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Research Article

High-Titer Glutamic Acid Production from Lignocellulose Using an Engineered *Corynebacterium glutamicum* with Simultaneous Co-utilization of Xylose and Glucose

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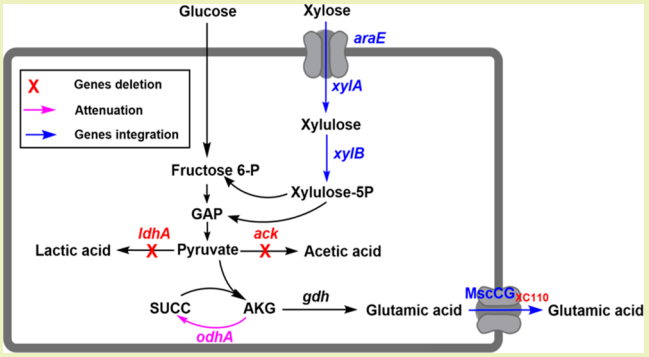
ABSTRACT: Xylose utilization by *Corynebacterium glutamicum* is an essential but unresolved issue in glutamic acid production from lignocellulose biomass. Coexistence of xylose with inhibitors requires a selective removal of inhibitors while the xylose is still well retained in the pretreated lignocellulose feedstock. Not only is xylose assimilation in *C. glutamicum* at low efficiency, but also there are unique challenges, which eliminate the generation of glutamic acid from xylose when lignocellulose is used. There include excessive biotin content in lignocellulose blocking intracellular secretion of glutamic acid, complicated organic acid generation pathways decreasing the glutamic acid conversion yield from xylose, and transmembrane resistance of xylose limiting the xylose utilization efficiency. Here, we applied a unique biodetoxification on pretreated wheat straw solids, which resulted in a complete removal of inhibitors and a high conservation of xylose sugar. The major focus of the study is a stepwise metabolic engineering of *C. glutamicum* to trigger high-titer glutamate production by coordinated assimilation of xylose and glucose from a typical lignocellulosic sugar. First, the secretion channel protein MscCG was modified to initiate glutamic acid secretion in biotin-rich environments from almost zero glutamic acid accumulation. Next, the byproduct generation pathways of lactate, acetate, and succinate were knocked out or attenuated to redirect carbon flux to glutamic acid accumulation. Further overexpression of the pentose transporter gene *araE* increased the xylose utilization rate and glutamic acid production. The finally obtained *C. glutamicum* GJ04 produced 39.8 g/L of glutamate from 60.3 g/L of glucose and 38.8 g/L of xylose in synthetic medium and produced 61.7 g/L of glutamate from 116.1 g/L of glucose and 39.6 g/L of xylose using wheat straw feedstock. This is the first example of the practical utilization of lignocellulose-derived xylose and glucose for cellulosic glutamic acid production.



KEYWORDS: *Glutamic acid, Xylose, Corynebacterium glutamicum, Lignocellulose, Metabolic engineering*

INTRODUCTION

Glutamic acid is a commodity amino acid with high potential as a polymer monomer.[1] However, food crop starch is currently used as the feedstock for glutamic acid production, which greatly restricts this potential. Among all available nonfood carbohydrate feedstocks, lignocellulose biomass is the most promising owing to its abundance and availability.[2,3] A major technical barrier of using lignocellulose as feedstock for glutamic acid production is the efficient utilization of xylose, which accounts for 30% of the lignocellulose-derived sugars.[4] To date, no major breakthrough in xylose assimilation to glutamic acid production by the dominant fermentation strain, *Corynebacterium glutamicum*, has been achieved.

The metabolic engineering of *C. glutamicum* has allowed xylose to be used for production of lactic acid,[5,6] succinic acid,[7−9] 3-hydroxypropionic acid,[10] γ-aminobutyric acid,[11] 5-aminovaleric acid,[12] and 1,5-diaminopentane.[13,14] In these xylose assimilation pathways, xylose is converted into xylulose by xylose isomerase (*xylA*), then to xylulose-5-phosphate by xylulokinase (*xylB*), and then assimilated into the pentose phosphate pathway to produce the target products.[15] However, this xylose conversion pathway does not work well in glutamic acid production when lignocellulose is used as feedstock. Gopinath et al. expressed the xylose isomerase gene *xylA* in *C. glutamicum* ATCC 13032 to utilize xylose from wheat bran.[16] Only part of the xylose and a small amount of glucose were converted to 11.8 g/L of glutamic acid while most glucan and

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Table 1. Strains and Plasmids Used

