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Engineering *Corynebacterium glutamicum* for efficient production of succinic acid from corn stover pretreated by concentrated-alkali under steam-assistant conditions

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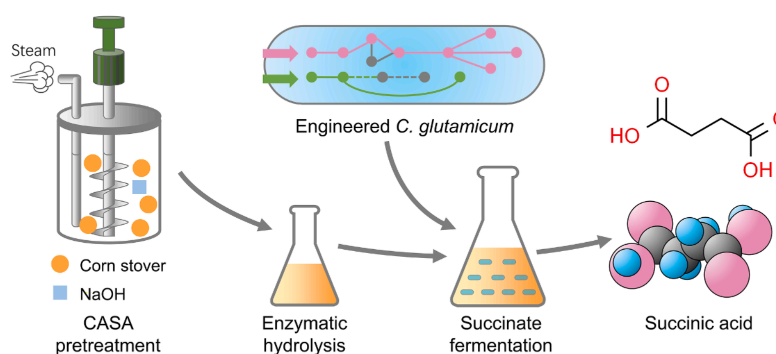
HIGHLIGHTS

- Constructing a succinate high-producing *C. glutamicum*.
- Introducing two xylose utilization pathways into *C. glutamicum*.
- Developing CASA pretreatment with high sugar yield and low black liquid.
- Achieving efficient succinate production from corn stover by engineered *C. glutamicum*.

GRAPHICAL ABSTRACT



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ABSTRACT

Corynebacterium glutamicum was developed for efficient production of succinic acid from corn stover (CS) pretreated by concentrated-alkali under steam-assistant (CASA) conditions. First, *C. glutamicum* was engineered by 1) blocking the by-products pathways (deletion of *ldh*, *pta-ackA*, and *cat*), 2) enhancing the carbon flux to succinate (overexpression of *pyc* and *ppc*), and 3) releasing the end-product inhibition (overexpression of *Ncgl0275*). The recombinant strain produced 117.8 g/L succinate in fed-batch fermentation. Second, to fully utilize xylose in lignocellulosic hydrolysate, two xylose utilization pathways—the isomerase pathway and the Weimberg pathway—were introduced into the recombinant strain. Third, CS was pretreated by CASA with a higher sugars yield and a lower black liquid. Finally, 64.16 g/L of succinic acid was obtained from 150 g/L CASA-pretreated CS by engineered *C. glutamicum*. These results showed a succinate high-producing *C. glutamicum* strain using glucose and xylose simultaneously as well as an effective and environmentally acceptable pretreatment strategy.

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1. Introduction

As one of the most important platform bulk chemicals, succinate is extensively used for a wide range of industrial applications (Bello et al., 2022). It has been listed by the Department of Energy of the United States (DOE) as one of the 12 bio-based platform compounds (Espro et al., 2021). The global succinate market will grow up to $218.14 Million by 2025, according to a report from Fior Markets (Newark, 2020). In comparison with chemical synthesis, succinate produced via microbial fermentation has garnered more attention due to the growing worries about environmental pollution and fossil fuels depletion. Meanwhile, succinate fermentation using lignocellulosic substrates has many benefits, including minimal emissions, affordable and renewable raw materials, and cost reductions of more than 50%. (Akhtar and Idris, 2014; Lu et al., 2021). Additionally, 1 mol succinate synthesis may fix 1 mol CO_2, which is a highly efficient approach to utilize CO_2 and achieve carbon emission reduction (Jin et al., 2023).

Some microorganisms naturally produce succinate like *Anaerobiospirillum succinogenes* (Putri et al., 2022), *Actinobacillus succinogenes* (Jokodola et al., 2022), and *Mannheimia succiniciproducens* (Lee et al., 2006). Besides, metabolically engineered bacteria were enabled to produce succinate, like *Vibrio natriegens* (Thoma et al., 2022), *Escherichia coli* (Yu et al., 2019), *Corynebacterium glutamicum* (Tenhaef et al., 2021), *Saccharomyces ccerevisiae* (Raab et al., 2010), and *Yarrowia lipolytica* (Cui et al., 2017). The greatest titer ever recorded for bacteria was created by a metabolically altered *C. glutamicum* strain with *NCgl0275* overexpression, which was able to release the succinate inhibition and produce more than 150 g/L of succinate from glucose (Chung et al., 2017). Cui et al. achieved 110 g/L succinate production from glycerol by deleting several genes that involved in acetic acid synthesis in *Y. lipolytica* (Cui et al., 2017). Succinate production from lignocellulosic biomass has received relatively little attention in publications. *E. coli* was genetically modified to produce a total of 11 g/L succinate from corn stalk (Liu et al., 2012). Additionally, *A. succinogenes* NJ113 was also capable of producing 70 g/L succinate from corn fiber (Chen et al., 2011). There are still several issues with the production of succinate from lignocellulosic substrate, including low sugar yield and poor xylose utilization.

Corynebacterium glutamicum is a typical industrial microorganism with great potential to over-produce succinic acid. It is generally considered safe (GRAS organism) (Ray et al., 2022). When oxygen is deprived, *C. glutamicum* will adjust its energy and carbon mostly on producing organic acids instead of accumulating biomass (Inui et al., 2004). *C. glutamicum* has a broad range of natural substrate spectrum, for instance, glucose, ethanol, and acetate. However, *C. glutamicum* naturally could not metabolize xylose, the second largest component in lignocellulose (Kawaguchi et al., 2006). So far, xylose catabolism occurs through three pathways in microorganisms: the isomerase pathway (Li et al., 2019), the oxidoreductase pathway, and the non-phosphorylative pathway (Domingues et al., 2021). The oxidoreductase pathway is predominantly found in eukaryotic microorganisms, the other two pathways are mainly carried out by bacteria. In recent years, a series of genetically modified *C. glutamicum* strains were studied for improving xylose utilization. Sasaki et al. developed a recombinant *C. glutamicum* via multi-locus chromosomal integration of the *xylAB* operon for simultaneous utilization of glucose and xylose (Sasaki et al., 2008). Tenhaef et al. explored the synthesis of α-ketoglutarate and succinate in xylose-using *C. glutamicum* by Weimberg pathway (Tenhaef et al., 2021).

