*APE::rocG* | 14.8/ ND | Shake flask; batch | Glucose | Deletion of *argF*, *proB*, *speE*::Ptac-M-*rocG*, *argR*::Ptac-M-*gapC* | (Jiang, Zhang, et al., 2013) |
| *C. glutamicum* ORN6 | 20.96/0.524 | Shake flask; batch | Glucose | Deletion of *argF*, *argR*, and *argG*; overexpression of *argBM*; attenuation of *pgi* | (Jensen et al., 2015) |
| *C. glutamicum* 1006 *[?]argR-argJ* | 31.6/0.396 | Shake flask; batch | Glucose | Deletion of *argR*; overexpression of *argJ* | (Hao et al., 2016) |
| *C. glutamicum* XAB03 | 41.5/ND | Shake flask; batch | Glucose, xylose | *C. glutamicum* SO26 with *xylAB* from *X. campestris*, expression of *araE*, overexpression of *pepc* | This study |

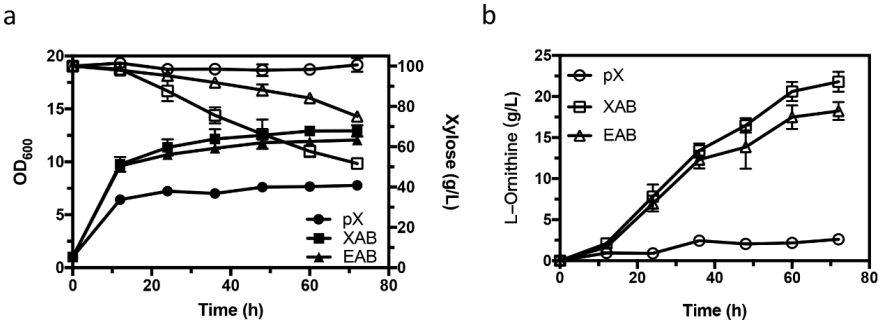


Fig.2. Comparison of the effects of *xylAB* genes from different strains.

a: Growth curve (solid) and xylose consumption (hollow) curves. b: L-Ornithine production curves. Strain

pX (hollow circular) is a control strain containing pXMJ19, Strain XAB (hollow square) overexpresses*xylAB* from *E. coil* , Strain EAB (hollow upper triangle) overexpresses *xylAB* from *X. campestris* .

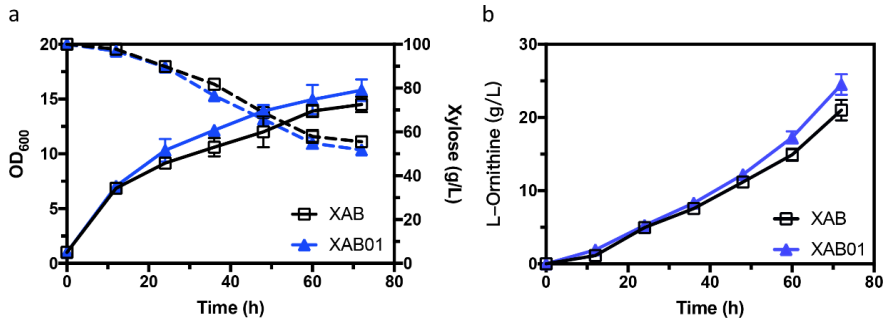


Fig.3. The promotion of *araE* on xylose and the effect on L-ornithine production.

a Growth and xylose consumption curves. b L-Ornithine production curves.

Strain XAB01 (blue solid triangle) integrated the *araE* gene from*B. subtilis* with a strong promoter Peftu, and XAB (black hollow square) is the control strain.