Strains	Characteristics	Sources
Escherichia coli DH5α	Host for plasmid construction	Lab stock
Escherichia coli BL21	Genes *xylAB_BL21* and *araE* source	Lab stock
Pediococcus acidilactici DSM 20284	Genes *xylAB_2911* source	DSMZ[a]
Amorphotheca resinae ZN1 (CGMCC 7542)	Biodetoxification fungus	Zhang et al.[20]
C. glutamicum S9114	Parental for glutamic acid fermentation	SIIM[b]
C. glutamicum-pPH36-*xylAB_BL21*	*C. glutamicum* S9114 harboring the plasmid pPH36-*xylAB_BL21*	This work
C. glutamicum-pPH36-*xylAB_2911*	*C. glutamicum* S9114 harboring the plasmid pPH36-*xylAB_2911*	This work
C. glutamicum-pPsod-*xylAB_BL21*	*C. glutamicum* S9114 harboring the plasmid pPsod-*xylAB_BL21*	This work
C. glutamicum-pPsod-*xylAB_2911*	*C. glutamicum* S9114 harboring the plasmid pPsod-*xylAB_2911*	This work
C. glutamicum-pPeftu-*xylAB_BL21*	*C. glutamicum* S9114 harboring the plasmid pPeftu-*xylAB_BL21*	This work
C. glutamicum-pPeftu-*xylAB_2911*	*C. glutamicum* S9114 harboring the plasmid pPeftu-*xylAB_2911*	This work
C. glutamicum-Δ*ldhA*::*xylAB* (GJ01)	*LdhA* knockout and integration of the expression cassette P*eftu_xylAB_BL21* in *C. glutamicum* S9114	This work
C. glutamicum-Δ*ldhA*::*xylAB*-ΔC110 (GJ02)	C-terminal truncation of MscCG in *C. glutamicum*-Δ*ldhA*::*xylAB*	This work
C. glutamicum-Δ*ldhA*::*xylAB*-ΔC110-RBS (GJ03)	RBS with 0.1 au substitution of *odhA* in *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110	This work
C. glutamicum-Δ*ldhA*::*xylAB*-ΔC110-RBS-Δ*ack*::*araE* (GJ04)	*Ack* knockout and the integration of the expression cassette P*H36_araE* in *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110-RBS	This work
C. glutamicum-ΔC110-RBS0.1	C-terminal truncation of MscCG and RBS with 0.1 au substitution of *odhA* in *C. glutamicum* S9114	Wen and Bao[24]

Plasmids	Characteristics	Sources
pTRCmob	Expression vector for *C. glutamicum*, TRC promoter, kanamycin resistance Km^R	Wang et al.[33]
pK18mobsacB	Mobilizable vector for selection of double crossover in *C. glutamicum*, kanamycin resistance Km^R	Wang et al.[33]
pPH36-*xylAB_BL21*	Vector for expression of *xylAB_BL21* by P*H36* promoter	This work
pPH36-*xylAB_2911*	Vector for expression of *xylAB_2911* by P*H36* promoter	This work
pPsod-*xylAB_BL21*	Vector for expression of *xylAB_BL21* by P*sod* promoter	This work
pPsod-*xylAB_2911*	Vector for expression of *xylAB_2911* by P*sod* promoter	This work
pPeftu-*xylAB_BL21*	Vector for expression of *xylAB_BL21* by P*eftu* promoter	This work
pPeftu-*xylAB_2911*	Vector for expression of *xylAB_2911* by P*eftu* promoter	This work
pK18-Δ*ldhA*::*xylAB*	Vector for replacement of *ldhA* by integrating the expression cassette P*eftu_xylAB_BL21* into the genome of *C. glutamicum* S9114	This work
pK18-ΔC110	Vector for truncation of C-terminal of MscCG in the genome of *C. glutamicum*-Δ*ldhA*::*xylAB*	Wen and Bao[24]
pK18-*odhA*RBS0.1	Vector for RBS with 0.1 au substitution of *odhA* in the genome of *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110	Wen and Bao[24]
pK18-Δ*ack*::*araE*	Vector for replacement of *ack* by integrating the expression cassette P*H36_araE* into the genome of *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110-RBS	This work

[a]DSMZ indicates the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. [b]SIIM indicates the collection center of Shanghai Industrial Institute of Microorganism, Shanghai, China.

xylan of wheat bran failed to be converted into fermentable glucose and xylose.

For complete utilization of xylose and glucose sugar for high-titer glutamate production, one of the major obstacles is the coexistence of glucose and xylose with inhibitors in the lignocellulose feedstock after pretreatment. Xylose is normally generated from hemicellulose in the process of pretreatment and is always mixed with inhibitors. This presents the dilemma that the presence of inhibitors completely suppresses the cell activity and production of fermenting strains, but removing these inhibitors by regular detoxification methods (water washing or overliming) causes significant xylose loss. Therefore, successful xylose utilization from a real and practical lignocellulose feedstock has rarely been demonstrated owing to the coexistence of xylose and inhibitors.

Until now, cellulosic glutamic acid production was still not established using the biorefining chain and is far below the requirement for practical application. The major technical barriers are as follows: (i) Selective removal of inhibitors occurring from pretreated lignocellulose feedstock while xylose is well conserved. (ii) Excessive biotin content in lignocellulose blocks intracellular secretion of glutamic acid from *C. glutamicum* cells.[17] (iii) Xylose metabolism generates multiple byproducts, such as lactic acid, acetic acid, and succinate.[15] (iv) Transport resistance limits efficient xylose transfer through the cell membrane.[18] (v) Genetic instability occurs when the *xylAB* cluster is expressed using plasmids.

To overcome these barriers, first, this study applied a unique biodetoxification on solid pretreated wheat straw feedstock particles by a biodetoxification fungus *Amorphotheca resinae* ZN1, in which process the inhibitors are completely removed, and the xylose sugar is well conserved for glutamic acid production in the fermentation step. Moreover, we further designed and conducted stepwise metabolic engineering of the dominant glutamic acid producing strain *C. glutamicum*, including (i) promoter screening for improved *xylAB* expression, (ii) modification of the glutamic acid transport protein MscCG to activate the glutamic acid secretion channel, (iii) knocking out the byproduct generation pathways, (iv) enhancement of xylose transmembrane transportation through xylose transporter overexpression, and (v) fixation of metabolic modifications through genome integration of genes with positive behaviors. The finally obtained strain was applied to high

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glutamic acid fermentation using wheat straw to confirm the cellulosic glutamic acid production performance.