Corn stover (CS) is one of the most accessible and plentiful agricultural residues that can be used for second-generation biofuel and bio-based chemicals production. The pretreatment is the first and essential step for CS bioconversion to break up the rigid structure of CS and separate three main components— cellulose, hemicelluloses, and lignin—so that the cellulose component can be hydrolyzed more effectively (Areepak et al., 2022). Among various pretreatment technologies (Zhao et al., 2022), alkaline pretreatment may remove lignin at moderate temperature efficiently, resulting in high cellulose recovery and low inhibitor generation (Kim et al., 2016). However, excessive black liquid generated during alkaline pretreatment leads to an environment pollution (Cha et al., 2016). As such, a new alkaline pretreatment that keep the superior pretreatment effect but with fewer black liquid was urgent needed.

In the present study, *C. glutamicum* was engineered to producing succinate efficiently, and the isomerase pathway and Weimberg pathway were combined to improve xylose utilization of *C. glutamicum* (Fig. 1). CS was pretreated by concentrated-alkali under steam-assistant conditions for succinate production.

2. Materials and methods

2.1. Culture media and conditions

C. glutamicum ATCC13032 (stored in our lab) were cultured in the brain heart infusion (BHI) medium. 91 g/L of sorbitol was added to BHI broth for preparing competent cells (Li et al., 2021). The CGXII minimal medium was used for batch and fed-batch fermentation containing 3-(N-morpholino) propanesulfonic acid 42 g/L, $(NH_4)_2SO_4$ 20 g/L, Urea 5 g/L, KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, $MgSO_4·7H_2O$ 0.25 g/L, $CaCl_2$ 10 mg/L, Biotin 0.2 mg/L, protocatechuic acid 0.3 mg/L, and trace elements ($FeSO_4·7H_2O$ 0.1 mg/L, $MnSO_4·H_2O$ 1 mg/L, $ZnSO_4·7H_2O$ 0.2 mg/L, $CuSO_4·5H_2O$ 20 mg/L, $NiCl_2·6H_2O$ 0.2 mg/L). *E. coli* DH5α was grown in LB medium with tryptone 10 g/L, NaCl 10 g/L, and yeast extract 5 g/L. BHI or LB plates supplemented with antibiotics (chloramphenicol 7.5 mg/L or kanamycin 50 mg/L) were used for *C. glutamicum* or *E. coli* transformants selection. BHI plates supplemented with 100 g/L sucrose was used for double selection according to previous description (Zhang et al., 2020).

High cell density was always used in organic acid fermentation of *C. glutamicum*. To avoid the problem of growth-decoupled succinate production in *C. glutamicum*, a two-stage fermentation process was developed: Cells growing aerobically are harvested and transferred to a production fermenter after resuspension in saline solution. A single colony of *C. glutamicum* was picked up into the first-degree seed medium (250 mL flask) containing 25 mL of BHI broth and cultured on a shaker (220 r/min, 30 °C) for 12 h. Then 5 mL first-degree seed culture was inoculated into a second-degree seed medium (250 mL flask) containing 25 mL of CGXII medium and 20 g/L glucose, and cultured on a shaker (220 r/min, 30 °C) for 24 h to an $OD_{600} \approx 25$. Finally, the cells in two flasks of second-degree seed culture were collected by centrifugation (4500 × g, 4 °C, 10 min), washed with CGXII medium, then transferred into fermentation medium (250 mL flask) containing 25 mL CGXII medium with the defined sugars and 200 mM $NaHCO_3$. 30 g/L $Mg_2(OH)_2CO_3$ was added in the medium to prevent acidification.

2.2. Construction of plasmids and recombinant

Plasmids used and strains constructed in this work are listed in Table 1. *C. glutamicum* ATCC13032 was used as host strain. To construct gene-knockout mutants, the suicide vector pK18mobsacB was used (Li et al., 2021). Fragments of 1000 bp for flanking genes to be deleted were amplified from *C. glutamicum*, and the recovered fragments were fused with enzymatically digested pK18mobsacB which were cloned into *E. coli* DH5α for amplification. The transformants were selected by chloramphenicol or kanamycin sensitivity and colony PCR. Then, the constructed plasmid was extracted from *E. coli*, and confirmed by sequencing. Subsequently, the plasmids were transformed into *C. glutamicum* through electroporation according to previous report (Li et al., 2021). The expression vector pEC-XK99E and pXMJ19 was used to overexpress genes in *C. glutamicum*. The target genes were colonized and amplified from *C. glutamicum* by PCR, and the recovered fragments were fused with enzymatically digested pEC-XK99E or pXMJ19. After verified by sequencing, the plasmid was transferred into *C. glutamicum*.