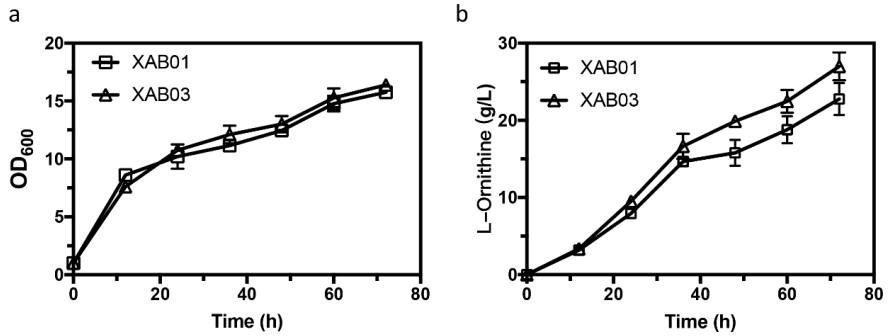


Fig.4. The PEPC modification reinforces the production of L-ornithine.

a: Growth curves. b: L-Ornithine production curves. XAB01 (hollow square) integrated the *araE* gene from *B. subtilis* with a strong promoter Peftu. XAB03 (hollow upper triangle) modified*pepc* on the basis of XAB01.

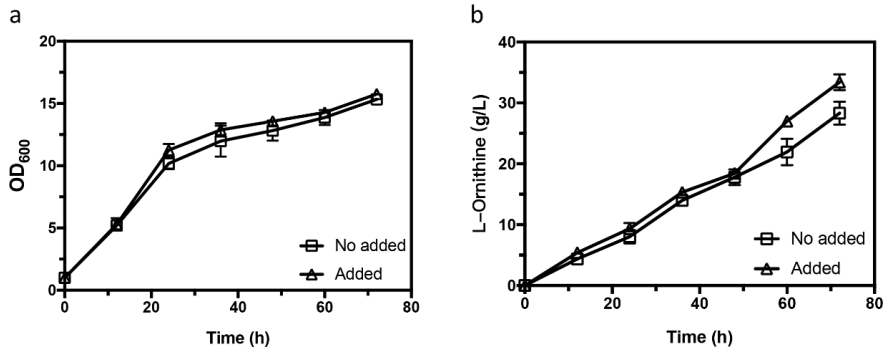


Fig.5. The effect of adding biotin and thiamine hydrochloride on L-ornithine production. a Growth curves for XAB03. b L-Ornithine production curves for XAB03.

Added (Biotin and thiamine hydrochloride, hollow upper triangle), No added (hollow square).

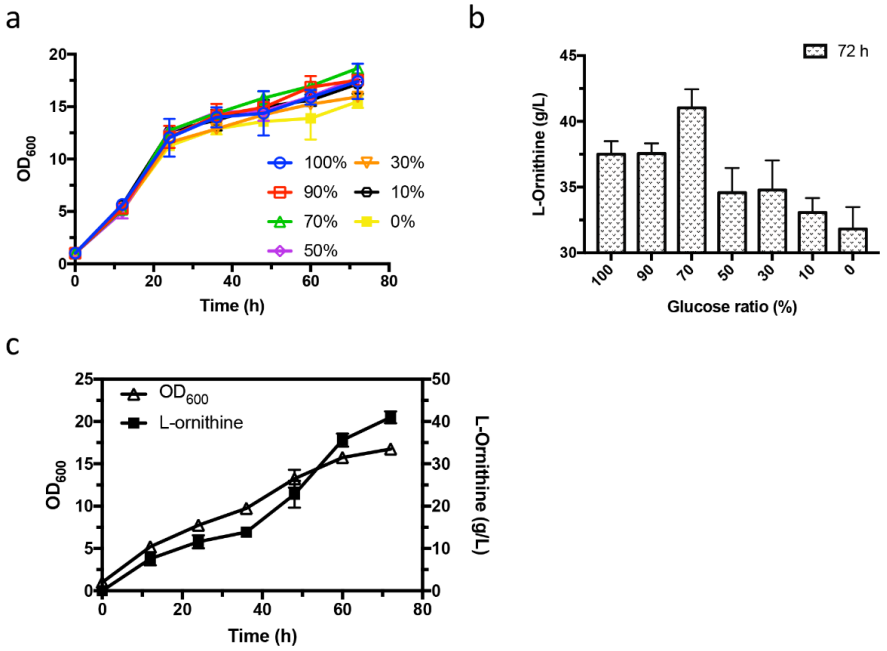


Fig.6. The effect of different ratios of glucose and xylose on L-ornithine production

a: Growth curves for *C. glutamicum* XAB03 at different glucose concentrations ratios (100%, blue circle; 90%, red hollow square; 70%, green upper triangle; 50%, purple diamond; 30%, orange lower triangle; 10%, black regular hexagon; 0%, yellow solid square.). b: L-Ornithine production curves. c: Optimal shake flask fermentation experiment of *C. glutamicum* XAB03 in $OD_{600}$(hollow upper triangle) and L-ornithine production (solid square) in glucose 70 g/L and xylose 30 g/L.