MATERIALS AND METHODS

Strain, Media, and Culture Conditions. The plasmids and strains are listed in Table 1. *E. coli* DH5α and *E. coli* BL21 were cultured at 37 °C in Luria−Bertani medium with 50.0 mg/mL of kanamycin.

C. glutamicum S9114 is storage B460 of the Shanghai Industrial Institute of Microorganism (SIIM, Shanghai, China, http://www.gsy-siim.com/). The culture condition was at 30 °C in the biotin-limited medium (1.0 g/L of potassium dihydrogen phosphate, 3.0 g/L of urea, 0.6 g/L of magnesium sulfate, 0.5 g/L of corn steep liquor) or in the biotin-rich medium (1.0 g/L of potassium dihydrogen phosphate, 3.0 g/L of urea, 0.6 g/L of magnesium sulfate, 25.0 g/L of corn steep liquor). Here, 60.0 g/L of glucose or xylose was added to the medium if needed.

Pediococcus acidilactici DSM 20284 is stored at Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and cultured according to the previous study.[19]

Amorphotheca resinae ZN1 is stored at Chinese General Microorganisms collection center (CGMCC, Beijing, China) with the storage code (CGMCC 7542) and cultured according to our previous study.[20]

Enzymes and Reagents. Cellulase Cellic CTec2 was obtained from Novozymes (China) (Beijing, China). The filter paper activity, cellobiase activities, and total protein concentration were 203.2 FPU/mL, 4900 CBU/mL, and 87.3 mg/mL, respectively, according to the NREL protocols LAP-006 and the method by Bradford.[21−23] T4 ligase and DNA polymerase were from Takara (Otsu, Japan). Seamless cloning kits were from Hanheng Biotech (Nanjing, China). Restriction endonuclease was from Thermo Scientific (Wilmington, DE, USA). Other general chemicals used were from local suppliers.

Plasmid and Recombinant Construction. The primers are listed in Table S1. The plasmids and the recombinants are listed in Table 1. Promoter P*H36* was synthesized by Shanghai Generay Biotech (Shanghai, China). Two endogenous promoters, P*sod* and P*eftu*, were obtained from *C. glutamicum* S9114 by PCR. Gene clusters *xylAB_2911* and *xylAB_BL21* were obtained from *P. acidilactici* DSM20284 and *E. coli* BL21 by PCR, respectively. The three promoters (P*H36*, P*sod*, and P*eftu*) were fused with the two *xylAB* clusters (*xylAB_BL21* and *xylAB_2911*), and then, the original TRC promoter was substituted in pTRCmob to obtain six expression plasmids.

Plasmid pK18-ΔC110, for deleting the C-terminal 110 amino acid residue of the glutamate exporter MscCG, and plasmid pK18-*odhA*RBS0.1, for alternating the ribosome-binding sequence (RBS) fragment of *odhA* with RBS0.1, were constructed by Wen and Bao.[24] The upstream (*ldhA*-up) and downstream (*ldhA*-down) fragments of the *ldhA* gene of *C. glutamicum* S9114 were overlapped with the cassette P*eftu-xylAB_BL21* by PCR. The PCR product, Δ*ldhA*::*xylAB*, was then ligated to *Eco*RI/*Hin*dIII of pK18mobsacB to generate plasmid pK18-Δ*ldhA*::*xylAB* for genome integration of *xylAB* at the *ldhA* gene locus. The upstream (*ack*-up) and downstream (*ack*-down) fragments of the *ack* gene were overlapped with the expression cassette P*H36-araE* by PCR. The PCR product, Δ*ack*::*araE*, was then ligated to XbaI/SphI of pK18mobsacB to obtain plasmid pK18-Δ*ack*::*araE* for genome integration of *araE* into the locus of the *ack* gene. Plasmids were transformed into *C. glutamicum* cells according to van der Rest et al.[25] Gene disruption and integration in the genome were according to the approved procedures.[26] The correct mutants were isolated by kanamycin-resistance selection, colony PCR, and sequence analysis.

Lignocellulose Feedstock and Biorefinery Processing. Wheat straw was from Nanyang, Henan, China, in fall 2018. The raw biomass was processed by air-drying and milling. The virgin wheat straw contained 34.6% of cellulose, 25.1% of hemicellulose, 18.4% of lignin, and 8.9% ash according to Sluiter et al.[27,28] Dry acid pretreatment was conducted according to our previous reports.[29,30] Before detoxification, the dissolved contents in the pretreated wheat straw included 21.2 mg/g DM (dry wheat straw matter) of glucose, 128.5 mg/g DM of xylose, 4.2 mg/g DM of furfural, 5.7 mg/g DM of 5-hydroxymethylfurfural (HMF), and 17.2 mg/g DM of acetic acid. The biodetoxifiation of pretreated wheat straw was conducted in a 5 L bioreactor equipped with a helical ribbon impeller as described by Zhang et al.[37] Then, 900 g of pretreated wheat straw was loaded in the reactor with 10% (w/w) inoculum of seed culture containing *A. resinae* ZN1. Biodetoxifiation was then carried out under an aeration rate of 1.0 vvm. The aeration rate (vvm) was defined as the air flow rate (L/min) per liter of loosely packed pretreated wheat straw material in the bioreactor. The wheat straw material was mixed slowly for 90 s prior to the samples being withdrawn, and the rest of the time of biodetoxifiation was still carried out in a solid state culture. After detoxification, the dissolved sugars in the wheat straw were 11.7 mg/g DM of glucose and 123.1 mg/g DM of xylose. The glucose loss accounted for 2.5% of the total glucose based on the cellulose content in the virgin wheat straw, and the xylose loss accounted for 1.9% of the total xylose based on the xylan content in the virgin wheat straw. No furfural, acetic acid, and HMF were detected. The pretreated and detoxified wheat straw was hydrolyzed at 50 °C for 48 h to obtain the wheat straw hydrolysate (116.1 g/L of glucose, 39.6 g/L of xylose, 1.3 g/L of acetic acid, 0.04 g/L of furfural, and 0.01 g/L of 5-hydroxymethylfurfural).