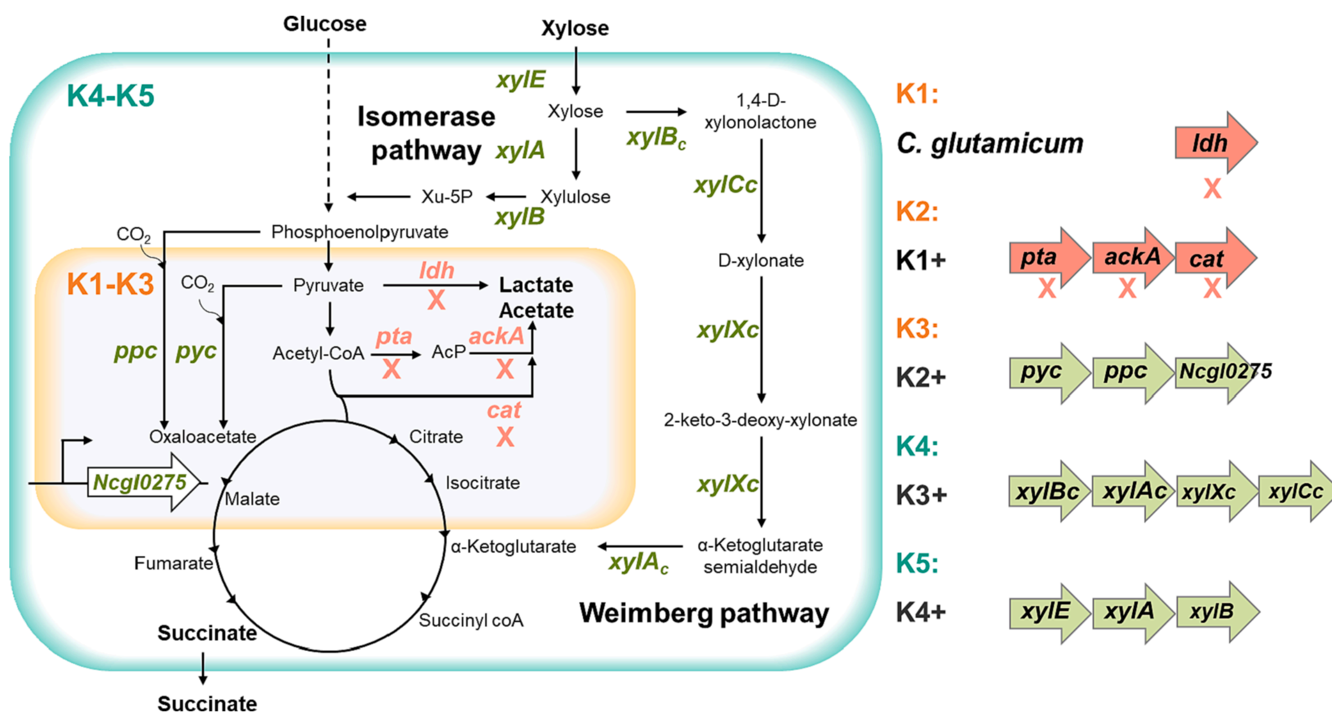

Fig. 1. Schematic diagram of the metabolic pathways for succinate production from glucose and xylose in *C. glutamicum.* Five engineered strains K1-K5 were constructed. The green color of genes indicates gene overexpression or heterologous expression. The red color of genes and the X sign indicate gene deletion.

Table 1
Strains and plasmids used in this study.

Strains	Description	Reference
DH5α	*E. coli* for plasmid amplification	Lab stock
ATCC13031	*C. glutamicum*	Lab stock
K1	*C. glutamicum*Δ*ldh*	This work
K2	K1Δ*pta-ackA*Δ*cat*	This work
K3	K2/pECXK99E-*ppc-pyc-cgl0275*	This work
K4	K3/pXMJ19-xylABE	This work
K5	K3/pXMJ19-xylABE-xylXABC	This work
Plasmids		
pk18mobsacb	Kan^R; vector for gene deletion	Lab stock
pEC-XK99E	Kan^R; *E. coli/C. glutamicum* shuttle vector	Lab stock
pXMJ19	Cm^R; *E. coli/C. glutamicum* shuttle vector	Lab stock
pk18-Δ*ldh*	pk18mobsacb carrying the flanking sequences of the *ldh* gene	This work
pk18-Δ*pta-ackA*	pk18mobsacb carrying the flanking sequences of the *pta-ackA* gene	This work
pk18-Δ*cat*	pk18mobsacb carrying the flanking sequences of the *cat* gene	This work
pECXK99E-*ppc-pyc*	Drived from pEC-XK99E, for expression of *ppc* and *pyc*	This work
pECXK99E-*ppc-pyc-cgl0275*	Drived from pEC-XK99E, for expression of *ppc*, *pyc*, and *cgl0275*	This work
pXMJ19-xylABE	Drived from pXMJ19, for expression of *xylA*, *xylB*, and *xylE*	This work
pXMJ19-xylABE-xylXABC	Drived from pXMJ19, for expression of *xylA*, *xylB*, *xylE*, *xylAc*, *xylBc*, *xylXc*, and *xylCc*	This work

2.3. Analytical methods

The biomass of strains was determined by measuring OD_{600}. Glucose, xylose, lactate, acetate, and succinate were measured by HPLC (Waters 1525, Waters, USA) equipped with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad). The mobile phase was 5 mmol/L H_2SO_4, and the flow rate was set at 0.6 mL/min. The temperatures of the column and RI detector (Waters 2414)) were set at 65 °C and 50 °C (Li et al., 2016).

2.4. Feedstock and CASA pretreatment

The corn stover (CS) was collected from Changtu County, Liaoning Province, China, was washed, dried, and milled in a grinder before being sieved through a 50-mesh strainer. The filtered CS powder was kept dry until further use.

50 g CS were mixed with NaOH granules (5 g, 10 g, or 15 g) and H_2O (0 mL, 50 mL, 250 mL, or 500 mL) in 5 L reactor, whose temperature was maintained at 130 °C for 30, 60, or 90 min by providing high-temperature steam from a steam generator. The pretreatment tank was equipped with a spiral paddle for better heat and mass transfer. The treated-CS were washed by tap water until pH became neutral, and dried in an oven (50 °C, 72 h). The steam consumption can be calculated by the lost water volume in steam generator.