# Supplementary Information

Table S1. Primers involved in this article

| Primers | Sequence (5'-3') |
|---|---|
| XAB-F | ggatcttccagagat*aagctt*aaggagatatacatgagcaacaccgttttcatc |
| XAB-R | caaaacagccaag***gagctct***gatgaatcagccggcgtagag |
| EAB-F | caggaaacagaataatt*aagctt*gggttgacattccgcggcattacctgattatgg |
| EAB-R | caaaacagccaagct*gaattc*ttacgccattaatggcagaagttgc |
| up-eftu-F | ccaccgaagctccgctc*gatatc*cacagggtagctggtagtttgaaaatc |
| araE-eftu-R | ccctcccgaatgttgagtaaagcacttcgtggtggctacgactttc |
| araE-F | gaaagtcgtagccaccacgaagtgctttactcaacattcgggaggg |
| araE-R | gaggcgcatatggaattc*gatatc*aacagcccttcccgtagaaagg |
| up-HA-F1 | cctgcaggtcgac*tctaga*gcccttccaaaggaatacttcgagaag |
| up-HA-H36-R2 | ccagatagaggtacccagcttttgaactactttaaacactctttcacattgaggg |
| up-HA-H36-F3 | ccctcaatgtgaaagagtgtttaaagtagttcaaaagctgggtacctctatctgg |
| pepc-H36-R4 | gatgtcatcgcgtagaaaatcagtcatggatcccatgctactcctaccaac |
| pepc-H36-F5 | gttggtaggagtagcatgggatccatgactgattttctacgcgatgacatc |
| D299N-R6 | ctcattcatgcg*gttc*gacaggctcagctcatg |

| Primers | Sequence (5'-3') |
|---|---|
| D299N-F7 | catgagctgagcctgtcg*aac*cgcatgaatgag |
| down-HA-pepc-R8 | actacccacccggctggactagccggagttgcgcag |
| down-HA-pepc-F9 | ctgcgcaactccggctagtccagccgggtgggtagt |
| down-HA-R10 | tgaccatgattacgaattcagatgtacaccaggctgatgattcc |
| mutant-(K-R)-F | gggttgccgta*gcg*agctgagatgatttcg |
| mutant-(K-R)-R | cgaaatcatctcagct*cgc*tacggcaaccc |
| pXMJ19-F | ttgacaattaatcatcggctcgtataatgtgtg |
| pXMJ19-R | ctgatttaatctgtatcaggctgaaaatcttctctc |
| M13 fwd | cgttgtaaaacgacggccagt |
| M13 rev | acaatttcacacaggaaacagctatgac |

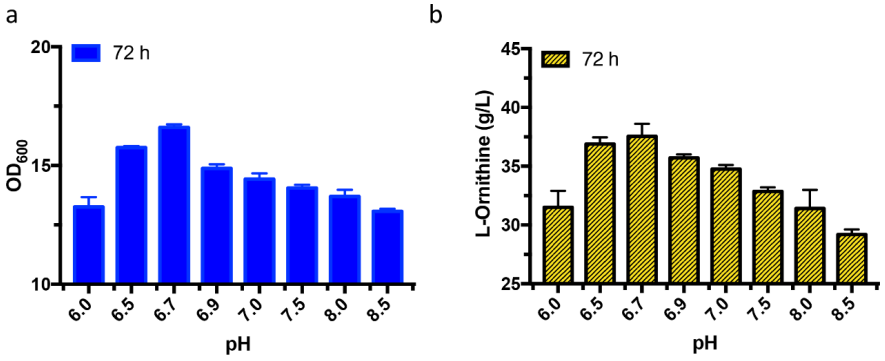


Fig. S1. Effect of different pH on L-ornithine yield

a Growth condition of *C. glutamicum* XAB03 at 72 h under different pH. b L-Ornithine production of *C. glutamicum* XAB03 at 72 h under different pH.