Glutamic Acid Fermentation. *C. glutamicum* cells were cultured on LB ager at 30 °C for 36 h, and then, a single colony was picked for seed culture preparation as described before.[17] The batch fermentation was performed in a 3 L fermentor (3BG-4, Baoxing Biotech Co., Shanghai, China) at 32 °C, 1.4 vvm of aeration, and 600 rpm. The seed culture was inoculated into 800 mL of wheat straw hydrolysate at a 10% (v/v) inoculum ratio. The pH was maintained at 7.2 by automatically adding 25% (w/v) ammonium hydroxide solution.

Analytical Methods. Glucose, xylose, and typical inhibitor compounds were detected according to He et al.[31] Glutamic acid was detected by the SBA-90 biosensor (Shandong Academy of Sciences, Shandong, China). Cell growth was detected by a UV−visible spectrophotometer (BIOMATE 3S; Thermo, Waltham, MA, USA).

RESULTS AND DISCUSSION

Constructing Xylose Assimilation Pathway in *C. glutamicum* S9114. Gene cluster *xylAB*, which encoded xylose isomerase and xylulokinase, was responsible for xylose assimilation into the pentose phosphate pathway in *C. glutamicum*. Three promoters were tested for improved *xylAB* expression, including synthesized P*H36*,[32] and endogenous promoters P*sod* and P*eftu* from *C. glutamicum* S9114. The two *xylAB* gene clusters were from *P. acidilactici* DSM 20284 (*xylAB_2911*) and *E. coli* BL21 (*xylAB_BL21*). The six expression cassettes were ligated to plasmid pTRCmob by substitution of its original pTRC promoter. The plasmids were then separately introduced into *C. glutamicum* S9114 to obtain six recombinants (Table 1). The xylose utilization capacity of the recombinants was evaluated (Table 2), with the results showing that the maximum xylose consumption (32.6 g/L) resulted from the combination of promotor P*eftu* and cluster

Table 2. Alternating Promotors and Xylose Assimilation Gene Clusters *xylAB* in *C. glutamicum*[a]

Promoters	Gene clusters	Cell growth (OD_{600})	Xylose consumed (g/L)
P*H36*	*xylAB_BL21*	7.6 ± 0.3	2.6 ± 0.1
	xylAB_2911	6.2 ± 0.1	0.3 ± 0.0
P*sod*	*xylAB_ BL21*	17.2 ± 0.9	15.5 ± 0.5
	xylAB_2911	8.4 ± 0.3	3.1 ± 0.1
P*eftu*	*xylAB_BL21*	31.3 ± 0.6	32.6 ± 0.7
	xylAB_2911	11.6 ± 0.4	6.1 ± 0.2

[a]*C. glutamicum* recombinants were cultured at 30 °C and 200 rpm in 250 mL flasks containing 30 mLof biotin-rich medium. The medium contained 40.0 g/L of xylose as the sole carbon source and 25.0 μg/mL of kanamycin. All the data were collected after 48 h of fermentation.

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xylAB_BL21 originating from *E. coli* BL21 (cassette P*eftu_xylAB_BL21*).

To obtain the genetically stable strain, cassette P*eftu_xylAB_BL21* was integrated into the *C. glutamicum* S9114 chromosome by substituting gene *ldhA* (Figure 1) to obtain