2.5. Enzymatic hydrolysis and separate hydrolysis fermentation

Enzymatic hydrolysis was performed by commercial cellulases (Cellic CTec2, Novozyme) in a citric acid-Na_2HPO_4 buffer (0.1 mol/L sodium citrate and 0.2 mol/L dibasic sodium phosphate, pH = 4.8). The CS and buffer at a ratio 5% (w/v) were added into a 50 mL flask with cellulases at 20 FPU/g-CS. The mixture was incubated in a shaker (50 °C, 220 r/min) for 72 h. Then cells were collected by centrifugation (4500 g, 4 °C, 10 min) and washed twice using CGXII medium, then transferred into the CS hydrolysate. 200 mM $NaHCO_3$ and 30 g/L $Mg_2(OH)_2CO_3$ were added to provide CO_2 and prevent acidification. Finally, the 250 mL flasks equipped with anaerobic plugs were incubated in a shaker (30 °C, 220 r/min) for 72 h.

2.6. Chemical composition analysis

According to the NREL laboratory analytical procedures, the compositions of CS were analyzed using the two-step acid hydrolysis method (Sluiter et al., 2008). Simply, CS was hydrolyzed by 72% H_2SO_4 at 30 °C for 1 h, and after that, the mixture was diluted with water to 4% H_2SO_4 and hydrolyzed at 121 °C for 1 h. The supernatant was then harvested by centrifugation (10000 × g, 10 min) to analyze the compositions of CS,

which including cellulose, hemicelluloses, and acid soluble lignin. Lastly, the solid precipitation was obtained by filtration and dried using a muffle furnace at 550 °C to quantify the acid insoluble lignin and ash.

2.7. Morphology analysis and thermogravimetric analysis

Scanning electron microscopy (SEM) was used to observe the morphological changes of CS before and after CASA-pretreatment. The CS was sputtered with gold before being fixed on aluminum sample stubs. Images were acquired under a 20 kV acceleration voltage. The differential scanning calorimetry (DSC 8500, PerkinElmer, USA) was used to perform thermal stability analysis. With a nitrogen flow rate of 80 mL/min, 5 mg CS was heated from 30 to 600 °C at 10 °C/min heating rate. The derivative thermogravimetric (DTG) curves were obtained using the first derivation from the weight loss curve.

3. Results and discussion

3.1. Engineering a succinate high-producing C. glutamicum

The first step, in order to increase the precursor availability of succinate, the *C. glutamicum* was modified by inactivating competing pathways to reduce by-product formation such as lactate and acetate. Lactate dehydrogenase encoded by *ldhA* catalyzes pyruvate to lactate, which determines the lactate formation. Phosphate acetyltransferase and acetate kinase encoded by *pta-ackA* catalyze acetyl-CoA to acetate in PTA-ACK pathway in most acetate-producing microorganisms. Acetate coenzyme encoded by *cat* catalyzes the one-step reaction of acetate from acetyl-CoA. Therefore, *C. glutamicum* Δ*ldh* (K1) and *C. glutamicum* Δ*ldh* Δ*pta-ackA* Δ*cat* (K2) were constructed to block biosynthesis of lactate, and lactate + acetate, respectively (Fig. 1). Under anaerobic condition with 45 g/L glucose (Fig. 2A), the deletion of *ldh* facilitated K1 significantly decrease lactate from 27.3 g/L to 1.0 g/L. The saved carbon flux consequently increases the titers of succinate and acetate by 197% and 53%. Based on K1, K2 was further deleted the acetate related genes *pta-ackA* and *cat*. K2 produced only 1.2 g/L acetate comparing to wild type 3.3 g/L, and achieved 4.0 g/L more succinate titer compared to K1.

The pyruvate carboxylase and phosphoenolpyruvate carboxylase were demonstrated as two main anaplerotic enzyme in succinate formation under oxygen deprivation in *C. glutamicum* (Ghiffary et al., 2022). They catalyze oxaloacetate (C4) synthesis from pyruvate and phosphoenolpyruvic acid (C3), the native *pyc* and *ppc* gene were thus overexpressed in *C. glutamicum* K1 to shift the carbon flux from the C3 pathway to the C4 pathway, enhancing succinate accumulation. Furthermore, in some cases, the glucose consumption and succinate production were initial high but decline gradually when the succinate concentration increased during the anaerobic fermentation (Sánchez et al., 2005). Recently, *Ncgl0275,* a WhiB family transcriptional regulator, has been reported involved in release of end-product inhibition for succinate production (Chung et al., 2017). Thus, this gene was overexpressed together with *pyc* and *ppc* under the Ptac promoter. *C. glutamicum* K3 produced 33.6 g/L succinate with 0.6 g/L lactate and 1.2 g/L acetate. It performed even better than K2 (Fig. 2A), indicating that more carbon flux flow into succinate biosynthesis.

The best recombinant strain K3 was further evaluated using higher glucose (100 g/L) to achieve more succinate production under an anaerobic condition. The result showed that all glucose was consumed at 40 h and 73.1 g/L of succinate was obtained (Fig. 2B) with main by-product acetate (8.0 g/L) and neglectable lactate. The succinate yield under higher glucose was 0.73 g/g which is similar with the fermentation under 45 g/L glucose (Table 2).