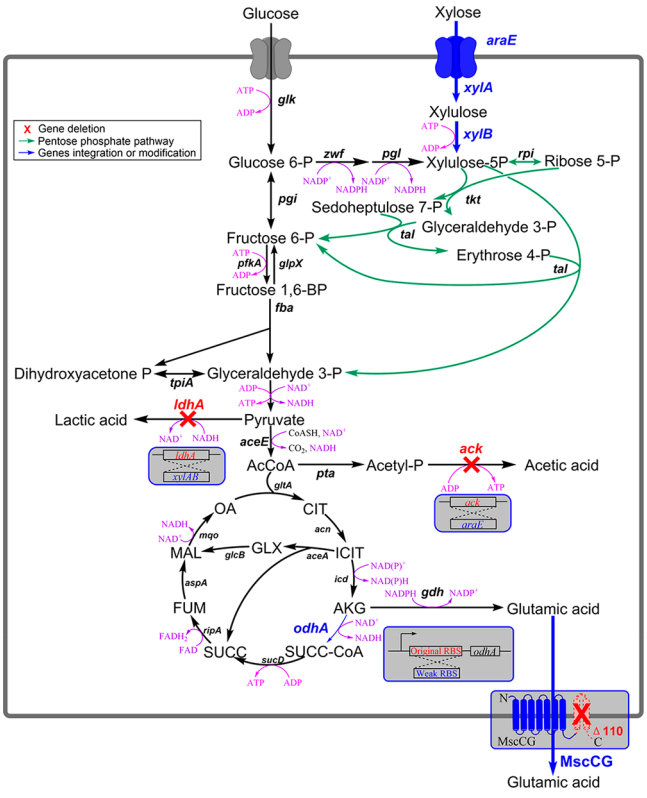

Figure 1. Metabolic engineering strategies for *C. glutamicum* on xylose assimilation to glutamic acid. Red crosses, gene knockout; blue arrow, genome integration of genes; green arrow, pentose phosphate pathway; *araE*, pentose transporter; *xylA*, xylose isomerase; *xylB*, xylulose kinase; *glk*, glucokinase; *zwf*, glucose-6-phosphate dehydrogenase; *pgl*, 6-phosphogluconolactonase; *pgi*, glucose-6-phosphate isomerase; *pfkA*, 6-phosphofructokinase; *glpX*, fructose 1,6-bisphosphatase; *fba*, fructose-bisphosphate aldolase; *tpiA*, triosephosphate isomerase; *aceE*, pyruvate dehydrogenase E1 component; *ldhA*, lactate dehydrogenase; *pta*, phosphate acetyltransferase; *ack*, acetate kinase; *gltA*, citrate synthase; *acn*, aconitate hydratase; *icd*, isocitrate dehydrogenase; *odhA*, 2-oxoglutarate dehydrogenase E1 component; *sucD*, succinyl-CoA synthetase subunit alpha; *ripA*, succinate dehydrogenase; *aspA*, aspartate ammonia-lyase; *mqo*, malate:quinone oxidoreductase; *aceA*, isocitrate lyase; *glcB*, malate synthase G; *gdh*, glutamate dehydrogenase; CIT, citrate; ICIT, isocitrate; AKG, α-oxoglutarate; SUCC-CoA, succinyl-CoA; SUCC, succinate; FUM, fumarate; Mal, malate; OA, oxaloacetate; MscCG, glutamate exporter.

C. glutamicum-Δ*ldhA*::*xylAB* (assigned as *C. glutamicum* GJ01). *C. glutamicum* GJ01 utilized 38.4 g/L of xylose, producing 23.5 g/L of glutamate in biotin-limited medium (Figure 2b), indicating that genome integration improved xylose assimilation. However, when *C. glutamicum* GJ01 was cultured in the biotin-rich medium, no glutamic acid was accumulated (Figure 2a) owing to the blockage of glutamate secretion.[17]

Triggering Glutamic Acid Accumulation from Xylose under High Biotin Conditions. Although *C. glutamicum* GJ01 enabled xylose assimilation, excessive biotin in the lignocellulose hydrolysate blocked glutamic acid secretion, leading to the failure of glutamic acid accumulation.[17] First, we focused on

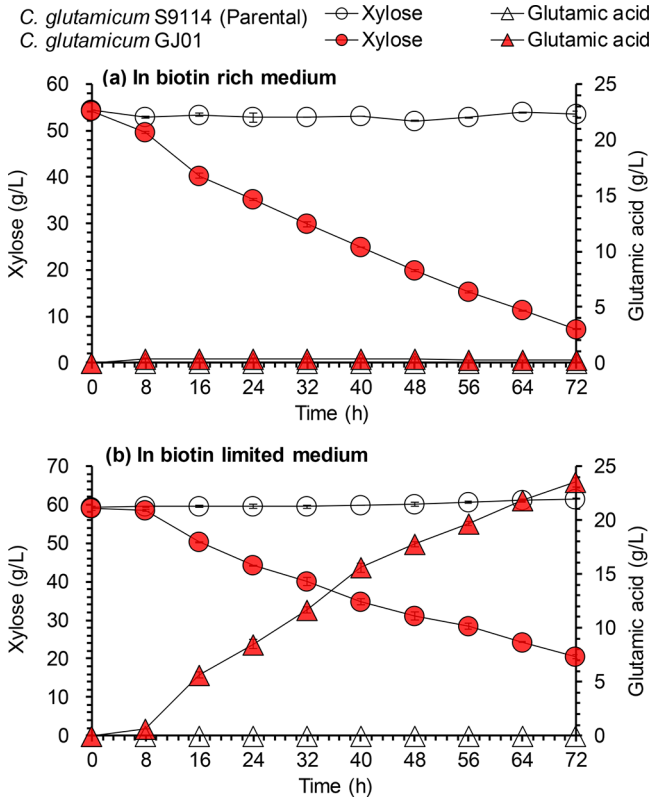

Figure 2. Evaluation of biotin content on glutamic acid accumulation using xylose as sole carbon source by *C. glutamicum* GJ01: (a) in biotin-rich medium and (b) in biotin-limited medium. *C. glutamicum* S9114, parental strain; *C. glutamicum* GJ01, engineered strain with integration of *xylAB* in *C. glutamicum* S9114. The glutamic acid fermentations were carried out at 30 °C and 200 rpm in 250 mL flasks containing 30 mL of medium. Mean values are presented with error bars representing the minimum and maximum values.

activating glutamic acid secretion by deleting the C-terminal 110 amino acid of the glutamate secretion channel protein MscCG (Figure 1).[33] The obtained strain, *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110 (assigned as *C. glutamicum* GJ02), was fermented in the biotin-rich medium, and 10.0 g/L of glutamic acid accumulation was obtained, compared with only 0.3 g/L by *C. glutamicum* GJ01 (Figure 3c).

Decreasing the α-oxoglutarate dehydrogenase activity redirects the α-oxoglutarate flux to glutamic acid synthesis.[34] Next, we reduced the ODHC activity by substituting the original RBS (CAAGGAAAAGAGGCGAGTACCTGCC) of *odhA* with the sequence RBS0.1 (CTCACCCACGAGTTCAATAACTAGG). The resulting strain, *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110-RBS (assigned as *C. glutamicum* GJ03), afforded 31.3 g/L of glutamic acid accumulation, which was approximately three times that afforded by *C. glutamicum* GJ02 (10.0 g/L) (Figure 3c).

An adaptive evolution of *C. glutamicum* GJ03 was also conducted to increase the xylose utilization rate. *C. glutamicum* GJ03 was cultured for 30 days by successive transfer into the biotin-rich medium with 45.0 g/L of xylose every 48 h. The xylose utilization rate of *C. glutamicum* GJ03 was improved from 0.5 to 0.8 g/L/h, and the cell growth was increased 4-fold (OD_{600} increased from 12.3 to 45.5). However, glutamic acid accumulation sharply reduced from 22.8 to 4.1 g/L, indicating that adaptive evolution might not be an appropriate method for the glutamic acid-producing strain.

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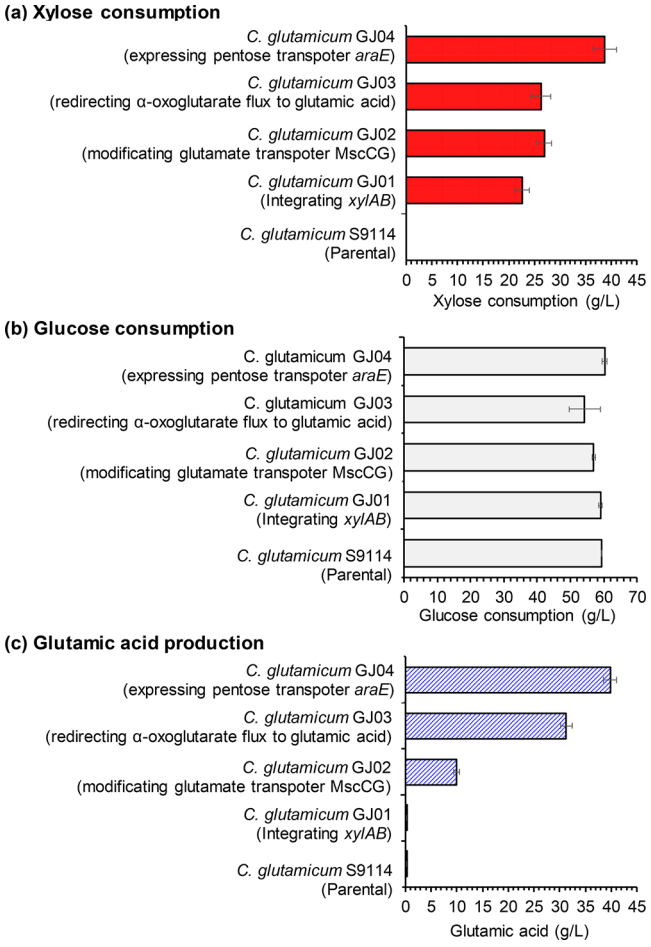

Figure 3. Increasing glutamic acid production from xylose by four steps metabolic modifications of *C. glutamicum*: (a) xylose consumption, (b) glucose consumption, and (c) glutamic acid production. *C. glutamicum* S9114, parental strain; *C. glutamicum* GJ01, strain with integration of *xylAB* in *C. glutamicum* S9114; *C. glutamicum* GJ02, strain with truncation of 110 amino acid of MscCG in *C. glutamicum* GJ01; *C. glutamicum* GJ03, strain with substitution of RBS of *odhA* by 0.1 au RBS in *C. glutamicum* GJ02; *C. glutamicum* GJ04, strain with integration of *araE* in *C. glutamicum* GJ03. The experiment was processed in a 3 L fermentor containing 800 mL of biotin-rich medium at 1.4 vvm of aeration, 600 rpm, 32 °C, and pH 7.2. The biotin-rich medium contained 60.0 g/L of glucose and 40.0 g/L of xylose. Mean values are presented with error bars representing the minimum and maximum values.

Finally, we increased the transmembrane transportation rate of xylose. Pentose transporter gene *araE* from *E. coli* BL21 was inserted into the genome by substituting the *ack* gene, giving strain *C. glutamicum*-Δ*ldhA*::*xylAB*-ΔC110-RBS-Δ*ack-araE* (assigned as *C. glutamicum* GJ04). Figure 3 shows that *C. glutamicum* GJ04 increased the xylose utilization rate approximately 1.5-fold and glutamic acid accumulation 1.3-fold compared with those of *C. glutamicum* GJ03. Notably, glucose and xylose were consumed simultaneously by *C. glutamicum* GJ04. Obtained strain *C. glutamicum* GJ04 was practically used for cellulosic glutamic acid fermentation.