To further improve the succinate titer, fed-batch fermentation was then carried out by using K3 (Fig. 2C). After 3 times of feeding 5 mL CGXII medium with 500 g/L glucose, K3 consumed about 200 g glucose

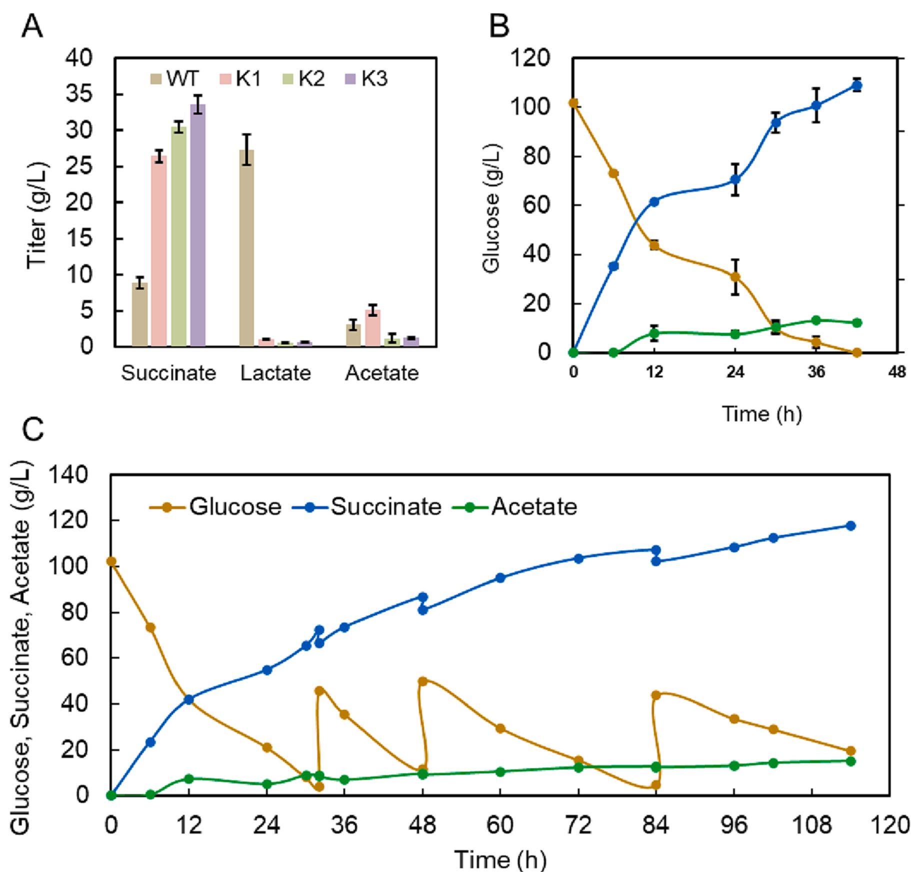

Fig. 2. Succinate fermentation of the engineered *C. glutamicum*. A: Products titers of strains K1-K3 in CGIXII medium with 45 g/L glucose. B: Batch fermentation of strain K3 in CGIXII medium with 100 g/L glucose under an anaerobic condition with initial OD_{600} = 30. C: Fed-batch fermentation of strain K3 under an anaerobic condition with initial OD_{600} = 50. K1: *C. glutamicum* Δ*ldh*, K2: K1Δ*pta*Δ*ackA*Δ*cat*, K3: K2/*ppc*/*pyc*/*cgl0275*. 200 mM $NaHCO_3$ and 30 g/L $Mg_2(OH)_2CO_3$ were added in the hydrolysate to provide CO_2 and prevent acidification.

Table 2
Succinate fermentation of *C. glutamicum* under different glucose condition.

Initial glucose	45 g/L				100 g/L	200 g/L
	WT	K1	K2	K3	K3	K3
Titer (g/L)	8.8	26.4	30.4	33.6	72.7	117.8
Yield (g/g)	0.19	0.58	0.67	0.75	0.72	0.59
Productivity (g/L/h)	0.44	1.32	1.52	1.68	1.73	1.04

and the final succinate titer is 117.8 g/L with the volumetric productivity of 1.04 g/L/h. Totally 14.98 g/L of acetate was accumulated. The yield of succinate is 0.59, which is much lower than batch fermentation. One possible reason is the decreasing cell viability during the long-time anaerobic fermentation.

3.2. Engineering a xylose-utilizing C. glutamicum

C. glutamicum cannot metabolize xylose as xylose negative bacterium. Some xylose positive bacterium such as *Caulobacter crescentus* utilize xylose by forming α-ketoglutarate through a five-step Weimberg pathway, which involved 5 enzymes: xylose dehydrogenase (*xylBc*), 2-keto-3-deoxy-d-xylonate dehydratase (*xylXc*), 1,4-xylono lactonase (*xylCc*), xylonate dehydratase (*xylDc*), and α-ketoglutarate semi aldehyde dehydrogenase (*xylAc*). We therefore cloned these genes into plasmid pXMJ19 under the Ptac promoter, and then introduced the plasmid pXMJ19-*xylBc-xylXc-xylAc-xylCc* into *C. glutamicum* K3, obtaining a strain K4.

Compared to Weimberg pathway, xylose isomerase pathway was more common found in broader microorganisms. According to the genome annotation of *E. coli*, xylose isomerase (*xylA*), xylulokinase (*xylB*), and xylose transporter (*xylE*), are the essential genes responsible for xylose metabolism. Thus, we cloned these three genes from *E. coli* DH5α into plasmid pXMJ19-*xylBc-xylXc-xylAc-xylCc* with the promoter Ptac, and then introduced the plasmid pXMJ19-Tac-*xylA-xylB-xylE*-Tac-*xylBc-xylXc- xylAc- xylCc* to *C. glutamicum* K3, obtaining strain K5 with Weimberg pathway and isomerase pathway (Fig. 1).