Glutamic Acid Fermentation in Biotin-Rich Xylose Medium and Wheat Straw Hydrolysate. Xylose is normally generated from hemicellulose in the process of pretreatment and is always mixed with inhibitors. This presents the dilemma that the presence of inhibitors completely suppresses the cell activity and production of fermenting strains, but removing these inhibitors by regular detoxification methods (water washing or overliming) causes significant xylose loss. Therefore, successful xylose utilization from a real and practical lignocellulose feedstock has rarely been demonstrated owing to the coexistence of xylose and inhibitors. Significantly, this study applied a unique biodetoxification on solid pretreated wheat straw feedstock particles by a biodetoxification fungus *Amorphotheca resinae* ZN1, resulting in a complete degradation of the inhibitors without the loss of xylose in the pretreated wheat straw feedstock. Using this biodetoxification method, the inhibitors are completely removed, and the xylose sugar is well conserved for glutamic acid production in the fermentation step.[35,36]

We compared the glutamic acid fermentation performance of *C. glutamicum* GJ04 with that of control strain *C. glutamicum*-ΔC110-RBS0.1 (without xylose utilization) in two biotin-rich environments: high biotin-containing synthetic medium and wheat straw hydrolysate. The biotin content of the synthetic medium was 18.6 μg/L, which was approximately 3−4-fold that of the upper level of the suboptimal biotin concentration (2.0−5.0 μg/L). Figure 4 shows that *C. glutamicum* GJ04 simultaneously and completely consumed 60.3 g/L of glucose and 38.8 g/L of xylose, with 39.8 g/L of glutamate accumulated. For comparison, control strain *C. glutamicum*-ΔC110-RBS0.1 consumed 53.6 g/L of glucose, but no xylose, and 25.8 g/L of glutamate was produced.

The biotin content of wheat straw hydrolysate was 45.0 μg/L, which was approximately 1 order of magnitude greater than the suboptimal level. When *C. glutamicum* GJ04 and the control strain *C. glutamicum*-ΔC110-RBS0.1 were used as glutamic acid-fermenting strains in wheat straw, Figure 5 shows that *C. glutamicum* GJ04 simultaneously and completely consumed 116.1 g/L of glucose and 39.6 g/L of xylose, while the control strain showed no observable xylose consumption and consumed 117.4 g/L of glucose. The glutamic acid production by *C. glutamicum* GJ04 was 61.7 g/L, which was approximately 25% greater than that of control strain *C. glutamicum*-ΔC110-RBS0.1 (49.2 g/L). However, the glutamate yield of co-utilizing glucose and xylose (0.40 g/g) was lower than that of utilizing glucose (0.42 g/g), because of the insufficient NADPH supply when xylose is utilized. In the case of glucose utilization, partial glucose is oxidized to xylulose-5P and NADPH is produced, but no such an oxidation occurs in the case of xylose utilization.

CONCLUSION

Xylose utilization is an important topic that has been widely investigated in various fermentation strains because xylose consists of 30% of the sugars in lignocellulose. This study provides the first meaningful result of high-titer glutamate production from both glucose and xylose using a real and practical wheat straw feedstock, instead of pure xylose and glucose sugars. In the stepwise metabolic engineering of *C. glutamicum* S9114, we enabled xylose assimilation by expressing *xylAB* (*C. glutamicum* GJ01) and then modified the glutamic acid secretion channel protein MscCG to activate glutamic acid secretion (*C. glutamicum* GJ02) and initiate glutamic acid accumulation (10.0 g/L) in the biotin-rich medium. To further improve the glutamic acid yield and accumulation titer, we knocked out the *ldhA* and *ack* genes to block byproduct generation from xylose (lactic acid and acetic acid). The α-oxoglutarate dehydrogenase activity was also reduced (*C. glutamicum* GJ03) to redirect α-oxoglutarate flux to glutamate

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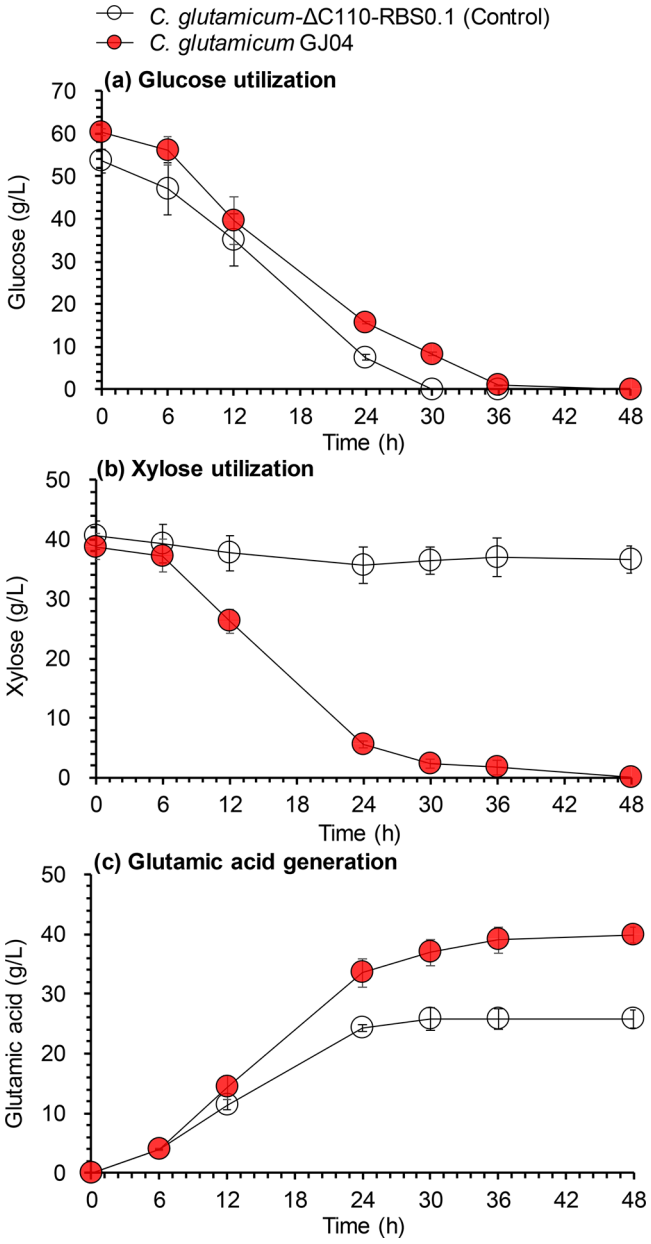

Figure 4. Glutamic acid fermentation of *C. glutamicum* GJ04 from pure xylose and glucose sugars: (a) glucose utilization, (b) xylose utilization, and (c) glutamic acid generation. The experiment was carried out in a 3 L fermentor (3BG-4, Baoxing Biotech Co., Shanghai, China) containing 800 mL of biotin-rich medium at 32 °C, 1.4 vvm of aeration, 600 rpm, and pH 7.2 regulation automatically using 25% (w/w) aqueous ammonium and 2 M H_2SO_4. The biotin-rich medium contained 40.0 g/L of xylose and 60.0 g/L of glucose. Mean values are presented with error bars representing the minimum and maximum values.

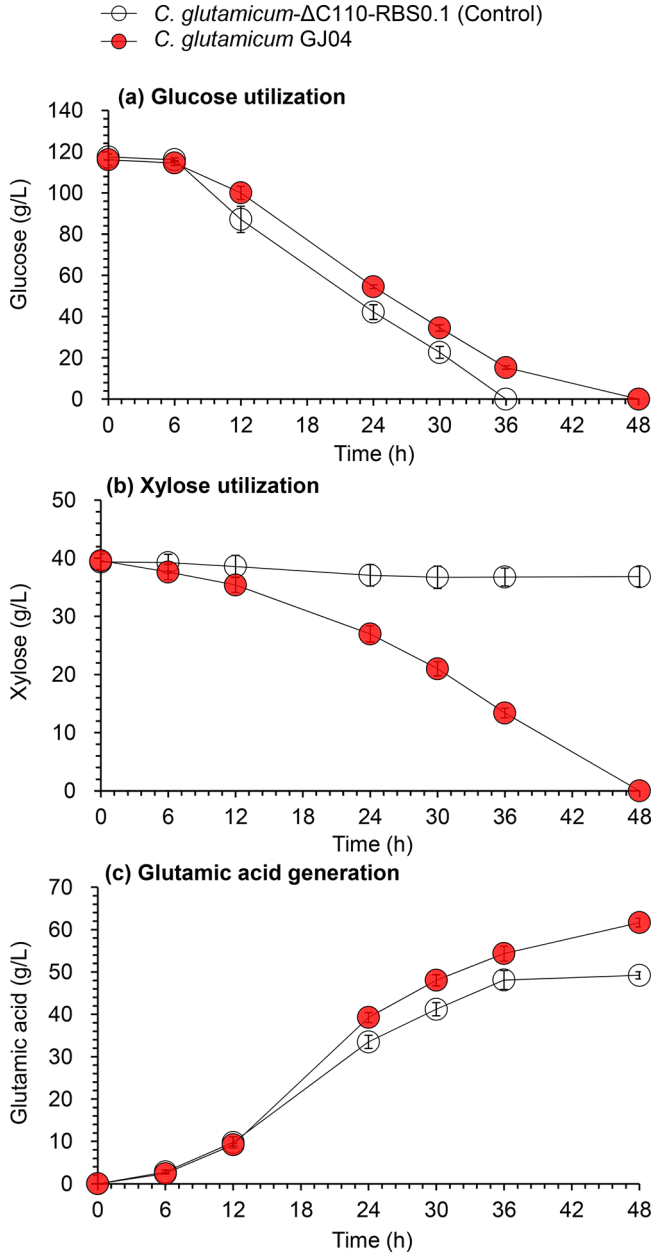

Figure 5. Glutamic acid fermentation of *C. glutamicum* GJ04 from wheat straw feedstock: (a) glucose utilization, (b) xylose utilization, and (c) glutamic acid generation. The experiment was processed in 3 L of fermentor containing 800 mL of wheat straw hydrolysate at 1.4 vvm of aeration, 600 rpm, 32 °C, and pH 7.2. Wheat straw hydrolysate contained 116.1 g/L of glucose and 39.6 g/L of xylose. Mean values are presented with error bars representing the minimum and maximum values.

generation. In the final step, we overexpressed the pentose transporter gene *araE* to increase xylose transmembrane transportation (*C. glutamicum* GJ04). All positive metabolic modifications were integrated into the genome to obtain a genetically stable strain, with the finally obtained strain, *C. glutamicum* GJ04, producing 61.7 g/L of glutamate from 116.1 g/L of glucose and 39.6 g/L of xylose using wheat straw feedstock. In addition to the dry acid pretreatment used in this study, there are some common pretreatment methods, such as alkaline pretreatment,[38] steam explosion pretreatment,[39] and ammonia fiber explosion pretreatment.[40] The dry acid pretreatment is unable to remove biotin from lignocellulose,[17] and the effect of other pretreatments on biotin removal remains to be studied. Therefore, whatever pretreatment was used, the knockout of byproducts and the resolution of biotin inhibition are effective methods to improve cellulosic glutamic acid production. This represents the first report of the efficient utilization of lignocellulose-derived xylose with simultaneous glucose utilization for cellulosic glutamic acid production and provides the basis for commodity glutamic acid production from lignocellulose biomass.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.9b07839.

Further experimental details and information (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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