The ability of the engineered *C. glutamicum* K3-K5 to metabolize xylose was assessed. When only providing 16 g/L xylose as sole carbon source under an aerobic condition, K3 did not grow in CGXII medium due to lacking of key enzymes involved in xylose utilization (Fig. 3A). K4 and K5 harboring xylose utilization pathway consumed all xylose in 18 h. K5 with two xylose utilization pathway showed faster cell growth than K4, which only possesses the one Weimberg pathway. Thus, the xylose consumption rate of K5 was significantly faster than K4 (Fig. 3B). Unfortunately, succinate cannot be detected, which may be caused by aerobic condition. To solve this problem, the anaerobic condition was changed for next assessment, K5 thus can produce 13.11 g/L succinate (Fig. 3C), but it cannot completely consume all xylose during the fermentation process. K5 consumed around 19.50 g/L xylose from initial 30.0 g/L xylose after 96 h fermentation. We hypothesize that the heterologously expressed xylose isomerase and the Weimberg pathway empower *C. glutamicum* xylose utilization, but the energy generation efficiency might be not enough for maintaining cell viability. Therefore, glucose as the rapid energy provider was added into the medium (Fig. 3D). After 30 h fermentation, totally 70.0 g/L of glucose was depleted while only 11.1 g/L of xylose was consumed. Totally 23.11 g/L of xylose was consumed after 72 h fermentation. Although the carbon catabolite repression (CCR) was observed using mixture sugars, the xylose consummation rate of the medium with mixture sugars (0.24 g/L/h) is faster than previous experiment with sole xylose addition (0.2 g/L/h) due to extra energy support by glucose. At the 60 h of the fermentation, totally 64.15 g/L succinate was accumulated, with a productivity of 1.17 g/L/h. The results indicated that xylose utilization pathway expression improved xylose utilization of *C. glutamicum.*

3.3. Pretreatment of corn stover by concentrated-alkali under steam-assistant (CASA) conditions

Conventional alkali pretreatment needs to prepare the alkali solution and then mix the solution with the biomass. Different form conventional alkali pretreatment, CASA pretreatment developed by our lab applies alkali powder (not alkaline solution) to reduce the addition of water,

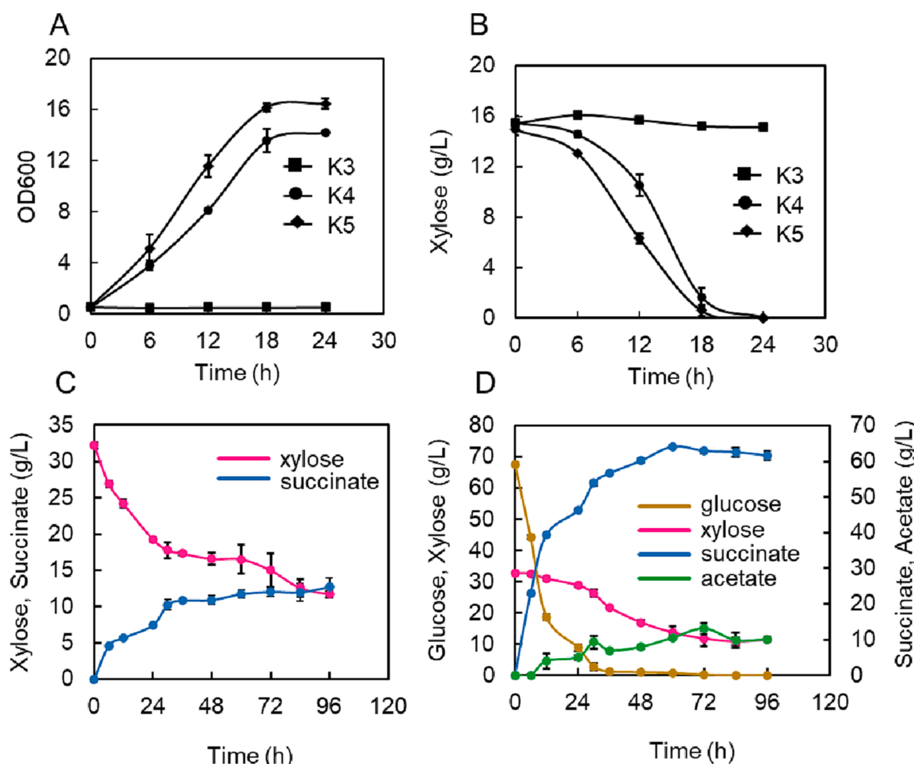

Fig. 3. Batch-fermentation of strain K3 and its engineered strains with xylose utilization pathway (K4, K5). Profiles of cell growth (A) and xylose consumption (B) in CXGII medium with 16 g/L xylose under an aerobic condition. C: Profiles of K5′ succinate production and xylose consumption in CGXIII medium with 30 g/L xylose under an anaerobic condition with initial OD_{600} = 30. D: Profiles of K5′ succinate and acetate production, xylose, and glucose consumption with initial OD_{600} = 30 in CGXII medium with 30 g/L xylose and 70 g/L glucose under an anaerobic condition. K1: *C. glutamicum* Δ*ldh*, K2: K1Δ*pta*Δ*ackA*Δ*cat*, K3: K2/*ppc*/*pyc*/*cgl0275*, K4: K3/*xylBc*/*xylAc*/*xylXc*, K5: K4/*xylA*/*xylB*/*xylE*. 200 mM $NaHCO_3$ and 30 g/L $Mg_2(OH)_2CO_3$ were added in the hydrolysate to provide CO_2 and prevent acidification.

which consequently can save steam consumption during the heating process because the specific heat capacity of water is much higher than lignocellulosic biomass. In addition, with the temperature rising, the condensed water from steam further dissolves the solid alkali forming a concentrated alkaline solution to remove the lignin efficiently, and the black liquid generation can be decreased.

3.3.1. Steam consumption and black liquid generation

Table 3 displayed that more alkaline solution required more steam to reach the set temperature, which increased the black liquid due to more condensed water from condensation. It consumed around 600 mL water to produce steam for maintaining 130 °C for 30 min under the ratio of 1:10 (biomass: alkaline solution), where the consumed steam is six times more than that under 1:0 condition (only adding NaOH powder without water). Besides, the pretreatment under 1:10 condition produced more than 1000 mL black liquid, while the pretreatment under 1:0 condition had only 100 mL black liquid. Therefore, the extreme condition of 1:0 without water addition can save steam consumption and reduce black liquid generation. It demands alkali powder rather than alkaline solution.

The sugar released during the enzymatic hydrolysis of CASA pretreated-CS showed no significant difference of glucose and xylose under various ratios of biomass and alkaline solution (Table 3). The average concentration of glucose and xylose were 28.6 g/L and 7.4 g/L, respectively. It reflected that CASA pretreatment could maintain the efficiency of enzymatic hydrolysis while reducing steam consumption and black liquid production.

3.3.2. Optimization of CASA pretreatment

The pretreatment time and NaOH dosage were further optimized for CASA pretreatment. The pretreatment time presents two-side effects. On the one hand, the prolonged time results in a significant quantity of lignin breakdown and certain hemicelluloses disintegrated, which benefits the following enzymatic hydrolysis. On the other hand, the longer time required more steam, and thus generated more black liquid. Fig. 4A showed the volume of water consumed for steam supply increased gradually as the pretreatment time prolonged. The water used for producing steam in 90 min was approximately 1100 mL, which was twice as the water volume in 30 min. Although the pretreatment times were extended from 30 min to 60 and 90 min, the accumulation of glucose and xylose from pretreated-CS was unaffected by the longer pretreatment time (Fig. 4B). Therefore, 30 min was good enough to produce sugar and save the steam and reduce black liquid in the CASA pretreatment.

The dosage of NaOH is essential for destroying the compact structure of lignocellulose. As Fig. 4C showed, when the amount of alkali was increased to 10 g, the concentration of glucose and xylose were increased to 27.7 g/L and 7.5 g/L, respectively. However, there was no further improvement of sugar concentration when the amount of alkali was increased to 15 g. Under the CASA pretreatment, the low concentration of NaOH was insufficient to completely dissolve all CS. Therefore, the optimal amount of alkali for CASA pretreatment is 10 g.

Table 3
Steam consumption and black liquid generation during the CASA pretreatment and sugars production from pretreated CS by enzymatic hydrolysis.

Ratio[1]	1:10	1:5	1:2	1:0[4]
Biomass (g)	50	50	50	50
Alkaline solution (mL)	500	250	100	0
Steam[2] (mL)	560	280	150	90
Black liquid (mL)	1150	600	270	100
Increased volume [3] (mL)	90	70	20	10
Glucose (g/L)	30.4	30.6	27.7	28.7
Xylose (g/L)	7.2	7.4	5.3	6.2

[1] Biomass: Alkaline solution (g: mL).
[2] Steam can be converted by lost water volume in steam generator.
[3] Increased volumes, Black liquid – Alkaline solution – Steam.
[4] We use 10 g alkali powder to replace alkaline solution.

3.3.3. Compositional and morphological changes

CS sample contains 37.3% cellulose, 22.0% hemicelluloses, and 19.1% lignin. After the optimized CASA pretreatment condition (30 min, 5 g NaOH), the content of cellulose increased to 78.3% because of the extensive removal of lignin (63%) and the exposure of cellulose. Compared to the untreated CS, significant morphological alterations after the CASA pretreated-samples can be observed by SEM (Fig. S1). Microscopically, the raw CS had a somewhat compact surface and a regular form, on the other hand, pretreated-CS losing lignin component left many holes across the smooth surface. In addition to creating numerous porosity hollows, the CASA pretreatment changed the microstructure of CS by disrupting the tight, regular, and cross-linked native structure, which impeded enzymatic hydrolysis of the cellulose component. Due to the delignification by CASA pretreatment, cellulose was transformed into loose state, which improved accessibility by cellulases.

The thermal stability of raw/pretreated CS in nitrogen were studied using TGA and DTG. The TGA curve revealed that the entire process could be divided three stages according to mass loss (Fig. S2). The first stage took place between 40 °C and 100 °C. The lignocellulose was stable at this stage, and the mass loss was primarily due to water evaporation, which are similar between the raw and pretreated-CS. The second stage started the lignocellulose decomposition between 100 °C and 500 °C, with rapid mass loss. The first decomposed component was hemicellulose at 200 °C–300 °C because of its poor thermal stability. The decomposition of cellulose occurred at 240 °C–350 °C. Because the cellulose content was relatively high in the pretreated-CS, it clearly showed that the mass of pretreated-CS in this range significantly decreased.

Overall, the SEM and TGA/DTG analysis both showed that the compact structure of CS was destroyed after CASA pretreatment. And the cellulose and hemicelluloses become exposed and more accessible to cellulases. Thus, CASA pretreated-CS is a promising substrate for enzymatic hydrolysis to produce the sugars.

3.4. Efficient succinate production from CASA pretreated-corn stover

We combined engineered stains and CASA pretreatment to test possibility of succinate fermentation by using the real CS. 150 g/L CASA-pretreated CS was hydrolyzed by cellulases Cellic CTec2 at a loading of 25 FPU/g substrate. CS hydrolysate containing 67.68 g/L glucose and 21.73 g/L xylose was obtained after 72 h enzymatic hydrolysis. The engineered strain K5 consumed all glucose and 16.50 g/L xylose during 72 h fermentation. The succinate titer and productivity at the end of the fermentation were 64.16 g/L and 1.07 g/L/h (Fig. 5). The by-products acetate was 10.06 g/L. The yield and productivity were similiar with results using mixture of glucose and xylose (Fig. 3D). However, the xylose consumption in lignocellulose hydrolysate was lower than that in the mixture of glucose and xylose, because of the limilted nutrients and possible inhibitors in the hydrolysate. Howerver, this result showed the promising applicartion to produce platform chemical succinate from lignocellulosic biomass. Among the few existing studies on succinate production from CS (Table S1), 67.68 g/L succinate was producced in this study. Although the titer of 67.68 g/L succinate in this work was not the highest value due to the lower CS loading, the titer and productivity also showed excellent potential for industrial production.

4. Conclusion

By metabolically engineering *C. glutamicum* and developing CASA pretreatment method, a productive fermentation process to produce succinate from corn stover was developed. A titer of 117.8 g/L succinate was obtained through fed-batch fermentation by using 200 g/L glucose.

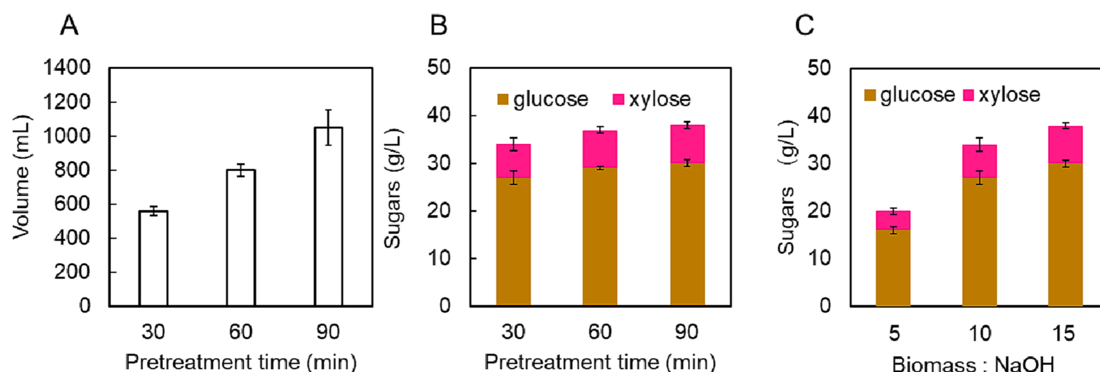

Fig. 4. Optimization of the pretreatment time and NaOH dosage in CASA pretreatment. A: The consumed water for producing steam used in CASA pretreatment under different time. B: Sugars production from 50 g/L pretreated corn stover under different time. C: Sugars production from pretreated corn stover under different NaOH dosage (g/g biomass) at 130 °C for 30 min.

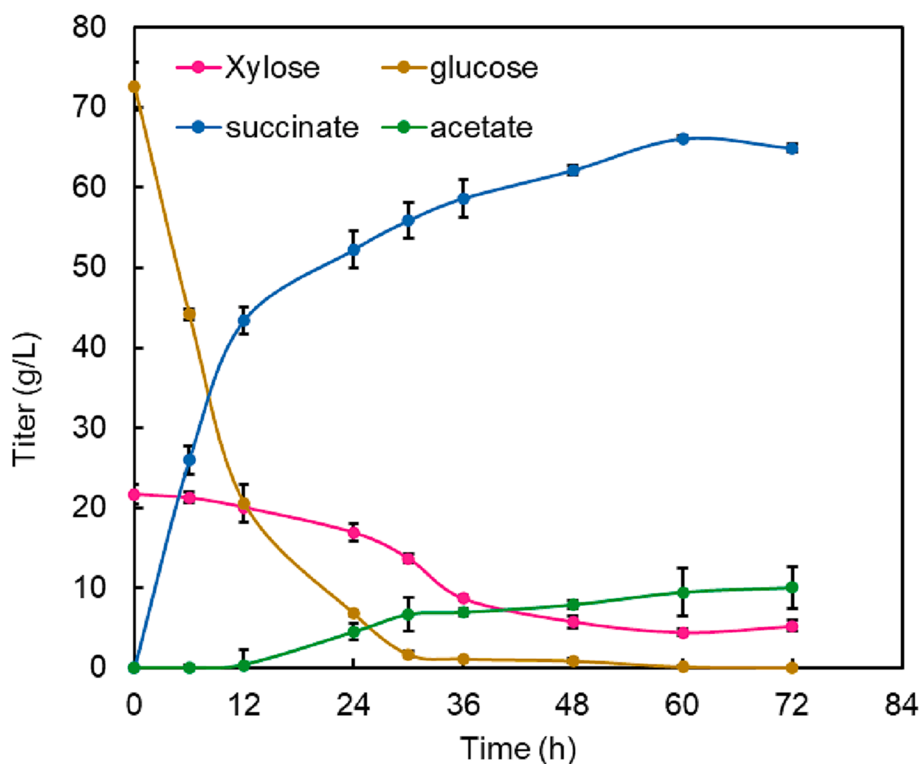

Fig. 5. Succinate production from CASA pretreated-corn stover hydrolysate with initial OD_{600} = 30 under anaerobic condition. 200 mM $NaHCO_3$ and 30 g/L $Mg_2(OH)_2CO_3$ were added in the hydrolysate to provide CO_2 and prevent acidification.

Additionally, the two xylose engineered pathways in *C. glutamicum* enabled the synthesis of 13.11 g/L succinate from 19.50 g/L xylose. Less steam consumption and low black liquid production was achieved through CASA pretreatment. Finally, the engineered *C. glutamicum* performed excellent utilization of CASA-pretreated CS to produce succinate under anaerobic condition. This study provides a reference for the bio-based chemicals production from lignocellulose substrate.

CRediT authorship contribution statement

Kai Li: Conceptualization, Methodology, Writing – original draft. **Cheng Li:** Writing – review & editing. **Xin-Qing Zhao:** Writing – review & editing. **Chen-Guang Liu:** Investigation, Supervision, Writing – review & editing. **Feng-Wu Bai:** Supervision, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128991.

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